Small Molecular Weight Inhibitors of Stress-Activated and Mitogen-Activated Protein Kinases

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Abstract: The stress-activated protein kinase (SAPK) and mitogen-activated protein kinase (MAPK) sub-families are crucial to environmental stress responses and responses to growth factors that cause transcriptional activation of genes required for cell proliferation, differentiation and programmed cell death. Small molecular compounds with specific structure/activity characteristics have been developed that competitively block SAPK/MAPK binding to ATP. Chemically modified compounds based on ATP binding pocket characteristics have improved selectivity and specificity for SAPK/MAPK isoforms. In addition, site-specific mutagenesis of MAPKs has helped identify the MAPK structures required for binding recognition and selectivity of these inhibitors. A group of extracellular-signal regulated protein kinase (ERK) inhibitors has been constructed based almost exclusively on their ability to inhibit the ERK activation cascade. Inhibitors have been employed *in vitro* to identify protein targets and mechanism of action of SAPKs/MAPKs. The efficacy of SAPK/MAPK inhibitors in animal models of inflammation, arthritis, heart failure, cancer and neurological degeneration has provided the impetus for using them in human studies of inflammation and in clinical trials.

Keywords: Stress-activated protein kinase, mitogen-activated protein kinase, c-jun-N-amino-terminal kinase, p38 kinase, extracellular signal-regulated kinase, anthrapyrazolone, pyridinyl imidazole, methoxyflavone.

INTRODUCTION

Stress-activated protein kinases (SAPKs) and mitogen-activated protein kinases (MAPKs) play a critical role in eukaryotic intracellular signal transduction pathways that are responsible for normal cellular proliferation, differentiation, cytokine gene regulation and cytokine responses as well as matrix metalloproteinase (MMP) gene expression, cellular responses to mechanical signals and programmed cell death (i.e. apoptosis) [1, 2]. SAPKs and MAPKs are grouped into protein kinase (PKs) sub-families based on the environmental stimuli required to initiate specific PK activation. Thus, the SAPKs, c-jun-amino-terminal kinase (JNK) and p38 kinase (p38) are generally activated *in vitro* by environmental stressors such as ultra-violet radiation, heat, osmotic shock and cytokines whereas the extracellular signal-regulated kinases (ERKs) respond *in vitro* mainly to serum, growth factors, some G-protein coupled ligands and other stresses and transforming agents [3]. Although SAPKs and MAPKs exhibit approximately 60-70% amino acid sequence homology between them, significant differences in their mechanism of activation residing in the PK activation loop specific for each PK as well as the level to which each SAPK or MAPK is activated by a specific stimulus have been elucidated [reviewed in 3].

Based on recent advances in bioinformatic techniques, SAPKs and MAPKs can also be characterized according to their enzyme structure and specific activation patterns of multiple SAPK/MAPK isoforms. For example, there are 10 apparent JNK isoforms encoded by 3 genes, namely, JNK1, 2, 3; Five p38 isoforms, namely, p38α, p38β1, p38β2, p38γ, p38δ (also known as ERK-6) and several additional ERK isoforms, namely, ERKp42/p44 (i.e. ERK1/2) and ERK-3, -4, -7 [4, 5]. Activation of specific SAPK/MAPK isoforms is likely to be stimulus-specific, tissue-specific and cellular localization-specific. This appears to be the case during inflammatory responses [3], in developing organs, in normal mouse, rabbit and human adult tissues as well as in diseased organs, such as rabbit cardiac myocytes following induction of ischemia [6]. In this case, confocal microscopy showed that p38α/β was distributed throughout the cytosol and nucleus, but p38γ was solely non-nuclear [6].

SAPKs and MAPKs are generally activated by one of 3 non-overlapping pathways. In general, MAPKs require the activity of upstream MAPKs, namely, MKK and MAPKKK for full MAPK activation. With a few specific exceptions, such as the recently clarified MAPKK-independent autophosphorylation and activation of p38α by transforming growth factor-β-activated protein kinase-1 (TAK-1) binding protein-1 (TAB1) (7), MAPKKK phosphorylates MKK [4]. Activated phospho-MKK causes MAPK phosphorylation in the threonine-X-tyrosine (i. e. the TPY motif) sequence within the MAPK loop of activation [8, 9], where X is a Pro, Gly or Glu in JNK, p38 and ERK, respectively. However, under specific conditions, MAPKs may also be activated by enzyme-enzyme interactions followed by dissociation mediated by downstream PKs, or by MAPK interactions at the site of ligand-receptor binding [4] that also indirectly regulate signal transduction.

Experiments designed to elucidate the requirements for specific and selective inhibition of SAPK and MAPK activity by small-molecular weight compounds have provided insights into the intracellular and physiologically

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relevant signaling pathways regulated by these PKs. The purpose of this mini-review is to discuss and evaluate experimental studies employing the most promising SAPK and MAPK inhibitors as evidenced by their *in vitro* inhibition profiles which have also been tested in animal models of allergy and inflammation, cerebral ischemia/reperfusion injury, spinal injury as well as in clinical trials of arthritis, stroke, heart disease and neurological degeneration disorders.

INHIBITORS OF JNK

JNK Activity

JNK1 and JNK2 are widely expressed in human tissues, but JNK3 is almost exclusively expressed in brain [10, 11] with low levels expressed in kidney and testis. Once activated JNK is translocated to the nucleus where JNK phosphorylates one of several transcription factors, including, activator protein-1 (AP-1), activating transcription factor-2 (ATF-2), Elk-1, nuclear factor of activated T-cells (NFAT) and p53 that regulate matrix metalloproteinase (MMP) gene expression and apoptosis protein gene expression, respectively [2]. Recent evidence linked interleukin-1-induced JNK activation [12] to IL-1β-induced MMP-13 gene transcription in cultured human chondrocytes [13], MEKK2 activation in human rheumatoid arthritis (RA) synovium and RA fibroblast-like synoviocytes [14], aberrantly activated JNK1 associated with the filamentous tau protein in the brain tissue of Alzheimer's disease patients and other neurological degeneration diseases [15] in the phenyl-1, -2, 4, 6 tetrahydropyridine (MPTP) mouse model of Parkinson's disease in which both JNK2 and JNK3, but not JNK1 are required for c-jun activation and nigrostriatal dopaminergic-cell apoptosis [16] that is closely linked to caspase-3 and caspase-9 activation [17], as well as IL-1β-induced c-jun and c-fos expression that are critical mediators of pancreatic beta-cell apoptosis [18], cerulein-induced experimental murine pancreatitis [19] and murine ovalbumin-induced allergic airway inflammation [20]. At the molecular level, JNK inhibition suppressed c-jun phosphorylation as well as the expression of pro-inflammatory molecules, namely, cyclooxygenase-2 (COX-2), interleukin-2 (IL-2), interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α), but not intracellular adhesion molecule-1 (ICAM-1), IL-1β or IL-8 in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells or cell differentiation (CD) protein-14+ monocytes [21]. Based on the results of these studies, experimental blocking of JNK activity might be expected to blunt MMP gene expression and apoptosis that are characteristic of several human degenerative disorders [2].

SP600125

[Anthra [1-9-cd] Pyrazol-6-(2H)-one]; JNK Inhibitor II]]

JNK activation is dependent on MEKK upstream activity [reviewed in 8]. MEKK phosphorylates JNKK at ser^{219} and ser^{223} resulting in JNKK activation. Activated JNKK phosphorylates JNK at the TPY motif activating JNK.

Small chemical inhibitors of activated JNK such as the anthrapyrazolone JNK inhibitor SP600125, (Fig. **1**) inhibit JNK activity by competing with JNK for binding to ATP [22]. Recent crystallographic evidence suggested that the inhibitor profile and specificity for JNK isoforms could be improved upon by deciphering the structure of the JNK-1/ATP binding site [23] because earlier studies indicated that JNK inhibitors like SP600125 did not show preferential inhibition of specific JNKs isoforms; rather SP600125 had relative specificity for JNKs compared to p38 kinase and ERKs. Indeed, Bennett *et al.* [21] showed that SP600125 reversibly inhibited JNK1,-2 3 with a K_i of 0.19 μM, but SP600125 also inhibited ERK2 and p38β, although SP600125 had a >20-fold potency towards the JNKs [21].

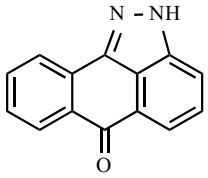


Fig. (1). Structure of SP 600125.

SP600125-mediated JNK inhibition was also shown to activate other signal transduction pathways. In this regard, SP600125 induced cyclic AMP response-element binding protein (CREB) activation at 20μM which was also the SP600125 concentration that blocked c-jun phosphorylation [24] in MIN6 cells (a mouse pancreatic beta and islet cell line). In that study [24], CREB activation was accompanied by elevated levels of activated phospho-p38. CREB activation also inhibited c-jun transcription suggesting that over-expression of CREB could protect pancreatic cells from cytokine-induced apoptosis.

AS601245

(1, 3 Benzothiazol-2-yl) (2-[[2(3-Pyridinyl) Ethyl] Amino]-4 Pyrimidinyl) Acetonitrile

AS601245, (Fig. **2**) is also a potent and relatively selective inhibitor of JNK activity [25]. Investigations on the structure-activity relationship of AS601245 showed that the presence of an aromatic group, two carbons away from the aminopyrimidine moiety with substitutions conferring hydrogen bond acceptor characteristics improved the biological activity of AS601245 against JNK compared to the parent compound, AS007149 [26]. Oral administration of AS601245 to mice with Type II collagen-induced arthritis (CIA) reduced paw swelling, but was not as effective as indomethacin. However, interest in the activity of AS601245 as an *in vivo* JNK inhibitor has been heightened by recent results showing that AS601245 inhibited JNK signaling and, in turn, promoted neuronal cell survival after experimental induction of cerebral ischemia in the gerbil [27]. Analysis of gerbil hippocampal CA1 neurons showed that AS601245 inhibited c-jun expression and c-jun phosphorylation [27] as well as exhibiting an anti-apoptosis effect comparable to caspase inhibition with AS600292 (compound 50a) [27]. The aromatic amide group in AS600292 was required for maximal JNK inhibition. However, *in vitro* JNK inhibition failed to correlate with *in vivo* potency in this animal model of stroke due to the relatively poor solubility of AS600292 in phosphate-buffered saline. Nevertheless, JNK inhibition resulted in a

delay of the loss of hippocampal neurons characteristic of transient global ischemia. The significant reduction in neuronal apoptosis after administration of AS601245 or AS6010292 [2-benzoylaminoethyl) thiophene sulfonamide benzotriazole] [27, 28] in either the focal or global cerebral ischemia model in gerbils, suggested that AS601245 was a "neuroprotectant" and therefore was of considerable interest as a potential novel therapy for stroke and Parkinson's disease [28].

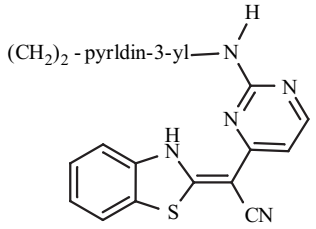


Fig. (2). Structure of AS601245.

AS601245 was shown to inhibit JNK-1, -2, and 3 with IC_{50} values of 0.15, 0.22 and 0.07 μM, respectively using *in vitro* PK assays [29]. Based on these results, AS601245 was tested in a mycocardial ischemia reperfusion model in rats and was shown to reduce infarct size without altering hemodynamics or reducing ST-segment displacement 4 [29]. A reduction in infarct size was accompanied by reduced c-jun phosphorylation as well as the number of terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) positive cells showing that AS601245 reduced apoptosis frequency in cardiac myocytes after induction of ischemia. In contrast, AS601245 did not alter phopho-p38 or phospho-ERK in the post-ischemic heart.

JNK Inhibition Based on Interactions Between JNK and its Substrates

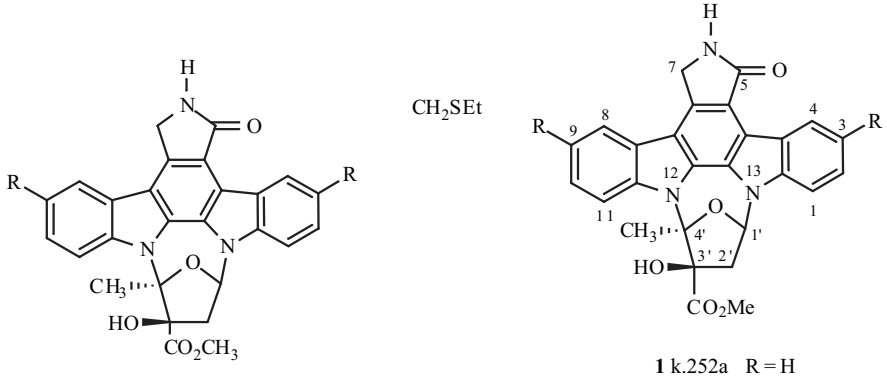
The JNK signaling pathway is also regulated by the JNK-interacting protein-1 (JIP1), a scaffolding protein that promotes JNK interaction with its substrates. For example, over-expression of JIP1 deactivated the JNK pathway by selectively causing retention of JNK in the cytoplasm. Recent studies of the ternary structure of JIP1-bound (i.e. pep-JIP1) JNK in complex with SP600125 provided a basis for the mechanism underlying the relative JNK1 specificity of SP600125 [30]. These analyses indicated that distortion of the ATP-binding site induced by pep-JIP1 binding created an allosteric-type inhibition of JNK activity. Thus, it is quite possible that SP660125 could block JNK1 when bound to JIP1 *in vivo* [30]. By exploiting an experimentally-induced alternative modification in JNK residues, Ile^{70} and Val^{196} also critical in the binding of SP600125 to JNK, Fricker *et al*. [31] were able to markedly improve the specificity of SP600125 for JNK-3, a critical JNK in normal brain tissue allostasis.

CEP-1347/KT7515: an Inhibitor of JNK Activation

CEP-1347/KT7515 (Fig. **3a**) is the *bis*-ethylthiomethyl ($R = CH_2SEt$) derivative of the natural product indolocarbazole alkaloid, K-252a isolated from *Nocardiopsis* sp. (Fig. **3b**). K-252a was originally characterized as an inhibitor of protein kinase C and then subsequently found to inhibit several serine/threonine PKs [32]. The physiologic effects of direct overlaid administration of CEP-1347/KT7515 were first demonstrated on chick embryo chorioallantoic membrane where the agent rescued 40% of lumbar motor neurons that normally die during development [33]. In addition, peripheral subcutaneous administration of CEP-1347/KT7515 reduced lumbar motor neuron death in the spinal nucleus of bulbocavernosus in postnatal rats with an efficacy equivalent to testosterone [33].

Saporito *et al.* [34] showed that CEP-1347/KT7515 was effective in suppressing neuronal death in response to MPTP in mice. Subsequent studies demonstrated that the mechanism of action of CEP-1347/KT7515 was to modulate JNK1 activity induced by mixed lineage kinases (MLK) [35]. Blocking MLK activity at the same concentration required for JNK inhibition [36] also suppressed neuronal apoptosis by reducing cytochrome c release and Bax-dependent caspase activation [37]. Of note, Bozyczko-Coyne *et al.* [35] showed that CEP-1347/KT7515 also blocked β-amyloid induced activation of MKK4 in cortical neurons *in vitro* as well as additional downstream signaling dependent on JNK, namely, phospho-c-jun, cyclin D and DP5, but only partially suppressed c-fos and fosB gene expression. In addition, CEP-1347/KT7515 had anti-apoptosis activity characterized by suppression of cytochrome c release from mitochondria, caspase-3 activity and calpain release [35]. CEP-1347/KT7515 suppression of β-amyloid-induced

3a. CEP-1347 **3b. K-252a**



1 k.252a R = H

Fig. (3). Structure of the K-252a derivative, CEP-1347 [32].

neuronal apoptosis [35] suggested that this compound might be of therapeutic benefit in Alzheimer's disease.

The anti-apoptosis effects of CEP-1347/KT7515 [38, 39] that promoted neuronal survival *in vitro* as well as the potential efficacy of CEP-1347/KT7515 in the treatment of Alzheimer's disease and Parkinson's disease [40] led to a small randomized, double-blind, placebo-controlled study of 30 patients with Parkinson's disease [41]. In this short-term 4 week study [41], CEP-1347 was judged to be safe and well-tolerated. CEP-1347 administered orally at 50 mg did not interfere with L-dopamine pharmacokinetics but had no effect on Parkinson's disease rating scale.

INHIBITORS OF p38 KINASE

p38 Kinase Activity

Among the isoforms of p38, the most extensive structural and inhibition data exists for p38α because of its involvement in the inflammatory response, including its critical role in regulating COX-2 and iNOS gene expression as well as NO synthesis induced by cytokines [42]. Although the functional role and tissue distribution of p38α and p38β2 is well documented, much less information exists about the functional roles of p38β1, p38γ or p38δ. In terms of tissue distribution, the p38δ isoform is found widely in adult and developing organs, but p38γ appears restricted to skeletal muscle [3].

The activation of p38 is dependent on upstream MEK/MKK activity. In that regard, MMKK1 phosphorylation of MEKK-3, MEKK-4 and MEKK-6 have all been implicated in p38 activation [43, 44]. Thus, apoptosis induced by oxidative stress was shown to occur via MKK-4 and MMK-7 activation of p38 as well as through MMK-3 and MMK-6 activation [44], suggesting significant redundancy in the p38 activation cascade.

SB203580

[(4-(4-Flurophenyl)-2-(4-Methylsulfinylphenyl]-5-(4-Pyridyl) Imidazole]

SB203580 (Fig. **4a**) was originally characterized as a highly selective, cell permeable inhibitor of p38α and p38β, with IC_{50} of 50 and 500nM, respectively. SB203580 inhibits p38 activity by interacting with the p38/ATP-binding pocket. By employing site-specific mutagenesis, Eyers *et al.* [45] established that the size of the Thr^{106} side chain in the p38α and p38β2 ATP binding region was essential for SB203580 inhibitory activity. This study [45] also showed that the side chain could be no larger than a threonine residue but could be smaller. In fact, SB203580 activity could be enhanced by substituting serine, alanine or glycine at the threonine site.

Activation of p38 has been implicated in several animal models of inflammatory disease. However, poor oral availability was associated with p38 imidazole inhibitory compounds *in vivo* unless the imidazole was methylated on the nitrogen. Thus, the oral bioavailability of imidazole-based p38 inhibitors was markedly improved by substituting the 4-aryl ring with m-trifluromethyl and substitution of the 5-heteroaryl ring with a 2-amino substitution (Fig. **4b**) [46]. This pyridine analog (i.e. compound, 48) was highly selective as a p38 inhibitor (IC_{50}, 0.19nM) compared to JNK2α_1 (IC_{50}, 1170nM). In addition, compound 48 was shown to have acceptable oral bioavailability in the rhesus monkey as well as in experimental treatment of rat adjuvant-induced arthritis (AIA) with a significant reduction in arthritis progression and serum TNF-α [46].

Correlative evidence for the anti-inflammatory effect of SB203580 was obtained by demonstrating a synovial joint sparing effect of SB203580 in the AIA Lewis rat model and the CIA model of murine arthritis [47, 48]. Thus, SB203580 administered to rats at a prophylactic dose of 60 mg/kg reduced the bone mineral density loss associated with AIA in addition to suppressing paw edema [48]. Badger *et al.* [48] also showed that the more selective p38 inhibitor SB242235 (Fig. **4c**) inhibited LPS-stimulated TNF-α in the normal rat as well as suppressing paw inflammation while normalizing bone loss in the rat AIA model even after AIA joint cartilage and bone had become prominent. Of note, indomethacin had no effect on bone mineral density in this model. Taken together, these results lend credence to the potential clinical efficacy SB242235 for treating human arthritis and chronic inflammation.

Further studies employing SB242235 *in vitro* were necessary to establish the specificity of SB242235 for the p38-associated inflammatory profile. Moreover, SB242235 failed to inhibit protein kinase C, protein kinase A, MEK-1, nitric oxide (NO) or nitric oxide synthase (iNOS) in IL-1-treated human chondrocytes, but did suppress prostaglandin E_2 (PGE_2) production [49]. These results, however, conflicted with earlier studies by Badger *et al.* [50] where SB203580 ($IC_{50,}$ 1μM) inhibited NO production by reducing iNOS mRNA in bovine chondrocytes.

In other animal disease models, SB203580 suppressed cardiomyocyte apoptosis in an experimental myocardial infarction/reperfusion model in the rat and improved post ischemic cardiac recovery exemplified by improvement in cardiac contractility [51], induced caspase-3-mediated apoptosis in a p38-transformed follicular lymphoma cell line [52] and suppressed the loss of hind limb function in a rat thoracic spinal injury model, accompanied by a decrease in the number of apoptotic cells in the lateral funiculi [53], therefore establishing a direct link between p38 activation and apoptosis [reviewed in 54].

FR167653

{1-[7-(4-flurophenyl)-1, 2, 3, 4-tetrahydro-8-(4-pyridyl) pyrazolo [5, 1-c][1, 2, 4] triazin-2-yl]-2-phenylethane-dione sulfate monohydrate}

FR167653 was first identified not as an antagonist but as an inhibitor of IL-1 and TNF-α synthesis in LPS-stimulated monocytes and in phytohemagglutinin-stimulated human lymphocytes *in vitro* as well as an effective treatment for LPS-induced septic shock and renal damage in rabbits [55]. The FR167653 ring structure (Fig. **4d**) resembles that of other potent p38 and SAPK inhibitors such as SB203580 (Fig. **4a**) and [(4-[4-(4-flurophenyl)-1-(3-phenylproply)-5-4-pyridinyl)-1H-imidazol-2-yl)-3-butyn-1-ol)] also known as RWJ67657 (RW Johnson Pharmaceutical Research) [3]. Using *in vitro* PK assays, Takahashi *et al*. [56] established

that FR167653 inhibited p38α, but not p38γ activity in a manner similar to the results obtained in previous studies with SB203580 and RWJ67657 where p38α and p38β were inhibited but ERK2 and other tyrosine PKs were not [57]. This study [57] also showed that RW67657 was an effective inhibitor of TNF-α synthesis when orally administered to LPS-injected mice.

The results of these *in vitro* PK assay studies were consistent with a structural analysis of FR167653 and its mechanism of action as well as its mode of interaction with p38 as reported by Gum *et al.* [58]. This study first demonstrated that the p38 α and β isoforms were sensitive to pyridinyl imidazoles. The binding of p38 to pyridinyl imidzoles involve Thr^{106}, His^{107} and Leu^{108} in the p38-ATP binding pocket. However, the p38 α and β isoforms were pyridinyl imidazole-insensitive presumably because they lacked homology to this amino acid triplet structure. Subsequently, Franz *et al.* [59] established that pyridinyl imidazole p38 inhibitors blunted stimulus-induced p38 phosphorylation at the Thr-Gly-Tyr activation motif. At physiologically relevant concentrations of FR167653, ATP cannot compete with FR167653 binding to either the non-phosphorylated or phosphorylated p38. This result suggested that pyridinyl imidazoles could block p38 activity by maintaining p38 in a configuration that could not bind to ATP.

Although FR167653 has structural similarity to SB203580, the latter was previously reported to be a potent COX-1 and COX-2 reversible inhibitor and also inhibited thromboxane synthase activity [60]. An earlier study had showed that FR167653 did not inhibit COX-1 or COX-2 measured *in vitro* even at concentrations as high as 10μM [56].

The potent p38 inhibiting activity of FR167653 resulted in its being tested for efficacy in animal models of inflammation. Thus, FR167653 was also shown to be an inhibitor of LPS-induced plasma leakage and carrageenan-induced paw edema in a mouse model of vascular inflammation [61]. LPS-induced vascular permeability was also suppressed by FR167653. This change in vascular permeability correlated with reduced serum and skin TNF-α, as well as PGE_2 levels at the LPS-injection site. However, FR167653 had no effect on arachidonic acid-induced plasma leakage in the mouse suggesting that FR167653-mediated inhibition of p38 was not related to COX-1 activity. Rather, FR167653 may inhibit cytokine-induced COX-2-synthesis but was not likely to be a COX-1 or COX-2 activity inhibitor.

It was also shown that FR167653 suppressed *Heliobacter pylori*-induced muscosal inflammation in a model of gastritis in the gerbil, in part, by suppressing chemokine production dependent on p38 activation [56]; however, gastric mucosal COX-1 and COX-2 levels were not reported. More recently, FR167653 was shown to suppress rat CIA which was characterized by reduced TNF-α and IL-1β serum levels as well as reduced IL-1 levels in the ankle joints and altered helper/suppressor (i.e. CD4-/CD8+) T-lymphocyte ratios in bone marrow [62]. Of note, FR167653 also inhibited osteoclast-like differentiation induced by soluble receptor-activated nuclear factor κB ligand (RANKL) and TNF-α *in vitro* [62] suggesting a mechanism for potential bone destruction-sparing effect associated with FR167653 treatment of inflammation *in vivo*.

Additional studies of FR167653 in animal models of heart failure have also been conducted. Thus, FR167653 significantly improved cardiac contractile function during reperfusion in a rat heart ischemia-reperfusion model and also attenuated cardiomyocyte apoptosis [63]. Infarct size was also considerably reduced by pretreatment with FR167653 in a mouse model of myocardial ischemia-reperfusion injury produced by ligation of the left anterior descending coronary artery [64]. In that study [64] phospho-p38α and β, nuclear factor κB (NF-κB), TNF-α, IL-1β and monocyte chemotactic factor (MCF) were significantly decreased in the heart tissue from animals pre-treated with FR167653 but activated JNK and ERK1/2 were not altered by FR167653. Taken together, these results indicated that FR167653 was a potent inhibitor of p38 activity and pro-inflammatory cytokines *in vitro* as well as in animal models of arthritis, gastric inflammation and heart failure.

SB202190

4-[(4-Flurophenyl)-5-(4-Pyridinyl)-1H-Imidazole-2-yl] Phenol

SB202190 (Fig. **4e**) bears a strong structural similarity to SB203580 (Fig. **4a**). Early in the experimental studies employing these inhibitors, Davies *et al.* [65] concluded that the p38 inhibitory specificity profile of pyridinyl imidazole inhibitors such as SB202190 and SB203580 could not be adequately determined because the p38 isoforms were so similar in primary structure. In addition, most of the PK inhibitors, even those in the pyridinyl imidazole class, inhibited more than one PK. Nevertheless, SB202190 has been employed in hundreds of experimental *in vitro* and *in vivo* studies since 2000 and many of these studies have probed the structural specificity of SB202190 for p38 as well as for p38 isoforms. Thus, Davies *et al.* [65] showed that SB202190 was a more potent inhibitor of p38β2 (IC_{50}, 100nM) than SB203580 (IC_{50}, 500nM), but SB202190 was more effective against p38α than p38β2. Other studies have compared p38 inhibition using SB202190 with biologically inactive structural analogs of SB202190 such as SB202474 [66]. In that regard, SB202190 induced apoptosis in an LPS-stimulated macrophage-like cell line J774.1 via p38 inhibition but SB202474 did not; neither SB202190 nor SB202474 suppressed phospho-ERK1/2 [66].

Several *in vitro* studies demonstrated that SB202190 could be employed to elucidate the role of p38 in intracellular inflammatory and apoptosis pathways, as well as in cell proliferation and MMP gene transcription. Consistent with this hypothesis, SB202190 inhibited LPS/TNF-α/IFN-γ-induced NO and iNOS gene dexpression in C6 glioma cells [67], strongly induced transcription of the cyclin DK inhibitor p21 [i. e. waf1/cip1] in a rat chondrosarcoma cell line [68], potentiated fibroblast growth factor-2-induced astroglial neurite extension [69], attenuated IL-1β-induced MMP-9 (i. e. 92kDa gelatinase) transcription in rat brain astrocytes [70] and transforming growth factor β_1-induced MMP-9 in human MDA-MB-231 breast cancer cells [71].

SB202190 was also used to explore p38-associated events in LPS-induced acute endotoxemia in mice. Thus, Peng *et al.* [72] showed that SB202190 suppressed endotoxemia and associated elevated myocardial TNF-α as well as significantly reversing LPS-induced left ventricular depression as measured by the first derivative left ventricular pressure but without altering heart rate, mean arterial pressure or left ventricular systolic pressure.

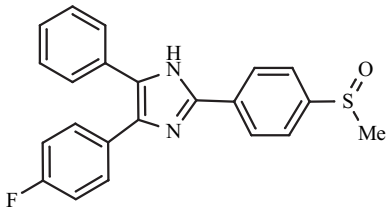
BIRB796

1-(5-Tert-Butyl-2-p-Tolyl]-2H-Pyrazol-3-yl]-3-[2-Morpholin-4-yl-ethoxy) Napthalen-1-yl] Urea

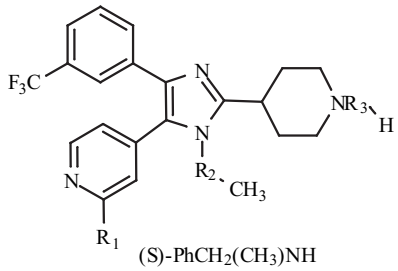
BIRB796 [73] (Fig. **4f**), an N-pyrazole-N'-naphthly urea inhibitor of all the p38 isoforms [74] binds to p38 with both slow association and dissociation rates [75] and has been employed to examine which of many potential protein targets are actually physiological substrates for p38γ [74]. BIRB796 has also been orally administered to human volunteers to establish a treatment for human endotoxemia induced by LPS [76]. BIRB796 significantly inhibited p38 activation in leukocytes, diminished leukocyte responses and down-regulated both L-selectin and C-reactive protein release. In a follow-up study, van den Blink *et al.* [77] showed that BIRB796 administered at 600 mg to normal volunteer subjects with LPS-induced endotoxemia showed suppressed chemokine receptors CXC receptor 1 and 2 as well as their ligands, IL-8 and growth-related oncogene-α compared to individuals receiving either a lower BIRB796 dose (50 mg) or placebo. The results of these studies indicated that BIRB796 suppressed p38 activity *in vivo* which was required for neutrophil activation and chemotaxis.

Novel p38 Kinase Inhibitors with Improved Specificity for p38 Isoforms

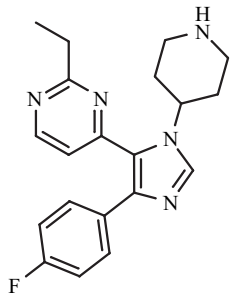
Other diverse scaffolds have been developed to improve the specificity of inhibitors for p38 kinase isoforms [78]. Compounds such as VX-745 (Vertex Pharmaceuticals) were reported to display unique specificity for p38 due to their



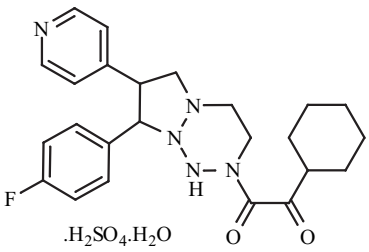
4a. SB203580 (GlaxoSmithKline)



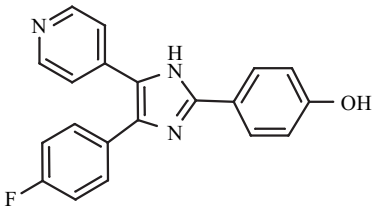
4b. Compound 48 (Merck Research Laboratoires)



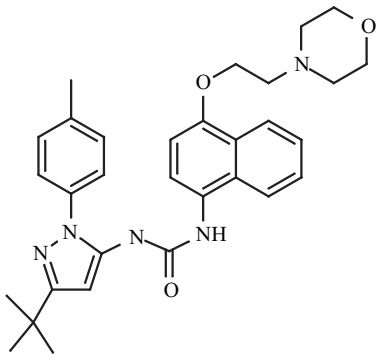
4c. SB242235 (GlaxoSmithKline)



4d. Structure of FR167653



4e. Structure of SB202190



4f. Structure of BIRB796 (Boehringer Ingelheim)

Fig. (4). p38 kinase inhibitors.

specific mode of binding to the p38 kinase active site [79]. VX-745 was reported to be active against all p38 kinase isotypes [80] and was associated with suppressed release of inflammatory mediators, including IL-1β, IL-6 and TNF-α [81]. Because of this inhibitory profile, VX-745 was employed in experimental animal studies to suppress inflammation [82], but these studies were discontinued because of serious neurological side effects in animals given high doses of the compound [83]. VX-702, another p38 kinase-specific inhibitor was shown to inhibit p38 kinase activation by human platelets treated with thrombin, PAR1 and PAR4 thrombin receptors, thromboxane A2 and collagen, but did not affect thromboxane A2 production [84]. Indeed, preliminary phase IIa clinical trial data released by Vertex Pharmaceuticals on October 18, 2004 showed that VX-702 demonstrated tolerability and reduced C-reactive protein levels when administered orally to patients with cardiovascular disease. A phase II clinical trial is also underway to investigate the safety, tolerability and clinical activity of VX-702 in RA. Recently, a novel p38 kinase inhibitor, TAK-715 (Takeda Pharmaceuticals) was developed to inhibit p38 kinase but also structured to maintain cytochrome P450 activity. TAK-715 showed acceptable bioavailability when administered orally to mice and rats as well as showing significant efficacy in the rat AIA model of arthritis [85].

INHIBITION OF ERKS

ERK Activation Cascade

ERK activation initiated by growth factors such as epidermal growth factor (EGF) is the most well studied of all the SAPK/MAPK activation cascades [2, 4, 5]. In the first step of the ERK activation cascade, EGF binds to its tyrosine kinase receptor resulting in the recruitment of the adapter proteins, Grb2 and Son-of-sevenless (Sos) to the plasma membrane [86]. In turn, Sos enhances GDP release activating Ras via a GTP for GDP exchange so that Ras-GTP binds to Raf which then activates the Raf serine/threonine kinase. Raf kinase phosphorylates one of several potential dual specificity MKKs (usually MEK1) activating ERK by phosphorylation of either a serine or tyrosine residue resulting in MEK1-ERK dissociation [87]. Monophosphorylated ERK then re-interacts with MEK1, so that activated MEK1 phosphorylates ERK1/2 on threonine[183] and tyrosine[185] to complete ERK1/2 activation. Major protein targets of ERK include pp90 ribosomal S6 kinase and cytoplasmic phospholipase A2 as well as nuclear transcription factors such as Elk-1 [88]. Dual phosphorylated ERK is a more potent promoter activator than non-phosphorylated ERK [1]. ERK-3, -5 and -7 phosphorylate separate target proteins distinct from those phosphorylated by ERK1/2 [89].

ERK INHIBITORS

PD98059 - 2'amino-3'-methoxyflavone

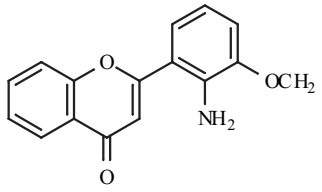
U0126 - 1, 4-diamino-2, 3-dicyano-1, 4,-bis (2-aminophenylthio) butadiene

SL327 - α-[amino[(4-aminophenyl) thio] methylene]-2-(trifluromethyl) benzeneacetonitrile

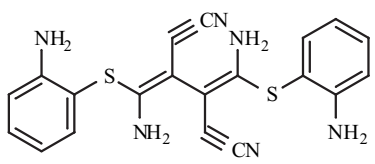
Arctigenin - [(3R, 4R)-4-[3, 4-dimethoxyphenyl) methyl] dihydromethoxyphenol) methyl-2-(3H)- furanone]

The development of ERK inhibitors was predictable upon completion of the elucidation of the upstream ERK activation cascade. Thus, development of ERK inhibitors focused on blunting MKK activities which activate ERKs. In this regard, PD98059 (Fig. **5a**) is a potent MEK1 inhibitor that does not inhibit the ERKs [90]. Alessi *et al.* [91] were among the first to show that the mechanism of action of PD98059 involves inhibiting (IC_{50}, 2-7 μM) MAPKK1activation by the oncogenic protein, raf or MEK1. Furthermore, while PD98059 enhanced c-raf basal levels, PD98059 did not inhibit raf-activated MAPKK1 itself in response to platelet-derived growth factor, serum and phorbol esters in Swiss 3T3 fibroblasts indicating that PD98059 binds to the inactive form of MAPKK1.

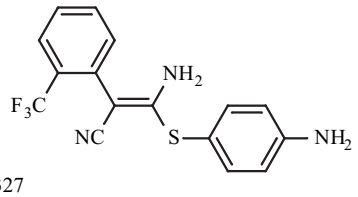
PD98059 inhibition of MEK1 and downstream ERK1 also resulted in suppressed cyclin D1 mRNA in platelet-derived growth factor-stimulated Chinese hamster embryo fibroblasts [92]. Suppression of a sustained ERK1 response by PD98059 was required for progression through the G_1 phase of the cell cycle [88]. Favata *et al.* [93] showed that U0126 (Fig. **5b**) was a non-competitive selective inhibitor of the MEK1, -2 substrates ATP and ERK with virtually no activity towards Raf, MEKK, ERK, MKK-3, -4 and -6 and



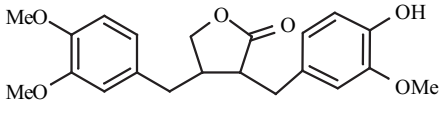
5a PD98059



5b U0126



5c SL327



5d Arctigenin

Fig. (5). Structure of ERK inhibitors.

in that respect had a similar inhibition profile to that of PD98059 [91]. Furthermore, PD98059 and U0126 both suppressed ras-transformed cell growth in soft agar, but neither altered the growth of normal cells.

SL327 (Fig. **5c**) is also an effective MEK1,-2 inhibitor with IC_{50} values of 180nM and 220nM, respectively. SL327 is capable of penetrating the blood brain barrier and has been employed in studies looking at the role of ERKs in mammalian learning. SL327 administered intraperitoneally to mice inhibited hippocampal ERK1/2 and blocked contextual fear conditioning and attenuated cue learning [94]. Finally, Arctigenin (Fig. **5d**), a phenylpropanoid dibenzylbutyrolactone lignan MKK1 inhibitor with anti-oxidant and anti-inflammatory properties was shown to inhibit ERK1/2 (IC_{50}, 1nM) and iNOS gene expression induced by LPS in Raw264.7 cells [95]. Arctigenin also suppressed NF-κB as well as AP-1 activity and blocked TNF-α mRNA expression. However, Arctigenin also inhibited p38 and JNK suggesting that MKK1 inhibition also blunts p38 and JNK activities in addition to ERK1/2 in these cells.

Suppression of ERK activity by upstream MKK inhibition was studied in animal models of inflammation. Inhibition of the ERK activation cascade by PD98059 reduced ovalbumin-induced peptidoleukotriene levels in guinea pigs but did not directly inhibit 5-lipoxygenase. Leukotriene inhibition was linked to relaxation of anaphylactic bronchial contraction [96].

PD98059 weakly inhibited histamine, IL-4 and IL-13 in human basophils *ex vivo* following immunoglobulin E (IgE) receptor stimulation but effectively suppressed leukotriene C4, ERK1, -2 activation as well as phosphatidyl-inositol-3-kinase (PI 3-kinase) [97]. These results indicated that PI 3-kinase activation which regulates apoptosis, proliferation and cytoskeletal reorganization/adhesion was ERK-dependent [97].

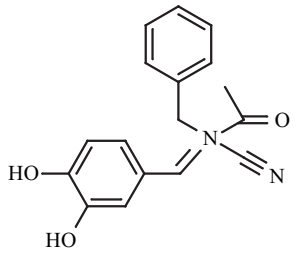


Fig. (6). Structure of JAK inhibitor, AG490.

PI 3-kinase operates downstream of tyrosine receptor-mediated proliferation and ERK activation. Of note, the PI 3-kinase inhibitors, wortmannin or LY294002 mimicked another PI 3-kinase inhibitor, namely imatinib (an ATP binding site inhibitor) by suppressing chronic myeloid leukemia (CML) progenitor cells proliferation *in vitro*, but AG490 [N-benzyl-3, 4-dihydroxy-benzylidenecyano-acetamide; Tyrphostin] (Fig. **6**), a Janus kinase (JAK) inhibitor did not alter CML cell proliferation [98]. The JAK/signal transducer and activator of transcription (STAT) pathway has been implicated in EGF receptor autophosphorylation [99] and cyclin (i.e. cdk2) activation [100]. Recently, AG490 was shown to block JAK2 activity and ablate rat astrocyte death after hydrogen peroxide, IL-6 or IFN-γ stimulation [101]. AG490 also blocked JAK3 activity and human T-cell activation by suppressing IL-2 and IFN-γ production which were stimulated by alloantigen or with cell differentiation protein-3 (CD3) or CD28 suggesting that AG490 may have potential benefit for inducing transplantation tolerance [102].

PD98059 has also been tested in animal models of pain and arthritis. PD98059 (10 μg) administered by intrathecal injection transiently blocked spinal nerve ligation-induced mechanical allodynia in dorsal horn neurons, suggesting that ERK activation in astrocytes contributed to neuropathic pain [103]. PD98059 also reduced nocioceptive pain characteristic of mono-articular arthritis induced by complete Freund's adjuvant (CFA) in rats [104]. Although U0126 did not alter basal pain sensitivity induced by CFA injected into the rat hindpaw, U0126 was non-toxic and suppressed the initiation of, and sustained heat and mechanical hypersensitivity components of CFA-induced inflammation [105], possibly by altering transcriptional activation of the dorsal neuron protein, prodynorphin and the substance P receptor protein, neurokinin-1. Of note, Pelletier *et al.* [106] showed that a selective MEK1/2 inhibitor, PD198306, partially suppressed cartilage degradation in the anterior cruciate ligament transection model of osteoarthritis in the rabbit which was associated, in part, with lower MMP-1 (collagenase-1) levels.

CONCLUSIONS

The capacity of certain SAPK/MAPK small molecular weight inhibitors to directly blunt PK activity *in vitro*, (especially those that block upstream MKK activity such as PD98056 and U0126) must be interpreted with caution [107]. Although PD98056 and U0126 contain two or more aromatic rings they do not otherwise share any structural similarities. Both PD98056 and U0126 increase cellular ADP to ATP and AMP to ATP ratios [108] by activating AMP-activating kinase in human embryonic kidney (HEK) cells expressing the upstream kinase, LKB1, required for AMP-activating kinase activity. In addition, previous results obtained with pan-p38 or pan-JNK inhibitors should be re-performed now that p38 isoform-specific inhibitors and the 1, 9 pyrazoloanthrone JNK inhibitor, SP600125 (i.e. JNKI2) with as much as a 300-fold increase in specificity for JNK-2, and -3 compared to ERK1 or p38 have been developed [21, 74, 109, 110]. Even with these caveats, structural substitutions in small molecular weight SAPK/MAPK inhibitors have improved their oral bioavailability and biodisposition making them more amenable for testing in animal models of inflammation, arthritis, heart failure, stroke and cancer and metastasis as well as in neurological degeneration. The ability of SAPK/MAPK inhibitors to effectively reach target tissues and further dampen inflammatory responses activated by cytokines [111] remains to be established in clinical trials especially in light of the available biological drugs such as the TNF-α monoclonal antibodies which have proved efficacious in treating RA and psoriatic arthritis as well as Crohn's disease. The extent to which these SAPK/MAPK inhibitors will be efficacious in treating neurological diseases also remains to be determined, but preliminary clinical trials with some of these inhibitors

have shown that they are relatively non-toxic and well-tolerated.

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