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Perspective

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Discovery and Development of Spleen Tyrosine Kinase (SYK) Inhibitors

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INTRODUCTION

Allergic and autoimmune disorders share significant functional overlap in the biologic pathways responsible for the activation of signal transduction events leading to production of numerous proinflammatory factors involved in disease initiation and progression. Given the reciprocal connections in these mechanistic pathways, it would be advantageous to target strategic master regulators with novel therapeutics to treat allergic and autoimmune diseases. One such crucial regulator is spleen tyrosine kinase (SYK), a member of the cytoplasmic protein tyrosine kinase (PTK) family. Geahlen et al. initially reported SYK as a proteolytically derived fragment (p40) from a parent p72 protein extracted from bovine thymus.[1,2] The isolated p40 protein was found to be catalytically active, possessing an adenosine 5′-triphosphate (ATP) binding site, and capable of intramolecular autophosphorylation of tyrosine residues.[1,2] Further studies have led to the observation that antibodies to p40 concomitantly showed cross-reactivity with a 72 kDa protein-tyrosine kinase from the spleen and thymus.[1,2] Yamamura et al. corroborated the earlier work, employing immunoblot analysis using anticytosolic protein-tyrosine kinase-40 antibody made for the N-terminal portion of the 40 kDa kinase, and identified a 72 kDa protein, which is now known as the SYK protein.[3,4] Application of a partial sequence oligonucleotide probe for the 40 kDa kinase led to a clone comprising the complete coding sequence for the 40 kDa kinase; the clone was made up of 628 amino acids with a molecular weight of 71 618 Da and corresponded to acidophilic SYK kinase.[5−8] Subsequent to its initial discovery, SYK has garnered substantial attention because of its importance as a key signal transduction regulator through antigen and Fc receptors in hematopoietic cells.[9−11] The validity of SYK as a target for therapeutic intervention has progressed over the past 15 years, and SYK has recently entered the mainstream of potential pharmaceutical targets with a number of inhibitor classes being reported in the literature.[12−17] The true potential as a validated target for inhibition by small molecules has also been investigated in the past few years, with advanced clinical trial data only emerging during this period of time (vide infra).

Expression of SYK is found extensively in hematopoietic cells, such as mast cells, basophils, B-cells, T-cells, neutrophils, dendritic cells (DC), macrophages, monocytes, erythrocytes, and platelets, as well as nonhematopoietic cells, such as osteoclasts, epithelial cells, fibroblasts, hepatocytes, and neuronal and vascular endothelial cells.[6,9−11,16,18−21] Another member of the nonreceptor tyrosine family is ζ-chain-associated protein kinase 70 of 70 kDa (ZAP-70), which is present in the cytoplasm and is closely related to SYK in both homology and function.[9,22−25] However, ZAP-70 expression is limited to T lymphocytes and natural killer (NK) cells.[9,19,22−25]

SYK and ZAP-70 constitute the cytoplasmic PTK family, possessing tandem N-terminal Src homology-2 domains (SH2) referred to as N-SH2 and C-SH2 domains. These are separated by interdomain A, and the C-SH2 domain is followed by interdomain B which precedes the C-terminal kinase domain (Figure 1).[11,25−29] The SH2 domains bind phosphorylated tyrosines, and the tandem SH2 domains of the SYK family neatly dock into a conserved peptide motif containing two phosphorylated tyrosines separated by 9−11 amino acids.[25,30−34] These motifs are found in signaling chains of multimeric receptors that respond to foreign and self-antigens in immune cells. Such motifs are referred to as immunoreceptor tyrosine-based activation motifs (ITAMs).[25,30−34] Importantly, the tandem SH2 domains also help to maintain SYK family kinases in an inactivated state via intramolecular interactions in resting immune cells.[11,24,26,27,29] Consequently, the binding of the two SH2 domains to ITAMs of receptors plays a critical role in SYK activation and frees the activated kinase domain for substrate interaction.[11,21,25]

This mode of activation also serves as a guiding principle to recognize the receptors that activate SYK family kinases. Essentially, the activation of these kinases is mediated by ITAM-containing receptors.[9−11,25,26] SYK, for example, transduces signals from antigen receptors such as the B-cell antigen receptor (BCR) and Fc receptors (FcR) that bind the Fc portion of immunoglobulins, which in turn bind antigens (Figure 2).[9−11,25,26] ZAP-70, consistent with its narrow expression profile, transduces signals from the T cell receptor (TCR) and ITAM-bearing NK cell receptors.[22−25] These multimeric receptors invariably contain one or more subunits possessing ITAM motifs.[9−11,26]

It has been recognized that ITAM-bearing subunits are shared with other receptors. For example, specific leukocyte integrins, receptor activator of nuclear factor-κB ligand (RANKL), and C-type lectin receptors (CLR), such as Dectin-2 or Mincle, have been shown to co-opt FcRγ and DNAX-activated protein (DAP12), which contain ITAMs, and when engaged they activate SYK.[10,11]

SYK PTK intrinsic activity has been reported to be up to 100-fold more active than ZAP-70,[19] and this difference probably reflects that SYK is easier to activate than ZAP-70. SYK, for example, is not as reliant on phosphorylated ITAM architecture for enzymatic activation as ZAP-70.[19,25] Interestingly, it has become clear in recent years that incomplete ITAMs, referred to as hemITAMs, are capable of interacting with and mediating SYK activation.[11,34] In this way, a number of C-type lectin receptors, such as Dectin-1, CLEC2, and CLEC9A, possess hemITAMs and transduce signals through SYK.[11,13,34] Altogether, SYK is activated through ITAMs present in BCRs

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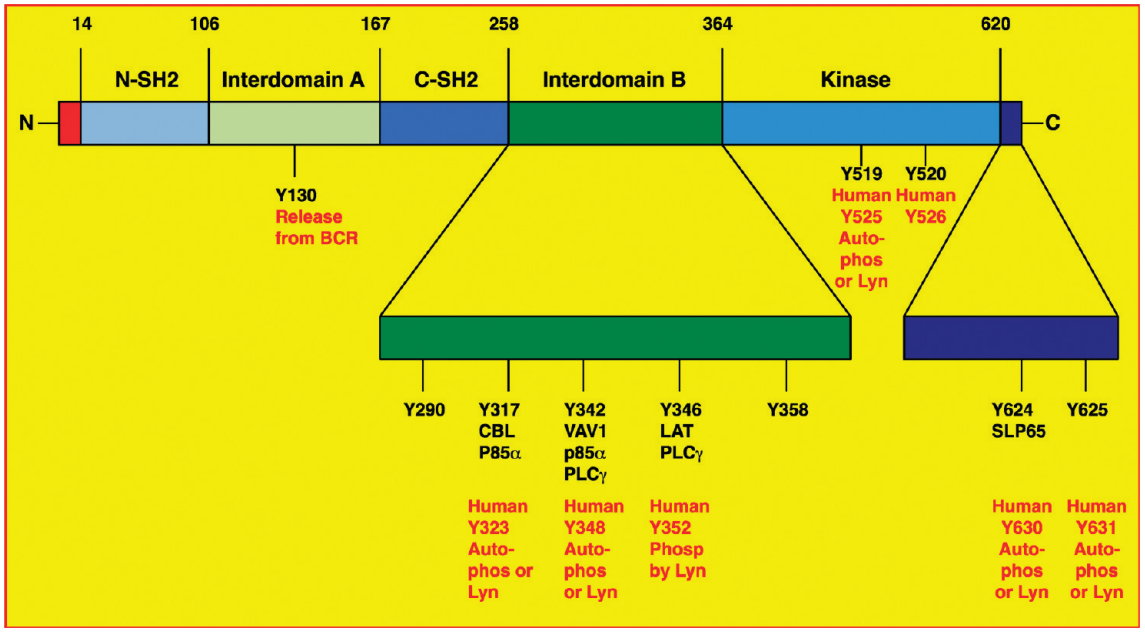

Figure 1. Structural sequence of SYK kinase. SYK is composed of tandem N-SH2 and C-SH2 domains, separated by interdomain-A and interdomain-B (activation loop) leads to kinase catalytic domain. The sites on SYK involved in phosphorylation through trans- and autophosphorylation, leading to activation and substrate docking and regulation of kinase activity, are shown.

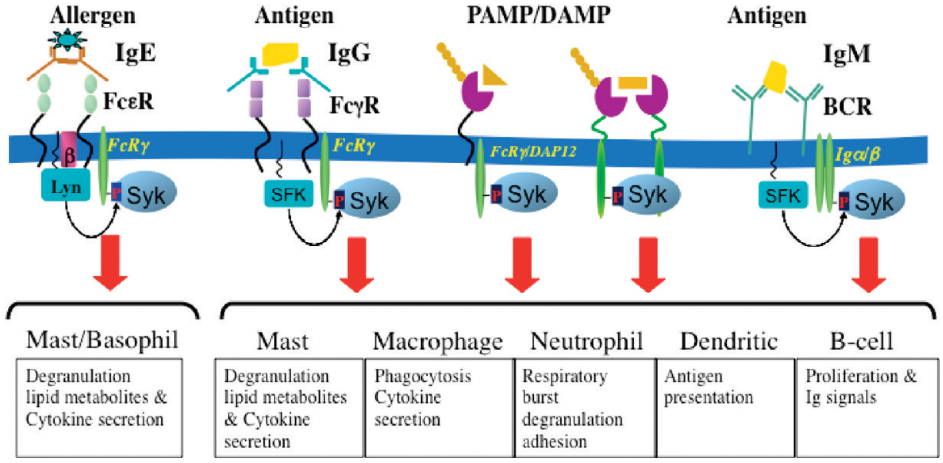

Figure 2. SYK in ITAM receptor signaling in multiple hematopoietic cells.

and FcRs that respond to antigen, in C-type lectin receptors that respond to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), and in receptors that have co-opted ITAM-bearing signaling chains (Figure 2).[11] Most, if not all, of the triggering ligands, in one way or another, have been associated with the inflammatory process in response to pathogens, antigens, and/or cellular damage.

SYK possesses multiple phosphorylation sites[11,25−29] that are fundamental structural prerequisites to the diverse functional activity of SYK (Figure 1). These phosphorylation sites contribute to maintaining the kinase in an inactivated state and are implicated in events leading to primary activation of the kinase catalytic domain, amplification of this activity, and the orchestrated phosphorylation of multiple substrates.[11,25,27,28] Regarding this last point, in particular, several of the phosphorylated SYK residues serve as docking sites for downstream-signaling proteins that orchestrate overall cellular responses together.[26−29] Despite considerable overlap, each specific cell type possesses a distinct set of effector molecules that respond to signals generated from SYK. In this way, activation of SYK can lead to varied cellular end points depending on the cell type, such as B cell survival, degranulation in mast cells, oxidative burst in neutrophils, antigen presentation in dendritic cells, proinflammatory cytokine production by macrophages, and so on (Figure 2).[9−11,25] In other words, utilization of the numerous sites of phosphorylation on SYK leads to the activation of a multitude of signaling networks relevant to immune and autoimmune maladies.[9−11,25−29] SYK, therefore, serves as a master regulator of inflammatory responses by a multitude of immune cells, and as a result, inhibiting SYK activity dampens inflammation broadly and comprehensively.

In addition to SYK and ZAP-70, there is an additional spliced variant known as SYKB[15,19,20] that lacks a 23-amino acid sequence in interdomain-B preceding the kinase domain. ZAP-70 also lacks this 23-amino acid sequence in interdomain-B. This similarity between SYKB and ZAP-70 implies functional overlap between the two kinases. SYKB is broadly expressed in numerous tissue types, such as spleen, lung, and bone

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marrow.[15,19,20] This makes SYKB the third family member with tandem SH2 domains, although with reduced proficiency in binding to ITAM.[30−34] Intriguingly, SYKB is more stable to protease degradation in lysates of RBL-2H3 basophil cells than SYK,[20] which may influence functional outcomes and provide further differentiation between SYK and SYKB.[20] Little else is known about the function of SYKB in diverse tissue types, and it has not been reported whether small molecule inhibitors differentiate between SYK and SYKB in cell-based assays.[12−17] Biochemical SYK assays are typically conducted with the kinase domain, which is the same for both SYK and SYKB. It is important to note that successful small molecule optimization has relied heavily on the application of functional cell-based assays (vide infra), and this will discussed throughout this review.

The murine genetic knockout of SYK has led to perinatal lethality caused by lymphatic vascular developmental abnormalities.[10,27,35−37] A recent study of SYK knockout mice avoided this difficulty by the use of bone marrow chimeras with $SYK^{-/-}$ hematopoietic cells, which established that SYK is necessary in the hematopoietic compartment for the development of autoimmune arthritis.[38] $SYK^{-/-}$ fetal liver cells were transplanted to wild-type mice that were lethally irradiated, producing $SYK^{-/-}$ bone marrow chimeras possessing a SYK-deficient hematopoietic system. Treatment of lethally irradiated wild-type mice with a single intraperitoneal injection of arthritogenic (K/BxN) serum induced arthritis, as assessed by both macroscopic and microscopic observation. No evidence or signs of arthritis were detected in these genetically modified mice lacking SYK.[38] Small-molecule inhibitors targeting SYK have shown preliminary efficacy in both animal models of arthritis and early phase rheumatoid arthritis (RA) clinical trials in humans, and recent genetic evidence in animals has validated this approach.[12−14,16,38]

The lethal hemorrhaging phenotype of the SYK-deficient mice led to initial concerns for SYK as a therapeutic target.[9−11,37] However, as mentioned, it was later shown that the perinatal lethality was due to a developmental defect and unlikely to occur in adults. Furthermore, mice reconstituted with SYK-deficient platelets exhibited normal bleeding times. This is likely due to redundant pathways impinging in platelet aggregation.[9−11] That is, SYK inhibition may block a specific platelet response, such as CLEC2 activation, but it is unable to stop aggregation resulting from the collection of inducers that control hemostasis.[10,11,13]

Another interesting genetic knockdown study of SYK was conducted in macrophages. Macrophages are a key player in both the innate and adaptive immune function and play an important role in inflammatory diseases through phagocytosis.[39,40] In a murine model, genetically modified SYK-deficient macrophages were unable to participate in phagocytosis and activation of downstream secondary messenger generation. However, these same macrophages were shown to have normal behavior upon stimulation by lipopolysaccharides (LPS).[39,40] Indeed, the SYK-deficient macrophages only had FcγR-mediated phagocytosis defects (no effects were seen on phagocytosis incurred by microorganisms or synthetic particles in innate immune functioning), thereby avoiding broad on-target immune deficiency.[39,40]

As mentioned above, SYK plays a role in mediating immunoreceptor-induced signals for cell growth and cell survival. In particular, SYK has been shown to activate PI3K, Akt, and mitogen-activated protein kinase (MAPK) signaling pathways in B cells, mast cells, monocytes, and NK cells.[10−16,41] The potential use of SYK inhibitors in hematological malignancies and virus-induced tumors has been reviewed elsewhere.[10−14,16,41] Conversely, it has been proposed that SYK, via an undefined mechanism, may suppress the growth and spread of certain solid tumors.[10,11,42,43] The original premise stemmed from the observation that SYK expression was inversely correlated with the invasiveness of breast cancer cell lines. However, in retrospect, it is now clear that breast cancer cell lines have well-defined gene signatures that correlate with their cell origin (luminal cells versus basal cells) and their invasiveness.[44−46] Consequently, hundreds of genes define the expression profile of these cell lines, and thus, the inverse correlation of invasiveness to SYK expression is not necessarily causal and is possibly coincidental.[10,46] Moreover, in the clinical setting, variation of SYK expression has not been associated with any of the molecular prognostic signatures that have been established in multiple gene-expression profiling studies in breast cancer.[47−51] Follow-up cell signaling experiments have been convoluted with forced SYK overexpression in cell lines that do not normally express SYK, and the results have been inconsistent and the effects unimpressive.[10,44,46] Thus, the data indicate that the association of SYK suppression with adverse prognosis of breast cancer is superficial, simplistic, and possibly completely wrong.

Several publications have described cell-based and biochemical assays for SYK activity inhibition.[52−55] Cell-based assays for functional evaluation of SYK inhibitory activity have included the use of basophils, cultured human mast cells (CHMC), B-cells, macrophages, neutrophils, and the monocytic cell line THP-1 using anti-immunoglobulin E (IgE), anti-IgG, and anti-IgM stimulation to assess activity on FcεR1, FcγR, and BCR, respectively.[52,56−64] For example, SYK-dependent inhibition of CHMC degranulation, as measured by reduction in tryptase release, is a classic readout of IgE[60] receptor-dependent activation. In parallel, evaluation of ionomycin-induced degranulation, through calcium mobilization, provides useful counter-screen data to ensure that inhibition of readout is not due to targets downstream of SYK.[56−64] Mast cells can also be used to ascertain inhibition of lipid mediators such as PGE_2 and LTC_4 and cytokines, such as TNFα, interleukin-8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, and IL-13. Inhibitors aimed at autoimmune disease need to show activity on key cell types involved with disease propagation to block multiple hubs of the underlying processes, which, if not inhibited, could lead to redundant pathways exploiting missed opportunities for inhibition.[9−11]

SYK ACTIVATION

SYK possesses multiple phosphorylation sites that participate in regulation and activation through trans- and autophosphorylation (Figure 1).[11,25−29,65−70] In addition, these sites are involved in amplification of enzymatic activity, resulting from conformational changes, and serve as docking sites for multiple interactive signaling proteins, thus promoting phosphorylation of numerous substrates responsible for downstream signal transmission (Figures 1−3).[11,60,65,68−70]

Recent work, including the elucidation of the three-dimensional crystal structure of the complete ZAP-70 kinase, has shed light on the molecular basis of the regulation of SYK PTK family activity.[71] A key step in the activation of most eukaryotic protein kinases is the correct positioning of a flexible C-helix in the kinase domain so that it forms a salt bridge with a catalytic lysine, which coordinates with the phosphate group of ATP.[72] In ZAP-70, this C-helix is kept away in an "inactive" position by the concerted interaction of several residues from

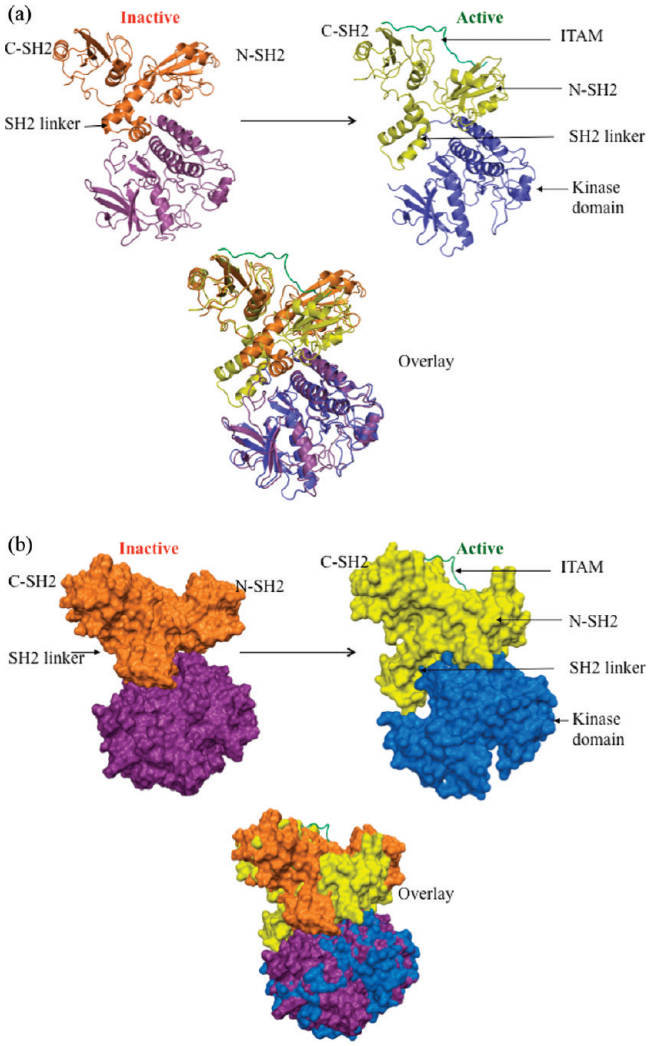

Figure 3. (a) SYK kinase is maintained in an inactive conformation, which entails the N-SH2 and C-SH2, along with C-terminus blocking access to the kinase ATP pocket. (b) Activation can be initiated through ITAM binding to the dual SH2 domains of SYK, resulting in conformational change and access to the kinase ATP pocket opening. Release from ITAM following phosphorylation of tyrosine residues (not shown) on SYK leaves the kinase in fully activated form.

interdomain-A and -B and from the kinase domain, as well as from the carboxy terminus.[69,71] This structure has been termed the "linker–kinase sandwich", and the disruption of this "sandwich" is essential for enzymatic activation of ZAP-70.[69,71]

On the basis of the structural details defined for ZAP-70, all evidence points to SYK being regulated in an analogous mode.[66,69−71] Most residues involved in the regulation of their activity are conserved. A low-resolution, 24 Å, three-dimensional structure of full-length SYK using single-particle electron microscopy (EM) indicates that SYK acquires a closed auto-inhibited conformation similar to that of ZAP-70 (Figure 3).[73,74] In addition, mutational studies of SYK interdomain-B residues (Y348 and Y352 in human SYK) substantiate the existence of autoinhibition, involving a mechanism corresponding to the "linker–kinase sandwich" region of ZAP-70.[69,75] Likewise, mutations in the C-terminal tail of SYK corroborate that residues in this region, including Y630 on SYK, play similar roles to those in ZAP-70.[69]

Classical activation of SYK occurs through interaction of its two SH2 domains with single dual phosphorylated tyrosine residues on ITAM subunits in the intracellular space. Tyrosine residues on the ITAM are separated by 12 amino acids. The entire length of the ITAM is composed of 18 amino acid residues with the consensus motif composed of D/ExxYxxL/Ix_{6-8}YxxL/I, and the two conserved tyrosines are separated by a 9−11 amino acid sequence.[11,26,30−34,65,69,70]

Although details remain to be elucidated in any one system, the prototypical activation of SYK can be envisioned to result from the following sequence.[66,67] First, the engagement and cross-linking of receptors by antigen or ligand result in the unmasking of ITAM tyrosine residues that can be phosphorylated by Src kinases such as Lyn (Figures 1−3).[68] The dual-phosphorylated ITAM then recruits SYK by binding its SH2 domains in a head-to-tail fashion (where the N-terminal phosphorylated tyrosine of the ITAM binds the C-SH2 and the C-terminal one binds the N-SH2).[11,69,70] This binding strains the conformation of the interdomain-A region, disrupting the autoinhibitory "linker–kinase sandwich", which in turn allows the repositioning of the C-helix into an "active" conformation (Figure 3). Subsequent phosphorylation of tyrosine residues Y348 and Y352 on interdomain-B further disrupts the linker–kinase interaction, making the reversion to autoinhibition unachievable.[11,69−71] As mentioned above, in support of this mechanism, the mutation of tyrosine residues Y348 and Y352 into phenylalanine residues locks the enzyme in a "closed state", resulting in marked reduction in ability to signal downstream.[69,76]

The phosphorylation of residues Y348 and Y352 has been associated with the action of Src kinases, such as Lyn, and can occur on catalytically inactive SYK.[11,26,27,69,70] The significance of trans- and autophosphorylation on these sites has also been assessed using small-molecule inhibitors.[76] Compound **1** (BIRA766, structure not disclosed), a Src kinase inhibitor, completely blocks phosphorylation of Y352 (Y346 in murine), implicating this as a major site for transphosphorylation by Lyn.[77] In addition, compound **1** shows no biochemical inhibition of SYK and potently inhibits Src kinase members Lyn, Fyn, Src, Fgr, and Yes.[77] SYK inhibitor, compound **2** (BAY61-3606, Figure 4), on the other hand, causes no reduction in phosphorylation of Y352, ratifying this site as not being susceptible to autophosphorylation.[77] Both compounds **1** and **2** abrogate the phosphorylation of Y348 (Y342 in murine) by an equivalent magnitude.[77] Compound **3** (R406, Figure 4) shows no inhibition of phosphorylation of Y352 but inhibits the phosphorylation on Y191 of human linker for activation of T-cells (LAT), a substrate for SYK, confirming that compound **3** works directly through SYK in primary cell lines.[12,77,78]

For activation of SYK, the phosphorylation of carboxy-terminus residues Y630 and Y631 has been extensively investigated.[79,80] These two residues probably interact with a proline residue from interdomain-A and also form part of the "linker–kinase sandwich" structure that autoinhibits the kinase.[69] Subsequent phosphorylation of Y630 prevents its interaction with interdomain-A, helping to disrupt the "sandwich" and keeping SYK in the active conformation.[79,80] Finally, the activation of SYK activity allows the autophosphorylation of Y525 and Y526 in the activation loop of the SYK kinase domain supporting an extended conformation of the loop, driving it out of the active site, and making space for substrate phosphorylation (Figure 1). In this way, the binding of the tandem SH2 domains to phosphorylated ITAM, followed by multiple tyrosine

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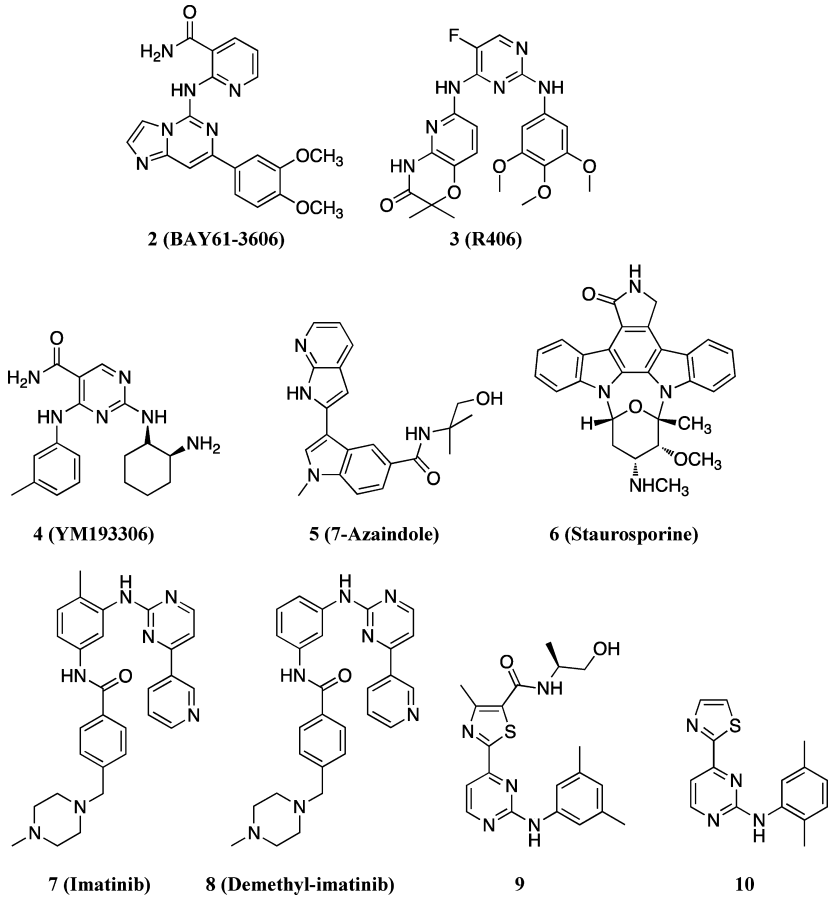

Figure 4. Structures of SYK kinase inhibitors **2**–**10**.

phosphorylations, results in the activation of SYK.[70,79,80] Mast cell degranulation, as measured by β-hexosaminidase release or activation of nuclear factor of activated T-cells (NFAT) and nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB) following antigen-mediated stimulation, was used to assess the functional cell-based impact of these mutations on SYK activity.[79,80]

The conformational changes involved in SYK activation from the resting state are depicted in Figure 3. Appreciable conformational changes in the N-SH2 and C-SH2 domains, interdomain-A and -B, and kinase domain occur in transition from inactive to active form,[66,69−71,79,80] resulting in a significant reduction in the interaction of peripheral residues with the kinase domain, thus exposing the ATP binding pocket. The inactive SYK model is based on the homology model developed from the crystal structure of inactive ZAP-70 (2OZO). The active SYK kinase model is based on the overlay of the crystal structure of the SYK kinase domain (1XBC) and the SH2 domain of SYK (1A81) on the crystal structure of inactive ZAP-70 (2OZO).[71,81−84]

Notably, this mode of activation does not explain how hemITAMs are able to activate SYK.[11,85,86] Remarkably, in these cases, both SYK SH2 domains are still required for SYK activation. This requirement became more understandable with the demonstration that phosphorylated hemITAMs interact with SYK with a 2:1 stoichiometry[11,85,86] (i.e., two hemITAMs, each contributing one pITAM, bind to the SH2 domains of one SYK protein). However, whether extracellular ligand interactions cross-linking hemITAM-containing receptors transmit signals that result in disruption of the SYK "linker−kinase sandwich" and whether this leads to SYK activation remain unknown.

In summary, a delicate balance exists in the amount of activated and autoinhibited SYK.[68,86] The numerous sites of phosphorylation on the SYK sequence contribute to both activation and regulation of its enzymatic activity.[11,26−28,66−70] The regulation of SYK will be discussed in the next section.

■ SYK REGULATION

SYK plays a pivotal role in the signal transduction of ITAM-dependent receptor activation. In fact, it has been argued that SYK acts as "signaling switch" while Src kinases act as "rheostats" that can be dialed in or out in a graded manner.[66,67,86] This is likely due to SYK kinase's unique positive autoregulation where, once activated, SYK can autophosphorylate residues in its linker domains that disrupt the autoinhibition and perpetuate an open conformation.[66,67,69,70,79,80]

Furthermore, the phosphorylation of tyrosine residues in SYK produces additional regulatory roles, as they serve as docking sites for proteins interacting with SYK.[11,27,60] This binding not only ensures proximity of substrates for downstream signaling but also protects the phosphorylated tyrosines from phosphatases. The various direct binding partners of SYK, including other signaling intermediates forming various signaling complexes in different hematopoietic cells, have been described extensively in recent excellent reviews.[6,9−11,26,27,69,70] For example, phosphorylated interdomain-B residues Y348 (pY348) and pY352 are important for binding and activating Vav and phospholipase C-γ (PLCγ) proteins.[11,79] These are necessary events for coupling to NFAT pathways by way of

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calcium mobilization and to NF-κB pathways through the CARD (caspase-recruitment domain)-MALT1-BCL10 complexes.[11,86] These activations result in the production and release of inflammatory cytokines. The sustained activation of SYK is significant, as it has been shown that SYK activity is required throughout the process of cytokine production and release, and SYK inhibitors are able to block ongoing cytokine production.[16,78]

Phosphorylated Y352 has also been shown to bind growth factor receptor-bound protein 2 that couples to extracellular signal-regulated kinase (ERK) signaling, as well as the p85 subunit of PI3K, leading to Akt activity.[10,11,41,69] The engagement of these two pathways leads to cellular survival and proliferation. Thus, it appears that SYK activation can lead to different outcomes (cytokine release or cell proliferation) in part by the differential docking of signaling molecules to pY352, directed by divergent intracellular circumstances.[41,69] The formation of these differential complexes can also make use of multipartite mutual interactions for multiple reasons. The phosphorylation of SYK Y630, for instance, generates a docking site for the SH2 domain containing leukocyte protein (SLP) family.[11,69,79] In B-cells, pY630 binds SLP-65, a membrane protein that itself binds PLCγ and the Tec family kinase Btk.[69] Interestingly, this interaction allows for the maintenance of active SYK close to the cell membrane, even after BCR internalization. It is also noteworthy that phosphorylation of Y130 of interdomain-A in SYK results in reduced affinity of the SH2 domains for pY of ITAMs, at the same time disrupting the autoinhibitory "linker–kinase sandwich".[25,87] This mechanism possibly accounts for the release of SYK from membrane-bound ITAMs while maintaining an active conformation for subsequent interactions in different intracellular compartments.[87,88]

Alternatively, the disengagement of SYK from ITAMs may be part of its eventual deactivation.[87,88] In this regard, much remains to be elucidated of how SYK returns to its basal state. However, it does appear that differential interactions play a role in turning off SYK. pY323 in interdomain-B has been shown to be a docking site for either PLCγ or the E3 ubiquitin ligase Casitas B-lineage lymphoma (Cbl) family of proteins.[64,89−91] The interaction of pY323 with Cbl proteins is important in the ubiquitination and degradation of SYK. Mutation of Y323 results in enhanced SYK-dependent signaling as shown in mast cells and basophils, as well as in B-cells.[89−91]

Consistent with SYK acting as a "signaling switch", the major regulatory step for its activity is during the initial steps of its activation.[66,67] It appears that SYK responds to a threshold of pITAMs set by the collective work of activating and inhibitory signals. As mentioned, ITAMs are initially phosphorylated by the Src family of kinases, which can be dialed in or out in a gradual fashion. Counteracting ITAM signaling are membrane-bound receptors that signal through conserved immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of sequence I/V/L/SxYxxL/V.[62,63,86,92−95] Fittingly, ITIMs are also phosphorylated by Src family kinases, but instead of recruiting SYK, pITIMs recruit phosphatases such as SH2 domain-containing tyrosine phosphatase or SH2-containing inositol phosphatase, which dephosphorylate signaling molecules, resulting in the down-regulation of signaling responses.[62,86,95] Thus, the nature of the immunoreceptors engaged on the surface of the cells determines the balance between pITIMs and pITAMs that determine whether SYK is activated or not. Curiously, depending on the quality of ligation of some immunoreceptors, pITAMs have been occasionally reported to also mediate inhibitory signals; however, the pITAMs appear to recruit phosphatases rather than SYK. SYK activity is invariably associated with activating ITAM-dependent signaling, and thus, SYK inhibitors do not affect inhibitory signaling.[62,64,86,96]

Recently, the phosphorylation of S291 (as opposed to tyrosine), found on the 23-amino acid sequence of interdomain-B in SYK, was reported to be catalyzed by the action of protein kinase C (PKC).[97] Upon BCR activation, PKC is activated as a direct consequence of the upstream activation of SYK. Combined, the mutual interplay by these two kinases provides the framework for a positive regulatory loop, where SYK activity leads to the activation of PKC, which in turn phosphorylates the solvent-exposed S291 on the flexible 23-amino acid sequence of SYK. This phosphorylation appears to promote interaction with prohibitin, a chaperone protein, thereby enhancing the ability of SYK to induce NFAT and Elk-dependent transcription.[97]

In summary, SYK can be inhibited following receptor activation conformational change to the fully active form and also in its inactive state.[64,65,79,80,89−91] Subsequent to activation of SYK through BCR signaling, interaction of residue Y323 with Cbl attenuates SYK activity.[89−91] Independent of any association with BCR through phosphorylation of S291 by PKC, SYK can be additionally regulated. In BCR activation, PKC is inherently linked to downstream signaling through SYK. Signal amplification is controlled through auto- and transphosphorylation of SYK, and therefore, SYK is continually available to activate PKC. The activated PKC is then available to participate in phosphorylation of S291 on interdomain-B of SYK and to contribute to SYK regulation to function as a signaling switch.[97]

SYK STRUCTURE

The high-resolution crystal structure of full-length SYK has not been reported; however, the solution structure of SYK C-terminal SH2 (SYK-C) domain of the human kinase interacting with a pentapeptide, mimicking the ITAM, has been determined.[98] For high-affinity binding to ITAM of Fc receptors, both the C- and N-SH2 domains of SYK need to participate in binding. However, in in vitro experiments, SYK-C possesses the capacity to bind to phosphorylated ITAM autonomously. Only SYK-C domain is able, through affinity purification to pull down the IgE-receptor complex adequately.[98] During mast-cell activation, SYK principally binds through sequential interaction of C- and N-SH2 domains to the γ-subunit of FcεR1 receptor ITAM domain.[98,99] FcεR1 has no enzymatic activity by itself and is reliant on Src and PTK for activation.[26,62,79] The C-terminal SH2 domain can bind in vitro unilaterally to tyrosine phosphorylated ITAM of FcεR1. In combination with SYK, containing only the C-SH2 domain, solution-based structural studies using phosphotyrosine peptide designed to mimic the γ-subunit of FcεR1 have been carried out to understand this interaction.[98] The most potent peptide Ac-Thr-pTyr-Glu-Thr-Leu-NH_2, based on the pY76 SH2-binding motif, was found to bind with IC_{50} = 20 μM, as determined by ligand concentration necessary to inhibit 50% of the binding of SYK-C to bound ITAM peptide covalently.[98] This study was the first attempt at elucidating the structure of SH2 and ITAM binding, suggesting that SYK-C can bind independently to ITAM. However, for strong in vivo binding, both SYK-C and SYK-N are obligatory.[98] For this peptide-based approach to be useful in inhibitor discovery, acceptable potency for SYK-C inhibition must translate to inhibition of full-length SYK found

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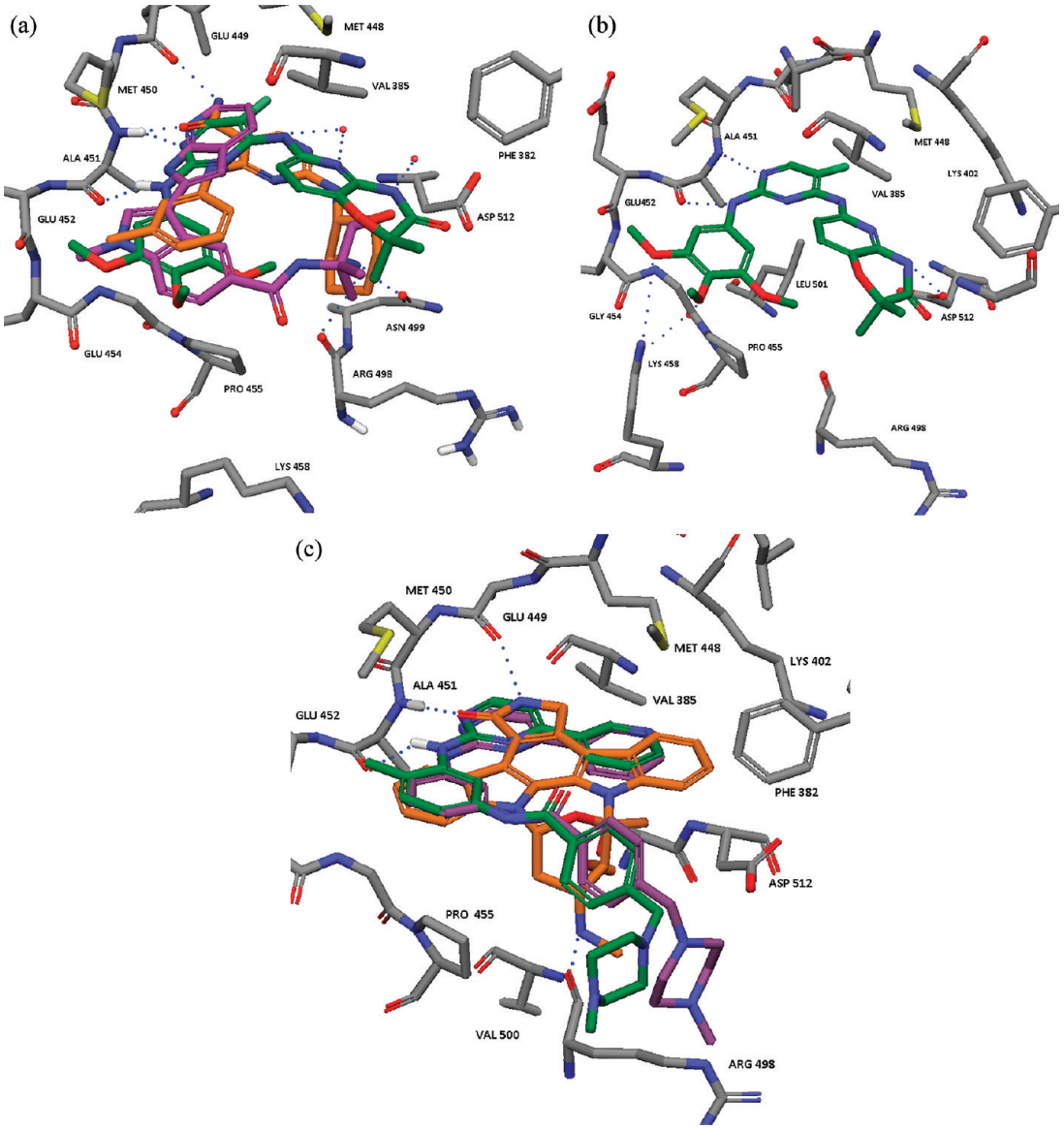

Figure 5. (a) Cocrystal image of compounds **3** (green), **4** (orange), and **5** (purple). (b) Cocrystal image of compound **3**. (c) Cocrystal image of compounds **6** (orange) and **7** (green) and overlap with docked compound **8** (purple).

in the intracellular milieu. Full-length SYK adopts a more complex conformation in vivo compared to artificial constructs.[66,73,74] Additionally, for such a peptide approach to be pursued successfully, rapid metabolic clearance would have to be overcome for meaningful therapeutic application.

In contrast to its family homologue ZAP-70,[71,81,82] SYK displays more conformational elasticity, accounting for its role in functional binding to multiple substrates.[11,69] The ability of the C- and N-SH2 domains in SYK to work in an independent and highly flexible manner has been investigated using X-ray crystallography.[84] The C- and N-SH2 domain sequence, from residues 7 to 269, was prepared and isolated for cocomplex formation with the CD3ε-chain diphosphorylated ITAM 20mer peptide sequence PDpYEPIRKGQRDLpYSGLNQR.[84] By use of the tandem SH2 domain as the docking site and phosphorylated ITAM peptide as ligand, the X-ray crystal structure was determined to a resolution of 3 Å.[84] Inhibitors blocking binding of the tandem SH2 domain of SYK with ITAM subunit could give more selective inhibition than small molecules targeting the kinase ATP pocket.

Several research groups have independently reported the crystal structure of the SYK catalytic domain, cocrystallized and ligand soaked with small molecule inhibitors (Figure 4).[78,83,100,101] The Roche group obtained the cocrystal structures of SYK kinase domain with three inhibitors **4** (YM193306),[102] **5** (7-azaindole),[103] and **3**[78] to a resolution of 2.5, 2.25, and 2.1 Å,[101] respectively (Figure 5a). SYK inhibition varies considerably for the three inhibitors. Compound **4** inhibits SYK with K_i = 0.005−0.02 μM. **5** has IC_{50} = 0.57 μM, and **3** shows IC_{50} = 0.041 μM.[78,101] The biochemical potency of compound **3** is rationalized by interaction of the pyrimidine nitrogen at the 1-position and the N−H linker from the trimethoxyphenyl at the 2-position participating in hydrogen bonding with hinge residue A451, and the trinitrogen assembly from the dimethylpyridoxazinone bicyclic system interacting with two water molecules and its participation in hydrophobic interactions. The solution side of the catalytic pocket has some flexibility, accounting for the subtle differences from a previously determined cocrystal structure with compound **3** (Figure 5b).[78,101] Inhibition of SYK by *cis*-cyclohexyldiamine containing pyrimidine

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compound **4**[101] can be explained by combination of hydrogen bond-donating interactions with residues R498, N499, and D512 from the primary amine and pyrimidine linking secondary amine. Furthermore, the salt bridge resulting from primary amine hydrogen bond interaction to the D512 carboxyl moiety aids in potency.[101] The weakly active SYK inhibitor **5** has the crucial interaction with hinge A451 but does not penetrate the ATP pocket significantly, in contrast to compounds **3** and **4**.[101]

The structures of compounds **6** (staurosporine)[100] and **7** (imatinib) with SYK kinase catalytic domain have also been reported (Figure 5c).[83] Compound **7** adopts a structure similar to the promiscuous kinase inhibitor **6**.[83] The reported crystal structures of compounds **6** and **7** indicate that the ligands bind the activated form of SYK. Thus, the SYK binding modes of ligands **6** and **7** resemble the cocrystal structure elucidated with compounds **3**, **4**, and **5** (Figure 4). The *o*-methyl group on compound **7** reduces the effectiveness for SYK binding (displaying K_i = 5 μM) because of steric hindrance blocking the formation of a U-shaped cis-conformation required for hydrogen bonding of the N−H from the ligand and SYK hinge residue A451.[83] Indeed, an analogue lacking this *o*-methyl group, compound **8** (demethylimatinib), is more potent for SYK inhibition with a K_i of 0.16 μM.[83] An analogous effect was observed in the cocrystal structure with potent inhibitor *N*-phenyl-4-(thiazol-2-yl)pyrimidin-2-amine compound **9**, which has K_i = 0.009 μM for SYK inhibition.[104] Introduction of an *o*-methyl on the 3,4-dimethylaniline linked at the 2-position of the pyrimidine, compound **10** (Figure 4), substantially reduces SYK inhibition with K_i = 1.817 μM, but also it must be taken into account that the methyl substituent and alanol side chain are not present in this analogue. The coplanarity of the pyrimidine ring and the 3,5-dimethylaniline substituent at the 2-position is critical to avoid a steric clash with hinge residue A451 (Figure 6).[104] All the structures obtained

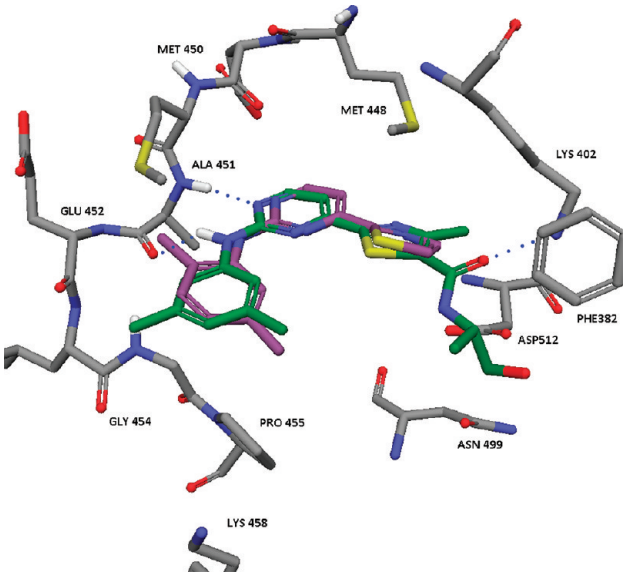

Figure 6. Cocrystal image of compound **9** (green) and overlap with docked compound **10** (purple).

with the SYK kinase domain show the ligands binding through distinguished U-shaped conformations because the pocket is unable to bind these ligands in alternative forms (Figures 5 and 6).[78,83,101,104] Ligands capable of inducing conformational changes in the ATP-binding pocket would allow alternative binding modes, thus potentially improving selectivity for SYK over other protein kinases. All the current inhibitors reported bind to SYK in the active form, but SYK in vitro activity does not necessitate phosphorylation of tyrosine residues.[83] Future efforts directed at binding to the inactive form to derive better selectivity should be investigated, with continued use of cell-based functional assays to investigate in vivo selectivity.[77,78]

■ SYK KINASE AND DISEASE

SYK plays an indispensable role in acute and chronic inflammation.[10,11,56,105−107] As outlined previously, activation of SYK occurs through preliminary extracellular antigen-induced cross-linking of FcεR1 and FcγRs I, IIA, and IIIA.[10,11,59] Upon activation, SYK, a master upstream regulator of signal transduction, propagates downstream signaling molecules, resulting in initiation of disease.[10,11,105−108]

Inflammation is a fundamental response to challenges on the body, instigated by injury sustained through physical tissue damage, exposure to chemicals, and pathogens. The immediate acute response is to eradicate the initial offense and launch repair mechanisms directed at damaged tissue through a complex series of steps. Exacerbated reaction to the primary insult leads to the development of chronic inflammatory processes, which remain less well understood and, if unrestrained, lead to stepwise pathogenesis of a plethora of ailments. Chronic inflammation with persistent efforts at repair may produce structural and dysfunctional tissue damage. Maladies resulting from chronic inflammation include asthma, RA, Crohn's disease, systemic lupus erythematosus (SLE), idiopathic thrombocytopenic purpura (ITP), and multiple sclerosis.[10,56,59,62,105−126] Control of inflammation has become a mainstay in the management of these chronic inflammatory conditions.[62,106−111,117,118]

Through the action of pathogens, chemicals, or physical injury, inflammatory responses are implemented by the coordinated action of leukocytes residing close to or within the distressed tissue. These immune cells have the ability to differentiate between harmful and harmless mediators and crucially between self- and nonself-molecular constituents of the body. PAMPs, DAMPs, and virulence factors (products of damaged cells) initiate innate immune responses leading to inflammation (Figure 2).[10,11,85,86] The innate immune response, however, does not provide long-lasting immunologic memory and is complemented by the specificity of the adaptive immune system. This involves recognition of both intact pathogenic antigens by antibodies and fragment antigens presented on major histocompatibility (MHC) molecules. The inflammatory response by adaptive immunity is generated through BCRs and TCRs, and the respective interaction with the Fc portion of antibodies is denoted as activating FcR.

Controlling these important activation events in immune cells can be used as a broad mechanism for the treatment of autoimmune and inflammatory diseases at pivotal stages of disease development. An attractive avenue to pursue is the upstream mitigation of key targets such as SYK.[10,11,105−108,110−112,114]

Allergic Rhinitis, Asthma, and Atopic Dermatitis. Up to 25% of the population in the developed world is stricken by anaphylaxis, allergic rhinitis (or hay fever), atopic dermatitis (or eczema), and allergic (or atopic) asthma.[116−119,127−129] These conditions are generally recognized as allergic disorders. At a basic level, these maladies have a significant impact on quality of life, and at their worst they can culminate in po ssibly fatal systemic allergic reaction, usually referred to as

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anaphylaxis.[116,129] On a molecular level, these disorders are driven by allergen-specific IgE and T helper 2 (T_H2) cell responses that recognize allergen-derived antigens.[116,130] Single exposure of a sensitized subject to a specific allergen leads to the manifestation of early phase reaction or a type I immediate hypersensitivity reaction (Figure 2).[116] This is the result of allergen cross-linking IgE-bound FcεR1 on mast cells and basophils, leading to degranulation.[94,95,99,116−118,127,128] Degranulation of mast cells and basophils leads to secretion of preformed mediators such as histamine, tryptase, 5-hydroxytryptamine (5-HT), and heparin, which is followed minutes later with the production and release of eicosanoids, lipid mediators such as leukotrienes (LTs) and prostaglandins, then hours later with the production of chemokines and inflammatory cytokines (Figure 2). Collectively, these mediators stimulate recruitment and activation of leukocytes for late-phase allergic reactions, which encompass recruitment and activation of T_H2 cells, eosinophils, basophils, and other leukocytes in conjunction with prolonged mediator release by mast-cell degranulation.[94,95,99,116] Unrelenting exposure to specific allergens leads to chronic allergic inflammation through both innate and adaptive immune cells, leading to tissue remodeling.[116,131]

Current treatments of asthma, allergic rhinitis, and atopic dermatitis include limiting exposure to the offending allergen(s) and use of antihistamines, decongestants, LT inhibitors, topical chromones, and corticosteroids.[117,118] Notwithstanding this success with existing therapies, symptoms still persist in some patients and there is ample opportunity for the development of further novel therapies.[117,118] Targets for inhibition include mediator inhibitors, mast-cell stabilizers, neural pathway inhibitors, allergen-specific peptide-based and deoxyribonucleic acid (DNA) based immunotherapies, blocking of IgE cross-linking of FcεR1 receptor, cytokine and chemokine inhibition, enhancement of T_H1 responses, blocking adhesion molecules, and phosphodiesterase inhibitors.[10,11,117]

Mast cell overactivation and degranulation leading to release of multiple proinflammatory mediators are key components of allergic diseases.[94,95,127−134] Thus, inhibitors of these processes represent a potential route to developing novel therapies.[127−134] In this endeavor, SYK inhibitors have been developed that show efficacy in animal models of allergic disease and in clinical studies of allergic rhinitis and asthma (vide infra).[127,128]

A humanized monoclonal antibody to IgE, better known as omalizumab, administered through the intravenous route has been shown to be effective in treating the early- and late-phase response in asthma, initiated through inhalation of allergen.[130,135] Interestingly, omalizumab was ineffective through the aerosolized route, speculated to result from lack of exposure, but the study did indicate a greater chance of adverse immunogenic reaction.[136] The relatively high cost of treatment and reported adverse events of anaphylaxis in a limited number of treated patients has meant the drug has to be administered in an office setting under physician monitoring.[117,135,136]

More recently, application of tyrosine kinase inhibitors for the treatment of allergic disorders has steadily gained momentum and represents a therapeutic intervention strategy for the future.[127−129]

Autoimmune Disorders: ITP, RA, SLE, and Type 1 Diabetes (T1D). In autoimmune disorders, components of adaptive immunity mistakenly recognize self-antigens as foreign and initiate a program for the elimination of agents containing such antigens.[107] ITP and autoimmune hemolytic anemia (AIHA) are diseases characterized by destruction of platelets and red blood cells, directed by autoantibodies recognizing cell-specific components that lead to mucocutaneous bleeding and anemia, respectively.[107,137] This antibody-targeted destruction is mediated by FcR-signaling and, by extension, SYK, which induces phagocytosis by macrophages of the reticuloendothelial systems. Indeed, SYK inhibition has been shown to be protective in murine models of ITP and AIHA using autoantibodies that recognize antigens in platelets and erythrocytes.[137]

In another set of autoimmune disorders, autoantibodies recognize self-antigens in anatomical structures too large to be engulfed by phagocytes, namely, macrophages and neutrophils.[9−11,39,40,59] In these cases, the follow-up defensive action by the "frustrated" phagocytes is to release toxic agents such as reactive oxygen species (ROS), proteases, and phospholipases into the environment, causing local destruction of cells and tissues, with the intent to neutralize "injurious" agents at the expense of some collateral damage. This is eventually followed by the production and release of lipid mediators, such as LTs and prostaglandins, to signal pain.[95,106,110,115,116,123,131] The production of chemokines and cytokines that recruit additional immune cells to the site of injury establishes a concerted and sustained inflammatory response, which intensifies tissue destruction. Subsequent steps include efforts of containment to ward off the "foreign" agents, followed by tissue repair. These measures include scar tissue formation, which can result in the remodeling of tissues, resulting in more permanent structural impairments.[10,62,106−115]

Several of these inflammatory conditions are partially reproduced in animal models using antibodies specific for particular tissue components. The intradermal injection of immune complexes in the Arthus reaction, for example, leads to the typical signals of acute inflammation, including swelling, induration, edema, hemorrhage, and pain.[78,138] Similarly, in rodent models of RA, injections of joint-specific antibodies elicit arthritic pathology in rodent paws, as evidenced by inflammation of joint synovial lining, pannus formation, and infiltration of inflammatory cells. These responses are also mediated by FcR signaling, implicating SYK as playing a pivotal role in the induction of inflammation.[38] Consistent with this suggestion, joint inflammation is observed in these antibody-directed arthritis models whether the antibody recognizes collagen in the collagen-antibody induced arthritis (CAIA) model or glucose 6-phosphate isomerase in the passive K/BxN model. Importantly, treatment with SYK inhibitor compound **3** (which has been shown to potently block FcR-dependent ROS production by neutrophils and cytokine production by mast cells and macrophages) ameliorates all inflammatory responses in the Arthus reaction, CAIA and K/BxN models.[78,138] Moreover, as mentioned above, SYK deficiency in the hematopoietic compartment in chimeric mice prevented arthritis in the K/BxN serum transfer model, providing genetic evidence for SYK's crucial involvement in the elicitation of joint pathology by autoantibodies.[38] Compound **3** and its methylene phosphate prodrug **11** (R788-fostamatinib) (Figure 7) are also able to inhibit disease progression and severity of the more complex model induced by collagen immunization (as opposed to passive injection of antibodies) in the collagen-induced arthritis model.[12−14,16,78,138] Treatment with compounds **3** and **11** not only blocked joint inflammation but also showed protection from bone destruction as determined by radiography and micro-CT (computed tomography) scans.[138] The suppression of joint pathology was complemented by reduction of serum of chemokine and cytokine levels, as well as cartilage degradation products.[138] However, there was little effect on anticollagen antibody levels.[138]

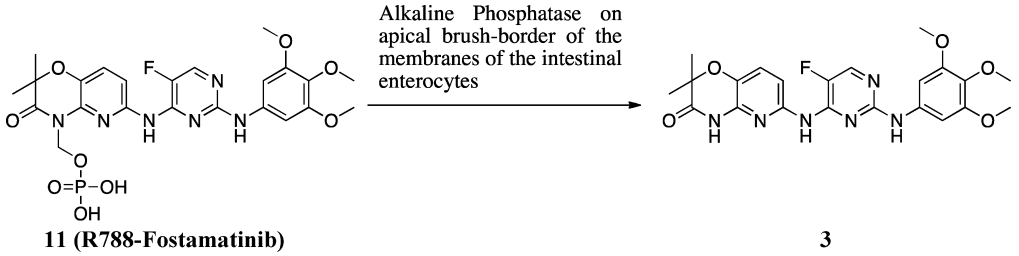

Figure 7. Conversion of methylene phosphate prodrug **11** to biologically active parent compound **3**. The conversion is catalyzed by the action of alkaline phosphatases present on the apical brush-border of the membranes of the intestinal enterocytes.

An important aspect of SYK is its role in osteoimmunology. This refers to the coordinated relationship of the immune system and bone compartments,[139−145] which share common signal transduction pathways and precursor cells in bone marrow. Pathology of RA leads to irreversible bone erosion, carried out through the process of osteoclastogenesis, and culminates in increased propensity for bone fracture.[139−141] One mechanistic pathway leading to bone erosion is directly correlated to SYK activity.[139−142] Macrophage colony-stimulating factor binds to the c-Fms receptor on osteoclasts, resulting in proliferation and survival of osteoclast precursor cells.[139−141] The interaction of osteoblasts (responsible for bone formation) with osteoclasts (responsible for bone resorption) occurs through RANKL or tumor necrosis factor (TNF) related activation-induced cytokine on the former cell binding to receptor activator nuclear factor-κB (RANK) on the latter cell type.[139−141] This RANKL/RANK interaction recruits TNF receptor-associated factor 6.[139−141] An unknown ligand on osteoblasts binds with ITAM subunits on DAP12/FcRγ[142,143] in the intracellular region, leading to enzymatic activation of SYK. This precedes the phosphorylated activation of PLCγ,[144] in conjunction with RANK, leading to calcium mobilization and interaction with calcium/calmodulin-dependent protein kinase type IV and calcineurin.[139−141] Several pathways are involved in osteoclast differentiation and osteoclastogenesis through NFAT, cytoplasmic 1 (NFATc1).[139−141] The ITAM-signaling events involved in osteoclastogenesis have been referred to as costimulatory to the RANK/RANKL pathway because both are necessary for the process to occur successfully.[139−141] The role of SYK in osteoclastic bone resorption has been examined using $SYK^{-/-}$ bone marrow macrophages (BMM) in both in vitro and in vivo studies.[142,143,145] The conclusions from these studies are that $SYK^{-/-}$ osteoclasts have diminished bone resorption ability of osteoclasts but display no effects on osteoclast differentiation.[142,143,145] Thus, inhibition of SYK could have utility in preventing bone damage in RA. Overall, the case for the importance of SYK in the development of RA is very strong, based on mechanism of action studies, informative genetic knockout investigations, and indications from early clinical trial data.[38,105−110,142−145]

Chronic autoimmune diseases, such as RA, are multifaceted, and their etiology is complex and, for the most part, ambiguous. It is generally believed that RA is genetically determined but that environmental factors play a role in the outcome of this disorder.[106,115] Some of the factors, which can serve as environmental triggers and misdirect immune response, could be infectious agents (e.g., viruses, bacteria, and fungi) or harmful chemicals (e.g., pollutants and tobacco smoke).[11,85,86] An interesting rodent model highlighting this aspect is the Sakaguchi (SKG) mouse, which is genetically prone to develop autoimmune arthritis.[146] Interestingly, these mice do not develop disease in a clean environment, but a single injection of β-glucan, a substitute for fungal antigens, can elicit severe chronic arthritis. It turns out that blocking Dectin-1, a major receptor for β-glucan, prevents disease induction.[146,147] Dectin-1, as mentioned above, is a CLR that signals through SYK, employing hemITAM motifs.[146,147] Whether SYK inhibition blocks SKG arthritis induced by fungal membrane components remains to be examined. However, it is tempting to speculate that, like in the SKG mice, PAMPs may also influence the course of autoimmune disorders and that SYK inhibition can be beneficial in preventing the establishment and re-establishment of some of these chronic inflammatory conditions.[11,78,138,146]

Antibody-mediated glomerulonephritis is another chronic disorder with far reaching clinical implications. Antibodies and/or immune complexes (ICs) under certain unfavorable conditions can initiate and sustain an inflammatory response in the glomeruli of the kidney, leading to its dysfunction characterized by protein in the urine (proteinuria).[148−151] In murine models, this ailment is dependent on Fc receptors, as shown by the reduction of disease severity in FcR deficient mice. These results also implicate SYK activity in the pathogenesis of the disorder, and indeed, treatment with SYK inhibitor compound **11** prevented crescentic glomerulonephritis in the nephrotoxic nephritis (NTN) model in Wistar−Kyoto rats.[151] This well-characterized model is elicited by the injection of nephrotoxic serum containing antibodies directed at kidney components. Notably, compound **11** treatment averted disease without blocking deposition of the ICs in the glomeruli;[151] i.e., SYK inhibition limited inflammation induced by ICs but not the deposition of ICs in the kidney. Additionally, treatment with compound **11** was able to reverse already established severe proteinuria even after the onset of disease.[151] Furthermore, compound **11** reduced the number of macrophages that had already infiltrated the glomeruli and ameliorated tissue injury as observed by the reduction of serum creatinine and the number of glomerular crescents.[151]

Glomerulonephritis is one of the most serious manifestations of SLE, a major chronic inflammatory disease affecting multiple tissues and organs including skin, joints, kidneys, small blood vessels, and the brain.[120,148−150] SLE is characterized by the presence of high-affinity autoantibodies against a plethora of intracellular and cell surface targets. Such targets include tissue-specific proteins and ubiquitously expressed cellular components (e.g., anti-DNA, anti-Ro, anticardiolipin, anti-NMDA receptor antibodies) and have all been implicated in the clinical manifestations of SLE. In the murine model of SLE NZB/W F1 mice, FcRγ-deficiency protected animals from disease without affecting the generation and deposition of ICs. This implicates autoantibodies, FcR signaling, and SYK in the downstream inflammatory events, and as expected, administration of compound **11** prior to or after onset of disease reduces renal

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pathology, delays the onset of proteinuria, and significantly prolongs survival of lupus-prone NZB/W F1 mice.[150] Similarly, SYK inhibition with **11** also suppressed established skin and kidney disease in the MRL/lpr and BAX/BAK murine models of SLE.[150]

A complex autoimmune disease like SLE is multifactorial and the autoantibody-FcR-SYK axis need not be the only path to tissue damage and pathology.[120,148−150] In fact, T-cells in SLE patients have been reported to have an overactive phenotype, and this hyperactive state is claimed to be due, in part, to a "rewiring" of the T-cell receptor (TCR) signaling complex, where the ITAM-bearing ζ chain of the TCR associated with ZAP-70 is replaced by the "trigger-happy" FcRγ-SYK.[120,148−150] Interestingly, SYK inhibition selectively reduced TCR-induced calcium responses in SLE T-cells and may represent an avenue to repress their hyperactive state. Furthermore, consistent with the prevalence in SLE of antibodies against intracellular and nuclear components, incomplete clearance of dying cells is thought to play an inflammatory role and to be a major source of autoantigens. Recently, another cellular response to DAMPs has been recognized. Mincle (also known as CLEC4E), for example, is a C-type lectin receptor on macrophages that senses necrotic cells by recognizing spliceosome-associated protein 130 in the extracellular compartment. CLEC4E associates with FcRγ, and its engagement results in ITAM-dependent activation of proinflammatory cytokines IL-1β and IL-18 by caspase-1 via a SYK-CARD9 pathway.[85,86] Similarly, C-type lectin receptor CLEC9A (also known as DNGR-1) on DCs is capable of sensing a necrotic signal that becomes exposed upon disruption of membrane integrity of the cells. Notably, CLEC9A possesses a hemITAM motif and signals through SYK to present antigens associated with cell death to activate CD8 cytotoxic T-cells.[11,85,86] Significantly, blocking CLEC9A in DCs reduced this cross-antigen presentation but without affecting the uptake of necrotic material. Thus, the emerging paradigm is that SYK-coupled CLRs are able to initiate inflammatory responses by both PAMPs and DAMPs. In the case of SLE, SYK inhibition may be beneficial by dampening responses to DAMPs and to self-antigen autoantibody ICs, as well as by suppressing hyperactive T-cells.[85]

T1D is a chronic autoimmune disease that targets and destroys pancreatic β-cells, resulting in severe insulin deficiency.[152] This disease is characterized by the early expression of anti-islet autoantibodies, where the presence of these autoantibodies allows prediction of T1D disease development and progression.[152] This association is mirrored in the nonobese diabetic (NOD) mouse, which also displays anti-insulin autoantibodies prior to overt diabetes. Moreover, NOD mice deficient in activating FcR receptors were protected from disease despite comparable production of autoantibodies. Correspondingly, administration of SYK inhibitor **11** to NOD mice delayed onset of spontaneous disease and progression of established early diabetes.[152] This salutary effect is likely due to suppression of diabetogenic T cell priming. Interestingly, treatment with compound **11** in this NOD model was found to increase the relative numbers of IL-10-expressing B-cells, a subset population capable of modulating down immune responses.[152]

In a set of insightful experiments, Clynes and colleagues demonstrated that genetic SYK deficiency in DCs resulted in impaired cross-priming of diabetogenic CD8 T-cells in the RIP-mOVA mouse model of diabetes, a process that was phenocopied by treatment with SYK inhibitor compound **11**.[152] In this model, which is dependent on FcRγ signaling, autoantibodies enhance antigen uptake and presentation to pathogenic T-cells to cause insulitis and diabetes development.[152] Interestingly, SYK inhibition with compound **11** impaired peptide loading into MHC when antigen was provided in the context of ICs but had no effect when provided as free peptides.[152] SYK inhibition did not disrupt the binding or internalization of ICs, suggesting a block in antigen processing. Treatment with compound **11** also blocked BCR-dependent antigen presentation by B-cells, a process known to be dependent on ITAMs present in the BCR.[152]

In summary, SYK inhibition has great potential for the treatment of allergic inflammatory disorders, as it blocks IgE-mediated activation of mast cells and basophils, a hallmark of atopic diseases that include asthma, allergic rhinitis, and atopic dermatitis.[127−129] Rather than inhibiting a single class of mediators such as histamine and LT, as most current therapies entail,[117,118] SYK inhibition blocks the generation of all preformed granule contents, including lipid metabolites and cytokines, potentially imparting broadly beneficial anti-inflammatory effects.[10−16] Inhibition of SYK is a logical strategy to prevent autoantibody-mediated disease given its pivotal role in FcR signaling in leukocytes, like macrophages and neutrophils.[77,78,105−112,114,137,138,150−152] These indications include autoantibody-mediated elimination of cells (e.g., ITP and AIHA), tissue-specific autoantibody-directed disorders (e.g., pemphigus in skin, glomerulonephritis in kidney), antineutrophil cytoplasmic autoantibodies-associated vasculitides in blood vessels, and systemic inflammatory diseases associated with autoantibodies (e.g., RA and SLE).[77,78,105−112,114,137,138,150,151] In myeloid cells, SYK also mediates ITAM-dependent CLR inflammatory responses to pathogens and cellular damage through PAMPs and DAMPs, respectively.[86] Thus, inhibiting SYK activity may prevent, delay, and diminish the intensity of the inflammation initiation, as well as relapse, awakened by infections and dying cells in autoimmune disease-prone individuals. SYK in fact plays a key role in the ITAM-mediated antigen presentation by B-cells to helper T-cells that amplify and perpetuate adaptive immune responses (in the autoimmune context, responses to self) and in the cross-presentation of antigens to CD8 cytotoxic T-cells that proceed to destroy invaded or supposedly invaded cells. Likewise, SYK plays pivotal roles in other harmful effector mechanisms including oxidative burst by neutrophils and bone resorption by osteoclasts.[139−142,145] Given the broad anti-inflammatory effects, SYK inhibition is likely to be efficacious in other varied indications associated with inflammation and detrimental antibodies.[150−152] Recent examples include ischemia−reperfusion, atherosclerosis, type 2 diabetes, and antibody-mediated cancer progression.[153−163]

B-Cell Lymphomas: Non-Hodgkin Lymphomas (NHL). SYK is necessary to transduce constitutive BCR signals[158,159] and participates in the survival and growth of B-cells in response to BCR stimulation (Figure 2). In specific contexts, BCR signaling via SYK is activated in an uncontrolled manner leading to lymphomas and leukemia. Indeed, murine studies have shown that SYK expression is required for the survival of NHL-like tumors in vitro.[160] Furthermore, pharmacologic inhibition of SYK-induced apoptosis in murine B-cell lymphomas in vitro and resulted in regression of NHL-like B-cell lymphomas.[160] Taken together, these results suggest that SYK expression and function are necessary for the survival of NHL cell lines[160] and highlight the potential use of SYK inhibition in the treatment of B-cell lymphomas. The use of SYK inhibitors in these indications has

been recently summarized in detail and is discussed in the clinical section of this article.[161−163]

DISCOVERY OF SYK INHIBITORS

Structure−activity relationship (SAR) work directed toward inhibition of SYK has lagged behind the elegant insights gained into its biology, mechanism of action, and potential therapeutic utility.[9−17] This section will provide, in chronological order, the discovery of SYK inhibitors. Early inhibitors were employed as probe compounds for the study of biologic mechanism of action. Interestingly, optimization programs directed toward SYK inhibitors have gained great value from reliance on cell-based functional assays for both on-target and off-target activity assessment. Initial assessment of SYK as a target for therapeutic intervention relied on the application of RBL-2H3 rat tumor basophils and CHMC cell lines.[56−64] Selectivity determination employed the use of CHMC, primary human T-cells, Jurkat T-cells, Ramos B-cells, and A549 epithelial, H1299 lung cancer, and HeLa cell lines.[9−11,78]

Over the past decade, diverse biologic activities of SYK in disease have been complemented by maturation in SAR programs and generation of pharmacologically relevant agents.[12−17] These agents have paved the path toward efficacy in animal models mimicking human disease. More recently, SYK inhibitors have advanced into clinical trials for the treatment of human maladies.[16,105,107−110,112,114]

FIRST GENERATION OF SYK INHIBITORS

Compound **12** (piceatannol, 3,4,3′,5′-tetrahydroxy-*trans*-stilbene; Figure 8) isolated as a plant secondary natural product

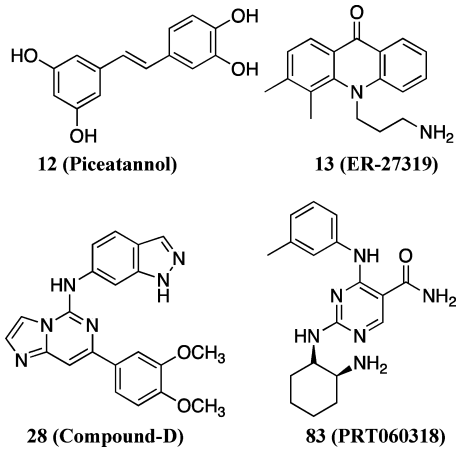

Figure 8. Structures of SYK inhibitors **12**, **28**, **83** and compound **13**.

inhibits antigen-induced activation of SYK in RBL-2H3 rat tumor basophils, allowing mechanistic elucidation into the involvement of SYK in mast cell and basophil degranulation.[95,164] The compound showed less effect on Lyn kinase activity, as measured by autophosphorylation in antikinase ICs and additionally by phosphorylation of peptide substrate angiotensin I.[164]

Assessment of SYK inhibition was evaluated through effects on antigen-induced FcεR1 cross-linking leading to mast cell degranulation and release of 5-HT.[60,95,127] Compound **12** only partially blocked the corresponding, non-SYK mediated, ionomycin-induced degranulation.[164] However, it showed discrepancy in concentrations necessary to effect anti-IgE mediated histamine release in human basophils, EC_{50} = 5 μM, versus human lung mast cells, EC_{50} = 25 μM.[164,165] Mechanistically, this disagreement suggests that the compound is inhibiting different molecular targets. Further variability was found, with compound **12** inhibiting SYK independent phorbol myristate acetate induced histamine release in human basophils but not in RBL-2H3 cells.[164] The phosphorylation of SYK is not inhibited in human basophils at 30 μM, which is effective for completely blocking IgE-mediated degranulation in the RBL-2H3 cell line.[164−166] The weak margin of potency observed with **12** for SYK inhibition has led to selective application as a chemical reagent for the study of RBL-2H3, human mast cells, and basophil function and not for therapeutic intervention.

Another first-generation inhibitor, compound **13** (ER-27319, 10-(3-aminopropyl)-3,4-dimethylacridin-9(10*H*)-one; Figure 8), has been reported as an inhibitor of antigen or anti-IgE-mediated degranulation of rodent and human mast cells.[95,167] Human mast cells were generated using methodology developed by the Saito group.[168] Inhibition of SYK activity by compound **13** was assessed by effects on 5-HT, inositol phosphate, arachidonic acid, and TNFα release, with IC_{50} of 10 μM, in RBL-2H3 cells stimulated by 4-dinitrophenylated bovine serum albumin (DNP-BSA).[167] Given the high concentration of compound **13** required to cause half-maximal inhibition of degranulation, targets other than SYK may also be inhibited. Inhibition of SYK by compound **13** was examined in RBL-2H3 and human mast cells through its effects on tyrosine phosphorylation of SYK using immunoprecipitation at an IC_{50} of 10 μM.[167] Measurement of tyrosine phosphorylation in Jurkat T-cells showed selectivity of compound **13** for SYK over family member ZAP-70.[167] By use of anti-CD3 stimulation, no inhibition of PLCγ phosphorylation by compound **13** was observed up to 100 μM. In contrast, phosphorylation of PLCγ in RBL-2H3 cells was inhibited. Antigen-induced cross-linking of FcεR1 leads to Lyn activation, which is a prerequisite for the phosphorylation of β and γ ITAM subunits of FcεR1. In RBL-2H3 cells, compound **13** did not abrogate the activity of Lyn, as phosphorylation of PKCδ was still detected up to 30 μM compound **13**.[167]

The action of compound **13** appears to be specific to mast cells, as inhibition of tyrosine phosphorylation of SYK in B-cells induced with anti-IgM was not blocked.[166] High concentrations of compound **13** are essential for antagonistic effects through SYK activation in mast cells and basophils.[166,167] However, the potential for inhibition of and/or binding to other targets, contributing to the observed phenotype, must not be overlooked.[166,167]

Following the natural product trend, flavonoids were screened as inhibitors of SYK activity in both biochemical and CHMC assays.[169] The activity of flavones **14** (luteolin) and **15** (apigenin) in a biochemical SYK assay is weak, with IC_{50} of 12.0 and 4.2 μM, respectively (Table 1).[169] Compounds **14** and **15** were both more potent in the inhibition of mast cell degranulation, as determined by tryptase release, with EC_{50} of 4.5 and 3.0 μM.[169] A group of flavonols, **16** (quercetin), **17** (fisetin), **18** (myricetin), and **19** (morin), were also tested and showed weak inhibition for both SYK and mast cell degranulation (Table 1). Activity correlated with CLogP values, which can contribute to permeability, but given the weak activity in biochemical SYK assays, the lack of translation to high potency in the mast cell assay is not surprising (Table 1).[169]

These initial forays into SYK inhibitors relied on the application of cell-based functional assays with sporadic confirmation

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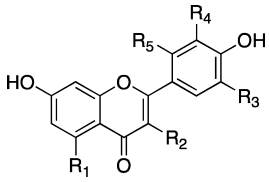
Table 1. SAR of Isolated Flavones and Flavonols, Showing SYK and CHMC Activity



compd	R_1	R_2	R_3	R_4	R_5	SYK, IC_{50} (μM)	CHMC, EC_{50} (μM)	CLogP
14 (luteolin)	OH	H	H	OH	H	12.0	4.5	1.41
15 (apigenin)	OH	H	H	H	H	4.2	3.0	2.01
16 (quercetin)	OH	OH	H	OH	H	20.0	35.0	0.63
17 (fisetin)	H	OH	H	OH	H	15.0	19.0	1.27
18 (myricetin)	OH	OH	OH	OH	H	6.4	54.0	−0.03
19 (morin)	OH	OH	H	H	OH	28.0	>300	1.73

through biochemical assays. Structures **12**−**19** do not fit pharmacophores associated with kinase inhibition through ATP pocket binding. Compound **13** targets the activation of SYK mediated through FcεR1γ ITAM, suggesting there is no direct interaction with the SYK ATP pocket. Additionally it lacks the ability to cause inhibition of activated SYK following immunoprecipation of SYK from activated cells.[167]

SECOND GENERATION OF SYK INHIBITORS

Optimization of second-generation SYK inhibitors appeared to be more dependent on the application of biochemical assays. This may have been driven by the availability of diverse biochemical kinase screening panels. As such, structural features indicative of binding to the ATP pocket of SYK became more apparent, following the screening of in-house libraries leading to the identification of diverse scaffold classes.[12−17,77,78,83,101−104]

A series of oxindoles were examined employing a homogeneous time-resolved fluorescence assay, and analogue **20**, with the sulfonamide side chain, proved to be the most potent derivative reported from this SAR.[170] The functional activity of compound **20** was ascertained using RBL-2H3 rat basophil cell IgE/FcεR1-mediated degranulation inhibition, measured as 5-HT release (EC_{50} = 0.11 μM).[170] Similarly, the SYK biochemical activity for compound **21** also translated to the cell-based assay (Table 2).

Oxindole derivatives showed good potency in the biochemical SYK assay. However, because of high polar surface area (PSA)[171] values, low solubility correlation with activity in functional RBL-2H3 cells did not consistently translate, as exemplified by compounds **22** and **23**.[170] The most potent compounds, displaying both SYK biochemical and functional RBL-2H3 activity, have PSA values ranging between 70 and 108 Å^2. Analogues with improved solubility also introduced additional polar moieties, with increase in heteroatom count, leading to an increase in PSA and ultimately decreased cell permeability.[170] The geometry at the 3-position of the oxindole is important for maintaining activity, with the *Z*-analogue **24** showing significant loss of activity. Reduction of the double bond in analogue **25** also shows loss in potency.[170] The sulfonamide functional group adds considerably to the total PSA. Bioisosteric acid (**26**) and amide (**27**) functionalities were used to circumvent this increase, and these analogues showed a wide range in cell-based activity, EC_{50} of 3.5 and 0.10 μM, respectively.[170]

The cell-based potency is rationalized through PSA but can be attributed to lack of selectivity. Control experiments to detect non-SYK dependent downstream ionomycin-induced degranulation were not reported, and thus, the degranulation phenotype could in fact be a result of promiscuous kinase inhibition. The oxindole scaffold, along with other SYK inhibitors, has been employed in pharmacophore model studies to identify novel scaffold classes and thus represents a useful class to lead-hop to other chemotypes.[172]

Compound **2** (Figure 4) has a bicyclic imidazo[1,2-*c*]-pyrimidine core, with nitrogen-linked nicotinamide and C−C linked 3,4-dimethoxyphenyl side chains at the 5- and 7-positions, respectively, and has been one of the most extensively profiled, potent SYK inhibitors in both biochemical and cell-based assays.[173] Compound **2** showed potent biochemical inhibition of SYK with K_i = 0.0075 ± 0.0025 μM and selectivity over a limited panel of Lyn, Fyn, Src, Itk, Btk, PKCα, and PKCθ kinases.[173] In human-cultured mast cells compound **2** inhibited FcεR1-mediated release of histamine, tryptase, prostaglandin D2, LT C_4/D_4/E_4, and de novo synthesis of cytokine GM-CSF.[168,173]

For an inhibitor to be broadly relevant as an anti-inflammatory agent, inhibition of both FcεR1 and Fcγ receptors is highly desirable.[174] Compound **2** effectively inhibited FcγR-mediated production of superoxide in a human monocytic cell line (U937) and freshly isolated monocytes from peripheral blood, with EC_{50} of 0.052 and 0.012 μM, respectively.[52,173] Furthermore, B-cell receptor function is extremely important in the development of disease resulting from overactivity in the immune system, and in human Ramos B-cells and mouse splenic B-cells, **2** inhibited [Ca^{2+}] release and proliferation with EC_{50} of 0.081 and 0.058 μM, respectively.[173]

Compound **2** inhibited the passive cutaneous anaphylaxis (PCA) reaction in rats in a dose-dependent manner and had ED_{50} = 8 mg/kg.[173] In addition, this compound showed efficacy in the DNP-BSA-induced acute asthma bronchoconstriction and bronchial edema model in male Wistar rats at 3 mg/kg.[173] In Brown Norway rats at 30 mg/kg, eosinophil buildup of bronchoalveolar lavage (BAL) fluid was largely mitigated in ovalbumin (OVA) induced chronic airway inflammation model. The substantial discrepancy between the dosing level in the acute and chronic models can be accounted for by the pharmacokinetic profile of compound **2**, maximum exposure of 0.167 mg/L being reached 2 h after administration of a single 10 mg/kg po dose and short half-life of 1.78 h.[173]

Compound **2** affects both IgG- and IgE-mediated pathways, implying application for both allergic and autoimmune diseases. At the time compound **2** was being investigated, the literature suggested that inhibiting SYK could lead to serious on-target side effects, such as tumor causation[42,43] and severe systemic hemorrhaging.[35−37] To date, however, there have been no reports of such on-target liabilities caused by compound **2** or

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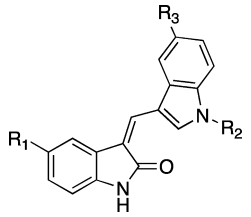
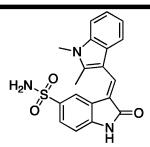
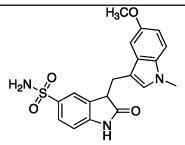
Table 2. Oxindole SAR for SYK and RBL-2H3 Activities



Cpd	R_1	R_2	R_3	SYK, IC_{50} (μM)	RBL-2H3, EC_{50} (μM)	Sol (mg/L)	PSA (Å^2)
20	CH_3NHSO_2	CH_3	OCH_3	0.020	0.11	< 0.1	105[a]
21	H_2NSO_2	CH_3	H	0.014	0.313	NA	108[a]
22	H_2NSO_2	$(CH_2)_3CONH_2$	OCH_3	0.012	> 10	5	164[a]
23	H_2NSO_2	$(CH_2)_3OH$	OCH_3	0.032	> 10	< 0.1	142[a]
26	HOOC	CH_3	OCH_3	0.273	3.5	5.6	81[a]
27	CH_3NHCO	CH_3	OCH_3	0.145	0.1	0.5	70[a]
24				Inactive at 4	NA	NA	98[b]
25				8.1	NA	NA	103[b]

[a]From ref 170. [b]Calculated using ref 171.

other SYK inhibitors, and this may, in part, be due to the fact that such side effects, including platelet activation and thrombus development, are controlled by a number of pathways.[12−17,41,46,78,173] As SYK has been shown to play a pivotal role in platelet activation through collagen receptor (glycoprotein VI) and some specific integrins (αII$\beta\beta$3),[153,175−177] it is, however, imperative to assess the activity of any SYK inhibitor on platelet function. This can be accomplished by measuring hemostasis in vivo, as assessed by effects on bleeding times following tail-tip amputation in mice, as demonstrated by compound **3**.[78] Indeed, no increase in bleeding time of mice is observed, following treatment with compound **3**, compared to control groups dosed with aspirin, which causes a substantial increase in bleeding time, thus confirming that SYK inhibition by compound **3** is not likely to cause severe systemic hemorrhaging.[78]

Compound **28** (Compound-D, Figure 8), is from the same class as compound **2**, with the introduction of a 1*H*-indazol-6-amine side chain in place of the 2-aminonicotinamide group at the 5-position of the core imidazo[1,2-*c*]pyrimidine scaffold.[173,178] The compound was utilized to probe the relationship of SYK inhibition to antigen presentation in mouse bone marrow DCs. Internalization of IC plays an important role in linking innate and adaptive immunity.[39] Upon internalization the antigen undergoes degradation by lysosomes, then loaded onto MHC and presented to T-cells as a MHC-peptide complex. This initiates the immune reaction to the internalized antigen. The activation of IgG Fcγ receptors in macrophages, monocytes, neutrophils, and DCs mirrors the activation of IgE FcεR1 in mast cells and basophils.[39,40,59] Both involve upstream intracellular events mediated by interaction of SYK with phosphorylated ITAM.

Compound **28** inhibits SYK with K_i = 0.01 μM and less potently inhibits Lyn, Fyn, Src, Itk, Btk, PKCα, and PKCθ.[178] Inhibition of IL-4 production (EC_{50} = 1.08 μM) was measured to establish inhibition of antigen presentation of IC by DCs to T-cells.[178] To prove that the effect of **28** on IL-4 production was not through either direct impairment of T-cells or non-IgG-mediated internalization, compound **28** was screened using a high concentration of conalbumin (10 μg/mL) or conalbumin peptide and showed no inhibition under these respective conditions.[178] Studies with compound **28** confirm and complement previous knockdown studies in Fcγ-receptor-mediated presentation and internalization of IC in DCs.[39,40]

A set of 5,7-disubstituted[1,6]naphthyridines with promising SYK biochemical activity have been reported.[179] Initial analogues **29** and **30** showed SYK activity with IC_{50} of 1.5 and 0.49 μM, respectively (Table 3). Replacement of the [1,6]naphthyridine ring system by pyrido[2,3-*d*]pyrimidine (compound **31**), isoquinoline (compound **32**), and quinazoline (compound **33**) led to greatly diminished SYK activity. Going from the ethylenediamine (compound **29**) to a propylenediamine (compound **34**) substituent at the 5-position of the [1,6]naphthyridine enhanced potency by 10-fold, with SYK

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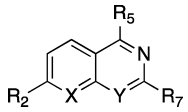
Table 3. SAR for 5,7-Disubstituted[1,6]naphthyridines



compd	X	Y	R_2	R_5	R_7	SYK, IC_{50} (μM)
29	N	C	H	$NH(CH_2)_2NH_2$	4-$CH_3OC_6H_4$	1.5
30	N	C	H	$NHCH_3$	4-$CH_3OC_6H_4$	0.49
31	N	N	H	$NHCH_3$	4-$CH_3OC_6H_4$	>30 μg/mL
32	C	C	H	$NHCH_3$	4-$CH_3OC_6H_4$	>30 μg/mL
33	C	N	H	$NHCH_3$	4-$CH_3OC_6H_4$	40
34	N	C	H	$NH(CH_2)_3NH_2$	4-$CH_3OC_6H_4$	0.14
35	N	C	H	$NH(CH_2)_2NH_2$	4-$(CH_3)_2NC_6H_4$	0.19
36	N	C	OH	$NH(CH_2)_3NH_2$	4-$CH_3OC_6H_4$	>30 μg/mL
37	N	C	CF_3	$NH(CH_2)_3NH_2$	4-$CH_3OC_6H_4$	>30 μg/mL
38	N	C	H	$NH(CH_2)_3NH_2$	4-F-C_6H_4	1.1
39	N	C	H	$NH(CH_2)_3NH_2$	4-Cl-C_6H_4	1.2
40	N	C	H	$NH(CH_2)_3NH_2$	3-F-C_6H_4	0.85
41	N	C	H	$NH(CH_2)_3NH_2$	3-Cl-C_6H_4	0.26
42	N	C	H	$NH(CH_2)_3NH_2$	4-(*N*-morpholinyl)C_6H_4	0.008
43	N	C	H	$NH(CH_2)_3NH_2$	4-$(N(CH_3)(CH_2)_3N(CH_3)_2)C_6H_4$	0.42
44	N	C	H	$NH(CH_2)_3NH_2$	4-BrC_6H_4	0.26
45	N	C	H	$NH(CH_2)_3NH_2$	4-$(CH_3)_2NC_6H_4$	0.034
46	N	C	H	$NH(CH_2)_4NH_2$	4-$(CH_3)_2N$-C_6H_4	0.019
47	N	C	H	$N(CH_3)(CH_2)_3NH_2$	4-$(CH_3)_2N$-C_6H_4	0.072
48	N	C	H	$NH(CH_2)_3NHCH_3$	4-$(CH_3)_2N$-C_6H_4	0.078
49	N	C	H	$NH(CH_2)_3NH_2$	4-$CH(CH_3)_2$-C_6H_4	0.090
50	N	C	H	$O(CH_2)_4NH_2$	4-$(CH_3)_2N$-C_6H_4	0.16
51	N	C	H	$NH(CH_2)_4OH$	4-$(CH_3)_2N$-C_6H_4	0.20

IC_{50} = 0.14 μM.[179] Replacement of the 4-methoxyphenyl ring on compound **29** at the 7-position of the central heterocyclic core with a 4-dimethylaminophenyl group (compound **35**) is tolerated and improves SYK activity to 0.19 μM.[179] Introduction of a hydroxyl (compound **36**) or trifluoromethyl (compound **37**) group at the 2-postion of the [1,6]naphthyridine led to complete loss of activity. Electron-withdrawing groups such as fluoro and chloro on the 7-phenyl ring decreased potency.[179] The 4-halogen analogues **38** (SYK IC_{50} = 1.1 μM) and **39** (SYK IC_{50} = 1.2 μM) showed activity similar to that of 3-substituted analogues **40** (SYK IC_{50} = 0.85 μM) and **41** (SYK IC_{50} = 0.26 μM), with **41** showing the best activity in this subset.[179] In fact, the 7-phenyl ring could be substituted with a larger 4-morpholino group with enhanced potency, **42** (SYK IC_{50} = 0.008 μM).[179] This position is relatively open to diverse substitution, possibly because substituents sit on the solution side of the ATP-binding pocket. Not all moieties maintained potency, as shown by compounds **43** and **44**. Compounds **45**, **46**, **47**, and **48** confirmed the preference for terminal basic nitrogen-containing propylenediamine and butylenediamine side chains at the R_5 position, with the presence of a dimethylaminophenyl group at the R_7 position.[179] In combination, with a propylenediamine group at the 5-position, the 7-position tolerates a hydrophobic isopropyl group on the phenyl ring (compound **49**). Replacement of the nitrogen connection to the [1,6]naphthyridine ring system with oxygen, as in compound **50**, showed significantly less activity compared to compound **46**. Likewise, substitution of the terminal amine with a hydroxy (compound **51**) moiety led to loss in activity. The R_5 position is limited to acyclic side chains with basic amines being preferred and aromatic containing side chains not tolerated.[179]

The [1,6]naphthyridine compounds have shown potent on-target biochemical SYK inhibition.[179] Unfortunately, no data-profiling selectivity over other kinase families in biochemical or cell-based functional assays and in pharmacokinetic, metabolism evaluation, efficacy, or toxicity studies have been reported. Interest has picked up again on this scaffold class with the generation of updated patents.[180,181]

Pyrimidine-5-carboxamide compound **52** was discovered by employing a SYK biochemical high-throughput screening (HTS) assay. The molecule displayed potent SYK inhibition, with IC_{50} = 0.041 μM and 273-fold selectivity over ZAP-70 (Table 4).[182,183] Replacement of the 2-ethylenediamino side chain at the 2-position of the pyrimidine with hydrogen, 2-methyl, and 2-phenyl led to complete loss of activity, as shown by compounds **53**, **54**, and **55**, respectively. Increased chain length from 2-ethylenediamino (compound **52**) to the 2-butylenediamino (**56**) analogue maintained activity. However, going to a secondary amine (compound **57**) diminished activity.[183] Introduction of a piperazine ring at the 2-position of the pyrimidine in analogue **58**, with secondary terminal basic nitrogen, completely abolishes activity, confirming the importance of a primary amine at the terminus. As exemplified by 2-ethylenehydroxy analogue **59**, an alcohol functional group is not tolerated.[183] Substitution on the phenyl ring and aromatic character of the side chain at the 4-position of the pyrimidine play valuable roles in maintaining activity, as proven by analogues **60**, **61**, **62**, and **63**; however, the cyclohexyl compound **64** loses significant activity.[183] Just as important is the role of the carboxamide, with the secondary amide (**65**) and carboxylic acid (**66**) analogues losing activity.[183] The SAR was rationalized employing a published cocrystal structure of compound **7** with SYK[83] and also the crystal structure of Lck,

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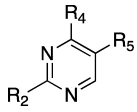
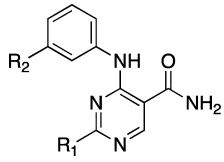
Table 4. SAR of Pyrimidine-5-carboxamides



compd	R_2	R_4	R_5	IC_{50} (μM) SYK	IC_{50} (μM) ZAP70
52	$NH(CH_2)_2NH_2$	3-$CF_3C_6H_4$	$CONH_2$	0.041	11.2
53	H	3-$CF_3C_6H_4$	$CONH_2$	>5	>5
54	CH_3	3-$CF_3C_6H_4$	$CONH_2$	>5	>5
55	C_6H_5	3-$CF_3C_6H_4$	$CONH_2$	>5	>5
56	$NH(CH_2)_4NH_2$	3-$CF_3C_6H_4$	$CONH_2$	0.047	10.5
57	$NH(CH_2)_2NHCH_3$	3-$CF_3C_6H_4$	$CONH_2$	0.21	>10
58	piperazin-1-yl	3-$CF_3C_6H_4$	$CONH_2$	>5	>5
59	$NH(CH_2)_2OH$	3-$CF_3C_6H_4$	$CONH_2$	0.35	>10
60	$NH(CH_2)_2NH_2$	C_6H_5	$CONH_2$	0.28	13.2
61	$NH(CH_2)_2NH_2$	2-$CH_3C_6H_4$	$CONH_2$	0.23	>30
62	$NH(CH_2)_2NH_2$	3-$CH_3C_6H_4$	$CONH_2$	0.03	5.2
63	$NH(CH_2)_2NH_2$	4-$CH_3C_6H_4$	$CONH_2$	0.15	6.5
64	$NH(CH_2)_2NH_2$	cyclohexyl	$CONH_2$	>10	>10
65	$NH(CH_2)_2NH_2$	3-$CF_3C_6H_4$	$CONHCH_3$	>10	>10
66	$NH(CH_2)_2NH_2$	3-$CF_3C_6H_4$	CO_2H	>5	>5

Table 5. Selectivity Profiling of Pyrimidine-5-carboxamides



compd	R_1	R_2	IC_{50} (μM) SYK	Itk	Btk	PKCα	PKCβ2	EC_{50} (μM) 5-HT release
52	$NH(CH_2)_2NH_2$	CF_3	0.041	22.6	15.5	5.1	11	0.46
56	$NH(CH_2)_4NH_2$	CF_3	0.047	4.0	2.9	1.5	16	1.45
62	$NH(CH_2)_2NH_2$	CH_3	0.03	29.3	9.6	6	13	0.73
67	$NH(CH_2)_2NH_2$	OCH_3	0.066	28.0	8.7	9	19	2.0
68	$NH(CH_2)_2NH_2$	OCH_2CH_3	0.081	23.1	10.7	8.5	27	0.93

because of the high degree of similarity of the respective ATP pockets.[183]

Potent analogues **52**, **56**, **62**, **67**, and **68** were screened for selectivity using a limited panel of six biochemical kinase assays and also an on-target functional assay assessing the inhibition of 5-HT release in RBL-2H3 cells (Table 5).[183] The initial HTS hit **52** proved to be the most potent compound in the RBL-2H3 cell-based assay. The in vitro on-target potency translated to an animal model, with analogue **52** showing efficacy in the PCA reaction with ED_{50} = 13.2 mg/kg.[183]

HTS screening led to the identification of potent *N*-phenyl-4-(thiazol-2-yl)pyrimidin-2-amine SYK inhibitors (Table 6).[104] Compound **69** inhibits SYK with K_i = 0.63 μM.[104] SAR produced a set of potent analogues with biochemical activity, corroborated in cell-based mast cell degranulation assays. Selectivity was assessed by counterassays targeting ZAP-70, cyclin-dependent kinase 2, Rho-associated serine/threonine kinase, and Src kinases. A range of substituents on the *N*-phenyl group at the 2-position of the pyrimidine were made, leading to noticeable improvements in SYK biochemical and mast cell degranulation potency.[104]

Interestingly, chirality influences activity, as shown by dissimilarities between (*S*)- and (*R*)-alanol derivatives **9** and **70**, showing SYK K_i of 0.009 and 0.21 μM, respectively.[104] A cocrystal structure of the SYK ATP pocket with potent analogue **9** aided in rationalizing this difference in potency (Figure 9).[104] The pyrimidine ring nitrogen at the 1-position provides a hydrogen bond acceptor interaction with SYK hinge residue A451, and the NH linker at the 2-position has hydrogen bond donor interaction with the carbonyl group of A451.[104] Also, the (*S*)-alanol side chain sits in the vicinity of the salt bridge formed between K402 and D512, and the geometry of two enantiomers **9** and **70** is slightly different resulting in a change in interaction and accounting for the difference in activity (Figure 9). The crystal structure indicates the importance of the hydrophobic binding interactions of 3,5-disubstituted phenyl ring in the ATP pocket and accounts for the loss of potency of the much simpler compound **10**, where an *o*-methyl group disrupts the coplanarity of the pyrimidine and phenyl rings (Figure 6). Solubility and permeability of compounds may also account for differences in the activity observed in the cell-based assay.[104]

A homology model for ZAP-70[24,81,82] based on a reported crystal structure of activated Lck kinase domain was employed for docking pyrimidine-5-carboxamide inhibitors and to lead-hop to other scaffold classes (Table 7).[184] This work led to the

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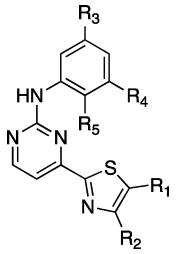
Table 6. SAR of *N*-Phenyl-4-(thiazol-2-yl)pyrimidin-2-amine Compounds



Cpd	R_1	R_2	R_3	R_4	R_5	K_i (μM)					EC_{50} (μM)
						SYK	ZAP-70	CDK2	ROCK	SRC	Mast Cell
69	H	H	H	C_6H_5O	H	0.63	NA	NA	NA	NA	NA
9		CH_3	CH_3	CH_3	H	0.009	0.24	> 3.33	2.3	> 3.33	0.07
10	H	H	CH_3	H	CH_3	1.82	NA	> 5	NA	0.15	NA
70		CH_3	CH_3	CH_3	H	0.21	1.5	> 3.33	> 2.5	> 3.33	4.2

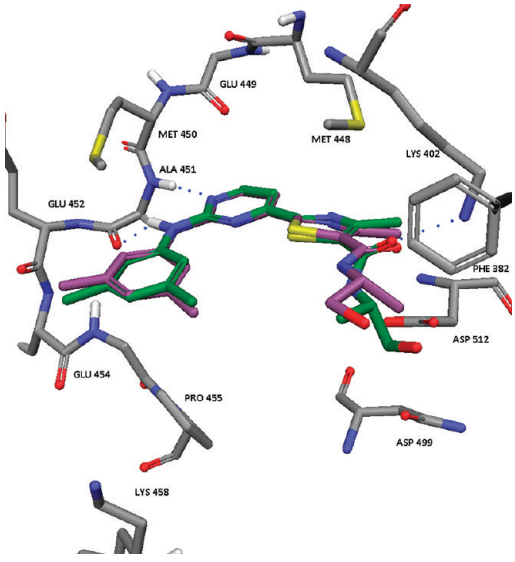


Figure 9. Cocrystal image of compound **9** (green) and docked compound **70** (purple) in SYK. In contrast to enantiomer **9**, hydroxyl group on enantiomer **70** is facing away from ASP512.

initial optimization of 1,2,4-triazolo[4,3-*c*]pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine scaffolds directed at SYK and ZAP-70 inhibition.[184] In general, compounds from these scaffold classes possessed greater potency for SYK. Given the close homology of SYK and ZAP-70[24,81,82] (vide supra), structure **52** was used as the initial scaffold for docking, along with the promiscuous kinase inhibitor **6**.[184] Docking studies of compound **52** in the ZAP-70 homology model point to a cavity where a ring could be constructed between the 1- and 6-positions of the pyrimidine ring (Table 7). This proposal was initially explored through screening of the 1,2,4-triazolo[4,3-*c*]pyrimidine central bicyclic scaffold and subsequently extended to the 1,2,4-triazolo[1,5-*c*]pyrimidine class (Table 7). The 1,2,4-triazolo[4,3-*c*]-pyrimidine chemotype can be converted to the 1,2,4-triazolo-[1,5-*c*]pyrimidine by a Dimroth rearrangement (Table 7).[184]

The 1,2,4-triazolo[4,3-*c*]pyrimidine scaffold compound **71** shows improved SYK activity compared to pyrimidine compound **52** (Table 4), although the ZAP-70 activities remain similar (Table 7).[184] Replacement of 2-ethylenediamino by a *cis*-cyclohexyldiamino group (compound **72**) retains SYK potency, but selectivity over ZAP-70 is decreased. Dimroth rearrangement of 1,2,4-triazolo[4,3-*c*]pyrimidine (compound **72**) generated 1,2,4-triazolo[1,5-*c*]pyrimidine compound **73**, which maintains similar SYK potency and activity for ZAP-70.[184] Aromatic substitution at the 3-position on the 1,2,4-triazolo-[4,3-*c*]pyrimidine ring (compound **74**) leads to loss of both SYK and ZAP-70 activity. However, introduction of an aromatic ring at the 2-position of the 1,2,4-triazolo[1,5-*c*]pyrimidine ring system (compound **75**) leads to a 10-fold reduction in SYK activity but with an improvement in ZAP-70 potency.[184] Further reduction in SYK activity is observed by introducing a 3-methoxyphenyl ring (compound **76**) at the 2-position of the triazole ring. The most potent ZAP-70 compound is produced by introduction of a 2-chlorophenyl ring (compound **77**) on the triazole ring. Cell-based activity of analogues was assessed by measurement of the inhibition of IL-2 production in peripheral blood mononuclear cells (PBMCs) and whole blood (WB) cells. The most potent compounds in these functional assays were **72** and **73**, with PBMC inhibition of 0.046 and 0.076 μM and WB inhibition of 0.44 and 0.48 μM, respectively.[184] Interestingly, compound **77**, a potent SYK and ZAP-70 inhibitor, shows activity of 1.76 μM in IL-2 production inhibition. Lack of solubility and cell permeability may account for the weaker IL2 inhibition

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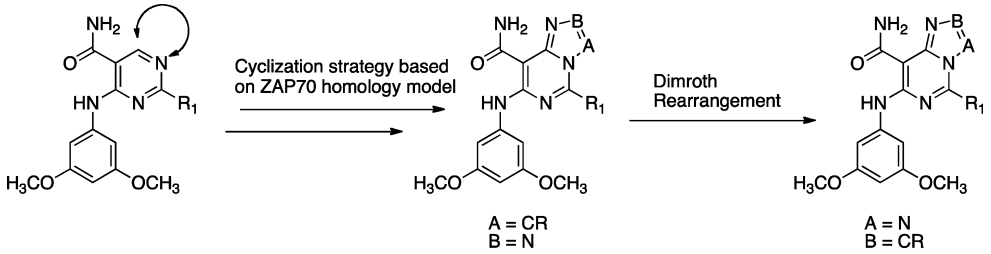
Table 7. Dimroth Rearrangement for Conversion of 1,2,4-Triazolo[4,3-*c*]pyrimidine to 1,2,4-Triazolo[1,5-*c*]pyrimidine Scaffold and SYK and ZAP-70 SAR Summary of 1,2,4-Triazolo[4,3-*c*]pyrimidine and 1,2,4-Triazolo[1,5-*c*]pyrimidine Compounds

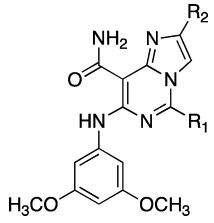


Cpd	R_1	A	B	IC_{50} (μM)				EC_{50} (μM)	
				SYK	ZAP-70	Lck	PKCβII	PBMC	WB
71	$NH(CH_2)_2NH_2$	CH	N	0.009	8.3	–	–	–	–
72		CH	N	0.009	0.12	0.18	0.68	0.046	0.44
73		N	CH	0.004	0.33	0.59	2.66	0.076	0.48
74		CC_6H_5	N	15.2	26	–	–	–	–
75		N	CC_6H_5	0.083	0.11	–	–	0.21	2.0
76		N	C-(3-CH_3O-C_6H_4)	0.12	0.12	–	–	–	–
77		N	C-(2-Cl-C_6H_4)	0.05	0.097	–	–	1.76	NA

exhibited by compound **77**. Taken together, these data suggest that these compounds show good potency for SYK and consistent selectivity over ZAP-70 and that many kinases upstream and downstream are involved in the IL-2 pathway leading to IL-2 production. However, more extensive selectivity assessment of these potent SYK inhibitors in both biochemical and cell-based assays is warranted.

The imidazo[1,2-*c*]pyrimidine scaffold class followed from the previously reported 1,2,4-triazolo[4,3-*c*]pyrimidine and from 1,2,4-triazolo[1,5-*c*]pyrimidine modulators of SYK and ZAP-70 activity (Table 8).[185,186] Both 1,2,4-triazolo[4,3-*c*]-pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine have high PSA values and low Caco-2 permeability,[185] ultimately leading to low oral bioavailability and limiting the chances of success in animal efficacy models. The 1,2,4-triazolo[4,3-*c*]pyrimidine compound **72** and 1,2,4-triazolo[1,5-*c*]pyrimidine analogue **73** have low Caco-permeability, 0.01×10^{-6} and 0.33×10^{-6} cm/s, respectively.[185] The imidazo[1,2-*c*]pyrimidine scaffold has one fewer nitrogen atom, leading to a lower PSA and improved Caco-2 permeability.[171,185]

Table 8. SAR of Imidazo[1,2-*c*]pyrimidine Scaffold



Cpd	R_1	R_2	IC_{50} (μM)		EC_{50} (μM)	
			SYK	ZAP-70	PBMC	WB
78	$NH(CH_2)_2NH_2$	H	0.23	3.5	NA	NA
79		H	0.006	0.23	0.058	0.28
80		H	1.2	> 10	NA	NA
81		C_6H_5	0.38	0.60	NA	NA
82		C_6H_5	0.028	0.041	0.20	2.0

The initial compound **78** with a 2-ethylenediamino moiety did not potently inhibit SYK (Table 8). Introduction of the *cis*-cyclohexyldiamine side chain, as in compound **79**, significantly improved activity. The *trans*-cyclohexyldiamine compound **80** shows loss in activity. In addition, isomers (1*R*,2*S*)-**81** and (1*S*,2*R*)-**82** show a significant difference in activity for both SYK and ZAP-70 but with little separation between SYK and ZAP-70 activities.[185] Compounds **79** and **82** were active, with EC_{50} of 0.058 and 0.20 μM in the PBMC and 0.28 and 2.0 μM in the WB assays.[185] In contrast to 1,2,4-triazolo[4,3-*c*]-pyrimidine and 1,2,4-triazolo[1,5-*c*]pyrimidine compounds, imidazo[1,2-*c*]pyrimidine **79** shows improved aqueous solubility of 930 μg/mL and Caco-2 permeability of 3.33×10^{-6} cm/s. Oral dosing of compound **79** shows a dose-dependent increase in exposure with 3, 10, 30, 100 mg/kg providing exposures of 40, 191, 770, and 4031 ng/mL, respectively.[185] Furthermore, compound **79** shows efficacy in the PCA reaction at doses of 3, 10, 30, and 100 mg/kg, with ED_{50} between 3 and 10 mg/kg.[185] In addition, concanavalin A induced IL-2 production suppression was only significant at doses higher than 10 mg/kg. This class of compound provides another validation of the translation of SYK inhibition to animal model efficacy.[185]

The pyrimidine-5-carboxamide[182,183] SYK inhibitors originated from the Yamanouchi group and were in-licensed by Portola.[187] Detailed SAR on this scaffold class led to optimization in potency

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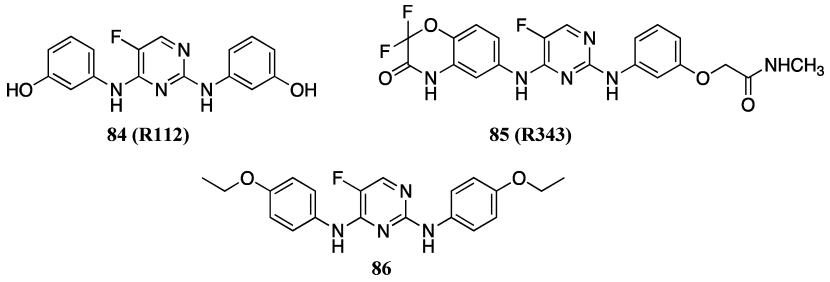

Figure 10. Structures of SYK inhibitors **84** and **85** and HTS hit **86**.

and selectivity and produced a number of analogues, including compound **83** (PRT060318; Figure 8).[187] Animal efficacy data for the prevention of heparin-induced thrombocytopenia (HIT) and thrombosis in a transgenic mouse model have recently been reported for compound **83**.[187] Compound **83** inhibited SYK with an IC_{50} of 0.004 μM and displayed functional selectivity over Src family kinase Lyn.[187] This was confirmed by showing no inhibition of Lyn mediated phosphorylation of Y352 on human SYK in WB, up to 10 μM. The compound showed biochemical selectivity as determined through screening in a panel of 138 kinases.[187] On-target convulxin-induced aggregation of human platelet-rich plasma (PRP) activation through GPVI/FcRγ was inhibited with EC_{50} = 2.5 ± 0.2 μM, and non-ITAM based platelet activation through adenosine diphosphate was not effected at concentrations up to 50 μM.[187] To gain additional insight for on-target mode of action through the FcγRIIA–ITAM–SYK pathway, compound **83** inhibited platelet activation by HIT IC at concentrations ranging between 0.3 and 3 μM in vitro and PRP from HIT mice treated with anti-CD9 antibody mimicking HIT IC acting through FcγRIIA was completely ablated ex vivo at inhibitor concentrations of ≥0.75 μM.[187] In animal efficacy studies, compound **83** at 30 mg/kg b.i.d. showed improvement in HIT IC induced thrombocytopenia, in treated animals (89.7 ± 3.7% of baseline) versus control (47 ± 10.1% of baseline) as determined by nadir platelet count. Finally, the compound also showed preventative activity on HIT IC-induced thrombosis in mouse lung, as determined by near-infrared imaging.[187]

The biochemical selectivity of compound **83** translated to cell-based activity, and the in vitro SYK activity has manifested itself in in vivo animal model studies.[187] More detailed cell-based profiling, efficacy, and toxicity evaluation is continuing with this class of compound, along with efficacy studies in man.

Rigel SYK inhibitors (compounds **84** [R112], **85** [R343], and **3** and its prodrug compound **11**) have been the most thoroughly investigated SYK inhibitors to date (Figures 4, 7, and 10).[12−14,16,78,137,138,150−155,157−163,188−201] Extensive evaluation of the effects of these compounds on the abrogation of FcR and BCR signaling has been undertaken. Also, preliminary signs of efficacy in the treatment of allergic rhinitis, asthma, RA, ITP, lupus, T1D, and B-cell lymphoma have been reported.[78,137,138,150−155,157−163,188−201] Compound **3** has been investigated as a prodrug **11** to improve its solubility. Prodrug derivative **11** rapidly converts to parent drug **3** through the action of alkaline phosphatase on the apical brush-border of the membranes of intestinal enterocytes when dosed orally (Figure 7).[194,195]

Previously, the use of cell-based assays such as RBL-2H3 and human mast cells has been reported for the investigation of SYK function.[164,202] HTS screening campaigns with CHMCs led to the identification of hit structure **86** (Figure 10), which inhibited anti-IgE-mediated tryptase release with EC_{50} = 1.67 μM, and this compound was optimized using CHMC assays.[201] The CHMC screens, combined with biochemical and Western blot assays, proved to be extremely useful in mechanism of action studies.[78,188,189] The generation of large numbers of primary human mast cells has been challenging,[202] but the Saito group has reported methodology that was further optimized.[168,188] Improvement relied on expanding cord blood $CD34^+$ progenitor cells with recombinant human FMS-like tyrosine kinase 3 (Flt3), recombinant human stem-cell factor, and human IL-6.[188] Primary human mast cells could be differentiated, resulting in the production of 1×10^9 cells through this methodology.[188] Discovery of compound **84** relied on the optimization of HTS hit **86**, identified from CHMC functional screens. In CHMCs, compound **84** inhibited anti-IgE-mediated tryptase release, which is a reliable measure of upstream activation of degranulation, with EC_{50} = 0.353 ± 0.117 μmol/L.[188,189] In contrast, compound **84** showed no inhibition of downstream degranulation mediated through ionomycin-induced mobilization of calcium. Inhibition of anti-IgE-mediated histamine release from human basophils by treatment with compound **84** occurs with EC_{50} = 0.28 μmol/L. Also, compound **84** inhibits dust-mite stimulation of histamine release with EC_{50} = 0.49 μmol/L, which more realistically reflects disease in humans.[188] Additionally, compound **84** inhibited multiple downstream biomarkers of SYK activities, such as LTC_4 (EC_{50} = 0.115 ± 0.021 μmol/L) secretion and TNFα (EC_{50} = 2.01 ± 0.97 μmol/L), GM-CSF (EC_{50} = 1.58 ± 0.87 μmol/L), and IL-8 (EC_{50} = 1.75 ± 0.88 μmol/L) production.[188,189] Notably, compound **84** inhibits SYK with K_i = 0.096 μM. The variation in concentration required for the inhibition of downstream markers in cell-based assays may be attributable to augmentation of different signals, variability in expression level of molecules, time required during incubation for inhibitor effect, positive feedback loops, and autoinhibitory effects on SYK. These factors also apply to the biologic effects of other compounds inhibiting SYK such as **2**, **3**, and **83**.[78,173,187] Biochemical screening of **84** in a panel of 25 kinases showed that SYK and Src kinases were most potently inhibited.[188] The mechanism of action of compound **84** was narrowed down by in vitro analysis of the events upon Lyn phosphorylation at Y352 on interdomain-B of SYK and subsequent SYK phosphorylation of Y191 on LAT and Y783 on PLCγ. Phosphorylation of LAT and PLCγ, both mediated by SYK, is inhibited with EC_{50} = 0.4 μmol/L. Compound **84** did not inhibit the phosphorylation of Y352 on SYK by Lyn in phosphoblot assays, thus showing selective inhibition of SYK over Lyn.[188]

Onset of action for compound **84** is rapid, as shown by in vitro inhibition of tryptase release from mast cells stimulated with anti-IgE antibody, following introduction of compound **84** in parallel or in 30 and 60 min increments.[188] Given the aim of

treating allergic rhinitis and prophylactic application, rapid onset of action represents an improvement from existing therapies.[117,118]

Extensive optimization of compound **84** led to more potent analogues suitable for inhaled and oral administration.[12−16,78,190,191] Thus, compound **85** has been developed for inhaled treatment of asthma, and compounds **3** and **11** have been developed for oral treatment of autoimmune and inflammatory diseases.[13,78,137,197−201] Compound **85**[201] inhibits SYK with K_i = 1.8 nM in the biochemical assay and SYK-dependent tryptase release in CHMC (EC_{50} = 27 nM). Other kinases most potently inhibited by compound **85** include Lyn, EGFR, Lck, Jak2, and FGFR4 with K_i of 30, 35, 116, 130, and 217 nM, respectively. In functional assays compound **85** is 100-fold more selective for SYK over Lyn kinase. This is demonstrated by **85**[201] not inhibiting phosphorylation of SYK Y352 mediated by Lyn but inhibiting the phosphorylation on Y191 of LAT in CHMC,[77,78] a substrate for SYK, confirming that compound **85** works directly through SYK.

Autoimmune diseases, such as RA, are inherently heterogeneous in character, involving numerous cell types such as macrophages, mast cells, DCs, B-cells, neutrophils, fibroblasts, and osteoclasts.[10,11] The association of SYK as an upstream regulator of signal transduction in these diverse cell types has been substantiated, and its role in autoimmune diseases has gained considerable attention.[10,11,78,138,150−156,187,193,197−200]

Compound **3** inhibits SYK, with K_i = 30 nM, and SYK-dependent FcεR1 and FcγR signaling in various cell types: CHMC (EC_{50} = 56 nM), macrophages derived from human monocytes (EC_{50} = 111 nM), monocytic THP-1 (EC_{50} = 171 nM), neutrophils (EC_{50} = 33 nM), and B-cells (EC_{50} = 48 nM).[78] Thus, cross-linking induced through anti-IgE, anti-IgG, and anti-IgM stimulation of FcεR1, FcγR, and BCR, respectively, is potently abolished following treatment with compound **3**.[78] Selectivity data for compound **3** were generated through screening in a panel of over 90 biochemical kinase assays employing a fixed ATP concentration (10 μM) and in over 15 on-target and 20 off-target cell-based assays.[78] Other kinases inhibited potently in biochemical assays include Ret, Flt 3, Jak, and Lck.[38,78,203] The resulting profile indicated that in cells, compound **3** generally inhibited other kinases at 5- to 100-fold less potency than SYK as judged by phosphorylation of target proteins and functional readouts.[78] After SYK, compound **3** inhibited Flt3, Jak, and c-kit most potently at about 4- to 5-fold less potency in cell-based functional assays.[78] In a separate study, compound **3** was also shown to potently inhibit Ret in biochemical (IC_{50} = 5 nM) and in SK-N-SH cells with EC_{50} = 80 nM) and in in vivo animal toxicity studies.[38,163,203] At the clinical doses of 100 mg in RA trials (vide infra), where the SYK activity in blood biomarker assays is inhibited by 50%, it is possible that inhibition to these other minor targets contributes to the overall pharmacological effect of compound **3**.[38,78,138,197−199]

Assessment of SYK-independent signaling through a variety of off-target cell-based assays relying on stimulation that are not dependent on SYK has been carried out. Compound **3** is at least 10-fold less potent in inhibiting LPS-mediated release of TNFα production through the toll-like receptor 4 (TLR-4).[78] Because of the involvement of macrophages and neutrophils in innate immune reaction, it is a prerequisite that an inhibitor of SYK does not affect phagocytosis, oxidative burst, chemotaxis, or microbicidal activity.[78] These defensive mechanisms not only are reliant on FcR signaling through SYK but also are activated through TLRs and complement those that are not regulated by SYK.[78,204,205] Preferably, inhibition of SYK should provide a sufficient window over these necessary immune responses. Indeed, compound **3** up to 50 μM had no significant activity on monocyte and granulocyte induced phagocytosis of fluorescein-labeled opsonized *Escherichia coli* bacteria or the oxidative burst of human leukocytes.[78] Compound **3** at 10 μM shows no effect on granulocyte migration toward the chemotactic peptide *N*-formyl-Met-Leu-Phe or the microbicidal clearance of *Staphylococcus aureus* by purified primary neutrophils up to 20 μM.[78] Overall, treatment with compound **3** shows no interference with the important innate function of pathogen clearance or with the ability to mount a response to infection.[78,192]

Compound **3**, a potent SYK inhibitor, has been evaluated in animal models reflecting human disease. Compound **3** in IC-mediated disease models such as the Arthus reaction, CAIA, and K/xBN models has shown significant efficacy.[78,138] The presence of phosphorylated SYK has been identified in the synovial intimal lining of RA synovial tissue.[193] Activation of SYK in fibroblast-like synoviocytes is initiated by proinflammatory cytokines, such as TNFα, and leads to downstream activation of MAPK member c-Jun N-terminal kinase (JNK).[78,138,193] Compound **3** reduced the cutaneous reverse passive Arthus reaction by 72% and 86% at doses of 1 and 5 mg/kg, respectively.[78,138] In the IC-mediated clinical arthritis CAIA mouse model (induced with passive immunization with arthrogenic anticollagen antibodies), administration of compound **3** at 5 and 10 mg/kg b.i.d. led to a significant reduction in hind paw clinical scores in comparison to vehicle, naive, and IgG-treated animals.[78,138] Histologic appraisal of the ankle joints of drug treated mice at the end of the study (day 14) confirmed complete absence of chronic synovitis, joint thickening, synovial hyperplasia, pannus formation, and infiltration of neutrophils, lymphocytes, and macrophages.[138] No improvement in vehicle-treated control animals was observed. These results were the first validation of SYK inhibition in ablating IC-mediated arthritis. The application of arthritogenic (K/BxN) serum has been reported in $SYK^{-/-}$ mouse chimeras to validate the critical importance of this kinase in the development of clinically relevant autoimmune arthritis.[38,138] Induction of arthritis in C57BL/6 mice through use of K/BxN serum followed by treatment with 10 mg/kg compound **3** b.i.d. delayed arthritis onset and reduced hind limb clinical scores by 50%.[138]

RA transitions from disease initiation (afferent phase) to propagation (effector phase) and culminates in bone and cartilage tissue damage.[10,62] In RA patients, the presence of cartilage oligomeric matrix protein (COMP) in serum correlates with the degradation of bone. In the collagen-induced arthritis (CIA) rat arthritic model both parent drug **3** and prodrug **11** reduced COMP levels following b.i.d. treatment and this level of reduction was consistent with complete reduction in bone degradation, as confirmed by radiography and micro-CT imaging of hind joints in rats.[138]

In animal studies, compounds **3** and **11** blocked the physical signs of arthritis: tissue swelling, joint space narrowing, inflammatory cell infiltration into the synovial fluid and tissue, and bone destruction.[138] Importantly, these improvements in clinical arthritis also correlated with a decrease in serum biomarkers such as IL-6, matrix metalloproteinase 3 (MMP3), and TNFα, relevant to human clinical studies.[138,193,197]

As discussed earlier, SLE occurs as result of overactivation of inflammatory conduits through IC presentation and complement. Clinical indicators in human disease include

glomerulonephritis, ITP, hemolytic anemia, polyserositis, and vasculitis.[137,150] Compound **11** was evaluated, at doses of 10, 20, and 40 mg/kg b.i.d., in a lupus NZB/NZW murine model for assessment of efficacy in a model reflective of human SLE.[150] This model is characterized by glomerular sclerosis and tubular damage, proteinuria, and elevated blood urea nitrogen levels (BUN).[150] Treatment of lupus-prone NZB/NZW mice with compound **11** prevents the development of renal disease and also treats established murine lupus. Overall, compound **11** shows diminution of renal disease as shown by reduction in glomerular sclerosis and tubular damage and proteinuria and BUN levels.[150]

Current therapy for glomerulonephritis is inadequate and relies on the use of broadly active immunosuppressive drugs; however, such treatment regimens result in serious side effects.[150,151] The pathogenesis of glomerulonephritis is an example of a disease mediated through the interaction of antibody or ICs with Fc receptors and represents a potential application of SYK inhibitors for disease amelioration. In the NTN model of antibody mediated glomerulonephritis in Wistar−Kyoto rats, compound **11** reduces proteinuria and tissue damage as measured by reductions in glomerular macrophage and CD8+ cell numbers and renal monocyte chemoattractant protein-1 (MCP-1) and IL-1β.[151] Interestingly, compound **11** demonstrates efficacy when used both prophylactically and during active glomerulonephritis, resulting in histological reversal of disease.[151] Doses of compound **11** required for efficacy in glomerulonephritis are in the range of 15−40 mg/kg b.i.d. and overlap with those applicable to RA and SLE animal studies.[78,137,138,150,151]

T1D is an autoimmune disease characterized by the presence of anti-islet antibodies before pathologic signs of disease activity.[152] Initial stages of T1D are referred to as the effector phase, primarily mediated by T-cells through ZAP-70, and are not reliant on SYK. Experimentally, this has been deciphered through the failure of prodrug **11** of SYK inhibitor **3** to protect NOD/SCID mice that had received diabetogenic splenocytes.[152] However, mice that received **11**-treated splenocytes were protected from diabetes.[152] Compound **11** has been tested in T1D RIP-mOVA and NOD spontaneous T1D animal models.[152] Treatment of NOD mice with compound **11** delayed the development of diabetes as confirmed by diminished appearance of invasive insulitis through histopathology analysis and by extended survival.[152] Indeed, compound **11** does not obstruct the diabetogenic effector phase but rather exerts efficacy through inhibition of islet-reactive T cell priming.[152]

Compound **11** was tested in a murine ITP model that can be used to assess effects on cytopenia.[137] Upon treatment of mice with an antibody targeting integrin αIIb, thrombocytopenia development was confirmed by the changes in platelet count after 24 h. Efficacy was examined by pretreatment of mice with 25 or 40 mg/kg compound **11**.[137] Animals showed significant improvement in thrombocytopenia development following treatment with compound **11**. In contrast, the vehicle treated group showed intense thrombocytopenia. The parent compound of **11** prevents engulfment of platelets and, hence, their destruction.[137]

SYK is the upstream regulator of mast cell and IgE-mediated airway hyperresponsiveness (AHR) in asthma and additionally regulates T_H2 cells and eosinophil response.[190,191] These cells are responsible for the release of cytokines and chemokines, resulting in airway inflammation with changes in airway function. Thus, SYK inhibitors could be effective agents in treating bronchial asthma.[190,191] This has been evaluated in animal models, such as allergen-induced airway inflammation, leading to AHR in BALB/c mice. As in in vitro screens, compound **3** inhibited downstream events transpiring as a result of mast cell activation. Thus, in OVA-challenged IgE-sensitized cells, compound **3** inhibited the release of IL-2, IL-6, IL-13, and TNFα in addition to MAPK-ERK1/2, JNK1/2, and p38 with EC_{50} = 0.01−0.1 μM.[190,191] Importantly, compound **3** inhibited AHR induced by OVA exposure in doses between 3 and 30 mg/kg.[190,191] The importance of SYK in other cell lineages relevant to AHR cannot be overlooked, and the potent inhibition in vivo could be attributable to B-cells, DCs, and eosinophils, which rely on SYK for key signal transduction leading to proinflammatory factor production and release.[190,191]

The relative SYK activities of compounds **3**, **84**, and **85** for SYK can be rationalized through cocrystal structural data (Figure 11) obtained with SYK kinase catalytic domain.[78,206]

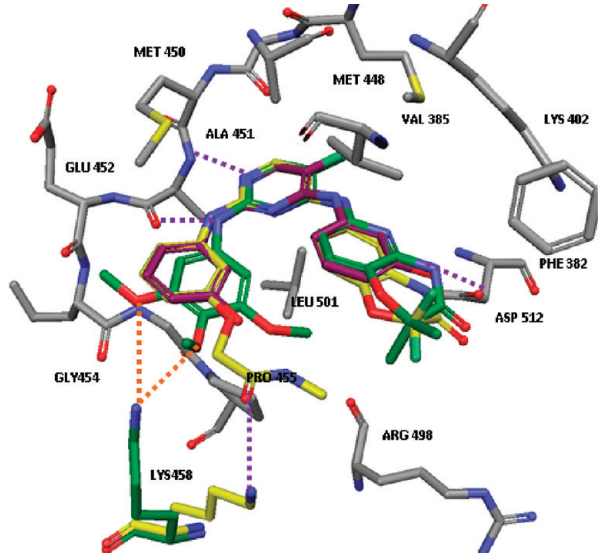

Figure 11. Cocrystal image of compounds **3** (green), **84** (purple), and **85** (yellow). Compound **84** shows hydrogen bonds with hinge residues ALA451 and GLU452 but no hydrogen bonds with ASP512 and LYS458. In contrast to compound **84**, compound **3** has hydrogen bonds with hinge ALA451, GLU452 and ASP512, LYS458 residues. Compound **85** forms hydrogen bonds with hinge ALA451, GLU452. Difluorobenzoxazinone ring also forms a hydrogen bond with ASP512, and the carbonyl moiety from the phenoxyacetamide side chain interacts with LYS458.

Compound **84** shows hydrogen bond interactions with hinge residue ALA451 and GLU452 but no hydrogen bonds with ASP512 and LYS458. In contrast, compound **3** has hydrogen bonds with hinge ALA451, GLU452 and ASP512, LYS458 residues with the hydrophobic dimethylpyridoxazinone and trimethoxyphenyl rings, respectively.[78,101] The pyrimidine nitrogen at the 1-position and the N−H linker from the phenoxyacetamide at the 2-position of **85** form hydrogen bonds with hinge ALA451 and adjacent residue GLU452. The 4-position pyrimidine substituent, the difluorobenzoxazinone ring, forms hydrogen bonds with ASP512, and the carbonyl group from the phenoxyacetamide moiety interacts with LYS458. The 5-fluoropyrimidine substituent is generally required to maintain selectivity over other kinases and also translation to cell-based functional assays.[78] The tighter binding manifested by numerous interactions with residues in SYK with inhibitors **3**

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and **85** accounts for the potency difference versus compound **84**.[78,188,206]

The discovery of compounds **3**, **84**, **85** and other reported inhibitors has been achieved through reliance on primary functional cell-based assays. In addition, data obtained from biochemical assays need to be confirmed with functional cell-based assays, as the former can produce misleading results when relying on screening paradigms using just the kinase catalytic domain. In addition, treatment of complex diseases may need obligatory inhibition of several targets best examined in parallel through functional assays and animal models with appropriate biomarker benchmarks.

CLINICAL DEVELOPMENT OF SYK KINASE INHIBITORS

Compounds **3**, **11**, and **84** are the first SYK inhibitors to transition from research to clinical development. Compound **85** has been evaluated in asthma patients through the inhalation route in phase 1 studies; however, clinical efficacy has not yet been published for this compound.

Compound **84** has shown preliminary signs of efficacy in a study involving the treatment of symptoms of seasonal allergic rhinitis in a park environment.[196] A double-blind, placebo-controlled study was conducted with patients randomized to receive intranasal 3 mg/nostril b.i.d. of compound **84** or vehicle control.[196] The patients were assessed for symptoms of allergic rhinitis (sneezes, runny nose/sniffles, and itchy/stuffy nose), with a global symptom complex (GSC) of 32 as the highest possible score.[196] Assessments were made by comparison of GSC over a period of 8 h from baseline of **84** treatment and placebo groups. Following treatment with compound **84**, there was a statistically significant (p = 0.005) reduction in GSC compared with placebo (7 units versus 5.4 units, respectively).[196] The GSC prior to start of treatment was ~18/32 in both groups. This was the first study conducted providing clinical proof that inhibition of SYK could lead to amelioration of a disease functionally related to SYK. Furthermore, the onset of action of compound **84** was rapid, with considerable improvements in symptoms occurring within 45 min subsequent to dosing, and lasting over a period of 4 h.[188,196] In clinical trials, compound **84** showed good tolerability, with adverse events similar between treatment and placebo groups.[196] As suggested, this study formed a proof of concept for the clinical use of SYK kinase inhibitors; however, given the potency of compound **84**, optimization was continued to acquire more potent compounds to advance to clinic and to recognize the full utility of SYK as a target.[188,196]

The second generation of analogues, related to compound **84**, is more potent and orally bioavailable. In the first human study with compound **3** designed to assess biomarker and pharmacodynamic (PD) effects linked to basophil degranulation induced upon ex vivo stimulation with IgE, CD63 cell-surface up-regulation was inhibited with EC_{50} = 500 ng/mL.[78] Phase 1 studies with compound **3** employed a suspension formulation, but a solid dosage form was more desirable for longer-term studies. Thus, compound **3** was converted to methylene phosphate prodrug **11** (Figure 7) to allow the delivery of the material in solid dosage form.[12−14,16,194,195]

Inhibition of SYK has gained greatest clinical prominence for the treatment of active RA, with the completion of three phase 2 trials with compound **11**.[197−199] Compound **11** has been examined in patients with active RA who have had incomplete clinical response to methotrexate (MTX) or disease modifiers. The primary efficacy end point in all three studies was American College of Rheumatology 20% improvement criteria (ACR20) response rates after 3 and 6 months of treatment.[197−199] ACR20 represents ≥20% improvement in swollen and tender joint counts and ≥20% improvements in three of the remaining five core measures (pain, C-reactive protein [CRP] level, modified health assessment questionnaire score, and patient and physician global assessment at week 12).[197] In addition, secondary end points are improvements in ACR50, ACR70, and disease activity score in 28 joints (DAS28). In the initial double-blinded randomized phase 2 dose-ranging study (TASKi1), patients with active RA who were unresponsive to MTX were randomized to receive 50, 100, or 150 mg b.i.d. of compound **11** or placebo for 3 months. Significant efficacy was observed in both 100 and 150 mg patient groups as determined by ACR20, ACR50, and ACR70 response rates (Table 9).[197]

Table 9. Phase 2 Data for Compound 11 in Three RA Clinical Trials

TASKi1, Response Rate at Week 12[a]				
treatment	ACR20	ACR50	ACR70	DAS28 < 2.6
placebo	38	19	4	8
11, 50 mg, b.i.d.	32	17	2	16
11, 100 mg, b.i.d.	65[b]	49[d]	33[c]	26[e]
11, 150 mg, b.i.d.	72[c]	57[c]	40[c]	49[c]
TASKi2, Response Rate at Month 6[a]				
treatment	ACR20	ACR50	ACR70	DAS28 < 2.6
placebo	35	19	10	7
11, 100 mg, b.i.d.	67[c]	43[c]	28[c]	31[c]
11, 150 mg, q.d.	57[c]	32[f]	14[g]	21[h]
TASKi3, Response Rate at Month 3[a]				
treatment	ACR20	ACR50	ACR70	DAS28 < 2.6
placebo	37	12	5	10
11, 100 mg, b.i.d.	38[i]	22[j]	9[k]	12

[a]Values are the number percent of patients who met the criteria. ACR20 = American College of Rheumatology 20% improvement criteria. DAS28 = disease activity score in 28 joints. b.i.d. = twice-daily dosing. q.d. = once-daily dosing. [b]p = 0.008. [c]p < 0.001. [d]p = 0.002. [e]p = 0.036. [f]p = 0.007. [g]p = 0.34. [h]p = 0.003. [i]p = 0.84. [j]p = 0.09. [k]p = 0.37.

The 50 mg treatment group showed a response similar to that of the placebo arm. Thus, the preferred dose for future studies was determined to be between 100 and 150 mg. Statistically significant improvements in clinical signs were also noticeable after just 1 week of treatment. This was verified with biomarker measurements, with reduction in serum IL-6 and MMP3 at doses of 100 and 150 mg in 1 week, and these were sustained through the duration of the study.[197] Adverse events from this study were manageable, with gastrointestinal effects and transient neutropenia (<1500/mm^3) occurring in a dose-dependent manner.[197] Hypertension was also observed in some patients.[197]

In a second double-blinded randomized phase 2 study (TASKi2), patients with active RA, despite treatment with MTX, were randomized to receive 150 mg q.d. or 100 mg b.i.d. of compound **11** or placebo for 6 months (Table 9).[198] A clinical response, as measured by ACR20, was observed in both active groups within 1 week following commencement of treatment. In this study, clinically significant efficacy compared to placebo was seen at 6 months in primary end point of ACR20 response rates, along with secondary assessments of ACR50,

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ACR70, and remission, as evaluated by DAS28. A greater response rate was seen in patients dosed with compound **11** at 100 mg b.i.d. in comparison to 150 mg q.d. The adverse events registered during this study were similar to what had been observed during the first phase 2 study.[197,198] In the second phase 2 study, of the 15% of patients recruited who had previously not responded to treatment with biologics, 43% of this group showed significant response in the 100 mg of compound **11** b.i.d., in contrast to 14% in the placebo arm.[198]

A parallel, a double-blinded randomized phase 2 (TASKi3) study in patients with active RA who had failed treatment with a biologic agent (including anakinra, abatacept, and/or rituximab) was conducted.[199] Patients were randomized to receive 100 mg b.i.d. of compound **11** or placebo for 3 months.[199] The primary end point (ACR20 response rate at month 3) was not met (Table 9), and this may in part be explained by baseline differences in the compound **11** and placebo treatment groups in terms of steroid use, prior biologic use, and synovitis score on magnetic resonance imagery (MRI).[199] A high ACR20 response rate was also observed in the placebo group (37%), which may have affected the ability to achieve the study's primary end point (Table 9).[199] However, secondary end point improvements in CRP and ESR were observed, along with a reduction in inflammation of the synovium membrane (synovitis) and bone (osteitis) as determined by RA MRI.[199] Improvements in these end points are of potential benefit, given that these measurements suggest remission within a very short treatment period.

The B-cell receptor has been an important drug target for autoimmune and inflammatory diseases.[6,10,11,57,70] Overactivation of BCR-mediated survival signals can lead to the development of B-cell lymphoma, and SYK is an upstream protein tyrosine kinase involved in this pathway.[161−163] The SYK inhibitor compound **3** has been evaluated in in vitro diffuse large B-cell lymphoma (DLBCL) cell lines, which demonstrate both tonic- and ligand-induced BCR signaling, as shown by basal phosphorylation of SYK348/352 and the SYK-dependent linker protein (BLNK).[41,162,163] Compound **3** causes apoptosis in DLBCL cell lines in concentrations between 1 and 4 μM.[162,163] Thus, SYK inhibitor **3**, as its prodrug **11**, was evaluated in patients with B-cell NHL and chronic lymphocytic leukemia (CLL).[163] The phase 1/2 clinical study included patient cohorts afflicted with B-NHL, DLBCL, follicular lymphoma (FL), and other NHL, including mantle cell lymphoma (MCL), marginal zone lymphoma, mucosa-associated lymphoid tissue lymphoma, lymphoplasmacytic lymphoma, and small lymphocytic leukemia/chronic lymphocyte leukemia (SLL/CLL).[41,163] In phase I of the clinical study, 13 patients with refractory DLBCL, FL, MCL, CLL/SLL were treated at doses of 200 mg or 250 mg b.i.d.[163] Objective response rates were 22% (5 of 23) for DLBCL, 10% (2 of 21) for FL, 55% (6 of 11) for SLL/CLL, and 11% (1 of 9) for MCL.[41,163] The 200 mg b.i.d. dosage was deemed the optimal dose and employed in the phase 2 portion of the study.[163] Adverse events from this phase 1/2 study were in line with observations from several RA trials and were consistent with the patient population of highly refractory/relapsed patients with cancer.[163,197−199]

Compound **11** has also been evaluated in an open-label dose-escalation phase 2 study in patients with chronic ITP who were refractory to conventional therapy, including corticosteroids, intravenous γ-globulin, monoclonal anti-CD20 antibodies, and thrombopoeitin agonists.[121,122,137,200] In summary, 8 of 16 patients attained continued responses with platelet counts exceeding 50 000 mm^3 on more than 67% of follow-up visits.[137,200] A total of three patients who showed a sustained increase in platelet counts had been refractory to thrombopoietic agonists. Adverse events were similar to what has been observed in other clinical trials with compound **11**.[137,163,197−199]

On the basis of the results of the RA early phase clinical studies, compound **3** and its prodrug **11** have demonstrated preliminary signs of improved outcomes in a dose-dependent manner, with manageable side effects. Compound **11** is currently in phase 3 RA studies to validate further the premise that autoimmune and inflammatory diseases are amenable to treatment through SYK inhibition.

■ CONCLUSIONS AND FUTURE DIRECTIONS

The validation of SYK as an effective target for therapeutic intervention in autoimmune and inflammatory disorders has picked up pace over the past decade. In particular, this has been attributable to the recognition of key biologic findings, coupled with successful small-molecule inhibitors of SYK that have shown early indications of clinical efficacy in diverse autoimmune and inflammatory diseases. Thus, compounds **3**, **11**, **84**, and **85** have been examined in the clinic for RA, ITP, B-cell lymphoma, allergic rhinitis, and asthma, with compound **11** currently in phase 3 studies for RA. Kinase programs initially had broad appeal as attractive targets for treatment of oncologic diseases. However, this has matured further with applications encompassing chronic autoimmune and inflammatory diseases. Selectivity has been cited as a major concern in developing drugs targeting ubiquitously expressed kinases. The debate about the specificity of kinase inhibitors has been a major point of discussion and has still not reached a suitable resolution since the first kinase inhibitors entered into medicinal chemistry optimization.[207,208] The question that needs to be asked is how specificity is determined and in what biologic context. In the circumstance of complex diseases like RA and other autoimmune diseases discussed in this review, with multiple pathways operating in concert as the disease progresses, specificity may be a relative attribute. A suitable treatment may need to target multiple biologic pathways, initiated by an upstream enzyme receptor complex. SYK is such a target, and because of the complexity of its intracellular function, diverse appropriate functional assays must be employed in parallel with biochemical assays in inhibitor optimization.

Currently, broadly exploited kinase biochemical assays use isolated catalytic domains in combination with peptide substrates during in vitro primary screening in drug discovery efforts. These formats suffer from the inability to address intracellular intricacies and inter- and intramolecular interactions seen in complex signaling cascades. Only by using cell-based assays in primary cells with disease-specific stimuli can the biochemical complexity of these systems be simulated. The latter can account for the dynamic regulation of kinase activity, influence of local environment such as localization, pH, ionic strength, protein−protein interactions, posttranslational modifications, various conformational states, and enzyme isoforms. When inhibitors for enzymes such as SYK kinase are discovered and optimized, biochemical assays may not account for the latent inactivated state present in the cellular environment and prerequisite activation required by ITAM binding, as well as auto- and transphosphorylation steps leading to intensified enzymatic activity. Dissection of these intricate steps for activation is best examined through functional cell-based assays supplemented

with measurement of the activation of kinases using antibodies against functionally unique phosphorylated sites of specific substrates through phosphoblot analysis. More stringent methodology applies cell-based functional assays to assess SYK functional phenotype output and employs complementary biochemical assays to get more refinement for the application of structure-based drug design techniques during SAR. Compounds identified through biochemical screens necessitate optimization for translation of potency to the intracellular environment, improvement in poor membrane penetrability, and/or low metabolic stability.

The application of functional assays is becoming more widely recognized, with the ability to measure complex interactions in signal transduction pathways and importantly improved assay reliability.[209] SYK inhibition has relied on the application of functional assays since its initial discovery, and only now is this approach being validated from discovery to development.[209] The discovery of SYK inhibitors (compounds **2**, **3**, **28**, **83**, **84**, and **85**) has been inherently possible because of the reliance on a functional phenotype based screening paradigm.

Recently, SYK has been described as a "switch" kinase as opposed to Src family kinases, which are akin to "rheostat" kinases.[66,67,86] Translation of this to drug discovery and development relays the point that ameliorating SYK activity will have a defined outcome. This principle has gained reinforcement with the successful completion of several phase 2 clinical studies in RA, ITP, and B-cell lymphoma. Additional translational studies, using PD biomarkers that interrogate compound activity at SYK and kinase targets for which cellular activity has been reported, are part of clinical studies underway that may further emphasize the position of SYK as a therapeutically relevant target.

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ABBREVIATIONS USED

5-HT, 5-hydroxytryptamine; ACR20, American College of Rheumatology 20% improvement criteria; AHR, airway hyperresponsiveness; AIHA, autoimmune hemolytic anemia; ATP, adenosine 5′-triphosphate; BAL, bronchoalveolar lavage; BCR, B-cell antigen receptor; BMM, bone marrow macrophage; BUN, blood urea nitrogen level; CAIA, collagen-antibody induced arthritis; CARD, caspase-recruitment domain; Cbl, Casitas B-lineage lymphoma; CHMC, cultured human mast cells; CIA, collagen-induced arthritis; CLR, C-type lectin receptors; CRP, C-reactive protein; CT, computed tomography; DAMP, damage-associated molecular pattern; DAP12, DNAX-activated protein; DAS28, disease activity score in 28 joints; DC, dendritic cell; DNA, deoxyribonucleic acid; DNP-BSA, dinitrophenylated bovine serum albumin; EM, electron microscopy; FcR, Fc receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSC, global symptom complex; HIT, heparin-induced thrombocytopenia; IC, immune complex; IL, interleukin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITP, idiopathic thrombocytopenia purpura; JNK, c-Jun N-terminal kinase; kDa, kilodalton; LAT, linker for activation of T-cells; LT, leukotriene; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MHC, major histocompatibility; MMP3, matrix metalloproteinase 3; MTX, methotrexate; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor κ-light-chain-enhancer of activated B-cells; NHL, non-Hodgkin lymphoma; NK, natural killer; NOD, nonobese diabetic; NTN, nephrotoxic nephritis; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PCA, passive cutaneous anaphylaxis; PKC, protein kinase C; PRP, platelet-rich plasma; PSA, polar surface area; PTK, protein tyrosine kinase; RA, rheumatoid arthritis; RANK, receptor activator nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species; SAR, structure−activity relationship; SH2, Src homology-2 domains; SKG, Sakaguchi; SLE, systemic lupus erythematosus; SLP, SH2 domain containing leukocyte protein; SYK, spleen tyrosine kinase; T1D, type 1 diabetes; TCR, T cell

receptor; T_H2, T helper 2; TLR-4, toll-like receptor 4; TNF, tumor necrosis factor; WB, whole blood; ZAP-70, ζ-chain-associated protein kinase 70 of 70 kDa

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