



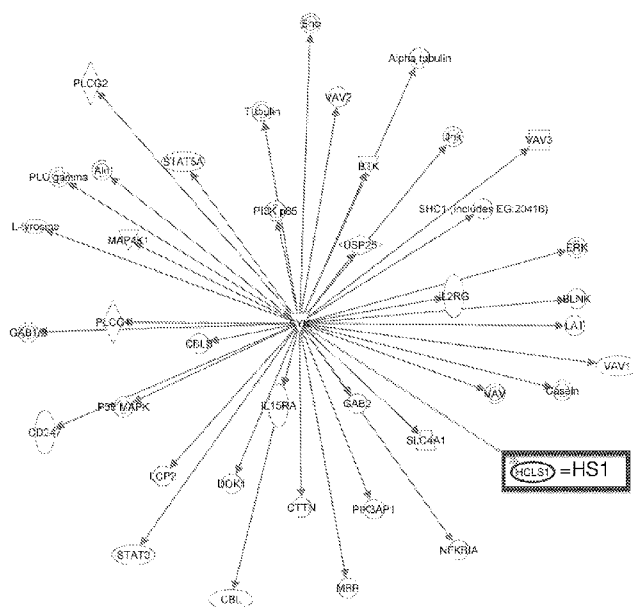
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- (54) Title: SYK-DEPENDENT HS1 TYROSINE PHOSPHORYLATION AND USES THEREOF

FIGURE 1



- (57) Abstract: The invention provides diagnostic methods for Syk-mediated diseases, and methods for treating Syk-mediated diseases, such as rheumatoid arthritis. One aspect of the invention provides a method for determining the status of a disease or condition in a mammal, the method comprising measuring the amount of a tyrosinephosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal. In certain embodiments, the method further comprises assessing the degree or severity of the disease or condition based on a substantially linear relationship between the amount of the pY-HS1 and the degree or severity of the disease or condition.

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SYK-DEPENDENT HS1 TYROSINE PHOSPHORYLATION AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No. 62/007,969 filed on June 5, 2014, the entire contents of which are incorporated herein.

BACKGROUND OF THE INVENTION

Spleen Tyrosine Kinase (Syk) is a 72 kDa non-receptor protein tyrosine kinase that functions as a key signaling regulator in most hematopoietic cells. Its closest homolog, and the only other member of the Syk-family kinases, is zeta-associated protein 70 (ZAP-70). Like Zap70, full-length Syk carries two N-terminal SH2 domains. These domains allow Syk to bind di-phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMS) on the intercellular portion of a variety of receptors involved in immune regulation. A variant of Syk, SykB, which lacks a 23 amino acid “linker insert” from the linker B region due to alternative splicing of exon 7, also is expressed in a variety of cell types.

Aggregation of the B cell antigen receptor leads to its association with Lyn and the subsequent phosphorylation of the first ITAM tyrosines of Ig- α and Ig- β . The recruitment of Syk, likely via its C-terminal SH2 domain, to the phosphorylated ITAM allows Syk to catalyze the phosphorylation of the more C-terminal tyrosines of each ITAM allowing the recruitment of additional Syk molecules to the clustered BCR complexes. This binding of Syk to the dually phosphorylated ITAM and its phosphorylation by Lyn and/or its autophosphorylation on Y342 and Y346 in linker B (and perhaps of Y519 and 520 in the activation loop) fully activates the kinase. Depending on the sites that are phosphorylated and the stoichiometry of their phosphorylation, the phosphotyrosines within linker B serve as sites for protein-protein interactions that help to amplify weak signals. The phosphorylation of Y317 by Lyn, in turn, dampens signaling in a Cbl-dependent manner, but is important for signaling to PI3K through other receptors involved in such processes as phagocytosis and motility.

Upon activation and recruitment to immunoreceptors, Syk catalyzes the phosphorylation, on tyrosines located within highly acidic regions, of numerous protein substrates that are important for transducing the antigen-receptor interaction into the appropriate physiological response. These include enzymes whose activity is enhanced as well as adaptor proteins whose phosphorylation promotes the assembly of signaling complexes through the recruitment of proteins containing phosphotyrosine-interacting domains. Known Syk substrates include, but are not limited to, Linker for Activator of T-cells (LAT), B-cell Linker (BLNK), Vav, Bruton's Tyrosine Kinase (BTK), Gab, Bcap, SH2-domain containing Leukocyte Protein-76 (SLP-76) and Phospholipase C γ .

For some Syk substrates, phosphorylation induces conformational changes that lead to alterations in the intrinsic activity of the phosphorylated substrate, such as PLC- γ 2, Btk, hematopoietic progenitor kinase-1 (HPK1) and the Vav1 guanine nucleotide exchange factor. For many other substrates, tyrosine phosphorylation by Syk instead promotes protein-protein associations by generating docking sites that are recognized by proteins that have SH2 domains or other phosphotyrosine-binding motifs. Syk appears to demonstrate a preference for the phosphorylation of tyrosines within motifs that can then be recognized by group I SH2 domains. Thus, the phosphorylation of many substrates for Syk including BLNK/SLP-65, LAB/NTAL/LAT2, 3BP2, BCAP, BANK and GCET generates scaffolds for the assembly of larger signaling complexes. For example, the phosphorylation of BLNK/SLP-65, a major Syk substrate in B cells, creates docking sites that bind Btk and PLC- γ to generate a protein complex that regulates the mobilization of calcium.

In some cases, tyrosine phosphorylation by Syk of some substrates can inhibit rather than promote protein-protein associations. For example, in red blood cells, the acidic cytoplasmic tail of the anion transport channel protein, band 3, binds to and inhibits the activities of several of the glycolytic enzymes including aldolase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The phosphorylation of Y8 on band 3 by Syk blocks these interactions and relieves the inhibition. It is likely that similar mechanism may be important to the regulation of additional protein-protein interactions. It is interesting to note that the acidic C-terminus of α -tubulin also binds glycolytic enzymes and is an excellent substrate for Syk.

Activated Syk can also dissociate from the receptor and appear in an active form in locations within the cell other than the plasma membrane including the nucleus. The phosphorylation of Y130 provides one mechanism for this dissociation. Signaling is terminated through the down-regulation of membrane-bound receptors and through the dephosphorylation of Syk and its substrates by one or more of several candidate phosphatases. Thus, multiple factors act in concert to influence the activity of Syk in order to regulate the quality and quantity of the signal that is sent from the BCR, which ultimately determines the physiological outcome of receptor engagement.

The role of Syk in cellular signaling was first identified in B lymphocytes, but Syk is expressed in many other cell types including most cells of the hematopoietic system, and at lower levels in some epithelial cells, fibroblasts, hepatocytes, vascular smooth muscle cells, endothelial cells and neuronal cells.

In B-cells, Syk is essentially involved in B-cell Receptor (BCR) signal initiation, leading to development and survival of B lymphocytes in both bone marrow and periphery (Cheng *et al.*, 1995, *Nature*, 378:3003; Turner *et al.*, 1995 *Nature*, 378:298). It is activated by the Src-family kinase (SFK) Lyn after Syk binds to doubly phosphorylated ITAMs on Ig α / β chains on the BCR. The downstream effects of BCR engagement include Ca²⁺ flux, mitogen-activated protein (MAP) kinase activation and Akt activation. Signaling through the BCR is critical for development and survival of B lymphocytes in both bone marrow and periphery.

In mast cells and basophils, Syk is a critical component of Fc ϵ R1 signaling where downstream effects of activation include degranulation, release of cytokines such as tumor necrosis factor α and interleukin-6 and release of lipid mediators such as LTC₄ (Costello *et al.*, 1996 *Oncogene*, 13:2595). Similar Syk-dependent signaling is driven by IgG-antigen crosslinking via Fc γ receptors in macrophages, neutrophils & dendritic cells (Kiefer *et al.*, 1998 *Mol. Cell Biol.*, 18:4209; Sedlik *et al.*, 2003 *J. Immun.*, 170:846).

In macrophages, Syk activity is believed to regulate phagocytosis of opsonized foreign (and self) antigens via the Fc γ R, and Syk is important for antigen presentation from and maturation of dendritic cells. A role for Syk has been proposed for osteoclast maturation and in DAP12 receptor signaling in these cell types involved in bone metabolism.

Reviews of these finding can be found in *Expert Opin. Invest. Drugs*, 2004, 13(7):743 and *Expert Opin. Invest. Drugs*, 2008, 17(5):641.

HS1 (hematopoietic cell-specific Lyn substrate-1) is a cytoskeletal interactor in the B-cell receptor (BCR) signaling pathway. HS1 phosphorylation has previously been correlated with prognosis in Chronic Lymphocytic Leukemia (CLL), in that hypophosphorylated HS1 (HS1^{hypo-p}) is associated with benign clinical course, and hyperphosphorylated HS1 (HS1^{hyper-p}) with poor outcome (Scielzo *et al.*, “HS1 protein is differentially expressed in chronic lymphocytic leukemia patient subsets with good or poor prognoses,” *J. Clin. Invest.*, 115(6):1644-1650, 2005). The semiquantitative analysis of HS1 differential phosphorylation, as originally carried out by 2DE by Scielzo *et al.*, however, left unsolved questions on the nature of the differentially phosphorylated sites and the activity of the HS1 protein.

Surprisingly, in a recent study by Hacken *et al.* (*Blood*, 121(12):2264-2273, 2013), using a newly available antibody specific for the pY397 residue of HS1, it was determined that, in CLL primary samples, 2DE-detected HS1^{hypo-p} cells showed high LYN and HS1 activation (*i.e.*, higher level of pY397 HS1), while HS1^{hyper-p} cells showed reduced activation of both molecules (*i.e.*, lower level of pY397 HS1). This result suggests that increased HS1 phosphorylation at Tyr 397 is correlated with a benign outcome in CLL patients (and lower overall HS1 phosphorylation), while decreased HS1 phosphorylation at Tyr 397 is correlated with a poorer prognosis in CLL patients (and higher overall HS1 phosphorylation).

SUMMARY OF THE INVENTION

One aspect of the invention provides a method for determining the status of a disease or condition in a mammal, the method comprising measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal, (1) wherein the disease or condition is characterized by increased B cell activation as measured by increased pY-HS1 (mediated by Syk activation); (2) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (3) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and (4) wherein the mammal is diagnosed as having a more advanced state of the disease or condition if

the amount of the pY-HS1 in the whole blood sample is increased as compared to that of a reference range; and/or wherein the mammal is diagnosed as having a better state of the disease or condition or as being in remission, if the amount of the pY-HS1 in the whole blood sample is within the reference range.

In certain embodiments, the disease is an autoimmune disease or an inflammatory disease. For example, the disease may be rheumatoid arthritis (RA), or the disease may be any of: lupus nephritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS), or type I hypersensitivity reactions (such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis, allergic asthma, and systemic anaphylaxis).

In certain embodiments, the mammal is a human, a rodent (*e.g.*, a rat or a mouse), or a non-human mammal.

In certain embodiments, the extent of binding between the antibody and the pY-HS1 is detected using flow cytometry or Western blot.

In certain embodiments, the extent of binding between the antibody and the pY-HS1 is detected using a strip test, a lateral flow device, or a dipstick.

In certain embodiments, the extent of binding between the antibody and the pY-HS1 is detected using Enzyme-linked immunosorbent assay (ELISA).

In certain embodiments, the extent of binding between the antibody and the pY-HS1 is detected in B cells or in platelets of the whole blood sample.

In certain embodiments, the method further comprises administering to the mammal either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition.

In certain embodiments, the mammal has previously been administered either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition.

In certain embodiments, the reference range represents the amount of the pY-HS1 in a baseline whole blood sample from the mammal. For example, in certain embodiments, the baseline whole blood sample is obtained from the mammal before the agent or test agent is administered to the mammal. In other embodiments, the baseline whole blood sample is obtained from the mammal after at least one dose of the agent or test agent has been administered to the mammal.

In certain embodiments, the agent or test agent inhibits HS1 phosphorylation at the tyrosine residue corresponding to Y397 of human HS1. For example, the agent or test agent may be an inhibitor of a kinase, such as an inhibitor of Syk.

In certain embodiments, the antibody binds to an epitope comprising a phosphorylated tyrosine corresponding to Y397 of human HS1, and does not bind an unphosphorylated tyrosine corresponding to Y397 of human HS1.

In certain embodiments, the whole blood sample is first contacted with an antibody that activates a B cell receptor (BCR), before determining the amount of the pY-HS1. For example, the antibody that activates BCR is an anti-IgD antibody, or an anti-IgM antibody.

In certain embodiments, the reference range represents the amount of HS1 protein phosphorylated at the tyrosine residue corresponding to Y397 of human HS1 in a matching whole blood sample from the middle 90%, 80%, 70%, 60%, or 50% of a healthy population.

In certain other embodiments, the reference range may represent the amount of HS1 protein phosphorylated at the tyrosine residue corresponding to Y397 of human HS1 in a matching whole blood sample from the middle 90%, 80%, 70%, 60%, or 50% of a population diagnosed with the disease or condition. This may be useful to gauge the severity or to predict the outcome of the disease or condition. For example, the population may be patients diagnosed to have moderate to severe rheumatoid arthritis (RA).

In certain embodiments, the method further comprises assessing the degree or severity of the disease or condition based on a substantially linear relationship between the amount of the pY-HS1 and the degree or severity of the disease or condition. The disease or condition may be lupus nephritis or SLE, and wherein the degree or severity of the disease or condition is measured by survival rate, or by onset of proteinuria.

In certain embodiments, the method further comprises administering to the mammal a therapeutic agent efficacious to treat the disease or condition, preferably based on the degree or severity of the disease or condition.

Another aspect of the invention provides a method to adjust the dose of a therapeutic agent useful for treating a disease or condition in a mammal in need of treatment, the method comprising: (1) administering a first dose of the therapeutic agent to the mammal;

(2) measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal; and, (3) comparing the amount of the pY-HS1 with a reference range; (4) repeating step (1) with a dose higher than the first dose, if the amount of the pY-HS1 in the sample is higher than the maximum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range; or repeating step (1) with a dose lower than the first dose, if the amount of the pY-HS1 in the sample is lower than the minimum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range; wherein the disease or condition is mediated by Syk activation; wherein the HS1 protein is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; and, wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, wherein the whole blood sample is derived from the mammal, and wherein the antibody binds the pY-HS1 but does not bind HS1 not phosphorylated at the tyrosine.

In certain embodiments, the therapeutic agent is an inhibitor of Syk.

Yet another aspect of the invention provides a method to identify a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising: administering the agent to the mammal, and determining the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal, (1) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (2) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and (3) wherein the mammal is identified as having the disease or condition that may be susceptible or sensitive to treatment by the agent, if the amount of the pY-HS1 in the whole blood sample is decreased as compared to that of a reference range, wherein the reference range represents a baseline amount of the pY-HS1 in a whole blood sample derived from the mammal before the agent is administered to the mammal.

Yet another aspect of the invention provides a method of treating a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising: (1) using any of the methods of the

subject invention to identify the mammal that may be susceptible or sensitive to treatment by the agent; and, (2) administer the agent to the mammal, thereby treating the mammal having the disease or condition.

A further aspect of the invention provides a method to compare therapeutic efficacy of a 1st therapeutic agent and a 2nd therapeutic agent for treating a disease or condition, the method comprising: (1) administering the 1st therapeutic agent to a 1st population of mammals, and determining a 1st decrease, if any, in the average amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in samples derived from the 1st population of mammals, after administering the 1st therapeutic agent; (2) administering the 2nd therapeutic agent to a 2nd population of mammals, and determining a 2nd decrease, if any, in the average amount of the pY-HS1 in samples derived from the 2nd population of mammals, after administering the 2nd therapeutic agent; (3) comparing the 1st decrease to the 2nd decrease, wherein the larger decrease is indicative of a better therapeutic efficacy; wherein: (i) the disease or condition is mediated by Syk activation; (ii) the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (iii) the amount of the pY-HS1 is measured by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue.

Another aspect of the invention provides a kit for measuring Syk pathway activation, the kit comprising: (1) a reagent that activates B cell receptor (BCR); and, (2) an antibody specific for a phosphorylated tyrosine corresponding to Y397 of human HS1.

In certain embodiments, the kit further comprises: (3) an antibody specific for a B-cell surface marker, wherein the antibody is optionally labelled by a fluorescent dye (*e.g.*, FITC) or a radioactive moiety. For example, the antibody may be specific for the phosphorylated tyrosine is labelled by a fluorescent dye (*e.g.*, PE).

It is contemplated that all embodiments described in the instant specification, including those only described in the examples or drawings, and including those only described under one aspect (but not other aspects) of the invention, can be combined with any other embodiments unless explicitly disclaimed. All such combined embodiments, though not explicitly enumerated, are all within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: INGENUITY PATHWAY ASSIST™ was used to identify potential Syk substrates based on literature annotations. Well established substrates (*e.g.*, LAT & BLNK) were identified in addition to hypothetical substrates like HS1/HCLS1.

Figure 2: HS1 phosphorylation at Y397 can be detected by Western blotting or flow cytometry. Human Ramos B cells were serum-starved overnight, stimulated with 40µg/mL goat anti-human IgG/IgM and then lysed for Western blotting (top 2 panels) or fix-lysed for staining and acquisition by flow cytometry (bottom panel). Cell signaling antibody 8714 was custom-conjugated to PE for flow cytometry. “Cell Signaling 8714 (or 3890 or 11880)” stands for the commercially available antibody of the same Cat. No. from Cell Signaling Technology, Inc. (Danvers, MA).

Figures 3A & 3B: Increased Syk-dependent HS1 tyrosine phosphorylation is detectable in anti-IgD-stimulated whole blood from multiple species. (FIG. 3A) Representative FlowJo7.6.5 histograms of P-HS1 geometric means from human (top), mouse (middle) or rat (bottom) B cells stimulated (while in whole blood) with anti-IgD. (FIG. 3B) Anti-IgD-induced HS1 phosphorylation in human, mouse or rat B cells is inhibited by proprietary or tool Syk inhibitors. Filled circle: Compound 5; Open Triangle: Compound 4; Filled Square: Compound 6.

Figures 4A & 4B: HS1 tyrosine phosphorylation in rat B cells is Syk- but not Btk-dependent. (FIG. 4A) Geometric means and (FIG. 4B) percent inhibition of anti-IgD-induced HS1 phosphorylation in rat whole blood treated *ex vivo* with the proprietary Syk inhibitor Compound 1 (filled circle) or tool Btk inhibitor Compound 2 (filled diamond). In Figure 4B, the HillSlopes and IC₅₀ values on the left and right columns are for Compounds 1 and 2, respectively.

Figures 5A-5C: Inhibition of HS1 tyrosine phosphorylation correlates with efficacy in the rat collagen-induced arthritis model. (FIG. 5A) Inhibition of anti-IgD-induced HS1 phosphorylation in B cells from rats dosed with the tool Syk inhibitor Compound 3. (FIG. 5B) Dose-dependent inhibition of disease activity (paw swelling) in dosed rats. (FIG. 5C) Pharmacodynamic (HS1 phosphorylation) versus efficacy (paw swelling) correlation for data obtained using Compound 3.

Figures 6A-6C: PKPD modeling of P-HS1 inhibition to target engagement reveals that Fostamatinib, a non-selective kinase inhibitor, does not inhibit Syk at efficacious doses. Utilizing data from the *in vitro* whole blood assays and the efficacy studies, integrated PKPD modeling generated an effect vs. time profile of target engagement (FIG. 6A). Efficacious concentrations of Fostamatinib do not inhibit P-HS1 while achieving full efficacy (FIG. 6B). A selective Syk inhibitor (Compound 4) does inhibit P-HS1 at efficacious doses (FIG. 6C). This data indicates that the efficacy observed with Fostamatinib is being driven by off target inhibition.

Figures 7A-7D: Inhibition of P-HS1 corresponds to efficacy in a preclinical model of lupus nephritis. FIG. 7A shows the dosing regimen and analysis done at different time points. A selective Syk inhibitor, Compound 4, dose-dependently prevented the onset of proteinuria (FIG. 7B) and increased survival (FIG. 7C). The effect was dose responsive and corresponded to the level of pHS-1 inhibition (FIG. 7D).

Figures 8A & 8B: Basal (FIG. 8A) and anti-IgD-induced (FIG. 8B) HS1 phosphorylation are dose-dependently inhibited by the proprietary Syk inhibitor Compound 4 in B cells from Rheumatoid Arthritis subjects.

Figure 9: Basal or anti-IgD-induced HS1 tyrosine phosphorylation is increased in B cells from rheumatoid arthritis (RA) versus healthy subjects. Phospho-Y397 or total levels of HS1 were measured in CD19-positive B cells from n=18 RA or n=20 healthy subjects from two separate cohorts. Data are presented as the geometric mean of P-HS1 normalized to the geometric mean of total HS1.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

Using INGENUITY PATHWAY ASSIST™ software, hematopoietic lineage cell-specific protein 1 (HCLS1/HS1) was identified as a candidate biomarker downstream of Syk activation. HS1 phosphorylated can be detected in B-cells from human or rodents, and the Syk dependence of HS1 phosphorylation downstream of BCR activation has been confirmed by measuring the inhibition of HS1 tyrosine-phosphorylation using several reference and lead compounds. It was found that *ex vivo* treatment of whole blood from RA patients dose-dependently inhibits both basal and anti-IgD-induced HS1 Tyr-

phosphorylation in B cells. At least the anti-IgD-induced HS1 phosphorylation is Syk- but not Btk-dependent. In the rat collagen-induced arthritis (CIA) model, inhibition of anti-IgD-induced HS1 Tyr-phosphorylation correlates with efficacy. In contrast, PK/PD modeling of HS1 Tyr-phosphorylation inhibition to target engagement reveals that Fostamatinib, a non-selective kinase inhibitor, does not inhibit Syk at efficacious doses, and thus the efficacy is at least partly based on off-target inhibition. Dose-dependent inhibition of HS1 Tyr-phosphorylation also correlated with an improvement in kidney function as measured by proteinuria, indicating that Syk inhibition is important in pathogenic mechanisms in lupus-prone mice.

Thus, contrary to or inconsistent with the finding of Hacken *et al.* (*supra*), the data presented herein supports the notion that pY397 phosphorylation on HS1 is positively associated with human disease status and is linearly correlated with Syk activation, more advanced disease status, as well as disease activity in animal models of autoimmunity. Indeed, in two independent cohorts, HS1 Tyr-phosphorylation is elevated in circulating B cells of RA patients compared to healthy controls, suggesting that HS1 Tyr-phosphorylation represents a disease / efficacy biomarker. Thus HS1 Tyr397-phosphorylation can be used as a measure of Syk engagement in healthy individuals as well as in RA patients.

Therefore, in one aspect, the invention provides a method for determining the status of a disease or condition in an individual (*e.g.*, a mammal), the method comprising measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the individual (*e.g.*, mammal), (1) wherein the disease or condition is characterized by increased B cell activation as measured by increased pY-HS1, or wherein the disease or condition is mediated by Syk activation; (2) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; and (3) wherein the individual (*e.g.*, mammal) is diagnosed as having a more advanced state of the disease or condition if the amount of the pY-HS1 in the sample is increased as compared to that of a reference range or value; and/or wherein the individual (*e.g.*, mammal) is diagnosed as having a better state of the disease or condition or as being in remission, if the amount of the pY-HS1 in the sample is within the reference range or about the same as the reference value.

More specifically, the invention provides a method for determining the status of a disease or condition in a mammal, the method comprising measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal, (1)

wherein the disease or condition is characterized by increased B cell activation as measured by increased pY-HS1, or wherein the disease or condition is mediated by Syk activation; (2) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (3) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and (4) wherein the mammal is diagnosed as having a more advanced state of the disease or condition if the amount of the pY-HS1 in the whole blood sample is increased as compared to that of a reference range; and/or wherein the mammal is diagnosed as having a better state of the disease or condition or as being in remission, if the amount of the pY-HS1 in the whole blood sample is within the reference range.

As used herein, pY397-HS1 includes a human HS1 phosphorylated at Tyr 397. The human HS1 may or may not include additional Tyr / Ser / Thr phosphorylation at other residues. The pY397-HS1 may also include a non-human HS1 phosphorylated at a Tyr corresponding to human HS1 Tyr 397. See below for using sequence alignment between human HS1 and non-human HS1 for identifying Tyr residue in non-human HS1 proteins that corresponds to human HS1 Tyr 397.

In certain embodiments, the disease or condition is an autoimmune disease or an inflammatory disease. For example, the disease or condition may be rheumatoid arthritis (RA). In other embodiments, the disease or condition may be lupus nephritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS), or type I hypersensitivity reactions (such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis, allergic asthma, and systemic anaphylaxis). In certain embodiments, the disease or condition is not CLL (Chronic Lymphocytic Leukemia). In certain embodiments, the disease or condition is any one listed in a separate section below.

The method of the invention can be used for any individual. In certain embodiments, the individual is a mammal, such as a human, a rodent (*e.g.*, a rat or a mouse), or a non-human mammal.

In certain embodiments, the sample may be a whole blood sample derived from the individual / mammal. The whole blood sample may be first contacted with an antibody that activates a B cell receptor (BCR), before determining the amount of the pY397-HS1.

Such activating antibody may be an anti-IgD antibody, or an anti-IgM antibody. See section below for the various samples and sampling methods, as well as BCR activation.

The amount of the pY397-HS1 can be measured using a number of art-recognized methods. For example, the amount may be determined / measured based on the extent of binding between pY397-HS1 and an antibody specific therefor, using flow cytometry or Western blot. Alternatively, the extent of binding between the antibody and the pY397-HS1 can be detected using a lateral flow device, such as a strip test, or a dipstick. Other methods, such as ELISA or RIA can also be used. See detailed methods in a separate section below.

In certain embodiments, when antibody is used to measure the amount of pY397-HS1, the antibody may bind to an epitope comprising a phosphorylated tyrosine corresponding to Y397 of human HS1, and does not bind an unphosphorylated tyrosine corresponding to Y397 of human HS1. In a related embodiment, the antibody may recognize a specific conformation of HS1 phosphorylated at Y397, but not a conformation associated with unphosphorylated Y397, even though the antibody may not bind directly to pY397-HS1.

In certain embodiments, the total amount of HS1 protein in the sample may also be measured, using, for example, an antibody specific for all forms of HS1, including unphosphorylated HS1, and HS1 phosphorylated at one or more Tyr / Ser / Thr residues, such as Tyr 397. In certain embodiments, the level / amount of pY397-HS1 is normalized against total amount of HS1 in the sample, such that the increase of phosphorylation at Tyr 397 per unit amount of total HS1 can be determined.

In certain embodiments, the amount of pY397-HS1 in a sub-population of cells from the sample is determined / measured. For example, the sub-population of cells may be B cells or platelets, such as those derived from a whole blood sample. Such sub-population of cells may be isolated or identified by, for example, gating for cells with staining for a B cell surface marker (such as CD19), or for cells of a particular size range in the case of platelets.

The methods of the invention may be used to determine the status of a disease or condition in an apparently healthy individual (such as those providing data for establishing a reference range or value of pY397-HS1), an individual suspected of or at high risk of having the disease or condition, or an individual who has already undergone treatment for the disease or condition (such as one involved in a clinical trial using an

experimental drug, or one following up with a physician to assess the efficacy of a treatment).

Thus in certain embodiments, the individual / mammal has previously been administered either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition. In other embodiments, the method further comprises administering to the individual / mammal either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition.

In certain embodiments, the measured pY397-HS1 is compared to a reference range or value to assist the determination of disease status. For example, the reference range may represent the amount of the pY397-HS1 in a baseline whole blood sample from the individual / mammal.

As used herein, “baseline” refers to a state that serves as a reference comparison point. For example, to assess the effect of a particular drug or a candidate therapeutic agent, the baseline may represent the reference point where no relevant drug / therapeutic agent has been previously administered. In certain embodiments, the baseline may be established at the beginning of a clinical trial, such as a double blinded placebo controlled study to determine the efficacy of the drug / therapeutic agent. In other embodiments, the baseline may be established at the beginning of an open label study, after the conclusion of the placebo controlled stage, wherein all enrolled patients, including those originally in the placebo group, are given certain doses of the drug / therapeutic agent.

Thus in certain embodiments, the baseline whole blood sample is obtained from the individual / mammal before the agent or test agent is administered to the individual / mammal. In other embodiments, the baseline whole blood sample is obtained from the individual / mammal after at least one dose of the agent or test agent has been administered to the individual / mammal.

In certain embodiments, the reference range represents a relatively normal range observed in healthy control samples. For example, the reference range may represent the amount of pY397-HS1 in a matching whole blood sample from the middle 90%, 80%, 70%, 60%, 50% of a healthy population, or a patient population. As used herein, middle 90% refers to pY397-HS1 levels excluding the top / highest 5% and the bottom / lowest 5% of pY-397-HS1 in a population. Matching whole blood samples may be obtained from a control

/ disease / healthy population. For example, for RA patients, matching control may be a population of healthy individuals with similar age, gender distribution, ethnic group, geographic location, *etc.* Matching control can also be a population of RA patients, such as those having moderate to severe RA.

In certain embodiments, the agent or test agent inhibits HS1 phosphorylation at the tyrosine residue corresponding to Y397 of human HS1. In certain embodiments, the agent or test agent is an inhibitor of a kinase, such as an inhibitor of Syk.

In certain embodiments, the method further comprises assessing the degree or severity of the disease or condition based on a substantially linear relationship between the amount of the pY397-HS1 and the degree or severity of the disease or condition. While not wishing to be bound by any particular theory, it appears that the degree or severity of the disease or condition (*e.g.*, RA) seems to be positively correlated with the level of pY397-HS1, such that higher pY397-HS1 suggests more severe or advanced disease status, while lower pY-397-HS1 suggests less severe or better disease status.

For example, in certain embodiments, the disease or condition is lupus nephritis or SLE, and the degree or severity of the disease or condition is measured by survival rate, or by onset of proteinuria. In certain embodiments, the method further comprises administering to the individual / mammal a therapeutic agent efficacious to treat the disease or condition, preferably based on the degree or severity of the disease or condition.

Another aspect of the invention provides a method to adjust the dose of a therapeutic agent useful for treating a disease or condition in a mammal in need of treatment, the method comprising: (1) administering a first dose of the therapeutic agent to the mammal; (2) measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal; and, (3) comparing the amount of the pY-HS1 with a reference range; (4) repeating step (1) with a dose higher than the first dose, if the amount of the pY-HS1 in the sample is higher than the maximum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range; or repeating step (1) with a dose lower than the first dose, if the amount of the pY-HS1 in the sample is lower than the minimum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range; wherein the disease or condition is mediated by Syk activation; wherein the HS1 protein is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; and,

wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, wherein the whole blood sample is derived from the mammal, and wherein the antibody binds the pY-HS1 but does not bind HS1 not phosphorylated at the tyrosine.

In certain embodiments, the therapeutic agent is an inhibitor of Syk, such as those described herein below.

Another aspect of the invention provides a method to identify a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising: administering the agent to the mammal, and determining the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal, (1) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (2) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and (3) wherein the mammal is identified as having the disease or condition that may be susceptible or sensitive to treatment by the agent, if the amount of the pY-HS1 in the whole blood sample is decreased as compared to that of a reference range, wherein the reference range represents a baseline amount of the pY-HS1 in a whole blood sample derived from the mammal before the agent is administered to the mammal.

Yet another aspect of the invention provides a method of treating a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising: (1) using any of the methods of the subject invention to identify the mammal that may be susceptible or sensitive to treatment by the agent; and, (2) administer the agent to the mammal, thereby treating the mammal having the disease or condition.

A further aspect of the invention provides a method to compare therapeutic efficacy of a 1st therapeutic agent and a 2nd therapeutic agent for treating a disease or condition, the method comprising: (1) administering the 1st therapeutic agent to a 1st population of mammals, and determining a 1st decrease, if any, in the average amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in samples derived from the 1st population of

mammals, after administering the 1st therapeutic agent; (2) administering the 2nd therapeutic agent to a 2nd population of mammals, and determining a 2nd decrease, if any, in the average amount of the pY-HS1 in samples derived from the 2nd population of mammals, after administering the 2nd therapeutic agent; (3) comparing the 1st decrease to the 2nd decrease, wherein the larger decrease is indicative of a better therapeutic efficacy; wherein: (i) the disease or condition is mediated by Syk activation; (ii) the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; (iii) the amount of the pY-HS1 is measured by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, (a) wherein the whole blood sample is derived from the mammal; and, (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue.

Another aspect of the invention provides a kit for measuring Syk pathway activation, the kit comprising: (1) a reagent that activates B cell receptor (BCR); and, (2) an antibody specific for a phosphorylated tyrosine corresponding to Y397 of human HS1.

The term “kit” as used herein refers to a packaged product, optionally with labels and/or instructions for using the same in carrying out the methods of the invention, *e.g.*, kit with reagents necessary or helpful for carrying out the methods of the invention for measuring Syk activation. The kit preferably comprises a box or a container that holds the components of the kit. The box or container may be affixed with a label or a Food and Drug Administration approved protocol. The components of the invention, held in the box or container, are preferably contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles or any other suitable shape or form. The kit can also include instructions for carrying out the methods of the invention.

In certain embodiments, the kit may further comprise: (3) an antibody specific for a B-cell surface marker, wherein the antibody is optically labelled by a fluorescent dye (*e.g.*, FITC) or a radioactive moiety. For example, the antibody specific for the phosphorylated tyrosine may be labelled by a fluorescent dye (*e.g.*, PE).

With the invention generally described here, certain specific features of invention are further described in the separate sections below. Embodiments described in each specific feature can be combined with embodiments in any one or more other features.

2. *Diseases or conditions mediated by Syk activation*

Spleen tyrosine kinase (Syk) (*J. Bio. Chem.*, 1991, 266:15790) is a non-receptor tyrosine kinase that plays a key role in immunoreceptor signaling in a host of inflammatory cells including B cells, mast cells, macrophages and neutrophils. Syk is related to zeta associated protein 70 (ZAP-70) but also demonstrates similarity with JAK, Src and Tec family kinases.

A number of diseases or conditions are associated with, caused or affected by, mediated by, or otherwise worsened by abnormal Syk activation. Thus Syk activation in a broad range of inflammatory diseases and immunological disorders can be measured using the subject reagents and methods.

These diseases, disorders, or conditions may include any one of rheumatoid arthritis (RA) (including those with associated symptoms such as bony erosions, joint destruction and generalized osteopenia), juvenile rheumatoid arthritis (JRA), osteoarthritis, Crohn's disease, inflammatory bowel disease (IBD), ulcerative colitis (UC), ankylosing spondylitis (AS), interstitial cystitis, asthma, systemic lupus erythematosus (SLE), multiple sclerosis (MS), or type I hypersensitivity reactions such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis, allergic asthma and systemic anaphylaxis.

A more comprehensive list of diseases or conditions that may be associated with, caused or affected by, mediated by, or otherwise worsened by abnormal Syk activation include: rheumatoid arthritis, asthma, allergic asthma, osteoarthritis, juvenile arthritis, ankylosing spondylitis, an ocular condition, interstitial cystitis, a cancer, a solid tumor, a sarcoma, fibrosarcoma, osteoma, melanoma, retinoblastoma, a rhabdomyosarcoma, glioblastoma, neuroblastoma, teratocarcinoma, hypersensitivity reactions, hyperkinetic movement disorders, hypersensitivity pneumonitis, hypertension, hypokinetic movement disorders, aortic and peripheral aneurisms, hypothalamic-pituitary-adrenal axis evaluation, aortic dissection, arterial hypertension, arteriosclerosis, arteriovenous fistula, ataxia, spinocerebellar degenerations, streptococcal myositis, structural lesions of the cerebellum, Subacute sclerosing panencephalitis, Syncope, syphilis of the cardiovascular system, systemic anaphalaxis, systemic inflammatory response syndrome, systemic onset juvenile rheumatoid arthritis, T-cell or FAB ALL, Telangiectasia, thromboangitis

obliterans, transplants, trauma/hemorrhage, type III hypersensitivity reactions, type IV hypersensitivity, unstable angina, uremia, urosepsis, urticaria, valvular heart diseases, varicose veins, vasculitis, venous diseases, venous thrombosis, ventricular fibrillation, viral and fungal infections, viral encephalitis/aseptic meningitis, vital-associated hemaphagocytic syndrome, Wernicke-Korsakoff syndrome, Wilson's disease, xenograft rejection of any organ or tissue, heart transplant rejection, hemachromatosis, hemodialysis, hemolytic uremic syndrome/thrombolytic thrombocytopenic purpura, hemorrhage, idiopathic pulmonary fibrosis, antibody mediated cytotoxicity, Asthenia, infantile spinal muscular atrophy, inflammation of the aorta, influenza A, ionizing radiation exposure, iridocyclitis/uveitis/optic neuritis, juvenile spinal muscular atrophy, B-cell lineage malignancy, lymphoma (*e.g.*, B cell lymphoma), myeloma, leukaemia, malignant ascites, hematopoietic cancers, a diabetic condition such as insulin-dependent diabetes mellitus glaucoma, diabetic retinopathy or microangiopathy, sickle cell anaemia, chronic inflammation, glomerulonephritis, graft rejection, Lyme disease, von Hippel Lindau disease, pemphigoid, Paget's disease, fibrosis, sarcoidosis, cirrhosis, thyroiditis, hyperviscosity syndrome, Osler-Weber-Rendu disease, chronic occlusive pulmonary disease, asthma or edema following burns, trauma, radiation, stroke, hypoxia, ischemia, ovarian hyperstimulation syndrome, post perfusion syndrome, post pump syndrome, post-MI cardiomyopathy syndrome, preeclampsia, menometrorrhagia, endometriosis, pulmonary hypertension, infantile hemangioma, or infection by Herpes simplex, Herpes Zoster, human immunodeficiency virus, parapoxvirus, protozoa or toxoplasmosis, progressive *supranucleo* palsy, primary pulmonary hypertension, radiation therapy, Raynaud's phenomenon, Raynaud's disease, Refsum's disease, regular narrow QRS tachycardia, renovascular hypertension, restrictive cardiomyopathy, sarcoma, senile chorea, senile dementia of Lewy body type, shock, skin allograft, skin changes syndrome, ocular or macular edema, ocular neovascular disease, scleritis, radial keratotomy, uveitis, vitritis, myopia, optic pits, chronic retinal detachment, post-laser treatment complications, conjunctivitis, Stargardt's disease, Eales disease, retinopathy, macular degeneration, restenosis, ischemia/reperfusion injury, ischemic stroke, vascular occlusion, carotid obstructive disease, ulcerative colitis, inflammatory bowel disease, diabetes, diabetes mellitus, insulin dependent diabetes mellitus, allergic diseases, dermatitis scleroderma, graft versus host disease, organ transplant rejection (including but not limited to bone marrow and solid organ rejection), acute or chronic immune disease associated with organ transplantation, sarcoidosis, disseminated intravascular coagulation, Kawasaki's

disease, nephrotic syndrome, chronic fatigue syndrome, Wegener's granulomatosis, Henoch-Schoenlein purpura, microscopic vasculitis of the kidneys, chronic active hepatitis, septic shock, toxic shock syndrome, sepsis syndrome, cachexia, infectious diseases, parasitic diseases, acquired immunodeficiency syndrome, acute transverse myelitis, Huntington's chorea, stroke, primary biliary cirrhosis, hemolytic anemia, malignancies, Addison's disease, idiopathic Addison's disease, sporadic, polyglandular deficiency type I and polyglandular deficiency type II, Schmidt's syndrome, adult (acute) respiratory distress syndrome, alopecia, alopecia areata, seronegative arthropathy, arthropathy, Reiter's disease, psoriatic arthropathy, ulcerative colitic arthropathy, enteropathic synovitis, chlamydia, yersinia and salmonella associated arthropathy, atheromatous disease/arteriosclerosis, atopic allergy, autoimmune bullous disease, pemphigus vulgaris, pemphigus foliaceus, pemphigoid, linear IgA disease, autoimmune haemolytic anaemia, Coombs positive haemolytic anaemia, acquired pernicious anaemia, juvenile pernicious anaemia, peripheral vascular disorders, peritonitis, pernicious anemia, myalgic encephalitis/Royal Free Disease, chronic mucocutaneous candidiasis, giant cell arteritis, primary sclerosing hepatitis, cryptogenic autoimmune hepatitis, Acquired Immunodeficiency Disease Syndrome, Acquired Immunodeficiency Related Diseases, Hepatitis A, Hepatitis B, Hepatitis C, His bundle arrhythmias, HIV infection/HIV neuropathy, common varied immunodeficiency (common variable hypogammaglobulinaemia), dilated cardiomyopathy, female infertility, ovarian failure, premature ovarian failure, fibrotic lung disease, chronic wound healing, cryptogenic fibrosing alveolitis, post-inflammatory interstitial lung disease, interstitial pneumonitis, pneumocystis carinii pneumonia, pneumonia, connective tissue disease associated interstitial lung disease, mixed connective tissue disease, associated lung disease, systemic sclerosis associated interstitial lung disease, rheumatoid arthritis associated interstitial lung disease, systemic lupus erythematosus associated lung disease, dermatomyositis/polymyositis associated lung disease, Sjögren's disease associated lung disease, ankylosing spondylitis associated lung disease, vasculitic diffuse lung disease, haemosiderosis associated lung disease, drug-induced interstitial lung disease, radiation fibrosis, bronchiolitis obliterans, chronic eosinophilic pneumonia, lymphocytic infiltrative lung disease, postinfectious interstitial lung disease, gouty arthritis, autoimmune hepatitis, type-1 autoimmune hepatitis (classical autoimmune or lupoid hepatitis), type-2 autoimmune hepatitis (anti-LKM antibody hepatitis), autoimmune mediated hypoglycaemia, type B insulin resistance with acanthosis nigricans, hypoparathyroidism,

acute immune disease associated with organ transplantation, chronic immune disease associated with organ transplantation, osteoarthritis, primary sclerosing cholangitis, psoriasis type 1, psoriasis type 2, idiopathic leucopaenia, autoimmune neutropaenia, renal disease NOS, glomerulonephritides, microscopic vasculitis of the kidneys, Lyme disease, discoid lupus erythematosus, male infertility idiopathic or NOS, sperm autoimmunity, multiple sclerosis (all subtypes), sympathetic ophthalmia, pulmonary hypertension secondary to connective tissue disease, acute and chronic pain (different forms of pain), Goodpasture's syndrome, pulmonary manifestation of polyarteritis nodosa, acute rheumatic fever, rheumatoid spondylitis, Still's disease, systemic sclerosis, Sjögren's syndrome, Takayasu's disease/arteritis, autoimmune thrombocytopaenia, toxicity, transplants, and diseases involving inappropriate vascularization for example diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization due to age-related macular degeneration, and infantile hemangiomas in human beings.

In addition, the subject diseases, disorders, or conditions may also include ascites, effusions, and exudates, including for example macular edema, cerebral edema, acute lung injury, adult respiratory distress syndrome (ARDS), proliferative disorders such as restenosis, fibrotic disorders such as hepatic cirrhosis and atherosclerosis, mesangial cell proliferative disorders such as diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes, and glomerulopathies, myocardial angiogenesis, coronary and cerebral collaterals, ischemic limb angiogenesis, ischemia/reperfusion injury, peptic ulcer Helicobacter related diseases, virally-induced angiogenic disorders, preeclampsia, menometrorrhagia, cat scratch fever, rubeosis, neovascular glaucoma and retinopathies such as those associated with diabetic retinopathy, retinopathy of prematurity, or age-related macular degeneration.

Further, the subject diseases, disorders, or conditions may also include hyperproliferative disorders such as thyroid hyperplasia (especially Grave's disease), and cysts (such as hypervascularity of ovarian stroma characteristic of polycystic ovarian syndrome (Stein-Leventhal syndrome) and polycystic kidney disease since such diseases require a proliferation of blood vessel cells for growth and/or metastasis.

In certain embodiments, the disease or condition is not CLL (Chronic Lymphocytic Leukemia).

The methods and reagents of the invention can be used to measure the extent of Syk activation in any one or more of the diseases or conditions described herein.

3. *HS1 and pY-HS1*

Hematopoietic Cell-specific Lyn Substrate 1 (HCLS1, or HS1) cDNA was cloned by screening a hybridoma cDNA library with a probe to the transactivating region of adenovirus-2 E1A (Kitamura *et al.*, *Nucleic Acids Res.* 17: 9367-9379, 1989).

Human HS1 is a 486-amino acid hydrophilic protein that lacks a signal peptide, N-glycosylation sites, and a transmembrane region, but contains several potential phosphorylation sites. It has an N-terminal series of at least three 37-amino acid repeats (each of which includes 2 alpha helices) called HS1 repeats, which are also found in cortactin. HS1 also has a central region homologous to the adenovirus E1A probe and a C-terminal SH3 domain.

HS1 can associate with the SH2 and SH3 domains of Lck. Binding to the Lck SH3 domain occurs constitutively, while binding to the Lck SH2 domain occurs only upon TCR stimulation. HS1 is also directly associated with HAX1, through binding to its C-terminal region. HS1 further interacts with HS1BP3; with FES/FPS; and with FGR via SH2 domain. A multiprotein complex may be formed among HS1, Lyn, and ANKRD54.

HS1 can be phosphorylated by FES; by LYN, FYN, and FGR after cross-linking of surface IgM on B-cells. Phosphorylation by LYN, FYN and FGR also requires prior phosphorylation by Syk or FES.

Syk and Fes are capable of phosphorylating Tyr residues 378 and 397 of HS1, while FGR is capable of phosphorylating Tyr 222 of HS1. In addition, Tyr 103, Tyr 140, Tyr 198, Ser 275, and Thr 308 of human HS1 can also be phosphorylated.

The NCBI Ref Seq for human HS1 is NP_005326.2, which represents human HS1 isoform 1 encoded by HS1 transcript variant 1. Shorter HS1 isoforms encoded by other transcript variants may exist. For the instant invention, “human HS1 (protein)” refers to human HS1 isoform 1 having the sequence of NP_005326.2, as reproduced below:

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1  MWKSVVGHVDV  SVSVETQGDD  WDTDPDFVND  ISEKEQRWGA  KTIEGSGRTE  HINIHQLRNK
61  VSEEHDLVRK  KEMESGPKAS  HGYGGRFGVE  RDRMDKSAVG  HEYVAEVEKH  SSQTDAAKGF
121 GGKYGVERDR  ADKSAVGFDY  KGEVEKHTSQ  KDYSRGFGGR  YGVEKDKWDK  AALGYDYKGE
181 TEKHEQRDY  AKGFGGQYGI  QKDRVDKSAV  GFNEMEAPTT  AYKKTTPIEA  ASSGTRGLKA
241 KFESMAEEKR  KREEEKAQQ  VARRQQRKA  VTKRSPEAPQ  PVIAMEEPAV  PAPLPKKISS
301 EAWPPVGTPP  SSESEPVRTS  REHPVPLLP  RQTLPEDNEE  PPALPPRTLE  GLQVEEPPVY
361 EAEPEPEPEP  EPEPENDYED  VEEMDRHEQE  DEPEGDYEEV  LEPEDSSFSS  ALAGSSGCPA
421 GAGAGAVALG  ISAVALYDYQ  GEGSDELSFD  PDDVITDIEM  VDEGWWRGRC  HGHFGLFPAN
481 YVKLLE
(SEQ ID NO: 1)

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Additional mammalian HS1 protein sequences are known in the art, and can be retrieved from public database such as GenBank by, for example, performing a BLASTp search using SEQ ID NO: 1 as a query sequence.

The result of the BLASTp search also includes sequence alignments with SEQ ID NO: 1. Thus a tyrosine residue corresponding to Y397 of human HS1 in any non-human mammalian HS1 protein can be readily identified.

Representative non-human mammalian HS1 proteins include (but are not limited to):

XP_003894042 from *Papio anubis* (olive baboon); XP_003825220 from *Pan paniscus* (pygmy chimpanzee); XP_002813282 from *Pongo abelii* (Sumatran orangutan); XP_004316855 from *Tursiops truncatus* (bottlenose dolphin); XP_004278593 from *Orcinus orca* (killer whale); XP_003275552 from *Nomascus leucogenys* (northern white-cheeked gibbon); XP_005548084 from *Macaca fascicularis* (crab-eating macaque); XP_006218952 from *Vicugna pacos* (alpaca); XP_516684 from *Pan troglodytes* (chimpanzee); XP_006090999 from *Myotis lucifugus* (little brown bat); XP_005654114 from *Sus scrofa* (pig); NP_001030229 from *Bos taurus* (cattle); XP_001502333 from *Equus caballus* (horse); XP_003991734 from *Felis catus* (domestic cat); NP_001011898 from *Rattus norvegicus* (Norway rat); and NP_032251 from *mus musculus* (house mouse).

Thus the methods and reagents of the invention can be used for human and other non-human mammals, including but not limited to non-human primates, livestock (mammalian) animals, experimental / laboratory animals (e.g., rats, mice, hamsters, or other rodents), mammalian pets (e.g., cats or dogs), or marine mammals, such as those named above.

As used herein, “tyrosine-phosphorylated HS1 protein (pY-HS1)” generally includes an HS1 protein (human or other non-human mammalian species) that is phosphorylated on at least one tyrosine residue, such as Tyr 103, 140, 198, 222, 378, and/or 397. Preferably, the pY-HS1 is phosphorylated on Tyr 397 of human HS1, or a tyrosine residue corresponding to Y397 of human HS1. In certain embodiments, the HS1 (human or non-human mammal) may include additional phosphorylation on one or more other Tyr, Ser, or Thr residues. In certain embodiments, the HS1 (human or non-human mammal) is phosphorylated only on Tyr 397 of human HS1, or a tyrosine residue corresponding to Y397 of human HS1.

4. *Samples and sampling*

The methods of the invention involve analyzing one or more samples derived from an individual, such as a mammal. The sample may be any suitable type that allows for the analysis of HS1 phosphorylation status. For example, the sample may be obtained from tissues and cells of hematopoietic origin.

Samples may be obtained once or multiple times from an individual. Multiple samples may be obtained from different locations in the individual (*e.g.*, blood samples, bone marrow samples and/or lymph node samples), at different times from the individual (*e.g.*, a series of samples taken to monitor response to treatment or to monitor for return of a pathological condition), or any combination thereof.

These and other possible sampling combinations based on the sample type, location and time of sampling allows for the detection of the presence and the status of diseases, or pre-pathological or pathological conditions, the measurement of treatment response and also the monitoring for disease or conditions.

When samples are obtained as a series, *e.g.*, a series of whole blood samples obtained after treatment, the samples may be obtained at fixed intervals, at intervals determined by the status of the most recent sample or samples, or by other characteristics of the individual, or some combination thereof. For example, samples may be obtained at intervals of approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 hours, at 1, 2, 3, or 4 weeks, at intervals of approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 months, at intervals of approximately 1, 2, 3, 4, 5, or more than 5 years, or some combination thereof. It will be appreciated that an interval may not be exact, according to an individual's availability for

sampling and the availability of sampling facilities. Thus approximate intervals corresponding to an intended interval scheme are encompassed by the invention.

As an example, an individual who has undergone treatment for a subject disease or condition may be sampled (*e.g.*, by blood draw) relatively frequently (*e.g.*, every hour, day, week, month or every three months) for the first six months to a year or two after treatment. Then if no abnormality is found, less frequently (*e.g.*, at times between six months and a year) thereafter. If, however, any abnormalities or other circumstances are found in any of the intervening times, or during the sampling, sampling intervals may be modified.

Generally, the most easily obtained samples are fluid samples. Fluid samples include normal and pathologic bodily fluids and aspirates of those fluids. Fluid samples also comprise rinses of organs and cavities (lavage and perfusions). Bodily fluids include whole blood, bone marrow aspirate, synovial fluid, cerebrospinal fluid, saliva, sweat, tears, semen, sputum, mucus, menstrual blood, breast milk, urine, lymphatic fluid, amniotic fluid, placental fluid and effusions such as cardiac effusion, joint effusion, pleural effusion, and peritoneal cavity effusion (ascites). Rinses can be obtained from numerous organs, body cavities, passage ways, ducts and glands. Sites that can be rinsed include lungs (bronchial lavage), stomach (gastric lavage), gastrointestinal track (gastrointestinal lavage), colon (colonic lavage), vagina, bladder (bladder irrigation), breast duct (ductal lavage), oral, nasal, sinus cavities, and peritoneal cavity (peritoneal cavity perfusion).

In some embodiments, the sample is a blood sample. In some embodiments, the sample or samples is whole blood sample. In some embodiments, the sample is a bone marrow sample. In some embodiments, the sample is a lymph node sample. In some embodiments, the sample is cerebrospinal fluid. In some embodiments, combinations of one or more of a blood, bone marrow, cerebrospinal fluid, and lymph node sample are used.

In certain embodiments, solid tissue samples may also be used, either alone or in conjunction with fluid samples. Solid samples may be derived from individuals by any method known in the art, including surgical specimens, biopsies, and tissue scrapings, including cheek scrapings. Surgical specimens include samples obtained during

exploratory, cosmetic, reconstructive, or therapeutic surgery. Biopsy specimens can be obtained through numerous methods including bite, brush, cone, core, cytological, aspiration, endoscopic, excisional, exploratory, fine needle aspiration, incisional, percutaneous, punch, stereotactic, and surface biopsy.

In one embodiment, a sample may be obtained from an apparently healthy individual, such as during a routine checkup, and analyzed so as to provide an assessment of the individual's status of a subject disease or condition.

In another embodiment, a sample may be taken to screen for a subject disease or condition of interest. Such screening may encompass testing for a single disease, a family of related diseases or a general screening for multiple, unrelated diseases. Screening can be performed weekly, biweekly, monthly, bi-monthly, every several months, annually, or in multi year intervals and may replace or complement existing screening modalities.

In another embodiment, an individual with a known increased probability of disease occurrence may be monitored regularly to detect for the appearance of a particular disease or class of diseases. An increased probability of disease occurrence can be based on familial association, age, previous genetic testing results, or occupational, environmental or therapeutic exposure to disease causing agents.

Individuals with increased risk for specific diseases can be monitored regularly for the first signs of a symptom of a subject disease or condition. Monitoring can be performed weekly, bi-weekly, monthly, bi-monthly, every several months, annually, or in multi year intervals, or any combination thereof. Monitoring may replace or complement existing screening modalities. Through routine monitoring, early detection of the presence of disease causative or associated cells may result in increased treatment options including treatments with lower toxicity and increased chance of disease control or cure.

In a further embodiment, testing can be performed to confirm or refute the presence of a suspected genetic or physiologic abnormality associated with increased risk of disease. Such testing methodologies can replace other confirmatory techniques like cytogenetic analysis or fluorescent *in situ* histochemistry (FISH). In still another embodiment, testing can be performed to confirm or refute a diagnosis of a pre-pathological or pathological condition.

In certain embodiments, an individual treated to reverse or arrest the progression of a disease or pre-pathological condition can be monitored to assess the reversion rate of the

treatment. If the anticipated reversion rate is not seen, further treatment with the same or a different treatment regimen can be considered.

Certain fluid samples can be analyzed in their native state with or without the addition of a diluent or buffer. Alternatively, fluid samples may be further processed or derived to obtain enriched or purified cell populations prior to analysis. Numerous enrichment and purification methodologies for bodily fluids are known in the art. For example, a common method to separate cells from plasma in whole blood is through centrifugation using heparinized tubes. By incorporating a density gradient, further separation of the lymphocytes (such as B lymphocytes) from the red blood cells can be achieved. A variety of density gradient media are known in the art including sucrose, dextran, bovine serum albumin (BSA), FICOLL diatrizoate (Pharmacia), FICOLL metrizoate (Nycomed), PERCOLL (Pharmacia), metrizamide, and heavy salts such as cesium chloride. Alternatively, red blood cells can be removed through lysis with an agent such as ammonium chloride prior to centrifugation.

Whole blood can also be applied to filters that are engineered to contain pore sizes that select for the desired cell type or class. For example, rare pathogenic cells can be filtered out of diluted, whole blood following the lysis of red blood cells by using filters with pore sizes between 5 to 10 μm , as disclosed in U.S. patent application publication US 2002-0028431 A1 (incorporated by reference). Alternatively, whole blood can be separated into its constituent cells based on size, shape, deformability or surface receptors or surface antigens by the use of a micro fluidic device as disclosed in U.S. patent application publication US 2006-0134599 A1 (incorporated by reference).

Select cell populations can also be enriched for or isolated from whole blood through positive or negative selection based on the binding of antibodies or other entities that recognize cell surface or cytoplasmic constituents.

Solid tissue samples may require the disruption of the extracellular matrix or tissue stroma and the release of single cells for analysis. Various techniques are known in the art including enzymatic and mechanical degradation employed separately or in combination. An example of enzymatic dissociation using collagenase and protease can be found in Wolters *et al.*, "An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation," *Diabetologia*, 35:735-742, 1992. Examples of mechanical dissociation can be found in Singh, N P., "Technical

Note: A rapid method for the preparation of single-cell suspensions from solid tissues,” *Cytometry*, 31:229-232 (1998). Alternately, single cells may be removed from solid tissue through microdissection including laser capture microdissection as disclosed in Emmert-Buck, M. R. *et al.*, “Laser Capture Microdissection,” *Science*, 274(8):998-1001, 1996. All incorporated herein by reference.

5. *Modulators*

The methods and reagents of the invention can be used in samples from any individual, with or without pre-treating the sample or individual (prior to the sample being taken) with one or more modulators that may activate or inhibit Syk signaling pathway components, including Syk itself.

In certain embodiments, one or more samples may be taken from the individual, and subjected to treatment by a modulator, as described herein. In some embodiments, the sample is divided into sub-samples that are each subjected to a different modulator. In some embodiments, the analysis includes the determination of the level and/or amount of pY-HS1 protein phosphorylated at Y397 of human HS1, or an equivalent position in a non-human HS1 protein. Determination of the status may be achieved by the use of activation / phosphorylation state-specific binding elements, such as antibodies, as described herein.

In some embodiments, the sample may be treated with at least one modulator. Such treatment can yield information regarding the effect of such modulators on phosphorylation status of HS1 at Y397, and disease status determination.

In some embodiments, the sample is divided into subsamples which are each treated with a different modulator.

A modulator may be an activator or an inhibitor, *e.g.*, a modulator may activate one or more activatable elements in one or more cellular signaling pathways (such as Syk signaling pathway), or inhibit one or more activatable elements in one or more cellular pathways (such as Syk signaling pathway). In certain embodiments, the modulator is an inhibitor of Syk kinase activity. The inhibitor may be a small chemical molecule, such as one of no more than 200, 500, or 1000 Da, or a polypeptide, or a nucleic acid based inhibitor (such as siRNA, shRNA, antisense RNA, ribozyme, *etc.*) that inhibits the synthesis of a target protein in the Syk activation pathway, such as Syk itself or a protein

required for Syk activation. See, for example, WO 2007/121347, WO 2005/049838, and WO 2005/007623 (all incorporated by reference).

Cells or samples or the individual can be treated with a modulator as a single pulse, or with sequential pulses. With sequential treatment, a modulator can be used at the same concentration and duration of exposure or at different concentrations and exposures. In some embodiments, cells or samples or the individual are treated with two modulators. In some embodiments, cells or samples or the individual are treated with 3, 4, 5, 6, 7, 8, 9, 10, or more modulators. These modulators can both be activators, inhibitors, or one can be an activator and the other an inhibitor.

Treatment can consist of simultaneous or sequential exposure to a combination of modulators. For example, a cell / sample / individual can be treated simultaneously with a B cell receptor activator such as F(ab)₂ against IgM or IgD, and a phosphatase inhibitor like H₂O₂.

Modulation can be performed in a variety of environments. In some embodiments, cells or samples are exposed to a modulator immediately after collection. In some embodiments where there is a mixed population of cells, purification of cells from the sample is performed after modulation. In some embodiments, whole blood is collected to which is added a modulator. In some embodiments, cells are modulated after processing for single cells or purified fractions of single cells. For example, whole blood can be collected and processed for an enriched fraction of lymphocytes (such as B lymphocytes) that are then exposed to a modulator.

In some embodiments, cells are cultured post collection in a suitable media before exposure to a modulator. In some embodiments, the media is a growth media. In some embodiments, the growth media is a complex media that may include serum. In some embodiments, the growth media comprises serum. In some embodiments, the serum is selected from the group consisting of fetal bovine serum, bovine serum, human serum, porcine serum, horse serum, and goat serum. In some embodiments, the serum level ranges from 0.0001% to 30%. In some embodiments, the growth media is a chemically defined minimal media and is without serum. In some embodiments, cells are cultured in a differentiating media.

Modulators include chemical and biological entities, and physical or environmental stimuli. Modulators can act extracellularly or intracellularly. Chemical and biological modulators include growth factors, cytokines, neurotransmitters, adhesion molecules, hormones, small molecules, inorganic compounds, polynucleotides, antibodies, natural compounds, lectins, lactones, chemotherapeutic agents, biological response modifiers, carbohydrate, proteases and free radicals. Modulators include complex and undefined biologic compositions that may comprise cellular or botanical extracts, cellular or glandular secretions, physiologic fluids such as serum, amniotic fluid, or venom. Physical and environmental stimuli include electromagnetic, ultraviolet, infrared or particulate radiation, redox potential and pH, the presence or absences of nutrients, changes in temperature, changes in oxygen partial pressure, changes in ion concentrations and the application of oxidative stress. Modulators can be endogenous or exogenous and may produce different effects depending on the concentration and duration of exposure to the single cells or whether they are used in combination or sequentially with other modulators. Modulators can act directly on the activatable elements or indirectly through the interaction with one or more intermediary biomolecule. Indirect modulation includes alterations of gene expression wherein the expressed gene product is the activatable element or is a modulator of the activatable element.

Modulators that are activators include ligands for cell surface receptors such as hormones, growth factors and cytokines. Other extracellular activators include antibodies or molecular binding entities that recognize cell surface markers or receptors including B cell receptor (BCR) complex, B cell coreceptor complex or surface immunoglobulins.

In one embodiment, cell surface markers, receptors or immunoglobulins are crosslinked by the activators. In a further embodiment, the crosslinking activator is a polyclonal IgM / IgD antibody, a monoclonal IgM / IgD antibody, F(ab)₂ IgM / IgD, biotinylated F(ab)₂ IgM / IgD, biotinylated polyclonal anti-IgM / -IgD, or biotinylated monoclonal anti-IgM / -IgD. In some embodiments, the modulator is a B cell receptor modulator. In some embodiments, the B cell receptor modulator is a B cell receptor activator.

An example of B cell receptor activator is a crosslinker of the B cell receptor complex or the B-cell co-receptor complex. In some embodiments, cross-linker is an antibody or molecular binding entity. In some embodiments, the cross linker is an antibody. In some embodiments, the antibody is a multivalent antibody. In some embodiments, the antibody is a monovalent, bivalent, or multivalent antibody made more multivalent by attachment

to a solid surface or tethered on a nanoparticle surface to increase the local valency of the epitope binding domain.

In some embodiments, the cross-linker is a molecular binding entity. In some embodiments, the molecular binding entity acts upon or binds the B cell receptor complex via carbohydrates or an epitope in the complex. In some embodiments, the molecular binding entity is monovalent, bivalent, or multivalent, and can be made more multivalent by attachment to a solid surface or tethered on a nanoparticle surface to increase the local valency of the epitope binding domain.

In some embodiments, the cross-linking of the B cell receptor complex or the B-cell co-receptor complex comprises binding of an antibody or molecular binding entity to the cell and then causing its crosslinking via interaction of the cell with a solid surface that causes crosslinking of the BCR complex via antibody or molecular binding entity.

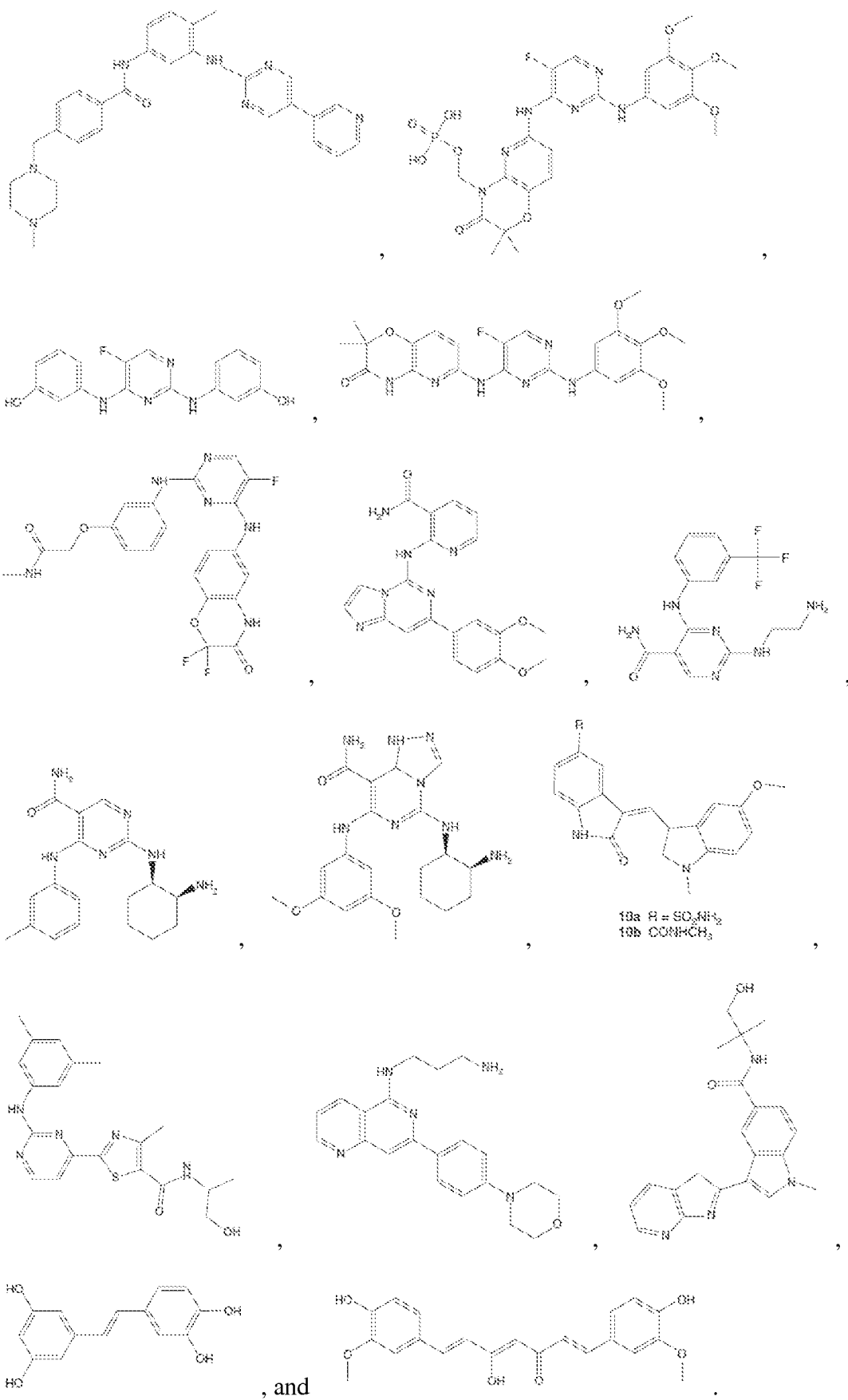
In some embodiments, the crosslinker is F(ab)₂ IgM, IgG, IgD, polyclonal BCR antibodies, monoclonal BCR antibodies, or Fc receptor derived binding elements. In some embodiments, the Ig is derived from a species selected from the group consisting of mouse, goat, rabbit, pig, rat, horse, cow, shark, chicken, or llama.

Inhibitory modulators include inhibitors of a cellular factor or a plurality of cellular factors that participate in a cell signaling pathway, such as the Syk signaling pathway. In certain embodiments, the inhibitor inhibits the activation of Syk, or inhibit the activity of a downstream target of activated Syk.

In certain embodiments, the inhibitor inhibits the kinase activity of Syk. For example, the inhibitor may be small-molecule inhibitors competing for the ATP-binding site of Syk.

Representative Syk kinase inhibitors may be any one of compounds disclosed in PCT/US2010/058598, filed on December 1, 2010, published as WO 2011/068899 (incorporated by reference), that have Syk kinase inhibitory activity.

Other Syk inhibitors that may be used in the subject methods include: fostamatinib (formerly R788) and the structurally related compounds R112, R406, and R343 developed by Rigel/Astra Zeneca (see Ruzza *et al.*, *Expert Opin. Ther. Patents* (2009) 19(10): 1361-1376, incorporated by reference), PRT062607 (Biogen Idec and Portola Pharmaceuticals); BAY 61-3606 and several pyrimidine-5-carboxamides compounds disclosed in Ruzza (*supra*). The structures of several representative Syk inhibitors are represented below.



In some embodiments, the methods of the invention provides for the use of more than one modulator. In some embodiments, the methods of the invention utilize a B cell receptor activator and a phosphatase inhibitor. In some embodiments, the methods of the invention utilize F(ab)₂ IgM / IgD or biotinylated F(ab)₂ IgM / IgD and H₂O₂.

6. *Determining / measuring pY-HS1 level*

The level of HS1 phosphorylated at Tyr 397 may be determined using any of many art-recognized means, including, but are not limited to: flow cytometry, Western blot, immunohistochemistry (IHC), immunofluorescent histochemistry with or without confocal microscopy, immunoelectronmicroscopy, mass spectrometry, 2-dimensional gel electrophoresis, differential display gel electrophoresis, microsphere-based multiplex protein assays, ELISA, Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and label-free cellular assays, or combination thereof.

In certain embodiments, the determining / measuring is carried out by a person, such as a lab technician. Alternatively, the determining / measuring is carried out using automated systems.

pY-HS1 can be detected and/or quantified by any method that detect and/or quantitates the presence of the same. Such methods may include radioimmunoassay (RIA) or enzyme linked immunoabsorbance assay (ELISA), immunohistochemistry, immunofluorescent histochemistry with or without confocal microscopy, reversed phase assays, homogeneous enzyme immunoassays, and related non-enzymatic techniques, Western blots, whole cell staining, immunoelectronmicroscopy, mass spectrometry, 2-dimensional gel electrophoresis, differential display gel electrophoresis, microsphere-based multiplex protein assays, label-free cellular assays, lateral flow device, and flow cytometry, *etc.* U.S. Pat. No. 4,568,649 describes ligand detection systems, which employ scintillation counting. These techniques are particularly useful for modified protein parameters. Readouts for proteins (*e.g.*, pY-HS1) and other cell determinants can be obtained using fluorescent or otherwise tagged reporter molecules (see below). For example, flow cytometry methods are useful for measuring intracellular parameters.

When using fluorescent labeled components in the methods and compositions of the present invention, it will be recognized that different types of fluorescent monitoring systems, *e.g.*, FACS systems, can be used to practice the invention. In some

embodiments, FACS systems are used or systems dedicated to high throughput screening, *e.g.* 96 well or greater micro titer plates. Methods of performing assays on fluorescent materials are well known in the art and are described in, *e.g.*, Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983); Flerman, B., "Resonance energy transfer microscopy," in: *Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology*, vol. 30, ed. Taylor, D. L. & Wang, Y.-L., San Diego: Academic Press (1989), pp. 219-243; Turro, N. J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Col, Inc. (1978), pp. 296-361.

Fluorescence in a sample can be measured using a fluorimeter. In general, excitation radiation, from an excitation source having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation that has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital computer. The computer also can transform the data collected during the assay into another format for presentation. In general, known robotic systems and components can be used.

In general, flow cytometry involves the passage of individual cells through the path of a laser beam. The scattering the beam and excitation of any fluorescent molecules attached to, or found within, the cell is detected by photomultiplier tubes to create a readable output, *e.g.* size, granularity, or fluorescent intensity.

The detecting, sorting, isolating, or measuring step of the methods of the present invention can entail fluorescence-activated cell sorting (FACS) techniques, where FACS is used to select cells from the population containing a particular surface marker, or the selection step can entail the use of magnetically responsive particles as retrievable supports for target cell capture and/or background removal. A variety of FACS systems are known in the art and can be used in the methods of the invention (see *e.g.*, W099/054494, filed Apr. 16, 1999; U.S. Ser. No. 20010006787, filed Jul. 5, 2001, each expressly incorporated herein by reference).

In some embodiments, a FACS cell sorter (*e.g.* a FACSVantage™ Cell Sorter, Becton Dickinson Immunocytometry Systems, San Jose, Calif.) may be used to sort, collect, and measure the amount of cells or fluorescent signals thereon based on their activation profile (positive cells) in the presence or absence of an increase in activation state in response to a modulator.

In some embodiments, the cells are first contacted with fluorescent-labeled activation state-specific binding elements (*e.g.* antibodies) directed against specific activation state of specific activatable elements (*e.g.*, pY-HS1 at Y397). In such an embodiment, the amount of bound binding element on each cell can be measured by passing droplets containing the cells through the cell sorter. By imparting an electromagnetic charge to droplets containing the positive cells, the cells can be separated from other cells. The positively selected cells can then be harvested in sterile collection vessels. These cell-sorting procedures are described in detail, for example, in the FACSVantage™ Training Manual, with particular reference to sections 3-11 to 3-28 and 10-1 to 10-17, which is hereby incorporated by reference in its entirety.

In another embodiment, positive cells or a selected population of cells (*e.g.*, B cells) can be sorted using magnetic separation of cells based on the presence of a marker on such cells. In such separation techniques, for example, cells to be positively selected (*e.g.*, B cells) are first contacted with a specific binding element (*e.g.*, an antibody or reagent that binds a B cell surface marker, such as CD19). The cells are then contacted with retrievable particles (*e.g.*, magnetically responsive particles) that are coupled with a reagent that binds the specific element (*e.g.*, marker specific antibody). The cell-binding element-particle complex can then be physically separated from cells without the marker, for example, using a magnetic field. When using magnetically responsive particles, the positive or labeled cells can be retained in a container using a magnetic field while the negative cells are removed. These and similar separation procedures are described, for example, in the Baxter Immunotherapy Isolex training manual which is hereby incorporated in its entirety.

Thus in some embodiment, cell analysis by flow cytometry on the basis of the activation state (*e.g.*, pY-HS1) may be combined with a determination of other flow cytometry readable outputs, such as the presence of surface markers (*e.g.*, on selected cell populations, such as B cells), granularity, and cell size (*e.g.*, platelets) to provide a correlation between the activation state (*e.g.*, pY-HS1) and other cell qualities measurable

by flow cytometry (*e.g.*, specific cell populations).

Thus, the invention provides methods of distinguishing cellular subsets within a larger cellular population. As outlined herein, these cellular subsets often exhibit altered biological characteristics (*e.g.* activation states, altered response to modulators) as compared to other subsets within the population. For example, as outlined herein, the methods of the invention allow the identification of subsets of cells from a population (*e.g.*, whole blood) such as primary cell populations, *e.g.*, B lymphocytes or platelets that exhibit altered responses (*e.g.* response associated with presence of a condition) as compared to other subsets. In addition, this type of evaluation distinguishes between different activation states, altered responses to modulators, cell lineages, cell differentiation states, *etc.*

In certain embodiments, the samples may be used directly. In other embodiments, the sample is first treated or prepared (*e.g.*, a sample “derived” from the individual) such that it is suitable for use in a specific detection method.

For example, for flow cytometry, a whole blood sample may first be fixed and then permeabilized, such that intracellular pY-HS1 can be detected by labeled antibodies. Optionally, there can be one or more intermediate washing, centrifugation, or blocking steps such that different reagents (such as fixing reagents and antibodies) do not interfere with one another's function, and to minimize any background signal.

Likewise, when necessary (such as for solid samples or biopsy), cells are dispersed into a single cell suspension, *e.g.* by enzymatic digestion with a suitable protease, *e.g.* collagenase, dispase, *etc.*, and the like. An appropriate solution is used for dispersion or suspension. Such solution will generally be a balanced salt solution, *e.g.* normal saline, PBS, Flasks balanced salt solution, *etc.*, conveniently supplemented with fetal calf serum or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from 5-25 mM. Convenient buffers include F1EPES1 phosphate buffers, lactate buffers, *etc.* The cells may be fixed, *e.g.* with 3% paraformaldehyde, and are usually permeabilized, *e.g.* with ice cold methanol; HEPESbuffered PBS containing 0.1% saponin, 3% BSA; covering for 2 min in acetone at -200°C; and the like as known in the art and according to the methods described herein.

In some embodiments, one or more cells are contained in a well of a 16-well, 48-well, 96-well plate or other commercially available multiwell plate. In an alternate embodiment,

the reaction mixture or cells are in a FACS machine. Other multiwell plates useful in the present invention include, but are not limited to 384 well plates and 1536 well plates. Still other vessels for containing the reaction mixture or cells and useful in the present invention will be apparent to the skilled artisan. The addition of the components of the assay for detecting the activation state or activity of an activatable element, or modulation of such activation state or activity, may be sequential or in a predetermined order or grouping under conditions appropriate for the activity that is assayed for. Such conditions are described here and known in the art.

Moreover, further guidance is provided below in the examples.

As will be appreciated by one of skill in the art, the instant methods and compositions find use in a variety of other assay formats in addition to flow cytometry analysis.

For example, in certain embodiments, the methods and compositions of the instant invention can be used in conjunction with a lateral flow device, which can qualitatively and/or quantitatively measure the concentration of an analyte (*e.g.*, pY397-HS1) in a biological fluid sample (*e.g.*, whole blood, serum, urine, saliva, and/or cellular extracts). See, for example, US 2014-0113280 A1 (incorporated herein by reference). This application describes a lateral flow test device for detecting the presence or quantifying the concentration of an analyte in a biological fluid, comprising: a housing defining a sample port in a surface of the housing; a test strip disposed within the housing; and a test well disposed within the housing between the sample port and the test strip; wherein the test well defines a flow path to the test strip and includes a mixer and a conjugate, the conjugate comprising a first antibody specific for a first epitope on the analyte and a signal entity; and wherein the test strip comprises a first end zone, a second end zone, and a trapping zone between the end zones, the trapping zone having irreversibly bound thereon a second antibody specific for a second epitope on the analyte.

The application also describes a lateral flow system for detecting the presence or quantifying the concentration of an analyte in a biological fluid, comprising: a lateral flow test device, the device comprising: a housing defining a sample port and a detection window in a surface of the housing; a test strip disposed within the housing, at least a portion of the test strip being associated with the detection window; and a test well disposed within the housing between the sample port and the test strip; wherein the test well defines a flow path from the sample port to the test strip and includes a mixer and a

conjugate, the conjugate comprising a first antibody specific for a first epitope on the analyte and a signal entity; and wherein the test strip comprises two end zones and a trapping zone between the two end zones, the trapping zone having irreversibly bound thereon a second antibody specific for a second epitope on the analyte; and a signal sensing device, the signal sensing device comprising: a processor; a detection head; a movable tray capable of holding the test device and moving the detection window under the detection head; and means for detecting a signal generated by the signal entity. Such lateral flow device and system can be readily adapted for use to detect and measure the amount of pY397-HS1 in a whole blood sample.

The system may further comprise a source for activating the mixer. In certain embodiments, the signal entity may be selected from the group consisting of an enzyme, fluorescent beads, fluorescent dots, and fluorescent water-soluble proteins. In certain embodiments, the means for detecting a signal is a voltage adjustable photomultiplier tube, a charge coupled device (CCD) system, or a Complementary Metal Oxide Semiconductor (CMOS) system. In certain embodiments, the signal sensing device may further comprise a light source and a light filter. In certain embodiments, the signal entity may be a fluorescent signal entity; and the signal sensing device may further comprise a light source and a light filter. In certain embodiments, the signal entity may be an enzyme capable of causing chemiluminescence or bioluminescence. In certain embodiments, the mixer may be a magnetic stirrer.

For example, in some embodiments, the methods and compositions of the instant invention can be used in conjunction with an "In-Cell Western Assay." In such an assay, cells of interest (such as B cells) may be plated at a desired density in an appropriate media and aliquoted into microwell plates (*e.g.*, Nunc™ 96 Microwell™ plates). Certain control wells are untouched, but experimental wells are incubated with a modulator, *e.g.* a Syk kinase inhibitor. After incubation with the modulator, cells are fixed and stained with labeled antibodies to the pY-HS1. Once the cells are labeled, the plates can be scanned using an imager such as the Odyssey Imager (LiCor, Lincoln Nebr.) using techniques described in the Odyssey Operator's Manual v1.2, which is hereby incorporated in its entirety. Data obtained by scanning of the multiwell plate can be analyzed and activation profiles determined.

In some embodiment, the methods of the invention (*e.g.*, multiplex version) include the use of liquid handling components. The liquid handling systems can include robotic

systems comprising any number of components. In addition, any or all of the steps outlined herein may be automated; thus, for example, the systems may be completely or partially automated, at least for the measuring, data collection, data comparison and storage, *etc.*

As will be appreciated by those in the art, there are a wide variety of components which can be used, including, but not limited to, one or more robotic arms; plate handlers for the positioning of microplates; automated lid or cap handlers to remove and replace lids for wells on non-cross contamination plates; tip assemblies for sample distribution with disposable tips; washable tip assemblies for sample distribution; 96 well loading blocks; cooled reagent racks; microtiter plate pipette positions (optionally cooled); stacking towers for plates and tips; and computer systems.

Fully robotic or microfluidic systems include automated liquid-, particle-, cell- and organism-handling including high throughput pipetting to perform all steps of screening applications. This includes liquid, particle, cell, and organism manipulations such as aspiration, dispensing, mixing, diluting, washing, accurate volumetric transfers; retrieving, and discarding of pipet tips; and repetitive pipetting of identical volumes for multiple deliveries from a single sample aspiration. These manipulations are cross-contamination-free liquid, particle, cell, and organism transfers. This instrument performs automated replication of microplate samples to filters, membranes, and/or daughter plates, high-density transfers, full-plate serial dilutions, and high capacity operation.

In some embodiments, platforms for multi-well plates, multi-tubes, holders, cartridges, minitubes, deep-well plates, microfuge tubes, cryovials, square well plates, filters, chips, optic fibers, beads, and other solid-phase matrices or platform with various volumes are accommodated on an upgradeable modular platform for additional capacity. This modular platform includes a variable speed orbital shaker, and multi-position work decks for source samples, sample and reagent dilution, assay plates, sample and reagent reservoirs, pipette tips, and an active wash station. In some embodiments, the methods of the invention include the use of a plate reader.

In some embodiments, thermocycler and thermoregulating systems are used for stabilizing the temperature of heat exchangers such as controlled blocks or platforms to provide accurate temperature control of incubating samples from 0°C to 100°C.

In some embodiments, interchangeable pipette heads (single or multi-channel) with single or multiple magnetic probes, affinity probes, or pipettors robotically manipulate the liquid, particles, cells, and organisms. Multi-well or multi-tube magnetic separators or platforms manipulate liquid, particles, cells, and organisms in single or multiple sample formats.

In some embodiments, the robotic apparatus includes a central processing unit which communicates with a memory and a set of input/output devices (*e.g.*, keyboard, mouse, monitor, printer, *etc.*) through a bus. Again, as outlined below, this may be in addition to or in place of the CPU for the multiplexing devices of the invention. The general interaction between a central processing unit, a memory, input/output devices, and a bus is known in the art. Thus, a variety of different procedures, depending on the experiments to be run, are stored in the CPU memory.

These robotic fluid handling systems can utilize any number of different reagents, including buffers, reagents, samples, washes, assay components such as label probes, *etc.*

7. *Binding Elements (e.g., antibodies)*

In some embodiments, the level of HS1 phosphorylated at Tyr 397 is determined / measured by contacting a sample with a binding element that is specific for HS1 phosphorylated at Tyr 397 (*e.g.*, the binding element does not bind HS1 with an unphosphorylated Tyr 397). In certain embodiments, the binding element binds to an epitope comprising a phosphorylated tyrosine corresponding to Y397 of human HS1, and does not bind an unphosphorylated tyrosine corresponding to Y397 of human HS1. In other embodiments, the binding element specifically recognizes a conformation of HS1 having Tyr phosphorylation at residue 397, but does not recognizes another conformation of HS1 having no Tyr phosphorylation at residue 397.

The term “binding element” as used herein includes any molecule, *e.g.*, peptide, nucleic acid (such as aptamer selected to bind to an pY-397 epitope), small organic molecule which is capable of detecting HS1 phosphorylated at Tyr 397, but not HS1 not phosphorylated at Tyr 397. When the binding element is an antibody, the term “activation state-specific antibody” or “activation state antibody” or grammatical equivalents thereof may be used to refer to such an antibody that specifically binds to pY-HS1.

In some embodiments, the binding element is a peptide, polypeptide, oligopeptide or a protein (such as an antibody or an antigen-binding fragment thereof, see below). The peptide, polypeptide, oligopeptide or protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus in certain embodiments, “amino acid,” or “peptide residue,” as used herein include both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine may be considered amino acids for the purposes of the invention. In other embodiments, “amino acid,” or “peptide residue,” as used herein include only naturally occurring amino acids. The amino acid side chains may be in either the (R) or the (S) configuration. In some embodiments, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradation. Proteins including non-naturally occurring amino acids may be synthesized or in some cases, made recombinantly: see van Hest *et al.*, *FEBS Lett* 428:(1-2) 68-70 May 22, 1998; and Tang *et al.*, *Abstr. Pap Am. Chem. S218: U138 Part 2* Aug. 22, 1999, both of which are expressly incorporated by reference herein.

In some embodiments, the binding element is an antibody. In some embodiment, the binding element is an activation state-specific antibody.

The term “antibody” includes full length antibodies and antibody fragments, and may refer to a natural antibody from any organism, an engineered antibody, or an antibody generated recombinantly for experimental, therapeutic, or other purposes as further defined below. Examples of antibody fragments, as are known in the art, such as Fab, Fab', F(ab')₂, Fv, scFv, or other antigen-binding subsequences of antibodies, either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA technologies. The term “antibody” comprises monoclonal and polyclonal antibodies. Antibodies can be antagonists, agonists, neutralizing, inhibitory, or stimulatory. The antibodies useful for the present invention may be nonhuman, chimeric, humanized, or fully human. The antibodies useful for the present invention may also be aglycosylated antibodies, which may be a deglycosylated antibody, a nonglycosylated or unglycosylated antibody.

In a specific embodiment, the antibody specifically bind to a Tyr phosphorylated HS1 (*e.g.*, at Tyr 397 of HS1) but do not bind to the corresponding nonphosphorylated HS1 (phospho-substrate antibodies).

Many antibodies suitable for the invention, such as antibodies that specifically bind to HS1 phosphorylated at Tyr 397 but do not bind to the corresponding HS1 not phosphorylated at Tyr 397, are commercially available. See, for example, Cell Signaling Technology Phospho-HS1 (Tyr397) (D12C1) XP® Rabbit mAb #8714, which is suitable for both Western blotting, immunofluorescence, and flow cytometry. Also see HS1 (D83A8) XP® Rabbit mAb (Human Specific) #3890. Such antibodies have been produced which specifically bind to Tyr 397 phosphorylated isoforms of HS1.

In some embodiments, an epitope-recognizing fragment or antigen-binding fragment of an activation state antibody (rather than the whole antibody) is used. In some embodiments, the epitope-recognizing fragment is immobilized. In some embodiments, the antibody light chain that recognizes an epitope is used. A recombinant nucleic acid encoding a light chain gene product that recognizes an epitope may be used to produce such an antibody fragment by recombinant means well known in the art.

In certain embodiments, non-activation state antibodies may also be used in the present invention. Non-activation state antibodies bind to epitopes in both activated and nonactivated forms of an element. Such antibodies may be used to determine the amount of HS1 phosphorylated and not phosphorylated at Tyr 397 in a sample. In some embodiments, non-activation state antibodies bind to epitopes present in HS1 non-phosphorylated at Tyr 397 but absent in HS1 phosphorylated at Tyr 397. Such antibodies may be used to determine the amount of non-phosphorylated HS1 in a sample. Both types of antibodies may be used to determine a change in the amount of pY-HS1, *e.g.*, from samples before and after treatment with a candidate bioactive agent or modulator as described herein.

In some embodiments the binding element is a nucleic acid, such as aptamers selected to bind pY-HS1 at Tyr 397 but not the non-phosphorylated form. The term “nucleic acid” includes nucleic acid analogs, for example, phosphoramidate (Beaucage *et al.* *Tetrahedron* 49(10): 1925 (1993) and references therein), phosphorothioate (Mag *et al.*, *Nucleic Acids Res.* 19:1437 (1991); and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu *et al.*, *J. Am. Chem. Soc.* 111:2321 (1989)), O-methylphosphoroamidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid (PNA) backbones and linkages (see Egholm, *J. Am. Chem. Soc.* 114:1895 (1992), incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6097 (1995)); non-

ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins *et al.*, *Chem. Soc. Rev.* (1995) pp. 169-176). These modifications of the ribosephosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. In some embodiments, peptide nucleic acids (PNA) which includes peptide nucleic acid analogs are used. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids.

8. *Labels*

The methods and compositions / kits of the invention may provide binding elements (*e.g.*, antibodies) comprising a label or tag. By label is meant a molecule that can be directly (*i.e.*, a primary label) or indirectly (*i.e.*, a secondary label) detected; for example a label can be visualized and/or measured or otherwise identified so that its presence or absence can be known. A compound can be directly or indirectly conjugated to a label which provides a detectable signal, *e.g.* radioisotopes, fluorescers, enzymes, antibodies, particles such as magnetic particles, chemiluminescers, or specific binding molecules, *etc.* Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin *etc.* Examples of labels include, but are not limited to, optical fluorescent and chromogenic dyes including labels, label enzymes and radioisotopes.

In some embodiments, one or more binding elements are uniquely label. Using the example of two activation state specific antibodies, by “uniquely labeled” is meant that a first activation state antibody recognizing a first binding target (*e.g.*, pY-HS1) comprises a first label, and second activation state antibody recognizing a second binding target (*e.g.*, the corresponding non-phosphorylated HS1) comprises a second label, wherein the first and second labels are detectable and distinguishable, making the first antibody and

the second antibody uniquely labeled.

In general, labels fall into four classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal labels; c) colored, optical labels including luminescent, phosphorous and fluorescent dyes or moieties; and d) binding partners.

Labels can also include enzymes (horseradish peroxidase, *etc.*) and magnetic particles. In some embodiments, the detection label is a primary label. A primary label is one that can be directly detected, such as a fluorophore.

Labels include optical labels such as fluorescent dyes or moieties. Fluorophores can be either “small molecule” fluors, or proteinaceous fluors (*e.g.* green fluorescent proteins and all variants thereof).

Suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malachite green, stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, IAEDANS, EDANS, BODIPY FL, LC Red 640, Cy 5, Cy 5.5, LC Red 705 and Oregon green. Suitable optical dyes are described in the 1996 *Molecular Probes Handbook* by Richard P. Flaugland, hereby expressly incorporated by reference. Suitable fluorescent labels also include, but are not limited to, green fluorescent protein (GFP; Chalfie *et al.*, *Science* 263 (5148):802-805 (Feb. 11, 1994); and EGFP; Clontech - Genbank Accession Number U55762), blue fluorescent protein (BFP; Quantum Biotechnologies, Inc. 1801 de Maisonneuve Blvd. West, 8th Floor, Montreal (Quebec) Canada H3H 1 J9), enhanced yellow fluorescent protein (EYFP; Clontech Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, Calif. 94303), luciferase (Ichiki, *et al.*, *J. Immunol.*, 150(12): 5408-5417 (1993)), β -galactosidase (Nolan, *et al.*, *Proc. Natl. Acad. Sci. USA*, 85(8):2603-2607 (April 1988)) and Renilla WO 92/15673; WO 95/07463; WO 98/14605; WO 98/26277; WO 99/49019; U.S. Pat. No. 5,292,658; U.S. Pat. No. 5,418,155; U.S. Pat. No. 5,683,888; U.S. Pat. No. 5,741,668; U.S. Pat. No. 5,777,079; U.S. Pat. No. 5,804,387; U.S. Pat. No. 5,874,304; U.S. Pat. No. 5,876,995; and U.S. Pat. No. 5,925,558). All of the above-cited references are expressly incorporated herein by reference.

In some embodiments, labels for use in the present invention include: Alexa-Fluor dyes (Alexa Fluor 350, Alexa Fluor 430, Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 660, Alexa Fluor 680), Cascade Blue, Cascade Yellow and R-phycoerythrin (PE) (Molecular Probes) (Eugene, OR), FITC,

Rhodamine, and Texas Red (Pierce, Rockford, 111.), Cy5, Cy5.5, Cy7 (Amersham Life Science, Pittsburgh, PA). Tandem conjugate protocols for Cy5PE, Cy5.5PE, Cy7PE, Cy5.5APC, Cy7APC can be found at [www dot dmr dot com slash abcon slash index dot html](http://www.dot.dmr.com/slash/abcon/slash/index.dot.html). Antibodies and labels are commercially available at Becton Dickinson.

Quantitation of fluorescent probe conjugation may be assessed to determine degree of labeling and protocols including dye spectral properties are also well known in the art. In some embodiments, the fluorescent label is a GFP and, more preferably, a Renilla, Ptilosarcus, or Aequorea species of GFP.

In some embodiments, a secondary detectable label is used. A secondary label is one that is indirectly detected; for example, a secondary label can bind or react with a primary label for detection, can act on an additional product to generate a primary label (*e.g.* enzymes), *etc.* Secondary labels include, but are not limited to, one of a binding partner pair; chemically modifiable moieties; nuclease inhibitors, enzymes such as horseradish peroxidase, alkaline phosphatases, luciferases, *etc.*

In some embodiments, the secondary label is a binding partner pair. For example, the label may be a hapten or antigen, which will bind its binding partner. For example, suitable binding partner pairs include, but are not limited to: antigens (such as proteins (including peptides) and small molecules) and antibodies (including fragments thereof (FABs, *etc.*)); proteins and small molecules, including biotin/streptavidin; enzymes and substrates or inhibitors; other protein-protein interacting pairs; receptor-ligands; and carbohydrates and their binding partners. Nucleic acid-nucleic acid binding proteins pairs are also useful. Binding partner pairs include, but are not limited to, biotin (or imino-biotin) and streptavidin, digoxin and Abs, and Prolinx™ reagents.

In some embodiments, the binding partner pair comprises an antigen and an antibody that will specifically bind to the antigen. By “specifically bind” herein is meant that the partners bind with specificity sufficient to differentiate between the pair and other components or contaminants of the system. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, the dissociation constants of the pair will be less than about 10^{-4} to 10^{-9} M⁻¹, with less than about 10^{-5} to 10^{-9} M⁻¹ being preferred and less than about 10^{-7} to 10^{-9} M⁻¹ being particularly preferred.

In some embodiment, the secondary label is a chemically modifiable moiety. In this embodiment, labels comprising reactive functional groups are incorporated into the molecule to be labeled. The functional group can then be subsequently labeled (*e.g.* either before or after the assay) with a primary label. Suitable functional groups include, but are not limited to, amino groups, carboxy groups, maleimide groups, oxo groups and thiol groups, with amino groups and thiol groups being particularly preferred. For example, primary labels containing amino groups can be attached to secondary labels comprising amino groups, for example using linkers as are known in the art; for example, homo- or heterobifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference).

In some embodiments, multiple fluorescent labels are employed in the methods and compositions of the present invention. In some embodiments, each label is distinct and distinguishable from other labels. As will be appreciated in the art antibody-label conjugation may be performed using standard procedures or by using protein-protein/protein-dye crosslinking kits from Molecular Probes (Eugene, OR).

Alternatively, detection systems based on FRET, discussed in detail below, may be used. FRET finds use in the instant invention, for example, in detecting activation states that involve clustering or multimerization wherein the proximity of two FRET labels is altered due to activation. In some embodiments, at least two fluorescent labels are used which are members of a fluorescence resonance energy transfer (FRET) pair.

FRET is phenomenon known in the art wherein excitation of one fluorescent dye is transferred to another without emission of a photon. A FRET pair consists of a donor fluorophore and an acceptor fluorophore. The fluorescence emission spectrum of the donor and the fluorescence absorption spectrum of the acceptor must overlap, and the two molecules must be in close proximity. The distance between donor and acceptor at which 50% of donors are deactivated (transfer energy to the acceptor) is defined by the Forster radius (R_0), which is typically 10-100 Å. Changes in the fluorescence emission spectrum comprising FRET pairs can be detected, indicating changes in the number of that are in close proximity. This will typically result from the binding or dissociation of two molecules, one of which is labeled with a FRET donor and the other of which is labeled

with a FRET acceptor, wherein such binding brings the FRET pair in close proximity. Binding of such molecules will result in an increased fluorescence emission of the acceptor and/or quenching of the fluorescence emission of the donor.

FRET pairs (donor/acceptor) useful in the invention include, but are not limited to, EDANS/fluorescein, IAEDANS/fluorescein, fluorescein/tetramethylrhodamine, fluorescein/LC Red 640, fluorescein/Cy 5, fluorescein/Cy 5.5 and fluorescein/LC Red 705. In certain embodiments, a primary antibody is labeled by one member of a FRET pair, and a secondary antibody is labeled by the other member of the FRET pair.

In some embodiments when FRET is used, a fluorescent donor molecule and a nonfluorescent acceptor molecule ("quencher") may be employed. In this application, fluorescent emission of the donor will increase when quencher is displaced from close proximity to the donor and fluorescent emission will decrease when the quencher is brought into close proximity to the donor. Useful quenchers include, but are not limited to, TAMRA, DABCYL, QSY 7 and QSY 33. Useful fluorescent donor/quencher pairs include, but are not limited to EDANS/DABCYL, Texas Red/DABCYL, BODIPY/DABCYL, Lucifer yellow/DABCYL, coumarin/DABCYL and fluorescein/QSY 7 dye.

The skilled artisan will appreciate that FRET and fluorescence quenching allow for monitoring of binding of labeled molecules over time, providing continuous information regarding the time course of binding reactions.

Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "rationing." Changes in the absolute amount of substrate, excitation intensity, and turbidity or other background absorbances in the sample at the excitation wavelength affect the intensities of fluorescence from both the donor and acceptor approximately in parallel. Therefore the ratio of the two emission intensities is a more robust and preferred measure of cleavage than either intensity alone.

In certain embodiments, activation state-specific antibodies can also be labeled with quantum dots as disclosed by Chattopadhyay, P.K. *et al.*, "Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry," *Nat. Med.* 12:972-977 (2006). Quantum dot labels are commercially available through, for example, Invitrogen.

Quantum dot labeled antibodies can be used alone or they can be employed in conjunction with organic fluorochrome-conjugated antibodies to increase the total number of labels available. As the number of labeled antibodies increase so does the ability for sub typing known cell populations (such as B cells).

Additionally, activation state-specific antibodies can be labeled using chelated or caged lanthanides as disclosed by Erkki, J. *et al.*, "Lanthanide chelates as new fluorochrome labels for cytochemistry," *J. Flistochemistry Cytochemistry*, 36:1449-1451, 1988; and U.S. Pat. No. 7,018,850, entitled "Salicylamide-Lanthanide Complexes for Use as Luminescent Markers." Other methods of detecting fluorescence may also be used, *e.g.*, Quantum dot methods (see, *e.g.*, Goldman *et al.*, *J. Am. Chem. Soc.* (2002) 124:6378-82; Pathak *et al.* *J. Am. Chem. Soc.* (2001) 123:4103-4; and Remade *et al.*, *Proc. Natl. Sci. USA* (2000) 18:553-558, each expressly incorporated herein by reference) as well as confocal microscopy.

The methods and composition of the present invention may also make use of label enzymes. By "label enzyme" is meant an enzyme that may be reacted in the presence of a label enzyme substrate that produces a detectable product. Suitable label enzymes for use in the present invention include but are not limited to, horseradish peroxidase, alkaline phosphatase and glucose oxidase. Methods for the use of such substrates are well known in the art. The presence of the label enzyme is generally revealed through the enzyme's catalysis of a reaction with a label enzyme substrate, producing an identifiable product. Such products may be opaque, such as the reaction of horseradish peroxidase with tetramethyl benzedine, and may have a variety of colors. Other label enzyme substrates, such as Luminol (available from Pierce Chemical Co.), have been developed that produce fluorescent reaction products. Methods for identifying label enzymes with label enzyme substrates are well known in the art and many commercial kits are available. Examples and methods for the use of various label enzymes are described in Savage *et al.*, *Previews*, 247:6-9 (1998); Young, *J. Virol. Methods*, 24:227-236 (1989), which are each hereby incorporated by reference in their entirety.

By "radioisotope" is meant any radioactive molecule. Suitable radioisotopes for use in the invention include, but are not limited to ^{14}C , ^3H , ^{32}P , ^{33}S , ^{125}I , and ^{131}I . The use of radioisotopes as labels is well known in the art. Methods for labeling of proteins with radioisotopes are known in the art. For example, such methods are found in Ohta *et al.*, *Molec. Cell*, 3:535-541 (1999), which is hereby incorporated by reference in its entirety.

As mentioned, labels may be indirectly detected, that is, the tag is a partner of a binding pair. By “partner of a binding pair” is meant one of a first and a second moiety, wherein the first and the second moiety have a specific binding affinity for each other. Suitable binding pairs for use in the invention include, but are not limited to, antigens/antibodies (for example, digoxigenin/anti-digoxigenin, dinitrophenyl (DNP)/anti-DNP, dansyl-X-anti-dansyl, Fluorescein/antifluorescein, lucifer yellow/anti-lucifer yellow, and rhodamine anti-rhodamine), biotin/avidin (or biotin/streptavidin) and calmodulin binding protein (CBP)/calmodulin. Other suitable binding pairs include polypeptides such as the FLAG-peptide (Flopp *et al.*, *BioTechnology*, 6:1204-1210 (1988)); the KT3 epitope peptide (Martin *et al.*, *Science*, 255:192-194 (1992)); tubulin epitope peptide (Skinner *et al.*, *J. Biol. Chem.*, 266:15163-15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:6393-6397 (1990)) and the antibodies each thereto. As will be appreciated by those in the art, binding pair partners may be used in applications other than for labeling, as is described herein.

As will be appreciated by those in the art, a partner of one binding pair may also be a partner of another binding pair. For example, an antigen (first moiety) may bind to a first antibody (second moiety) that may, in turn, be an antigen for a second antibody (third moiety). It will be further appreciated that such a circumstance allows indirect binding of a first moiety and a third moiety via an intermediary second moiety that is a binding pair partner to each.

As will be appreciated by those in the art, a partner of a binding pair may comprise a label, as described above. It will further be appreciated that this allows for a tag to be indirectly labeled upon the binding of a binding partner comprising a label. Attaching a label to a tag that is a partner of a binding pair, as just described, is referred to herein as “indirect labeling.”

EXAMPLES

Example 1 Identification of HS1 as a proximal target engagement biomarker as a measure of Syk activity

Comparative pathway analysis was conducted to identify potential Syk substrates based on literature annotations, using the INGENUITY PATHWAY ASSIST™ and Fisher's Exact Test method. For the analysis presented herein, the indicated pathways had a

significance of <0.05 and at least two regulated transcripts. Well established substrates (*e.g.*, LAT & BLNK) were identified to validate the method, in addition to hypothetical substrates identified, such as HS1/HCLS1.

Example 2 HS1 phosphorylation at Y397 as detected by Western blotting

To conduct Western blotting, Ramos human B cells were starved overnight, and then stimulated with 40 $\mu\text{g}/\text{mL}$ goat anti-human IgG and IgM (H+L) (Jackson Labs, Cat. No. 109006127) for 30, 20, 15, 10, 5 or 2 minutes (or 0 minutes = unstimulated) at 37°C. Cells were first washed in 0.5 mL cold 1XPBS with protease inhibitor (Calbiochem Cat. No. 539131) and PhosSTOP phosphatase inhibitor (Roche Cat. No. 04906837001). The cells were then lysed in 0.5 mL lysis buffer (Cell Signaling 9803 with protease inhibitor and phosphatase inhibitor). About 25 μg of lysate was combined with Novex 2X sample buffer (LC2676) and 10X reducing agent (NP0009), boiled, and resolved on a Novex 4-20% Tris-Glycine gradient gel (EC6028). Resolved proteins were transferred to an Immobilon PVDF membrane (IPV2000), blocked for 1 hr with PBS/0.5% Tween20/3% gelatin from cold water fish skin and then blotted overnight at 4°C with anti-phospho Y397 HS1 (Cell Signaling Cat. No. 8714) or total HS1 (Cell Signaling Cat. No. 3890) diluted 1/1000 with PBS-T. Membrane was then incubated at room temperature (RT) for about 1 hour with goat anti-rabbit IgG-HRP (Molecular Probes Cat. No. 987244) at 1:5000 in PBST/1% Tween 20. Membrane was then developed for 1 minute in Amersham ECL mixture (RPN2106) and Amersham Hyperfilm ECL (28906836) exposed. The results are shown in Figure 2, top 2 panels.

It is apparent that Y397 of HS1 was detectably phosphorylated in Ramos human B cells stimulated by IgG/IgM cross-linking within 2 minutes of stimulation. pY397 phosphorylation increased (adjusted based on same HS1 protein load) or peaked at 5 minutes post stimulation, and remained at about the same level through at least 30 minutes.

Example 3 HS1 phosphorylation at Y397 as detected by flow cytometry

Serum-starved Ramos B cells, or human, rat, or mouse whole blood were treated with compound for 45 minutes at 37°C, and then stimulated with (respectively) 40 $\mu\text{g}/\text{mL}$ goat

anti-human IgG and IgM (H+L) (Jackson Labs Cat. No. 109006127), 50 µg/mL anti-human IgD (Bethyl Labs A80-106A), 50 µg/mL anti-rat IgD (AbD Serotec MCA-190), or 50 µL of anti-mouse IgD antiserum (eBioscience 24-5093) for an additional 5 minutes at 37°C. Cells were then fix-lysed (BD 558049) for 5 minutes at 37°C, spun down and washed with BD stain buffer (554656) and spun down again. Rat or mouse cells were blocked for 15 minutes on ice with rat (BD 550271) or mouse (BD 553142) FC block diluted 1:100 in BD stain buffer. Cells were then permeabilised on ice for 30 minutes (BD 557885), and human cells were then blocked for 10 minutes on ice with permeabilisation buffer with 4% human serum. Cells were stained on ice for 1 - 2 hours with 1:50 anti-human Phospho-HS1-PE (Cell Signaling 11880BC), and 1:5 dilution of anti-human CD19-FITC (BD Biosciences 555412), or 1:100 anti-rat CD45R-FITC (eBioscience 11-0460-82) or 1:50 anti-mouse CD45R-FITC (BD Pharmingen 553088) in perm buffer. Cells were washed with 1 mL perm buffer, spun down, re-suspended in stain buffer, and acquired on a BD FACScalibur. Flow cytometric data was analyzed using FlowJo7.6.5. The Cell Signaling antibody 8714 was custom-conjugated to PE for flow cytometry. The results are shown in Figure 2, bottom panel, and Figure 3A (left side panels).

It is apparent that increased Syk-dependent HS1 tyrosine phosphorylation is detectable in anti-IgD-stimulated whole blood from multiple species.

Example 4 HS1 tyrosine phosphorylation is Syk- but not Btk-dependent

Figure 3B (right side panels) shows that anti-IgD-induced HS1 phosphorylation in human, mouse or rat B cells can be inhibited by various proprietary or tool Syk inhibitors. The IC₅₀ values of the various Syk inhibitors are provided in Figure 3B.

Geometric means and percent inhibition of anti-IgD-induced HS1 phosphorylation in rat whole blood, treated *ex vivo* with the proprietary Syk inhibitor Compound 1 or tool Btk inhibitor Compound 2, were presented in Figures 4A and 4B, respectively. It is apparent that the Syk inhibitor inhibited HS1 pY phosphorylation in a dose-dependent manner, while the BTK inhibitor is largely ineffective, except at the highest concentrations.

Example 5 Inhibition of HS1 tyrosine phosphorylation correlates with efficacy in the rat collagen-induced arthritis model

The rat collagen-induced arthritis (CIA) model was used to demonstrate the correlation between HS1 pY phosphorylation and efficacy of arthritis treatment.

Female Lewis rats aged 7 weeks were immunized with 600 µg of bovine type II collagen intradermally on day 0, and repeat boost was administered on day 6. Scoring was performed under isofluorane anesthesia on days 8, 11, 13, 15, and 18 post injection, and scoring was measured using micro-controlled volume meter where rats' ankles were dipped to the hair line and displacement was recorded in mL. Animals were housed 3 per cage. Trans-gel was provided beginning at first clinical signs of disease (day 11). Food and water were provided *ad libitum*.

The results shown in Figure 5 demonstrate that inhibition of HS1 tyrosine phosphorylation correlates with efficacy in the rat CIA model. Specifically, FIG. 5A shows inhibition of anti-IgD-induced HS1 phosphorylation in B cells from rats dosed with the tool Syk inhibitor Compound 3. FIG. 5B shows dose-dependent inhibition of a disease activity (*i.e.*, paw swelling) in dosed rats. FIG. 5C is pharmacodynamic (measure by HS1 phosphorylation) versus efficacy (measured by paw swelling) correlation.

Example 6 PKPD modeling reveals off-target inhibition by Fostamatinib

Fostamatinib is an experimental drug candidate for the treatment of a variety of diseases, and has been in clinical trials for treating rheumatoid arthritis, autoimmune thrombocytopenia, and lymphoma. The drug is administered orally as a disodium salt, and is a prodrug of the active compound taminib (R-406), which is a non-selective kinase inhibitor that inhibits the kinase activity of, for example, the enzyme spleen tyrosine kinase (Syk).

PKPD modeling of P-HS1 inhibition to target engagement reveals that Fostamatinib does not inhibit Syk at efficacious doses. Utilizing data from the *in vitro* whole blood assays and the efficacy studies, integrated PKPD modeling generated an effect vs. time profile of target engagement (FIG. 6A). Surprisingly, efficacious concentrations of Fostamatinib do not inhibit P-HS1, while achieving full efficacy (FIG. 6B).

Conversely, a known selective Syk inhibitor (Compound 4) does inhibit P-HS1 at its

efficacious doses (FIG. 6C).

This data indicates that the efficacy observed with Fostamatinib is likely being driven by off target inhibition.

Example 7 Inhibition of P-HS1 corresponds to efficacy in a preclinical model of lupus nephritis

This example demonstrates, via the mouse lupus model, that P-HS1 level inversely correlates to the severity of lupus nephritis.

Specifically, in the IFN α -accelerated mouse model of Lupus, NZB/W mice were *i.v.* injected with 5×10^9 IFN α adenovirus particles in 100 μ L PBS dose volume. Compound administration and scoring began one week later. Mice were assessed weekly for urine protein levels and body weight. Urine was manually expressed and individually tested for protein levels using Albustix reagent strips for urinalysis (Siemens 2191). Urine protein levels (PU) were graded visually according to the scale of: 0=Trace, 1=30 mg/dL, 3=100 mg/dL, 5=300 mg/dL and 7=2000+ mg/dL. Mice were considered to be proteinuric (elevated urine protein) if there were 2 consecutive Grades of PU > 5 (>300 mg/dL) or f Grade of PU > 5 prior to death or euthanasia.

A selective Syk inhibitor, Compound **4**, dose-dependently prevented the onset of proteinuria (FIG. 7A) and increased survival (FIG. 7B). The effect was dose responsive and corresponded to the level of pHS-1 inhibition (FIG. 7C).

Example 8 Basal and anti-IgD-induced HS1 phosphorylation are both dose-dependently inhibited by a Syk inhibitor in B cells from Rheumatoid Arthritis subjects

To demonstrate that HS1 phosphorylation level is correlated with Syk activity, whole blood samples were obtained from four donors / Rheumatoid Arthritis patients. B cells in the whole blood sample were either unstimulated (Figure 8A) or stimulated (Figure 8B) by anti-IgD antibody, before percent inhibition of HS1 phosphorylation at Y397 was plotted against a range of Syk inhibitor (Compound **4**) concentrations.

Figures 8A and 8B show that both basal and anti-IgD-induced HS1 phosphorylation at Y397 were dose-dependently inhibited by the Syk inhibitor in B cells from those

Rheumatoid Arthritis patients. The IC_{50} values for the inhibitor and Hill slope for each donor were provided in Figures 8A and 8B.

Example 9 Basal and anti-IgD-induced HS1 phosphorylation are both increased in B cells from Rheumatoid Arthritis (RA) versus healthy subjects

To demonstrate that RA patients have increased basal and anti-IgD-induced HS1 phosphorylation at Tyrosine residue 397 as compared to healthy control, HS1 phosphorylated at Tyr397 (pY-397 HS1) or total levels of HS1 were measured in CD19-positive B cells from 18 RA patients and 20 healthy subjects from two separate cohorts. Data is presented in Figure 9 as the geometric mean of pY-HS1 normalized to the geometric mean of total HS1.

It is apparent that the geometric mean of pY-397 HS1 (normalized to total HS1) in the RA patients is statistically significantly higher than that in the healthy individuals, for both Basal and anti-IgD-induced HS1 phosphorylation.

WE CLAIM:

1. A method for determining the status of a disease or condition in a mammal, the method comprising measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal,
 - (1) wherein the disease or condition is characterized by increased B cell activation as measured by increased pY-HS1 (mediated by Syk activation);
 - (2) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1;
 - (3) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1,
 - (a) wherein the whole blood sample is derived from the mammal; and,
 - (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and
 - (4) wherein the mammal is diagnosed as having a more advanced state of the disease or condition if the amount of the pY-HS1 in the whole blood sample is increased as compared to that of a reference range; and/or wherein the mammal is diagnosed as having a better state of the disease or condition or as being in remission, if the amount of the pY-HS1 in the whole blood sample is within the reference range.
2. The method of claim 1, wherein the disease is an autoimmune disease or an inflammatory disease.
3. The method of claim 1, wherein the disease is rheumatoid arthritis (RA).
4. The method of claim 1, wherein the disease is lupus nephritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS), or type I hypersensitivity reactions (such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis, allergic asthma, and systemic anaphylaxis).
5. The method of claim 1, wherein the mammal is a human, a rodent (*e.g.*, a rat or a mouse), or a non-human mammal.
6. The method of claim 1, wherein the extent of binding between the antibody and the pY-HS1 is detected using flow cytometry or Western blot.
7. The method of claim 1, wherein the extent of binding between the antibody and

- the pY-HS1 is detected using a strip test, a lateral flow device, or a dipstick.
8. The method of claim 1, wherein the extent of binding between the antibody and the pY-HS1 is detected using Enzyme-linked immunosorbent assay (ELISA).
 9. The method of claim 1, wherein the extent of binding between the antibody and the pY-HS1 is detected in B cells or in platelets of the whole blood sample.
 10. The method of claim 1, further comprising administering to the mammal either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition.
 11. The method of claim 1, wherein the mammal has previously been administered either an agent that is efficacious to treat the disease or condition, or a test agent that is potentially efficacious to treat the disease or condition.
 12. The method of claim 11, wherein the reference range represents the amount of the pY-HS1 in a baseline whole blood sample from said mammal.
 13. The method of claim 12, wherein the baseline whole blood sample is obtained from said mammal before the agent or test agent is administered to said mammal.
 14. The method of claim 12, wherein the baseline whole blood sample is obtained from said mammal after at least one dose of the agent or test agent has been administered to said mammal.
 15. The method of claim 10, wherein the agent or test agent inhibits HS1 phosphorylation at the tyrosine residue corresponding to Y397 of human HS1.
 16. The method of claim 15, wherein the agent or test agent is an inhibitor of a kinase.
 17. The method of claim 16, wherein the kinase is Syk.
 18. The method of claim 1, wherein the antibody binds to an epitope comprising a phosphorylated tyrosine corresponding to Y397 of human HS1, and does not bind an unphosphorylated tyrosine corresponding to Y397 of human HS1.
 19. The method of claim 1, wherein the whole blood sample is first contacted with an antibody that activates a B cell receptor (BCR), before determining the amount of the pY-HS1.
 20. The method of claim 19, wherein the antibody that activates BCR is an anti-IgD antibody, or an anti-IgM antibody.

21. The method of claim 1, wherein the reference range represents the amount of HS1 protein phosphorylated at the tyrosine corresponding to Y397 of human HS1 in a matching whole blood sample from the middle 90% of a healthy population.
22. The method of claim 1, further comprising assessing the degree or severity of the disease or condition based on a substantially linear relationship between the amount of the pY-HS1 and the degree or severity of the disease or condition.
23. The method of claim 22, wherein the disease or condition is lupus nephritis or SLE, and wherein the degree or severity of the disease or condition is measured by survival rate, or by onset of proteinuria.
24. The method of claim 22, further comprising administering to the mammal a therapeutic agent efficacious to treat the disease or condition, preferably based on the degree or severity of the disease or condition.
25. A method to adjust the dose of a therapeutic agent useful for treating a disease or condition in a mammal in need of treatment, the method comprising:
 - (1) administering a first dose of the therapeutic agent to the mammal;
 - (2) measuring the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal; and,
 - (3) comparing the amount of the pY-HS1 with a reference range;
 - (4) repeating step (1) with a dose higher than the first dose, if the amount of the pY-HS1 in the sample is higher than the maximum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range; or
repeating step (1) with a dose lower than the first dose, if the amount of the pY-HS1 in the sample is lower than the minimum of the reference range, until reaching a proper dose associated with an amount of the pY-HS1 within the reference range;wherein the disease or condition is mediated by Syk activation;
wherein the HS1 protein is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1; and,
wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1, wherein the whole blood sample is derived from the mammal, and wherein

the antibody binds the pY-HS1 but does not bind HS1 not phosphorylated at the tyrosine.

26. The method of claim 25, wherein the therapeutic agent is an inhibitor of Syk.
27. A method to identify a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising: administering the agent to the mammal, and determining the amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in a sample derived from the mammal,
 - (1) wherein the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1;
 - (2) wherein the measuring is performed by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1,
 - (a) wherein the whole blood sample is derived from the mammal; and,
 - (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue; and
 - (3) wherein the mammal is identified as having the disease or condition that may be susceptible or sensitive to treatment by the agent, if the amount of the pY-HS1 in the whole blood sample is decreased as compared to that of a reference range, wherein the reference range represents a baseline amount of the pY-HS1 in a whole blood sample derived from said mammal before the agent is administered to said mammal.
28. A method of treating a mammal having a disease or condition that may be susceptible or sensitive to treatment by an agent that disrupts B cell function, the method comprising:
 - (1) using the method of claim 27 to identify the mammal that may be susceptible or sensitive to treatment by said agent; and,
 - (2) administer said agent to said mammal, thereby treating the mammal having the disease or condition.
29. A method to compare therapeutic efficacy of a 1st therapeutic agent and a 2nd therapeutic agent for treating a disease or condition, the method comprising:
 - (1) administering the 1st therapeutic agent to a 1st population of mammals, and

- determining a 1st decrease, if any, in the average amount of a tyrosine-phosphorylated HS1 protein (pY-HS1) in samples derived from the 1st population of mammals, after administering the 1st therapeutic agent;
- (2) administering the 2nd therapeutic agent to a 2nd population of mammals, and determining a 2nd decrease, if any, in the average amount of the pY-HS1 in samples derived from the 2nd population of mammals, after administering the 2nd therapeutic agent;
- (3) comparing the 1st decrease to the 2nd decrease, wherein the larger decrease is indicative of a better therapeutic efficacy;

wherein:

- (i) the disease or condition is mediated by Syk activation;
- (ii) the pY-HS1 is phosphorylated at a tyrosine residue corresponding to Y397 of human HS1;
- (iii) the amount of the pY-HS1 is measured by contacting a whole blood sample with an antibody to detect the extent of binding between the antibody and the pY-HS1,
- (a) wherein the whole blood sample is derived from the mammal; and,
- (b) wherein the antibody binds the pY-HS1, but does not bind HS1 not phosphorylated at the tyrosine residue.
30. A kit for measuring Syk pathway activation, the kit comprising:
- (1) a reagent that activates B cell receptor (BCR); and,
- (2) an antibody specific for a phosphorylated tyrosine corresponding to Y397 of human HS1.
31. The kit of claim 30, further comprising:
- (3) an antibody specific for a B-cell surface marker, wherein the antibody is optionally labelled by a fluorescent dye (*e.g.*, FITC) or a radioactive moiety.
32. The kit of claim 30, wherein the antibody specific for the phosphorylated tyrosine is labelled by a fluorescent dye (*e.g.*, PE).

FIGURE 1

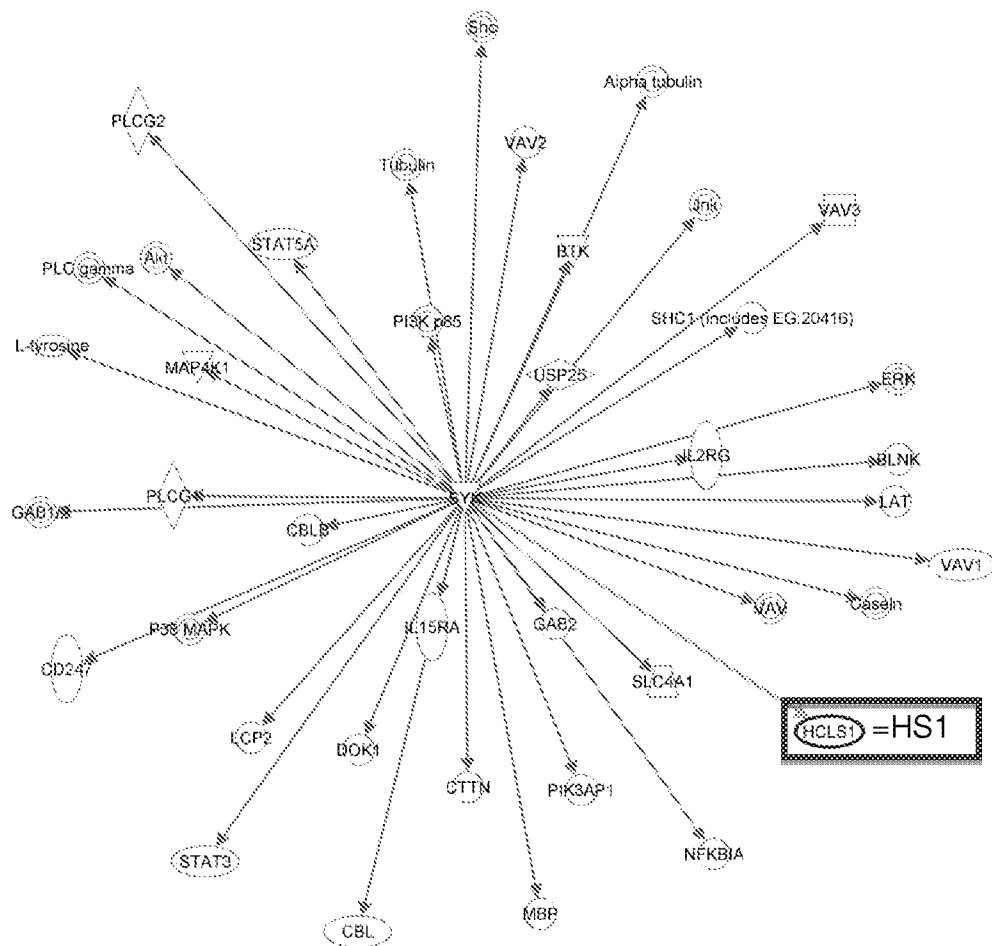


FIGURE 2

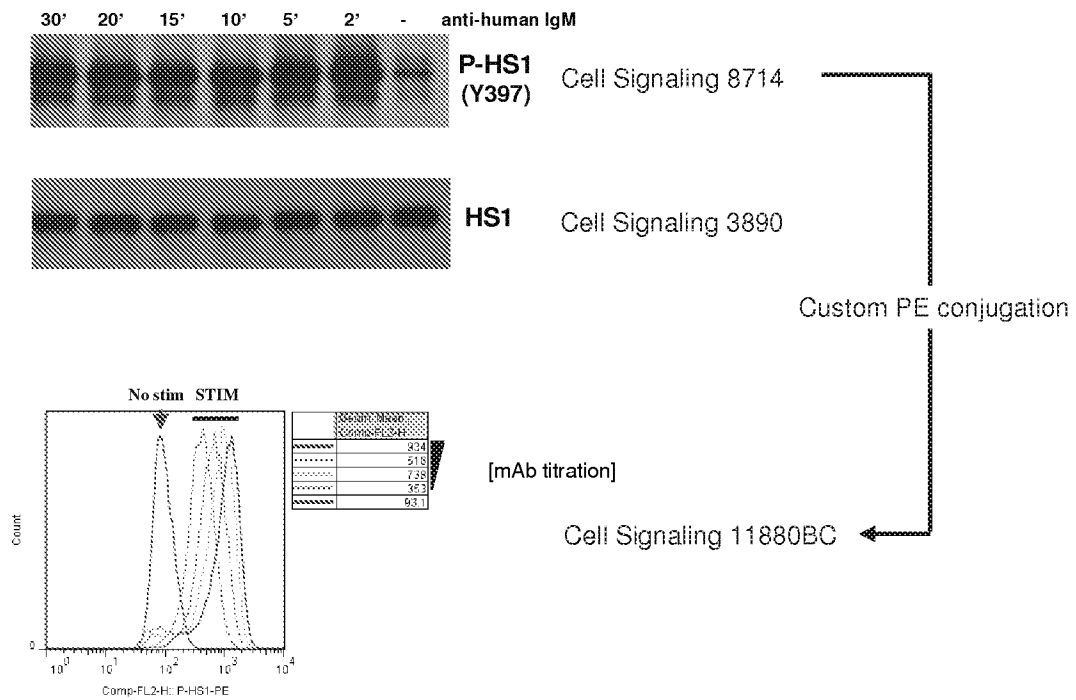


FIGURE 3

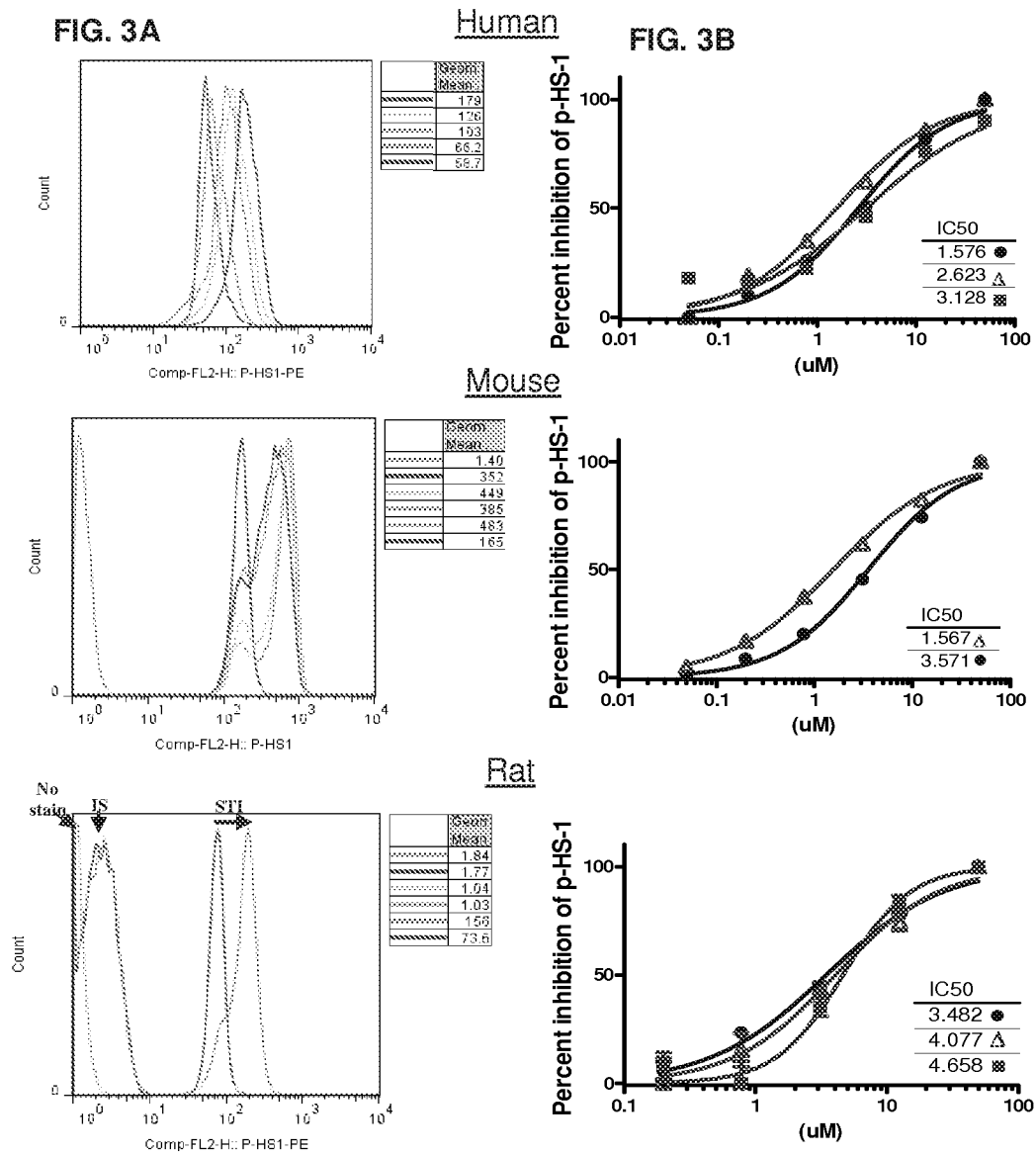


FIGURE 4

FIG. 4A

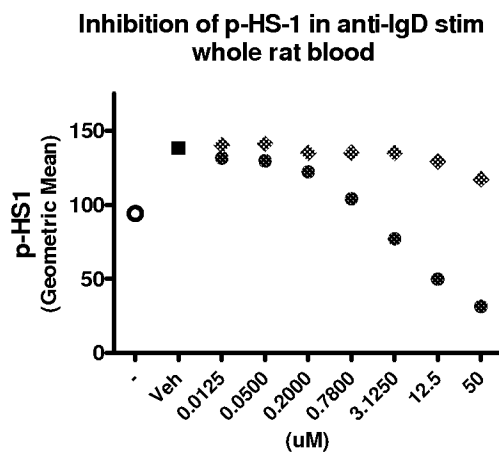


FIG. 4B

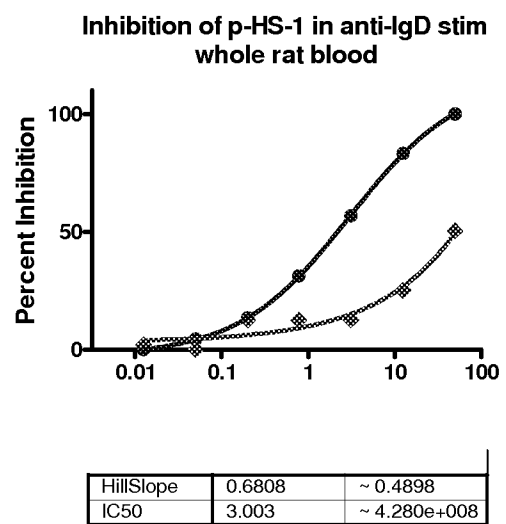


FIGURE 5

FIG. 5A

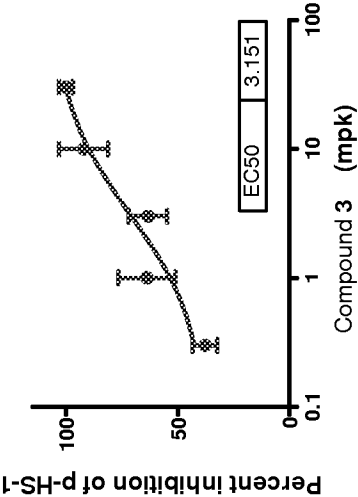


FIG. 5B

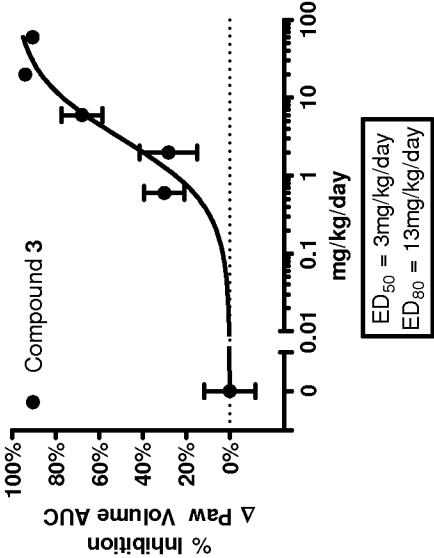


FIG. 5C

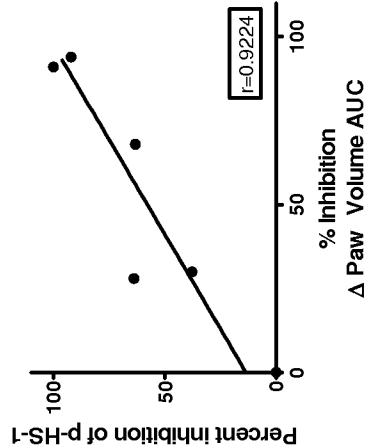


FIGURE 6

FIG. 6A

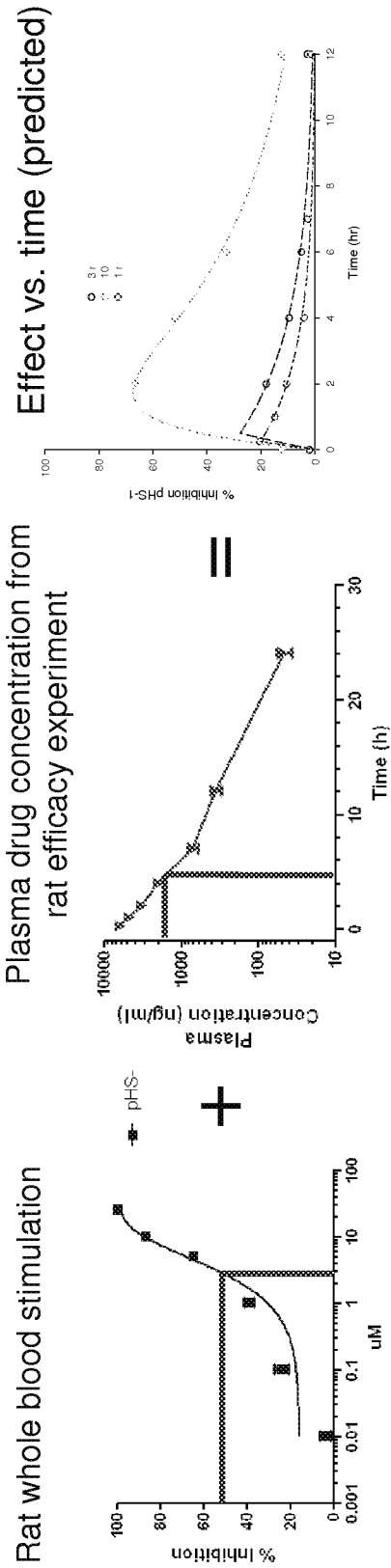


FIG. 6B Fostamatinib-pHS1

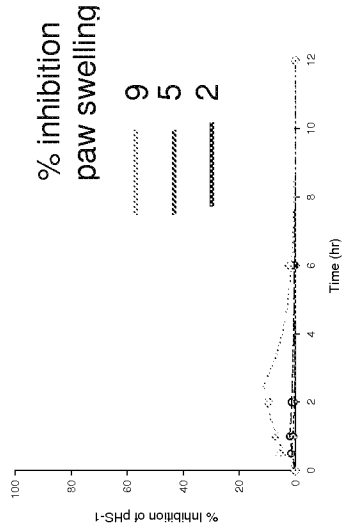


FIG. 6C Compound 4 [mAb titration]

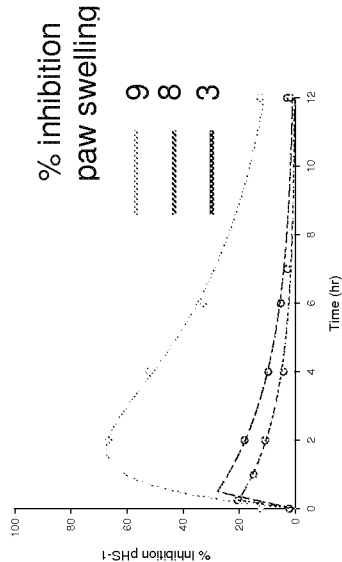


FIGURE 7

FIG. 7A

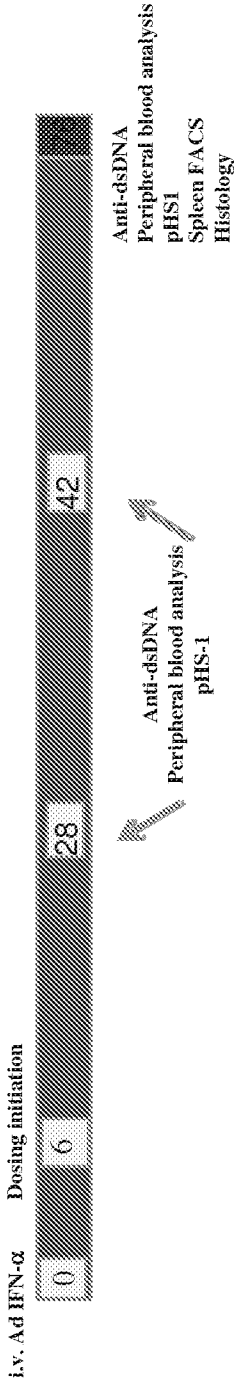


FIG. 7B

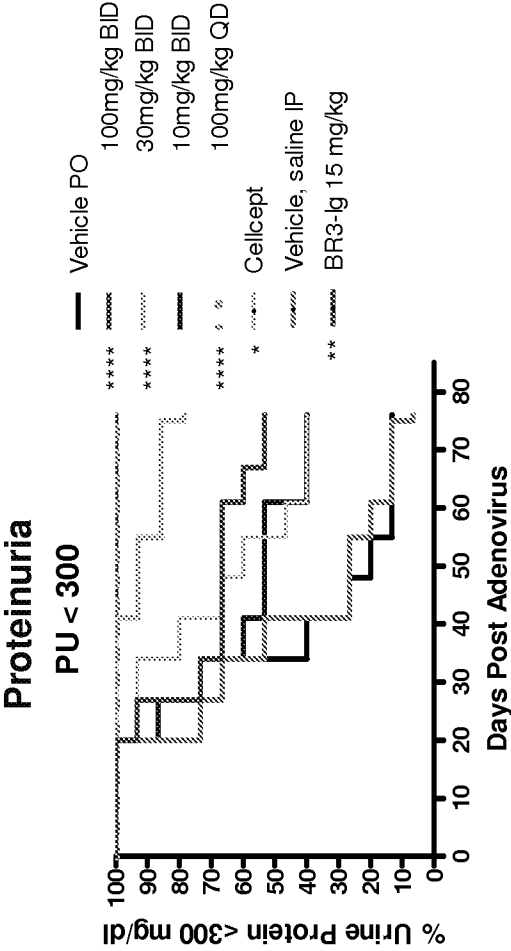


FIG. 7C

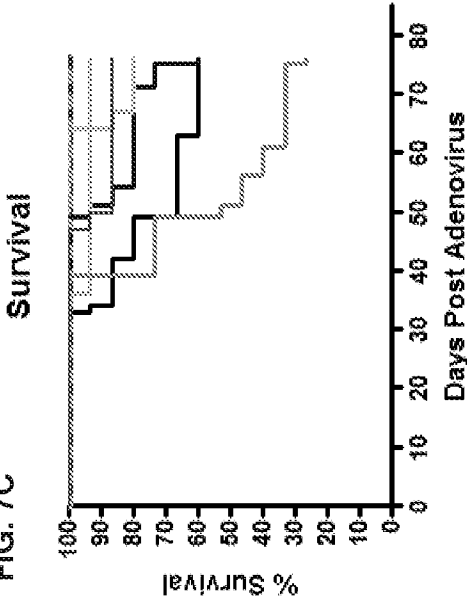


FIGURE 7 – CONT'D.

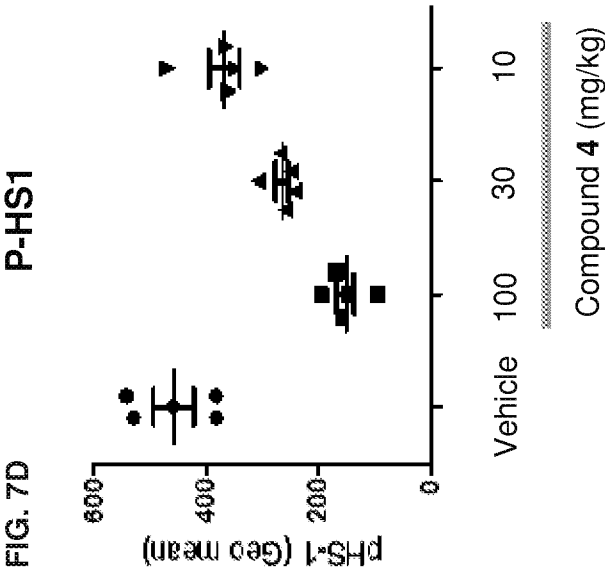


FIGURE 8

FIG. 8A

p-HS1 RA Compound 4 Titration
Unstimulated

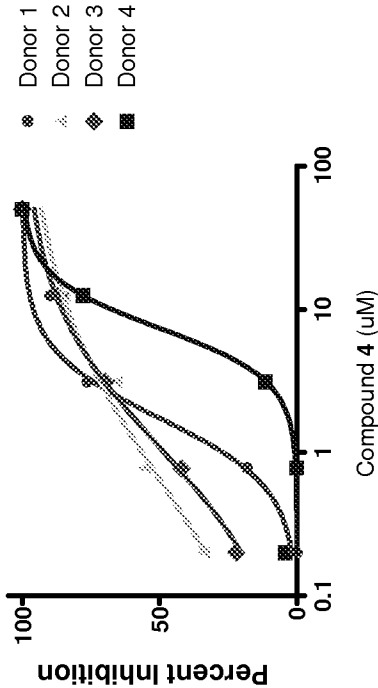
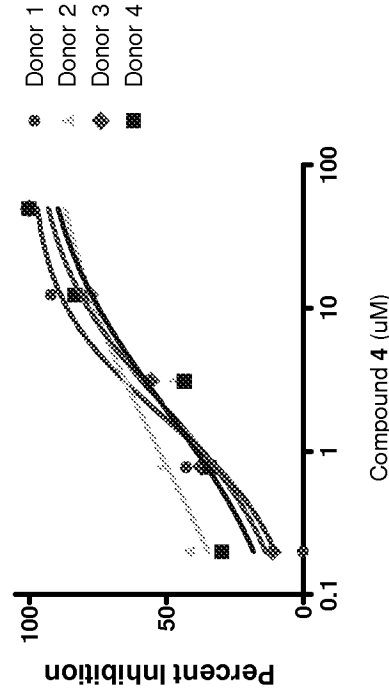
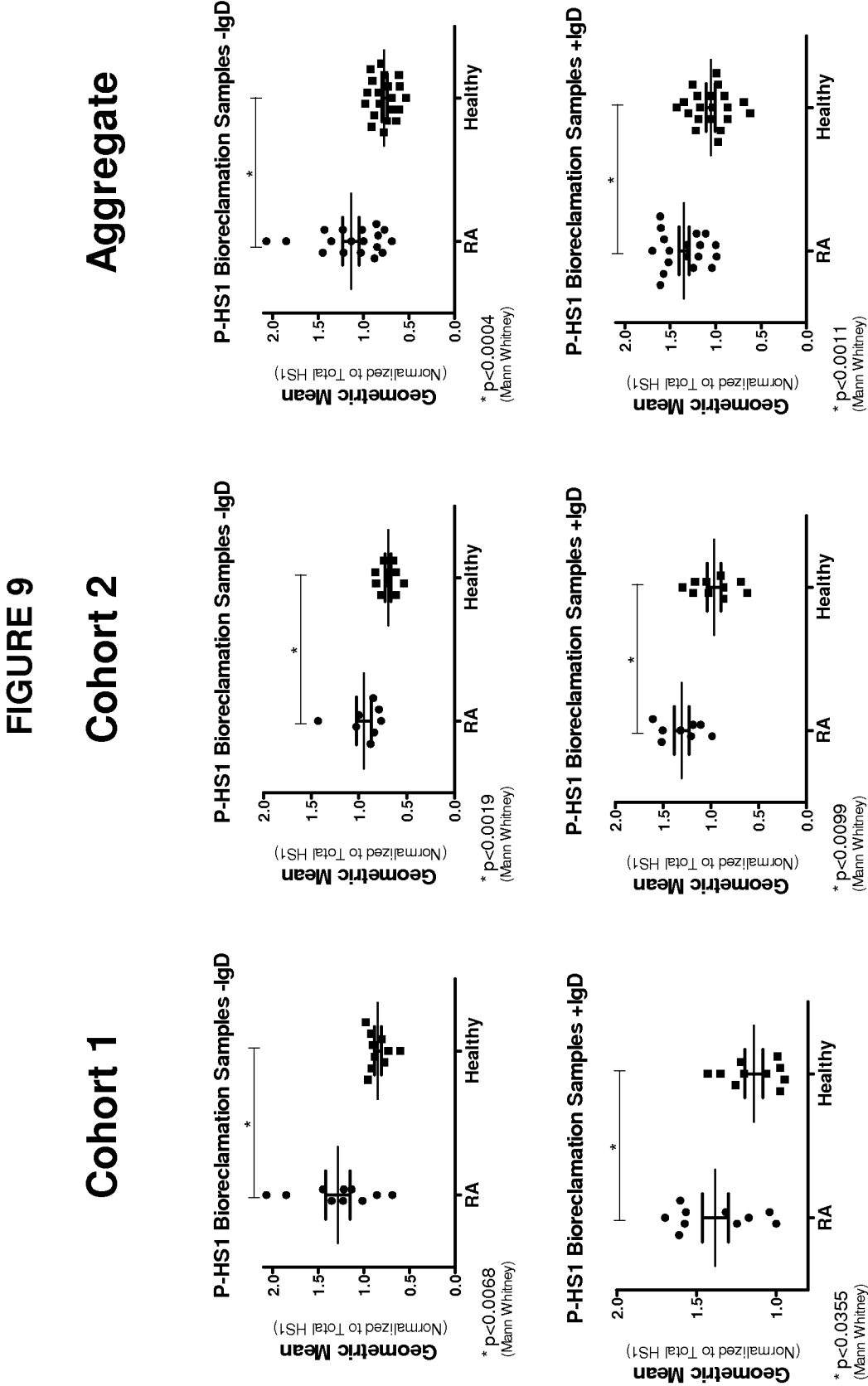


FIG. 8B

p-HS1 RA Compound 4 Titration
Stimulated





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/033890

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/53 (2015.01)

CPC - G01N 33/53 (2015.09)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C07K 14/47; G01N 33/53, 33/68 (2015.01)

CPC - C07K 14/47, 16/40; C12Q 1/00; G01N 33/53, 33/68, 33/5008 (2015.09)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - C07K 14/47, 16/40; C12Q 1/00; G01N 33/53, 33/68, 33/5008 (2015.09) (keyword delimited)

USPC - 435/7.1; 530/350

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google Scholar, Google, Pro Quest, Pub Med

Search terms used: Hematopoietic Cell-Specific Lyn Substrate 1 LckBP1 P75 HS1 Hematopoietic Lineage Cell-Specific Protein Cortactin-Like CTTNL phosphorylation Y397

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0297137 A1 (CALIGARIS-CAPPIO et al) 25 November 2010 (25.11.2010) entire document	30-32
Y	TEN HACKEN et al. "Targeting the LYN/HS1 signaling axis in chronic lymphocytic leukemia," Blood. 16 January 2013 (16.01.2013), Vol. 121, Pgs. 2264-2273 and Supplemental Information, Pgs. S1-S19. entire document	30-32
A	SCIELZO et al. "HS1 protein is differentially expressed in chronic lymphocytic leukemia patient subsets with good or poor prognoses," J Clin Invest. June 2005, Vol. 115, Pgs. 1644-1650. entire document	1-32
A	YAMANASHI et al. "Role of tyrosine phosphorylation of HS1 in B cell antigen receptor-mediated apoptosis," J Exp Med. 07 April 1997 (07.04.1997), Vol. 185, Pgs. 1387-1392. entire document	1-32
A	WO 2013/162651 A1 (US ARMY CENTER FOR ENVIRONMENTAL HEALTH RESEARCH (CEHR)) 31 October 2013 (31.10.2013) entire document	1-32

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 September 2015

Date of mailing of the international search report

16 OCT 2015

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