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Review Article

p38 mitogen-activated protein kinase as a target for drug discovery

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Introduction

p38 mitogen-activated protein kinase (MAPK) (p38/p38-α/CSBP2/RK) was originally identified as a serine/threonine kinase activated by stimulation of monocytes with bacterial lipopolysaccharide (LPS), and was later shown to regulate LPS-induced production of the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (1, 2). Overproduction of these cytokines has been implicated in a wide variety of diseases with an inflammatory component such as rheumatoid arthritis, endotoxic shock, inflammatory bowel disease, osteoporosis and many others, as discussed below (3, 4). This kinase has also been implicated in certain signaling pathways induced by IL-1β and TNF-α (5), suggesting that a small molecule p38 kinase inhibitor may inhibit both the production and biological effects of these cytokines. p38 has also been implicated in the regulation of other proinflammatory pathways such as IL-4-induced low affinity IgE receptor expression in human monocytes (6). The potential for inhibition of the production and signaling of multiple proinflammatory cytokines and, consequently, the downstream cascades induced by these cytokines, is a potential advantage for small molecule p38 kinase inhibitors over soluble TNF receptors or anti-TNF antibodies currently marketed or in clinical trials, and is one reason why such molecules have attracted wide attention within the pharmaceutical industry. This review is intended as a brief survey of the potential therapeutic utility of p38 kinase inhibitors and the structures and properties of published compounds. Detailed reviews of p38 MAPK and the inhibitor SB-203580 have recently been published (5, 7).

p38 expression and activation

The p38 MAPK family includes p38-α, CSBP1, p38-β, p38-β2/p38-2, p38-γ/ERK6/SAPK3, p38-δ/SAPK4 and Mxi2 (5). In human tissues, p38-α mRNA is widely expressed, p38-β and p38-δ expression is slightly more restricted (highest in brain and glandular tissues, respectively) and p38-γ is largely restricted to skeletal muscle (8). The p38-α isoform is the most extensively studied. All of the published p38 kinase inhibitors tested that inhibit p38-α are roughly equipotent against p38-β and do not inhibit p38-γ or p38-δ. The structural basis for this selectivity is discussed below.

Stimulation of p38 kinase activity occurs following dual phosphorylation on Threonine-180 and Tyrosine-182 in the p38 activation loop which causes a conformational change that exposes the enzyme active site. Upstream kinases thought to phosphorylate p38 *in vivo* include MKK3, MKK6 and several others (5, 9). Activation of p38 is induced during many cellular responses. The most well-studied are those induced by environmental stresses such as osmotic shock and UV light, and by proinflammatory stimuli such as LPS, IL-1β and TNF-α (5, 10-12). For example, in IL-1 receptor-associated kinase (IRAK)-deficient fibroblasts, IL-1β-induced p38 activation is reduced by 3- to 5-fold compared to wild-type cells (13), directly implicating IRAK as a proximal signaling molecule important for IL-1β-induced p38 activation. Activation of p38 is also induced by simultaneous signaling through the T cell antigen receptor and the costimulatory molecule CD28 in mouse T cells (14) and human T cell lines (15, 16), but can be activated via CD28 signaling alone in primary human T cells (17). Activation is also induced via the antigen receptor in human B cells and by coligation of the antigen receptor and CD19 in mouse splenic B cells (18, 19).

p38 functions

The requirement for p38 activation in cellular responses has been defined largely through the use of specific p38 kinase inhibitors, primarily the pyridinyl imidazoles. One widely used compound of this class, SB-203580 (2, 7, 10), blocks the activity of p38 by specifically binding to the p38 ATP-binding site (20). Pyridinyl imidazoles inhibit LPS-stimulated IL-1β and TNF-α biosynthesis in human monocytes by a mechanism involving inhibition of mRNA translation (2, 5, 7), although a study using macrophage cell lines has suggested inhibition of IL-1β production at the level of gene transcription as well (21). SB-203580 can also partially block interferon-γ production by mouse Th1-type T cells and completely block IL-4 production by human $CD4^+$ T cells at the transcriptional level (17, 22). Thus, p38 is involved in the production of various cytokines, and at multiple levels of biosynthesis.

Much of the focus on the function of p38 has centered on its role in cytokine synthesis and signaling in various *in vitro* systems. In addition to its central role in regulating TNF-α and IL-1β production in response to LPS and environmental stresses, p38 may be important for the production and/or signaling of several other cytokines or growth factors that may be important in disease and/or in the maintenance of normal physiological responses. Some of these roles may be secondary to activation by IL-1 or TNF-α. For example, SB-203580 inhibits IL-1-induced transcription of the inducible nitric oxide synthase gene in bovine articular chondrocytes (23). Others may be more direct, such as the activation of p38 by TGF-β in the 293 human embryonic kidney cell line (24). Recent data suggest that p38 inhibitors may have therapeutic utility beyond their well-known antiinflammatory properties. Several transgenic and knockout mouse models as well as *in vitro* studies using SB-203580 strongly implicate p38 in the regulation of IL-12 and interferon-γ gene transcription (22, 25), and hence, in the regulation of Th1-type immune responses. In contrast, in studies of human T cells, p38 inhibitors potently and selectively blocked IL-4 production, suggesting a predominant role in the regulation of Th2-type immunity (17). Another study of primary human T cells found evidence to support involvement of p38 in regulation of both Th1 and Th2 responses, as well as in the production of the antiinflammatory cytokine IL-10 (26).

In vitro evidence for therapeutic uses of p38 kinase inhibitors

In vitro evidence suggests that there may be other therapeutic uses for p38 kinase inhibitors in addition to their well-known potential for use as treatments of classic inflammatory diseases such as rheumatoid arthritis (RA). For example, p38 inhibitors can block replication of human immunodeficiency virus-1 (HIV-1) in primary human T cells (27) and IL-1-induced HIV-1 replication in a macrophage cell line (28). Evidence also suggests that SB-203580 may decrease myocardial TNF-α production and cardiomyocyte apoptosis while increasing myocardial function after ischemia and reperfusion (29, 30). Indeed, there is a growing literature that suggests TNF-α (and perhaps p38 kinase) plays a key role in ischemia/reperfusion injury and chronic heart failure.

In vitro data also suggest that p38 kinase may play an important role in inflammatory responses in the brain which appear to contribute to the progression and severity of Alzheimer's disease (31). Increased levels of activated (phosphorylated) p38 have been detected in the Alzheimer's brain relative to normal brain with high levels in the area of amyloid plaques (32). Treatment with β-amyloid fibrils activates p38 kinase in rat microglia freshly isolated from brain (33) and SB-203580 inhibits β-amyloid peptide-induced TNF-α production from microglia (34).

Another area of potential therapeutic utility (or potential source of side effects) for p38 kinase inhibitors is regulation of programmed cell death or apoptosis. Inhibition of apoptosis may be desirable for neurodegenerative diseases such as Alzheimer's disease, while induction of apoptosis may be desirable in cancers. SB-203580 blocked glutamate-induced apoptosis of rat cerebellar granule cells (35). A dominant negative MKK3 construct (an upstream activator of p38) blocked apoptosis of the rat neuronal-like cell line PC12 induced by growth factor withdrawal (36), and a p38 inhibitor had similar effects (37). In each case, induction of neuronal apoptosis was closely correlated with increased p38 kinase activity. SB-203580 can also inhibit adrenomedullin-induced apoptosis in rat glomerular mesangial cells *in vitro* (38) and fibroblast apoptosis induced by serum withdrawal (37) or sodium salicylate treatment (39). SB-203580 did not inhibit activation-induced death of mouse B cells or T cells *in vitro* (13), perhaps because inhibition of both p38 and JNK (c-Jun N-terminal kinase) is required to block apoptosis, as has been shown for Fas-induced apoptosis in the Jurkat human T cell line (40). On the other hand, SB-203580 can actually enhance apoptosis of cytokine-deprived human eosinophils (41) and SB-202190 at high doses (20-50 μM) induced apoptosis in Jurkat and HeLa cells (42).

An important consideration for interpreting the significance of *in vitro* data using p38 kinase inhibitors is the concentrations of inhibitors used. For example, many studies have used SB-203580 to inhibit a cellular response and thereby attribute the response to p38 kinase inhibition. It is not uncommon for the inhibitor to be used only at micromolar concentrations. However, the IC_{50} for SB-203580-mediated inhibition of p38 kinase activity in activated cells ranges from approx. 30-600 nM, depending on the system (2, 7, 10, 17, 28). Therefore, it is important that these inhibitors be titered to demonstrate that the IC_{50} for inhibition of the response being measured is within the expected range for inhibition of p38 kinase activity. Ideally, the IC_{50} for inhibition of the response should be compared to the IC_{50} for inhibition of p38

kinase activity within the cells being studied. There is a close correlation between these values in several studies (7, 17, 23, 43) and the comparison can easily be done by assaying the phosphorylation or activity of a p38 substrate, such as MAPKAPK-2, isolated from the cells of interest (11). This is an important issue, since SB-203580 has been shown to inhibit the tyrosine kinase lck (44), the related serine/threonine kinase JNK (45), COX-1, COX-2 and thromboxane synthase (46) and an uncharacterized target required for IL-2-dependent T cell proliferation (47) with IC_{50}s ranging from 0.28-20 μM, and to have complex effects on c-raf in cells (48). It is possible that potent p38 kinase inhibitors other than SB-203580 will also exhibit cross-reactivities at high (μM) concentrations.

Rheumatoid arthritis

Chronic inflammatory disorders such as rheumatoid arthritis (RA), inflammatory bowel diseases (Crohn's disease and ulcerative colitis) and multiple sclerosis are major causes of morbidity and mortality. As a representative example of the potential of p38 inhibitors for treatment of chronic inflammatory diseases, we will discuss RA in the most detail. RA affects about 1% of the adult population, causes more than 9 million physician visits per year and, if left untreated, results in progressive joint destruction and premature death (49, 50). Since RA typically occurs between the ages of 45 and 65, these numbers are likely to increase in the near future as the population ages.

RA is a systemic, chronic disorder of unknown etiology, characterized initially by joint inflammation and later by destruction of cartilage and bone. RA is typically remitting, but, if treated inadequately, results in progressive joint destruction. The disease course is highly variable between patients and many researchers believe that these differences reflect different etiologies or genetic effects on disease progression.

Histologically, the involved joints exhibit grossly edematous synovial membranes including hyperplasia and hypertrophy of the lining cells. The normally paucicellular stroma can be markedly infiltrated with mononuclear cells, including B and T lymphocytes and monocytes/macrophages. The organization of the infiltrating cells closely resembles the structure of lymph nodes including lymphoid aggregates around postcapillary venules lined by activated endothelial cells, as well as germinal centers, strongly suggesting that an immune-mediated response is important in the etiology (51). The synovial fluid often contains large numbers of neutrophils, as well as antigen-antibody complexes. These immune complexes may perpetuate the inflammation by stimulating the release of chemotactic factors and destructive enzymes from the infiltrating neutrophils or by activation of the complement cascade.

There is no known cure for RA. Because the etiology of the disease is unknown and since there is significant variability in symptoms and disease progression between patients, the medical management of RA is complex. Recent treatment guidelines published by the American College of Rheumatology emphasize early diagnosis and aggressive treatment in order to reduce the probability of irreversible joint damage (52). However, many of the agents currently used require weeks or months before clinical benefit is obtained and toxicity often limits the duration of their use. More effective and less toxic drugs are greatly needed for this prevalent and chronic disease.

TNF-α and IL-1β in rheumatoid arthritis

In human patients with RA or other chronic inflammatory diseases, as well as in animal models of these disorders, cytokines produced by activated macrophages (*e.g.*, TNF-α and IL-1) appear to be intimately involved in the pathology. Although a large number of cytokines and chemotactic factors secreted by lymphocytes, macrophages, fibroblasts and endothelial cells are found in increased quantities in the rheumatoid synovial membrane and fluid (53), it is generally agreed that TNF-α and IL-1 are of particular importance (54). These two cytokines have several effects that likely potentiate the chronic inflammatory reaction including stimulation of expression of adhesion molecules on endothelial cells, stimulation of the release of inflammatory eicosanoids, reactive oxygen radicals and metalloproteinases from fibroblasts and chondrocytes, increased bone resorption, decreased collagen and proteoglycan synthesis, stimulation of fibroblast proliferation and increased synthesis of other proinflammatory cytokines such as IL-6 and IL-8 (54). Neutralization of TNF-α bioactivity in synovial cell cultures from RA patients inhibited the release of other proinflammatory mediators including IL-1, suggesting that increased production of TNF-α may be one of the key primary events in RA (55). In addition, inhibition of TNF-α or IL-1 in several animal models of RA has been particularly effective (see below). Based on these results, it is reasonable to suggest that inhibition of the synthesis of IL-1 and, particularly, TNF-α, or inhibition of their effects on cells would be of significant therapeutic benefit in diseases involving chronic inflammation such as RA.

Animal models of rheumatoid arthritis

Injection of IL-1 or TNF-α protein (56) or the gene for IL-1 (57) directly into the joints of animals can mimic some, but not all, of the features of human RA. Importantly, a synergistic effect of the two proteins was observed (56). Transgenic mice overexpressing human TNF-α developed a severe, chronic inflammatory arthritis (58), even when expression of the human TNF-α was restricted to cell-surfaces and no soluble human TNF-α could be detected (59). Consistent with the synergistic effect of IL-1 and TNF-α proteins for induction of arthritis following intraarticular injection (56), the development of arthritis in transgenic mice expressing human TNF-α

Table I: Summary of in vivo *arthritis studies.*

Inducer of arthritis	Species	Intervention target	Specific intervention	Ref.
Type II collagen	Mouse	IL-1	Anti-IL-1; IL-1ra	61
		TNF	Anti-TNF; TNF-R/Fc	62-64
		p38	SB-203580	80
Type II collagen	Rat	IL-1	IL-1ra	65
		TNF	TNF-R	66
		p38		
Freund's adjuvant	Rat	IL-1	Anti-IL-1; IL-1ra	65, 67
		TNF	Anti-TNF	67
		p38	SB-203580; RWJ-68354; SB-210313	80-82

required signaling through the type I IL-1 receptor since antibodies to that receptor completely prevented disease (60).

The three most well-studied animal models for human RA are the type II collagen-induced models in mice and rats and the adjuvant-induced model in rats (Table I). In all three, inhibition of TNF-α and/or IL-1 activity is effective in preventing disease or decreasing symptoms (61-67). Depending on the model, interventions with antibodies to IL-1 or TNF-α, the IL-1 receptor antagonist (IL-1ra) or soluble receptors for TNF-α have been demonstrated to inhibit swelling and/or bone erosions. There is some evidence in these and other less frequently used models that reduction of TNF-α primarily inhibits the inflammatory response (61, 67), while inhibition of IL-1 activity may be more important in preventing cartilage destruction and bone erosion (61, 68).

Human therapeutics

There is now convincing evidence in human patients that inhibition of TNF-α or IL-1 bioactivity can be an effective treatment for chronic inflammatory diseases. Antibodies to TNF-α significantly improved symptoms in patients with either RA (69-71) or Crohn's disease (72, 73). Consistent with those results, a soluble TNF-α receptor (p75)-Fc fusion protein (etanercept) was also found to decrease disease activity in RA patients (74, 75). The clinical data on these modulators of TNF-α bioactivity have been recently reviewed (76, 77). Treatment of RA patients with the naturally occurring antagonist of IL-1 bioactivity, IL-1ra, is also effective (78, 79).

Although these exciting results demonstrate that protein molecules can be effective therapeutics for chronic inflammatory diseases, protein drugs have several potential drawbacks compared to small molecules with similar mechanisms of action. First, they are relatively expensive to manufacture. Second, even when the proteins are primarily of human origin, immune reactions are sometimes observed. For example, the occurrence of antibodies in human patients to the mouse/human chimeric monoclonal antibody cA2 has been documented (71). Such antibodies may neutralize the drug's effects and could conceivably cause immune complex nephritis or other complications. Third, protein drugs are typically given intravenously or subcutaneously; oral administration of proteins is very unlikely to be effective.

Several companies are currently investigating the use of small molecule inhibitors of p38 kinase for treatment of inflammatory disorders. Such compounds have several potential advantages over protein inhibitors of TNF-α or IL-1 bioactivity. First, it is clear that such inhibitors are often orally active. Second, as noted above, p38 inhibitors, in addition to inhibiting synthesis of both IL-1 and TNF-α, also inhibit signaling by these cytokines. This dual mechanism of action for p38 inhibitors is a major potential advantage over large molecule inhibitors of TNF-α or IL-1 bioactivity which can only inhibit signaling by the specific cytokine and cannot directly inhibit synthesis of either one. Finally, it is unlikely that small molecule inhibitors will induce an immune reaction in patients. One potential disadvantage of a small molecule p38 inhibitor is a relative lack of specificity for p38 compared to anti-cytokine antibodies or IL-1ra. Obviously, the less specific a compound is for p38 the more likely it is that the compound will cause significant side effects.

Based on the above theoretical advantages of a small molecule p38 inhibitor, it is encouraging that such inhibitors have shown efficacy after oral administration in the collagen-induced arthritis model in mice (80) and in adjuvant-induced arthritis in rats (80-82).

Inhibitors of p38 kinase

As seen in Figure 1, the majority of p38 inhibitors reported to date have been designed around the core structure of SKF-86002 (**1**). Workers at SmithKline demonstrated that the pyridyl and 4-fluorophenyl ring on the central imidazole are critical to binding, and most work started with this key piece of the pharmacophore. SKF-86002 itself is a fairly weak inhibitor of p38 (IC_{50} = 260 nM) (83) and was modified significantly by SmithKline to produce far superior analogs such as the triarylimidazole SB-203580 (**2**) (IC_{50} = 48 nM) and the aminopyrimidine SB-220025 (**3**) (IC_{50} = 19 nM) (84).

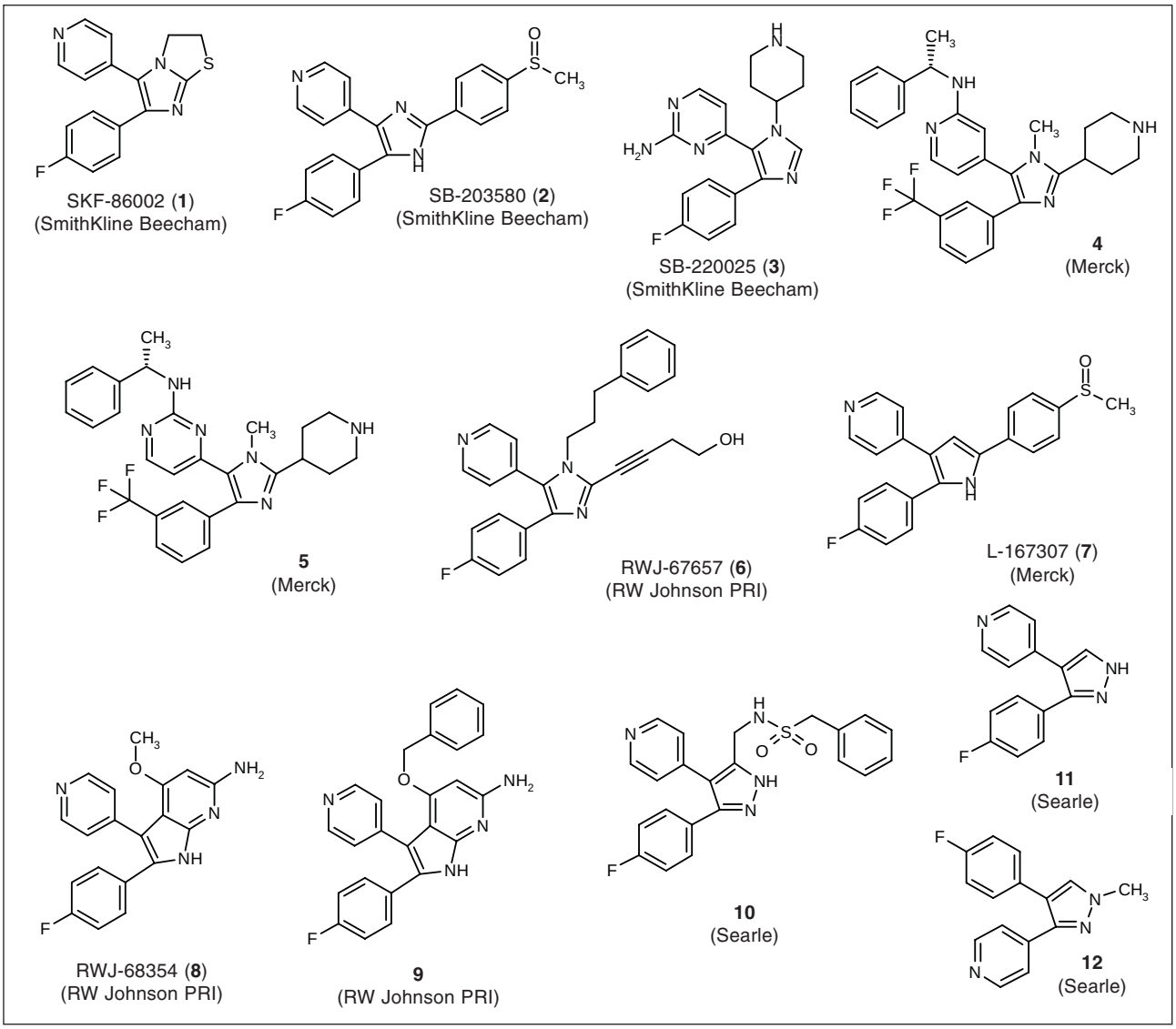

Fig. 1. Known p38 kinase inhibitors.

Research at Merck led to another series of novel imidazoles containing a 2-piperidinyl moiety. Compounds **4** and **5** showed IC_{50}s of 0.19 and 0.11 nM, respectively (85). RW Johnson has also reported a novel imidazole inhibitor of p38, RWJ-67657 (**6**), which had an IC_{50} of 3 nM in an assay measuring TNF-α release from LPS-stimulated human peripheral blood mononuclear cells (86).

Merck was first to report the successful replacement of the central imidazole core of SKF-86002 with other heterocycles like furans, pyrroles and pyrazolones (45). The pyrrole L-167307 (**7**) was shown to have an IC_{50} of 5 nM. Workers at RW Johnson have extended this concept and replaced the central imidazole with indole or pyrrolopyridine cores. In a cellular assay which measures a compound's ability to inhibit TNF-α release from LPS-stimulated PBMCs, RWJ-68354 (**8**) and compound **9** showed IC_{50}s of 6.3 and 0.91 nM, respectively (87). Finally, Searle has described compounds containing a central pyrazole core. Pyrazoles **10**, **11** and **12** were shown to inhibit p38 with IC_{50}s of 8, 49 and 400 nM, respectively (88).

The regiochemistry of substitutions on the central core of these p38 inhibitors has proven to be very important. For example, if the N-methyl group of imidazole **5** is shifted to the imidazole nitrogen proximal to the 3-trifluoromethylphenyl ring, the activity drops from 0.11 nM to 1220 nM, a 10,000-fold decrease (85). Significant drops in potency are also seen in pyrroles (*i.e.*, L-167307) when the regiochemistry of the pyrrole nitrogen is shifted, placing the nitrogen of the pyrrole proximal to the 4-pyridyl ring (45).

Recently, work has shifted from the original 4-pyridyl analogs like the imidazoles SKF-86002 and SB-203580

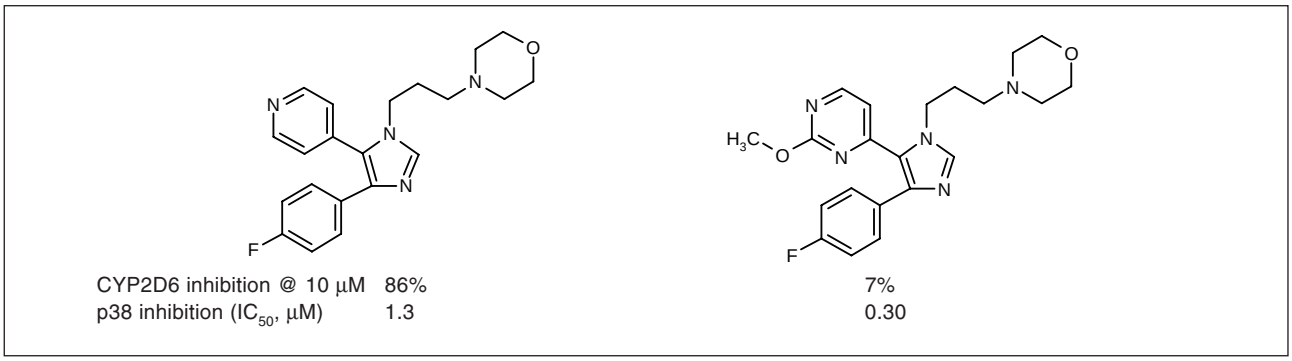

Fig. 2. Effects of 4-pyridyl substitution on CYP2D6 and p38 inhibition.

to substituted pyrimidine and pyridine analogs like SB-220025 and compound **4**. Adams *et al.* have reported that pyridinylimidazoles such as SB-203580 potently inhibited liver P450 isozymes. Also, SB-203580 caused increased liver weights and significant elevations of hepatic P450 enzymes in 10-day rat toxicological studies. It is postulated that the pyridine of SB-203580 binds to the heme iron of the P450 enzymes and is responsible for the observed liver effects. As seen in Figure 2, replacing the 4-pyridyl ring with a 2-methoxypyrimidine substituent results in reducing the inhibition of CYP2D6 by 10-fold, while achieving a 4-fold increase in potency against p38 (89).

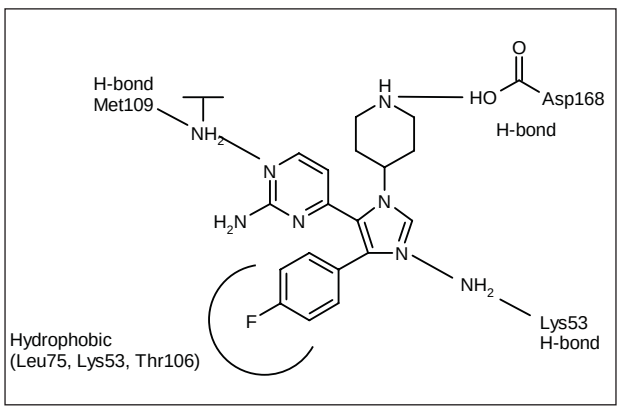

Fig. 3. Representation of imidazole SB-220025 bound at the active site of p38.

Mode and site of p38 inhibitor binding

Crystallographic and kinetic experiments have shown that all p38 inhibitors studied bind at the ATP site of p38 and compete with ATP for binding to active, phosphorylated p38 (20, 84, 90, 91). The crystal structures of 6 unique inhibitors bound to p38 have been reported. Figure 3 shows some of the key interactions between SB-220025 and p38 (84). The pyrimidine ring participates in a hydrogen bond with Met109 and the 4-fluorophenyl ring is deeply embedded in a hydrophobic pocket. These interactions are common among all the crystal structures reported to date, and are critical for anchoring the inhibitors into the ATP site. In fact, the hydrogen bond between Met109 and the N-4 nitrogen of the pyrimidine is analogous to the interaction seen with the N-1 adenine of ATP in all available kinase crystal structures.

Specificity of p38 inhibitors

Not surprisingly, members of the MAPK family share a high degree of conserved structural domains, particularly in the ATP site, and many of the residues that make contact with p38 inhibitors are conserved throughout the family. Thr106 is an exception, however, as it is replaced by methionine in p38γ and the JNK kinases, and by glutamine in ERK1 and ERK2 (92). The hydrophobic pocket that accepts the fluorophenyl ring, and in particular Thr106 (Fig. 3), has therefore been implicated as a critical determinant for the specificity of these compounds. It has been shown in several site-directed mutagenesis studies that replacing Thr106 with larger residues (*i.e.*, methionine) rendered the enzyme resistant to binding of p38 inhibitors, whereas replacing Thr106 with smaller residues (*i.e.*, alanine), enhanced binding (92, 93). It has also been shown that mutating position 106 in kinases insensitive to p38 inhibitors converts them into enzymes that are potently inhibited by SB-203580 (93). These results show the critical nature of Thr106 in imparting the impressive selectivity seen for p38 inhibitors as a class of compounds binding to the ATP site of a kinase.

A final issue concerning the activity of p38 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 unactivated p38 however, the two domains of the kinase are misaligned, suggesting that ATP cannot bind to unactivated p38. Researchers at Merck have shown that this is indeed the case by measuring the interaction between p38 and ATP using a competitive binding assay (94). It has also been

shown that p38 inhibitors bind equally well to both the activated and unactivated form of the enzyme. Therefore, ATP is noncompetitive with studied p38 inhibitors for the unactivated form of the enzyme, but upon phosphorylation of p38 at Thr180 and Tyr182, the enzyme undergoes a reorientation of its two major domains and significantly increases its affinity for ATP. In the active form of p38, ATP and inhibitor are competitive. By being able to bind to the inactive form of p38 noncompetitively with ATP, p38 inhibitors may be able to keep the enzyme in a configuration that cannot bind ATP. It is also possible that the inactive enzyme-inhibitor complex is a poorer substrate for activating kinases than is inactive p38 alone. This would suggest that p38 inhibitors may actually be inhibiting p38 activation rather than inhibiting enzymatic activity. Small molecule inhibitors of kinases that bind at the ATP site and are both selective and potent *in vivo* are quite rare, and this is an intriguing explanation for the observed activity of these compounds.

Conclusions

Clearly, a need exists for the treatment of chronic inflammatory diseases and inhibitors of p38 have the potential to fill this void. The large amount of information currently being generated around this target, coupled with the significant number of companies pursuing lead compounds, should provide a number of clinical compounds in the near future. In fact, Vertex has initiated a phase II trial in Europe to test the tolerability and pharmacokinetics of its p38 compound VX-745 in patients with rheumatoid arthritis (95).

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