

Chapter 2

Small-Molecule Protein and Lipid Kinase Inhibitors in Inflammation and Specific Models for Their Evaluation

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Abstract

The inflammatory response requires complex and coordinated cooperation of different signaling pathways and cell types. Therefore, more than 40 different protein or lipid kinases can be regarded as potential small-molecule inhibitor targets to approach a therapy of acute inflammation, such as septic syndrome, and especially chronic inflammation, such as rheumatoid arthritis or inflammatory bowel disease. Besides the general considerations about selectivity and potency of small-molecule kinase inhibitors, in this chapter special emphasis is put on the inflammation-specific methods and assays available for testing potential small-molecule inhibitors for their anti-inflammatory activity. Examples for human cell-based assays for characterization of the effect of inhibitors on contribution of various cell types, such as monocytes, neutrophils, mast cells, T-cells, and synovial fibroblasts, to the inflammatory scenario are given. It is further demonstrated how these assays are complemented by rodent models for septic syndrome, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and systemic lupus erythematosus. Finally, it is discussed how the results obtained by these methods can be further validated and which future strategies for the treatment of chronic inflammation will exist.

Key words: Animal models, Sepsis, Rheumatoid and collagen-induced arthritis, Crohn's disease, Ulcerative colitis, Lupus erythematosus

1. Introduction

Acute and chronic inflammation is based on a complex, multicellular scenario where protein and lipid kinases are involved in many steps and places (for recent reviews see refs. 1 and 2). Hence, there is a strong potential for the use of small-molecule kinase inhibitors to interfere with inflammation and, more relevantly, to contribute to therapy of chronic inflammation such as rheumatoid arthritis (RA) or inflammatory bowel disease (IBD). After identification of the first inflammation relevant protein kinases targeted by Smith Kline Beecham compounds of the SB203580-type, the p38 MAPKs

Table 1
Kinases involved in inflammation (for details and signaling pathways
see refs. 1, 4)

Kinase superfamily	Members involved in inflammation
Receptor tyrosine kinases	c-KIT (mast-stem cell growth factor receptor), recepteur d'origine nantais (RON), TYRO3, AXL, and MER (TAM) receptor family
Nonreceptor tyrosine kinases	Janus kinase (JAK)1/2/3, Tyrosine kinase 2 (TYK2), Lymphocyte cell-specific protein tyrosine kinase (LCK), T-cell-specific kinase (TSK or ITK), Zeta-chain-associated protein kinase (ZAP)70, Spleen tyrosine kinase (SYK), Bone marrow tyrosine kinase in chromosome X (BMX), Bruton's tyrosine kinase (BTK)
Protein-serine/threonine kinases	IL-1R-associated kinase (IRAK)1/2/4, TGF- β -activated protein kinase (TAK)1, MAPK kinase kinase (MEKK)3, TNFR-associated factor (TRAF) family member-associated (TANK)-binding kinase (TBK)1, inhibitor of κ B (I κ B) kinase (IKK) $\alpha/\beta/\epsilon$, Tumor progression locus 2 (Tpl2 or c-COT), Extracellular signal-regulated kinase (ERK)1/2, c-JUN N-terminal kinase (JNK)1/2, p38 MAPK α/β , MAPK-activated protein kinase (MAPKAPK or MK) 2/3, MAPK interacting kinase (MNK)1/2, Receptor-interacting serine-threonine kinase (RIP)
Dual specific protein kinases	MAPK kinase 1/2 (MEK1/2), 3/4/6/7 (MKK3/4/6/7)
Lipid kinases	Phosphoinositide 3-kinase (PI3K) γ/δ

α and β , more than 15 years ago (3), today a long list of more than 40 potential target kinases exists (see refs. 1, 4 and Table 1). Several small inhibitors against these targets are already in clinical trials, while others has to be identified, characterized, and optimized further before entering clinics.

Methods of molecular biology are mainly involved in analyzing selectivity and potency of small-molecule inhibitors by various in vitro assays. To monitor selectivity of protein kinase inhibitors, a screen of in vitro activity over a panel of recombinant protein kinases (5), competitive binding assays against a kinase expression library (6), or a proteomic analysis of the competitive affinity purification of kinases in the presence of inhibitor molecules (7) are carried out. Although some small-molecule inhibitors show rather high selectivity, with the exception of rapamycin, which uses a completely different mechanism of action via cyclophilin-binding, there is no monospecific kinase inhibitor identified so far. To increase selectivity and potency of kinase inhibitors, there is a general trend to shift from ATP-competitive inhibitors to molecules that, in addition, confer allosteric inhibition of the kinase, such as the p38 MAPK-inhibitor BIRB 796 (8), or, to inhibitors that target activator-kinase, such as the MEK1 inhibitors of the Parke Davis-family and the U0126 compound, or kinase-substrate interaction, such as the

recently described JNK-inhibitor BI-78D3 (9). Potency of inhibitors is usually characterized by the IC_{50} value – the inhibitor concentration that is necessary to reduce kinase activity in an in vitro assay to 50%. When comparing IC_{50} values, it should be taken into account that this value depends on assay conditions, such as ATP and substrate concentration, structure of the recombinant kinase, and the presence of further kinase-binding components.

Nevertheless, the above issues are not specific for targeting inflammation, but represent general considerations for all small-molecule kinase inhibitors. For targeting inflammation, the cell-based assays and the animal models, in which the inhibitory molecules has to be tested, are the specific issues and are discussed in more detail here.

2. Cell-Based Assays for Small-Molecule Inhibitors to Target Inflammation

As stated above, many cell types and cytokines are involved in the inflammatory response and, hence, the choice of the inflammatory stimulus, cell type and readout parameter for analyzing the anti-inflammatory action of an inhibitor is crucial. The cell type to be analyzed is often chosen according to the known cell-type specific function or, if the function is not known, to the expression and activity pattern of the kinase targeted by the small molecule. However, it may well be that a specific kinase is involved in regulation of inflammatory processes in more than one cell-type. At the moment, this is not reflected by the assays, which all try to use only one well-defined cell-type. Furthermore, since inhibitors are finally searched for therapeutic treatment of humans, preferential cellular assays of anti-inflammatory action should use human primary cells or cell lines to avoid effects due to structural variations of the kinase molecules between species. As a result of these considerations, only few cell-based assays seem suited. Owing to the various cellular players in inflammation, the cell-based assays always represent only a specific part of the inflammatory scenario (Table 2). Monocytes are preferred to monitor the effect of small-molecule inhibitors on TLR-mediated innate immunity. The prominent TLR4-ligand LPS is used as a sepsis-relevant stimulus and secretion of the “master”-cytokine TNF is an ideal readout parameter. In addition, sometimes the influence of the inhibitor on the phosphorylation of intracellular substrates of the kinase of choice is also monitored (10, 11). Other aspects of inflammation are better represented by mast cells (12), T-cells (13), or neutrophils (11), and appropriate assays have been developed using these cell types (Table 2). Also, disease-specific primary cells, such as synovial fibroblasts from RA patients, were used to monitor the disease-modifying properties of the compound more directly (14). IC_{50} values determined in these

Table 2
Examples of cell-based assays

Cells	Stimuli	Readout	Kinase inhibitors analyzed and IC ₅₀ (if available)
Cell line THP-1, human monocytic leukemia cell line	LPS	TNF	MK2, Compound 83, IC ₅₀ = 1.6 μ M (32) P38, BIRB 796, IC ₅₀ = 18 nM (8)
Cell line U937, human monocytic leukemia cell line	LPS	Phospho-Hsp27, TNF	MK2, Compound 23, IC ₅₀ = 4.8 μ M (10)
Primary human monocytes derived from human blood buffy coats by negative selection	LPS, IL-1b	TNF	Tpl2/C-COT, compound 1, IC ₅₀ = 0.6 μ M (33)
Human embryonic kidney cells (HEK293) overexpressing TLR4	LPS	NFkB-dependent reporter gene	BTK, Effect of LFM-A13 measured (34)
Human mast cells culture derived from cord blood CD34+ progenitor cells and expanded by Flt3, SCF, and IL-6 treatment (35)	Sensitization with IgE κ appa, stimulation with anti-IgE-antibodies	Degranulation by measurement of tryptase activity in supernatant	SYK, Compound 36, IC ₅₀ = 70 nM (12)
Human CD4+ T-cell purified from whole blood	Activation of TCR and CD28 by anti-CD3- and anti-CD28-antibodies	IL-2	ITK, Compound 8x, IC ₅₀ = 1.6 μ M (13)
Human RA synovial fibroblasts	IL-1 α	Phospho-ERK	Effect of PD184352 measured (14)
Human neutrophils purified from blood by Percoll gradient centrifugation	TNF priming, fMLP stimulation	Intracellular PIP3, Oxidative burst by oxidation of exogenous cytochrome C in the supernatant	PI3K γ , AS-252424, IC ₅₀ = 2 μ M (11)

Cells, stimuli, and readouts, kinases targeted and inhibitors

cell-based assays are usually higher than the values obtained from in vitro assays. This is mainly due to “bioavailability” of the compounds, which includes permeation to the relevant cellular compartment, intracellular solubility, competitive off-target binding to other cellular components, and intracellular stability of the compound.

3. Inflammatory Disease Models Appropriate for Testing of Small Molecules

3.1. Sepsis Model

Sepsis is a systemic response to infection that includes fever, enhanced heartbeat and respiration rate, decreased blood pressure, and multiple organ dysfunction. The septic syndrome is observed in intensive care units worldwide. It is a major cause of death, with mortality rates that range from 20% for sepsis to >60% for septic shock. Sepsis is commonly elicited by lipopolysaccharide (LPS), a constitutive component of the outer membrane of gram-negative bacteria. The part of LPS that causes septic shock is lipid A, which acts as bacterial endotoxin. The response to LPS occurs through Toll-like Receptors (TLRs) and results in the release of the proinflammatory cytokines TNF, IL-1, and IL-6 which trigger the inflammatory reactions. Overproduction of these cytokines causes pathological amplification of the inflammatory cascade leading to sepsis. For mice, two sepsis models are established, which are designated low-dose and high-dose LPS model.

In the low-dose LPS model, mice are sensitized by preceding administration of D-galactosamine, leading to severe depletion in hepatic UTP and inhibition of macromolecular synthesis, and subsequently (after 1–3 h) injected with low-dose LPS (15). Mice given 300 mg D-galactosamine/kg (typically 20 mg) have a lethal dose with 50% survival (LD_{50}) of 0.5 ng LPS per animal, with death occurring about 5–9 h later. In the low-dose model, lethality is due to massive hepatic necrosis in response to LPS by a process dependent upon TNF and IFN- γ . Sometimes, D-galactosamine is also combined with medium-dose LPS treatment (Table 3).

High-dose LPS challenge of mice is based on intraperitoneal or intravenous injection with LPS doses of 25–100 μ g per animal. The LD_{50} is around 150 μ g, with lethality after approximately 1.5 day. The observed mortality is due to cytokine induced endothelial cell injury and highly correlates with TNF, IL-1, and IL-6 levels. High-dose LPS is the most often used assay for analysis of inhibitory compounds and for characterization of mouse knockouts of potential target kinases (Table 3).

3.2. Models for Rheumatoid Arthritis

RA is an inflammatory polyarthritis of unknown autoimmune-based origin leading to joint deformation, destruction, and final loss of function. Its worldwide distribution has an estimated prevalence of 1–2%. In contrast to RA, the factors that induce experimental arthritis in mouse are well established. Such factors are living bacteria or bacterial components, adjuvants, cartilage specific proteins or other antigens used in different experimental models.

The collagen-induced arthritis (CIA) model (16) is the most commonly used and best described model so far. Immunization of mice with autologous or heterologous type II collagen with incomplete Freund's adjuvant leads to arthritis with a maximum severity

Table 3
Examples of animal models of inflammation for testing of inhibitors or mouse kinase knock outs

Animal model	Inhibitor application and inflammatory stimulus	Readout	References
Rat or mouse high dose LPS model	MK2- or p38-Inhibitors orally dosed prior LPS-challenge	Serum TNF measured by LC-MS or ELISA	(10, 36)
Mouse (low dose) LPS D-gal model	A combination of LPS (5–50 mg per kg body weight) and D-gal (0.4–1 g per kg body weight) simultaneously injected intraperitoneally into MK2-KO mice	Serum TNF level after 90 min measured by ELISA, Lethality between 1 and 24 h	(18, 37)
Collagen-induced arthritis in DBA/1LacJ mice	Kinase knockout (MK2) mice treated with bovine collagen type II	Arthritic score, IL-6 mRNA in paws,	(18)
Collagen-induced arthritis in DBA/1LacJ mice	Mice treated with bovine collagen type II and subsequently with daily doses of p38-inhibitor Org 48762-0	Arthritic score	(36)
Mouse DSS-induced chronic ulcerative colitis	MEK-inhibitor RDEA119 Orally dosed	Histological colonic damage score	(38)
MRL/lpr-mice spontaneously developing symptoms of SLE at about month 5	PI3Ky-inhibitor AS605240 administered intraperitoneally every 12 h starting from month 2 until month 5	Titer of DNA-specific autoantibodies or number of CD4+ memory T-cells	(28)

around day 30. Importantly, CIA is accompanied by expression of TNF and IL-1 β in the joints. Any blockade of these molecules leads to alleviation of arthritis. Accordingly, inhibition or deletion of protein kinase targets, such as p38 or MK2, results in decreased arthritic score in this model (17, 18) (Table 3).

In the serum transfer model of arthritis, mice expressing the KRN T-cell receptor transgene and the MHC class II molecule Ag7 (K/BxN mice) develop inflammatory arthritis, and serum from these mice, due to pathogenic autoantibodies to glucose-6-phosphate isomerase, causes similar arthritis in a wide range of mouse strains (19). Hence, this model could be extremely useful for the investigation of the development of autoimmune-induced arthritis.

3.3. Models for Crohn's Disease and Ulcerative Colitis

CD and UC are the two major forms of chronic inflammatory bowel disease (IBD). The clinical appearance of IBD is heterogeneous, which may reflect an uneven impact of genetic factors,

microbial factors in the enteric environment and altered immune response in the etiology of IBD. So far, there is no animal model which exactly reproduces human IBD. However, some animal models resemble certain aspects of IBD and can be used for both, further investigation of the underlying pathophysiological mechanisms and validation of therapeutic strategies.

The first suited model is dextran sodium sulfate (DSS)-induced colitis. Here, feeding of mice with DSS polymers (30–60 kDa) in the drinking water for several days induces an acute colitis characterized by bloody diarrhea, ulcerations and infiltrations with granulocytes (20). DSS is directly toxic to gut epithelial cells of the basal crypts and, hence, affects the integrity of the mucosal barrier. The DSS colitis model is particularly useful for studying the contribution of innate immune mechanisms of colitis.

Other models are trinitrobenzene sulfonate (TNBS)- and oxazolone-induced colitis. Here, in susceptible strains of mice colitis can be induced by administration of the hapten TNBS (21) or the organic compound oxazolone (22) in ethanol. As a result, modifications of otherwise nonimmunogenic autologous or microbial proteins transform them immunogenic to the host and causes autoimmune colitis with high lethality. In addition, various mouse knockouts (KOs) and transgenic mice develop IBD spontaneously due to direct or indirect modulation of T-cell function, such as IL-2- and IL-10-KO (23, 24), to TNF upregulation by a targeted TNF mRNA stabilizing mutation (Δ ARE, (25)) or perturbations in the gut epithelium, such as keratin 8-KO (26).

3.4. A Model for Systemic Lupus Erythematosus

SLE is a chronic autoimmune disease with deregulated T-cell mediated B-cell activation resulting in inflammation and tissue damage. This disease, which is nine times more frequent in women than in men and affects around 0.1% of the population, often harms skin, joints, heart, kidney, and nervous system. Owing to symptomatic treatment with corticosteroids, the mortality was decreased to around 20% after 20 years. A genetic model that reflects many aspects of SLE is the mouse inbred strain MRL/*lpr* (lymph proliferation), which carries a homozygote recessive Fas-antigen mutation (27). Together with an additional autosomal dominant mutation, which affects the induction of macroscopic skin lesions, the *lpr* mutation accelerates the progression of skin lesions to a severe systemic disease similar to SLE.

Male MRL/*lpr*-mice spontaneously develop symptoms of SLE at 5–6 month of age, while females develop SLE 1 month earlier. Hence, to analyze the effect of a small-molecule inhibitor of SLE, mice have to be treated repeatedly with the compound beginning between month 2 and 3.5. As disease-relevant readout, DNA-specific autoantibodies or CD4+ memory T-cells can be quantified after 5 months in these animals (28).

4. Conclusions

There are various cell-based and animal models of inflammation available for testing efficiency of small-molecule kinase inhibitors in inflammation. While the cell-based models of human origin represent only specific parts of the complex multicellular inflammatory scenario, the animal models mirrors the complex scenario much better – but 70 million years of divergent evolution between rodents and humans make direct transfer of the result to humans sometimes problematic. However, a combination of human cell-based and animal models in evaluation of small-molecule kinase inhibitors could be sufficient for characterization of compounds of interest before entering clinics. However, inhibitor studies should be complemented with studies using mouse knockouts or catalytic-dead knockins of the target kinase and target knockdown approaches in human cells.

Future developments of small-molecule inhibitors in inflammation should take into account that intracellular signaling proceeds in networks including feedback control (cf. refs. 1, 29). Hence, inhibition of a specific kinase target can even increase the long-term inflammatory response leading to adverse effects. Better understanding of signaling networks will facilitate the combination of targets and use of inhibitor cocktails for a specific anti-inflammatory therapy. Finally, since signaling involves kinase cascades with specific kinase–kinase and kinase–substrate interaction often based on docking motifs, which can be targeted by peptides as well (reviewed in refs. 30, 31), the targeting of catalytic activity and allosteric properties of protein or lipid kinases should be complemented with targeting specific protein–protein interaction.

Together, there is a strong need and a realistic perspective for orally available small-molecule inhibitors for the therapy of inflammation. However, in contrast to the use of small-molecule inhibitors for the treatment of cancer, the treatment of chronic inflammation by such molecules will be more difficult, since higher efficacy compared with classical treatments, fewer side effects, and extraordinary low toxicity are required for the long-term treatment of usually non-life-threatening nonmalignant diseases.

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Kinase Inhibitors

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