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Computational Insights for the Discovery of Non-ATP Competitive Inhibitors of MAP Kinases

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Abstract

Due to their role in cellular signaling mitogen activated protein (MAP) kinases represent targets of pharmaceutical interest. However, the majority of known MAP kinase inhibitors compete with cellular ATP and target an ATP binding pocket that is highly conserved in the 500 plus representatives of the human protein kinase family. Here we review progress toward the development of non-ATP competitive MAP kinase inhibitors for the extracellular signal regulated kinases (ERK1/2), the c-jun *N*-terminal kinases (JNK1/2/3) and the p38 MAPKs (α , β , γ , and δ). Special emphasis is placed on the role of computational methods in the drug discovery process for MAP kinases. Topics include recent advances in X-ray crystallography theory that improve the MAP kinase structures essential to structure-based drug discovery, the use of molecular dynamics to understand the conformational heterogeneity of the activation loop and inhibitors discovered by virtual screening. The impact of an advanced polarizable force field such as AMOEBA used in conjunction with sophisticated kinetic and thermodynamic simulation methods is also discussed.

INTRODUCTION

Protein kinases are important drug targets due to their critical roles in cellular signaling. A number of protein kinase inhibitors, such as Iressa (gefitinib), Tarceva (erlotinib), Gleevec (imatinib), and Tykerb (lapatinib) are in clinical use for the treatment of cancer [1, 2]. Imatinib, for example, is approved for the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). Each of these inhibitors competes with ATP for binding to the ATP binding site.

In this review we examine progress toward the discovery of drugs that target mitogen activated protein (MAP) kinases, mainly from a computational perspective. MAP kinases

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are serine/threonine-specific protein kinases that are activated in response to extracellular stimuli [3]. Important MAP kinases include extracellular signal regulated kinases (ERK1/2), the c-jun N-terminal kinases (JNK1/2/3) and p38 MAPK α , β , γ , and δ . MAP kinase signaling pathways are critical targets for a number of human diseases including cancer, diabetes, neurodegenerative and inflammatory diseases [3]. Here we focus on the potential for developing non-ATP competitive inhibitors that target sites such as the MAPK recruiting sites [4]. Such ligands have potential to regulate MAPK activities by blocking the ability of MAPKs to bind upstream activating kinases, scaffold proteins or downstream substrates.

A significant advantage of non-ATP competitive ligands over ATP competitive ligands is that they are not in competition with cellular ATP for binding to active sites. In addition, MAP kinase substrate binding sites may offer unique specificity against other families of kinases. On the other hand, it may be more difficult to design potent non-ATP competitive inhibitors due to protein docking sites being largely solvent exposed and relatively shallow [2]. It is also a challenge to identify inhibitors that are selective within the MAPK family. However, we see increasing evidence, including our own unpublished work that suggests that the substrate/upstream protein-docking sites of MAPKs are drugable. We suggest, that detailed computational studies when combined with NMR and X-ray crystallography can furnish insights into the subtle features of MAPK structure and dynamics that will be important for the design of potent non-ATP competitive MAPK inhibitors.

Below we first review computational and experimental studies that have contributed to our current understanding of MAP kinase structure and dynamics. Next, progress toward the design of non-ATP competitive inhibitors is discussed for ERKs, JNKs and p38 MAPKs. Emphasis is placed on the current and future roles of advanced computational modeling methods.

THE STRUCTURE AND DYNAMICS OF MAP KINASES

We begin this section by presenting the conserved features of MAP kinases using the combination of a multiple sequence alignment and a structure of JNK1. Following this introduction, we examine the ability of modern molecular biophysics theory to optimize structural models from X-ray crystallography experiments and discuss implications for the structure-based design of MAPK inhibitors. We then discuss conformational differences between inactive and active MAP kinases based on both structural evidence and molecular dynamics simulations. Finally, non-ATP competitive MAP kinase inhibitors that rely on interactions outside the ATP binding pocket are reviewed.

The Sequence and Structure of MAP Kinases

A multiple sequence alignment of ERK1/2, JNK1/2/3 and p38 MAPK α / β / γ / δ is shown in Figure 1 and serves as a guide to both the conserved and variable regions within the binding sites discussed throughout this review [5]. Although the structure of protein kinase family members has been extensively studied, only a single structure is available for many MAP kinase isoforms. Based on the profile hidden Markov model (HMM) Pkinase/PF00069 defined in Pfam v. 25, there are 1575 protein kinase family structures in the protein data bank (PDB) as of March 2011 [6]. The MAP kinase subset includes structures of ERK1 (1),

ERK2 (16), JNK1 (7), JNK2 (2), JNK3 (23), p38 MAPK α (129), p38 MAPK β (3), p38 MAPK γ (1) and p38 MAPK δ (1). Therefore, more than twice as much structural work has been completed for p38 MAPK α than the other MAP kinases combined (129 *versus* 53).

As a complement to the sequence overview, we introduce conserved structural features of MAP kinases based on the structure of JNK1 (PDB ID 3O17) shown in Figure 2A. As for all MAP kinases, it is composed of two domains. The N-terminal domain has ~135 residues and is made up mainly of β -sheets. The β_{1L0} and β_{2L0} strands of loop 0 (L0) precede a set of five conserved strands of the broader protein kinase family (β_1 to β_5) [7]. The final N-terminal strand β_5 leads into a linker region that connects the N-terminal domain with the C-terminal domain and controls their relative orientation. The C-terminal domain is composed of ~225 residues that mainly form α -helices. The active site of MAP kinases is located between the two domains and is heavily influenced by the conformation of loop 12 (L12), which has also been referred to as the activation loop or phosphorylation lip. The β_8 strand leads into three residues (DFG) whose conformation is critical for enzyme activity. Finally, we note the MAP kinase insert (helices α_{1L14} and α_{1L14}) that is absent in other protein kinases, but has been shown to contribute important interactions with both MAP kinase regulators [8, 9] and substrates [10].

Ewald X-Ray Crystallography Refinement of MAP Kinase Structures

The rationale design of potent and specific MAP kinase inhibitors benefits from precise protein-inhibitor structural models derived from X-ray crystallography diffraction data. The reliability of structure-based virtual screening for the discovery of lead compounds diminishes as the resolution of the X-ray data used to generate the structural model is reduced (~2.5 Å or better may be preferable) [11, 12]. Furthermore, once lead compounds are identified, their further optimization depends in part on understanding the water hydrogen-bonding network in the neighborhood of the MAP kinase-inhibitor binding site [13, 14]. For these reasons, we review recent advances in X-ray crystallography refinement and their impact on MAP kinase structure quality.

Over the last decade polarizable force fields [15, 16] have been developed that are more transferable across chemical environments (i.e. vacuum, liquid and crystalline phases) than fixed charged descriptions of biomolecular energetics traditionally used for X-ray crystallography refinement [17–19]. In addition, the particle-mesh Ewald (PME) method has revolutionized the efficient, rigorous evaluation of periodic, long-range electrostatic interactions [20–23]. For example, the polarizable Atomic Multipole Optimized Energetics for Biomolecular Applications (AMOEBA) force field [24, 25] evaluated using PME [26] has recently been coupled to X-ray crystallography refinement using the program Force Field X [26, 27]. This approach will simply be referred to as Ewald refinement because it represents the first application of PME electrostatics to biomolecular X-ray crystallography refinement. Ewald refinement has been shown to improve R/R_{free} statistics and model geometry for high resolution peptides [28] and proteins [29], the joint refinement of X-ray plus neutron diffraction data sets [30], and low-resolution biomolecular data sets [26].

To illustrate the usefulness of these recent advances in X-ray crystallography theory for structure-based design of MAP kinase inhibitors, Ewald refinement was performed for a

subset of five MAP kinase structures with an emphasis on complexes with non-ATP competitive inhibitors. The mean resolution of the data sets (shown in Table 1) was 2.5 Å and they were originally refined to mean R and R_{free} values of 22.6 and 27.4 respectively. The MolProbity structure validation tool [31, 32] was applied to the deposited structural models, which quantifies van der Waals clashes, side-chain rotamer orientation and polypeptide backbone (ϕ, ψ) conformation. The MolProbity score is calibrated to indicate the expected experimental resolution of the structural model. For the five deposited MAP kinase structures, the mean MolProbity score (2.9) is in the 51st percentile and indicates model quality that is approximately what is expected for experimental data to 2.9 Å (ie. half of the deposited structures at this resolution have better scores and half worse). After Ewald refinement, the mean R and R_{free} values improved to 20.3 and 26.0, respectively. In addition, the mean MolProbity score (1.9) was improved to the 97th percentile and indicates model quality consistent with experimental data collected to 0.6 Å higher resolution than what was actually measured. Consistent with previous findings, Ewald refinement of MAP kinases achieved more physical models that agree more closely with the experimental data.

For example, Ewald refinement changed the JNK1 model shown in Figure 2A by extending energetically favorable secondary structure elements to give the structure shown in Figure 2B while improving the agreement with the measured diffraction data (ie. reducing R_{free} from 30.6 to 27.9). The 3 β -strands and 4 α -helices that were extended are labeled. We expect that improvements seen near the DRS (Φ_{chg}) and the FRS (α_{g} , activation loop) will impact downstream computational methods, such as structure-based virtual screening of non-ATP competitive MAP kinase inhibitors. In a later section, we discuss refinement of the water hydrogen-bonding network around MAP kinase inhibitors.

Conformational Dynamics of MAP Kinases

The conformational dynamics of a MAP kinase is central to processes such as its activation as well as its interactions with ligands and substrates [33, 34]. Many of the dynamic processes associated with MAP kinases are on the micro- to millisecond timescale. While computational and theoretical methods for examining long time dynamics are under active development [35], large-scale conformational changes with timescales of microseconds and beyond are still difficult to capture with atomic detail. Thus, the computational studies of the intrinsic dynamics of protein kinases, including the MAP kinases have been limited. However, some protein kinases including Abl, Src, and CDK have been investigated using targeted MD, biased sampling, String and coarse-grained methods with useful insights [36–40]. For example, molecular dynamics simulations using the String Method with Swarms-of-Trajectories, allowed Roux and colleagues to propose a stepwise mechanism of conformational transitions associated with Src kinase activation where the opening of the activation loop is followed by rotation of the α_{C} helix [39]. Below we describe a number of conformational features of the MAP kinases, which have been described experimentally, but which lack significant detailed computational analysis.

Differences between active and inactive MAP Kinase conformations—A key conformational change of MAP kinases is driven by the phosphorylation of two residues within the activation loop between the conserved DFG and APE motifs (see the multiple

sequence alignment of Figure 1). Upon phosphorylation of the threonine and tyrosine residues of the T-X-Y motif, activation loop reorientation is accompanied by other alterations such as lobe closure, α_C helix rotation, and C-terminal reorganization [33]. It has been suggested that the allosteric coupling of these substructures prepares the kinase for ATP and substrate binding [41]. Structures of both inactive (PDB: 1ERK, Figure 3A) and active (PDB: 2ERK, Figure 3B) forms of ERK2 have been solved, which demonstrates the conformational changes of MAP kinases upon activation [42, 43]. The active structure is dual phosphorylated on the conserved T-X-Y motif ($^{183}\text{T-E-Y}^{185}$ for ERK2) within the phosphorylation lip (L12). The negative charges on the phosphorylated residues favor refolding of the phosphorylation lip via electrostatic interactions with surface arginine-rich binding sites. The phosphorylated tyrosine residue moves ~ 9 Å into the domain interface and contributes to a modest closure of the two domains [42, 43]. The phosphorylated tyrosine comes to the surface via a backbone rotation and forms the proline-directed P+1 specificity site [42, 43]. The amino acid sequence of ERK1 is 90% identical to ERK2 (see Figure 1) and is also a proline-directed Ser/Thr kinase that is activated via dual phosphorylation of the threonine and tyrosine residues of $^{202}\text{T-E-Y}^{204}$. While there are currently 16 structures of ERK2 available, only a single ERK1 data set has been deposited in the PDB; that of the mono-phosphorylated Y^{204} form (PDB: 2ZOQ). This structure demonstrates a novel conformation that is distinguishable from the un-phosphorylated and dual-phosphorylated forms [44]. Despite the similarities between ERK1/2, the specific inhibition of ERK2 is more promising therapeutically because studies of knockout mice have shown that ERK2 can largely compensate for the absence of ERK1 [45, 46].

Conformations associated with autophosphorylation—Protein kinases can be activated via phosphorylation by upstream kinases or through auto-phosphorylation (either through an inter- or an intra-molecular mechanism) [47]. While MAPKs are typically activated by an upstream MAP kinase kinase (MKK, MEK, or MAP2K), there has been increasing evidence that certain MAPKs are capable of auto-phosphorylation. For example, TAB1-mediated p38 MAPK α auto-phosphorylation in MEF cells accounted for a small portion of the total p38 MAPK α phosphorylation that was induced by hyperosmolarity and anisomycin [48]. It was further suggested, based on crystal structures of intrinsically active mutants, that the conformational change in loop 16 plays a critical role in inducing auto-phosphorylation through an intermolecular mechanism [49]. Similarly, ERK mutants capable of auto-phosphorylation have been reported [50]. Emrick *et al.* discovered that the mutation of a gatekeeper residue in ERK2 led to auto-phosphorylation. In this case phosphoryl transfer was suggested to occur through an intra-molecular mechanism [51]. It has been proposed that JNK2 α 2 auto-phosphorylates through an intermolecular mechanism [52].

Auto-phosphorylation may be stimulated through allosteric activation upon interaction with protein binding partners, such as scaffold proteins [47]. For example, a segment of Ste5 allosterically activated auto-phosphorylation of MAPK Fus3 [53]. Recently, we performed MD simulations of JIP1 peptide binding to JNK1 [54]. The simulations clearly demonstrated that the binding of pepJIP1 has a significant effect on the inter-domain motion and structure near the active site. Removal of pepJIP1 causes an increase in domain separation.

Interestingly, the activation loop in apo JNK1 is similar to the inactive form of apo ERK2, while in the JNK1•L-pepJIP1 complex it resembles the active form of apo ERK2, or the inactive form ERK2 complexed to a docking peptide derived from pepHePTP [55].

Although essential for understanding MAP kinase activities and regulation under different conditions, the auto-phosphorylation mechanism is not well understood. Due to the dynamic nature of this molecular mechanism, computational studies could potentially bring critical insights, which will in turn open up new opportunities for MAP kinase based therapeutics.

Conformations associated with the DFG motif—The conformational flexibility of the conserved Asp-Phe-Gly (DFG) motif at the beginning of the activation loop (see Figures 1 and 2) has been increasingly explored in the structure-based design of kinase inhibitors. In order to illustrate this flexibility and compare inhibitors that stabilize different DFG conformations we introduce structures of the c-jun N-terminal kinases (JNK) [56]. In 1998, the first JNK structure was solved by Su *et al.* of JNK3, which demonstrated that misalignment of the catalytic residues and occlusion of the active site by the phosphorylation lip are consistent with the low activity of un-phosphorylated JNK3 [57]. Of the two JNK2 structures in the PDB, the first (PDB: 3E7O) is of a complex of JNK2 with N-[3-[5-(1H-1,2,4-triazol-3-yl)-1H-indazol-3-yl]phenyl]furan-2-carboxamide (Figures 4a and 4b) with the activation loop in a ‘DFG-in’ conformation consistent with catalysis [58]. The second (PDB: 3NPC) shows the complex of JNK2 with BIRB-796 with the activation loop in a ‘DFG-out’ conformation, which does not support catalysis (Figures 4c and 4d) [59].

Ewald refinement was performed for both 3E7O and 3NPC in order to orient the water hydrogen-bonding network around the JNK2 inhibitor-binding site [14, 29]. This information can be used to optimize lead compounds by chemical modifications in order to displace water molecules that, for example, do not have access to a full complement of hydrogen bonding partners [14]. For example, Ewald refinement of JNK2 complexed with the carboxamide inhibitor (3E7O) orients three water molecules that hydrogen bond directly to the inhibitor (Figure 4B). Figure 4A show that waters 1 and 2 interact with three hydrogen-bonding partners, while water 3 only forms a single canonical hydrogen bond to the inhibitor. This suggests that water 3 may be in an energetically unstable environment, such that the appropriate chemical modification of the inhibitor could promote displacement of water 3 into bulk solvent, resulting in the tighter binding of the modified inhibitor. Similarly, Ewald refinement of 3NPC orients a bridging water molecule that may be displaced by the addition of a hydrogen-bond donating functional group to nitrogen N12 of BIRB-796 (Figure 4D). During the shift from ‘DFG-in’ to ‘DFG-out’, the phenylalanine (Phe-169) side chain is able to move from its buried conformation (Figure 4a) by about ~10 Å (Figure 4c) and create a new hydrophobic pocket, but this interferes sterically with ATP binding. In addition to the diaryl urea containing BIRB-796, imatinib, sorafenib and doramapimod have been shown to stabilize the DFG-out conformation of various protein kinases [60–64]. Inhibitors that stabilize DFG-out conformations are sought after, because they are generally more selective and display slower dissociation kinetics relative to inhibitors that stabilize the DFG-in conformation [65, 66].

Elcock and colleagues showed that in some 60 nanosecond explicit-solvent molecular dynamics simulations the DFG-in configuration transformed to DFG-out spontaneously [66]. This observation is consistent with an NMR study [67] that suggests the DFG-out configuration exists intrinsically, although rarely. Nonetheless, a steered molecular-dynamics study of p38 MAPK α suggested the inhibitors complex with the DFG-in or a transition state structure first, which then transforms to DFG-out. Furthermore, Kufareva and Abagyan [68] showed that key features of 'DFG-out' inhibitor binding are present in the DFG-in state such that it is possible to predict the bound state structure based on the DFG-in conformation alone. More recently, based on the combination of long MD simulations and experimental studies, Shan *et al.* proposed a mechanism where protonation of ASP triggers the conformational switch [40]. These calculations have brought unique insights and guidance that are beyond the reach of experiments albeit further studies of long-time dynamics under various conditions are needed to obtain a comprehensive picture of the DFG conformational properties.

Both activated and inactive MAPKs are important drug targets. While they each possess specific dynamic properties, they are intrinsically linked. Thus, the conformation of an activated MAPK may be viewed as a high-energy conformation of the inactive enzyme stabilized by phosphorylation. Some MAPKs appear capable of self-activating and whether this self-activation is through an intermolecular or intramolecular mechanism of autophosphorylation is generally not clear and requires further experimental investigation. There is evidence to suggest that some ligands may stabilize conformations of the inactive MAPK that more closely resemble the activated enzyme while some ligands may stabilize intrinsically inactive conformations where the DFG motif is incapable of supporting catalysis. Understanding the dynamics associated with the various conformational states of each MAPK are expected to be invaluable in the search for inhibitors that take advantage of the conformational flexibilities embedded within MAPK polypeptides. The computational modeling of these states is likely to be an important line of research over the next few years.

NON-ATP SITE INHIBITORS

MAP kinase inhibitors are considered a very important class of therapeutic. The majority of available MAP kinase inhibitors target the ATP binding site, which is highly conserved among more than 500 protein kinases. This conservation promotes cross reactivity and the possibility of undesirable toxicities that may limit their potential as drugs [69]. In 2007, Bain *et al.* profiled the selectivity of 65 compounds considered to be relatively specific kinase inhibitors against a panel of 70–80 protein kinases [70]. The p38 MAPK inhibitor SB 203580 was shown to inhibit c-Raf and GSK3 *in vitro* and the JNK inhibitor SP600125 was shown to inhibit 13 other kinases with similar potency including PKD1, CHK2, Aurora B and C, MELK, CK1, DYRK2, DYRK3 and HIPK3 [70]. This work highlights the difficulty of identifying highly specific ATP-competitive protein kinase inhibitors. Another limitation of such inhibitors is that they must compete with ~1 mM endogenous ATP, which has quite high affinity for MAP kinases [71]. Finally, more than 10,000 patents or patent applications for protein kinase inhibitors have been filed since 2001. Most of these are competitive with ATP, making it a challenge for drug discovery researchers to find novel chemical space [72].

In the last few years, drug discovery efforts by a number of groups have demonstrated progress towards validating kinase-binding sites that are distinct from the ATP binding site. MAPK interactions with upstream kinases and downstream substrates are mediated by recruitment sites found on the surface of MAPKs that are recognized by modular docking sequences [73]. When a canonical substrate-docking sequence binds a MAP kinase recruitment site, the effective concentration of the substrate Ser/Thr-Pro motif is locally increased near the active site. This, in turn, increases the rate of substrate turnover [74]. Disruption of the docking site interaction can lead to an overall reduction in the rate of substrate phosphorylation. For example, D-site peptides from MKK3 inhibit the ability of p38 MAPK to phosphorylate the transcription factors MEF2A and ATF2 [75]. Unlike peptide-based inhibitors, which cover a substantial surface area, the challenge for the development of small molecule inhibitors is to target recruitment sites located on the protein kinase's surface in such a manner that the docking process may be inhibited. A number of small molecule non-ATP competitive inhibitors have been described. In some cases *in silico* screening methods were the principal vehicle for their discovery.

In this section we describe the non-ATP competitive MAPK inhibitors that have been reported in the literature for ERK1/2, the JNKs and the p38MAPKs as shown in Figure 7. We also highlight the first model of a MAPK-substrate complex, which has proved to be useful in our laboratory for interpreting the mechanisms of non-ATP competitive inhibitors of ERK2.

Extracellular Signal-Regulated Kinases

ERK2-substrate model—In recent work, we have studied the ERK2 recruitment sites in detail utilizing a range of biochemical approaches [4, 10, 76-79]. ERK2 is known to have two recruitment sites called the D-recruitment site (DRS) and the F-recruitment site (FRS) (Figures 2 and 6), which bind the D-site and F-site of protein substrates, respectively [79, 80]. The DRS is an extended peptide-binding groove found on the rear face of the MAPK, while the FRS is a shallow hydrophobic pocket found adjacent to the Ser/Thr-Pro binding site [4]. The docking sequence that targets the D-site is a modular motif formed of both hydrophobic and basic residues (ϵ_{2-3} -X₂₋₆- ϕ_A -X- ϕ_B , where ϕ is a hydrophobic residue and ϵ is a basic residue). The F-site docking sequence ψ X ψ P is smaller, where ψ is an aromatic residue [81, 82]. The structural basis of the interaction between the ERK DRS and its docking sequence has been studied using structures from X-ray crystallography [83]. The basic residues of D-site docking motifs bind to negatively charged surface residues such as Asp-316 and Asp-319 in the region identified as Φ_{chg} in Figure 5. The hydrophobic residues of the D-site docking motif (ϕ_A -X- ϕ_B) bind the nearby hydrophobic pocket Φ_{hyd} .

No crystallographic structures of an active MAPK in complex with a substrate have been reported, possibly reflecting the high flexibility of these complexes. As an alternative approach towards understanding MAPK-substrate interactions we modeled the ERK2•Ets-1 complex using experimental observations to guide the modeling [79]. Modeling showed that although the N-terminus of Ets-1 does not include the consensus docking sequence (ϵ_{2-3} -X₂₋₆- ϕ_A -X- ϕ_B for ERK2, it makes an important contribution to the binding of Ets-1 to ERK2 [4]. This was confirmed by NMR [79]. Lig-D (FQRKTLQRRNLKGLNLNL) is a peptide that binds

the DRS of ERK2 and affects the K_m , but not the k_{cat} of Ets-1 phosphorylation by ERK2. Testing of the kinetic mechanism of inhibition by Lig-D showed a hyperbolic signature that suggests that Lig-D partially inhibits the ability of ERK2 to phosphorylate Ets-1 by displacing the *N*-terminus of Ets-1. In contrast, Lig-F (YAPRAPAKLAFQFPSR), which contains the F-site docking sequence ($\psi X \psi P$), exhibits a mechanism of full competitive inhibition and effectively blocks the binding of Ets-1 to ERK2. Understanding the mechanisms of MAPK-substrate binding should facilitate the utilization of computational methods to identify small molecule inhibitors.

In silico approaches identify small molecules that bind the ERK2 DRS—Unlike the ATP binding site, targeting of the DRS or FRS has the potential to identify inhibitors that are specific for ERK. In 2005, Hancock *et al.* reported the first non-ATP competitive small molecule inhibitor of ERK identified by computer-aided drug design (CADD) [84]. By using the program SPHGEN to detect the possible binding sites on the surface of inactive ERK2 in combination with mutagenesis experiments, they recognized important residues within the Φ_{chg} (Asp-316 and Asp-319) and Φ_{hyd} regions (Thr157 and Thr158) of the DRS (Figure 5A) involved in intermolecular interactions with protein substrates. These observations served as the basis for the *in-silico* screening of 800,000 compounds and ultimately the selection of 80 compounds for the *in vitro* testing of biological activity. Compounds were assayed for their ability to inhibit the phosphorylation of p90Rsk-1 by ERK2 in HeLa cells. Compounds **1** and **2** (Figure 7) were identified as ERK2 inhibitors with an ability to inhibit p90Rsk-1 phosphorylation by more than 50% at a concentration of 100 μ M. The binding of compound **1** to ERK2 was confirmed using a fluorescence-quenching assay ($K_d = 5 \mu$ M). However, the direct binding of **2** has not been established. Compound **1** is predicted to contact the polar groove between Asp-316 and Asp-319 of the Φ_{chg} region and Thr-157 and Thr-158 of the Φ_{hyd} region and is suggested to have additional π -charge interactions with Arg-133.

In 2006, Chen *et al.* reported the characterization of another 13 compounds discovered via *in silico* screening of the same library against active ERK2 using the same docking site as described above for **1** [85]. Many of the compounds that were identified as targets for inactive ERK2 were also identified as targets for active ERK2, consistent with the notion that minimal conformational changes occur within the DRS of ERK2 upon activation. We note that ERK2 is a monomer in both the active and inactive forms [86]. Compounds **3** and **4** showed 70% inhibition of Elk-1 and 50% inhibition of p90Rsk-1 phosphorylation in HeLa cells at a concentration of 100 μ M. Furthermore, these two compounds both showed clear selectivity for ERK2 inhibition *versus* p38 MAP kinase in HeLa cells. Compounds **3** and **4** were shown to directly bind ERK2 with a K_d of 13 and 16 μ M, respectively.

In 2011, Boston *et al.* used a computational search to identify additional compounds exhibiting similar chemical features to compound **1** [87]. MAC-BITS fingerprints in conjunction with a Tanimoto Similarity index were employed to search a virtual database of more than a million commercially available compounds. Ten compounds were identified with close similarity to compound **1** and tested for their biological activity. Compounds **5** and **6** inhibited caspase-9 and p90Rsk-1 phosphorylation by ERK in HELA cells at 50 μ M

and inhibited HeLa cell proliferation at 100 μ M. The inhibitory effect of these compounds on caspase-9 and p90Rsk-1, which are regulators of the pro-apoptotic Bad protein, caused induction of HeLa cell apoptosis.

In 2009, Li *et al.* synthesized a series of analogues of **1** in order to study its structure-activity relationship [88]. It was discovered that a shift of the ethoxy substitution from the 4 position to the 2 position on the phenyl ring (compound **7**) improved its biological activity and specificity towards ERK over JNK and p38 MAPK. Compound **7** demonstrated an enhanced ability to inhibit U937 cell proliferation (human leukemia cells) and Elk-1 phosphorylation in U937 cells.

Based on our understanding of how MAP kinase kinases recognize MAPKs it is expected that inhibitors that target the DRS of MAP kinases have potential to inhibit both the activation of MAP kinases as well as their catalytic function. Although compounds **1–7** were able to inhibit the phosphorylation of protein substrates such as Elk-1 and/or p90Rsk-1 by ERK2, they were not found to inhibit the phosphorylation of ERK2. Further work is necessary to understand this interesting observation. The biological evaluation of compounds **1–7** confirms the usefulness of both structure-based and ligand-based computational approaches to the discovery of selective MAP kinase inhibitors that target a protein-binding site and demonstrates that *in-silico* drug discovery can translate into cellular activity [89].

c-Jun N-Terminal Kinases

JNK-Jip interactions—The JNK Interacting Protein (JIP) scaffolds JIP1-4 bind to JNK and serve to enhance its activation *in vivo*. One of the best-known classes of non-ATP competitive inhibitors of JNK is the JIP-based peptide inhibitor (see Figure 5b). JIP-based peptide inhibitors are derived from the 11-residue sequence (¹⁵³RPKRPTTLNLF¹⁶³) that corresponds to the single DRS docking domain of JIP1. This peptide, called pepJIP1, acts as a specific inhibitor of JNK [90] and inhibits the phosphorylation of substrates such as c-Jun, Elk, and ATF2 in *in vitro* cell-free assays. JIP1 exhibits selectivity for the JNKs versus the closely related MAP kinases ERK and p38 MAPK [90].

Crystal structures of the pepJIP1-JNK1 complex provide insight into the mode of JIP1 binding to the DRS of JNK (Figure 5b) [90]. Molecular dynamics simulations and alanine-scanning identified four residues (R156, P157, L160 and L162) that are crucial for the binding of pepJIP1 to JNK [54]. In one of our recent reports we engineered new JIP-based peptide inhibitors that demonstrate specificity for the JNK2-isoform with an IC₅₀ ~90 nM. Using a flexible six carbon linker 6-aminohexanoyl (AHX) these peptides fused the JIP sequence to either the N-terminus of an inverted TAT sequence (JIP¹⁰-TATⁱ: Ac-PKRPTTLNLF-AHX-RRRQRRKKRG-amide) or to a poly-arginine sequence (JIP¹⁰-R₉: Ac-PKRPTTLNLF-AHX-RRRRRRRRR-amide) [91]. Both peptides were shown to significantly inhibit JNK activation and c-Jun phosphorylation in HEK293T cells. For example, a 5 μ M concentration of either peptide is sufficient to inhibit the activation/phosphorylation of cellular JNKs by ~90%. This confirms the hypothesis that the targeting of the DRS of JNK represents a viable approach for the inhibition of JNK signaling *in vivo*.

Small molecules that bind the JNK DRS and block JNK-Jip interactions—In 2008, Stebbins *et al.* identified the thiadiazole BI-78D3 (**8**) as the first small molecule to target the JNK-JIP interaction [92]. BI-78D3 was identified as a non-ATP inhibitor of JNK that efficiently displaces biotinylated pepJIP1 from GST-JNK1 with an IC₅₀ of 500 nM. Molecular modeling studies suggested that the benzodioxan moiety of BI-78D3 binds a small pocket in the DRS of JNK [92] characterized by an extensive hydrogen-bonding network to Arg-127 of JNK1. BI-78D3 exhibits dose-dependent inhibition of the phosphorylation of JNK substrates, both *in vitro* and in cells, with selectivity for JNK over p38 MAPK α , mTOR and PI3-kinase. BI-78D3 inhibits the TNF- α -induced phosphorylation of c-Jun in a dose-dependent manner in HeLa cells with an IC₅₀ of 12.5 μ M. Moreover, BI-78D3 abolishes ConA-induced liver damage and restores insulin sensitivity, compared to a vehicle control in a mouse model. In 2009, De *et al.* discovered a new series of 2-thio-benzothiazoles, which included BI-78G3 (**9**). It was suggested that like **8**, **9** binds the DRS of JNK1 [93] with the nitrothiazole group crossing the ridge near Arg-127 and Cys-163 (Figure 5B). Compound **9** displaces pepJIP1 from JNK1 with an IC₅₀ of 160 nM and inhibits c-Jun phosphorylation with an IC₅₀ of 1.8 μ M. While **8** and **9** were reported to more potent towards JNK compared to p38 MAPK α , their activity towards ERK2 was not reported [93]. Compound **10** is a derivative of **9** and is reported to have similar binding properties to **8** and **9** [94]. In 2010, De *et al.* introduced BI-90H9 (compound **11**), which has similar properties to compounds **8–10** albeit with a marginally higher plasma stability [95]. Compounds **8–11** represent an interesting starting point for the development of JNK and ERK inhibitors. However, their specificities need to be evaluated further and derivatives developed that lack reactivity towards thiols.

In 2009, Chen *et al.* used a fluorescence polarization assay to screen for compounds that disrupt the JNK-JIP interaction [96]. Compound **12** was found to inhibit the JNK1, JNK2 and JNK3 kinase activity with IC₅₀s of 6, 8 and 10 μ M respectively. Unfortunately, while compound **12** appears to be quite selective for JNK it exhibits limited cell permeability [96]. Thus, further development of this promising class of inhibitor is required.

Chen *et al.* also characterized compound **13** as a dual site JNK inhibitor; it is a competitive inhibitor of ATP, which is reported to allosterically inhibit the JIP-JNK interaction. Unlike **12**, however, it inhibits a number of protein kinases, consistent with the reduced selectivity expected of an inhibitor that targets the ATP binding site [96].

The thiophene-3-carboxamide derivative, **14**, was reported by De *et al.* [97] to be a dual site ATP and JIP inhibitor through its binding to both the ATP binding site and the JIP binding site. Compound **14** inhibits JNK1 kinase activity with an IC₅₀ of 1.3 μ M (LANTHA assay) and inhibits the binding of pepJIP1 to JNK1 with an IC₅₀ of 4.6 μ M (DELFI assay). Molecular modeling suggests that this compound binds within the ATP site and forms a hydrogen bond with the side chain of Gln-37. Binding to the DRS is favored by several hydrophobic contacts and hydrogen-bond interactions of the carboximide group with the backbone carbonyl of Asn-114 and amide nitrogen of Val-118. Compound **14** shows enhanced selectivity towards JNK (based on a panel of 26 kinases) and does not inhibit p38 MAPK α (2% inhibition at 25 μ M) [97].

A novel binding site on JNK near the MAPK insert—In 2011, Compound **15** was discovered in a high throughput screen of 500,000 compounds. A unique binding site was confirmed by NMR, utilizing ^{13}C -methyl-labeled JNK1 α 1 (residues 1–364, T183E and Y185E) and by crystallization of the complex of JNK1 and **15** [71]. The binding site for **15** was shown to be located in the region of the MAP insert region (Figure 6A). Interestingly, this compound binds exclusively to the inactive form of JNK1 and accordingly inhibits the phosphorylation of JNK1 by MKK7 with an IC_{50} of 7.7 μM .

p38 MAP Kinases

p38 MAPK is a promising target for the treatment of several inflammatory diseases due to its role in controlling the production of pro-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor [98].

Small molecules targeting the DRS of p38 MAPK—In 2004, Davidson *et al.* showed that targeting the non-ATP binding site of a MAP kinase could produce a substrate selective inhibitor [99]. They discovered a non-ATP p38 MAPK α inhibitor (**16**) that inhibits the phosphorylation of MK2a with a K_i of ~330 nM. In contrast, this compound inhibits the phosphorylation of ATF2 with a K_i of more than 20 μM . Isothermal titration calorimetry analysis confirmed the binding of **16** to p38 MAPK α , but showed that this compound does not prevent the binding of MK2a. While a deuterium exchange mass spectrometry (DXMS) study suggested that **16** binds in the vicinity of the p38 MAPK α active site and disrupts the ATP binding site [99], the basis for its substrate selectivity is unknown.

A lipid-binding site on p38 MAPK—Recently, a novel lipid-binding site was noted in the C-terminal lobe of p38 MAPK formed by the MAP kinase insert [71, 100, 101]. In 2009, Perry *et al.* applied AutoLigand to examine both the optimal ligand size and the affinity of compounds that may bind this site [101], which represents a promising target for the design of inhibitors since it is a hydrophobic pocket furnishing hydrogen-bond acceptors. In 2011, Comess *et al.* reported the discovery of several inhibitors that bind this site in p38 MAPK α [71]. High-resolution NMR and crystallographic studies provided details on the binding mode for compound **17** (Figure 6B). Interestingly, the binding of **17** induces conformational changes in the region of the binding site, which have the potential to be explored in the future. Compound **17** inhibits both the ability of MKK6 to phosphorylate p38 MAPK α as well as the ability of activated p38 MAPK α to phosphorylate MK2 cascade with IC_{50} 's of 1.2 and 0.8 μM , respectively. Surprisingly, **17** exhibits significantly less activity against p38 MAPK β , γ or δ ($\text{IC}_{50} > 40 \mu\text{M}$). However, unfortunately it shows marked activity against a number of other protein kinases including Pim1 ($K_i = 4.5 \mu\text{M}$), Pim3 ($K_i = 1.9 \mu\text{M}$), Map4k4 ($K_i = 4.7 \mu\text{M}$), KHS ($K_i = 5.2 \mu\text{M}$), Gsk3 β ($K_i = 4.9 \mu\text{M}$) and BRSK1 ($K_i = 8.5 \mu\text{M}$).

CONCLUSIONS

An increasing number of non-ATP competitive MAPK inhibitors are being reported in the literature. Several sites found on the surfaces of MAPKs have been shown to bind small molecule inhibitors and in some cases structures of these complexes have been reported. However, given the low throughput of X-ray crystallography and the potential difficulties of

determining the structures of MAP kinases complexed to surface bound ligands, *in silico* modeling approaches may prove to be useful, especially if prior knowledge of binding sites are available, for example through the use of high-field NMR (e.g. ERK2) [79].

The available evidence suggests that the development of small molecule non-ATP inhibitors of MAPKs represent a viable approach towards the inhibition of MAPK pathway signaling in cells. While no highly potent, i.e. $K_i < 10$ nM, inhibitors have been developed to date further advances in computational methods should aid in the development of such inhibitors.

Over the last decade, the polarizable atomic multipole AMOEBA force field has been developed in order to provide a more accurate and transferable, albeit computationally more expensive, alternative to fixed charge descriptions of molecular electrostatics [24, 25]. In spite of their neglect of polarization and point charge approximation, fixed charged force fields have proven their usefulness for sampling the conformational heterogeneity of MAP kinases via molecular dynamics [54] and given insight into MAPK-substrate docking [4]. However, some domains of molecular modeling for the discovery of non-ATP competitive inhibitors of MAP kinases benefit from chemical accuracy (i.e. prediction of binding free energy to experimental accuracy of 1.0 kcal/mole), which has been an important goal of the AMOEBA force field.

For example, Ewald crystallography refinement of MAP kinases based on the prior chemical information in the AMOEBA force field evaluated using Ewald's method for electrostatics improves secondary structure and orients the water hydrogen-bonding networks. Precise structural models enhance the reliability of molecular docking to predict the binding modes of new lead compounds [85, 102–106]. Understanding water structure and energetics within the relatively shallow binding sites on the surface of MAP kinases may be critical to the further optimization of the non-ATP competitive inhibitors reviewed here [91].

Once the docking pose is known and water hydrogen-bonding networks have been surveyed, the quantitative *in silico* prediction of relative binding affinities becomes a valuable approach for lead optimization. Progress in the sampling of molecular configurations [107], the physical model describing atomic interactions [108, 109] and more powerful computer hardware [110] all contribute to advancements in lead optimization. Our strategy has been based on coupling the AMOEBA polarizable force field to both explicit [24, 108] and implicit solvent [109, 111, 112]. For example, initial work on trypsin-benzamidine systems predicted a strong correlation ($R=0.9$) between binding affinity predictions and experiment. In ongoing work on MAP kinases, we are exploring enhanced sampling methods such as λ -dynamics [113], metadynamics [114] and the orthogonal space random walk (OSRW) method [115, 116] in order to compute binding free energies as efficiently as possible.

Advancements in computational technology is critically needed for us to fully understand the conformational dynamics of protein kinases, which underlie structure, function and inhibitor binding. Approaches such as Milestoning [35, 117] and a Markovian state model (MSM) based adaptive seeding method [118] are promising for achieving long-time sampling. For example, the former has been successfully applied to elucidate complex conformational dynamics such as allosteric transitions in hemoglobin [119] and the myosin

stroke [35]. The later was demonstrated to recover RNA folding kinetics. In both approaches, the process of a slow conformational transition can be modeled as a series of smaller steps that can be taken in a highly parallel fashion. Coupling polarizable force fields with such efficient dynamic sampling algorithms has the potential to bring novel insight into the role of conformational dynamics in MAP kinase activities, which is essential for designing innovative strategies targeting MAP kinases.

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Figure 1.

Shown is a multiple sequence alignment of human ERK1, ERK2, JNK1α2, JNK2α2, JNK3α2 and p38 MAPKα (CSBP2), p38 MAPKβ, p38 MAPKγ and p38 MAPKδ. The shades of blue indicate the degree of conservation for each residue and darker shades correspond to greater conservation. Note that the first 38 n-terminal residues of JNK3α2 and a variable number of c-terminal residues on each MAP kinase are not shown.

Figure 2.

A.) JNK1 (PDB ID: 3O17) is shown with each segment of secondary structure labeled. The subscripts for secondary structure elements that are not conserved for all protein kinases refer to loop number. For example, the first β -strand of Loop 0 is β_{1L0} . B.) Ewald refinement of the original experimental data using prior chemical information defined by the polarizable AMOEBA force field improves the quality of the structural model via extension of energetically favorable secondary structure elements and by improving agreement with the measured diffraction data (ie. reducing R_{free} from 30.6 to 27.9). The 3 β -strands and 4 α -helices that were extended are labeled. Changes near the DRS-site (Φ_{chg}) and FRS-site (α_g , activation loop) may impact downstream computational methods such as virtual screening of non-ATP MAP kinase inhibitors.

Figure 3.

A.) The inactive structure of ERK2 (PDB ID: 1ERK) shows the unphosphorylated T-X-Y motif (cyan) located on the activation loop (L12) of protein kinases ($^{183}\text{T-E-Y}^{185}$ for ERK1/2). B.) The active structure of ERK2 (PDB ID: 2ERK) has been phosphorylated on both the threonine and tyrosine residues that favor conformational rearrangements stabilized by electrostatic interactions between the negatively charged phosphorylated residues and positively charged arginine residues (grey).

Figure 4.

Shown in each panel is a MAP kinase structure complexed with an inhibitor (cyan, spacefill) that targets DFG-in or DFG-out (magenta, ball & stick) and the corresponding conformation of the activation loop (magenta, backbone only). A.) JNK2 in the DFG-in conformation is shown in a complex with type-I inhibitor N-[3-[5-(1H 1,2,4-triazol-3-yl)-1H-indazol-3-yl]phenyl]furan-2-carboxamide (PDB ID 3E7O). B.) Ewald refinement of A orients the water hydrogen-bonding network around the JNK2 inhibitor-binding site. C.) JNK2 in the DFG-out conformation in a complex with type-II inhibitor BIRB-796 (PDB ID: 3NPC). D.) Ewald refinement of C orients the water hydrogen-bonding network around the JNK2 inhibitor-binding site. E.) p38 MAPK α in the DFG-out conformation in a complex with BIRB-796 (PDB ID 1KV2). Ewald refinement was not performed for E because no diffraction data was deposited.

Figure 5.

The D-recruitment sites of A.) ERK2 with pepHePTP (PDB ID: 2GPH), B.) JNK1 with pepJIP1 (PDB IDs: 1UKH, 1UKI, 2G01, 2GMX, 2H96, 2NO3) and C.) p38 MAPK α with pepMKK3B (PDB ID: 1LEZ) / pepMEF2A (PDB ID: 1LEW) are shown. Qualitatively, to the right of the conserved C163 residue is a hydrophobic patch that contributes affinity to the interaction of MAP kinases with scaffolding proteins and to the left are interactions with negatively charged aspartate and/or glutamate residues. The superposition of JNK1 structures shows consistency for pepJIP1 residues critical to binding, but significant conformational heterogeneity otherwise.

Figure 6.

Shown in A and B are Ewald refinements of two MAP kinase complexes with non-ATP inhibitors that bind at the FRS-site. A.) The structure of the α isoform of JNK1 in complex with inhibitor 46A bound (PDB ID: 3O2M), which exhibits extensive interactions with a second copy of itself within the asymmetric unit of the crystal. B.) A promising structure of p38 MAPK α with inhibitor 3NE bound (PDB ID: 3NEW).

Figure 7.
2D structures of non-ATP competitive MAP kinase inhibitors discussed throughout the text.

TABLE 1

Shown are improvements in JNK1 and JNK2 protein-inhibitor structural models using Ewald refinement. The R/R_{free} statistics for the deposited PDB structures are shown as originally reported and after recalculation using the Force Field X program. After Ewald refinement, the final structural models improved the mean R_{free} value relative to the recalculated R_{free} by 1.4% on average. In addition, the mean MolProbity score was improved from the 51st percentile to the 97th percentile (see the text for a description of the MolProbity structure validation tool).

	Original Refinements				Ewald Refinements			
	PDB / Ligand	Res (Å)	R / R _{free}	MolProbity Score %	Recalculated R / R _{free}	Final R / R _{free}	MolProbity Score %	RMSD (Å)
	3O17 / Apo	3.0	26.3 / 30.8	3.9 17	25.5 / 30.6	22.9 / 27.9	2.3 97	0.38
	3O2M / 46A	2.7	23.3 / 26.6	3.3 30	22.8 / 26.4	19.7 / 24.9	2.3 94	0.25
	3NEW / 3NE	2.5	21.9 / 30.6	3.0 42	22.0 / 30.6	21.6 / 29.2	1.6 99	0.40
	3E7O / 35F	2.1	21.9 / 25.0	2.1 77	21.7 / 24.6	18.4 / 23.0	1.6 97	0.39
	3NPC / B96	2.4	21.4 / 25.4	2.1 88	21.0 / 24.7	18.8 / 25.0	1.7 98	0.16
	Mean	2.5	23.0 / 27.7	2.9 51	22.7 / 27.4	20.3 / 26.0	1.9 97	0.31