Structure-Activity Relationships of p38 Mitogen-Activated Protein Kinase Inhibitors

Jordi Bolós*

Prous Institute for Collaborative Biomedical Research; Barcelona Science Park, Laboratory P1C41, 08028 Barcelona, Spain

Abstract: Rheumatoid arthritis and other chronic inflammatory diseases constitute a major therapeutic challenge, usually not sufficiently met by the classical antiinflammatory medications. Recent research efforts provided new insights into the molecular basis of these pathologies and disclosed new opportunities for developing improved drugs directed to the chemical mediators of the disease. The enzyme p38 MAP kinase plays a central role in the signal transduction cascade that leads to the production of both the proinflammatory cytokines, TNF-α and IL-1β, thus representing an attractive therapeutic target for novel antiinflammatory therapies. A number of p38 inhibitors belonging to different structural families have been developed as potential antiinflammatory drugs, and some of them progressed into clinical trials. The initial pyridinyl imidazole inhibitors contributed to the identification and characterization of p38 MAP kinase as the molecular target of these new drugs, and were found to act as competitive inhibitors at the ATP binding site of the enzyme. A number of variations in the pyridine and imidazole rings were subsequently introduced. Other inhibitors structurally unrelated to the pyridinylimidazoles have also been developed, such as the pyridopyridazinones, diaryl ureas, aminobenzophenones and aromatic amides. One of these structural classes, the *N,N'*-diarylureas, has been found to interact with a distinct allosteric site of p38 MAP kinase and requires a deep conformational change prior to binding.

Keywords: p38 MAP kinase inhibitors, cytokine-suppressing drugs, antiinflammatory drugs, pyridinyl imidazoles, triaryl azoles, pyridopyridazinones, diaryl ureas, aromatic amides.

INTRODUCTION

Cytokines as Key Mediators in Inflammatory Diseases

Classical medical treatments for rheumatoid arthritis (RA) and other chronic inflammatory diseases usually provide only partial efficacy, and their long-term use is frequently associated with tolerance-limiting side effects [1]. In fact, conventional antiinflammatory drugs can be regarded as basically symptomatic treatments, being unable to modify the progress of the underlying disease. In recent years, although the pathogenesis of rheumatoid arthritis is not completely elucidated, significant advances have been made to our understanding of the mechanisms involved. These insights disclosed new opportunities for improved therapeutic approaches targeted to the chemical mediators of the inflammatory pathology [2].

Cytokines are small molecular weight proteins secreted mainly by cells of the immune system, which act as key mediators in physiological functions, such as host defense and inflammatory responses. The overproduction of cytokines is, however, associated to a number of pathologic conditions [3]. The proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β) are two of the most important mediators involved in the pathophysiology of several chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, septic shock, and osteoporosis. TNF-α is mostly produced by monocytes and tissue macrophages activated either by infectious or inflammatory stimuli, and elicits a variety of inflammatory effects [4]. IL-1β is another key element of inflammatory and immunologic reactions, playing a major role in bone resorption and erosive cartilage damage in the affected joints [5].

The crucial roles of TNF-α and IL-1β in chronic inflammatory diseases are supported by studies in animal models as well as therapeutic experience in humans. Blockade of TNF-α in animal models of RA has been shown to suppress joint swelling, while neutralization of IL-1 prevents cartilage damage and reduces the inflammatory cell recruitment [6]. Although TNF-α and IL-1β represent separate targets for antiinflammatory therapies, an *in vivo* synergistic interaction between the effects of these cytokines has been demonstrated [7,8]. According to this, combination therapy strategies against both TNF-α and IL-1β have been proposed as an advantageous treatment approach [8,9]. Furthermore, the current therapeutic experience in humans has provided definitive evidences of the beneficial effects of counteracting the increased cytokine levels through several different mechanisms [10]. The chimeric monoclonal antibody, infliximab [11], which neutralizes TNF-α, was approved in 1998 for the treatment of Crohn's disease and rheumatoid arthritis. Also the fully humanized antibody against TNF-α, adalimumab [12], was recently introduced into human therapeutics. A different strategy for blocking this cytokine is represented by etanercept, a soluble TNF-α receptor fusion protein which acts by binding competition with the endogenous TNF receptors [13], and was approved by the FDA in 1998 for the treatment of RA. Alternatively, blocking the effects of IL-1β by means of either the naturally occurring IL-1 receptor antagonist or its recombinant analogs

*Address correspondence to this author at the Prous Institute for Collaborative Biomedical Research; Barcelona Science Park, Laboratory P1C41, 08028 Barcelona, Spain; E-mail: JORDI-BOLOS@terra.es

1389-5575/05 $50.00+.00

showed significant improvements in patients with inflammatory diseases. The recombinant IL-1 receptor antagonist, anakinra [14], was recently approved for its use in cases of moderate to severe RA.

Whereas the above treatments proved to be effective in inflammatory diseases, and contributed to validate these cytokines as useful therapeutic targets, these protein drugs are still associated with important drawbacks limiting their use. Some of these are their high costs, lack of oral efficacy, short half-life, and potential immune reactions. Further potential limitations for these protein therapies are the increased risks for infections, malignancies and congestive heart failure [15]. Taken together, these facts suggest that small molecules displaying both inhibition of IL-1β and TNF-α would represent a highly desirable alternative for the treatment of RA. Indeed, inhibition of the effects of TNF-α by non-protein, small molecules is currently being explored through several different strategies [16,17]. Occupying a central position in the signal-transduction pathway, the mitogen-activated protein (MAP) kinase p38 [18] is a key enzyme involved in the production of several proinflammatory cytokines, including TNFα and IL-1β. Inhibition of p38 MAP kinase by small molecules was thus regarded as a promising target for the development of novel antiinflammatory drugs directed towards the chemical mediators of inflammation, and currently available evidences seem to confirm the validity of this approach.

The Mitogen-Activated Protein Kinase p38

The mitogen-activated protein kinases are a family of ubiquitously distributed enzymes which intervene in the signal transduction pathways from many cell surface receptors, and participate in the regulation of a number of physiological, as well as pathophysiological cellular processes, including cell growth, differentiation, and apoptosis. MAP kinases transfer a phosphoryl group from ATP to the hydroxyl groups of serine and threonine residues in a variety of protein substrates characterized by containing proline at the P+1 position. These enzymes are in turn activated upon phosphorylation by specific kinases (termed MAPKK or MKK) in response to diverse extracellular stimuli such as cytokines, hormones, and environmental stress [19,20]. The common mechanism of activation of MAP kinases involves an unusual double phosphorylation on both the threonine and tyrosine residues in a Thr-X-Tyr motif. The aminoacid residue X is specific for each MAP kinase subtype, being glycine for p38 MAP kinases [19,21].

A number of MAP kinases have been identified and cloned in mammals and yeasts. Each one is activated by different extracellular stimuli and participates in the signaling pathways of distinct physiological processes, characterized by their specific upstream activators and downstream substrates. The three main MAP kinase families are the extracellular signal-regulated protein kinases (ERK), the c-Jun amino-terminal kinases (JNK) and the p38 kinases (reactivating kinases, RK or CSBP2) [19,21]. The ERK are activated by growth factors and mitogenic stimuli, and participate in the control of cell proliferation and differentiation. In contrast, JNK and p38 kinases are activated by environmental stress factors, including the proinflammatory signals, and are often referred to as the stress-activated protein kinases (SAPK).

p38 MAP kinase, a 38 kDa protein, is a member of the MAP kinase family, whose activation occurs in response to different proinflammatory stimuli such as osmotic shock, endotoxin (bacterial lipopolysaccharide, LPS), UV light, or cytokines [22,23]. This activation is accomplished by double phosphorylation both on threonine^{180} and tyrosine^{182} [22] by specific kinases (mainly MKK3, MKK6) which are themselves activated by other kinases (MKKK) [24]. Among the downstream substrates in the p38 signaling pathways, some transcription factors and MAP kinase-activated protein kinases (MAPKAP) have been identified, which transduce signals to specific genes as well as to other cellular elements [23]. The transduction cascades mediated by activated p38 MAP kinase lead to the production of proinflammatory cytokines, mainly TNF-α and IL-1β (Fig. **1**) [18]. The inflammatory signal transduced by p38 also results in the activation of important enzymes involved in the inflammatory response, such as cyclooxygenase-2 and matrix metalloproteinases [25]. Not surprisingly, inhibition of this enzyme has attracted great interest as therapeutic target for intervention in different types of inflammatory diseases [26,27]. In addition to the mentioned role of p38 in the pathogenesis of RA [28], the enzyme has been proposed to participate in a number of physiological and pathological processes involving inflammation, angiogenesis and immune functions. Inhibition of p38 has demonstrated activity in a number of experimental disease models, including myocardial injury [29], stroke [30], cancer [31], Alzheimer's disease [32], and HIV replication [33], which could represent additional therapeutic applications for p38 MAP kinase inhibitors.

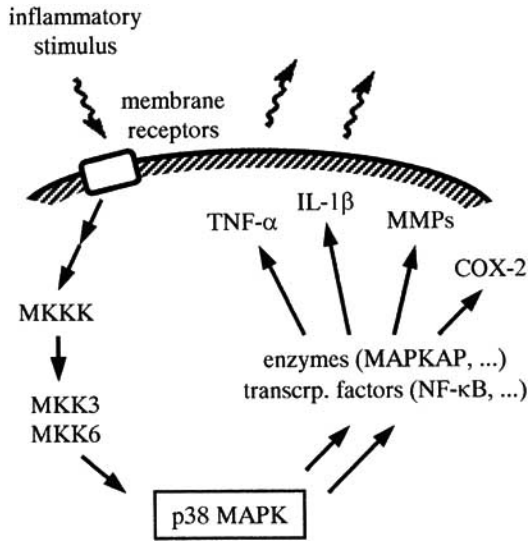


Fig. (1). Simplified representation of inflammatory signal transduction pathway showing the key position of p38 MAP kinase.

Further advances revealed the existence of at least four members of the p38 MAPK family, with different tissue distribution and different affinities for their activators, substrates, and also for many small molecule inhibitors [34,35]. Thus, most p38 inhibitors are active against p38α and p38β isoforms, but display poor or no activity against p38γ and p38δ. The physiological significance of these isoforms is not completely clear; however, MAP kinase

p38α, which is expressed in a wide variety of tissues and activated in response to inflammatory stimuli, is generally considered the most relevant p38 isoform involved in the inflammatory signal transduction pathway, and the appropriate target for antiinflammatory intervention.

Progression into the knowledge of the physiological role and molecular structure of p38 MAP kinase has advanced in parallel with the development of selective inhibitors of this enzyme. These early inhibitors, which were shown to suppress the release of proinflammatory cytokines, were initially designed as cytokine-suppressing antiinflammatory drugs (CSAID). Thus, the enzyme was formerly referred to as the CSAID binding protein (CSBP).

INHIBITORS OF P38 MAP KINASE

Due to their potential therapeutic applications, considerable effort is being devoted to the discovery of small molecule inhibitors of p38 MAP kinase. Several pharmaceutical companies have been involved in the research for novel p38 inhibitors, and a number of compounds, representing a variety of chemical structures, have been developed in this field. The main structural families of selective p38 inhibitors are the following.

Pyridinyl Imidazoles

A main pharmacophore for p38 MAP kinase inhibitors is constituted by 4-pyridinylimidazoles. The early reports of a series of antiinflammatory bicyclic imidazoles [36], represented by the imidazothiazole SK&F 86002 (1) (Fig. **2**), provided a starting point for these structures. Although these compounds were originally conceived as hybrid structures from the antiinflammatory imidazole derivative tiflamizole and the bicyclic immunomodulator levamisole, they were later found to display a different mechanism of action. Thus, the new bicyclic imidazoles were only weak inhibitors of the metabolism of arachidonic acid (dual inhibitors of cyclooxygenase and 5-lipooxygenase), and their antiinflammatory and immunoregulatory effects in animal models were rather correlated with the inhibition of IL-1 and TNF production in human monocytes at low micromolar

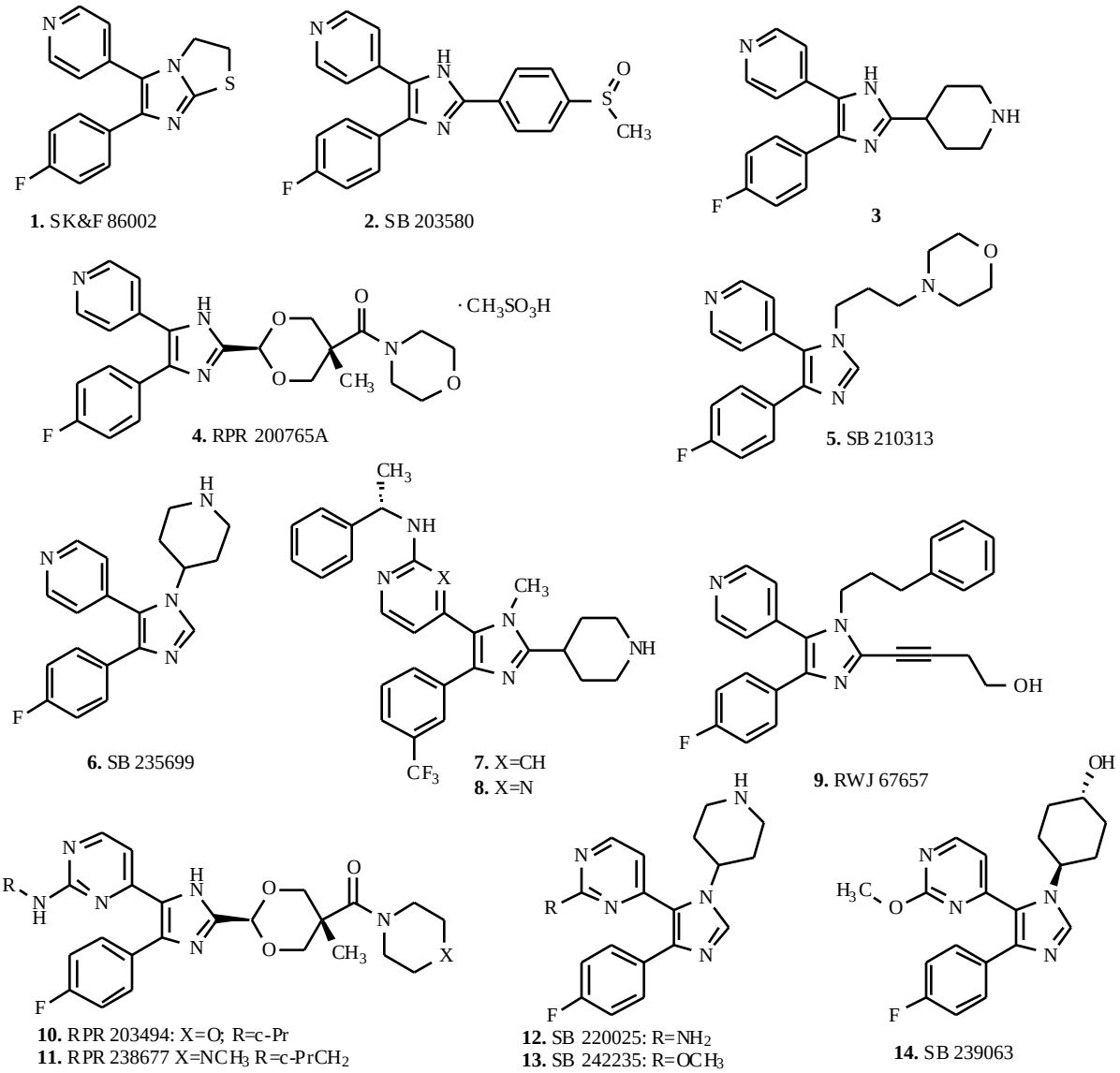


Fig. (2). Structures of pyridinyl and pyrimidinyl imidazole inhibitors of p38 MAP kinase.

concentrations [37,38]. Further studies led to identification of the molecular target of these new cytokine-suppressing antiinflammatory drugs (the CSAID-binding proteins) as two splicing variants of a protein kinase, termed CSBP1 and CSBP2, respectively [18]. The CSBP2 form exhibited high homology with a previously identified murine p38 kinase [39] and was subsequently recognized as the human p38 MAP kinase [40].

Subsequent modifications of the structure of SK&F 86002 led to the more potent and selective 2,4,5-triaryl imidazoles [41]. This core structure gave rise to a number of pharmacological tools and drug candidates. Within this series, compound SB 203580 (**2**) displayed oral efficacy in several models of cytokine inhibition and inflammatory diseases and became a prototypical standard for other p38 inhibitors [42]. The *in vivo* pharmacological profile of SB 203580 reinforced the evidences that inhibition of p38 MAP kinase could represent an important advance in the therapy of inflammatory diseases. Unfortunately, progression of SK&F 86002 and SB 203580 into the clinical practice was hampered by severe toxicological properties. Thus, these first generation inhibitors have been found to be hepatotoxic and potential carcinogens [43,44]. The origin of these toxic effects has been related to their potent induction of some cytochrome P-450 isoenzymes [44,45], a profile which had previously been recognized as an indicator of potential carcinogenesis [46]. Interactions of P-450 with either the pyridine or imidazole rings are among the models proposed to be involved in this toxic mechanism, and different strategies to modify or replace these rings have been devised in order to dissociate the p38 inhibitory potency from the undesired interaction with cytochrome P-450.

In the search for improved inhibitors of p38 MAP kinase, a number of variations around the triaryl imidazole structure have been conducted by different research groups, contributing to define the major structural requirements for inhibition of the enzyme, and providing improved inhibitors. A key structural element in these compounds is the presence of a 4-pyridinyl group. This group is essential for the activity of p38 inhibitors and its replacement for either a phenyl ring [41,47] or the positional isomers 2-pyridinyl and 3-pyridinyl [47] leads to practically inactive compounds. Moreover, in the original series of bicyclic imidazoles, as well as in other series of *N*-substituted imidazoles, a strong positional preference for the pyridinyl group is observed. Thus, the corresponding regioisomers substituted at the imidazole N in position beta to the pyridinyl group are always considerably less potent [36]. The electron density at the pyridine nitrogen is another important factor for the affinity to the enzyme, and it has been shown to correlate with p38 inhibitory potency in several series of pyridinyl as well as pyrimidinyl derivatives [48,49]. Accordingly, electron-donor substituents, such as alkoxy or amino groups, in position 2 of the pyridine ring give rise to stronger inhibitors, whereas electron-withdrawing groups result in a reduction of the inhibitory properties [45,48-50].

The aryl group in position 4 of the imidazole ring also plays an important role for the potency and selectivity of these inhibitors, and the analogous compounds with lower alkyl groups or halogen atoms in this position are almost inactive as p38 inhibitors [41,47]. Within the different substitution patterns on the 4-phenyl group, a *p*-fluoro substitution leads to some of the most potent compounds. Other small, lipophilic substituents on the 4-phenyl ring, such as the chloro or trifluoromethyl groups in positions *m*- or *p*- have led in some cases to improved p38 inhibitors [41,47,50].

Conversely, variations in the 2-aryl substituent of SB 203580 showed that this moiety does not define a key structural feature for p38 inhibitors. Indeed, the 2-(*p*-methylsulfinyl)phenyl moiety has been replaced for a number of diverse groups without loss of activity. Thus, the 2-cyclohexyl analogue of SB 203580 displays a similar potency, and the 2-unsubstituted derivative exhibits only a modest decrease in its potency and selectivity over other kinases [50]. Variations in the substituents in position 2 thus offer the possibility to modulate some physicochemical properties of the molecules while keeping their affinity and selectivity profiles. Replacement of the 2-aryl group in SB 203580 for a 2-(4-piperidinyl) group in (**3**) resulted in improved p38 inhibitory potency, combined with enhanced selectivity against other kinases [50]. The 2-aryl group has also been replaced for a stable, cyclic ketal in RPR 200765 (**4**), a trisubstituted imidazole derivative which demonstrated an *in vivo* pharmacological profile similar to that of the standard SB 203580 in several models of arthritis, along with good oral bioavailability [51,52].

Similarly, a large variety of substituents are tolerated at the 1N of the imidazole ring, either in the presence or the absence of the 2-aryl group. Among the 1,4,5-trisubstituted imidazoles, a number of compounds with diverse aliphatic and aromatic substituents in position 1 demonstrated *in vitro* binding potency superior to that of SK&F 86002. A preferred compound within this series was the morpholinopropyl derivative SB 210313 (**5**) [53], which also displayed potent *in vivo* oral activity and reduced interaction with most cytochrome P-450 isoenzymes. The low lipophilicity of this molecule has been related to its reduced affinity for cytochrome P-450, although it still retains a potent inhibition of isoenzyme CYP2D6 [44]. The piperidinyl imidazole SB 235699 (also designated as HEP 689 and VK 19911) (**6**), which was active in several animal models of skin inflammation, entered phase I clinical trials as topical antiinflammatory for skin disorders such as psoriasis and atopic dermatitis [54], although its development has been reported to be discontinued. In a series of tetrasubstituted imidazoles, the position 2 in the pyridinyl group showed to tolerate relatively bulky amino groups, such as in (**7**), which displayed increased potency and selectivity over related kinases as well as improved pharmacokinetic properties [50]. RWJ-67657 (**9**) is another tetrasubstituted imidazole with 10-fold higher potency than the standard SB 203580 [55]. It underwent early phase clinical trials, showing to inhibit the cytokine release and to counteract the clinical effects induced by endotoxin.

Structural Aspects of the Binding of Pyridinyl Imidazoles to p38 MAP Kinase

Elucidation of the tridimensional structure of p38 MAP kinase and its mode of interaction with selective ligands by means of site-directed mutagenesis and X-ray crystallography has been of crucial importance to understand the key

structural requirements and to assist in the rational design of more potent and selective inhibitors. The crystal structure of the unphosphorylated, low activity form of p38 MAP kinase has been determined by X-ray crystallography [56,57]. This enzyme exhibits important similarities with the previously solved MAP kinase ERK2, with some differences that account for their different specificity to peptide substrates, activating enzymes, and small molecule inhibitors. The protein is constituted by two domains, forming a small N-terminal lobe of 135 aminoacid residues and a large C-terminal lobe of 225 aminoacid residues. A deep channel exists at the junction of the two domains, being the docking site for the peptide substrate. In the vicinity of this site, a cleft is formed between the two lobes, where the ATP cofactor can be accommodated. In the unphosphorylated form, a surface loop (known as activation lip or phosphorylation lip) constituted by residues Gly^{170} to Tyr^{185} occludes the substrate binding channel. In addition, in this low activity form, the two domains are twisted as compared to other related kinases, which results in a misalignment of some catalytic residues in the active site. Phosphorylation of Thr^{180} and Tyr^{182} residues in the activation lip is assumed to cause a conformational change enabling the protein for its enzymatic activity.

Additionally, structural data on the binding mode of several pyridinyl imidazoles to p38 MAP kinase have become available, contributing to the understanding of the molecular basis for ligand recognition and the structure-activity relationships for the small molecule inhibitors. Early studies showed that pyridinyl imidazoles bind competitively into the ATP site of the enzyme [58]. The highly conserved sequence motifs in the ATP binding region throughout a number of protein kinases contrast with the selectivity of these ligands. In fact, the design of specific kinase inhibitors targeted to the ATP site is still regarded as a challenge in medicinal chemistry, their selectivity relying on the exploitation of less conserved surrounding areas, not used by ATP itself [59]. Crystallographic data have been reported for the unphosphorylated p38 MAP kinase complexed with several pyridinylimidazole inhibitors [54,60,61]. Unlike ATP, these inhibitors bind with similar affinity to both the phosphorylated and unphosphorylated forms of p38 kinase, suggesting that their binding mode to both forms should be closely related [54,62]. The pyridine N atom mimics the N-1 nitrogen of the ATP adenine, forming a H bond with the backbone NH of Met^{109}. A further H bond is established between the basic imidazole N and the amino group of Lys^{53}. The spatial arrangement of the pyridine and imidazole nitrogen atoms required by this binding mode accounts for the positional preference of the pyridyl group in the imidazothiazole series. An important additional binding interaction is established with the *p*-fluorophenyl ring, docking into a hydrophobic pocket not occupied by ATP, usually known in the kinase field as the specificity pocket. This pocket is able to admit significantly bulkier substituents compared to related kinases, which constitutes a key determinant for the potency and selectivity of these compounds. The relevance of the Thr^{106} residue for the selectivity versus other kinases has been shown by directed-site mutagenesis studies [63]. Accordingly, isoforms p38α and p38β with a Thr residue in this position, display similar affinities to pyridinyl imidazole inhibitors, whereas p38γ and p38δ, having the bulkier Met residue, and ERK2, which has a Gln residue, are significantly less sensitive to pyridinyl imidazoles [61,63].

Substituents at positions 1 and 2 of the imidazole ring have been found to fit within the ATP phosphate-binding region, which proved able to accommodate a wide variety of structures. For each particular inhibitor, specific interactions have been shown with different aminoacid residues in this region. For example, the (methylsulfinyl)phenyl group in SB 203580 produces a stacking interaction with Tyr^{35}, while the piperidine N of SB 235699 forms a salt bridge with Asp^{168} [59,61].

A further hydrophobic area has been identified near the pyridine binding site, being able to accommodate bulky substituents in position 2 of the pyridine ring [45,59]. This binding pocket, which is not occupied by the unsubstituted pyridine derivatives, has been exploited in some recent inhibitors, providing compounds with enhanced potency and selectivity.

Pyrimidinyl Imidazoles

The 4-pyridinyl substituent was considered an essential element in the early p38 inhibitors, and replacement of this moiety for several structurally diverse groups resulted in compounds practically devoid of affinity for the enzyme [47]. Indeed, the strong H bond established between the pyridine nitrogen and the amide NH of Met^{109} is a key determinant of binding affinity, common to all pyridinyl imidazole inhibitors of p38. On the other side, the hepatic changes leading to induction of P-450 enzymes and subsequent toxicity of these first generation pyridinyl imidazoles were related to their potent *in vitro* inhibition of some cytochrome P-450 isoenzymes [43]. In the origin of this inhibition, interaction of the pyridine N with the ferric ion of the heme cofactor of P-450 has been regarded as one possible participating mechanism [64]. In the attempts to dissociate both effects, an important modification was introduced with the successful replacement of the 4-pyridyl moiety by related heterocycles. Based on the known fact that pyrimidine is a weaker P-450 inhibitor compared to pyridine [65], a pyrimidinyl group was claimed to retain the key H bond with Met^{109} in p38, whereas minimizing the interaction with cytochrome P-450 [44]. Replacement of the pyridine ring in SB 210313 for an unsubstituted 4-pyrimidinyl group led to a potent p38 inhibitor with reduced P-450 interaction, although it still showed some inhibition of isoenzyme 2D6. Inhibition of this isoenzyme was further reduced by the introduction of amino and methoxy substituents at position 2 of the pyrimidine ring, with no appreciable loss of p38 inhibitory potency [44]. Notably, placement of these functional groups on position 6 of the pyrimidine, despite higher structural analogy with the 6-membered ring of the ATP adenine, leads to substantially less potent compounds [44]. Replacement of the pyridine ring for a pyrimidine in a number of previous series of pyridinyl imidazoles has been an active area in the search for improved p38 inhibitors, and this has frequently resulted in analogues with reduced CYP-450 interaction. Introduction of electron-donating groups on position 2, which is related to increased nucleophilicity at the pyrimidine N, has led to potent p38 inhibitors. Furthermore, bulky substituents linked to a 2-amino group

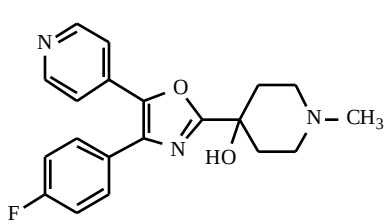
provide enhanced affinity for p38, whereas the steric hindrance with these substituents seems to confer higher selectivity versus other kinases and reduced interaction with CYP-450. The 1-phenylethylamino group in L 790070 (**8**) (Fig. **2**) was the preferred substituent in a series of tetrasubstituted imidazoles, showing marked enantioselectivity for the *S* configuration [50]. Other pyrimidinyl imidazoles are the aminopyrimidinyl analogues of RPR 200765A: RPR 203494 (**10**) [66] and RPR 238677 (**11**) [67], with minimal inhibition of cytochrome P-450, and the aminopyrimidinyl derivative of SB 235699: SB 220025 (**12**) [61,68] as well as its methoxypyrimidinyl analogues SB 242235 (**13**) [69,70] and SB 239063 (**14**) [69,71]. Compound SB 242235 has been reported to enter phase I clinical trials.

Heterocyclic Replacements of the Imidazole Scaffold

The search for alternative scaffolds to the central heterocyclic ring in the triaryl imidazoles has been another active area in the design of improved p38 inhibitors. The imidazole ring plays a structural role by keeping the appropriate spatial distribution of the three aryl rings, (which are not planar with the central heterocycle, but have rather been compared to a propeller's blades) as well as establishing a key H bond between the nitrogen in beta to the pyridinyl group and the amino group of Lys[53] in the enzyme. However, the core imidazole ring proved not to be essential, and other heterocyclic scaffolds have been shown to maintain similar electronic and spatial features. Replacement of the central imidazole ring with other five-membered nitrogen heterocycles, including pyrazoles, oxazoles, isoxazoles, thiazoles and triazoles, has been extensively explored [72-74], leading in some cases to interesting compounds. The pyridinyl oxazole (**15**) is an orally active compound devoid of inhibition of P-450 isoenzymes [74]. Among the pyrazole analogues, different possible substitution patterns exist for the aryl groups. The 3-(p-fluorophenyl)-4-pyridinylpyrazoles (**16**) are far more potent than their 4-(p-fluorophenyl)-3-pyridinyl counterparts (**17**) [73]. The isomeric 1-aryl-5-pyridinylpyrazoles, such as (**18**), have also been reported to display potent *in vitro* inhibitory potency [75]. In a series of 5-membered heterocyclic derivatives including furans, pyrroles and pyrazolones [76], the N atoms in the imidazole scaffold proved not to be indispensable. Thus, the furan derivatives still retained some p38 inhibitory potency, although fivefold less potent than the analogous imidazoles. Notably, in some potent compounds containing pyrrole and pyrazolone rings, the NH group would be required to directly interact with Lys[53] in the active site of p38, acting as H bond donor, as opposed to the previously assumed role for the imidazole N. Further development of the 2,5-diaryl-3-(4-pyridinyl)pyrrole series led to the potent compound L 167307 (**19**) [76]. Interestingly, computer modeling of L 167307 failed to reveal favorable interactions with p38 MAP kinase [63].

The imidazole ring has also been replaced by six-membered heterocycles possessing a basic nitrogen atom, such as pyridines, pyrazines, and pyrimidines. Pyridinyl pyrimidines showed similar structure-activity relationships to the analogous imidazoles, although with a lower potency, a fact that was attributed to their less favorable geometry [77]. Low *in vitro* potency was also reported for a series of 2-aryl-3-(4-pyridinyl)pyrazines, [78] and 2-aryl-3-(4-pyrimidinyl)pyridines [79].

Some interesting compounds have arisen from the replacement of the imidazole ring with certain fused polycyclic scaffolds. Pyrrole-fused bicyclic structures include indoles, and several isomeric pyrrolopyridines (azaindoles). Among them, the pyrrolo[2,3-*b*]pyridine derivative RWJ 68354 (**20**) [49,80] entered phase I clinical trials as potential antiinflammatory. Other related analogues include imidazo[1,2-*a*]pyrimidines [81] and the *in vitro* highly potent pyrrolo[3,2-*b*]pyridines [82]. Crystallographic studies with these latter compounds revealed a H bond with Lys[53] at



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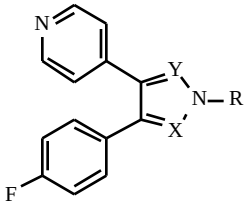
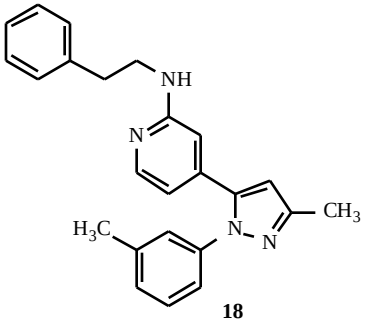


16. X=N, Y=CH
17. X=CH, Y=N



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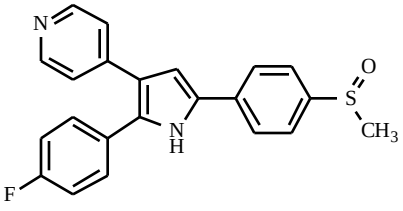
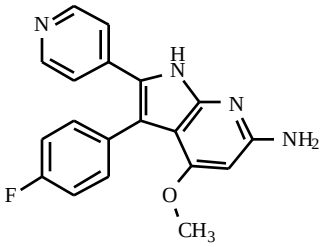
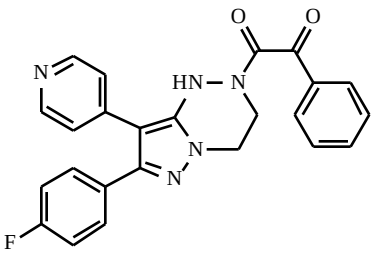


19. L 167307

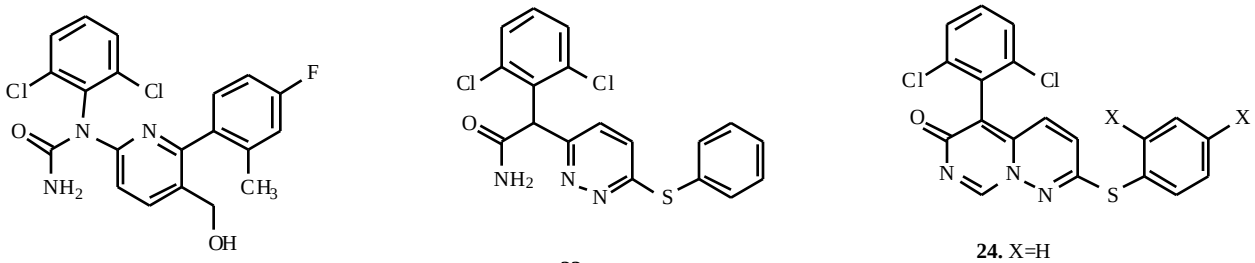


20. RWJ 68354



21. FR 167653

Fig. (3). Structures of non-imidazole pyridinyl heterocycle inhibitors of p38 MAP kinase.



22. VRT 034465 **23** **24.** X=H **25.** X=F (VX 745)

Fig. (4). Structures of pyrimidopyridazinones and some ring-opened precursors.

a more favorable distance than other analogues [82]. Another interesting bicyclic system is represented by the pyrazolo[5,1-*c*][1,2,4]triazine FR 16765 (**21**) [83], which demonstrated antiinflammatory activity in a number of preclinical disease models.

Pyrimidopyridazinones and Related Structures

A different structural family is constituted by compounds with a pyrimidopyridazinone nucleus and other related bicyclic analogues, sometimes referred to as azanaphthalenones. The acyclic compounds 2,2-diarylacetamides and *N*,*N*-diarylureas, described in an early patent as p38 inhibitors [84], can be regarded as structural precursors of this class. Among these compounds, the *N*,*N*-diarylurea derivatives were generally more potent as p38 inhibitors than the analogous diarylacetamides. A representative compound in the diarylurea series is VRT 034465 (**22**) (Fig. **4**), with nanomolar affinity for p38 MAP kinase [84]. Derivatization of the closely related 2-(3-pyridazinyl)-2-aryl acetamide (**23**) upon treatment with dimethylformamide dimethylacetal resulted in the spontaneous cyclization to the pyrimido[3,4-*b*]pyridazinone (**24**), which displayed a promising pharmacological profile as p38 inhibitor. Further elaboration of the phenylsulfanyl ring in order to improve its metabolic stability led to the clinical candidate VX 745 (**25**), with significant anti-inflamatory activity in a number of animal models, high selectivity, and minimal inhibition of CYP-450 [85]. In fact, this compound was the first p38 inhibitor to undergo clinical trials in patients with RA and, for some time, the p38 MAP kinase inhibitor in most advanced development stage. In human patients, VX 745 produced a good improvement in the clinical manifestations of RA, being in general well tolerated and free of CNS toxic effects at therapeutic doses. However, safety concerns about some adverse neurological effects observed in dogs in long-term toxicological studies led in 2001 to the discontinuation of two phase II clinical trials on rheumatoid arthritis and myelodysplastic syndrome, respectively. This potential nervous system toxicity was related to extensive penetration across the blood-brain barrier, which has been attributed to the high lipophilicity of VX 745. The clinical results obtained with VX 745 encouraged the proprietary company, Vertex, to advance the clinical development of a second generation of orally active p38 inhibitors, VX 702 and VX 850, compounds reported to belong to a chemically distinct family and to be devoid of the capacity to cross the blood-brain barrier.

A number of derivatives containing heterocyclic scaffolds analogous to the pyrimidopyridazinone nucleus, such as dihydroquinazolin-2-ones [86,87], napthyridinones [87], and pyrido[3,2-*d*]pyrimidin-2-ones [88] have been further disclosed. Introduction of piperidino or piperazino groups in position 7 of the bicyclic scaffold provided improved activity, although these compounds still displayed poor pharmacokinetic properties. Pyridazinone (**26**) (Fig. **5**) belongs to a new class of p38 inhibitors designed as hybrid structures from VX 745 and the pyridinyl imidazoles [89].

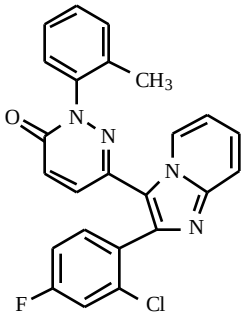


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Fig. (5). Pyridazinone inhibitor of p38 MAP kinase.

Crystallographic studies of VX 745 and related compounds complexed with p38 MAP kinase revealed that these compounds bind competitively to the ATP site, in a similar mode to the triaryl imidazoles [86,89,90]. The arylsulfanyl ring of VX 745 matches into the lipophilic specificity pocket, whereas the accessory lipophilic pocket next to the pyridine site in the pyridinylimidazoles is occupied by the dichlorophenyl group. Notably, the heterocycle carbonyl establishes hydrogen bond interactions with the backbone NH groups of both Met^{109} and Gly^{110}. Optimal interaction in this binding mode requires a peptide flip at Gly^{110}. Other kinases with bulkier residues at this position show rotational restriction that reduces the binding affinity. The more than 1000-fold selectivity of VX 745 for p38α compared to JNK [85] could be explained by the particular binding mode of this family of p38 inhibitors.

N,N'-Diarylureas

The *N,N'*-diaryl ureas constitute a distinct structural class of p38 inhibitors, exhibiting a unique binding mode to the enzyme. An early patent described some *N*-phenyl-*N'*-thienyl ureas and thioureas as in general poor specificity inhibitors of diverse tyrosine kinases, although noreference was made to p38 or other serine/threonine kinases [91]. Some *N,N'*-diphenylurea derivatives were later reported as *in vitro* moderately potent inhibitors of p38, with no apparent

advantage over their *N,N*-diaryl-substituted counterparts [92]. An important improvement in these structures was introduced with the replacement of one phenyl group for a five-membered heterocycle bearing a bulky lipophilic substituent. Thus, the related *N*-phenyl-*N'*-pyrazolyl urea (**27**) (Fig. **6**), was reported as a screening hit from a combinatorial library, with potent, reversible p38 MAP kinase inhibitor properties [93].

Efforts to optimize the *N*-phenyl-*N'*-pyrazolyl urea lead compounds (**27**) [93] and (**28**) [94] have been conducted independently by two research groups. Systematic modification of these molecules revealed that the urea functionality constitutes a key structural element in this class of inhibitors. Both urea NH groups proved to be essential, and methylation of either urea N or replacement for a methylene group yielded compounds practically devoid of p38 inhibitory activity. The analogous thiourea derivatives also showed an important decrease in the inhibitory potency [94]. Whereas the *tert*-butyl group on the heterocyclic ring is essential for the potency in this family of p38 inhibitors, the pyrazole nucleus can be replaced with some other five-membered heterocycles, such as isoxazoles and thiophenes [93,95]. Improvement of the potency was further achieved by replacement of the pyrazole *N*-methyl substituent with various aryl groups [94,96]. Alternatively, modifications of the urea *N*-phenyl ring revealed that this group cannot be successfully replaced for H, cycloalkyl or pyridinyl groups, whereas a naphthyl moiety brought about improved inhibition of p38 in both *in vitro* and cellular assays [94]. A different direction of optimization consisted in the introduction of an additional aryl substituent on the phenyl ring, linked through an O, S or CH_2 bridge. A representative compound in this series is the pyridinylmethyl derivative (**29**) [97]. Subsequent optimization of the *N*-naphthyl series culminated in the clinical candidate BIRB 796 (doramapimod) (**30**), with sub-nanomolar affinity for p38 MAP kinase, which recently entered phase III clinical trials for the treatment of RA [94,98].

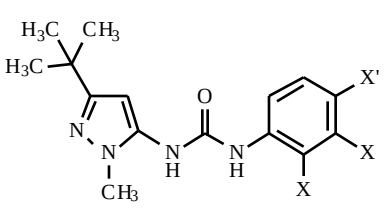
The crystal structure of p38 complexed with diaryl urea (**29**) revealed a different binding mode for this compound [94,99]. The new allosteric binding site identified for the *N,N'*-diarylureas is located near to the ATP pocket but spatially distinct from it, and is only exposed after a large conformational change of the enzyme. Thus, no overlap exists between the binding sites of ATP and the most simple diaryl ureas. When comparing the binding modes of urea (**29**) and a iodinated analogue of SB 203580, overlap only exists at the specificity pocket, which is occupied by either the fluorophenyl group of the triarylimidazole or the chlorophenyl group of urea (**29**) [60]. A bidentate hydrogen bond is established between the urea nitrogens and the side chain carboxylate group of Glu^{71}. Notably, the pyrazole N atoms seem not to establish any binding interactions with the enzyme.

This particular binding mode requires an important conformational change, mainly involving the Asp^{168}-Phe^{169}-Gly^{170} residues (DFG) of the enzyme. In all serine/threonine kinases, this highly conserved DFG motif adopts a preferred conformation in which the side chain of Phe^{169} is embedded into a hydrophobic pocket in the groove between the two lobes ("DFG-in" conformation). To allow for binding to the diaryl urea inhibitor, the Phe^{169} side chain moves away, exposing a large lipophilic pocket ("DFG-out" conformation), which is occupied by the *tert*-butyl group of (**29**). In its new position, the aromatic ring of Phe^{169} generates a hydrophobic interaction with the urea *N*-phenyl ring. Whereas, in this conformation, the diaryl urea inhibitor does not directly occupy the ATP site, its binding is obstructed by steric hindrance between the ATP phosphate groups and the side chain of Phe^{169} [99]. Not surprisingly, such a large conformational reorganization is associated to much slower binding and dissociation rates as compared to triaryl imidazoles [100]. This slow dissociation kinetics has been hypothesized to provide enhanced therapeutic benefit.

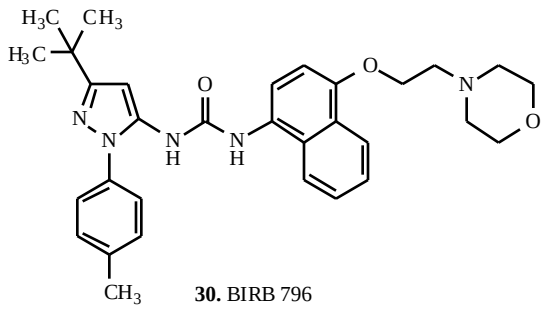
Diaryl Ketones

A series of 4-anilinobenzophenones has been reported as potent inhibitors of LPS-stimulated release of TNF-α and IL-1β from human peripheral blood mononuclear cells. Optimization studies on the lead compound, 4-[(2-aminophenyl)amino]benzophenone, revealed that the ketone function as well as the secondary amino group are essential structural elements, whereas the primary amine can be successfully replaced with several other groups. Substituents in *ortho*- to the carbonyl group enhance the *in vitro* potency [101]. A representative compound in this series is EO 1428 (**31**) (Fig. **7**), which demonstrated potent antiinflammatory effects in a guinea pig model of UVB-induced acute dermatitis [102]. Based on molecular modeling simulations, these compounds have been suggested to bind in the ATP site of p38. In the proposed binding model, a H bond is established between the benzophenone carbonyl and the NH of Met^{109} while the 2-methylphenyl group matches the kinase specificity pocket [101].

Additionally, heterocyclic diaryl ketones have been explored as p38 inhibitors. A series of orally active benzoyl pyridines, benzophenones, and benzimidazolyl pyridinyl



27. X=Cl; X'=H
28. X=H; X'=Cl
29. X=H; X'=CH_2(4-Pyr)



30. BIRB 796

Fig. (6). Structures of *N,N'*-diarylurea inhibitors.

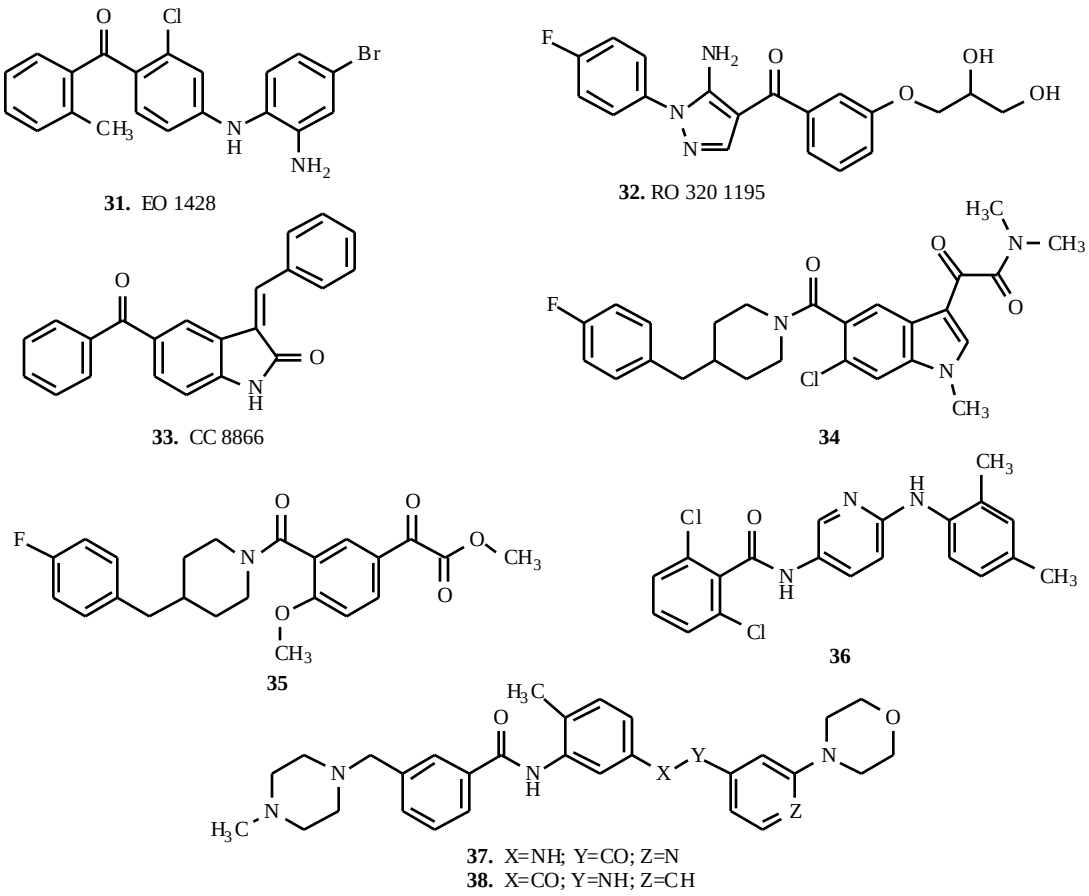


Fig. (7). Structures of benzophenone and aromatic carboxamide inhibitors.

ketones was developed as ring-opened analogues of a weakly potent 4-aryl-pyrido[2,3-*d*]pyrimidine precursor [103]. The pyrazolyl ketone RO 320 1195 (**32**) [104] has been reported to enter phase I clinical trials for RA. The binding mode of RO 320 1195 has also been related to the ATP site, with the carbonyl group forming a H bond with the NH of Met[109], and an additional H bond between the pyrazole amino group and the backbone carbonyl of His[107]. The fluorophenyl group of (**32**) occupies the kinase specificity pocket. The oxindole ketone CC 8866 (**33**) is another heteroaryl ketone under development as potential anticancer drug [105].

Aromatic Carboxamides

Several different families of compounds containing as the common structural feature amides of aromatic carboxylic acids have been developed as p38 MAP kinase inhibitors. These compounds can be regarded as analogs of the aryl ketone inhibitors, in which the keto group has been replaced by an amide function. Within this group, considerableefforts have been focused on amides of *N*-benzylpiperazine or 4-benzylpiperidine with several regioisomers of indolecarboxylic acid. Whereas indole-7-carboxamido derivatives [106] are virtually devoid of inhibitory potency, the corresponding carboxamides in positions 4 [107], 3 [108], 5 and 6 [106] of the indole nucleus have been reported as moderately potent inhibitors. Introduction of substituents in *ortho*- to the carbonyl group, which is related to loss of planarity between the aromatic ring and the carbonyl group, usually enhances the inhibitory potency. Furthermore, in the indole-5-carboxamides, a glyoxyl ester or amide group in position 3 leads to potent compounds, such as (**34**) [109]. The inhibitory potency seems not to be associated to the indole ring, and in related compounds it has been successfully replaced with related heterocycles or phenyl rings, such as in the benzamide SCIO 323 (**35**), which progressed to phase I clinical trials [110]. SCIO 469 is another compound, presumably related to the arylcarboxamide class, reported to be in phase II clinical trials on RA patients.

A structural family closely related to the anilinobenzophenones is constituted by mono-amides of aromatic diamines, such as (**36**) [111]. Similarly, replacement of the diaryl amine with a second amide function gives rise to the diamide class [112]. Both the diamides of 1,3-phenylenediamine [113,114] and their reversed-amide isomers, the benzoylamino benzamides [115], such as (**37**) and (**38**), respectively, have been disclosed as p38 inhibitors with comparable inhibitory potencies. Further structural variations of the amide moiety include the cyclic analogues, 3,4-dihydroquinazolin-4-ones [116], and the heterocyclic bioisosters of the amide group, the aminopyrimidine derivatives [117].

CONCLUSION

p38 MAP kinase, occupying a central position in the inflammatory signal transduction pathway, is an attractive

target for the development of new antiinflammatory medications, since inhibition of this enzyme provides a mechanism for controlling the overproduction of TNF-α and IL-1β. Besides the proven crucial role of these cytokines as key mediators in inflammatory pathologies, the synergistic effects observed when blocking simultaneously TNF and IL-1 provide an additional value to this approach. With the first small molecule p38 inhibitors, intervention at this level of the inflammatory signaling cascade has been validated as an interesting therapeutic option, and other vital functions controlled by MAP kinases seem not to be adversely affected by this blockade. Severe liver toxicity encountered with the early p38 inhibitors was however a serious hurdle delaying their clinical development. Notwithstanding this, toxicity appears not to be linked itself to the mechanism of p38 inhibition. Currently, there is abundant structural information available to assist in the rational design of new p38 inhibitors, and it seems possible to dissociate the antiinflammatory from toxic effects in new molecules. Success probabilities are enhanced by the wide structural diversity of these inhibitors, including compounds that bind to different sites of the enzyme. Improved selectivity versus other kinases, as well as between the different isoforms of the enzyme could provide further advantages for more selective inhibitors. A number of compounds representative of different structural families are being developed as p38 inhibitors, and some of them have been advanced to clinical development. Final clinical results are eagerly awaited in this field. These are expected to provide definitive evidences of the benefits of this therapeutic mechanism and, hopefully, a better medical treatment approach to chronic inflammatory diseases.

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