Mitogen Activated Protein (MAP) Kinases: Development of ATP and Non-ATP Dependent Inhibitors

Chad N. Hancock[2], Alba T. Macias[1], Alexander D. MacKerell, Jr.[*] and Paul Shapiro[*]

[1]*Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy,* [2]*Molecular and Cell Biology Program, University of Maryland, Baltimore, MD 21201, USA*

Abstract: Extracellular signals regulate most of the body's physiological functions through the MAP kinase signaling pathways. These MAP kinase signaling pathways are normally under tight regulation such that activation and inactivation occurs only when needed. However, aberrant regulation observed with naturally occurring mutations in specific signaling proteins often results in constitutive activation of the MAP kinases and is involved in several pathophysiological conditions, such as cancer, neurodegeneration, and inflammation. As such, much effort has been expended to develop inhibitory molecules of the MAP kinase signaling pathways. Several compounds have been identified that inhibit MAP kinase signaling by targeting receptors or other proteins upstream of the MAP kinases. The development of specific inhibitors of the MAP kinases themselves has been less successful and only a few compounds, which interfere with ATP binding, have been identified. A common problem with kinase inhibitors that compete with ATP binding is their lack of specificity. Thus, alternative approaches to inhibit MAP kinase function are being sought. The MAP kinase proteins contain docking domains that direct the interactions with a variety of substrate proteins. Using the 3-dimensional structure of MAP kinases and computer modeling, molecules that target specific docking domains and selectively disrupt substrate interactions are being developed. This non-ATP interfering approach may allow the selective inhibition of MAP kinase substrates involved in disease processes while preserving MAP kinase functions associated with normal cells.

Key Words: Mitogen activated protein kinase, extracellular signal-regulated kinase, signal transduction, computer-aided drug design, database screening.

I. INTRODUCTION

The mitogen activated protein (MAP) kinases play a central role in the regulation of most biological processes including cell growth, proliferation, differentiation, inflammatory responses and programmed cell death. The three major MAP kinase signaling proteins consist of the extracellular signal-regulated kinases (ERK), c-Jun N-termnal (JNK), and p38 MAP kinases. Unregulated activation of the MAP kinases is involved in inflammatory diseases, neurodegeneration, and cancer cell proliferation. Thus, there is much interest in identifying and developing novel and specific MAP kinase inhibitors that may find utility as chemotherapy, anti-inflammatory, or anti-neuro-degenerative agents. The MAP kinases, and their regulation and function in disease, are extensively studied; a search of PubMed using the keyword 'MAP kinases' yields over 16,000 references! Yet, despite the wealth of data on the function and regulation of MAP kinases, only inhibitors of the p38 MAP kinases have been developed and show promise as research tools and in the clinic. To date, there are no specific inhibitors of the ERK or JNK MAP kinases.

The common approach to developing a kinase inhibitor is to target the ATP binding site. While this is effective, there are associated problems with a lack of specificity. Moreover, MAP kinases mediate a wide array of diversity of normal and pathological biological functions by targeting dozens of protein substrates. Thus, it might be advantageous to prevent MAP kinase regulation of substrates involved in disease processes while maintaining the regulation of substrates involved in normal function. This review will highlight some of the regulatory and functional aspects of the MAP kinase signaling pathways and then discuss the current status of some MAP kinase inhibitors. Finally, new approaches that use computer modeling to identify low molecular weight molecules that target unique MAP kinase substrate binding domains but do not interfere with ATP binding will be discussed.

A. The MAP Kinase Signaling Modules

The MAP kinase signaling pathways share several common features involved in their regulation. The MAP kinase signaling pathways are organized into kinase signaling modules that function to transmit extracellular signals into a regulated cellular response [1]. In most cases, binding of an extracellular ligand to its receptor on the plasma membrane, through a variety of adaptor and G-proteins, leads to the sequential activation of the kinase signaling module (Fig. **1**). The MAP kinase kinase kinases (MKKKs) phosphorylate and activate the MAP kinase kinases (MKKs), which in turn phosphorylate and activate the MAP kinases themselves. MAP kinase proteins are proline-directed kinases and phosphorylate serine or threonine residues within an S/TP motif on dozens of cytoplasmic and nuclear proteins [1].

B. The Extracellular Signal-Regulated Kinase (ERK) MAP Kinases

The ERK1 and ERK2 isoforms are activated by a variety of growth factors, cytokines and stress signals that affect cell

*Address correspondence to this author at the Department of Pharmaceutical Sciences, University of Maryland, Baltimore, MD 21201, USA Tel: 410-706-8522 (PS), 410-706-7442 (AM); Fax: 410-706-0346; Email: amackere@rx.umaryland.edu, pshapiro@rx.umaryland.edu

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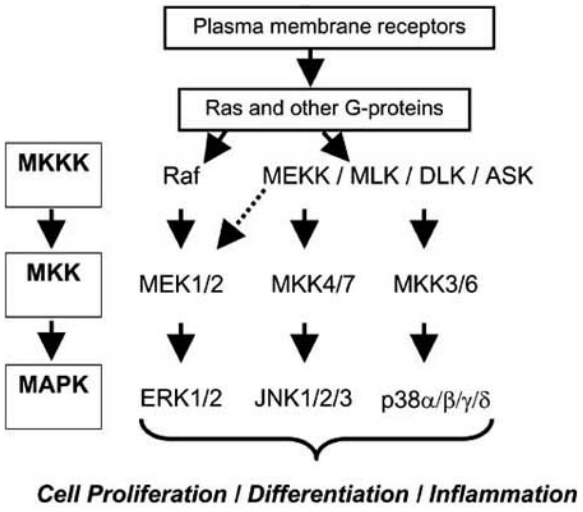

Fig. (1). Overview of MAP Kinase Signaling Pathways.

MAP kinase signaling pathways. The three major MAP kinases signaling proteins (ERK, JNK, and p38) are regulated by receptor activation and G-proteins through a three kinase cascade. The MAP kinases phosphorylate and regulate dozens of substrates involved in most physiological responses, including cell proliferation, differentiation, and inflammation. The solid lines represent well-documented regulatory mechanisms. The dotted line represents potential regulation by upstream kinases.

growth, proliferation, and differentiation [2]. This MAP kinase pathway is often activated by growth factor receptors with intrinsic tyrosine kinase activity (receptor tyrosine kinase or RTK). Growth factor ligand binding to the extra-cellular domain results in receptor dimerization, activation of tyrosine kinase activity, and receptor autophosphorylation. RTK autophosphorylation promotes the recruitment of adaptor proteins containing SH2-domains. This in turn leads to activation of the Ras G-protein, which activates Raf kinases and MAP or ERK kinases 1 and 2 (MEK 1/2) and ERK 1/2. ERK proteins may phosphorylate and regulate the function of more than 50 different substrate proteins in both the cytoplasm and nucleus [2].

As a key regulator of cell proliferation, the ERK pathway contains several proto-oncogenes involved in tumor formation. Oncogenic mutations in Ras G-proteins have been identified in approximately 30% of all human cancers [3-5], and oncogenically mutated B-Raf has been found in greater than 60% of all malignant melanomas and in lower frequencies in other cancers [6]. Receptor tyrosine kinases that regulate ERK activity and nuclear transcription factors targeted by the ERK pathway have also been identified as oncogenes that contribute to uncontrolled cell proliferation of cancer cells [7-12]. Thus, specific inhibition of proteins in the ERK pathway is an important goal. Several reviews that will not be discussed give a nice description on clinically relevant inhibitors of receptor tyrosine kinases (Herceptin [13], Iressa [14]), Ras (farnesyl transferase inhibitors) [15], or Raf (BAY43-9006) [16, 17].

C. The p38 MAP Kinases

There are four p38 MAP kinase isoforms that have been identified in mammals (α, β, γ, and δ). P38 MAP kinase is activated by MKK3 and MKK6, and these are in turn activated by several kinases including, MKKK1-4, Mixed Lineage Kinases (MLK), Apoptosis Signal-regulating Kinase (ASK), and Dual Leucine Zipper-Bearing Kinase (DLK) [18]. The p38 MAP kinase pathway is often activated in response to environmental stresses such as osmotic shock and ionizing radiation, as well as by inflammatory cytokines such as Tumor Necrosis Factor-α (TNFα) and several interleukins (IL). The p38 MAP kinase signaling pathway also regulates the expression and production of TNFα and IL-1. These cytokines act to promote inflammation in autoimmune disorders, and their inhibition has been shown to alleviate the symptoms of chronic inflammation [19]. IL-1 and TNFα have an additive effect on tissue destruction, and together with IL-6 are responsible for the chronic inflammation seen in rheumatoid arthritis [20, 21]. IL-1 and TNFα increase the expression of adhesion molecules and increase neutrophil recruitment into joints, which release proteases that degrade collagen [22, 23]. These are, in brief, some of the reasons that extensive efforts for developing new treatments for chronic inflammation have focused on p38 MAP kinase signaling.

D. The c-Jun N-terminal Kinase (JNK) MAP Kinases

The JNK MAP kinase proteins consist of 3 gene products and a total of ten splicing variants, each of which has different substrate affinity *in vitro* [24]. Whereas JNK1 and 2 have a broad tissue distribution, JNK3 is expressed primarily in the brain and testes [25]. Thus, depending on the stimulus and cellular environment, JNK activity can contribute to cell survival, apoptosis, cell proliferation, or differentiation [26]. JNK proteins are activated by MKK4 and MKK7, which are in turn activated by a number of MKKK proteins as was the case with the p38 MAP kinases. Thus, stimuli that activate p38 MAP kinase signaling often will activate JNK signaling.

As with the p38 MAP kinases, JNK has been implicated in chronic inflammatory diseases. Similar to p38, expression of TNF-α and interleukins are regulated by the JNK MAP kinase pathway [27]. JNK activity may also be involved in the pathology of neurodegenerative diseases such as Alzheimers, Parkinsons, and Amyotrophic Lateral Sclerosis (ALS, or Lou Gehrig's disease). JNK3 is specifically expressed in brain tissue, and JNK3 knockout mice show a decrease in the phosphorylation of the JNK substrate, c-Jun, and reduced apoptosis following nerve growth factor (NGF) deprivation [28]. One approach for decreasing JNK activity and neurodegeneration through inhibition of MLK proteins is currently being tested in clinical trials [29].

The role of JNK signaling in cancer formation and progression is not well defined and perhaps controversial. JNK has an established role in promoting apoptosis [30] and may suppress cancer cell metastasis [31-33]. Similarly, loss of JNK3 expression in human brain tumors supports a tumor-suppressor role for this protein [34]. However, others have suggested that constitutive activation of JNK is required for the transformation of certain cell lines [35-37].

Thus, a better understanding of JNK pathway regulation and function in the context of cell proliferation is needed to establish the legitimacy of JNK as a target for the treatment of cancer.

II. CHALLENGES OF DEVELOPING MAP KINASE INHIBITORS

Significant efforts have been made to identify selective inhibitors of the MAP kinase pathways for the treatment of chronic inflammation, neurodegeneration, and cancer cell proliferation. An extensive list of inhibitors has been developed to target receptor proteins and signaling proteins upstream of MAP kinases. A summary of all of these compounds is beyond the scope of this review. For excellent reviews of these compounds, see Boldt and Kolch, 2004 [38] and Jackson and Bullington, 2002 [39]. The focus of this part of the review will be on the development of inhibitors that directly target MAP kinases. The first part of this section will summarize the general approach for identifying inhibitor compounds and the associated pitfalls. The middle section will highlight compounds designed to interfere with ATP binding on MAP kinases. Finally, the last section will cover new approaches for developing MAP kinases inhibitors that do not interfere with ATP binding.

A study into the history revealed two approaches to the identification of MAP kinase inhibitors. The first is the screening of large chemical libraries to assess inhibition of the activation of certain substrates, followed by modification and refinement of lead compounds to reach greater inhibition in both *in vitro* and *in vivo* models [39]. This approach has the advantage of identifying potentially specific compounds with a desired effect from a large pool of lead compounds. However, this approach has usually placed the understanding of mechanistic information about the compound as secondary. As crystal structures of the MAP kinases were reported, and computer modeling of protein-inhibitor interactions became possible [40], a second approach became available in which compounds were designed to bind to specific regions on the MAP kinases, these being either the ATP-binding domain or non-catalytic substrate binding domains [41-43]. This approach allows for specific, directed modifications to be made to the lead compounds based on the modeling information. This, in combination with testing in biological assays, may produce highly specific compounds with better information on mechanism of action. These two approaches are by no means mutually exclusive, and recent reports have used both lead compound screening and detailed molecular modeling to identify MAP kinase inhibitors [41-44].

The vast majority of MAP kinase inhibitors identified to-date bind to the ATP-binding pocket. Kinase activity is inhibited through competition of the compound with ATP and the inhibition of phosphoryl transfer to the substrate protein. While inhibition of ATP is certainly effective, one potential problem with this approach is that these inhibitors must compete with relatively high concentrations of ATP found *in vivo*, thereby increasing the inhibitor's IC_{50}. In addition, because the ATP-binding domain is a necessary and relatively well-conserved region among many kinases, questions have been raised regarding the specificity achievable by targeting this domain [45-47]. The potential problems associated with targeting ATP binding sites underscores the importance of assaying a broad range kinase families when testing for compound specificity. To overcome the potential limitations of targeting ATP binding, recent efforts to design inhibitors of the MAP kinases have attempted to enhance compound specificity by focusing on non-ATP binding domains.

A. Inhibitors of p38 MAP Kinase

SB203580/SB202190

Pyridinlyl imidazole compounds were the first to be identified that potently inhibited p38 MAP kinases [39, 48]. SB203580 and SB202190 are ATP-competitive inhibitors of p38α and p38β, but not p38γ or p38δ [49]. Both compounds have been shown to inhibit inflammatory responses in animal models. SB203580 inhibited inflammatory cytokine production in a lipopolysaccharide (LPS)-induced model of inflammation in both mice and rats, with an IC_{50} of 15-25 mg/kg, reduced mouse mortality in lipopolysaccharide (LPS)-induced shock, and inhibited paw inflammation in a collagen-induced arthritis model at 50 mg/kg [50]. SB203580 has also been shown to block IL-1-stimulated p38 activation and subsequent induction of inducible nitric oxide synthase (iNOS) and NO production [51]. A structurally related compound, SB220025 has also been shown to block LPS-induced TNFα production with an ED_{50} of 7.5 mg/kg, and suppressed progression of collagen-induced arthritis in the mouse model [52]. SB203580, being the best characterized of the p38 inhibitors, will be the focus in this section. Additional information on the pyridinyl imidazole-based inhibitors is available elsewhere [39].

The mechanism of SB203580 and related pyridinyl imidazoles binding to p38 MAP kinase has been characterized. Initial reports identified 14 conserved and non-conserved residues in the ATP binding pocket of p38 MAP kinase that could participate in binding the structurally similar compound VK-19911 [53]. Subsequent mutational analysis determined which of the non-conserved residues played a role in the specificity of the pyridinyl imidazoles for p38α and p38β [54]. The Thr106 in the ATP binding pocket was shown to be critical for the binding of di- and tri-arylimidazole as well as triarylpyrrole inhibitors of p38α. Mutation of this residue to glutamine or methionine, the equivalent amino acids in ERK and JNK MAP kinase, respectively, rendered p38α insensitive to SB203580 but did not affect its kinase activity [54]. In fact, the Thr at position 106 on p38 MAP kinase has been shown to be sufficient for binding of SB203580 [55]. As such, replacement of Met106 in JNK1 with a threonine conferred sensitivity to SB203580. The necessity of Thr106 for binding also explains the insensitivity of p38γ and p38δ to SB203580, as they have a methionine at this position [56]. As with JNK, replacement of Met106 with threonine in p38γ and p38δ confers sensitivity to SB203580.

It was further determined that the size of the side chain of residue 106 played a substantial role in binding, with smaller side chains (serine, alanine, and glycine) greatly enhancing sensitivity, while sidechains larger than threonine decreased

the levels of inhibition [55]. Additional amino acids may also participate in the binding of SB203580 and related compounds. For example, Met109, Thr157, and the conserved residues Lys53 and Asp168 in the ATP-binding pocket may all be involved in the binding of pyridinyl imidazole inhibitors [57].

While most kinases have a large hydrophobic residue at the position equivalent to Thr106 of the ATP-binding pocket, a number have been identified that also have a threonine at this site. These kinases, including LCK, GSK3β, and PKBα, have been shown to also be sensitive to SB203580, although at somewhat higher concentrations than what is needed to inhibit p38 MAP kinase [45]. This raises the questions of SB203580 non-specificity through the inhibition of additional unrelated kinases.

BIRB 796

While less clinical data is available as compared to the pyridinlyl imidazoles, the binding mechanism of the ATP-competitive p38 inhibitor BIRB 796 has also been determined (Fig. **2**). BIRB 796 was identified as a candidate for clinical trials from a series of N-pyrazole, N'-aryl urea compounds [41]. Chemical modifications were made to one lead compound, which was identified to have moderate binding to p38 MAP kinase and the ability to inhibit TNFα production. Three structural modifications in the lead compound were found to increase inhibitor activity. Replacement of the methyl group on the pyrazole ring with a tolyl group, of the chlorophenyl group with a naphthyl moiety, or introduction of an ethoxymorpholine on the napthyl ring resulted in ~140, 15, or 11 fold increases in p38 MAP kinase inhibition, respectively.

The mechanism through which BIRB 796 inhibits p38 MAP kinase was identified using X-ray crystallography data of p38 MAP kinase in complex with the inhibitory compound. BIRB 796 caused a shift in the residues of Asp168-Phe169-Gly170 of the activation loop, moving the phenol ring of Phe169 10 angstroms away from a hydrophobic binding pocket. This unique conformation allowed for insertion of the tert-butyl group on carbon 5 into the hydrophobic pocket. Thus while this compound binds in a position adjacent to the ATP-binding pocket, this formation stabilizes a conformation of p38 MAP kinase that is unable to bind to ATP [42]. The increase in inhibition by the addition of the ethoxymorpholine was due to hydrogen bonding between this group and Met109. The formation of this bond prevents interactions between this residue and the adenine base of ATP.

BIRB 796 was found to inhibit TNFα production in cell culture with an IC_{50} of 18 nM and, in animal models, inhibit LPS-induced TNFα production and collagen-induced arthritis severity [42]. As with SB203580, BIRB 796 has also been found to inhibit a number of kinases from unrelated kinase families, including tyrosine kinases and receptor tyrosine kinases [47]. Although these additional kinases were less sensitive to BIRB 796 than p38 MAP kinase, this again raises the question of non-specificity with competitors of ATP.

B. Inhibitors of JNK MAP Kinase

SP600125

A number of attempts have been made to develop inhibitors of JNK MAP kinase [58]. However, the lack of specific inhibitors has prevented the full realization of their

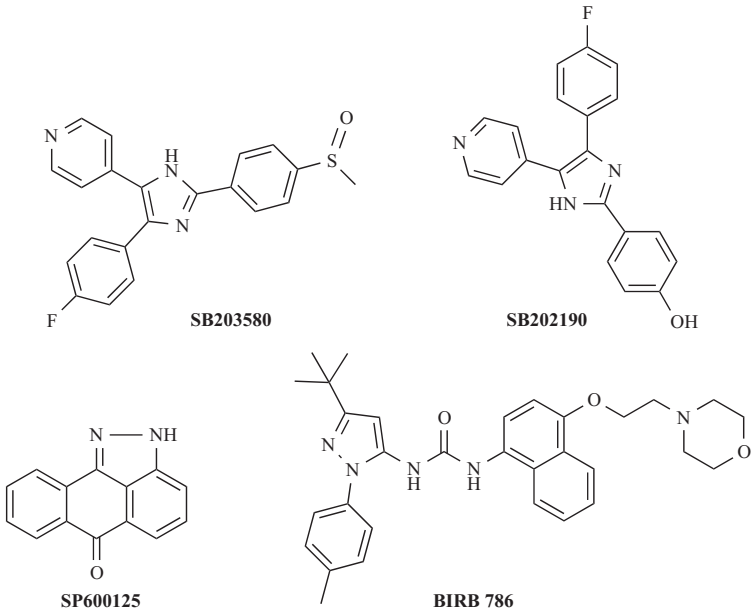

Fig. (2). p38 and JNK MAP Kinase Inhibitors.

Structures of commonly used p38 and JNK MAP kinase inhibitors that target ATP binding. SB203580, SB2020190, and BIRB 796 target p38 MAP kinases. SP600125 is a putative JNK MAP kinase inhibitor.

utilization for research or clinical use. The best described JNK inhibitor, SP600125 (Fig. **2**), was identified from a proprietary series of anthrapyrazolone series of compounds to be ATP-competitive. Initial results showed a 10-fold selectivity for JNK1-3 *in vitro* (Ki = 0.19 μM), but a much higher IC_{50} *in vivo* (5-10 μM) [59].

SP600125 has been shown to inhibit expression of IL-2 and IFN-γ in Jurkat T-cells (IC_{50} = 6 and 7 μM for IL-2 and IFN-γ, respectively), and cyclooxygenase-1 (COX-2) and TNF-α in $CD14^+$ primary human monocytes (IC_{50} = 5 and 10 μM for COX-2 and TNF-α, respectively) [59]. Intravenous or oral administration of SP600125 also inhibited TNF-α production in an LPS-induced inflammation model in mice [59] and reduced joint damage in a rat rheumatoid arthritis model [60].

The 3D structure of SP600125 bound to JNK1 has been subsequently reported [61]. SP600125 is surrounded by hydrophobic residues in the ATP-binding pocket; residues Ile32, Val40, Ala53, Ile86, Met108, Leu110, Val158, and Leu168 all make effective van der Waals contacts. In addition, there are two hydrogen bonds formed between residues Glu109 and Met111 of JNK with the N-atom and NH group at positions 1 and 2, respectively, of SB600125.

Despite efforts to show specificity of SP600125 for JNK but not other kinases [59, 60], recent studies suggest SP600125 is less specific than originally thought [46]. In addition, 13 of 28 other kinases tested were inhibited with equal or greater potency than JNK, including p70 ribosomal S6 kinase (S6K1) cyclin-dependent protein kinase 2 (CDK2), and AMP-activated protein kinase (AMPK) [46]. The increased non-specificity of SP600125 may be due to a higher IC_{50} reported to be 5.8 μM rather than 0.19 μM as originally reported [59].

JNK Interacting Proteins (JIP)

One mechanism for keeping MAP kinase signaling modules organized is through interactions with scaffolding proteins [62]. For JNK MAP kinases, the JIP proteins (JIP1-3) have been identified as scaffolds that interact specifically with the mixed lineage kinase family of kinases (MLK) and with MKK7 [63]. The JIP proteins may therefore act to organize JNK pathway members into modules that can respond to specific stimuli with greater efficiency [64]. The JIP proteins share a conserved region for JNK binding referred to as the JNK binding domain (JBD) [63, 65], and peptides derived from the JBD of JIP1 have been identified as a potential means by which to inhibit the JNK pathway. The importance of this structure is that JIP1 interacts with JNK on a region thought to be involved in substrate interactions, suggesting regions of JNK that are non-ATP binding may be targeted for inhibiting kinase function. This concept will be developed in a later section.

A peptide consisting of residues 143-163 of the JNK binding domain (JBD) of JIP1 was found to inhibit JNK activity towards c-Jun, Elk, and ATF2 *in vitro* [66]. Similarly, over-expression of the JBD of JIP1 prevented apoptosis of pancreatic β-cell lines [67]. A variety of studies support a role for JIP1 inhibition of JNK activation in protecting cells from apoptosis [68, 69]. In the context of Parkinsons disease, over-expression of the JBD sequence provided protection from apoptosis in animal models and further lends support for targeting JNK in neurodegenerative disorders [68,70].

The structure of the 11 C-terminal amino acids of the JBD of JIP1 complexed with JNK1 has subsequently been reported, and the significance of these residues has been confirmed [61]. The van der Waals contacts of Pro157, Leu160, and Leu162 as well as a hydrogen bonding between Arg156 of JIP and Glu329 of JNK1 were identified as necessary for the selective binding of JIP [61, 66]. Glu329 is within the common docking (CD) domain of the JNK kinases [71] and was cited as one structural determinate of the specificity of JIP for JNK. A second structural factor was the α2 helix of JNK, which contains additional residues critical for JIP binding. This is a 1 turn helix in JNK but is a two turn helix in p38 MAP kinase, suggesting that this region may be important in determining substrate selectivity for the different MAP kinases [61].

While the JIP1 peptides are not low molecular weight inhibitors amenable to development into therapeutic agents, the structural interactions between JNK and JIP1 illustrate how alternative approaches for developing inhibitors of the MAP kinase proteins can be exploited.

III. MAP KINASE INHIBITION OF NON-ATP BINDING DOMAINS.

A. Docking Domains on MAP Kinase Substrates

In the development of inhibitors of non-ATP binding regions, it is important to examine what is known about the interactions between MAP kinases and their substrates. As mentioned, the MAP kinases are proline-directed serine/threonine kinases [2]. In addition, substrate phosphorylation is directed by the interactions of unique sequence motifs on the MAP kinases and on the substrate proteins. Substrate specific regions include the D-domain, or D-Box, which is a sequence conserved in the ETS family of transcription factor substrates of ERK proteins [72-74]. This domain consists of a Leu-X-Leu sequence C-terminal to a cluster of basic arginine or lysine residues [75]. Similar sequences are found in MEK proteins [76, 77], ribosomal S6 kinase (Rsk-1) [78], and several phosphatases that regulate MAP kinases [79-81]. A similar sequence is found on JIP1 and the transcription factor c-Jun and is thought to direct interactions with JNK [72, 73, 82, 83]. The D-domain is believed to direct interactions with substrates and enhance phosphorylation by MAP kinases on consensus phosphorylation sites in their target proteins [72, 73, 84].

Another motif defined by the amino acid sequence Phe-X-Phe-Pro has been identified in a variety of ERK substrates such as transcription factors, putative scaffolding proteins, and phosphatases, and also has been shown to direct substrate interactions with and phosphorylation by ERK proteins [84]. The phenylalanines in this sequence appear to be critical for promoting phosphorylation by ERK. Mutations within this sequence in Elk1 reduces binding affinity with ERK by 3-fold. The addition of a Phe-X-Phe-Pro sequence into peptide substrate is sufficient to direct ERK phosphorylation. The Phe-X-Phe-Pro sequence and D-domain have

also been shown to act cooperatively to direct phosphorylation of substrates by ERK [85]. Mutation of the D-box in the transcription factor Elk1 reduced binding to ERK by 14-fold, whereas mutations of both the Phe-X-Phe-Pro sequence and D-box resulted in a 50-fold reduction in binding affinity. Thus, specific peptide motifs on substrate proteins are important for interactions with MAP kinases and efficient phosphorylation events.

B. Docking Domains on MAP Kinases

In addition to docking domains on the substrate proteins, docking domains on MAP kinases themselves direct substrate-specific interactions. An understanding of these docking regions will be important for the development of substrate specific inhibitors. As mentioned previously, one substrate binding domain on the MAP kinases is the common docking or CD domain. This consists of the C-terminal residues Asp316 and Asp319 in ERK2, Asp313, Asp315, and Asp316 of p38α, and Asp326 and Glu331 of JNK1 and 2. These negatively charged residues interact with clusters of positively charged lysine/arginine residues on the interacting protein [71, 86]. The CD domain of ERK2 is a common binding site for MKK1, the MAP kinase-activated protein kinase (MAPKAPK) MNK-1, and MAP kinase phosphatase 3 (MKP-3) (Fig. **3**). Mutation of D316 and D319 to asparagines greatly inhibited ERK interactions with these proteins, as did mutations of the lysines and arginines of the docking site in the interacting substrate proteins [86]. Additional mutational analysis showed that altering the aspartates of the CD domains inhibited binding of MKK6, MKP5, and MNK1 with p38, and MKK4 and MKP5 with JNK. These data indicated that all of the major MAP Kinase family members use a common docking region for upstream activators, downstream kinases, and inactivating phosphatases [86].

Fig. (3). The CD and ED docking Domains on ERK2.

Ribbon diagram of the 3D structure of the unphosphorylated form of ERK2 showing the spatial relationship of the ERK2 phosphorylation sites and the docking region. Phosphorylation residues Thr183 and Tyr185 are red spheres, common docking (CD) residues Asp316 and Asp319 are blue spheres, and the ED residues Thr157 and Thr158 are green spheres. The putative docking groove is located between the CD and ED residues.

Another docking domain close to the CD domain in ERK2 and p38MAP kinase has been identified (Fig. **3**). This domain, consisting of Thr157 and Thr158 in ERK2 and Glu160 and Asp161 in p38 MAP kinase, is referred to as the ED domain [87] and directs ERK2 and p38 MAP kinase specificity towards the MAPKAPK substrate. Mutations of Thr157 and Thr158 of ERK2 to the ED sequence of p38 MAP kinase converted the substrate specificity of ERK2 to that of p38 MAP kinase substrates. However, mutations of the CD and ED domains of p38 MAP kinase to the sequence found in ERK2 did not change the substrate specificity, indicating that additional amino acids are involved in the recognition of these substrates [87]. The region between the CD and ED domains forms a groove in the steric structure of ERK2 and p38 MAP kinases. Zhang, *et al*. have subsequently used site-directed mutagenesis to map the docking interactions of ERK2 with MKP3 and Elk1 substrates and found that while both utilize a number of the same residues, each substrate also had a number of unique contacts with ERK2 [88].

C. Inhibition Based on Disrupting Protein-Protein Interactions

The identification of docking domains on MAP kinases indicates that there are specific regions that direct the interactions with specific substrates. Although the understanding of these docking regions is limited, these sites represent regions that may be targeted for selective inhibition of substrates. Because of the wide range of MAP kinase substrates and the ubiquitous role of MAP kinases in most physiological functions, exploitation of these docking domains may identify unique inhibitor compounds that selectively interfere with MAP kinase regulation of substrates involved in pathological process but not substrates involved in normal metabolism.

Accordingly, we have identified low molecular weight compounds that interact with specific ERK docking domains and selectively disrupt ERK interactions with protein substrates [43]. This approach for inhibiting protein-protein interactions has recently been shown to be feasible in a variety of systems [89, 90]. For example, compounds that block Src homology-2 (SH2) domains inhibit interactions between active tyrosine phosphorylated receptors and SH2 domain containing signaling proteins such as Src, Grb2, and PI3 kinase, which are involved in cancer cell proliferation and survival [91] and with $p56^{lck}$, which is involved in T-cell activation [92]. Another example is the identification of small molecules that bind to Bcl-2 thereby blocking Bcl-2 anti-apoptotic function [93]. Thus, it is feasible to use low-molecular weight compounds to block protein-protein interactions, although such interactions are anticipated to involve extended molecular surfaces that are significantly larger than the inhibitors themselves.

Rational identification of novel compounds targeting a specific site in a protein may be performed *via* computer-aided drug design (CADD) [40]. In target-based CADD the 3D structural information of a putative binding site is analyzed and compounds that structurally complement that binding site are selected from an in silico or virtual chemical database. Structural complementarities may be determined

via a variety of criteria, including best fit, interaction energies, hydrogen bonding, and other criteria such that the compounds are expected to have an enhanced probability of binding to the site on protein. CADD selected compounds are then experimentally screened for binding and regulation of the target protein. CADD database screening methods typically increase hit rates of 0.01% (i.e. 1 in 10,000) or less using only experimental high-throughput screening to 5% or more [40, 94]. A particular advantage of in silico database screening of commercially available compounds over, for example, de novo drug design for identifying new compounds is it eliminates the need for chemical synthesis in the initial phases of the study, thereby greatly reducing the costs and time. Once the CADD selected compounds have been experimentally screened and biolo-gically active compounds identified (i.e. lead compounds), chemical modifications can be systematically introduced into the lead compounds to improve their effectiveness in targeting the protein of interest. Among the many successes of CADD database screening are identification of inhibitors of protein-protein interactions [89, 90], indicating that such an approach is appropriate for blocking ERK interactions with protein substrates.

Essential for the successful application of target-based CADD is the availability of the necessary experimental data on the system, including the three-dimensional structure of the protein and information on residues involved in the binding interaction being targeted. 3D structures of proteins may be obtained from NMR and/or X-ray crystallography experiments; a large number of protein 3D structures have been determined and are available in the protein databank (PDB) [95]; 3D structures may be readily download from the PDB *via* its web site. Alternatively, 3D structures of proteins may be obtained *via* homology with other similar types of proteins, although the quality of such structures is largely dictated by the extent of conservation between the target protein and the protein being used as the basis of the model. Once the 3D structure of the protein is available it is necessary to identify putative binding sites on the protein surface. In the case of a catalytic site, such as the ATP site on kinases, such identification is trivial; however, in the case of protein-protein interactions exact identification of a putative binding site is often more difficult. In such cases computational approaches, such as the SPHGEN program associated with the database screening program DOCK [96] may be used to locate putative binding pockets (e.g. concave surfaces) on the protein surface. Selection from these putative sites is then performed based typically on mutational studies that have identified residues important for a protein-protein or other bimolecular interaction.

D. Design of ERK-Specific Docking Domain Inhibitors

ERK2 represents an ideal system for the application of CADD methods to identify novel inhibitors of protein-protein interactions. For the protein the 3D crystal structures of both the unphosphorylated (inactive) and phosphorylated (active) forms have been solved. A CADD database search of an in silico database of over 800,000 compounds was undertaken targeting a putative binding pocket between the ED and CD docking domains discussed above. The search targeted the unphosphorylated of ERK2 and employed the program DOCK [97, 98] along with the CHAOS suite of programs developed in the MacKerell laboratory [99].

From the initial screen, 20,000 compounds were selected based on the van der Waals (VDW) attractive energy normalized for the size of the compound [100]. The goal of the initial screen is to identify any compound that has an approximate structural complementarity to the target binding site using tractable computer resources. Use of the VDW attractive energy assures that only compounds that have significant shape complementarity to the binding pocket are selected; if the electrostatics were included in the scoring at this stage, compounds with highly favorable electrostatic interactions but potentially not a good fit to the binding site may be selected. In addition, a size based normalization procedure to the scoring is used to avoid the bias of DOCK scoring towards higher molecular weight compounds, allowing compounds with lead-like or drug-like properties [101, 102] to be selected from the screen. The initially selected 20,000 compounds are then subjected to a second screen that includes additional relaxation of the ligand during the docking process. Selection of compounds from the secondary screen was based on the total interaction energy and included size normalization. Use of the total interaction energy for scoring is appropriate as only compounds that sterically complement the binding site are included in the search such that it is desirable to include the electrostatic interactions in the scores. From the secondary screen a total of 500 compounds were selected. 500 compounds are typi-cally still too many compounds for performing experimental assays in timely fashion. Thus, a subset of these compounds with maximal chemical diversity was selected for testing in biological assays. This procedure involved clustering based on chemical fingerprints from which clusters of chemically similar compounds are identified. Individual compounds are then selected for the biological assays from each cluster. This final selection involves consideration of solubility, size and number of hydrogen bond acceptors and donors [103], although the rules are not rigorously followed to insure that all interesting compounds are tested. From this chemical diversity analysis 80 compounds were selected for biological testing.

We have applied the principles outlined above to identify novel low molecular weight chemical entities that bind to ERK2 and specifically inhibit downstream phosphorylation of ERK substrates [104]. Two compounds identified are shown in Fig. (**4**). This successful effort strongly supports the hypothesis that inhibitors of ERK-substrate proteins can be identified *via* a combination of CADD and biological assays. Ongoing studies using the above approaches targeting the active, phosphorylated ERK2 as the target have identified additional compounds with the desired biological profile (P. Shapiro and A.D. MacKerell, Jr. Submitted); further supporting the strength of the CADD based lead compounds identification approach.

E. A p38 MAP kinase inhibitor that targets non-ATP binding sites

A potential p38 MAP kinase inhibitor called CMPD1 may also affect p38 MAP kinase function by targeting the docking domains. Davidson, et. al used a high throughput

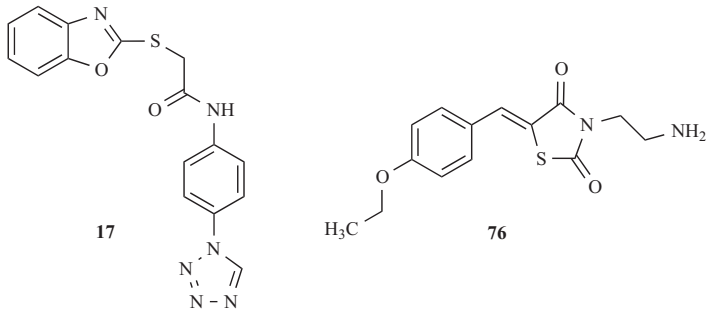

Fig. (4). ERK2 Docking Domain Inhibitors.

Non-ATP dependent ERK2 inhibitors. Compounds 17 and 76 are recently developed ERK docking domain inhibitors that disrupt ERK-mediated phosphorylation of Rsk-1 and Elk-1 but do not affect ERK activation [43].

screen of compounds to identify CMPD1 [44]. CMPD1 was shown to inhibit p38 MAP kinase phosphorylation of MAP kinase-activated protein kinase 2 (MAPKAP2) (Ki = 330 nM). MAPKAP2 is a kinase substrate of p38α and p38β that is required for maximal TNFα and IL-1β production.

CMPD1 was found to be non-competitive with ATP, bound to the inactive and active forms of p38 MAP kinase, and did not reduce the binding affinity of MAPKAP2 for p38α. To provide insight into the mechanism of action, deuterium exchange mass spectroscopy (DXMS) was used to identify regions on p38 that were affected by CMPD1. Increases in deuterium exchange rates indicate regions that are more accessible whereas decreases in exchange rates suggest regions that are protected. Both situations provide information about conformational changes within the protein. The protected regions on p38 MAP kinase included residues 107-115 near the active site, residues 153-163, which include the ED docking domain residues, and residues 165-171, which includes the hydrophobic DFG loop residues [44]. Thus, while CMPD1 binds near the active site, it does not inhibit ATP binding. However, structural changes that occurred near the docking domain regions suggest that CMPD1 may be interfering with substrate interactions. Interestingly, CMPD1 was less effective in preventing p38 MAP kinase phosphorylation of the transcription factor ATF2 (Ki > 20 uM), indicating that CMPD1 may be a substrate-selective inhibitor of p38 MAP kinase [44].

CONCLUSIONS

Selective inhibition of the MAP kinases is a goal for the development of new treatments for a variety of pathophysiological situations including chronic inflammation, neurodegenerative disorders, and cancer. While inhibitors aimed at blocking ATP binding will certainly block MAP kinase activity, they tend to be non-specific and thus have limited clinical use. New approaches aimed at developing inhibitors that target non-ATP binding sites on MAP kinases are being developed. These studies take advantage of the 3D structures of the MAP kinases to identify unique regions or docking domains that may regulate substrate interactions. Computer aided drug design approaches have been used to identify low molecular weight inhibitors that target ERK2 docking domains and disrupt substrate interactions. These findings suggest that it may be possible to design inhibitors of the MAP kinases that prevent activation of specific substrates. This will be furthered realized once additional information on the MAP kinase regions involved in substrate interactions is discovered. The goal of targeting substrate docking domains is to design molecules that specifically disrupt MAP kinase interactions with substrates involved in disease processes while preserving regulation of substrates involved in normal cellular functions.

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