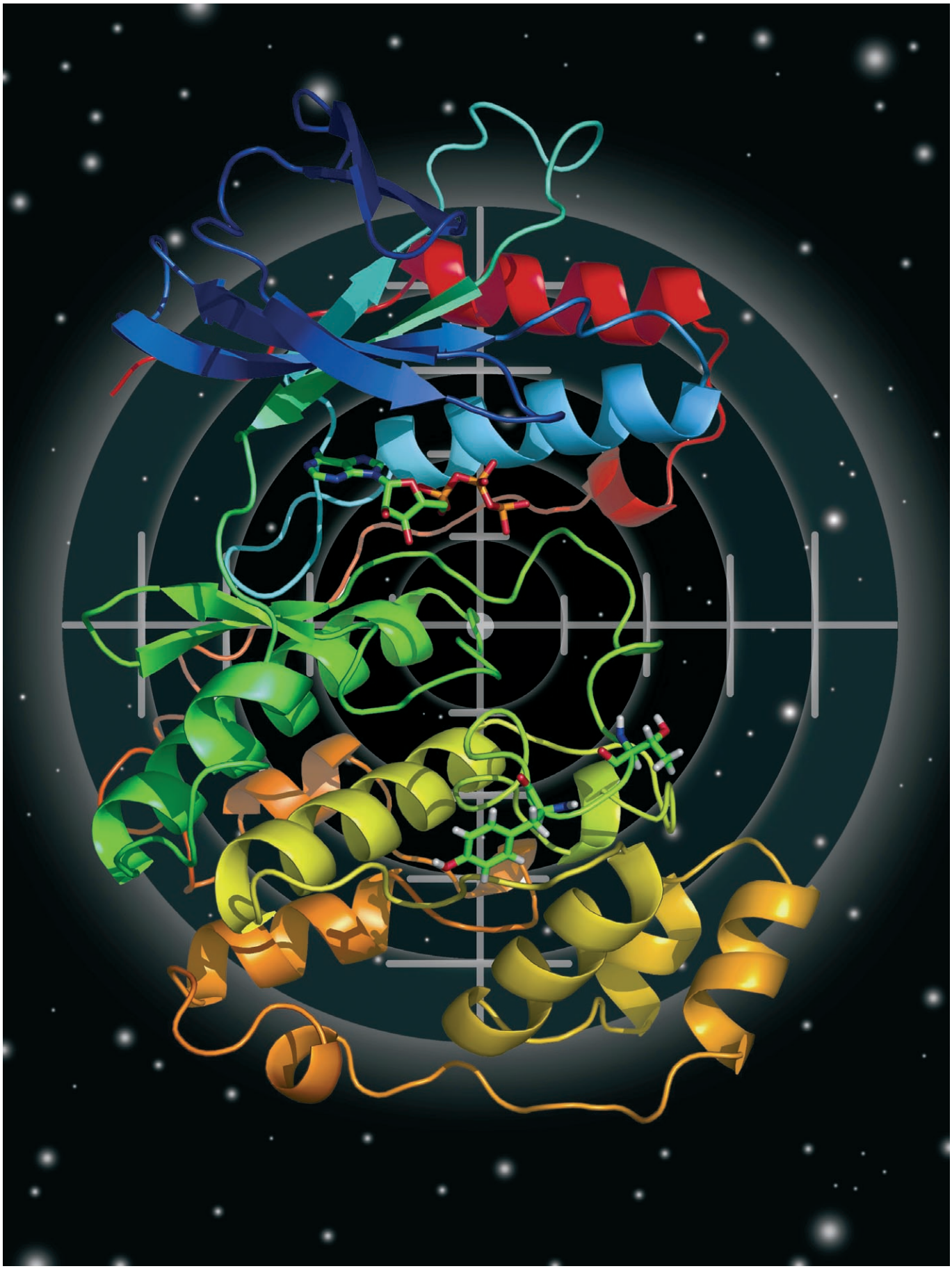

 DOI: 10.1002/cmdc.200600271

DOI: 10.1002/cmdc.200600271

Are MAP Kinases Drug Targets? Yes, but Difficult Ones

Simona Margutti and Stefan A. Laufer*[a]

Pharmaceutical companies are facing an increasing interest in new target identification and validation. In particular, extensive efforts are being made in the field of protein kinase inhibitors research and development, and the past ten years of effort in this field have altered our perception of the potential of kinases as drug targets. Therefore, in the drug discovery process, the selection of relevant, susceptible protein kinase targets combined with searches for leads and candidates have become a crucial approach. The success of recent launches of protein kinase inhibitors (Gleevec, Imatinib, Sutent, Iressa, Nexavar, Sprycel) gave another push to this field. Numerous other kinase inhibitors are currently undergoing clinical trials or clinical development. Some questions are nevertheless unanswered, mostly related to the great number of known kinases in the human genome, to their similarity with each other, to the existence of functionally redundant kinases for specific pathways, and also because the connection between particular pathways and diseases is not always clear. The review is leading the reader through a panoramic view of protein kinase inhibition with a major focus on MAPK, successful examples and clinical candidates.

1. Introduction

The pharmaceutical industry is undoubtedly one of the world's largest industries and has a constantly growing market. It faces steadily growing pressure to release more new chemical entities (NCEs) each year that will potentially evolve into commercial drugs and, ideally, innovative drugs with novel mechanisms of action that target therapeutic areas of unmet medical need. These attributes summarise the short term expectations of the upper management team of almost every pharmaceutical company. In contrast to these expectations, and despite the steady growth of R&D investment, the field of drug discovery and development has not evolved as efficiently as expected.[1]

Although most companies have increased their R&D expenditure considerably over the last few years, no notable increase in output of NCEs has occurred for the industry as a whole. In fact, the rise in R&D expenditure has been accompanied by a steady decline in new drugs reaching the market. In 2001, the output of the global biopharmaceutical industry in terms of new drugs was the lowest in ten years, and the number of FDA approvals for the first half of 2006 is as low as 11. Moreover, with the launch of only two or three compounds per year against novel targets, the majority of approvals for new drug candidates continues to be those directed against well-established target molecules (see Figure 1).[2]

The pharmaceutical industry still suffers from two persisting bottlenecks; one closely related to target validation, and the other related to the overall quality of compounds in terms of their ability to progress through the value-chain of drug development. A recent emphasis on the "front-loading" of research has tackled these issues at an earlier stage in the drug discovery pathway. In particular, interest has increased in target discovery both for the identification of novel targets (target identification) and for reduction of subsequent failures from incorrect biological hypotheses through early validation (target validation)- the so-called "first to fail" principle. Szymkowski from Roche Bioscience[3] underlined the crucial role of target validation in the pharmaceutical industry, emphasising its role in strengthening correlative data (from gene arrays, EST libraries, and proteomics) by demonstrating a pivotal role for the candidate in a disease model.

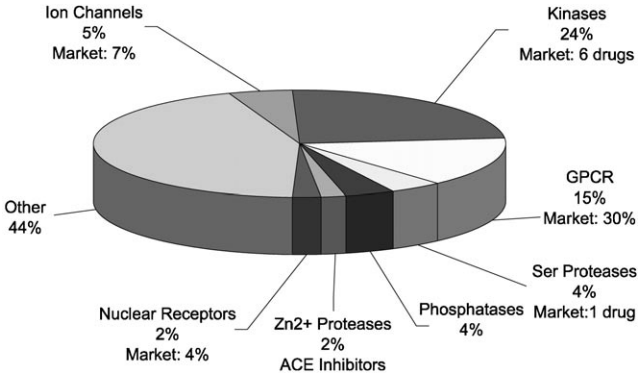

Figure 1. Schematic presentation of the target family distribution within the human "druggable genome".[1]

2. The Druggable Genome

A comprehensive analysis of the drug targets underlying current drug therapy undertaken in 1996 by Jürgen Drews, the former head of global research for Hoffmann-La Roche, showed that less than 500 molecular targets were addressed.

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The result was a far cry from the potential total number of targets, which Drews originally estimated at somewhere between 3000 and 10000. At that time human genes were thought to number at least 100000. Over the following years the number of discrete drug targets has not grown appreciably; on the contrary, the number of human genes had shrunk, with most estimates suggesting about 30000 genes. Thus, a greater slice of the human genome was being targeted than previously believed (Figure 2).

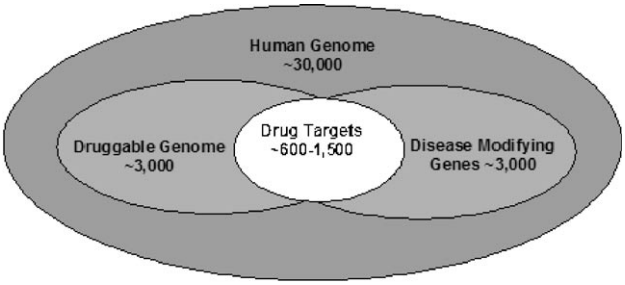

Figure 2. Number of Drug targets. The effective number of drug targets can be determined by the intersection of the number of genes linked to disease and the "druggable" subset of the human genome.[4]

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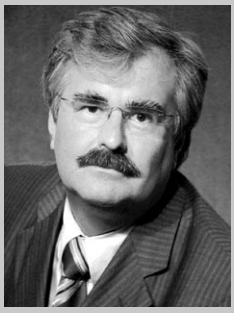


Stefan Laufer received his scientific training at the Universities of Regensburg and Mainz. (Degree in Pharmacy, PhD in Pharmaceutical Chemistry, Habilitation for Pharmaceutical Chemistry). After a postdoc period at the institute of pharmaceutical chemistry and biopharmacy in Frankfurt, Germany, his professional appointments started in 1990 with Head Dept Drug Research at Merckle Pharmaceutical GmbH, Germany, which was followed by R&D Director and Member of the Board at the same company. Since 1999 he holds the chair for pharmaceutical chemistry and is head to the department of pharmaceutical/medicinal chemistry at the Eberhard-Karls-University Tuebingen, Germany. His research fields include anti-inflammatory drug research and discovery (Molecular targets: COX-1/2/3, Lipoxygenases, Cytokine-Release inhibitors, Kinase-inhibitors, Transcription factors).

Great excitement was generated by publications from Celera Genomics[5] and the international Human Genome sequencing Consortium[6] describing the draft sequence of the human genome only five years after Drews' analysis, and the crucial question for every biopharma business became: "How many potential drug targets are encoded in the human genome?", leading to the concept of "the druggable genome," that is, that subset of the ~30000 genes in the human genome that express proteins capable of binding drug-like molecules.

Different companies conducted surveys on the druggable genome. In 2002 Pfizer Inc. researchers Andrew Hopkins and Colin Groom reported the identification of 399 nonredundant molecular targets that are members of just 130 protein families. Pfizer researchers identified several proteins targeted by experimental drugs and eliminated other protein targets that did not bind Lipinski rule-of-five[7] compliant compounds with binding affinities below 10 μM. Although that ability of a protein to bind a small molecule with the appropriate chemical properties at the required binding affinity might make it druggable, it doesn't necessarily make it a potential drug target. That honour belongs only to those proteins that are also linked to disease. Moreover, whereas not all proteins can be modulated by small-molecules (undruggable targets), occasionally an unexpected allosteric binding site for a drug or chemical tool is discovered. Therefore, methods to discover ligands for unpredicted binding sites could improve the cost effectiveness of searches for chemical tools. The 2002 Pfizer survey confirmed that the potential for compound development is being increasingly addressed at earlier stages of discovery. The drug-like character of compounds has been assessed by means ranging from the intuition and experience of the chemist to sophisticated computational methods; the latter includes machine learning algorithms that generalise from various chemical descriptors of known good drugs[8] and expert systems that adopt a rule-based approach using easily measured properties.[9] The ranges that according to the Lipinski rule-of-five establish windows of drug-likeness have been extended and refined with parameters such as the number of rotatable bonds.[10] To date there have been few such general heuristics for predicting the target-likeness or the inherent tractability of targets to intervention, independent of their disease relevance; in this contest the Hopkins and Groom study has the merit of considering the set of targets comprising the druggable genome in aggregate terms, such as their drug-binding domain content.

3. Signal Transduction as Drug Target

In the field of target identification there has been a great deal of enthusiasm for the prospect of identifying novel drug targets based on knowledge of key signal transduction components and their links to disease. As signalling disorders represent a major cause for the pathological states and as most of the recently validated target molecules of drug research are signal transduction related macromolecules (mostly kinases), signal transduction therapy has become one of the most important areas of drug research.[11,12] Efforts to map signal trans-

duction pathways have intensified over the recent years. In this regard, Isis Pharmaceuticals employs an interesting method for mapping pathways using antisense oligonucleotides that can selectively reduce target gene expression. If this target gene is in a key pathway, the lower level of protein expression will result in pathway interruption and cellular abnormalities, thus potentially validating the protein and its gene as a therapeutic target of interest.

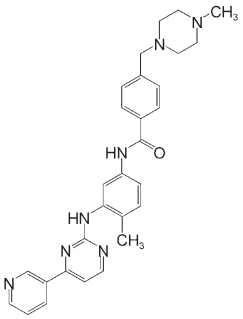
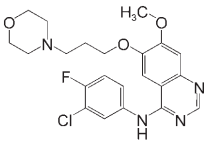
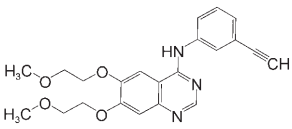
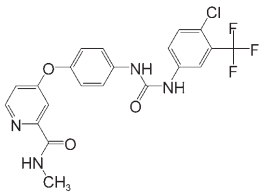
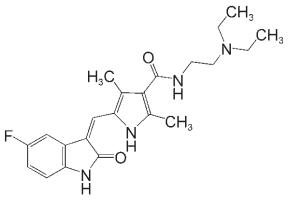
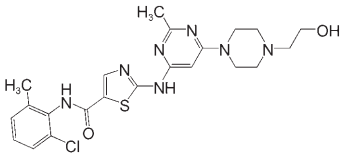
4. Kinases as Drug Targets

Approximately 20–25% of the druggable genome consists of kinases involved in signal transduction. Currently, however, only six kinase inhibitors are being used in clinical practice (Table 1), which yields a wide perspective for drug discovery.

The eukaryotic protein kinases form the largest superfamily of homologous proteins and genes of the human genome. Within this family, there are now hundreds of different members whose sequences are known. Although there is a rich diversity of structures, regulation modes, and substrate specificity among the protein kinases, there are also common structural features.

The common feature conserved throughout the entire protein kinase family is the catalytic domain with its associated catalytic centre, which consist of ~250–300 amino acid residues. The chemical activity of a kinase involves removing a phosphate group from ATP and covalently attaching it to one of three amino acids that have a free hydroxyl group. Most kinases act on both serine and threonine (serine/threonine kinases), others act on tyrosine (tyrosine kinases), and a number (dual specificity kinases) act on all three, for example, MEK

Table 1. Protein kinase inhibitors currently approved as drugs, their predominantly targeted kinases, and clinical use.

Drug	Structure	TM	Year	Target Kinase(s)	Clinical use
Imatinib mesylate STI571		Gleevec	2001	Bcr-abl tyrosine kinase, c-kit kinase	Chronic myelogenous leukaemia (CML), gastrointestinal stromal tumors (GISTs) and a number of other malignancies.
Gefitinib ZD 1839		Iressa	2003	Epidermal growth factor receptor (EGFR) tyrosine kinase (Her1 or ErbB-1)	Locally advanced or metastatic non-small cell lung cancer (NSCLC) in patients who have previously received chemotherapy
Erlotinib HCl OSI-774 CP-358,774		Tarceva	2004	EGFR tyrosine kinase	In combination with gemcitabine for treatment of locally advanced, unresectable, or metastatic pancreatic cancer
Sorafenib Bay 439006		Nexavar	2005	c-Raf/b-Raf kinase, VEGF receptor 2/3 tyrosine kinase, mPDGFR-β, FLt3 and p38α	Advanced renal cell cancer
Sunitinib SU11248		Sutent	2006	Multiple tyrosine receptor kinases, including PDGF- and VEGF receptor tyrosine kinase.	GIST and renal cell carcinoma (RCC)
Dasatinib (Spycell) BMS-354825		BMS	2006	Src-Abl tyrosine kinase	Chronic myelogenous leukaemia (CML), Philadelphia chromosome-positive acute lymphoblastic leukaemia (Ph + ALL)

(MAPKK), which, as will be further discussed is involved in the MAP kinase cascade, is a mixed serine/threonine and tyrosine kinase. Structurally distinct from most other protein kinases are histidine kinases which are found mostly in prokaryotes as part of two-component signal transduction mechanisms. A phosphate group from ATP is first added to a histidine residue within the kinase, and later transferred to an aspartate residue on a receiver domain on a different protein, or sometimes on the kinase itself. The aspartyl phosphate residue is then active in signalling. Some investigations[13,14] seem to indicate the presence of eukaryote-like kinases and phosphatases in prokaryotes as well.

Different studies were undertaken on kinase classification and homology, about twenty years ago Hanks and collaborators[15] undertook a comparative analysis of all the available sequences of protein kinase catalytic domain. The homologous catalytic-domain amino acid sequences of 65 distinct eukaryotic protein kinases were aligned and their overall relationship was visualised by the construction of a phylogenetic tree demonstrating the overall similarity among the catalytic domains (Figure 3).

The catalytic domains were shown not to be uniformly conserved but, rather, consist of alternating region of high and low conservation. Such an arrangement of alternating regions of high and low conservation is a common feature of homologous globular proteins and gives some clues to higher order structure. A protein structure will provide a close general model for other proteins with which its sequence homology is

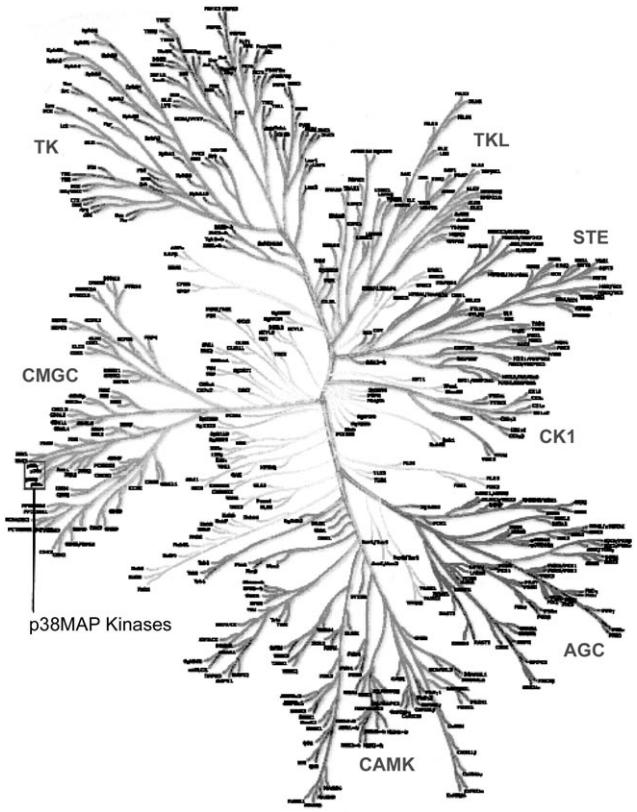


Figure 3. The human kinome.[16]

$>50\%$.[17] On the basis of amino acid sequence similarity, seven groups of protein kinases are identified which are further divided into 90 families and 145 subfamilies.[16] Relevant for this survey, as will be discussed, are the mitogen-activated protein kinases (MAPKs) and, in particular three well-characterised subfamilies of MAPKs: extracellular signal-regulated kinases ERK1 and ERK2; the c-Jun NH_2-terminal kinases, JNK1, JNK2, and JNK3; and four p38 enzymes, p38α, p38β, p38γ, and p38δ. A fourth MAPK, ERK5, is a relatively recently identified[18] MAPK and is being studied intensely.[19–21] Sequence alignments with other human kinases, define for the ATP binding site of p38α a range of amino acid sequence conservation of 40–100%. Within the ERKs, ERK1 and ERK2 share respectively 51.6 and 55% of sequence homology with p38α; for JNK1, JNK2, and JNK3 the percentages are: 52.5, 55, and 44.5% , finally for the p38 isoforms the following data are reported: p38β 78.6%, p38γ 68.6%, and p38δ 67.1% homology. Data within members of the ERK-family and JNK-family are also available and defined as 85 and 78–84% respectively.[22–27]

The individual family members, approximately 518 kinases, constitute a functional basis for basically every physiological process, kinases playing a central role in propagation of signal transduction in every type of cell[28] and are reported to be involved in a plethora of diseases. In particular, tumourogenesis has been linked to the aberrant function of protein kinases that play roles in cell proliferation, migration, and invasion. Thus, kinases have become of particular interest as potential targets for anticancer agents. As a result, protein kinases account for 20–30% of the drug discovery programmes of many companies, even though some problems are related to the definition of kinases as drug target.

The discussed identical catalytic mechanism, together with a high degree of sequence homology, identical protein folding topologies, and the common co-substrate ATP initially led to the assumption that protein kinases constitute a nondruggable family of protein targets. Thus, it is possible that one small molecule inhibitor may bind to several different kinases, in addition to the target, blocking normal signalling that is needed for normal cellular function. However, it is possible that the potential to block a multitude of kinases may prove to be useful in several types of cancers, as long as the inhibitor is specific to malignant or premalignant cells. Moreover, virtually all of the protein-kinase inhibitors that have been developed were ATP competitive, and the difficulties that were involved in developing compounds with sufficient potency to compete with the ATP concentrations that are present in the intracellular milieu (2–10 mM) were becoming apparent, especially for the many protein kinases that have a Michaelis–Menten constant (K_m) for ATP of 10 μM or less. The challenge is for an inhibitor to compete at nM concentrations against mM ATP. Indeed, it might be no coincidence that a plethora of potent inhibitors that target the p38 MAPK have been developed, because its K_m for ATP is above 0.1 mM. It is surprising that virtually no compounds that compete for binding with the protein-substrate binding site have progressed to late development stages. Unlike ATP competitive inhibitors, such compounds might have the potential to prevent a protein kinase from

phosphorylating some substrates but not others. A much greater emphasis on developing compounds that bind preferentially to inactive forms of protein kinases, or which prevent one protein kinase from activating another, might well pay dividends in the development of anti-inflammatory drugs; nevertheless, because of drug resistance issues, a similar approach could be not optimal in anticancer research.

5. Kinase Inhibition

Taking into consideration kinase inhibition principles, it is possible to distinguish four types of inhibitors: substrate-competitive inhibitors; ATP-competitive inhibitors; activation inhibitors/allosteric modulators; and irreversible inhibitors.

5.1. Types of Inhibitors

Substrate-competitive inhibitors. The substrate-binding site seems to have obvious advantages over the ATP-binding site as a target for inhibiting kinase activity. First, substrate-binding inhibitors are not affected by the high ATP concentration found in cells. Second, the substrate-binding site of a kinase controls selectivity, whereas the ATP-binding site is highly conserved throughout all kinase family members. Whereas substrate-competitive inhibition has been applied successfully in enzyme classes such as the proteases, its use for kinase inhibition has not been successful thus far. As the kinases have rather large substrate binding sites,[29] they lack the specific, compact hydrophobic pockets that could serve as targets for small molecule inhibitors.

ATP-competitive inhibitors. As previously discussed in this review, for a long time the development of a potent and selective ATP-competitive inhibitor was considered impossible. The main concerns are selectivity, because of the shared highly conserved ATP-binding site, and, potency; as a consequence of intracellular ATP concentrations up to 10 mM. Thus, the concentrations required for an inhibitor to reach 50% inhibition are two or three orders of magnitude higher than the inhibition constant itself. Experience collected over the last ten years shows that fairly selective ATP-site specific kinase inhibitors can be generated, because of the suitability of the binding site to drug design, with different examples showing that specificity and selectivity can be achieved by the derivatisation pattern of an underlying core structure.[11] On the other hand, it must be underscored that many specific protein-kinase inhibitors, such as PD98059 and SB203580, are not developable because of toxicity or ADME problems. Nevertheless, from a chemical biology perspective, these compounds could be extremely useful research agents.

Selectivity of an inhibitor can theoretically be explored by kinase profile assay. The standard biochemical approach to addressing this problem relies on the specificity screens with an in vitro inhibition assay against panels of purified kinases.[30] A recent Ambit Bioscience publication[31] describes a map method to determine kinase inhibitor specificity by measuring binding of small molecules to the ATP site of kinases. The binding assay was used to profile 20 kinase inhibitors against a panel of 119 protein kinases. The low concentration of kinases used in this method allows measurement of binding affinities as low as 1–10 pM; furthermore, binding affinity provides a common denominator that allows direct comparisons between all kinases in the panel. As it is not yet possible to biochemically assay every kinase in the genome, it will become more important to show that a drug is exerting its effect through inhibition of a particular protein kinase, by showing that the effect of the drug disappears when a drug-resistant mutant is overexpressed or replaces a wild-type enzyme. This approach is based on the evidence that every protein kinase can be mutated into an active, inhibitor-insensitive mutant, often by changing the gatekeeper residue into a bulkier residue. This method is used for highly stringent target validation.

Irreversible inhibitors. Irreversible inhibitors are not usually used in the pharmaceutical industry because of the possible toxicity they could incur. An example of this type of inhibitor is Canertinib, an irreversible small-molecule tyrosine kinase inhibitor that blocks signal transduction through all four members of the ErbB (or epidermal growth factor [EGF]) family and is in development for the treatment of advanced nonhematological cancers.[32]

Activation inhibitors/allosteric modulators. More relevant and interesting are allosteric modulators, which as previously pointed out, could potentially solve the selectivity issues related to protein kinase inhibition.[33] A first example of an inhibitor exploiting such a mechanism was Gleevec (Imatinib, STI-571). The structural mechanism for its inhibition of Abels tyrosine kinase (Abl) was described by researchers from the Rockefeller University in 2000.[34] As already pointed out, whereas conformations of protein kinases that are fully active are very similar, striking differences exist among the various inactive conformations of kinases from different subfamilies. A crucial aspect of the conformational transition between the active and inactive states is the activation loop segment, which is of varying length and sequence, and is often the site of activating phosphorylation in the kinase domain. In structures of protein kinases that are in a fully active state, the activation loop is in an extended or open conformation. Two crucial aspects define this active conformation of the activation loop. First, an aspartic acid residue (Asp 381 in Abl) within a strictly conserved Asp-Phe-Gly (DFG) motif at the NH_2-terminal base of the activation loop is positioned so as to interact properly with a magnesium ion that coordinates the phosphate group of ATP. Second, the rest of the loop is positioned away from the catalytic centre so that the COOH-terminal portion of the activation loop provides a platform for substrate binding. In the structure of Abl–STK-571,[34] and the AblK–STK-571[35] complex, the NH_2-terminal portion is rotated drastically with respect to the active conformation, so that the activation loop adopts a conformation that mimics substrate binding to the enzyme and prevents its activation by other kinases.[36] Critical to the binding of Gleevec is the adoption by the kinase of an inactive conformation, in which the centrally located activation loop is not phosphorylated. The conformation of this loop is distinct from that in active protein kinases, and from the inactive form of the closely related Src kinases. As a result, the inhibitor has a high affinity for

Abl kinase, yet being essentially inactive against Ser/Thr-kinases and most of the tyrosine kinases. These selective results suggest that compounds that exploit the distinctive inactivation mechanism of individual protein kinases can achieve both high affinity and high specificity.

Another example of this allosteric type of binding mode is the Boehringer Ingelheim compound BIRB-796, which will be discussed further.

5.2. Structural Basis for Inhibitor Selectivity

To further prove that specificity could be achieved by ATP-competitive inhibitors, crucial experimental data has been published by researchers at Merck.[37] Comparison of the crystal structures of p38 bound to different compounds showed that binding of more specific molecules is characterised by a peptide flip between Met 109 and Gly 110. Gly 110 being a residue specific to the α, β, and γ isoforms of p38. The σ isoform and the other MAP kinases have bulkier residues in this position. These larger residues would likely make the peptide flip energetically unfavourable, thus explaining the selectivity of binding. This stabilised peptide flip is caused by a switching of the hydrogen-bond donor and acceptor distribution around the peptide plane.

In p38α, the peptide flip changes the (Φ,ψ) peptide bond angles of Met 109 (*i* residue) and Gly 110 ($i+1$ residue) from a (β, α_R) conformation to a (α_R, α_L) conformation, without affecting the orientation of adjacent peptide planes or side chains. This flip belongs to the group two flips, which are characterised by having ψ(*i*) values that are in the α_R or α_L regions when Φ($i+1$) is positive. As Φ($i+1$) must take on positive values if peptide-plane flipping is to occur, the residue at position $i+1$ is often a glycine; the X-Gly motif seems to be quite common among different cases. If the residue $i+1$ is not a glycine, the presence of a residue other than glycine would probably reduce the likelihood that the peptide flip would occur.

6. MAPK

Cells recognise and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade that leads to activation of the mitogen-activated protein kinases (MAPKs). All eukaryotic cells possess multiple MAPK pathways, which co-ordinately regulate diverse cellular activities, running the gamut from gene expression, mitosis, and metabolism to motility, survival and apoptosis, and differentiation. To date, five distinct groups of MAPKs have been characterised in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1,2, and 3, p38 isoforms α, β, γ, and δ, ERKs 3 and 4, and ERK5.[38,39] As *Saccharomyces cerevisiae* possesses six different MAPKs, the relatively greater complexity of the human genome suggests that there are probably several additional vertebrate MAPK subfamilies. The most extensively studied groups of vertebrate MAPKs to date are ERK1/2, JNKs, and p38 kinases.

MAPKs can be activated by a variety of different stimuli (Figure 4), but in general, ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters, whereas the JNK and p38 kinases are most responsive to stress stimuli ranging from osmotic shock and ionising radiation to mechanical wear and cytokine stimulation.

6.1. ERK1 and ERK2 Signalling Pathway

The mammalian ERK1/2 module, also known as the classical mitogen kinase cascade, consists of the MAPKKKs A-Raf, B-Raf, and Raf-1; the MAPKKs, MEK1 and MEK2; and the MAPKs, ERK1

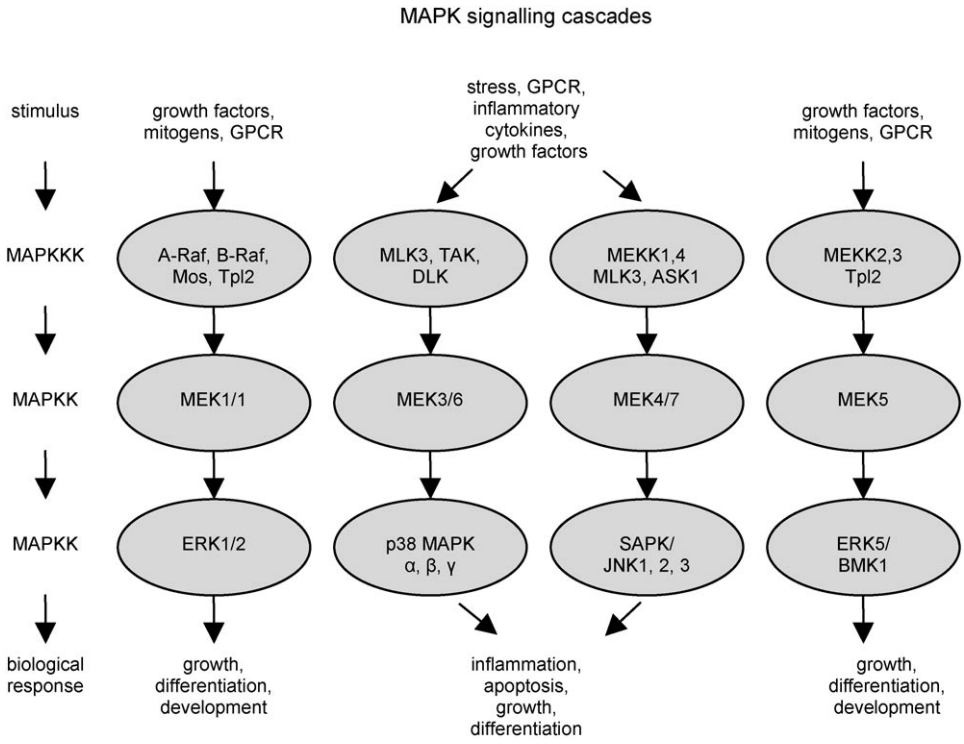


Figure 4. MAPK signalling cascades. Although each MAPK has unique characteristics, a number of features are shared by the MAPK pathways. Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs, which are serine/threonine kinases are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues located in the activation loop of the kinase. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues followed by a proline; however, substrate selectivity is often conferred by a specific interaction motif located on the physiological substrate.

and ERK2. The ERK pathway, also known as the p42/p44 MAP kinase pathway, is a major determinant in the control of cell growth, cell differentiation, and cell survival. This pathway, which operates downstream of Ras, is often upregulated in human tumours and as such represents an attractive target for anticancer therapy.

ERK1 and ERK2 have 83% amino acid similarity and are expressed to varying extents in all tissues, including terminally differentiated cells, and have been estimated to be present in the range of 100–500 nM in tissues of greatest abundance.

6.1.1. Mechanism of Activation

ERK pathway is outlined in Figure 5. Cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein

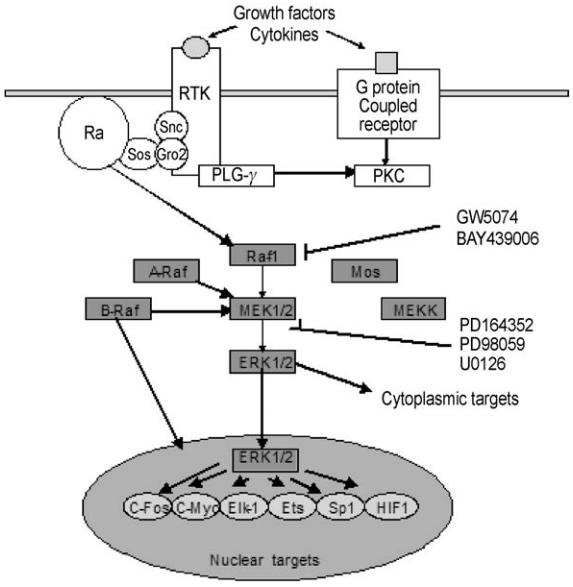


Figure 5. Schematic representation of the ERK pathway focusing on its function at the convergence of diverse intracellular signalling pathways. The MAP kinase cascade composed by three sequential kinases (MAPKKK, MAPKK, MAPK) is reported. Well-characterised inhibitors of Raf-1 and MEK1/2 are also shown in bold type.[47]

Ras.[40,41] The exact mechanism of Raf activation is still elusive[42,43] but is known to require Ras binding and multiple phosphorylation events at the membrane. Regulation of both Ras and Raf is crucial for the proper maintenance of cell proliferation, as activating mutations in these genes lead to oncogenesis. Activated Raf binds to and phosphorylates the dual specificity kinases MEK1 and -2, which in turn phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. Like MAP kinases, MEKs are activated over 1000-fold by phosphorylation of two residues.[44–46] However, both phosphorylations are on either serine or threonine, and each of the two will partially increase the activity of MEK1/2.

In general, MEK family members are among the most selective protein kinases known in that they phosphorylate very few substrates. MEK1/2 have not been shown to phosphorylate any other MAP kinases or other proteins of physiologic relevance. However, MEK4 has been shown to phosphorylate at least one protein other than MAP kinases, suggesting that other MEKs may also have a few non-MAP kinase substrates.

ERK1/2 are distributed throughout cells. In unstimulated fibroblasts the majority of them are associated with the microtubule cytoskeleton. Upon stimulation, a significant population of ERK1/2 accumulates in the nucleus; the nuclear localisation is essential for morphological transformation of 3T3 fibroblasts and for neurite extension in PC12 cells, a model system for neuronal differentiation.[38]

By amplification through the signalling cascade, it is estimated that activation of only 5% of Ras molecules is sufficient to induce full activation of ERK1/2.[48] ERK1/2 signalling has been implicated as a key regulator of cell proliferation,[49] and for this reason, inhibitors of the ERK pathway are entering clinical trials as potential anticancer agents.[47,50] An example is Sorafenib launched as a drug in 2005 and recently reviewed by Wilhelm et al.[51] In fact, the ERK pathway represents a convergence point for the majority of mitogenic signalling pathways, so that there are many upstream signalling molecules including receptor tyrosine kinases (RTKs) Grb2, Sos, Shc, Ras, and protein kinase C whose abnormal activation could culminate in the constitutive activation of the ERK pathway and could represent potential targets for the development of anticancer drugs.

The ERK pathway inhibitors are divided in two classes: Raf-1 inhibitors and MEK1/2 inhibitors (Figure 6).

6.1.2. Raf-1 Inhibitors

The major cause of constitutive activation of the ERK pathway in human tumours is a disorder in Raf, Ras, or other signalling molecules upstream of Ras.[52] Specific inhibitors of Raf are expected to efficiently block aberrantly activated mitogenic signalling. Examples of Raf-1 inhibitors are GW5074 and Sorafenib (BAY 43-9006).

GW5074 inhibits Raf-1 kinase activity in vitro with an IC_{50} of 9 nM. It also inhibits EGF-stimulated ERK activation quite effectively without inhibiting the EGF receptor tyrosine kinase.

Sorafenib[51,53–55] was identified as a potent inhibitor of Raf-1 by screening chemical libraries using a combination of an in vitro Raf kinase biochemical assay and a tumour cell-based mechanistic assay.[56] Sorafenib inhibits Raf-1 kinase activity in vitro with an IC_{50} of 12 nM. It also suppress tumour growth in human tumour xenograft models with mutant K-ras genes (HTC116 colon carcinoma, MiaPaca-2pancreatic carcinoma, and H460 non-small cell lung carcinoma). However, Sorafenib, as a single agent, failed to show therapeutic activity in the treatment of malignant melanoma, despite positive results in renal carcinoma.[57] This is probably because it also inhibits a number of other kinases (such as VEGFR, PDGFR), which are also overexpressed in renal carcinoma. The reason for the failure in malignant melanoma may be attributed to its inability

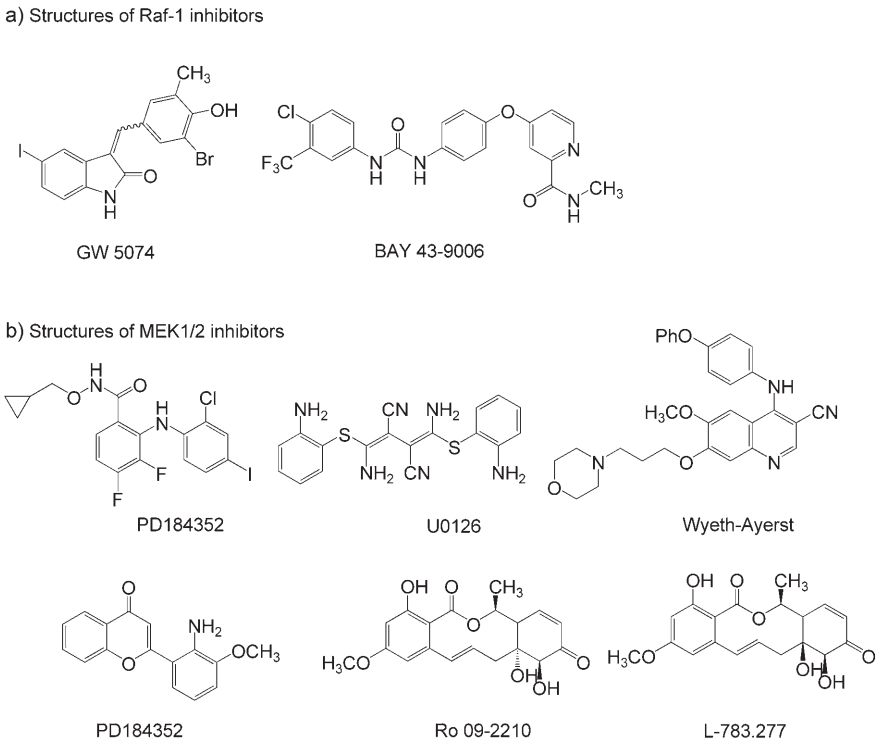


Figure 6. Structures of inhibitors of the ERK pathway.[47]

to reach a concentration in melanoma cells sufficient to inhibit Raf-1. Niculescu-Duvaz et al.[58] recently presented improved Raf-1 inhibitors based on a disubstituted pyrazine scaffold.

6.1.3. MEK1/2 Inhibitors

To date no substrates for MEK1/2[47] have been identified other than ERK1 and ERK2. This apparent selectivity, in addition to an ability to phosphorylate both Tyr and Thr residues, is consistent with MEK1/2 playing a central role in the integration of mitogenic signals into the ERK pathway.[59–61] MEK1/2 have not been identified as oncogene products, but they stand at the focal point of many mitogenic signalling pathways, and constitutive activation of MEK1 has been detected in a variety of human tumour cells.[52] These findings suggest MEK1/2 as a good target for anticancer agents development. In Figure 5 some MEK1/2 inhibitors are reported and their structures are illustrated in Figure 6. As previously discussed, the majority of protein kinase inhibitors developed so far are competitive with ATP. PD98059, U0126, and PD184352 differ in this respect as they do not compete with ATP, a characteristic that may allow those compounds to function as more specific inhibitors.

As the Ras-MAPK signalling pathway is of the central importance with regard to cell proliferation, transformation, and invasion, inhibitors of the kinase components of this pathway would appear to have great potential as anticancer agents. As pointed out by Berger and Mallon,[62] despite the central nature of this pathway, selective inhibitors of Raf and MEK appear to cause no toxic effects in normal cells. A potential issue for a selective kinase inhibitor is resistance. As there is a large degree of cross-talk between the various signalling pathways, it is possible that some tumours cells might be able to overcome inhibition of this pathway.[63] Thus, it is likely that these kinase inhibitors will be more effective in combination with agents acting by other mechanisms.

6.2. JNK pathway

The JNKs were first identified by their activation in response to a variety of extracellular stresses and their ability to phosphorylate the N-terminal transactivation domain of the transcription factor c-Jun. JNK1, -2, and -3, are also known as SAPKγ, SAPKα, and SAPKβ, which exist as ten or more spliced forms.[24,64] The JNK1 and JNK2 genes are expressed ubiquitously. In contrast, the JNK3 gene has a more limited pattern of expression and is largely restricted to brain, heart, and testis. They are involved in cytokine production and other aspects of the inflammatory response, more generally in the function of the immune system, stress-induced and developmentally programmed apoptosis, actin reorganisation, and in cell transformation.[65]

Activation requires, like ERK1/2 and p38, dual phosphorylation on Tyr and Thr within the tripeptide motif Thr-Pro-Tyr (TPY) located in the kinase subdomain.[24] This phosphorylation is mediated by a dual specificity protein kinase. JNK activators are MKK4 (SEK1/JNKK) which has been characterised[66–68] and MKK7. In turn, MKK4/7 are activated by phosphorylation mediated by several MAPKKKs, including MEKK1-4, MLK2 and-3, Tpl-2, DLK, TAO1 and -2, TAK1, and ASK1 and -2.[39] The mechanism of MEKK1 activation is not understood, but this pathway may involve the phosphorylation of MEKK1 by the PAK protein kinase that is activated by the small GTP binding proteins Rac1 and Cdc42. The structure of the JNK signalling pathway is therefore similar to the ERK pathway that is activated by the small GTP binding protein Ras via the Raf-1 and the MEK1 protein kinase cascade (Figure 7).[69]

Like ERK1/2, the activities of JNK/SAPKs are influenced by nuclear hormones. Swantek initially demonstrated that activation of JNK/SAPKs in macrophages and other cell types is inhibited by glucocorticoids in a manner that depends on the presence of nuclear receptors but not on transcription.[70] The mechanism has not been elucidated, but the site of inhibition appears to lie upstream of MEK7. Dexamethasone also inhibited TNFα-induced JNK phosphorylation in HeLa cells, based on immunofluorescence with antiphosphoJNK antibodies.[71] A

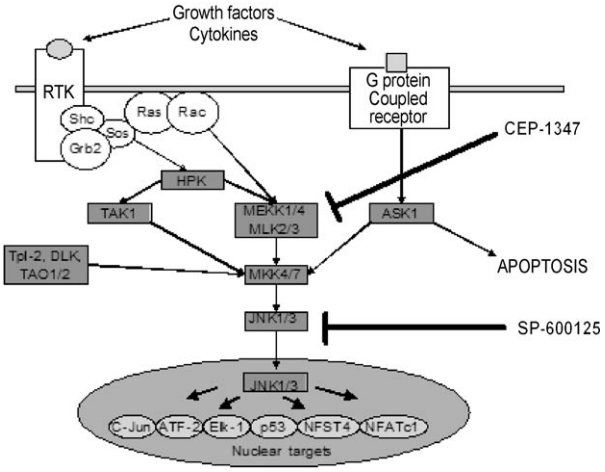


Figure 7. Schematic representation of the JNK pathway. JNKs respond to stress signals including heat shock, osmotic stress, inflammatory cytokines, growth factors and GPCR agonists. Stress signals are delivered to this cascade by members of small GTPases of the Rho family (Rac, Rho, cdc42).As with the other MAPKs, the membrane proximal kinase is a MAPKKK, typically MEKK1/4, or a member of the mixed lineage kinases (MLK) that phosphorylates and activates MKK4 (SEK) or MKK7, the SAP/JNK kinases. Alternatively, MKK4/7 can be activated by a member of the germinal centre kinase (GCK) family in a GTPase-independent manner. Activation of JNK is by dual phosphorylation (at Thr-Pro-Tyr motif) by MAPKK4/7. Activated JNKs dimerize and translocate to the nucleus where they activate (phosphorylate) transcription factors including c-Jun, ATF-2, Elk-1, and p53. Activation of the JNK signalling cascade generally results in apoptosis and is involved in inflammation.

possibly similar effect has been observed with retinoic acid.[72] The transcriptional activation function of the retinoic acid receptor was required for the long term but not the acute suppression of JNK/SAPK activity. The effect could be detected at the level of MKK4, which was also inhibited. At least part of the suppression was due to induction of MKP-1.

6.2.1. JNK Function

Compared with ERK, JNK activity is more strongly induced in response to proinflammatory stimuli, and there is preliminary evidence that inhibition of JNK activity can retard or prevent tissue damage in animal models of rheumatoid arthritis.[73]

One approach to study the function of JNKs has included in vivo gene knockouts of each of the three JNK genes. Whereas loss of either JNK1 or JNK2 alone appears to have no serious consequences, their combined knockout is embryonic lethal.[74] In contrast, the loss of JNK3 is not embryonic lethal, but rather protects the adult brain from glutamate-induced excitotoxicity.[75] This latter example has generated considerable enthusiasm for JNK3, considered an appropriate target for the treatment of diseases in which neuronal death should be prevented (for example, stroke, Alzheimer's, and Parkinson's diseases). In contrast to the expression pattern of JNK3, the expression of JNK1 and JNK2 proteins is not tissue-restricted. It has therefore taken greater efforts and more complex studies to understand the role of these two JNK isoforms in physiological and pathophysiological processes. In the first studies to evaluate JNK1/1 and JNK2 –/– mice, the loss of either JNK isoform was shown to alter T cell differentiation.[76–78] As the inappropriate regulation of T cells underlies many autoimmune diseases, attention has been directed to the inhibition of JNKs in diseases such as rheumatoid arthritis. In more recent studies, roles specifically for JNK2 as a mediator of inflammatory responses have been explored. In the passive collagen-induced arthritis model, the JNK2–/– animals showed modestly decreased joint damage (cartilage erosion and proteoglycan depletion) but little effect on inflammation, leading to a conclusion that JNK2 was not the sole mediator of arthritic changes.[79] Gene knockout animals have been used to demonstrate that JNK could provide a suitable target for the protection against obesity and diabetes and that JNKs may act as tumour suppressors. The role of JNKs as therapeutic targets has been recently reviewed by Manning and Davis.[80]

In parallel to these studies, other approaches to inhibit expression of JNKs such as antisense techniques and the more recently adopted RNA-mediated interference approaches should accelerate discoveries of the cellular events mediated by JNKs, and will determine whether JNKs are druggable targets.

6.2.2. Pathway Inhibitors

Specific inhibitors for the JNK/SAPK pathway are not yet available. Most of the JNK inhibitors reported thus far come from synthetic efforts to design p38 inhibitors. The p38 inhibitors SB203580 (described earlier) and SB202190 also block JNK activity at concentrations above those necessary to block p38. There are, however, some naturally occurring peptide inhibitors that have been reported.[81,82] Park et al. showed that selenite was a direct inhibitor of JNK1 activity both in vitro and in vivo, having an in vitro IC_{50} of between 10 nM and 50 nM.

CEP-1347, a MLK inhibitor. One of the first compounds to be discovered that inhibits the JNK pathway but without effect on p38 is Cephalon's CEP-1347 that inhibits the JNK pathway at the level of the MLK (Figure 11). The compound inhibits all five members of the MLK family (MLK1, MLK2, MLK3, dual leucine zipper kinase, and leucine zipper-bearing kinase) and members of the GCK family (GCK, KHS, and NIK). CEP-1347 prevents death of neurons that had been deprived of growth factors and also inhibits JNK1 activation with equivalent IC_{50} values;

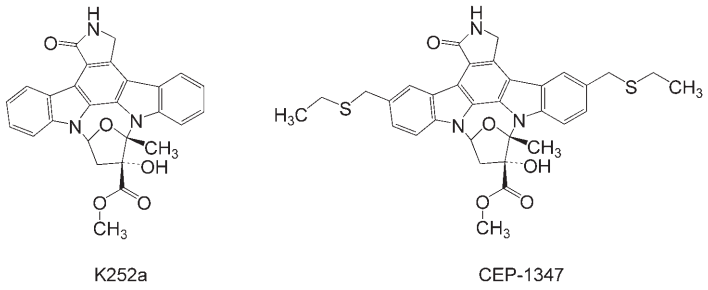
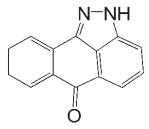


Figure 8. Chemical inhibitors of the JNK pathway. CEP-1347 was discovered during attempted optimisation of the natural product K252a.

ERK1/2 activity is unaffected. This inhibitor also inhibits JNK1 activation in fibroblasts caused by ultraviolet irradiation, osmotic shock, and glycosylation inhibitors.

CEP-1347, originally called KT7515 (or 3,9 bis[(ethylthio)-methyl]-K252a), was identified in a medicinal chemistry approach directed to optimise the drug-like properties of the naturally occurring compound K252a[83] (Figure 8). Specifically, a series of 3,9-disubstituted [(alkylthio)-methyl]- and (alkoxymethyl)-K252a derivatives were tested for an increase in neurotrophic response. This response was specifically evaluated by the survival of cholinergic neurons in spinal neuron cultures and their maintenance of choline acetyl transferase activity. At the same time, the aim was to also reduce biochemical properties that limited its use as therapeutic agent. For example, for a compound to be ultimately used in neuronal applications, it should have low inhibitory activity toward the high affinity nerve growth factor-receptor, trk. CEP-1347 met these initial criteria, and was also demonstrated to have decreased ability to inhibit protein kinase C, cAMP-dependent protein kinase, and myosin light chain kinase.[83,84]



SP-600125

Figure 9. Reversible ATP competitive JNK inhibitor: SP600125. SP-600125 acts as a reversible ATP-competitive inhibitor of equal potency towards three JNK gene products, JNK1, JNK2 and JNK3. Specifically IC_{50} values calculated for JNK1, JNK2 and JNK3 were 40, 40 and 90 nM, respectively.[25]

The neuroprotective and neurorestorative role of CEP-1347 makes it interesting, as pointed out in a recent review on Parkinson's disease treatment,[85] as a therapeutic agent targeting the mechanism involved in the pathogenesis of Parkinson's disease. The PRECEPT study is currently in progress to assess the neuroprotective role of CEP-1347 in the early phase of the disease.

SP-600125, an ATP-competitive inhibitor of JNK MAPK. Different research groups have been focused their attention toward inhibition of JNK MAPK rather than the upstream components of the pathway, with the aim of achieving greater specificity of effects. One of the major players in this area has been Signal Pharmaceuticals (now part of Celgene). Signal has described pyrazoloanthrone derivatives, such as SP-600125 (Figure 9), as inhibitors of JNK.[86] SP-600125 inhibits JNK1/2 with an IC_{50} of 110 nM and JNK3 with an IC_{50} of 150 nM, but is much less active against p38 ($IC_{50} = 30\ \mu M$).[73] Anthrapyrazoles have not been previously reported as kinase inhibitors; however, the presence of nitrogens within the highly planar, fused-ring structure of SP-600125 is consistent with features of other known kinase inhibitors.[25] Such ring structures exhibit poor solubility in water, (only 0.0012 $ng\,mL^{-1}$) as originally described by Bennett and co-workers.[25] Chemical modification studies undertaken to define the critical features of SP-600125 required for its inhibition of JNK have shown that N-alkyl substitution lowered inhibitory activity, whereas a chlorine added at position 8 produced a more potent analogue.[25] It may thus be possible to further enhance the solubility of SP600-125 derivatives yet retaining the inhibitory capability.

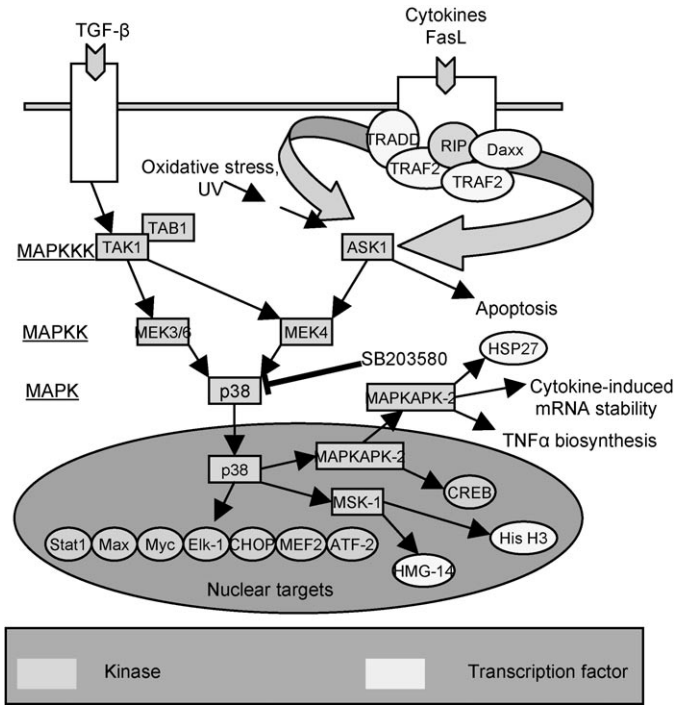


Figure 10. p38 MAPK signalling pathways. P38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines. As with other MAPK cascades, the membrane-proximal component is a MAPKKK, typically a MEKK or a mixed lineage kinase (MLK). The MAPKKK phosphorylates and activates MKK3/6, the p38 apoptotic stimuli. p38 MAPK is involved in regulation of HSP27 and MAPKAP-2 and several transcription factors including ATF-2, Stat1, the Max/Myc complex, MEF-2, Elk-1 and, indirectly, CREB by activation of MSK1. Localization of p38 is controversial; in fact, p38 has been shown to be present in both the nucleus and cytoplasm of quiescent cells, but upon cell stimulation, the cellular localization of p38 is not well understood. Some evidence suggests that, following activation, p38 translocates from the cytoplasm to the nucleus, but other data indicate that activated p38 is also present in the cytoplasm of stimulated cells.

SP-600125 was shown to block IL-1-induced accumulation of phosphorylated JNK and expression of c-jun in cultured synoviocytes. In vivo, administration of SP-600125 was shown to inhibit JNK activation and collagenase expression in the joints of rats with adjuvant arthritis. Animals also showed a reduction in paw swelling and bone and cartilage damage. Thus, inhibition of JNK could be a potential therapy for diseases as rheumatoid arthritis.

Concerns arise from the IC_{50} values for the three JNK isoforms (see Figure 9). For some applications, an inhibitor with this ability to interfere with all JNK isoforms will be desirable, but as the previously discussed results of JNK gene knockout studies have suggested, isoforms perform specific roles. Therefore, the discovery of new compounds (or SP-600125 derivatives) showing isoform selectivity would be highly desirable. The best clinical prospect for JNK inhibitors would appear to be as agents with a neuronal target such as stroke or Parkinson's disease, where a specific JNK3 inhibitor could be given without effects on non-neuronal cells.

6.3. p38 MAPK Pathway

The p38 (also known as CSPB, mHOG1, RK, and SAPK2) are serine/threonine kinases that play a central role in numerous proinflammatory responses.[87] p38 kinases are widely expressed in many cell types, including immune, inflammatory, and endothelial cells (Table 2).

Table 2. p38 isoform expression in cells of the immune system and endothelium.[97]

p38 isoforms	Tissues expression	Cellular expression
p38α	Ubiquitous Mainly: spleen, bone marrow, heart, brain, pancreas, liver, skeletal muscle, kidney, placenta, lung	All cell types Mainly: peripheral leukocytes,
p38β	Ubiquitous Mainly: brain and hearth	Endothelial cells, T cells
p38δ	Lung, kidney, endocrine organs, small intestine	Macrophages, neutrophils, T cells, monocytes
p38γ	Skeletal muscles, hearth	Little or no expression in immune system

α-Isoform of p38[88] is 50% identical to ERK2 and bears significant identity to the yeast kinase Hog1p involved in the response to hyperosmolarity.[22,89] The p38α isoform has been associated most closely to inflammatory responses.[90] A variety of factors, including stress, endotoxin, cytokines such as tumour necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β), and cigarette smoke, activate the p38 kinases. Once activated, p38 phosphorylates downstream substrates to initiate a signal cascade that regulates synthesis of a variety of proinflammatory mediators. TNFα, IL-1β, and COX-2 are among the most important proinflammatory mediators regulated by p38. The inhibition of each of these inflammatory mediators has been demonstrated to lead to clinical benefit in rheumatoid arthritis (RA), based on approved biologics and NSAIDs. In addition to regulating the production of mediators such as TNFα and IL-1β, p38 is activated following the binding of TNFα, IL-1β, and RANKL to their receptors and is responsible for some of their effects. p38 inhibition therefore offers opportunities to intervene in processes involving these cytokines. In addition to inhibiting production of the cytokines themselves, p38 inhibition has the potential to block subsequent deleterious effects caused by the cytokines.

Three additional p38 family members, p38β,[26,91,92] p38γ,[93,94] and p38δ,[26,91,92] share 47% to 42% sequence identity to ERK2 but are 75%, 62%, and 64% identical to p38α, respectively. In mammalian cells, the p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines, but not appreciably by mitogenic stimuli.

Most stimuli that activate p38 also activate JNK, and both are inhibited by the anti-inflammatory drug SB203580, which has been extremely useful in delineating the function of p38.[22] Within the p38 family, the ubiquitously expressed p38α and p38β are inhibited by pyridinyl imidazole drugs, whereas the other two p38 kinases, p38γ and p38δ, are insensitive to these drugs.[95] p38γ and p38δ, with 67% identity to each other, have distinct expression patterns. p38γ is primarily expressed in skeletal muscle, and its expression is upregulated during muscle differentiation.[93] p38δ expression is also developmentally regulated and is most highly detected in lung, kidney, endocrine organs, and small intestine.[96]

Activation of p38 in cells is mainly mediated by MEK3 and MEK6. MEK4 also displays activity toward p38 in vitro.[66] In MEK4-/- fibroblasts both JNK and p38 lost their responsiveness to TNFα, intereukin-1, and hyperosmotic stress, suggesting that crosstalk exists between these two stress-sensitive pathways.[98] Activation of the p38 isoforms results from the MEK3/6-catalysed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loop. The structures of inactive and active (phosphorylated) p38α have been solved by X-ray crystallography. The phosphorylated TGY motif and the length of the activation loop were found to differ in ERK2 and JNK, which likely contributes to the substrate specificity of p38.[99,100]

Owing to the specificity of MEKs and the relatively low sequence identity among the p38 isoforms, selective activation of different p38 isoforms by distinct MEKs was observed. This signalling specificity is crucial for the generation of appropriate biological responses by the p38 pathway.

MEKs are activated by a plethora of MAPKKKs, typically a MEKK or a mixed lineage kinase (MLK), which becomes activated in response to various physical and chemicals stresses such as oxidative stress, UV irradiation, hypoxia, ischemia, and various cytokines including IL-1 and TNFα (Figure 9). Details regarding the contributions of MEKKs and MLKs to the p38 pathway remain poorly understood. MEKKs 1–3 have been implicated in p38 activation, although they preferentially regulate JNKs and ERKs.

Members of the MLK family contain an SH3 domain, leucine zippers, and a small GTPase binding domain.[101] Protein–protein interaction through these domains facilitates integration of signals by MLKs from upstream regulators to the downstream MAP kinases. Other kinases that may regulate p38 include TPl2, ASK1, and TAK1 (Figure 10).

6.3.1. p38 Activity and Inhibition

A large body of evidence indicates that p38 activity is critical for normal immune and inflammatory responses. p38 is activated in macrophages, neutrophils, and T cells by numerous extracellular mediators of inflammation, including chemoattractans, cytokines, chemokines, and bacterial lipopolysaccharides.[102] p38 participates in macrophage and neutrophil functional responses, including respiratory burst activity, chemotaxis, granular exocytosis, adherence, apoptosis, and also

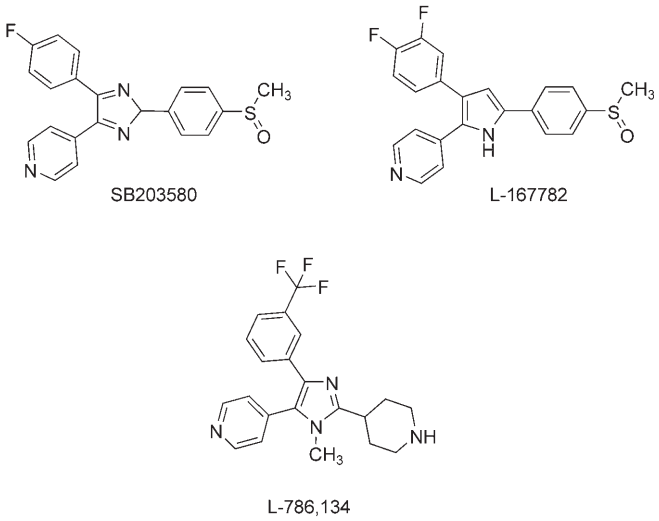


Figure 11. Structures of triarylimidazole SB203580, triarylpyrrole L-167782 and diarylimidazole L-786,134.

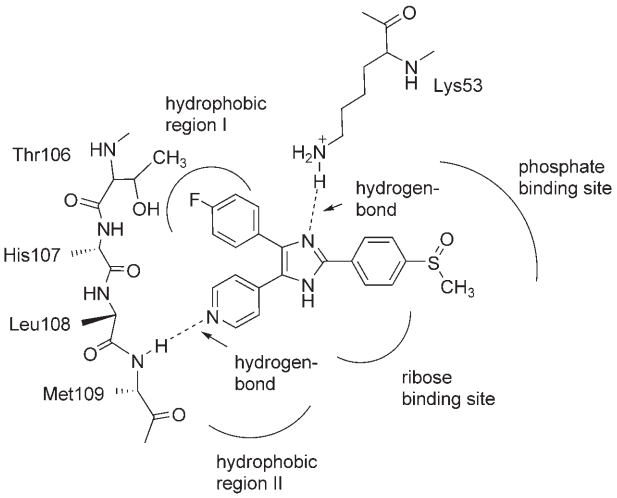


Figure 12. Important interactions between the prototypical pyridine-4-yl imidazole inhibitor SB203580 and the ATP binding site of p38α; the hydrophobic area below the linker region is not occupied by SB203580

mediates T-cell differentiation and apoptosis by regulating gamma interferon production.

The requirement for p38 activation in cellular responses has been defined largely through the use of experimental pyridinyl-imidazole anti-inflammatory drugs, the cytokine-suppressive anti-inflammatory drugs (CSAIDs), the most extensively characterised of which is the compound SB203580.

p38 inhibitors are potent inhibitors of LPS-mediated TNF-α production in macrophages.[22] The ability of p38 inhibitors to block TNFα synthesis can be exploited in the treatment of inflammatory diseases.

6.3.2 Pyridinyl-imidazole Inhibitor: SB203580

The basis for p38 inhibition was revealed in the crystal structure of p38α in complex with SB203580. As previously discussed, in vitro assays demonstrated that only p38 α and β are inhibited by CSAIDs; p38 δ and γ are completely unaffected by this class of drugs in vitro or in transfected cells.[91] Davies and co-authors[30] published a study on specificities of different commercially available compounds with relatively selective inhibition of particular serine/threonine-specific protein kinases, describing the preferential inhibition of the α and β isoforms over the γ and σ by SB203580.

Crystallographic,[103–105] mutational,[106,107] and biochemical[108] studies demonstrated that SB203580 binds in the ATP binding site of p38. The 4-fluorophenyl ring of the inhibitor is located in a hydrophobic pocket with walls formed by the N-terminal domain core at the back of the active site (Figure 15). Thr106 in the hinge of the p38α ATP binding pocket is the factor determining the nature of the residue that can be accommodated in the hydrophobic region; groups such as *p*-fluorophenyl, m,p-difluorophenyl, and *m*-trifluoromethylphenyl substituents on either imidazole or pyrrole scaffolds can occupy a space near Thr106 (Figure 14). The selectivity of compounds as SB203580 for p38α has been attributed to the presence of Thr106 in the ATP-binding site. Other MAP kinases (Table 3),

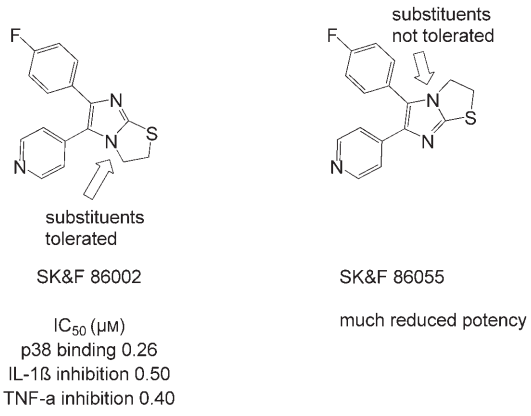


Figure 13. The correct regiochemistry is required at the imidazole nucleus for bioactivity.

except p38β, have either a Met or a Gln residue in this position and the larger side chains of these residues prevent the binding of the fluorophenyl ring of the inhibitors. A site-directed mutagenesis study undertaken by Lisnok et al.[107] showed that mutation of Thr106 to Gln, the residue present at the corresponding position in ERK-2, or Met, the corresponding residue in p38γ, p38δ, and JNKs, renders the inhibitor ineffective. Thr106 orients the drug to interact with His107 and Leu108 of the ATP binding pocket (Figure 11). Substitution of Thr106, alone or in combination with His107 or Leu108, with the corresponding, more bulky residues from p38γ or p38δ (Met and Pro or Phe, respectively, in both cases) abolishes SB203580 binding. Conversely, if the amino acid of p38γ, p38δ, or even SAPK γ which corresponds to p38α Thr106 is replaced with Thr, the resulting mutants display at least partial sensitivity to SB203580.[106,109]

Amino acid comparison between residues in p38α and homologous Ser/Thr kinases. Bold residues in Table 3 represent amino acids different from those in p38α. Sequence differences at the primary level are most prominent at residues 106,

Table 3. Amino acid comparison between residues in p38α and homologous Ser/Thr kinases.[a]

	35	38	51	53	75	84	86	104	106	107	108	109	155	157	168
p38α	Y	V	A	K	L	I	L	L	T	H	L	M	N	A	D
p38 β	Y	V	A	K	L	I	L	L	T	**T**	L	M	N	A	D
p38γ	Y	V	A	K	L	I	L	L	**M**	**P**	**F**	M	N	A	D
p38δ	Y	V	A	K	L	I	L	L	**M**	**P**	**F**	M	N	A	D
JNK2β	Q	V	A	K	L	I	L	L	**M**	**E**	L	M	N	**V**	D
JNK2α	Q	V	A	K	L	I	L	L	**M**	**E**	L	M	N	**V**	D
ERK-2	Y	V	A	K	L	I	**I**	**I**	Q	**D**	L	M	N	**L**	D
cAPK	F	V	A	K	L	**V**	L	**L**	**M**	**E**	**Y**	**V**	N	**L**	D

[a] Bold residues represent amino acids different from those in p38α. Sequence differences at the primary level are most prominent at residues 106, 107, 108 and 157. Even though residue 109 is highly conserved, it has been suggested that Met 109 is a key contributor to inhibitor specificity. Site-direct mutagenesis studies[107] show that mutation of Ala 157 in p38α caused diarylimidazoles to be more potent as compared to wild-type p38.

107, 108, and 157. Even though residue 109 is highly conserved, it has been suggested that Met 109 is a key contributor to inhibitor specificity. Site-direct mutagenesis studies[107] show that mutation of Ala 157 in p38α caused diarylimidazoles to be more potent as compared to wild-type p38.

In addition to the interaction with Thr 106, the binding mode of SB203580 shares a common set of features with various described pyridin-4-yl imidazole derivatives, notably, the formation of a hydrogen bond between the backbone NH group of Met 109 in the linker region and the 4-pyridine nitrogen atom of the inhibitor (Figure 12).[100, 103, 105]

As observed with various inhibitors of p38 MAP kinase,[110, 111] replacement of the pyridin-4-yl moiety with a pyridin-3-yl ring results in a 500-fold decrease in the inhibition of cytokine release. This loss of potency could be explained by an unfavourable geometry of the pivotal hydrogen bond with Met 109, thus underscoring the crucial importance of this pyridine ring for biological activity. Furthermore, according to Lisnock's data,[107] mutation of Met 109 to Ala caused the inhibitor to be less potent compared to wild-type p38. Still open to debate is the relevance of the hydrogen bond between N3 of the imidazole ring and Lys 53 of p38 MAP kinase. Although several studies indicate the imidazole as a critical determinant for the binding of pyridinylimidazoles to p38 MAP kinase,[87, 103, 105, 106] some authors[37, 106, 112] suggest a sole role for the imidazole as a scaffold for positioning the fluorophenyl and pyridine rings.

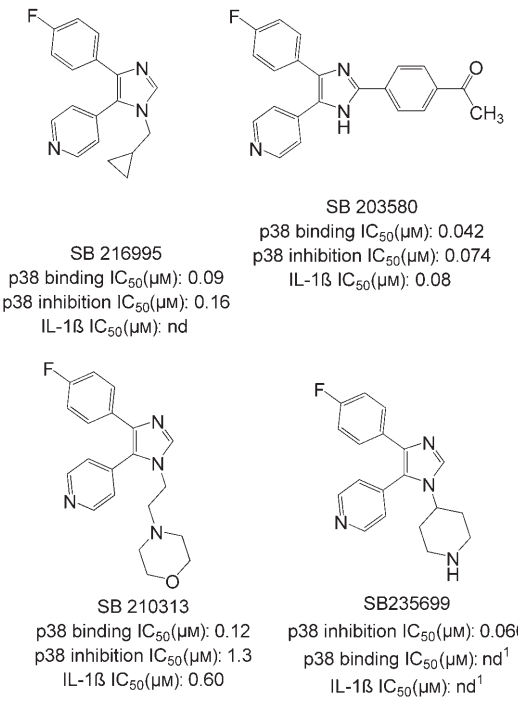


Figure 14. N-substituted pyridinyl imidazoles: SB216995, SB235699, and SB210313; [1]not determined.

For ATP competitive inhibitors to show in vivo efficacy, they must maintain their bioactivity in the presence of millimolar levels of ATP. To understand how exactly this is accomplished, several studies were undertaken.[113] Crystallographic and kinetic experiments have shown that all pyridinyl-imidazole p38 inhibitors bind at the ATP binding site of p38 and compete with ATP for binding to active, phosphorylated p38. In crystal structures of kinases solved with bound ATP, the N-terminal and the C-terminal domains work together to form a catalytic pocket capable of binding all substrates in the proper orientation. In the crystal structure of inactivated p38 however, the two domains of the kinase are misaligned, suggesting that ATP cannot bind to inactivated p38. It has also been shown that p38 inhibitors as SB203580, bind equally well to both the activated and inactivated forms of the enzyme. Therefore, when p38 is in its inactivated form, ATP is noncompetitive with those inhibitors. This fact leads to a thermodynamic advantage for the inhibitor in vivo, where the high ATP concentration would require very high inhibitor concentrations to effectively compete with ATP. Experimental data suggest a mechanism by which a kinase inhibitor that competes with ATP can function in vivo at concentrations approximately equal to its K_i value. The inhibitor may bind to a form of the enzyme that is inaccessible to ATP and thereby prevents transformation of the enzyme into its ATP accessible form.

SB203580 is a reasonably selective and cell permeable inhibitor that inhibits p38α kinase activity in vitro with an IC_{50} of 50 nM against 100 μM ATP. Using SB203580, several SAR studies leading to selectivity and potency improvements and novel p38 chemotypes discovery programs had been undertaken and recently reviewed.[114–118]

The development of SB203580, and of first generation p38 inhibitors in general, into anti-inflammatory drug was obstructed by its severe liver toxicity, as the pyridinyl imidazole were found to interact with hepatic cytochrome P450 (CYP450) enzymes involved in drug metabolism.[119] It remains unclear

whether this unwanted side effect is caused by the pyridine or the imidazole ring. Strategies to dissect inhibition of p38 from interference with cytochrome P450 have included the replacement of the pyridine ring with other hydrogen bond acceptors,[119,120] the introduction of sterically demanding substituents at the 2 position of the pyridine ring,[120,121] the introduction of substituents at the imidazole ring nitrogen adjacent to the pyridine ring,[120,121] and the replacement of the imidazole ring with other 5- or 6- membered heterocycles.[111,112,122–124]

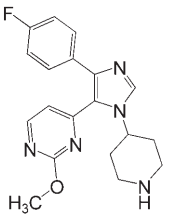
Table 4. Reduced Inhibition of Human CYP450 Isoforms by the N1-Substituted Pyridinylimidazole SB210313.

Code	Inhibition of CYP450 isoforms [%][a]				
	1A2	2C9	2C19	3A4	2D6
SK&F 86002	85	80	64	19	22
SB203580	61	75	85	61	67
SB210313	<50	<50	<50	<50	86

[a] Inhibition (%) of CYP450 isoforms at test compound concentration of 10 μM.

6.3.3. N-Substituted Imidazoles Inhibitors

In addition to 2,4,5-triarylimidazoles, well represented by SB203580, the group at GSK has also prepared a vast number of 1,4,5-substituted imidazole inhibitors of p38 MAP kinase and cytokine release.[110,125] Originally it had been observed with bicyclic imidazoles such as SK&F 86002 and its analogues that the correct regiochemistry at the core heterocycle is crucial for efficient binding to p38 (Figure 13).[125] For various N-substituted imidazole inhibitors of p38 it was confirmed that only at the imidazole ring nitrogen adjacent to the pyridine ring are substituents tolerated without loss of activity.[110,120,125] Initially N-substituted imidazoles were mainly prepared to reduce interaction with CYP450 enzymes; however, appropriate substituents at this position were found to contribute to enhanced p38α binding[104,126] and oral anticytokine activity.[126]



SB 242235
p38 inhibition IC_{50} (μM): 0.019

Figure 15. Structure of SB242235.

Boehm and Gallagher have reported that N-substituted pyridinyl imidazoles bind to p38 MAP kinase with generally lower affinity than their N-unsubstituted counterparts[110,125] (Figure 14). The lower binding affinity also translated into weaker inhibition of IL-1β release from PBMC (peripheral blood mononuclear cells), although the more potent compounds (that is, SB216995, SB210313) still displayed anti-TNFα activity in vivo.

SB210313, furthermore, interferes slightly less with most of the CYP450 enzymes than either SK&F86002 or SB203580 (Table 4).[119]

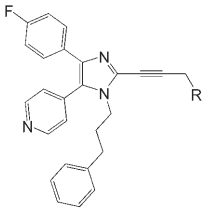
The basic piperidine substituent of SB235699 (VK-19911, HEP 689)[104] interacts with Asp 168, and this additional interaction may account for the outstanding bioactivity observed for SB235699 compared to other N-substituted pyridinylimidazoles (Figure 15).

Several groups have investigated the properties of this compound. Vertex investigators have reported the co-crystallisation of p38 MAP kinase with SB235699.[104] GSK and Leo Pharmaceuticals have jointly developed SB235699 as a topical anti-inflammatory agent and have advanced this drug candidate into phase 1 clinical trials for the treatment of psoriasis, contact eczema, and atopic dermatitis.

Several groups have undertaken studies on other N-substituted pyridinylimidazoles. A series of piperidin-4-ylimidazoles and N-methylpiperidinylimidazole were realised;[126] those investigations showed that substitution on the 2-aminopyrimidine nitrogen in some cases reduced p38 inhibition, but simultaneously improved oral activity. The 2-alkoxypyrimidine SB242235[127] (Figure 15) has been used as a pharmacological tool in various models of inflammation and it was selected as a clinical development candidate.

Besides SB242235 and SB235699, another N-substituted imidazole is at present in clinical development, namely RWJ67657. Workers at the R.W. Johnson Pharmaceutical Research Institute investigated the binding kinetics to p38 in a series of 1,2,4,5-tetrasubstituted imidazoles exemplified by RWJ67657 and RWJ67671.[128] For these compounds, the same rank order of potency was found in the isolated p38α assay and in the cell-based TNF-α release assay (Table 5). The weaker binding inhibitors (cf. RWJ67671) differ from the more potent ones, such as

Table 5. Binding Kinetics of SB203580, ATP, and 1,2,4,5-Tetrasubstituted Imidazoles Developed at RW Johnson Pharmaceutical Research Institute.



Code	R	IC_{50} [nM]		K_d [s^{-1}][c]	K_a(M-1XS^{-1})[d]	K_d [nM][e]
		p38α[a]	TNF-α[b]			
SB203580	na[f]	79 ± 12	23 ± 1	0.017	8.05×10^5	21
RWJ67657	CH_2OH	30 ± 3	3.2 ± 0.1	0.003	7.12×10^5	5
RWJ67671	C_5H_{11}	1700 ± 222	81 ± 6	0.010	1.09×10^5	89
ATP	na	na	na	0.016	1.24×10^4	1280

[a] Inhibition of activated p38α MAP kinase. [b] inhibition of LPS-stimulated TNF-α release from human PBMC. [c] dissociation rate. [d] association rate. [e] dissociation constant. [f] not applicable.

RWJ67657 and SB203580, mostly in their association rates. The difference between the most potent inhibitors, in turn, is determined by their dissociation rate (RWJ67657 versus SB203580), that is, the strength of protein–inhibitor interactions. Interestingly, the binding characteristics of ATP are similar to those of the weak inhibitors. With regard to the correlation between K_d and IC_{50} values in the cell-based assay, the authors conclude that these imidazoles bind to the unactivated form of the enzyme. ATP, on the other hand, has only weak affinity for unactivated p38,[128] and this crucial difference may explain why inhibitors binding in the ATP cleft of p38 are able to maintain their efficacy in cell-based assays. The pharmacology of RWJ67657 as the most potent inhibitor from this series was further evaluated and the compound was advanced into early clinical trials with indications as an antiarthritic drug and agent for inflammatory bowel disease.[129]

6.3.4. *Allosteric* N,N′*-Diarylurea-based Inhibitors: BIRB796*

Research groups at Vertex,[130,131] Bayer, and Boehringer have independently described *N*,*N*′-diarylureas as inhibitors of p38 cytokine release.[132–134] While binding to the ATP cleft of p38, inhibitors of this class adopt a binding mode distinct from that of both ATP competitive and the diaryl heterocycles-based inhibitors.

The group at Boehringer has demonstrated that the potent N-aryl-N′-pyrazolylurea BIRB796 (Figure 16) stabilises a conformation of p38 which cannot be accessed by ATP, and BIRB796 is therefore best described as a noncompetitive, allosteric inhibitor of p38.

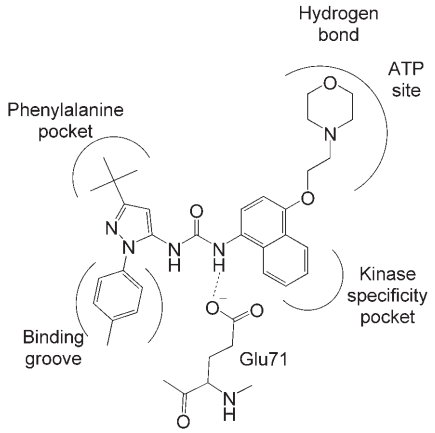


Figure 16. Structure of BIRB796 and its main interaction with p38α.

Upon binding of BIRB796 to p38, a considerable conformational change takes place in the conserved Asp 168/Phe 169/Gly 170 (DFG) region. The switch from the "DFG-in" to the "DFG-out" conformation arranges the side chain of Phe 169 in a way that leads to a steric clash with the phosphate groups of ATP. At the same time a large hydrophobic pocket is revealed, which is capable of accommodating the *tert*-butyl group characteristic for potent *N*,*N*′-diarylurea inhibitors. The tolyl substituent on the pyrazole ring has favourable interactions with the hydrophobic portions of the side chain of the conserved Glu 71 in helix αC. This tolyl group also causes a conformational change in the Glu 71 side chain such that only one of the urea NH groups can hydrogen bond with this residue (Figure 16). As previously pointed out, it has been speculated that this conformational rearrangement of the protein is the rate determining step responsible for the slow binding kinetic observed for these allosteric p38 inhibitors. The morpholino ether oxygen makes a hydrogen bond with the backbone NH of residue 109, equivalent to that made by the N1 atom of the adenine base of ATP. In addition to establishing interactions in the ATP pocket, the morpholino group also improves the physical-chemical properties of the otherwise lipophilic inhibitor.[132]

BIRB796 is in development by Boehringer Ingelheim as a treatment for rheumatoid arthritis and other inflammatory conditions such as Crohn's Disease and psoriasis. BIRB796 has a picomolar affinity for p38α ($K_d = 0.1$ nM) and inhibits the enzyme with an $IC_{50} = 63$ nM.[135]

By September 2001, the in vivo effects of BIRB796 on neutrophil activation had been tested in phase I clinical trials. In a single-escalating dose, randomised, placebo-controlled, double blind, 64-patient trial, BIRB796 was well tolerated at all dosages.

In 2001, a randomised, parallel-group, double-blind, placebo controlled study into the efficacy and safety of different doses (5, 10, 20, and 30 mg) of BIRB796, taken orally twice daily during 4 weeks in patients with active rheumatoid arthritis in whom at least one DAMARD has not been effective was initiated. A potentially dose limiting side effect, liver transaminase values above the upper limit of normal, has been reported.

6.3.5 *Structurally Diverse Clinical Candidates*

Apart from the imidazole nucleus, a multitude of other monocyclic and fused heterocycles was employed as scaffolds for the essential diaryl pharmacophore. The trisubstituted pyrazole developed at Pfizer, SD06 (Table 6), is an example. Pfizer recently presented data[136] on the synthesis and development of this orally active p38 inhibitor. The compound exhibits significant functional selectivity for p38α ($IC_{50} = 80$ nM) over p38β ($IC_{50} = 26\,000$ nM). The binding modus of SD06 has been described. As the substituent at the 3-position of the pyrazole ring approaches the ATP binding site of p38 kinase, a hydrophobic cavity in the p38 kinase develops around the 3-position substituent at the binding site. This hydrophobic cavity is believed to form as the 3-position substituent binds to a specific peptide sequence of the enzyme. In particular, it is believed to bind to the side chains of Lys 52, Glu 59, Leu 73, Ile 82, Leu 84, Leu 101, and the methyl group of the Thr 103 side chain of p38 kinase at the ATP binding site. The pyrimidine ring at the 4-position of the pyrazole ring, as already described for diarylimidazoles, brings a suitable hydrogen bond acceptor functionality. This acceptor bonds to the backbone N-H of the Met 106 residue while one edge of this substituent is in contact with bulk

Table 6. p38 MAPK Inhibitors in Clinical Development.

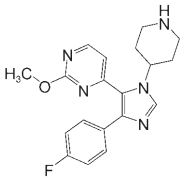
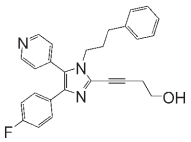
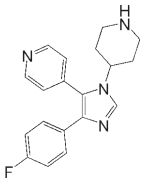
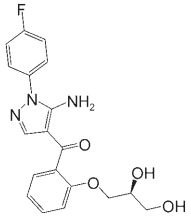
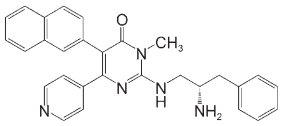
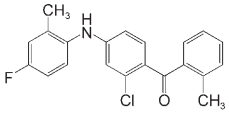
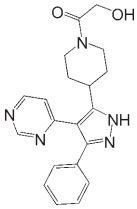
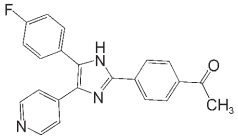
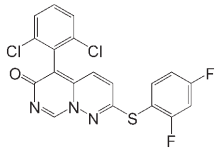
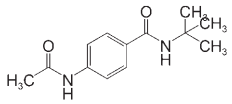
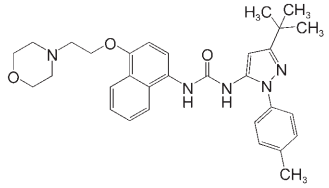
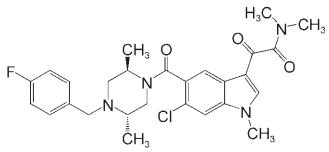
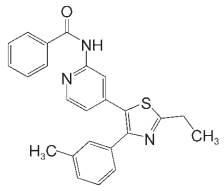
Drug name	Structure	Therapeutic group	Mechanism of action	Organization
SB242235		Antiarthritic Drugs	p38MAPK Inhibitors	GlaxoSmithKline (Originator)
RWJ67657		Antiarthritic Drugs Inflammatory Bowel Disease, Agents for	IL-1β Production inhibitors, TNFα production Inhibitors, p38MAPK Inhibitors	R.W. Johnson (Originator)
SB235699		Antipsoriatics, Atopic Dermatitis, Agent for. Topical Antiinflammatory agents	p38MAPK Inhibitors	GlaxoSmithKline (Originator) Leo
RO3201195		Rheumatoid Arthritis, treatment of	IL-1β Production inhibitors, IL-6 Production inhibitors, inhibitors of Signal Transduction pathways TNFα production Inhibitors, p38MAPK Inhibitors	Roche (Originator)
SB281832	NA[a]	Antiallergy/Antiasthmatic Drugs. Inflammatory Bowel Disease, agent for. Rheumatoid Arthritis, Treatment of	p38MAPK Inhibitors	GlaxoSmithKline (Originator)
SCIO323	NA[a]	Antiarthritic Drugs	p38MAPK Inhibitors	Scios (Originator)
AMG528		Antiarthritic Drugs	Inhibitors of Signal transduction pathways TNFα production Inhibitors, p38MAPK Inhibitors	Amgen (Originator)
EO1606		Acne Therapy Atopic Dermatitis, Agent for	p38MAPK Inhibitors	Leo (Originator)
SD06		Antiarthritic Drugs	Inhibitors of Signal transduction pathways, p38α MAPK Inhibitors	Pfizer (Originator)
PS540446	NA[a]	Rheumatoid Arthritis, Treatment of	Inhibitors of Signal transduction pathways, p38 MAPK Inhibitors	Bristol-Myers Squibb (Originator)
KC706	NA[a]	Antipsoriatics Rheumatoid Arthritis, treatment of	Inhibitors of Signal transduction pathways, p38 MAPK Inhibitors	Kemia (Originator)
SB203580		Antiarthritic Drugs, Inflammatory Bowel Disease, agent for	Calcium Channel Activators Stress-Activated Protein (SAP/Jun) Kinase Inhibitors p38 MAPK Inhibitors	GlaxoSmithKline (Originator)

Table 6. (Continued)

Drug name	Structure	Therapeutic group	Mechanism of action	Organization
VX745		Myelodysplastic Syndrome Therapy Rheumatoid Arthritis, Treatment of	Antiinflammatory Drugs p38 MAPK inhibitors	Kissei Vertex (Originator)
CPI1189		AIDS Dementia, Treatment of Alzheimer's Dementia, Treatment of Cognition Disorders, Treatment of Neuropatic Pain, Treatment of	Antioxidants Apoptosis Inhibitors p38 MAPK inhibitors	Centaur (Originator) Renovis
BIRB-746		Antipsoriatics Inflammatory Bowel Disease, Agent for Rheumatoid Arthritis, Treatment of	Inhibitors of Signal Transduction Pathways p38 MAPK Inhibitors	Boehringer Ingelheim (Originator)
SCIO469		Multiple Myeloma Therapy Myelodysplastic Syndrome Therapy Rheumatoid Arthritis, Treatment of	Inhibitors of Signal Transduction Pathways p38 MAPK Inhibitors	Scios (Originator)
VX702	NA[a]	Inflammatory Bowel Disease, Agent for Osteoarthritis, Treatment of Rheumatoid Arthritis, Treatment of Treatment of Disorders of the Coronary Arteries and Atherosclerosis	Inhibitors of Signal Transduction Pathways p38 MAPK Inhibitors	Kissei Vertex (Originator)
SB681323	NA[a]	Atherosclerosis Therapy Chronic Obstructive Pulmonary Disease (COPD), Treatment of Rheumatoid Arthritis, Treatment of	Inhibitors of Signal Transduction Pathways p38 MAPK Inhibitors	GlaxoSmithKline (Originator)
TAK715		Rheumatoid arthritis, Treatment of	Inhibitors of Signal Transduction Pathways TNF-α Production Inhibitors p38α MAPK Inhibitors	Takeda (Originator)
SB856553	NA[a]	Atherosclerosis Therapy Chronic Obstructive Pulmonary Disease (COPD), Treatment of Rheumatoid Arthritis, Treatment of	Inhibitors of Signal Transduction Pathways p38α MAPK Inhibitors	GlaxoSmithKline (Originator)

[a] Not available.

solvent. SD06 had been reported to bind to phosphorylated as well as unphosphorylated p38α (using surface plasmon resonance technology). SD06 inhibits LPS-stimulated TNFα production in HWB in vitro with an IC_{50} of 1.5 μM. The compound showed efficacy in a number of arthritis animal models (reduction of paw swelling, bone destruction, and cytokine suppression). In a three month toxicity study, SD06 showed no adverse CNS effects in monkeys and rats, despite its ability to cross the blood/brain barrier. Some adverse effects were observed on the skin and in the gastrointestinal tract with prolonged dosing in vivo. Pfizer advanced SD06 to phase I clinical studies and pharmacodynamic effects were reported in a human ex vivo model with an EC_{50} value = 50.6 nM for inhibition of LPS-induced TNFα production, corresponding to an ED_{50} = 11.3 mg. Currently, SD06 is being tested for its anti-TNFα activity in phase I clinical trials in healthy volunteers following endotoxin challenge.[136]

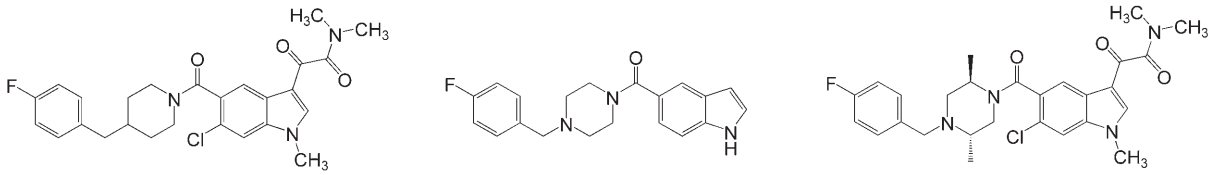
RO3201195 (Table 6) is a pyrazole ketone developed at Roche by applying a high throughput screening (HTS) approach to develop multiple leads series from distinct scaffolds.[137] This HTS campaign led to the identification of several pyrazole ketones which were found to inhibit p38α at low micromolar concentrations. Co-crystallisation of RO3201195 with unphosphorylated p38α revealed a novel binding motif in the ATP binding site that was previously unknown for p38 kinase inhibitors.[138] Particularly, two hydrogen bonds formed between the amine of the inhibitor and the backbone His 107 and with the side chain hydroxyl group of Thr 106. The latter residue is present in approximately 205 of human kinases and, according to authors, an inhibitor with the potential to form a

hydrogen bond to Thr 106 would confer improved selectivity versus other kinases which lack this residue. In addition, the pyrazole ketones make a hydrogen bond between the benzoyl oxygen and the main chain of Met 109. This key interaction is observed in all reported structures of ATP competitive p38 inhibitors. Additional binding energy is generated by the position of the N-phenyl ring into the hydrophobic pocket which is partially defined by the specificity residue Thr 106. The glycerol monoether group attached to the *meta* position of the benzoyl ring of RO3201195 confers good physicochemical properties (solubility and oral bioavailability) yet retaining potency against p38 and also affords good inhibition of LPS induced cytokine production in undiluted human whole blood, and efficacy in in vivo inflammation models. RO3201195 inhibits p38 with an $IC_{50} = 700$ nM. RO3201195 binds to p38α (both, non-phosphorylated and the Thr 180 and Tyr 182 phosphorylated) and p38β isoforms but does not bind to either the p38γ or p38δ isoform. Based on the range of efficacy in the biological models, the desirable metabolic profile, and the favourable preclinical safety pharmacology, RO3201195 was selected as a clinical candidate for the treatment of rheumatoid arthritis.

AMG548 (Table 6), in clinical development as an antiarthritic drug, contains the classical SB203580 diaryl interaction but changing the core heterocycle to a 6-membered ring. AMG548 was in phase I human trials and data supporting the advancement of this compound had been presented. The pyridinone inhibitor interacts with p38α through the classical Met 109-pyridine hydrogen bond. The C2 carbonyl and Lys 53 also form a hydrogen bond. Preclinical data showed that AMG548 is efficacious in both acute (LPS-induced TNFα production in mice) and chronic (CIA and adjuvant induced-arthritis (AdA) in Lewis rats) models of arthritis. In these in vivo models AMG548 ameliorated acute production of proinflammatory cytokines ($ED50 = 0.5$ mg kg^{-1}) and inhibited the symptomatic and structural manifestations of severe joint destruction in CIA and also AdA. Pharmacokinetic evaluation in both rat and dog revealed good terminal half-life ($t_{1/2}$) and suitable oral bioavailability for once-daily oral dosing in humans. Evaluation in 14 day rodent toxicology studies and dog QTc studies demonstrated the desired safety multiples with no QTc prolongation in dogs. Based on overall profile of AMG548, the molecule was advanced to safety assessment and subsequently to phase I clinical trials. AMG548 was the first internal small-molecule clinical candidate for Amgen and it progressed from clinical candidate portal to first-in-human (FIH) dosing in less than one year. In the FIH study, AMG548 was dosed orally at 0.3 to 300 mg (once daily). AMG 548 had linear pharmacokinetics with a mean terminal elimination $t_{1/2}$ of 24 h. The compound demonstrated 30 to 955 inhibition of ex vivo whole blood LPS-induced TNFα and IL-1β cytokine production at oral doses of 3, 10, 30, 60, 100, and 300 mg (once daily) in healthy male volunteers. At doses of 60 to 300 mg, $>85\%$ inhibition was observed beyond 24 h, following a single oral dose. These data demonstrate that AMG548 possesses suitable pharmacodynamics/pharmacokinetics for once-daily oral dosing. AMG548 was further evaluated in a 14 day multiple dose study in 54 healthy male volunteers. Isolated liver enzyme elevations were observed in nine out of 54 individuals randomised to AMG548, and one of 18 individuals randomised to placebo. These hepatic transaminase levels were not associated with increases in bilirubin or alkaline phosphatase. Further development of AMG548 was suspended because of random liver enzyme elevations that were not dose- or exposure-dependent.

In March 2005, Takeda reported the discovery of TAK715 (Table 6) as a potent and orally active antirheumatic agent.[137,139,140] The thiazole-benzamide was shown to selectively inhibit p38α ($IC_{50} = 7.1$ nM) and block LPS-induced TNF-α production in THP-1 cells (human monocytic cell line) ($IC_{50} =$ 48 nM). In an in vivo model of acute inflammation in mice, TAK715 inhibited the LPS-induced release of TNF-α by 88% after a 10 mg kg^{-1} oral dose. The compound was reported to have been advanced into phase II clinical trials.

Scios (a subsidiary of Johnson & Johnson) is developing a series of small-molecule, orally available, p38 inhibitors. Recently the structure of SCIO-469 (Table 6, Figure 17) was disclosed revealing that this molecule belongs to a class of indole amides explored by the company in a p38 inhibitors development program started from an HTS hit: an indole carboxamide derivative (Compound **9**, Figure 17), with weak inhibitory activity for p38α.[141] As shown by data of SCIO469 and SX011



SX011

p38a K_i = 9 nM
p38ß K_i = 90 nM
ERK2 > 300,000 nM
JNK1 > 300,000 nM

Compound **9**

SCIO469

p38a K_i = 9 nM
p38ß K_i = 98 nM
ERK2 > 5000 nM
JNK1 > 5000 nM
Lck > 5000 nM
WB TNF-a IC_{50} = 300 nM
Rat carrageenan ED50 ~ 30 mg/Kg
Rat $t_{1/2}$ = 30 min

Figure 17. Scios compounds; SX011, compound **9**, SCIO469.

(Figure 17), substitutions at both the 3-position of indole and the piperazine ring improved potency dramatically. The carbonyl amide forms the crucial hydrogen bond with NH of Met 109 while the polar α-keto amide moiety extends toward solvent, allowing the 6-Cl-indole and the F-phenyl moieties to bind into lipophilic pockets. SCIO469 is highly potent against p38α and p38β (Figure 17), blocking the LPS-induced release of TNF-α from human whole blood, and inhibiting LPS-induced IL-1β release from human PBMCs in a dose-dependent manner.

A phase I study investigated the safety, pharmacodynamics, and pharmacokinetics of single ascending oral solution doses from 0.03 to 5 mg kg^{-1} SCIO469 in healthy volunteers. The compound was safe and well tolerated, the only reported side effect being mild, transient light-headedness at highest doses which suggests that SCIO469 crosses the blood/brain barrier. By October 2004, a phase II study to determine the analgesic efficacy of SCIO469 in acute post-surgical dental pain had also been completed. All SCIO469 treated patients showed a significantly longer time to rescue medication compared with placebo. This study represents the first clinical demonstration of acute analgesic effects by inhibition of p38.

Scios is the first company to disclose clinical exploration of p38 inhibitors in multiple myeloma (MM). In 2004, Scios demonstrated the ability of SCIO469 to strongly reduce p38 phosphorylation in bone marrow stromal cells, and to inhibit the production of IL-6, VEGF, IL-1β, receptor of activated NF-κB ligand, and prostaglandin E2. In combination with proteosome inhibitors, SCIO469 enhanced the reduction in multiple myeloma cell proliferation and potential proteasome inhibitor-induced apoptosis. SCIO469 is at present still under phase II clinical investigation.

Vertex Pharmaceutical Inc was one of the first companies to disclose, in 2001, a nonpyridinylimidazole-based p38 inhibitor. VX745 (Table 6), a first-generation p38 inhibitor, is potent against p38α (IC_{50} = 10 nM) and blocks the synthesis of TNFα in human whole blood (IC_{50} = 177 nM)and PBMCs (IC_{50} = 56 nM) after LPS stimulation in vitro.[135,137] The compound VX745 binds to the ATP site of p38α by a hydrogen bond with the NH of Met 109 backbone, whereas the lipophilic "back pocket" is occupied by the difluorophenyl group.[142] VX-745 was shown to have anti-inflammatory activity in rodent models and to penetrate the blood/brain barrier. The compound was generally well tolerated with the most frequently reported adverse event being elevation in liver transaminases. No CNS side effects were seen in humans, however, the drug was subsequently suspended following the observation of neurological effects in dogs.

VX702 (Table 6), the structure of which has not been disclosed, was chosen as a back up compound because of its inability to cross blood/brain barrier. A phase I safety and pharmacokinetic study showed that VX702 (2.5 to 80 mg) was well-tolerated. In an ex vivo assay primed with LPS, VX702 inhibited IL-6, IL-1β and TNFα production (IC_{50} = 59, 122 and 99 ng mL^{-1}, respectively). In a phase IIa study completed in October 2004, VX702 demonstrated safety and tolerability in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI). VX702 significantly reduced serum levels of the inflammatory biomarker C-reactive protein (CRP) in patients undergoing PCI, and CRP remained significantly lowered up to four weeks after the five-day dosing period.

In June 2005, Vertex announced initiation of a three-month, double blinded, randomised, placebo-controlled, phase II study to assess two doses of VX702 in rheumatoid arthritis. The compound will be dosed once daily without concomitant methotrexate.

Ketone-based p38 inhibitors have been pursued by multiple companies. Leo pharmaceutical has explored aminobenzophenone p38α inhibitors.[143] The aminobenzophenone EO1606 (Table 6) is a potent and selective inhibitor of p38 MAPK (isoforms α and β) and of the upstream kinase MKK6. The compound was tested in an animal model of noninflammatory acne, the rhino mouse. Its efficacy in this model is comparable to that of the retinoids. The compound is at present in clinical development as agent for atopic dermatitis and acne therapy. The in vivo models tested were recently reviewed by Petersen.[144]

As reported in Table 6, additional compounds are undergoing clinical development. GlaxoSmithKline undertook clinical development of compounds such as SB-281832, SB-681323, SB-856553, for which no structures had been to date disclosed. Also Centaur, Bristol-Myers Squibb, and Kemia are developing p38 kinase inhibitors that are being clinically evaluated. As a result of the lack of specific information regarding the development status of those compounds, we chose not to include them in this review.

7) Discussion and Conclusion

Pharmaceutical companies are facing increasing interest in new target identification and validation as pointed out by a recent review.[145] In particular, extensive efforts are being made in the field of kinase inhibitors research and development and the past ten years of effort in this field have altered our perception of the potential of kinases as drug targets to the pharmaceutical industry. Therefore, in the drug discovery process, the selection of relevant, susceptible protein kinase targets combined with searches for lead drug candidates have become a crucial approach. It is evident that kinases, which play an integral part of controlling cellular signalling, would be of intrinsic interest as potential targets for drug design.

Recently, researchers have become especially adept at developing potent protein kinase inhibitors with different activities. Several examples of anticancer therapeutic agents are available, that is, STI-571 (Gleevec, Imatinib),[146] trastuzumab (Herceptin),[147] and the EGFR inhibitor ZD-1839 (Iressa). Numerous other kinase inhibitors are currently undergoing clinical trials or clinical development.

Some questions are nevertheless unanswered, mostly related to the great number of known kinases in the human genome, to their similarity with each other, to the existence of functionally redundant kinases for specific pathways, and also because the connection between particular pathways and diseases is not always clear. This situation is not unique for the kinases, which are relatively young targets in the drug discovery pro-

cess. Similar questions arise for many drug projects, and initially, all of them are unanswered, along with all of the other normal questions of drug development.

Unacceptable safety profiles have been the cause for clinical attrition of initially promising inhibitors. Liver enzyme elevations were reported with BIRB796 clinically.[148] The Vertex drug, VX-745, was discontinued because of an unanticipated CNS toxicity in dogs during a six month safety study. Unfortunately, based on the very limited information associated with these adverse effects, it is impossible to determine the basis of these issues. Nevertheless, several studies had been undertaken to overcome chemical-based toxicity problems and, particularly in the case of p38 MAPK inhibitors, it is now clear what type of modifications could result in an improvement.

Furthermore, as a consequence of the wide-ranging regulatory roles of kinases in diverse cellular processes, the possibility of an adverse event resulting from undesired pharmacological activity is the major concern for protein kinase inhibitor drug class. Added to this are safety issues related to the potential lack of specificity[31] that plague all kinase inhibitors. How selective is selective enough?

To improve kinase selectivity of certain inhibitors, development of compounds that bind preferentially to the inactive forms of protein kinases, or which prevent one protein kinase from activating another, is a promising approach. Detailed understanding of the catalytic and regulatory properties of kinases can contribute significantly to development of novel kinase inhibitors including more specific allosteric inhibitors. Specificity problems, as discussed, arise from the evidence that members of the MAPK family share a high degree of conserved domain structure, particularly in the ATP site, and many residues that make contact with p38 inhibitors are conserved throughout the family. The exception is Thr 106, which has therefore been implicated as a critical determinant for the specificity of these compounds. Many results emphasised the critical nature of Thr 106 in imparting the good selectivity seen for p38 inhibitors as a class of compounds binding to the ATP-binding site of a kinase.

A second issue concerning activity of ATP-competitive inhibitors is the level of activation of the enzyme. In crystal structures of kinases solved with bound ATP, the N-terminal domain and the C-terminal domain work together to form a catalytic pocket capable of binding all substrates in the proper orientation. In the crystal structure of inactivated p38 however, the two domains are misaligned, suggesting that ATP cannot bind to inactivated enzyme. The capability of inhibitors to bind equally well to both the activated and inactivated form of the enzyme could also represent a good opportunity to develop inhibitors with enhanced specificity and potency, underscoring the importance of studies on allosteric inhibitors.

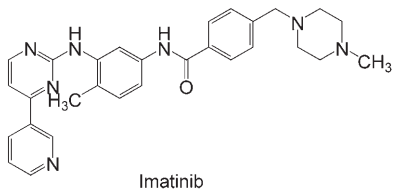
However, we must be aware of the fact that for protein kinases, it will be very unlikely to find a unique compound that specifically inhibits a selected single protein kinase, leaving the other more than 2500 protein kinases unaffected. The paradigm "one drug, one target, one disease" cannot hold true.

The potential use of protein kinase inhibitors for cancer treatment is increasing, as reviewed by Dancey and Sausville.[147] In particular, these authors discuss the need to diagnose the activation state of the kinase target in the tumours of patients entering clinical trials if the drug is to receive a fair assessment of its capabilities. Related to that is the consideration of when in the course of a tumour's evolution kinase-directed drugs are most likely to have an impact. Clinical data from STI571 in CML indicate that kinase inhibitors could have a great impact on preventing the evolution of tumours to a fully malignant state, or in treatment of "small volume disease" to prevent the development of clinically overt metastatic disease. The therapeutic inactivation of an essential protein kinase creates selective pressures, such that tumour cells evolve a variety of routes to resistance. These include producing a drug-resistant variant of the targeted protein, substituting its cellular function by upregulating alternate pathways, and by increasing the expression and function of transporters involved in drug efflux. A general drawback of target-specific monotherapy therefore derives from the fact that a single genetic alteration conferring target resistance to an individual tumour cell can eventually lead to relapse. The ability of kinases to mutate in response to the selective pressure created by drug treatment provides a strong rationale for hitting more than one essential target at the same time in the tumour cells.[149] Multitargeted therapy can be achieved with either a combination of medicines or single promiscuous drugs that act on a set of disease-relevant proteins. Protein kinases, which share a relatively conserved ATP-binding site, are amenable to the latter concept of targeted polypharmacology.

A possible strategy would be to combine agents that target components in parallel signal transduction pathways, such as a mitogen-activated protein kinase kinase (MEK) inhibitor and an Akt inhibitor. UCN-01, which inhibits the activation of Akt, and the MEK antagonist CI-1044 (PD184352), seem to synergistically interact following UCN-01 activation of mitogen-activated protein kinase (MAPK) signalling.[150] Of great interest is also the possibility to combine kinase inhibitors with standard cytotoxic agents.[151,152]

The idea of treating complex disease such as cancer by aiming for several targets at once is becoming more accepted,[153] and an increasing number of researcher claimed that "magic bullet" drugs designed to hit a single target might not be the answer to treat not only cancer but also cardiovascular diseases. Common disorders tend to result from multiple molecular abnormalities, not from a single defect. Drews[154] follows the case histories of five modern drugs (COX2 inhibitors, Natalizumab, Imatinib, Bevacizumab, and Enfurvirtide) as a basis of reflection on the magic bullets concept and, more in general, the actual state of drug discovery. Imatinib, a good example of a mixed-drug (see Figure 18) already discussed in this survey, represents a landmark in kinase drug discovery and development. The studies and investments undertaken by Novartis led not only to a different future for a drug that started with an "orphan drug" prospective, but also have changed the perspective on a not very common disease such as chronic myeloid leukaemia (CML).

From this point of view, the data published by Fabian[31] concerning the specificity profiles for different clinical kinase



Imatinib

p210BCR-^{ABL}: 0.25 μM
PDGFR: 0.1 μM
c-KIT: >0.1 μM
EGFR: >100 μM
c-ERBB: >100 μM

Figure 18. Chemical structure of Imatinib.[154] As well as inhibiting the BCR-ABL kinase, Imatinib is a potent inhibitor of c-KIT and the platelet-derived growth factor receptor. IC_{50} values for inhibition of autophosphorylation are given.

inhibitors are not so negative. The Ambit researchers undertook a study that led to an efficient way to determine kinase inhibitor specificity by measuring binding of small molecules to the ATP site of kinases.

Members of the p38 MAP kinase family, like the extracellular related (ERK) and c-jun N-terminal (JNK) kinase families, are ubiquitously present in all cell types. This evidence combined with their roles in different processes and in vitro evidences suggests that there may be other therapeutic uses for p38 kinase inhibitors in addition to their well-known potential for treatment of classic inflammatory diseases, thus underlying the need of further exploration of kinase inhibitors potential therapeutic utility. The characteristics that, at the same time, lead to potential side effects have already been mentioned. Dambach[155] from Bristol Myers Squibb recently reviewed those potential side effects that could be associated with inhibition of p38α/β MAP kinases. The outcome of this survey indicates which organs should be closely monitored for the evaluation of adverse pharmacological effects.

In conclusion, the large body of information currently being generated around protein kinases as drug targets, coupled with the significant number of companies pursuing lead compounds, should provide a number of clinical compounds in the near future. Nevertheless, many different aspects must be considered. Are protein kinases drug targets? Yes, but difficult ones!

Acknowledgements

The authors are grateful to Dr. David Domeyer for the molecular modeling data, and to Franey Nogueira for the graphic support. The authors would also to thank Frank Lehmann for proof reading.

Keywords: antitumor agents · drug targets · MAP kinase · new chemical entities

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Received: November 23, 2006
Revised: March 14, 2007
Published online on May 31, 2007

