Identification of the Syk kinase inhibitor R112 by a human mast cell screen

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Background: Activation of the IgE receptor, FcεRI, in mast cells is the key mechanism initiating and propagating pathophysiological responses in allergic rhinitis.
Objective: Identify and characterize a small molecule inhibitor of IgE-dependent mast cell activation for the treatment of allergic diseases.
Methods: A cell-based high-throughput screen for small molecules that block IgE signaling was performed in cultured human mast cells. A potent inhibitor, referred to as R112, was selected and characterized by using biochemical and cell-based assays. R112 effects on IgE-dependent degranulation and cytokine production was measured in mast cells and basophils and compared with other mast cell inhibitors.
Results: R112 inhibited degranulation induced by anti-IgE cross-linking in mast cells (tryptase release, effective concentration for 50% inhibition [EC_{50}] = 353 nmol/L) or basophils (histamine release, EC_{50} = 280 nmol/L), and by allergen (dust mite) in basophils (histamine release, EC_{50} = 490 nmol/L). R112 also blocked leukotriene C4 production and all proinflammatory cytokines tested. Subsequent molecular characterization indicated that R112 is an ATP-competitive spleen tyrosine kinase (Syk) inhibitor (inhibitory constant [K_i] = 96 nmol/L). Its onset of action was immediate, and the inhibition was reversible. Incubation of mast cells with R112 showed that cytokine production in mast cells was dependent on sustained activation of the FcεRI-Lyn–spleen tyrosine kinase pathway. Unlike other mast cell inhibitors, R112 was able to completely inhibit all three IgE-induced mast cell functions: degranulation, lipid mediator production, and cytokine production.
Conclusion: R112 potently, completely, and rapidly abrogated all mast cell activation cascades triggered by IgE receptor cross-linking.
Clinical implications: R112 and its analogues offer a new modality in the treatment of allergic rhinitis. (J Allergy Clin Immunol 2006;118:749-55.)

Key words: *Syk, Fc receptors, IgE, histamine, tryptase, cytokines, mast cells, allergic rhinitis, small molecule inhibitor, steroids*

Abbreviations used
CHMC: Cultured human mast cells
DMSO: Dimethyl sulfoxide
EC_{50}: Effective concentration for 50% inhibition
ERK: Extracellular signal-regulated kinase
FcRγ: Fc receptor γ
ITAM: Immunoreceptor tyrosine-based activation motif
LAT: Linker for activator of T cells
LRF: Leukocyte rich fraction
LT: Leukotriene
MT: Modified Tyrode's
Syk: Spleen tyrosine kinase

IgE plays a central role in atopic diseases such as allergic rhinitis.[1] Allergens cross-link and activate IgE-loaded FcεRI receptors expressed on the cell surface of mast cells and basophils, resulting in a rapid release of preformed granule contents that include neutral proteases (tryptases, chymases), proteoglycans (heparin, chondroitin sulfate), and biogenic amines (histamine). This degranulation is followed by synthesis and secretion of arachidonic acid end-products such as leukotriene (LT) B4, LTC4, and prostaglandin 2, and later by the synthesis and secretion of chemokines and cytokines including TNF-α, IL-4, IL-5, and IL-8.[2-5] Together, the action of these and other mediators and cellular factors result in early symptom expression and activation of the inflammatory cascade.[3,4,6]

Current therapeutic strategies for allergic rhinitis like antihistamines and anticysteinyl leukotrienes antagonize single mediators and provide effective but partial symptom relief, whereas the more effective corticosteroid agents inhibit multiple proinflammatory pathways.[6] Given that FcεRI-dependent activation of human mast cells results in substantial change in expression of more than 2400 genes, including several incompletely characterized genes with immunoregulatory potential,[7] preventing mast cell and basophil activation by antagonizing IgE-FcεRI signaling offers an attractive broad therapeutic approach.[1,8]

Here we describe the identification and characterization of R112, a novel small molecule inhibitor of the IgE-FcεRI signaling pathway. We show that R112 is a potent, fast, and reversible inhibitor of spleen tyrosine kinase (Syk) kinase, and describe the effects of Syk inhibition on production and release of multiple mediators and bioactive factors. These data complement the recent report

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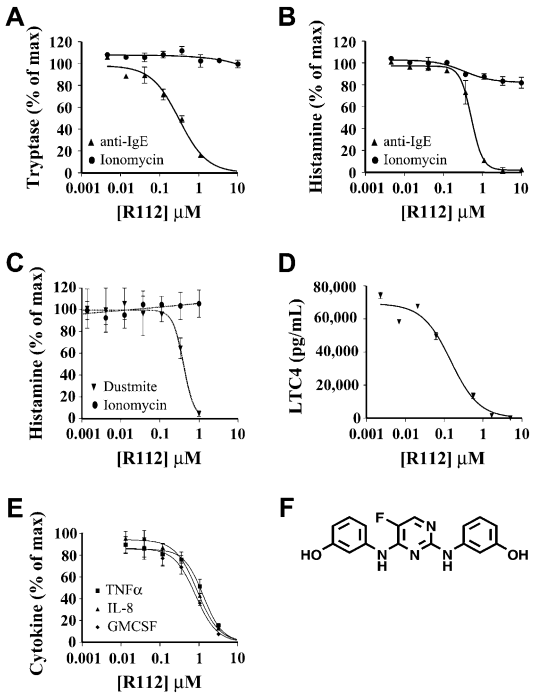

FIG 1. Representative EC_{50} curves showing the activity of R112 in CHMC and primary human basophils stimulated with anti-IgE, dust mite, or ionomycin. **A,** R112 inhibition of tryptase release by stimulated CHMC. The graph represents an average of more than 30 assays. **B** and **C,** R112 inhibition of histamine release by primary human basophils stimulated with anti-IgE or dust mite. **D,** R112 inhibition of LTC4 release by CHMC. **E,** R112 inhibition of cytokine production by CHMC. **F,** Chemical structure of R112.

indicating that R112, dosed intranasally, significantly improved all nasal symptoms in volunteers with seasonal allergic rhinitis in a phase II clinical study in a park environment.[9]

METHODS

R112

The small molecule R112 was synthesized by the Department of Chemistry of Rigel Pharmaceuticals, Inc. The structure of R112 is shown in Fig 1. The discovery and structure-activity relationship leading to R112 will be presented elsewhere (R. Singh, Manuscript in preparation, May 2006).

Human mast cell culture

On the basis of the method by Saito et al,[10] human mast cells were derived from cord blood $CD34^+$ progenitor cells as described in this article's supplementary text in the Online Repository at www.jacionline.org.

Human mast cell sensitization and stimulation

Cultured human mast cells (CHMCs) were primed for 5 days with 50 to 100 ng/mL IgE κ myeloma (Cortex Biochem, San Leandro, Calif) and 50 to 100 ng/mL IL-4 (Peprotech Inc, Rocky Hill, NJ) in mast cell (MC) culture medium (200 ng/mL human interleukin-6 (IL-6) [Peprotech Inc] and 200 ng/mL stem cell factor (SCF) [Peprotech Inc] in StemPro-34 SFM culture medium [Invitrogen, Carlsbad, Calif]) at a cell density of 350,000 cells/mL in a humidified atmosphere of 5% CO_2 in air at 37°C. Before stimulation, cells were washed in either Modified Tyrode's (MT) buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L $CaCL_2$, 1.0 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 20 mmol/L HEPES, 0.1% BSA; pH 7.4) or MC culture medium and stimulated with either 0.25 to 2 μg/mL anti-IgE (Bethyl Laboratories, Montgomery, Tex), or 2 μmol/L ionomycin (VWR International, San Francisco, Calif). For tryptase measurement, ~1500 cells per well were stimulated for 30 minutes in MT buffer. For leukotriene C4 (LTC4) and cytokine production, 100,000 cells per well were stimulated for 1 hour in MT buffer or for 7 hours in MC culture medium.

Tryptase, LTC4, and cytokine assays

The tryptase released in the supernatants of stimulated mast cells was quantified by cleavage of 10 to 50 mmol/L of synthetic fluorescent peptide substrate Z-Ala-Lys-Arg-AMC.2TFA (Enzyme Systems Products, Livermore, Calif) in tryptase buffer (0.1 mol/L HEPES, 10 wt/vol% glycerol, 10 mmol/L heparin sodium salt, 0.01% NaN_3; pH 7.5) for 30 minutes at room temperature. The luminescent readout was measured at 355 nm emission 460 nm excitation wavelength. LTC4 (Cayman Chemical, Ann Arbor, Mich) and cytokines (Upstate Biotechnology/Chemicon, Lake Placid, NY) were measured by using Luminex (Luminex Corp., Austin, Tex) multiplex technology according to the manufacturer's instructions. The amount of mediators released was determined with appropriate standard curves.

Primary basophil assay

Basophil activity was measured in leukocyte rich fraction (LRF) from dextran sedimented heparinized whole blood (GE Healthcare, Chicago, Ill). LRF was washed with PBS and resuspended in MT buffer containing 0.5% dimethylsulfoxide (DMSO) to its original volume. LRF was mixed with R112 or budesonide to a volume of 100 μL and incubated for 1 or 20 hours followed by a 30-minute stimulation with 0.005 to 5 μg/mL anti-IgE (Bethyl Laboratories). Histamine release was measured by ELISA according to the manufacturer's description (Beckman Coulter, Brea, Calif).

Western blot

Cultured human mast cells were sensitized for 5 days as described. Cells were then preincubated at 1×10^6 cells/mL with DMSO vehicle or R112 at different concentrations for 40 minutes, and stimulated with 2 μg/mL antihuman IgE for 5 minutes. The cells were spun down, washed in PBS, and resuspended in Tris-Glycine SDS Sample Buffer (Invitrogen, Carlsbad, Calif). Western blots were performed according to standard protocol by using 8% Tris-Glycine gels (Invitrogen), Immobilon P membrane (Millipore, Chicago, Ill), and ECL Western Blot detection reagent (GE Healthcare). Primary antibodies were purchased from Cell Signaling Technologies (Beverly, Mass). Membranes were reprobed with antibodies recognizing various other proteins to verify equal amount of protein in each lane (data not shown).

In vitro fluorescence polarization kinase assay and K_i determination

The fluorescence polarization reactions were performed as described elsewhere (see the Online Repository at www.jacionline.org). For K_i determination, duplicate 200 μL reactions were set up at 8 different ATP concentrations from 200 μmol/L (2-fold serial dilutions) in the presence of either DMSO or R112 at 5 μmol/L, 2.5 μmol/L, 1.25 μmol/L, 0.625 μmol/L, or 0.3125 μmol/L. At different time

points, 20 μL of each reaction was removed and quenched to stop the reaction.

For each concentration of R112, the rate of reaction at each concentration of ATP was determined and plotted against the ATP concentration to determine the apparent Michaelis constant (K_m) and V_{max} (maximal rate). Finally, the apparent K_m (or apparent K_m/V_{max}) was plotted against the inhibitor concentration to determine the K_i. All data analysis was performed by using Prism and Prism enzyme kinetics programs (GraphPad Software, Inc, San Diego, Calif).

Data analysis

All inhibitory and effective concentrations for 50% inhibition (IC_{50} and EC_{50}) values in biochemical and cell based assays were determined by using GraphPad Prism 3.0 software and Prism Enzyme programs (GraphPad Software, Inc). Results are presented as the averages ± SDs.

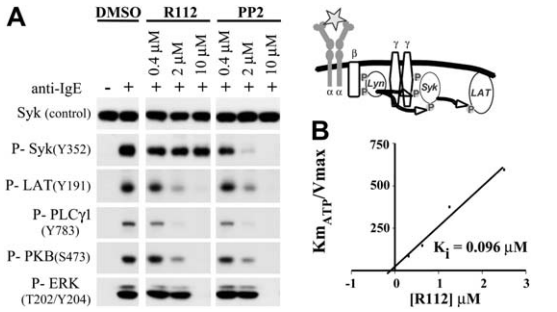

FIG 2. R112 inhibits Syk kinase activity. **A,** Western blots using phospho-specific antibodies on anti-IgE stimulated CHMC. P-Syk(Y352) phosphorylation indicates Lyn activity and P-LAT(Y191) phosphorylation indicates Syk activity. In the model, α, β, and γ designate the IgE receptor chains. **B,** K_i determination of R112 on recombinant Syk protein.

RESULTS

R112 inhibits IgE-mediated mast cell activation pathways

To identify mast cell activation inhibitors, we first developed a method to obtain as many as 1×10^9 primary human mast cells in culture from cord blood $CD34^+$ progenitor cells (see this article's Methods and Fig E1 in the Online Repository at www.jacionline.org for CHMC characterization). A high-throughput screen was then performed to identify small molecule compounds that inhibited anti-IgE–mediated tryptase release from CHMC. In parallel, a counterscreen using ionomycin-induced degranulation was performed to select for molecules that act upstream of calcium mobilization in the IgE pathway.[11,12]

Medicinal chemistry of initial hits from the screen resulted in the identification of R112 as a potent mast cell inhibitor. R112 caused a dose-dependent inhibition of anti-IgE–mediated tryptase release (EC_{50} = 0.353 ± 0.117 μmol/L) but showed no activity on ionomycin-triggered tryptase release (Fig 1, *A*). Likewise, R112 inhibited anti-IgE–mediated histamine release by basophils isolated from human blood (EC_{50} = 0.28 μmol/L, Fig 1, *B*), and importantly, histamine release by basophils stimulated with dust mite, a more physiological stimulus of IgE-FcεRI signaling (EC_{50} = 0.49 μmol/L; Fig 1, *C*).

R112 also inhibited in CHMC the secretion of LTC4 (EC_{50} = 0.115 ± 0.021 μmol/L; Fig 1, *D*) and of several proinflammatory cytokines including TNF-α (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; Fig 1, *E*).

Thus, consistent with Syk playing an essential role in the three major inflammatory cascades regulated by IgE receptor signaling, R112 completely inhibits degranulation, production of arachidonic acid metabolites, and production of cytokine and chemokines. Interestingly, as shown by the EC_{50} values, the R112 concentrations needed to inhibit 50% of the production and/or release of the different mediators do not always correlate with inhibiting 50% of Syk kinase activity. Differences in EC_{50} values indicate that the readouts, although completely dependent on Syk, measure endpoints of downstream signaling cascades with different complexities, which integrate multiple components such as signal amplifications, regulatory feedback loops, and accumulation of end products. Other Syk inhibitors, including R112 analogues, show the same tendencies in EC_{50} shifts.[13]

Mechanism of action of R112

Because R112 did not inhibit ionomycin-induced degranulation, we systematically tested R112 effects on signaling events upstream of calcium mobilization. Current understanding[12,14-16] indicates that on receptor cross-linking, Lyn phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the Fc receptor γ (FcRγ) subunit of the FcεRI receptor complex. Syk kinase then binds the diphosphorylated ITAM on FcRγ and becomes activated by phosphorylation. Syk subsequently phosphorylates a variety of substrates, including scaffolding proteins Vav, linker for activator of T cells (LAT), and SLP-76, that orchestrate a multitude of divergent downstream signaling events. The formation of signaling complexes results in the activation of phospholipase Cγ (PLCγ), which mediates calcium mobilization and protein kinase C (PKC) activation, resulting in degranulation and cytokine gene transcription. The production of lipid mediators and cytokines is in addition regulated by the activated mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38.

To identify the mechanism of R112 action, we examined the sequential activation of Lyn and Syk kinases. Phosphorylation of Syk on tyrosine 352 (Y352) is dependent on Src-family kinases, most likely Lyn, the first kinase to be activated.[17,18] Activated Syk then directly phosphorylates the adaptor molecule LAT on tyrosine 191 (Y191).[19] As shown in Fig 2, *A*, the control compound PP2, a general Src-family kinase inhibitor,[20] potently inhibited phosphorylation of Syk (Y352) and all subsequent phosphorylation events in the signaling cascade. R112, however, did not inhibit phosphorylation of the Lyn target Syk (Y352), but inhibited the phosphorylation of the Syk

TABLE I. R112 effects in different cell-based assays

Cell type	Stimulation	Receptor type	Readout	EC_{50} (μmol/L) ± SD
Mast cell, CHMC	Anti-IgE	ITAM (FcεRI)	Degranulation (tryptase)	0.353 ± 0.117
Primary human T cell	anti-CD3/anti-CD28	ITAM (TCR/CD28)	(IL-2) production	3.4 ± 0.4
Jurkat T cell line	C305 (α-TCR)	ITAM (TCR)	CD69 upregulation	3.3 ± 0.9
Jurkat T cell line	Phorbol 12-myristate 13-acetate	Enzyme (PKC)	CD69 upregulation	32.5
Primary human T cell	IL-2	Cytokine (IL-2R)	Cell proliferation	2.3 ± 0.13
A549 epithelial line	IFN-γ	Cytokine (IFNR)	CD54 upregulation	30
A549 epithelial line	IL-1	Cytokine (IL-1R)	CD54 upregulation	>30
A549 epithelial line	TNF	Cytokine (TNFR)	CD54 upregulation	>30
A549 epithelial line	Serum	General cell growth	Cell proliferation	>10
H1299 lung cancer line	Serum	General cell growth	Cell proliferation	>10
Mast cell, CHMC	Ionomycin	Intracellular protein	Degranulation (tryptase)	>10

TCR, T-cell receptor; *PMA*, phorbol 12-myristate 13-acetate; *IL-1R*, interleukin-1 receptor.

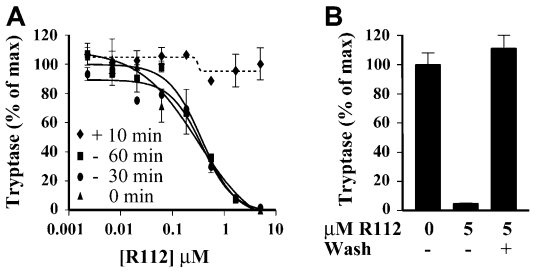

FIG 3. R112 inhibition is rapid and reversible. **A,** R112 was added to CHMC for 60 or 30 minutes before, simultaneously with, or 10 minutes after the stimulation with anti-IgE. **B,** CHMCs were preincubated for 30 minutes with DMSO or 5 μmol/L R112. One aliquot of R112-treated cells was washed 5 times with MT buffer before all aliquots were stimulated with anti-IgE.

target LAT (Y191), indicating that Syk kinase is the primary target of R112.

As expected, most phosphorylation events downstream of Syk[12,14] were inhibited with similar potency by R112 (Fig 2, *A*), including those of p38 and JNK (data not shown). The weaker inhibition of ERK phosphorylation, which is the most downstream phosphorylation tested, correlates with the less potent inhibition of cytokine production and may be a result of the integration of the signal transduction leading to their activation. In accordance with this notion, PP2 shows the same tendency with respect to inhibition of ERK phosphorylation. The inhibition of the Syk signaling cascade was equally potent in murine bone marrow–derived mast cells and Ramos B-cells stimulated through the B-cell receptor (data not shown).

To confirm that Syk was the cellular target of R112, *in vitro* kinase assays were performed by using purified Syk protein. R112 inhibited Syk kinase activity with an IC_{50} of 226 nmol/L. Moreover, R112 was shown to be an ATP-competitive inhibitor of Syk kinase with a K_i of 96 nmol/L (Fig 2, *B*). Also consistent with Syk being R112's primary target, screening of more than a thousand R112 analogues demonstrated a very close positive correlation between inhibition of IgE-mediated mast cell degranulation and inhibition of *in vitro* Syk kinase activity (data not shown).

Selectivity of R112

We performed *in vitro* kinase assays against a panel of 25 kinases, in which Syk and Src family kinases were inhibited most potently. However, we have found that *in vitro* kinase assays do not always correlate with the corresponding activity inside cells. For example, R112 inhibited the Lyn kinase *in vitro* assay with an IC_{50} of 0.3 μmol/L; however, as mentioned previously, in CHMCs, R112 did not inhibit the phosphorylation of Syk (Y352), and therefore most likely did not inhibit Lyn activity (Fig 2, *A*). Additional discrepancies were obtained with lymphocyte-specific tyrosine kinase (Lck) and epidermal growth factor receptor (EGFR) (see this article's Table E1 in the Online Repository at www.jaci-online.org). Rather than establishing clear correlations between *in vitro* and in-cell kinase activity for multiple kinases, we used the *in vitro* kinase data to help us select cell-based assays in which different and multiple kinases were involved (Table I). The results indicate that T-cell activation, which is dependent on the kinase activity of Lck and zeta-chain–associated protein kinase (Zap70) (the closest homologue of Syk),[17] was inhibited at roughly 10-fold higher concentrations of R112. Furthermore, almost no inhibitory activity by R112 was observed on IFN-γ, IL-1β, and TNF-α signaling as assessed by intercellular adhesion molecule 1 (CD54) upregulation in the human epithelial cell line A549, nor did R112 show any effect on cell proliferation in A549 or the H1299 lung cancer cell line. Moreover, 10 μmol/L of R112 did not inhibit epidermal growth factor (EGF)-induced activation of the mitogen-activated protein kinase pathway (ERK phosphorylation) in normal human dermal fibroblasts), SCF-induced ERK phosphorylation in bone marrow-derived mast cells (BMMC), or peptidoglycan-induced IL-8 production in CHMCs (data not shown). Taken together, the cellular, biochemical, and phosphorylation pathway analyses suggest that R112 is a biologically selective inhibitor of Syk kinase activity.

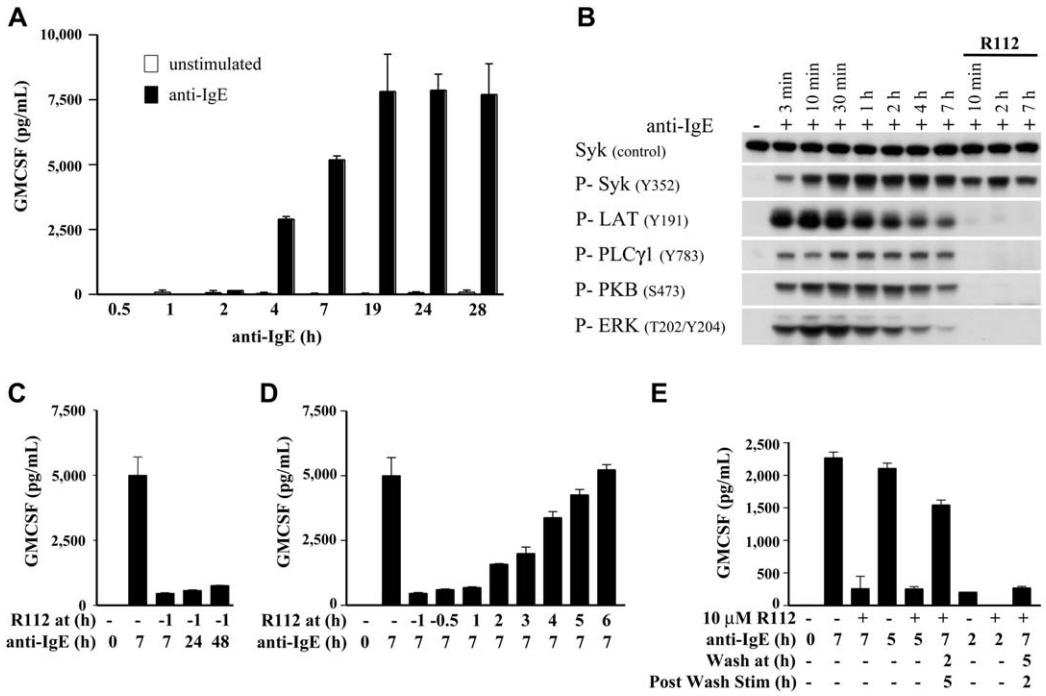

FIG 4. Sustained Syk activity is required in anti-IgE induced cytokine production in CHMC. **A,** Cytokine production kinetics by anti-IgE induced CHMC. All cytokines measured behave similarly; GM-CSF is shown as a representative. **B,** The FcεR pathway stays activated for at least 7 hours. **C,** R112 inhibition of cytokine production is long-lasting. **D,** Cytokine production is halted as soon as R112 is added. **E,** Inhibition of cytokine production by R112 is reversible.

Inhibition by R112 is fast and reversible

We tested the onset of action of R112 in inhibiting CHMC activation. Addition of R112 sixty or thirty minutes before or concomitant with anti-IgE antibody induction inhibited tryptase release at equal potencies (Fig 3, *A*). Thus, the results indicate that R112 is highly permeable and rapidly contacts the target site to inhibit FcεR signaling. As expected, addition of R112 ten minutes after stimulation did not inhibit tryptase release, consistent with the fact that the majority of degranulation happens within 10 to 30 minutes after stimulation.[21,22]

Consistent with R112 being a competitive inhibitor, its activity in cells was rapidly reversible. When CHMCs were preincubated with R112 at 5 μmol/L, a complete inhibition was observed. This inhibition was completely abrogated after the cells were washed 5 times before stimulation (Fig 3, *B*). Taken together, these results show that R112 is a rapid and reversible inhibitor of FcεR-mediated mast cell degranulation.

R112 inhibition of induced and ongoing cytokine production

Unlike mediators like tryptase or histamine, which are preformed and released by degranulation, most mast cell–derived cytokines are newly transcribed and translated on stimulation, and thus accumulate over a period of several hours.[4,5,23] To investigate Syk inhibition of cytokine production, we tested R112 effects on GM-CSF production by CHMCs by using concentrations of R112 of 3 to 10 μmol/L to ensure complete inhibition of Syk. Results with the production of other cytokines were comparable. On the basis of time courses of cytokine release (Fig 4, *A*), we chose to measure the effect of R112 inhibition at 7 hours after stimulation, a time point when all of the cytokines had accumulated to a measurable level but production was still actively ongoing. On the molecular level, the Syk pathway remained activated for at least 7 hours after stimulation, as seen by the phosphorylation of the signaling proteins (Fig 4, *B*). The kinetics of phosphorylation is unusually sustained and may relate to the method by which the cells were derived. R112 completely inhibited this sustained phosphorylation of all signaling components downstream of Syk.

To evaluate R112 duration of action, CHMC stimulation time was extended. R112 inhibition of cytokine production was sustained over a period of at least 48 hours (Fig 4, *C*). Next, we examined R112 effects on ongoing mast cell activation. Interestingly, cytokine production could be substantially inhibited even when R112 was added 1 hour after stimulation (Fig 4, *D*). When R112 was added 2 to 6 hours after stimulation, the cytokine that could be measured corresponded to the amount of cytokine that had already been produced before addition of R112 (Fig 4, *D*; compare with time course in Fig 4, *A*). Similarly, when R112 was washed out 2 hours or 5 hours after stimulation, and cytokine was measured 5 hours or 2 hours later, respectively; the cytokine measured correlated

to the amount produced in a comparable time without inhibition (Fig 4, *E*). Together, this indicates that cytokine production is stopped when the Syk inhibitor is added and, conversely, that cytokine production resumes once the Syk inhibitor is removed with the stimulus still present. Thus, sustained activation of the Syk pathway is required to sustain cytokine production. The results also indicate that R112 can block allergen-induced cytokine production even in cells that have already degranulated.

DISCUSSION

Allergic conditions such as allergic rhinitis are largely the manifestation of the effects of allergen-dependent activation of IgE-loaded FcεRI receptors on mast cells and basophils.[3,4,6] The Syk kinase plays an essential role in this IgE-dependent activation. Blocking Syk activity would therefore inhibit the production and release of multiple mediators and likely impart broad beneficial effects because each of these mediators play distinct but overlapping roles in the inflammatory response.[8]

Here, we characterized the small molecule R112 as a biologically selective and potent ATP-competitive inhibitor of Syk kinase activity with a K_i of 96 nmol/L (Fig 2). Consistent with Syk's central role in IgE-dependent mast cell activation, R112 blocked the release and production of the three main types of mast cell bioactive mediators: preformed mediators such as tryptase and histamine, lipid metabolites such as leukotrienes, and proinflammatory factors including chemokines and cytokines (Fig 1). R112 has recently been studied in volunteers with seasonal allergic rhinitis in a phase II clinical trial, showing a significant reduction in clinical symptoms (23% relative to placebo; $P = .0005$).[9] One notable outcome was the broad range of individual symptoms significantly improved by R112. Among others, these included stuffy, itchy, and runny nose; sneezes; cough; and headache.[9] It is likely that R112's wide-ranging effects are related to its comprehensive block of mast cell and basophil mediator release and production.

In addition, R112 effects might also be mediated via other cell types regulated by Syk activity.[8] FcεRI receptor expression has been observed in other cell types including dendritic cells, neutrophils, and eosinophils, although their functional significance in allergic responses is still unclear.[24] Moreover, Syk plays a central role in the signaling from all activating Fc receptors, including FcεRI, FcγRI, FcγRIIA, and FcγRIIIA, which populate the cell surface of most immune cells such as granulocytes, monocytes, macrophages, and dendritic cells.[8,25] Consequently, R112 has the potential to inhibit both IgE-mediated and IgG-mediated response, as was observed in the R112 inhibition of FcγRI-dependent mast cell activation (Table I). Finally, some selected integrins and the B-cell receptor are also known to signal through Syk. Thus, Syk inhibition offers broad anti-inflammatory activity potential.

Another notable R112 feature revealed by the clinical trial was its rapid onset of effect: rhinitis symptoms were significantly relieved within 30 to 45 minutes after dosing.[9] This observation correlates with the results of our cell-based assays. As expected from an ATP-competitive kinase inhibitor, we found that R112 onset of action was immediate (Fig 3, *A*). In addition, R112 inhibition was rapidly reversible (Fig 3, *B*), suggesting that R112 can be used to modulate mast cell responses by controlling its concentration at the site of action.

Importantly, our data show that the expression of proinflammatory cytokines in mast cells depends on continuous and sustained Syk signaling, and that R112 can inhibit this cytokine expression even after early responses such as degranulation have already occurred (see R112 added 1 hour after stimulation; Fig 4, *D*). Moreover, reflecting its rapid onset of action, R112 is also able to block ongoing cytokine production (see R112 added 2-5 hours after stimulation; Fig 4, *D*). Thus, R112 not only can prevent mast cell activation but also has the potential to inhibit an ongoing inflammatory signaling response. When assessed relative to other mast cell inhibitors, including steroids, only the Syk kinase inhibitor R112 was able to block potently, quickly, and completely the release of all allergen-induced mediators and cellular factors produced by mast cells and basophils (see this article's Table E2 and Fig E2 in the Online Repository at www.jacionline.org). Taken together, the R112 activity profile is consistent with its rapid onset of action and broad symptom relief observed in seasonal allergic rhinitis volunteers in a 2-day phase II clinical trial.

In a recent 7-day trial of R112 versus placebo and beclomethasone spray, R112 was indistinguishable from placebo (data not shown). We believe this to be a result of the short pharmacodynamic effect of the local R112 concentration (at least 4 hours in the first trial), which was not sufficient when R112 was dosed 2 times per day in the 7-day trial. To relieve chronic symptoms, future development candidates should possess longer duration of action than R112.

In summary, we present here the molecular and cellular characterization of the Syk kinase inhibitor R112. Syk inhibition represents an attractive new modality in the treatment of allergic inflammation.

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REFERENCES

1. Holgate S, Casale T, Wenzel S, Bousquet J, Deniz Y, Reisner C. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. J Allergy Clin Immunol 2005;115:459-65.
2. Bingham CO, 3rd, Austen KF. Mast-cell responses in the development of asthma. J Allergy Clin Immunol 2000;105:S527-34.
3. Boyce JA. Mast cells: beyond IgE. J Allergy Clin Immunol 2003;111: 24-32, quiz 3.
4. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005;6:135-42.

5. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997;77: 1033-79.
6. Holgate ST, Broide D. New targets for allergic rhinitis: a disease of civilization. Nat Rev Drug Discov 2003;2:902-14.
7. Sayama K, Diehn M, Matsuda K, Lunderius C, Tsai M, Tam SY, et al. Transcriptional response of human mast cells stimulated via the Fc(epsilon)RI and identification of mast cells as a source of IL-11. BMC Immunol 2002;3:1-5.
8. Wong BR, Grossbard EB, Payan DG, Masuda ES. Targeting Syk as a treatment for allergic and autoimmune disorders. Expert Opin Investig Drugs 2004;13:743-62.
9. Meltzer EO, Berkowitz RB, Grossbard EB. An intranasal Syk-kinase inhibitor (R112) improves the symptoms of seasonal allergic rhinitis in a park environment. J Allergy Clin Immunol 2005;115:791-6.
10. Saito H, Ebisawa M, Sakaguchi N, Onda T, Iikura Y, Yanagida M, et al. Characterization of cord-blood-derived human mast cells cultured in the presence of Steel factor and interleukin-6. Int Arch Allergy Immunol 1995;107:63-5.
11. Hide I, Bennett JP, Pizzey A, Boonen G, Bar-Sagi D, Gomperts BD, et al. Degranulation of individual mast cells in response to Ca2+ and guanine nucleotides: an all-or-none event. J Cell Biol 1993;123:585-93.
12. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. Curr Opin Immunol 2003;15:639-46.
13. Yamamoto N, Takeshita K, Shichijo M, Kokubo T, Sato M, Nakashima K, et al. The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents. J Pharmacol Exp Ther 2003;306: 1174-81.
14. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. Nat Rev Immunol 2002;2:773-86.
15. Nadler MJ, Kinet JP. Uncovering new complexities in mast cell signaling. Nat Immunol 2002;3:707-8.
16. Rivera J. Molecular adapters in Fc(epsilon)RI signaling and the allergic response. Curr Opin Immunol 2002;14:688-93.
17. Chu DH, Morita CT, Weiss A. The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol Rev 1998;165: 167-80.
18. Law CL, Chandran KA, Sidorenko SP, Clark EA. Phospholipase C-gamma1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. Mol Cell Biol 1996; 16:1305-15.
19. Saitoh S, Odom S, Gomez G, Sommers CL, Young HA, Rivera J, et al. The four distal tyrosines are required for LAT-dependent signaling in FcepsilonRI-mediated mast cell activation. J Exp Med 2003;198: 831-43.
20. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, et al. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 1996;271:695-701.
21. Benhamou M, Gutkind JS, Robbins KC, Siraganian RP. Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release. Proc Natl Acad Sci U S A 1990;87:5327-30.
22. Demo SD, Masuda E, Rossi AB, Throndset BT, Gerard AL, Chan EH, et al. Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay. Cytometry 1999;36:340-8.
23. Burd PR, Thompson WC, Max EE, Mills FC. Activated mast cells produce interleukin 13. J Exp Med 1995;181:1373-80.
24. Macglashan D Jr. IgE and Fc{epsilon}RI regulation. Ann N Y Acad Sci 2005;1050:73-88.
25. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol 2001;19: 275-90.