Spleen tyrosine kinases: biology, therapeutic targets and drugs

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Spleen tyrosine kinase (Syk) is an intriguing protein tyrosine kinase involved in signal transduction in a variety of cell types, and its aberrant regulation is associated with different allergic disorders and antibody-mediated autoimmune diseases such as rheumatoid arthritis, asthma and allergic rhinitis. Syk also plays an important part in the uncontrolled growth of tumor cells, particularly B cells. For these reasons, Syk is considered one of the most interesting biological targets of the last decade, as proved by the great number of papers and patents published, and the possibility of treating these pathologies by means of Syk kinase inhibitors has led to a great interest from the pharmaceutical and biotech industry.

In this review, we describe the structure of Syk and its role as key mediator of immune receptor signaling in host inflammatory cells. We review data describing the efficacy of novel Syk inhibitors in the treatment of a variety of disorders in animal models and clinical trials and, finally, we report the most relevant Syk small-molecule inhibitors developed so far.

Introduction

In the past decade, important progress has been made in understanding the pathogenic mechanisms and defining the roles of relevant cells involved in allergic and autoimmune disorders. These breakthroughs facilitated the development of novel and effective drugs, such as the anti-IgE monoclonal antibody omalizumab (Xolair), which proved to be capable of inhibiting both acute airway constriction and, later, inflammatory eosinophilia [1]. In addition, treatment of rheumatoid arthritis (RA) has been revolutionized by the introduction of tumor necrosis factor-alpha (TNF-α) inhibitor biologics, which have also been demonstrated to suppress Crohn's disease, psoriasis and other chronic inflammatory conditions [2]. Although these protein therapies are highly effective, they are difficult and expensive to develop, manufacture and administer. Moreover, some of these targeted biological treatments are associated with relevant side-effects, such as the reactivation of tuberculosis and other latent infections. For these reasons, the current research goal is to explore new therapeutic approaches and generate safer, more efficacious and more cost-effective therapies with improved dosing schedules. Several targets are candidates, and they include cytokines, chemokines and factors that participate in the signal transduction pathways, such as proteins of complement, adhesion molecules and kinases [3]. In particular, a kinase with high potential for discovering novel inhibitors for the treatment of inflammatory

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and autoimmune disorders is spleen tyrosine kinase. This cytoplasmic protein kinase, discovered in 1991 [4], associates with different receptors on the surface of different cells of immune system (including B cells, mast cells, macrophages and neutrophils) and non-immune cells (such as osteoclasts and breast cancer cells). The engagement of these receptors with their ligands activates Syk, which, in turn, orchestrates different cellular processes, including cytokine production (in T cells and monocytes), bone resorption (in osteoclasts) and phagocytosis (in macrophages) [5]. In addition, because Syk is positioned upstream in the cell signaling pathway, therapies targeting Syk might be more advantageous than drugs that inhibit a single downstream event [6].

This makes Syk a therapeutic target for an array of inflammatory diseases and, for this reason, many pharmaceuticals companies (including Rigel, Pfizer, Bayer and ZaBeCor), as well as many academic institutions, have been involved in the development of small-molecule inhibitors of Syk.

Biology
Structure of Syk
The non-receptor spleen tyrosine kinase, along with zeta-chain-associated protein kinase 70 (ZAP-70), is a member of the Syk family of cytosolic protein kinases implicated in antigen and Fc receptor signaling. The two enzymes share a characteristic dual Src homology 2 (SH2) domain separated by a linker domain. SH2 domains typically bind to phosphorylated tyrosine residues within a motif contained in a target protein. This interaction initiates a cascade of events inducing several cellular responses.

The Sky tandem SH2 domains specifically bind phosphorylated immunoreceptor tyrosine-based activating motif (ITAM) present in the cytoplasmic region of immunoreceptors (i.e. Fc receptors, T- and B-cell receptors, and natural killer [NK] cells). ITAM is a conserved sequence composed by two precisely spaced tyrosine residues surrounding a consensus motif carrying the specific binding site for SH2 domains.

The tandem SH2 domains of Syk possess a high conformational flexibility that enables them to 'evaluate' the relative distance between phosphotyrosines of ITAM belonging to different receptors and adjust the relative orientation of their SH2 domains to fit that spacing [7]. This particular feature enables Syk to interact and modulate the activity of several different immunoreceptors, such as B- and T-cell antigen receptors, NK-cell receptors and, possibly, some G-protein-coupled receptors. For this reason, Syk is considered not only a protein kinase but also a true protein adaptor.

An 80–100 amino acid motif (interdomain B) links the second SH2 domain to the kinase domain. This insert is not present in ZAP-70 and can also exist as a spliced variant lacking a sequence of 23 amino acids (SykB) [8]. SykB displayed intrinsic enzymatic activity comparable to that of Syk [9] but exhibited reduced ability to bind phosphorylated ITAM motif. Taken together, these results demonstrate that the linker domain is crucial for the ability of Syk to regulate immunomodulatory signals.

Function and role of Syk
Syk in hematopoietic cells
Syk is expressed ubiquitously in hematopoietic cells (such as macrophages, mast cells, leukocytes, platelets and erythrocytes) and at lower levels in epithelial, fibroblast, neuronal cells, hepatocytes and other cell types [10] (Table 1). In immunocompetent cells, Syk binding to the phosphorylated ITAM motif enables activation of

TABLE 1

Syk expression and functions in different cell types.

Cell type		Receptor	Ligand	Signal	Disease involvement	Syk functional role	Refs
T cells (TC)	Resting	TCR	MHC	CD3ζ	Autoimmunity	No involvement of Syk	[14]
	Effector	TCR	MHC	FcR-γ	Autoimmunity	Proliferation and differentiation?; releasing of mediators, self-antigen presentation to B cells	[14]
	Natural killer	FcγRIIIa; NKp30; NKp44; NKp46; KIR CD94/NKG2C	IgG; BAT3; HLA class I; HLA-E	DAP12	Autoimmunity	Elimination of antibody coated cells; surveillance of genotoxic stress/transformation; surveillance of mitotic cells	[15]
B cells		BCR	Membrane-bound antigen	Igα; Igβ	Autoimmunity	Pre-B cells development and activation	[12]
		FcgRIIB	Feedback		Autoimmunity	Inhibition of B cell activation	[13]
Red blood cell						Band 3 protein phosphorylation; cells removal from circulation, glycolysis, cell shape, membrane transport	[14]
Granulocytes	Neutrophil	FcγR1; FcγRII; FcεR1 FcγRIII; integrin	IgG; IgE	FcR-γ	Inflammation, autoimmunity	Releasing NO; reactive oxygen intermediates, adhesion, phagocytosis	[3]
	Basofil	FcεRI	IgE	FcR-β; FcR-γ	Allergy	Degranulation	[3]
	Eosinohil	FcγR; FcεR	IgG; IgE	FcR-γ	Allergy	Degranulation; reactive oxygen intermediates generation	[3]

TABLE 1 (Continued)

Cell type		Receptor	Ligand	Signal	Disease involvement	Syk functional role	Refs
Macrophages		FcgR1; FcγRII; FcεR1; integrin	IgG; IgE		Inflammatory; allergy; autoimmunity	Releasing NO; reactive oxygen intermediates, adhesion, phagocytosis	[3]
Mast cells		FcεRI	IgE	FcR-β; FcR-γ	Allergy; autoimmunity	Degranulation, cytokine production, arachidonic acid metabolism	[3]
Dendritic cells		FcRs	IgA; IgG; IgE	DAP12	Autoimmunity	Antigen internalization, presentation, cell maturation release of IL-12	[17]
Osteoclasts		RANK	RANKL	DAP12;FcRγ	Bone resorption	Osteoclastogenesis	[21]
Platelets		GpVI; GPIb-IX-V; CLEC-2; avb3	Collagen; von Willebrand factor; lectin; fibrinogen thrombin	FcR-γ;		Spreading, aggregation, arachidonic acid metabolite release; serotonin secretion	[3]
Fibroblasts	Embryonic			G protein		Differentiation to adipocytes; adopogenesis	[10]
	Nasal				Airway diseases?	LPS-induction of RANTES production; IL-1 induced chemokine production	[10]
Synoviocytes		TNF-R	TNF-α		Rheumatoid arthritis	TNF-a induced IL-32 release, metalloprotease 3 gene expression, matrix degradation and synovial fluid regulation	[22,23]
Epithelial cells	Breast				Tumor progression	Control of cell division	[10]
	Airway	β1-integrin			Lung inflammation?	Expression of ICAM-1 and IL-6	[3]
Hepatocytes		AT1; AT2	Angiotensin II	G protein	Viral hepatitis-induced carcinogenesis?	ERK activation; glycogen granule accumulation	[10]
Smooth muscle	Vascular	AT1; AT2	Angiotensin II; sICAM-1	G protein	Hypertension vascular injury	Protein synthesis; cell migration	[10]
Endothelial cells	Vascular				Hypertension vascular injury	Cell growth, migration, survival; separation of lymphatic vessels from blood vessels	[10]
Neuronal				CD3ζ?		Neuronal differentiation and neurite extension	[10]

several nodes of the inflammatory cascade. Allergic pathologies are characterized by enhanced production of IgE antibodies directed against antigens. IgE bound to the FcεR1 receptors present on mast cells and basophils cell membrane causes receptor aggregation. The FcεRI is a heterotetramer consisting of a single IgE-binding α-sub-unit, a β-subunit and two γ-chains [11]. The cytoplasmic tails of both the β and the γ subunit contain an ITAM motif. IgE receptor interaction causes receptor aggregation and the consequent activation of the cytoplasmic Src kinase, Lyn. Lyn binds to the β-subunit and phosphorylates the ITAM motif that is then bound by the two Syk SH2 domains (Fig. 1).

Syk activation induces a cascade of events causing cell activation with consequent synthesis and release of massive amount of inflammatory modulators responsible for the acute allergic reaction. In a similar way, Syk is involved in the activation of B cells and T cells induced by the interaction between the B-cell and T-cell receptors (BCR and TCR, respectively) and antigens.

B cells are fundamental for the adaptive immune response producing antibodies directed against foreign particles and cells

and presenting antigen coupled with the major histocompatibility complex (MHC) to T cells. The cells are activated by the interaction of BCR with antigens. BCR is constituted by an immunoglobulin coupled with Igα and Igβ, two proteins containing the ITAM motif able to interact with Syk. BCR activation induces a secondary messenger's cascade leading to B-cell development and maturation. After antigen stimulation, Syk$^{-/-}$ B cells accumulate at the early stage of maturation yet fail to express complement receptors and other markers [12].

B cells, like other immunocompetent cells, also express a peculiar immunoglobulin receptor, the FcγRIIB. FcγRIIB is constituted by a single subunit lacking the ITAM sequence and bearing an inhibitory motif called the immunoreceptor tyrosine-based inhibitory motif that, when phosphorylated and coupled to BCR, inhibits Syk activation [13]. FcγRIIB represents a feedback system that regulates lymphocyte activation.

T cells play a pivotal part in cell-mediated immunity. They are characterized by the presence on their surface of peculiar receptors, the TCR. The TCR is deputed to recognize antigens bound to
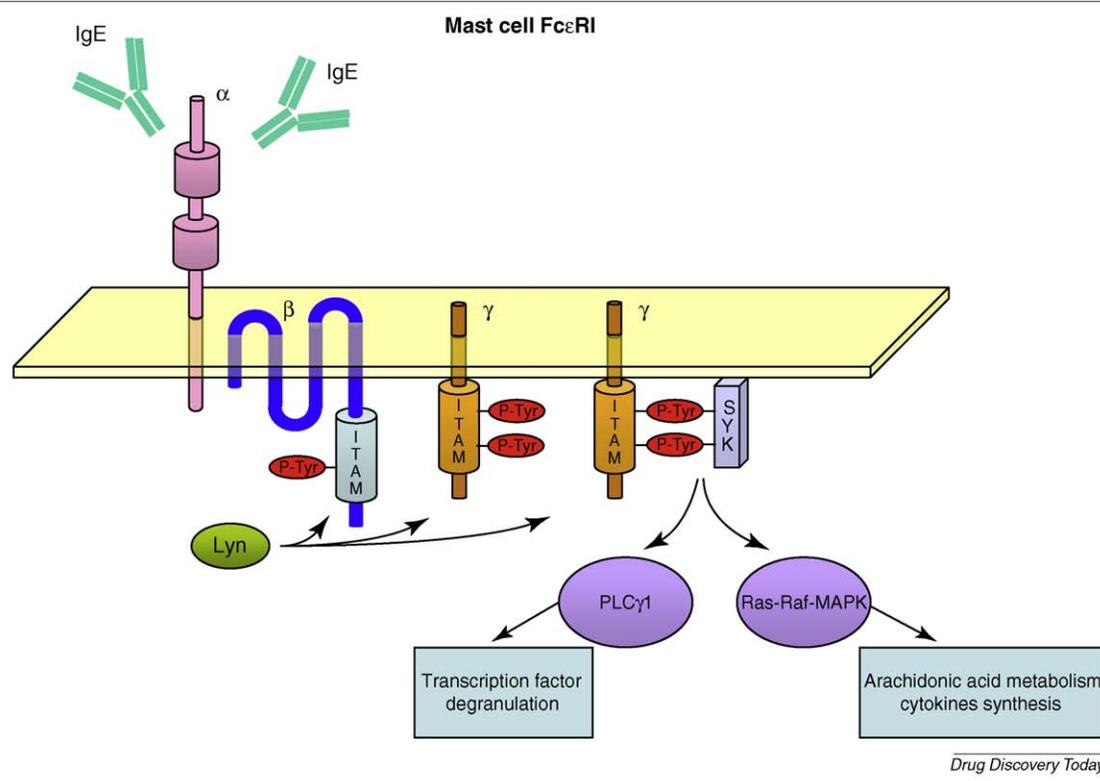
FIGURE 1

IgE/receptor interaction causes Lyn activation that phosphorylates β and γ ITAM motif. Syk binds to the γ subunit posphorylated tyrosine trough the tandem SH2 domains and after phosphorylation modulates the downstream pathways.

MHC and is constituted, in the majority of cases, by α and β and, to a lesser degree, by γ and δ heterodimer. The coupling between the receptor and the complex formed by antigen and MHC complex induces a cascade of events causing activation of resting T cells and differentiation to effector T cells. In the resting T cells, TCR complex contains another module formed by CD3γ, δ, ε and ξ. The ITAM motif of CD3ξ interacts with the tandem SH2 domains of ZAP-70. The TCR present in the effector T cells CD3ξ is replaced by the FcR-γ subunit, which is able to interact with Syk [14].

NK cells (the cells responsible for killing of tumor and infected cells) express a variety of different receptors, some of them containing the ITAM motif [15]. The IgG receptor FcγIIIa activates antibody-dependent cellular cytotoxicity, whereas NKp30, NKp44 and NKp46 enhance killing activity and, for this reason, are called natural cytotoxicity receptors. The nature of the endogenous ligands for these receptors is still to be clarified. The signals mediated by these receptors are transmitted by Syk; however, NK cells deriving from mice lacking the genes for Syk and ZAP-70 are still able to lyse various tumor targets *in vitro* and *in vivo*, suggesting that the two kinases are not essential for NK cytolytic activity [16]. These results strongly suggest that distinct and redundant signaling pathways are involved in NK natural toxicity.

The dendritic cells are the major antigen-presenting cells necessary for T-cell differentiation and activation. IgA, IgG and IgE bind to the respective receptors FcαR, FcγR and FcεR present on the surface of immature dendritic cells. The antigens are then internalized and processed and the cells upon maturation present antigens at their cell surface through MHC molecules. The cells migrate to the draining lymph nodes where they reside in the mature antigen-priming mode. Syk is not necessary for dendritic cell differentiation but is essential for immune complex internalization and the following antigen presentation to T cells. Syk also has a pivotal role for dendritic cell maturation and the subsequent interleukin (IL)-12 production [17].

Macrophages and neutrophils represent the first group of inflammatory cells to migrate toward the site infected by other organisms. They are phagocytes and after stimulation release several proteins having cytotoxic and chemoattractant activity that, in turn, causes the inflammation amplification. These cells are involved in both allergic and autoimmune reaction. Evidence in the literature indicates that Syk is implicated in integrin- and IgG-receptor-mediated signaling: Syk-deficient macrophages were defective in phagocytosis induced by FcγR activation by opsonized IgG, whereas the integrin-dependent, complement-mediated phagocytosis was unaltered. Induction of NOS2 in response to Lipopolysaccharides (LPS) and gamma-interferon was normal in mutant macrophages, suggesting that Syk is not involved in these signaling pathways [18]. Furthermore, Syk expression inhibition using antisense oligonucleotides or small interfering RNA caused a reduction in LPS-induced responses in rat alveolar macrophages *in vivo* and in rat peritoneal macrophages *in vitro* [19]. Syk-defective neutrophils were not able to generate reactive oxygen intermediates in response to opsonin-IgG, whereas the generation of reactive oxygen species in response to tetradecanoyl phorbol acetate was not affected. By contrast, Syk mutant neutrophils displayed normal degranulation and migration in response to the bacterial peptide formyl-Met-Leu-Phe [19]. Taken together, these results suggest that macrophages and neutrophils possess several different

redundant pathways involved in innate responses and that Syk is involved in some of them.

Syk is also crucial for platelet function; in particular, Syk is involved in the signal cascade induced by the activation of several platelet surface receptors, such as the collagen GpVI, integrin, αIIbβ3 and lectin CLEC-2 receptors. The selective Syk inhibitor R406 fully prevents human platelet shape change and aggregation induced by activation of Fcγ-R chain and CLCE-2 receptors [20].

Syk in other cells

In addition to immune response, ITAM-based activation is also involved in a variety of other biological functions. For example, Syk is crucial for osteoclast differentiation and maturation. Precursor myeloid cells express DAP12 and Fcγ-R chain, two proteins containing the ITAM motif. Bone marrow cells lacking both proteins are not able to differentiate into multinucleated osteoclasts and reabsorb bone *in vitro* and showed impaired phosphorylation of Syk. Osteoclast progenitors derived from Syk knockout mice exibited similar impairment in development and bone resorption [21]. Osteoclasts express high levels of αvβ3 integrin. This receptor, after interaction with bone matrix proteins, mediates the ability of the cells to organize the cytoskeleton, polarize, produce organelles containing H⁺ATPase and, finally, degrade bone. This process is under the control of a complex formed by αvβ3 integrin and c-Src kinase. Upon integrin occupancy, c-Src phosphorylates Syk through the mediation of DAP12 and Fcγ-R [22], leading to osteoclast bone resorption.

Synoviocytes are the main cells present in the luminal surface of the synovial membrane. Among them, the fibroblast-like synoviocytes are involved in the production of matrix constituents, such as collagens and fibronectin, for synovial fluid and intimal interstitium. These cells also have an important role in matrix degradation by producing enzymes such as collagenase and metalloprotease. In fibroblast-like synoviocytes deriving from RA patients, Syk is expressed and phosphorylated and was capable of activation by exposure to TNF-α. In synoviocytes, TNF-α induces c-jun N-terminal kinase activation and proinflammatory gene expression. The Syk inhibitor R406 was able to inhibit the increase of metalloprotease 3 gene expression induced by TNF-α exposure [23]. In addition, the Syk knockdown strongly inhibited TNF-α-induced production of IL-32, a potent proinflammatory cytokine that seems to have a crucial role in RA [24].

Syk kinases as a therapeutic target for disease

As described previously, Syk is a key mediator of immunoreceptor signaling in a host of inflammatory cells, including B cells, mast cells, macrophages and neutrophils. These immunoreceptors, including Fc receptors and BCR, are important for both allergic and antibody-mediated autoimmune diseases and, thus, interfering with Syk could conceivably represent a means of treating these disorders [6]. Several studies have highlighted Syk as a key player in the pathogenesis of a multitude of diseases (Fig. 2), including RA [25], asthma [26], allergic rhinitis [27], lymphoma [28], leukemia [29], carcinoma [30], metastasis [31], functional gastrointestinal disorders [32], idiopathic thrombocytopenic purpura [33], Wiskott-Aldrich syndrome [34], systemic lupus erythematosus [35], multiple sclerosis [36] and anaphylactic shock [37].

Rheumatoid arthritis

RA is a chronic, systemic, inflammatory disorder that affects many tissues and organs but principally attacks the joints, producing an inflammatory synovitis that often progresses to destruction of the articular cartilage and ankylosis of the joints [6]. Approximately 1% of the worldwide population is afflicted by RA, with an annual incidence of 25–50 new cases per 100,000 individuals [3].

Despite extensive efforts, the etiology and pathogenesis of RA remain poorly understood, and numerous cell populations have been implicated, including T cells, B cells, monocytes and macrophages, mast cells, dendritic cells, and fibroblasts. Chemokines, matrix metalloproteinases, adhesion molecules, angiogenic growth factors and dysregulated intra-articular expression of proinflammatory cytokines (in particular IL-1β and TNF-α) have key roles in the pathogenesis of RA [38].
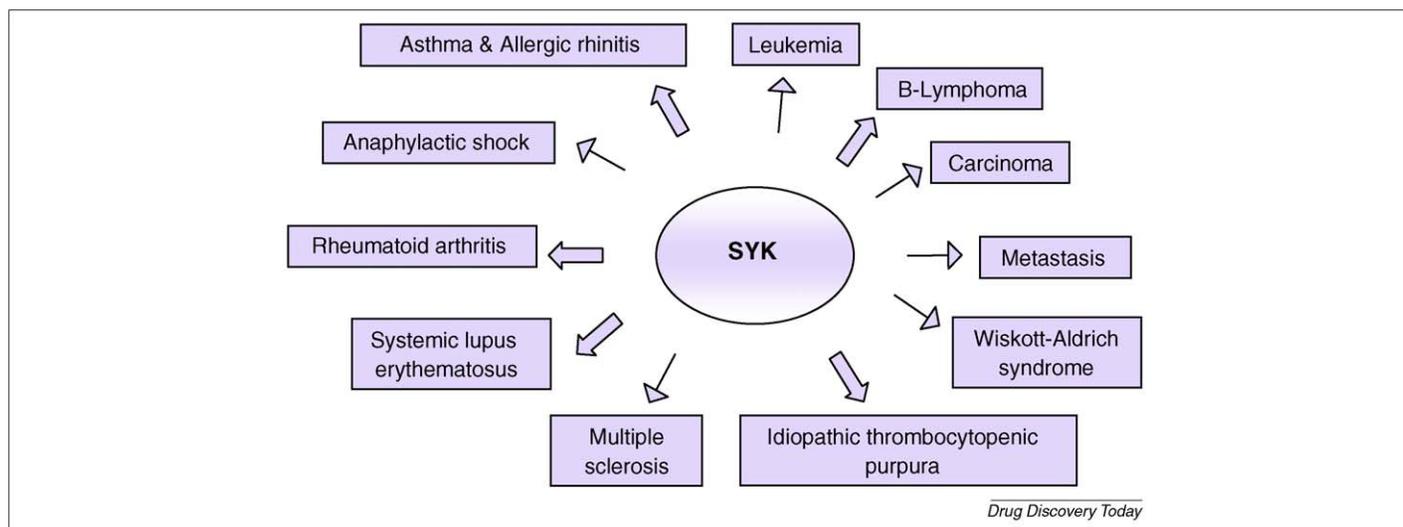

FIGURE 2

Spleen tyrosine kinases (Syk) are involved in different inflammatory and autoimmune disorders such as those reported in the figure (bold arrow: pathologies expanded in the paper).

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Pharmacological treatments for RA include non-steroidal anti-inflammatory drugs (NSAIDs, e.g. nimesulide, celecoxib and rofecoxib), steroids (e.g. prednisolone-dipyridamole, prednisolone farnesylate and Advantan), and disease-modifying antirheumatic drugs (DMARDs) (e.g. methotrexate, sulfasalazine, leflunomide, cyclosporine A and hydroxychloroquine) [3]. Nevertheless, these drugs are characterized by important adverse effects, such as gastrointestinal and renal side-effects (NSAIDs), bone loss (steroids) and liver toxicity (methotrexate).

The unmet needs in RA treatment triggered the development of biological agents. They show impressive clinical effects either alone or in combination with DMARDs. Such drugs include TNF-α blockers (etanercept, infliximab and adalimumab), IL-1 receptor antagonists (anakinra and AMG 108), monoclonal antibodies against B cells (rituximab, ocrelizumab and HuMax), T-cell co-stimulation blockers (abatacept) and IL-6 receptor antagonists (tocilizumab).

Despite the evident success of biological therapies, concerns remain regarding their immunosuppressive effects and the associated increased risk of infection [39]. Furthermore, biological therapies do not induce permanent remission, and they are expensive and are associated with the pain involved from long-term multiple injections. Other pathways and modes of drug delivery, therefore, need to be investigated for RA patients.

The use of small-molecule inhibitors against the signaling pathways that lead to the process of inflammation, cartilage and bone destruction has been a recent focus for the development of drugs for RA.

Rigel Pharmaceuticals Inc. is developing fostamatinib (R788), a prodrug of Syk inhibitor R406, for the potential treatment of RA. This compound is a potent inhibitor of IgE- and IgG-mediated activation of Fc receptor signaling (EC_{50} for degranulation = 56–64 nM). R406 inhibited phosporylation of Syk substrate linker for activation of T cells in mast cells and B cells linker protein SLP65 in B cells. Consistent with Syk inhibition, oral administration of R406 to mice reduced immune-complex-mediated inflammation in a reverse-passive Arthus reaction and two antibody-induced arthritis models [40]. Preclinical studies of R406 or R788 demonstrated a statistically significant reduction in major inflammatory mediators, leading to reduced inflammation and bone degradation in models of RA [40]. The phase II clinical trial data for R788 are impressive. The drug achieved ACR20 response rates of 65% and 72% when given twice daily at 100 mg and 150 mg, respectively (ACR20, a composite endpoint defined by the American College of Rheumatology to measure a drug's impact on clinical signs and symptoms of RA, requires 20% improvements in tender joint count, in swollen joint count and in least three of five other parameters [41]). Just 38% of patients in the placebo arm attained a similar response. R788 also demonstrated superior efficacy over placebo at ACR50 and ACR70, which are more stringent measures of patient responses to treatment. R788 is orally bioavailable and was well tolerated in phase I and II trials, with the most common side-effects being gastrointestinal intolerance, neutropenia and elevated liver alanine aminotrasferase levels. Those side-effects do not compare unfavorably with those of biologics. AstraZeneca and Rigel Pharmaceuticals have announced an exclusive worldwide license agreement for the global development and commercialization of fostamatinib disodium, or R788. R788, which has completed a comprehensive phase II program, is the most devel-

oped oral Syk inhibitor being evaluated for RA. AstraZeneca will design a global phase III program, anticipated to begin in the second half of 2010, with the goal of filing New Drug Applications with the US Food and Drug Administration and the European Medicines Agency in 2013 [42]. Thus, R788 seems to be a promising therapeutic for RA and a direct challenger to the TNF-α inhibitor biologics, even if data are required to establish the efficacy and long-term safety of the drug [41].

Allergic conditions

Bronchial asthma is a complex disease of the lung that affects the lower respiratory tract, characterized by reversible airway obstruction, chronic airway inflammation and airway hyperresponsiveness. An estimated 300 million people worldwide suffer from asthma, with 500,000 hospitalizations each year and 250,000 annual deaths attributed to the disease. It is estimated that the number of people with asthma will grow by more than 100 million by 2025. The annual economic cost of asthma is $19.7 billion [43].

The activation of the immunological pathway in response to environmental antigens (allergens), elevated levels of IgE and high-affinity binding to specific receptors on mast cells and basophils, followed by receptor crosslinking and activation of the pro-inflammatory signaling pathway, are key features of asthma pathophysiology in the majority of patients [44].

Allergic rhinitis, or allergy, is characterized by a hypersensitive immune response in the upper airways to seasonal or perennial allergens leading to episodes of sneezing, itching, nasal congestion and a runny nose. Allergic rhinitis is the most prevalent respiratory disorder in industrialized society, affecting approximately 40% of children and nearly a third of the adult population [27]. The prevalence of rhinitis is approximately 35% in Europe and Australasia, according to the European Community Respiratory Health Survey. From 2000 to 2005, the cost of treating allergic rhinitis almost doubled from $6.1 billion (in 2005 US dollars) to $11.2 billion [45].

Allergic rhinitis shares many cellular and molecular mechanisms with asthma. Moreover, there is growing evidence of a close link between allergic rhinitis and the subsequent development of asthma, and recent studies have suggested that effective treatment of rhinitis can reduce the proportion of patients that progress to develop asthma [46].

Extensive evidence has shown that the pathophysiology of human allergic conditions is closely associated with the production of allergen-specific IgE. IgE, in turn, binds tightly to its multimeric receptor, referred to as FcεRI, which is present on the surface of mast cells and basophils in the mucosal lining of the airways. This binding sensitizes the cells to specific allergens, stabilizes the receptor complex and causes a substantial increase in the expression of FcεRI on the cell surface. Subsequent exposures with the multivalent allergens result in the crosslinking of antigen-specific IgE–FcεRI complex inducing the release and production of hundreds of mediators. Collectively, these mediators are thought to play major parts in causing the symptoms of early and late allergic response [27].

The majority of drugs currently used to treat allergic disorders target only a single mediator released by mast cells. Examples include antihistamine H1 receptor antagonists (e.g. diphenhydramine, fexofenadine and azelastine), leukotriene modifiers (e.g. montelukast, zafirlukast, pranlukast and zileuton) and steroids

(e.g. ciclesonide, beclomethasone and fluticasone) that predominantly inhibit mast-cell mediator production [6]. Rather than antagonizing single mediators, an alternative approach is to inhibit the production and release of all mast cell mediators by antagonizing IgE action. In this respect, an important addition to drug therapies for allergic conditions is the recombinant, humanized IgG monoclonal antibody omalizumab, which binds selectively to human IgE. Omalizumab has demonstrated clinical efficacy in patients with allergic asthma and seasonal and perennial allergic rhinitis, confirming the central role of IgE in such diseases [47]. This biologic, however, is rather expensive and requires regular injections.

An alternative to blocking IgE action is to target the intracellular signaling cascade initiated by the antigen crosslinking of the multimetric IgE–FcεRI receptor complex. For this reason, Syk has been considered as a compelling target for therapeutic intervention [48]. Aerosolized Syk antisense oligodeoxynucleotide inhibits allergen-induced inflammation in rat model, indicating that this might be a target for drug development [49]. Several companies started developing Syk inhibitors for treating asthma, including Bayer, Novartis, Rigel Pharmaceuticals and Pfizer [26]. The Bayer researchers identified BAY 61-3606, a potent (K_i = 7.5 nM) and selective inhibitor of Syk kinase capable of blocking antigen-induced inflammation both in vitro and in vivo. Oral administration of BAY 61-3606 to rats statistically significant suppressed bronchoconstriction and bronchial edema. Furthermore, BAY 61-3606 attenuated antigen-induced airway inflammation in rats [50]. Efficacy of the Syk kinase inhibitor NVP-QAB205-AA (Novartis AG) was observed in vivo asthma models, as well [51]. Rigel developed the first Syk inhibitor to enter clinical studies: R112 was tested in a phase II double-bind, randomized, placebo-controlled study using an intranasal administration in volunteers with symptomatic seasonal allergic rhinitis [52]. R112 applied intranasally statistically significant improved symptoms over placebo, and duration of action exceeded four hours, demonstrating for the first time the clinical relevance of Syk inhibition in humans [42]. A further seven day efficacy study of intranasal R112 dosed twice daily failed to demonstrate an effect over placebo, however, suggesting a longer duration of action is necessary to show benefit [53]. Rigel Announces Pfizer Selection of R343, a Syk Kinase Inhibitor, for Advanced Preclinical Development in Allergic Asthma. Rigel Pharmaceuticals, Inc. Press release, May 17, 2006. In December 2007, Pfizer began a phase I clinical trial of an inhaled formulation of R343 for the treatment of allergic asthma [54].

Idiopathic thrombocytopenic purpura
Idiopathic thrombocytopenic purpura (ITP, also known as primary immune thrombocytopenic purpura) is an autoimmune hematological disorder in which antibodies attack and destroy platelets. Symptoms of ITP include the development of bruises (purpura), petechiae, the formation of hematomas, bleeding from the nostrils and at the gums, and internal bleeding. In the USA, ITP affects approximately 2 in 100,000 adults per year and 5 in 100,000 children per year [55]. Patients with ITP have accelerated clearance of circulating IgG-coated platelets via FcγR-bearing macrophages in spleen and liver. ITP is mediated by production of IgG against one or more antigens exposed on platelets. Specifically, antibodies related to platelet glycoproteins (GP) IIb–IIIa, GPIb–IX, GPIb and

GPIIIa, among others, have been shown to be potentiators of ITP [56]. The coating of platelets with IgG renders them susceptible to opsonization and phagocytosis by splenic macrophages. The IgG autoantibodies are also thought to damage megakaryocytes, the precursor cells to platelets, but this is thought to contribute only slightly to the decrease in platelet numbers.

A normal platelet count is considered to be in the range of 150,000–400,000 per cubic millimeter of blood for most healthy individuals; hence, one can be considered thrombocytopenic below that range, although the threshold for a diagnosis of ITP is not tied to any specific number. Several studies have shown that patients with platelet counts persistently below 30,000 per cubic millimeter are at risk of a life-threatening bleed [57].

As far as ITP treatment is concerned, a platelet infusion might be administered in an emergency-bleeding situation to attempt to rapidly raise the platelet count. First-line therapy includes steroids (dexamethasone or prednisone), intravenous immunoglobulin or a combination of these drugs. Second-line treatments include removal of the spleen (splenectomy). A new strategy is treatment with anti-D IgG (WinRho, Rhophylac or RhoGAM) to Rh-D positive patients [57]. Immunosuppresants, such as mycophenolate, mofetil and azathioprine, are also being shown to be effective and useful in treatment. Other agents, such as romiplostim and eltrombopag, have been developed to boost platelet production. The off-label use of rituximab, a chimeric monoclonal antibody against the B-cell surface antigen CD20, has been shown in preliminary studies to be an effective alternative to splenectomy in some patients [57].

Syk associates with the FcγR in various inflammatory cells, including macrophages, which are thought to be the cells responsible for platelet clearance in ITP. Ligand binding to FcγR I, IA and IIIA induces activation of the receptor complex and phosphorylation of the ITAMs with the downstream recruitment and activation of Syk. Given its central role in FcγR-mediated signal transduction and propagation of inflammatory response, Syk can be considered a reasonable target for ITP. Indeed, blocking FcγR signaling by inhibiting Syk would ameliorate platelet destruction in patients with ITP. To determinate whether inhibition of Syk would be useful in FcγR-dependant cytopenias, such as ITP, mouse models were used to evaluate R406 in treating cytopenia. Amelioration of ITP was dramatic and, thus, a phase II trial was initiated to study the effect of Syk inhibition in humans with ITP [33]. In November 2007, R788 (a novel, oral Syk kinase inhibitor) entered phase II clinical trial in patients with ITP. The single-center, open-label, dose-escalating study showed that R788 can improve platelet counts in this autoimmune disorder. The primary side-effects were gastrointestinal-related symptoms. R788 elevated blood pressure in some patients but did not seem to have a statistically significant effect on neutrophil counts [58]. In conclusion, inhibition of Syk was an efficacious means of increasing and maintaining the platelet count in half the patients with chronic refractory ITP.

Systemic lupus erythematosus
Systemic lupus erythematosus (SLE, or lupus) is a chronic autoimmune disease that can affect every organ system of the body including joints, skin, blood vessels, liver, kidney and nervous system. It initially manifests as fever, malaise, joint paints, myalgias and fatigue. The course of the disease is unpredictable, with periods of illness (called 'flares') alternating with remissions [59].

In the USA, the prevalence of SLE is estimated to be approximately 53 per 100,000, and in Northern Europe, the rate is approximately 40 per 100,000 people. The disease occurs nine times more frequently in women than in men [60].

Because of the variety of symptoms and organ system involvement in SLE, its severity in an individual must be assessed to treat SLE successfully. Current therapies for lupus treat the symptoms of the disease and include NSAIDS (e.g. ibuprofen), DMARDs (e.g. antimalarials, such as hydroxychloroquine), steroids (e.g. methylprednisolone and prednisolone) immunosuppressants (e.g. methotrexate, cyclophosphamide and azathioprine) and immune globulin intravenous (Gamimune, Gammagard and Sandoglobulin) [61].

SLE is characterized by multisystem microvascular inflammation with the generation of autoantibodies. Although the specific cause(s) of SLE is unknown, multiple factors are associated with the development of the disease, including genetic, racial, hormonal and environmental factors. The genetic contributions to disease susceptibility and severity are complex and involve multiple traits that implicate abnormalities of both innate and adaptive immune systems [59].

FcRγ-deficient (NZB/NZW) mice generate and deposit immune complex and activate complement but are protected from severe nephritis, suggesting that the downstream inflammatory events are dependent on FcRγ expression. FcRγ deficiency reduces experimental hemolytic anemia and thrombocytopenia in mice and modulates development of anti-glomerular basement membrane anti-glomerular basement membrane (anti-GBM) antibody-induced glomerulonephritis. NZB/NZW mice given soluble FcγRIII demonstrate reduced anti-double-stranded DNA antibody levels, decreased proteinuria and prolonged survival, providing a statistically significant rationale for targeting FcR pathways for the treatment of lupus [60].

B cells also have a considerable role in lupus pathogenesis via production of autoantibodies, resulting in antigen–antibody immune complex formation and stimulation of FcR. Because Syk is crucial for the signaling of B-cell receptors and activating FcR, a Syk-selective inhibitor would ameliorate disease in spontaneous lupus-prone mice [35].

The importance of Syk function in SLE is further substantiated in the literature, including reported affects on ITAM-bearing ezrin/radixin/moesin signal transduction in T-cell tissue invasion, and FcγRIIIa alleles predictive of progression to end-stage renal disease [62]. Bahjat et al. demonstrated that the Rigel Syk-selective inhibitor R406/R788 prevented the development of renal disease and treated established murine lupus nephritis [59].

A phase II, multicenter, double-bind, randomized, placebo-controlled clinical trial was planned to assess the efficacy and safety of R788 (150 mg p.o., bid) in patients with SLE (n = 225). At the time of publication, the trial had been suspended.

B-cell lymphoma

Lymphoma is the name given to a variety of blood cancers that result when lymphocytes, or white blood cells, grow uncontrollably and build up in the lymphatic system and bone marrow, giving rise to malignant tumors. In general, lymphomas are divided into two large groups of neoplasms, namely Hodgkin disease and non-Hodgkin lymphoma (NHL).

NHL is the most abundant lymphoma with extensive heterogeneity over 25 subtypes. NHL is the fifth most common cancer in the United States, and from 2002 to 2006, its incidence was 19.5 per 100,000 individuals per year, and its age-adjusted death rate was 7.1 per 100,000 men and women per year [63]. Eighty-five percent of NHL belongs to the B lineage, and the most commonly occurring variety includes the diffuse large B-cell lymphomas (DLBCLs). Follicular lymphoma (FL) is the second most common type of B-NHL and includes 35–40% of all adult lymphomas [64].

NHLs are treated by combinations of chemotherapy, monoclonal antibodies, immunotherapy, radiation and hematopoietic stem cell transplantation. Despite the important progress that has been made, mostly because of the introduction of rituximab (a chimeric monoclonal antibody against the protein CD20, sold under the trade names Rituxan and MabThera) combined with chemotherapy, further efforts are needed to identify new molecular targets in NHL. Indeed, the five-year survival rate for patients with NHL is estimated to be 50% and, even for patients who respond to anti-CD20 treatment, disease recurrence is common [28].

The uncontrolled growth of tumor cells, particularly B cells, is partially mediated by the Syk enzyme, which signals the growth and survive mechanism of aberrant B cells. By inhibiting Syk, the signal is curtailed and the aberrant cells cannot proliferate. Syk is involved in pre-B-cell development and maturation and seems to amplify B-cell antigen receptor signaling, which might be an important survival mechanism in certain B-cell lymphomas [55].

The mammalian target of rapamycin (mTOR) is emerging as a promising target for antitumor therapy. Leseux et al. have demonstrated that Syk has a central role in mTOR activation; they found that both expression and activity are elevated compared to normal or chronic lymphocytic leukemia B cells. In addition, they provided evidence that Syk operates through phospholipase-D- and phosphatidylinositol 3 kinase (PI3K)-independent pathways. Finally, Syk inhibition by piceatannol or siRNA plasmids resulted in a potent inhibition of mTOR activity in FL cells, as well as in mantle cell lymphoma, Burkitt lymphoma and DLBCLs. These findings suggest that the Syk–mTOR pathway has a crucial function in FL survival and, therefore, that Syk could be a promising new target for B-lymphoma therapy [28].

Gururajan et al. investigated the ability of curcumin to modulate the growth of B lymphomas. Curcumin inhibited the growth of both murine and human B lymphoma through inhibition of survival kinase Akt and its key target, Bad. In vitro kinase assays, however, showed that Akt is not a direct target of curcumin. Gururajan et al. identified Syk as a novel target for curcumin in B lymphoma. Syk is constitutively activated in primary tumors and B lymphoma cell lines, and curcumin down-modulated Syk activity accompanied by downregulation of Akt activation. Moreover, they showed that overexpression of Akt (a target of Syk) or Bcl-x_L (a target of Akt) could overcome curcumin-induced apoptosis of B lymphoma cells. These observations suggest a novel growth-promoting role for Syk in lymphoma cells [64].

Another study suggesting that targeting Syk might be a viable therapeutic strategy was reported by Yung et al. Using a genetic approach, they showed that Syk expression is required for the survival of murine NHL-like tumors in vitro and that tumor cells deficient in Syk failed to expand in vivo. In addition, a pharma-

cologic inhibitor (R788) of Syk was able to induce apoptosis of transformed B cells *in vitro* and led to tumor regression *in vivo*. Finally, they showed that genetic or pharmacologic inhibition of Syk activity in human NHL cell lines are generally consistent with results found in mouse models [65].

Therapeutic targeting of the BCR pathway in NHL is currently being explored in the clinic. For example, the immunoglobulin complexes on the surface of the B-cell lymphomas can be targeted by specifically generating monoclonal antibodies to an idiotype [66].

Another example involves Rigel's Syk inhibitor R788. The key advantage of a small molecule inhibitor of downstream signal from BCR, like R788, is that their relative lack of specificity compared with an immunological approach allows their use across a wider spectrum of tumors. A phase I/II, multicenter, open-label clinical trial in patients with refractory or relapsed B-cell NHL assessed the efficacy and safety of R788. Of the 68 patients enrolled, 23 had DLBCLs, 21 had FL, 11 had chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), 9 had mantle cell lymphoma (MCL), 1 had lymphoplasmacytic NHL and 3 had mucosa-associated lymphoid tissue (MALT) lymphoma. No response was observed in the patient with lymphoplasmacytic NHL or MALT lymphoma. Response rates for the other lymphomas were 21% (DLBCLs), 10% (follicular NHL), 54% (CLL/SLL) and 11% (MCL). The median progression survival was 4.5 months. This trial was ongoing at the time of publication [55].

Small molecules as Syk inhibitors

Several pathologies can be treated through the inhibition of Syk activity. The huge number of Syk-related patents and papers found in the literature is a further indication of its attractiveness as a therapeutic target. The great interest in this field is shown, not only by the increasing number of international applications in recent years but also by the noteworthy number of pharmaceutical companies and academic institutions that have been involved in the development of small-molecule Syk inhibitors. As already reported by Xie *et al.* [67], more than one hundred small molecules have been reported as having such activity. Some of the principal classes of these molecules are shown in Table 2.

The first three structures reported in Table 2 have represented key scaffolds in the search for more potent and selective Syk inhibitors. Staurosporine (Table 2, entry 1) [68] is a natural product that shows biological activities ranging from anti-fungal to anti-hypertensive. The main biological activity of staurosporine is the inhibition of protein kinases through the prevention of ATP binding to the kinase. Staurosporine binds to many kinases with high affinity, although with little selectivity. Imatinib mesylate (Table 2, entry 2) [69], marketed by Novartis as Gleevec, is used in the treatment of several types of tumors. It is the first member of a new class of agents that act by inhibiting Syk, rather than non-specifically inhibiting rapidly dividing cells. Piceatannol (Table 2, entry 3), a stilbene derivative, (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) has been reported to act as an anti-inflammatory, immu-

TABLE 2

**Structures of Syk inhibitors reported in the literature**

| Entry	Compound	Company	Structure	Reported biological activity as Syk inhibitor (IC$_{50}$)	Refs
1	Staurosporine			13 nM	[68]
2	Imatinib (Gleevec®, STI-571)	Novartis			[69]
3	Piceatannol				[70]
4	R112	Rigel		226 nM	[71–74]
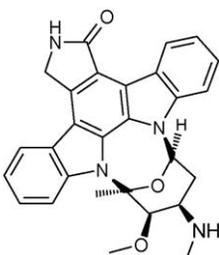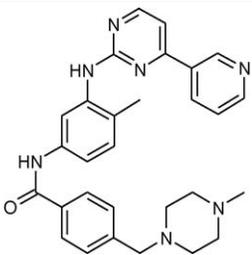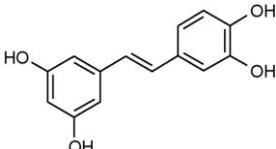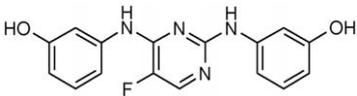
TABLE 2 (Continued)

Entry	Compound	Company	Structure	Reported biological activity as Syk inhibitor (IC$_{50}$)	Refs
5	R406	Rigel		41 nM	[75]
6	R788	Rigel			[55,76–79]
7	R343 (?)	Pfizer			[80]
8	BAY61-3606	Bayer		7.5 nM	[81,82]
9		Boehringer Ingelheim		90 nM	[83,84]
10		Aventis		5 nM	[85]
11		Astellas		41 nM	[86]
12		Kissei		9 nM	[87–89]
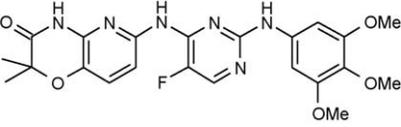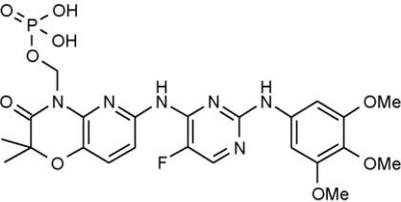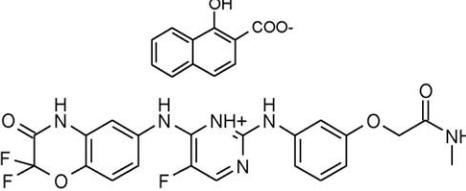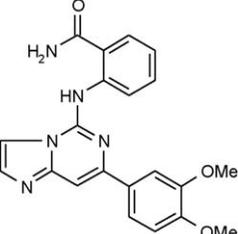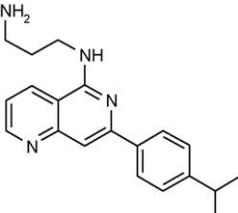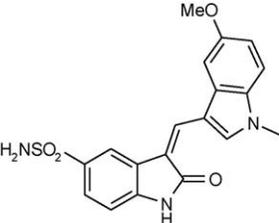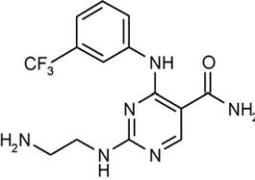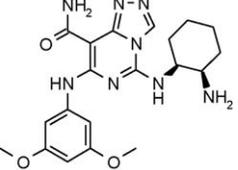
TABLE 2 (*Continued*)

Entry	Compound	Company	Structure	Reported biological activity as Syk inhibitor (IC_{50})	Refs
13		Kissei			[87–90]
14		Kissei			[88–90]
15		Vertex		630 nM	[91,92]
16		Bayer Yakuhin			[93]
17	ER-27319	Eisai		10 nM	[94]
18	NVP-QAB205-AA	Novartis		10 nM	[95]
19		Novartis			[96]
20		Hoffmann-La Roche			[97]
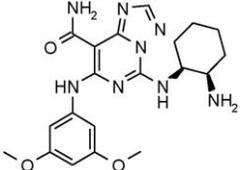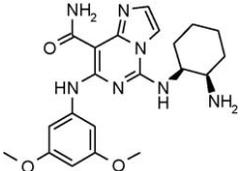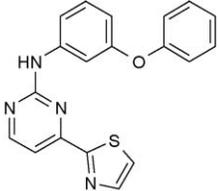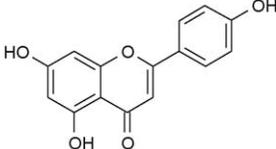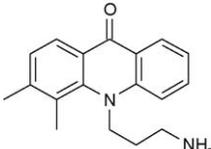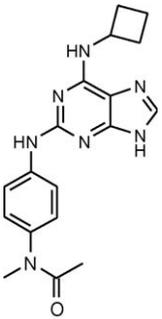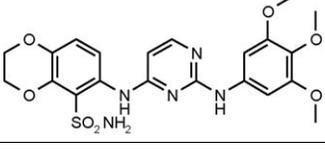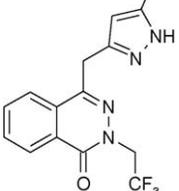
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TABLE 2 (Continued)

Entry	Compound	Company	Structure	Reported biological activity as Syk inhibitor (IC$_{50}$)	Refs
21		Glaxo Group Limited			[98,99]
22		Glaxo Group Limited			[100]
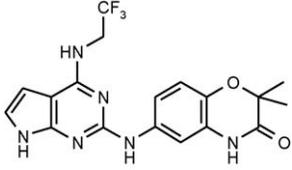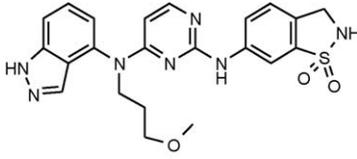
nomodulatory and anti-proliferative compound [70]. All these compounds, although affected by selectivity issues, served as valuable starting points for the structure-based drug discovery that, in recent years, has led to more potent and selective spleen tyrosine kinase inhibitors.

Rigel Pharmaceutical first claimed the use of 2,4-pyrimidine-diamine as a Syk inhibitor in WO03063794 [71] and WO2004/014382 [72]. Further claims by Rigel for the use of compounds with similar structures are made in WO2005/012294 [73], WO2006/068770 [74] and WO2007/120980 [75]. From these applications, structures of R112 and R406 (Table 2, entry 4 and 5) – whose properties as Syk inhibitors have been discussed previously – came forth. In this context, two more compounds are worth mentioning: Rigel's R788 (Table 2, entry 6), now undergoing clinical studies as Tamatinib Fosdium, or Fostamatinib [55], and R343. Structure of Fostamatinib, together with its salts and method of preparation, were originally claimed in WO2006/078846 [76]. Fostamatinib is a phosphate prodrug of R406 that undergoes *in vivo* metabolism to give back the original active compound. More recent international applications, in which specific uses of Fosta-matinib are also claimed, are WO2007/124221 [77], WO2008/061201 [78] and WO2008/064274 [79]. In 2009, Pfizer Limited claimed the preparation of xinafoate salt of N4-[(2,2-difluoro-4H-benzo [1,4]oxazin-3-one)-6-yl]-5-fluoro-N2-[3-(methylaminocar-bonylmethylene oxy)phenyl]-2,4-pyrimidinediamine (Table 2, entry 7) to be used for inhaled formulation in the treatment of asthma [80]. Among compounds that are reported to have benefits in the treatment of asthma by preventing antigen-induced broncho-constriction and airway inflammation, BAY 61-3606 [81] is also noteworthy. It is an orally available imidazo[1,2-c]pyr-imidine derivative (Table 2, entry 8) claimed by Bayer in WO2001/083485 [82]. In the same patent, triazolopyrimidine derivatives were also claimed for the same applications as imidazopyrimidine compounds. Both Boehringer Ingelheim Pharmaceuticals and Aventis Pharmaceuticals published, in 2003, the structure of small molecules as inhibitors of Syk. Boehringer's [1,6]naphthyridines were identified and claimed in WO2003/057695 [83]. In a paper published in the same year [84], it has been shown that 5,7-disubstituted-[1,6]naphthyridines (Table 2, entry 9) are potent inhibitors of the tyrosine kinase Syk. The structure–activity rela-tionship (SAR) around position 7 and 5 were also described in the

same paper. All evidence suggested that a 7-aryl, and preferably *para*-substituted aryl group, as well as 5-alkyldiammines, have synergistic effects on activity. Meanwhile, screening by Aventis led to the identification of oxindoles [85], whose further optimiza-tion led to the elucidation of the indole-substituted oxoindoline (Table 2, entry 10) with IC$_{50}$ = 5 nM. Similarly, researchers from Astellas identified a series of anilinopyrimidine derivatives (Table 2, entry 11) as Syk inhibitors [86]. The SAR indicated that an N–H group in the 2 position of the pyrimidine ring is necessary for Syk inhibitory activity, with the ethylendiammino group as the most favorable substituent. As for substitution in the 4 position, aniline groups were effective, and formation of a hydrogen bond with the 5-carboxamide group might be very important for main-tenance of planarity, which seems to increase activity. Starting from literature reports (based particularly on staurosporine and Astellas' anilinopyrimidine derivatives) and structure-based drug design, Kissei's researchers designed 1,2,4-triazolo[4,3-c]pyrimi-dine and 1,2,4-triazolo[1,5-c]pyrimidine [87]. Representative com-pounds of this class (Table 2, entries 12 and 13) exhibited strong inhibition of Syk and ZAP-70 kinase and also suppressed IL-2 production by peripheral blood mononuclear cells. The same authors, later, were able to optimize the bioavailability of these compounds by reducing their polar surface area; a nitrogen atom was removed, thus decreasing the polar surface area, and a SAR around the newly-found imidazo[1,2-c]pyrimidine (Table 2, entry 14) was also performed [88]. All the aforementioned structures were claimed in JP2004/238296 [89] and JP2004/203748 [90]. It is again from a high-throughput screening campaign that Farmer *et al.* [91] at Vertex Pharmaceuticals designed a novel class of 4-thiazolyl-2-phenylaminopyrimidines as potent and selective Syk inhibitors. In particular, N-(3-phenoxyphenyl)-4-(thiazol-2-yl)pyrimidin-2-amine (Table 2, entry 15) was found to have a good affinity for SYK (K_i = 630 nM). This novel phenyl amino pyrimidine pharmacophore-based analog served as the starting point for further optimization. It is worth mentioning that these types of structures were already claimed, by the same company, in a patent filed in 2002 [92]. Flavonoids, especially flavones and flavonols, are known to inhibit the activities of several kinases. In a study published in 2003 by Bayer Yakuhin in collaboration with Gifu Pharmaceutical University [93], their activity as inhibitors of Syk and mast cell degranulation was also highlighted. The potency

of flavonoids was characterized by a 7-hydroxy benzopyrone with a 4-carbonyl and 3- or 5-hydroxyl 2-phenyl ring (Table 2, entry 16). Moreover, the inhibitory potency of flavonoids in a Syk kinase assay was different from that of other type of kinases (namely, PKC and PI3K), suggesting some selectivity against tyrosine kinase. More than ten years ago, Eisai's researchers described ER-27319, an acridone-related compound (Table 2, entry 17), as a potent and selective inhibitor of antigen or anti-IgE-mediated degranulation of rodent and human mast cells [94]; Syk activation is inhibited with an $IC_{50} = 10\ \mu M$. The list of companies that worked or are working on Syk as a therapeutic target is even wider. In 2001, Novartis filed a patent in which purine derivatives (Table 2, entry 18) were claimed to have an inhibitory activity against the tyrosine protein kinase, Syk [95]; later, in 2005, the same company filed a patent claiming 2,4,di(hetero)-arylammino-pyrimidine (Table 2, entry 19) derivatives as ZAP-70 and/or SYK inhibitors [96]. In 2007, Hoffmann-La Roche found that the phthalazin-1(2H)-one compound (Table 2, entry 20) possessed Syk inhibitory activity: this formed part of the patent WO2007/107469 [97]. Pyrrolopyrimidine derivatives (Table 2, entry 21; WO2007/042299 [98] and

WO2007/042298 [99]) and 1H-indazol-4-yl-2,4-pyrimidine diammino derivatives (Table 2, entry 22; WO2007/085540 [100]), are the two classes of compounds that Glaxo Group Limited has declared as active in inhibiting Syk activity.

Concluding remarks

Syk is a cytosolic non-receptor tyrosine kinase present in many cells, principally presiding over the inflammatory process. Inhibition of Syk activity is a valuable therapy in many pathologies, and a large number of small molecules have been synthesized and tested as Syk inhibitors. Therapeutic activity of some of them has already been demonstrated (e.g. the use of R788 in the treatment of RA) and they are currently in the advanced phases of clinical trials. Despite these encouraging results, some issues are still related to these molecules (e.g. their long-term safety has not yet been demonstrated). In addition, because these compounds are known to block the activation of cells involved in the production of inflammatory mediators (e.g. macrophages and B cells), concerns have been raised about the possibility of side-effects related to the shutting down of the whole inflammatory process.

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