Published in final edited form as:

Toxicol Appl Pharmacol. 2014 April 1; 276(1): 47–54. doi:10.1016/j.taap.2014.01.002.

A systems toxicology approach identifies Lyn as a key signaling phosphoprotein modulated by mercury in a B lymphocyte cell model

Joseph A. Caruso¹, Paul M. Stemmer¹, Alan Dombkowski², Nicholas J. Caruthers¹, Randall Gill³, and Allen J. Rosenspire^{3,*}

¹Institute of Environmental Health Sciences, Wayne State University, Detroit MI; USA

²Department of Pediatrics, Wayne State University, Detroit MI; USA

³Department of Immunology and Microbiology, Wayne State University, Detroit MI; USA

Abstract

Network and protein-protein interaction analyses of proteins undergoing Hg²⁺-induced phosphorylation and dephosphorylation in Hg²⁺-intoxicated mouse WEHI-231 B cells identified Lyn as the most interconnected node. Lyn is a Src family protein tyrosine kinase known to be intimately involved in the B Cell Receptor (BCR) signaling pathway. Under normal signaling conditions the tyrosine kinase activity of Lyn is controlled by phosphorylation, primarily of two well known canonical regulatory tyrosine sites, Y-397 and Y-508. However, Lyn has several tyrosine residues that have not yet been determined to play a major role under normal signaling conditions, but are potentially important sites for phosphorylation following mercury exposure. In order to determine how Hg²⁺ exposure modulates the phosphorylation of additional residues in Lyn, a targeted MS assay was developed. Initial mass spectrometric surveys of purified Lyn identified 7 phosphorylated tyrosine residues. A quantitative assay was developed from these results using the multiple reaction monitoring (MRM) strategy. WEHI-231 cells were treated with Hg²⁺, pervanadate (a phosphatase inhibitor), or anti-Ig antibody (to stimulate the BCR). Results from these studies showed that the phosphoproteomic profile of Lyn after exposure of the WEHI-231 cells to low a low concentration of Hg²⁺ closely resembled that of anti-Ig antibody stimulation, whereas exposure to higher concentrations of Hg²⁺ led to increases in the phosphorylation of Y-193/Y-194, Y-501 and Y-508 residues. These data indicate that mercury can disrupt a key regulatory signal transduction pathway in B cells and point to phospho-Lyn as a potential biomarker for mercury exposure.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding Author: Allen J. Rosenspire, Department of Immunology & Microbiology, Wayne State University, 550 E. Canfield St., Detroit, MI 48201, arosenspire@wayne.edu, Phone: 313-577-6496.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest

Keywords

autoimmune disease; B cell; Lyn; mass spectrometry; mercury; multiple reaction monitoring; phosphoproteomics; systems biology; toxicology; WEHI-231

INTRODUCTION

Mercury is a potent neurotoxin with environmental exposure being associated with neurological deficits, particularly in children (Clarkson *et al.*, 2006). Recent work indicates that exposure to mercury at low concentrations with no clear neurotoxicity may contribute to idiosyncratic autoimmune disease (AD) in humans. Evidence from animal models has conclusively shown that Hg^{2+} triggers a genetically-restricted systemic lupus erythematosus (SLE)-like disorder known as mercury induced autoimmune disease (HgIA) at exposures that are not neurotoxic. Susceptibility to HgIA is primarily a function of the H-2 haplotype, but other genes are clearly involved. Characteristics of HgIA include hyperimmunoglobulinemia, generation of serum autoantibodies that target the nucleolar protein fibrillarin, and the production of systemic immune complex deposits leading to glomerulonephritis (reviewed in references Druet, 1995; Pollard *et al.*, 1997; Bagenstose *et al.*, 1999; Rowley *et al.*, 2005). In humans exposed to mercury, a single study has shown a significant correlation between occupational exposure to mercury and subsequent development of SLE (Cooper *et al.*, 2004). There is not, however, a human counterpart to murine HgIA as no human HLA loci appear to modulate susceptibility to mercury-induced frank AD as it does in susceptible rodents.

Most human environmental exposure to Hg^{2+} is in the form of organic mercury. However findings in mice show that exposure to mercury in this form produces many of the features of HgIA in susceptible strains of mice. It is thought that the metabolism of MeHg to divalent Hg^{2+} is responsible for the induction of AD (Havarinasab *et al.*, 2005b; Havarinasab *et al.*, 2005a). The clear implication of these results is that human exposure to either inorganic or organic mercury at current environmental levels may put individuals at risk for AD and increase the incidence of AD in the general population. Nevertheless, taken as a whole the available evidence suggests that environmental exposure to either Hg^{2+} or organic mercury does not cause AD directly in humans. Rather, it is likely that mercury interacts with other intrinsic and extrinsic factors in exacerbating development or progression of AD (Silbergeld *et al.*, 2005; Mayes, 1999).

This view is supported by experiments in mouse models of AD. In most genetically autoimmune-prone mouse strains exposures to Hg^{2+} , at concentrations that do not produce neurotoxicity, increase susceptibility to autoimmune disease (Hultman *et al.*, 2006; Pollard *et al.*, 1999; Pollard *et al.*, 2001; Brenden *et al.*, 2001). More recently it has been shown that mouse strains that are not otherwise naturally prone to AD or susceptible to Hg^{2+} , do have increased susceptibility to disease in several models of induced (acquired) autoimmunity when they are exposed to low levels of Hg^{2+} . For instance, chronic graft-versus-host disease (GVHD) is a murine model of acquired AD that is induced using normal, non-autoimmune prone donor and F1 recipient mice that are resistant to Hg^{2+} -induced autoimmunity. When

the donor and host mice are exposed to low concentrations of Hg^{2+} for short durations prior to GVHD onset they develop more severe disease (Via *et al.*, 2003). Likewise, in murine collagen-induced arthritis, another acquired autoimmune disease that cannot be induced by mercury alone, non-toxic levels of Hg^{2+} exacerbate the disease in a mercury-insensitive mouse strain (Hansson *et al.*, 2005). Other experiments have shown that exposure to low-level Hg^{2+} increases disease severity in a mouse model of experimental autoimmune myocarditis (Nyland *et al.*, 2012). Most recently we have found that low levels of Hg^{2+} exposure exacerbate disease in experimental autoimmune encephalitis, a well established model of multiple sclerosis, in the Hg^{2+} -weakly responding C57BL/6 mouse strain as well as the Hg^{2+} -responding SJL strain (AJR, unpublished data).

The cumulative data from animal models show that the immune response to Hg^{2+} is complex. After exposure to low levels of Hg^{2+} a susceptible H-2 background is necessary but not sufficient to induce HgIA, as non H-2 genes are also involved. In non- Hg^{2+} -susceptible H-2 backgrounds exposure to low levels of Hg^{2+} does not induce HgIA, but rather increases the risk and severity of clinical disease in the presence of other genetic or environmental triggering events. In humans, exposure to low level Hg^{2+} does not appear to induce AD directly. But, much like the Hg^{2+} -induced exacerbation of autoimmunity, the immune system is clearly perturbed by Hg^{2+} exposure (Queiroz *et al.*, 1997b; Queiroz *et al.*, 1997a; Dantas *et al.*, 1997; Perlingeiro *et al.*, 1994). More recent epidemiological studies have found correlations between mercury blood levels and increases in proinflammatory cytokine levels, anti-nuclear antibody titers, and alterations in Ig isotype profiles in populations which were exposed to mercury through diet (Nyland *et al.*, 2011b; Nyland *et al.*, 2011a).

Defects in function and signaling in immature B cells are often associated with autoimmunity (Yanaba *et al.*, 2008; Marino *et al.*, 2012; Konigsberger *et al.*, 2012). Exposure to low level mercury is one of many environmental triggers that correlate with disturbance in B cell signaling. In an effort to identify candidate biomarkers of Hg^{2+} exposure in the immune system that might be linked to AD, we have utilized a systems biology approach to explore the effects of Hg^{2+} on WEHI-231 B cells. WEHI-231 cells are a well characterized mouse B cell line that exhibits many of the characteristics of immature B cells (Jakway *et al.*, 1986). Our initial experiments utilized qualitative mass spectroscopic techniques to delineate global effects of mercury exposures on the WEHI-231 phosphoproteome, so as to identify potential protein targets of Hg^{2+} (Caruthers *et al.*, 2013). In the present study, bioinformatic data analysis algorithms identified the Src family protein phosphokinase Lyn as the “key node” and the B cell receptor signaling pathway as the primary pathway modulated by Hg^{2+} . Next we utilized a targeted and quantitative mass spectroscopic technique to characterize the effect of Hg^{2+} on the phosphorylation of individual amino acid residues in Lyn.

METHODS

Cell Culture

WEHI-231 cells were obtained from the American Type Culture Collection, Rockville MD. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM

glutamine, 50 μ M mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified 5% CO₂ atmosphere. Cells were passaged three times a week and harvested for experiments while in logarithmic growth.

Exposure and Phosphopeptide Preparation

Cells were pelleted and then resuspended at 5×10^6 cells per ml complete medium and exposed to the indicated concentrations of HgCl₂ (Hg²⁺) or pervanadate for 10 min. Cells were washed twice in ice cold Hanks' solution then pelleted and frozen. Cell pellets were lysed in 20 mM Tris (pH 8), 0.5% SDS, 1 mM LiF, and 0.1 mM Na₃VO₄. The supernatant from a 20,000 $\times$ g centrifugation was taken for analysis. Protein in lysates was determined using a BCA assay (Pierce). Samples were digested overnight with 1:100 trypsin (Sigma, TCPK treated) at a sample protein concentration of 1 mg/ml in buffer containing 20 mM Tris (pH 8), 0.1% SDS, 1 mM LiF, 0.1 mM Na₃VO₄, 1.0 mM CaCl₂ and 10% acetonitrile. All samples were evaluated by SDS-PAGE to ensure full digestion of the proteins before proceeding to phosphopeptide isolation. Phosphopeptides were isolated from 1 mg of digested cell protein using 6 mg of 5 μ m TiO₂ beads (GL Sciences). Cell digests were incubated with TiO₂ in 2% TFA saturated with glutamic acid in 60% acetonitrile. The beads were washed 3 times with 1% TFA in 60% acetonitrile before eluting phosphopeptides with NH₄OH in 50% acetonitrile. TiO₂ eluates were neutralized with formic acid, dried under vacuum and stored at -80°C until analysis by LC-MS/MS.

LC-MS/MS

Peptides were desalted on a trapping column (Peptide CapTrap, Michrom Bioresources) and separated using a Magic 0.1 mm $\times$ 150 mm AQ C18 column (Michrom) over a linear gradient of 5% to 35% acetonitrile. The top 5 ions in the MS1 scan (400–1800 m/z) were selected for fragmentation by collision-induced dissociation (2 amu isolation width; NCE=35; Activation Q=0.25; Activation time=30ms) using a LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific). Dynamic exclusion was turned on. A MS3 scan was triggered if a loss of 24.5, 32.7 or 49.0 was detected in the top 3 ions of the MS2 scan. Three types of sample were evaluated for Lyn phosphosites: Lyn immunoprecipitation from control and Ig-activated WEHI cells; TiO₂ phosphopeptide enrichment from WEHI cells; and recombinant murine Lyn (EMD Millipore). Samples were analyzed by LC-MS/MS after tryptic digestion.

Database Searching

Peak lists were generated using Proteome Discoverer (Thermo, version 1.4.0.288) and imported into Scaffold (Proteome Software, version 4.1). MS2 data were scored using Mascot (Matrix Science; version 2.4.0) and X! Tandem (version 2010.12.01.1) against mouse protein sequences within the SwissProt database (16611 entries) and simultaneously against a scrambled decoy database. Searches included up to 1 missed tryptic cleavage for Mascot and 2 missed cleavages for X!Tandem. The iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine, acetylation of the protein N-terminus and phosphorylation of serine, threonine and tyrosine were specified as variable modifications. X!Tandem searches also included a peptide N-terminal Gln to pyro-Gln variable modification. Peptides were considered a positive identification if they achieved a

0.1 false discovery rate (FDR) for Lyn identification experiments (87% probability), or 1.0 FDR for phosphopeptide enrichment experiments (95% probability). Phosphorylation site localization probability was calculated using Scaffold PTM (ver. 2.1.2; Proteome Software).

Protein-Protein Interaction and Pathway Analysis

Biological triplicate experiments with 250 μM HgCl_2 or quadruplicate experiments with 25 μM pervanadate were analyzed by LC-MS/MS as described above. Relative abundance of TiO_2 selected phosphopeptides was estimated by spectral counting. All the phosphopeptides that mapped to a protein were included in the count. A phosphoprotein was considered significantly altered by treatment if it scored $p < 0.05$ by the Student's T-Test, and if the cumulative spectral counts were at least 2-fold higher or lower in the treatment group versus the control group. This generated a set of 119 phosphoproteins out of 907 total for the mercury experiment, and 123 phosphoproteins out of a total of 620 total for the pervanadate experiment. Network analysis was performed using the Reactome Functional Interaction plugin (2012 FI Network) of Cytoscape (ver 2.8.3). Pathway analysis was performed using KEGG pathways via Enrichr (Chen *et al.*, 2013).

Lyn Immunoprecipitation

1×10^8 WEHI cells per 10 ml were untreated or treated with activating antibody (320 $\mu\text{g}/\text{ml}$) for the indicated times, or the indicated concentrations of pervanadate or HgCl_2 for 10 min at 37°C . All treatments were performed as biological triplicates. At the endpoint, cells were placed on ice, and then pelleted at 4°C , washed with ice-cold wash buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM NaF, 1 mM Na_3VO_4 , 2 mM β -glycerophosphate], and re-pelleted. Cells were lysed with wash buffer containing 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 mM MgCl_2 and 100 U Benzonase (Novagen). After a 5 min digestion at 37°C , 1X Protease Inhibitor Cocktail (Sigma) and 10 mM EDTA were added. Samples were centrifuged at high speed and supernatants transferred to new tubes, pre-cleared with Protein A/G-agarose, and then immunoprecipitated with 25 μl of anti-Lyn IgG (Santa Cruz) and 50 μl of Protein A/G-agarose overnight with end-over-end rotation at 4°C . The following day, the beads were washed then proteins were eluted by boiling with non-reducing SDS sample buffer. Proteins were separated by SDS-PAGE using 4–12% Bis-Tris gels (Life Technologies).

Multiple Reaction Monitoring

For each sample, the area of the gel lane where Lyn was pre-determined to localize, between 50 and 70 kDa, was excised. Gel slices were reduced with 5 mM TCEP, alkylated with 15 mM iodoacetamide and in-gel digested with 0.04 μg trypsin (Promega). Eluted peptides were resuspended in 5% acetonitrile, 0.1% formic acid, and 0.005% trifluoroacetic acid and separated by reverse phase chromatography as described above. 33% of each sample was analyzed in duplicate on a TSQ Vantage triple quadrupole mass spectrometer (Thermo). Instrument settings included a FWHM of 0.7 and cycle time of 0.5 s. Method optimization was performed to determine i) the transitions which gave the highest signal, ii) the optimal collision energy for each transition, and iii) the peptide retention times. 15 transitions were

monitored for 4 min each within a 45 min gradient. Transition lists can be found in the Supplemental Table 4. Data was analyzed using Skyline software (MacCoss lab, University of Washington, version 1.4.0). Peak areas for each transition were integrated, averaged for 2 runs, and then normalized against an un-modified control Lyn peptide to control for changes in Lyn abundance ($^{121}\text{VNTLETEESFFK}^{132}$). The untreated samples were then normalized to a value of 1 and the sample groups were adjusted accordingly.

RESULTS

We hypothesized that mercury exacerbates autoimmune disease by perturbing signaling networks during key points of B cell maturation. One of the major biochemical modalities cells utilize to transmit information is through tightly controlled processes of protein phosphorylation and dephosphorylation. Accordingly, we reasoned that Hg^{2+} intoxication of immature B cells might be associated with a unique bio-signature imprinted on the phosphoproteome. Our first approach was to ascertain whether a short term exposure to inorganic mercury alters the global phosphoproteomic profile of an immature B cell model, WEHI-231 (Coggeshall *et al.*, 1985; Scott *et al.*, 1986; Caruthers, Stemmer, Gill, and Rosenspire, 2013).

Accordingly WEHI-231 cells were exposed to various concentrations of Hg^{2+} or pervanadate for 10 minutes and then lysed. Pervanadate was used as a control since this compound is a well known inhibitor of tyrosine and serine phosphatases (Wright *et al.*, 2009). Therefore, we would expect treatment to lead to an increase in the number of phosphoproteins identified. After exposure to either HgCl_2 or pervanadate, phosphoproteome wide phosphopeptide analysis was performed by enriching phosphopeptides from cell extracts using TiO_2 -affinity chromatography followed by LC-MS/MS identification. A quantitative estimate of relative abundance for each identified protein was then calculated based on spectral counting. We found that HgCl_2 and pervanadate each had a concentration dependent effect to increase the number of phosphorylated proteins that are significantly changed in spectral counts in comparison to the untreated sample (Fig. 1).

Next, we repeated the protocol outlined in figure 1, with but with cells that were exposed to either 25 μM pervanadate or 250 μM Hg^{2+} , and employed a bioinformatics systems approach to analyze the phosphoproteomic data. Phosphoproteins whose abundance were significantly up- or down-regulated after 250 μM Hg^{2+} or 25 μM pervanadate treatment were identified (Supplemental Table 1) and mapped to KEGG pathways (Kanehisa *et al.*, 2012). Based on this analysis, the B Cell Receptor Signaling Pathway (HSA04662) was identified as the highest scoring pathway for both mercury and pervanadate treatments (Table 1). The next top 4 scoring pathways for each of the treatments were dissimilar for the two toxicants.

Environmental exposures to mercury generally involve low level exposures which are typically chronic. However in order to cast as wide a net as possible so as to identify proteins whose phosphorylation might be affected by Hg^{2+} , in this case WEHI-231 cells were exposed to 250 μM HgCl_2 . While an exposure to 250 μM HgCl_2 is not necessarily

environmentally relevant and is likely toxic, exposures were limited to 10 minutes so that the cells were harvested while still intact. Through high level acute exposures we wanted the initial proteome-wide exploratory experiments to achieve maximal effect in stimulating phosphorylation. By so doing we expected to identify all proteins whose phosphorylation state was altered by Hg intoxication, including those which otherwise might be missed upon short term exposures to lower concentrations of HgCl₂. However it is understood that once identified in a phosphoproteome wide experiment, the significance of any specific phosphoprotein in mediating the effects of environmental mercury exposures needs to be confirmed in more focused experiments conducted with environmentally appropriate HgCl₂ exposures.

In any event, in order to further illuminate the effect of Hg²⁺ and pervanadate on B cell signaling, proteins with significantly altered phosphorylation profiles after exposure to either reagent were mapped to protein-protein interaction networks using Cytoscape (Fig. 2). In this graphical visualization, proteins whose phosphorylation were significantly affected by Hg²⁺ (Fig 2A) or pervanadate (Fig 2B) are displayed as nodes and known interactions between nodes as edges. For mercury (Fig. 2A) there were 2 major foci: interactions predominantly associated with B cell receptor signaling pathway (*upper left*) and RNA processing (*upper right*). In contrast, pervanadate (Fig. 2B) elicited changes in a large network of proteins associated with B cell receptor signaling and Fc-ε receptor I signaling (*center-left*), as well as a smaller network involved with protein folding (*upper right*). The protein with the greatest number of interacting partners was designated as the 'key node'. This analysis demonstrates that in mercury intoxicated WEHI-231 cells, Lyn was the key node with 9 interactions. For pervanadate, PLCG2 and PTPN6 were the key nodes (12 interactions each), but notably Lyn was not found to be significantly altered.

In order to obtain a higher resolution profile of how mercury intoxication alters Lyn phosphorylation, we determined which residues in Lyn were phosphorylated or dephosphorylated after exposure to HgCl₂. To ensure that we obtained maximal and unbiased peptide sequence coverage, the results obtained from WEHI-231-derived Lyn were compared with results obtained with recombinant murine Lyn. Physiologically relevant phosphorylation sites in Lyn were identified from control and anti-Ig-stimulated WEHI-231 cells by TiO₂-affinity selection or by Lyn immunoprecipitation. Cumulatively, ~81% of the Lyn protein sequence was covered by peptides identified using mass spectrometry, and 7 phosphosites were identified. Those sites were localized to tyrosine residues 117, 193, 194, 460, 473, 501 and 508 with high confidence (Fig. 3B and Supplemental Figure 1). For murine Lyn, only pY-508 has previously been verified by site-specific methods (Hasegawa *et al.*, 1999), but all of these sites have been detected by discovery MS/MS in other laboratories (Hornbeck *et al.*, 2012). Phosphosites pY-193, pY-194 and pY-473 have been detected and discussed previously, but less is known about pY-117, pY-460 and pY-501 (Hornbeck, Kornhauser, Tkachev, Zhang, Skrzypek, Murray, Latham, and Sullivan, 2012).

MS/MS discovery data was used to develop a label free relative quantitation assay for site-specific Lyn phosphorylation. The specificity and sensitivity of the quantitation was enhanced by 3 steps in the sample preparation. First, Lyn was immunoprecipitated from control and treated cell extracts. Second, immunopurified proteins were separated by SDS-

PAGE, and only the gel region corresponding to the area of Lyn migration was excised (Supplemental Figure S2), thereby reducing sample complexity and ion suppression. Third, peptides were quantified by multiple reaction monitoring (MRM), a highly sensitive mass spectrometric technique which can monitor the abundance of several different peptides within a single chromatographic run. Since multiple peptides can have similar mass, MRM relies on the quantitation of individual MS/MS fragment ions, or ‘transitions’ (Liebler *et al.*, 2013). These 2 features (parent and fragment m/z), along with chromatographic retention time, lead to a specific signature for each peptide. Three co-eluting transitions were selected per peptide to ensure the correct peptide was selected, but only the most intense transition was utilized for quantitation, since these transitions provide the most reliable quantitation (Percy *et al.*, 2013). MRM protocols were developed for each of the 7 identified Lyn phosphosites, however, quantifiable measurements were only achieved for 4 phosphopeptides (Fig. 4 and Supplemental Table 4). These peptides included residues pY-117, pY-193/pY-194 (the optimized transition ions could not distinguish the precise phosphosite localization), pY-508, and a doubly phosphorylated peptide containing pY-501 + pY-508.

For the MRM experiments, concentrations of pervanadate and HgCl_2 were selected that produced slight, moderate and maximal changes in protein phosphorylation (Figure 1). We have previously shown that anti-Ig stimulates BCR signal transduction within two minutes of application (McCabe, Jr. *et al.*, 2007). Our results here show that anti-Ig induces maximal phosphorylation of Y-501 and Y-508 after 1 min, and levels remain elevated over the full 15 min for which they were measured (Fig. 4). Pervanadate stimulated the phosphorylation of these sites to a lesser extent, but HgCl_2 exposure led to increased abundance of the singly phosphorylated peptide by over 25-fold, and the doubly phosphorylated peptide by over 150-fold at the highest concentration. In contrast, the pY-117 phosphorylation event was non-responsive or slightly decreased with treatment. The relative abundance of pY-193/194 was marginally increased by anti-Ig, pervanadate or low levels of HgCl_2 , however, pY-193/194 levels increase over 50-fold in response to 50 or 100 μM HgCl_2 . These data show that mercury has the capacity to alter the phosphorylation profile of Lyn, and by extension its signaling activity, in a unique and profound way.

DISCUSSION

Mercury has well-documented adverse human health effects, most notably on neurological tissues. Mercury exposure during specific windows of fetal development has been shown to have deleterious effects later in life including impairment of cognitive thinking, memory, language, attentiveness and fine motor skills (Clarkson, 1997; Clarkson and Magos, 2006). Less is known about the role of mercury in immune disease, but we hypothesize that mercury may exacerbate autoimmune disease by perturbing signaling networks during B cell maturation. Our initial experiments showed that short-term exposures of HgCl_2 dramatically changes the composition of the B cell phosphoproteome, and as a result, likely interferes with phosphorylation-dependent signal transduction pathways in B cells (Fig. 1). Unanswered is the more important question as to whether or not specific bio-signatures of Hg^{2+} exposure might be found within the phosphoproteomic response.

Utilizing western blotting technology, previous investigators have shown that acute 0.2 – 2 mM Hg²⁺ exposures induced rapid (within 5 seconds) tyrosine phosphorylation in a large number of lymphocyte proteins. Considering that Hg²⁺ was known to affect cellular redox levels, it was suggested that the mechanism was redox-linked and independent of traditional ligand-cell surface receptor interactions (Rahman *et al.*, 1993; Nakashima *et al.*, 1994). If this is true, then the bio-signature of Hg²⁺, at least at higher exposure levels, might not be expected to be significantly different from that of pervanadate, a reagent clearly linked to a redox mechanism (Park *et al.*, 2000). We utilized bioinformatic approaches to address this question. By applying pathway analysis to analyze the panels of proteins with significantly changed phosphorylation, we found that the BCR pathway was the most highly affected signaling pathway for both Hg²⁺ and pervanadate exposures (Table 1). Considering that we were working with a B cell line, these results may not at first seem surprising, but they do suggest that the proto-typical signaling networks within the WEHI-231 B cell model remain functional. From this viewpoint it would appear that the biological effects of Hg²⁺ and pervanadate exposure are very similar, but the secondary and tertiary pathways modulated by these toxicants are different (Table 1).

We next used a more sophisticated algorithm to produce a graphical representation of the known individual pairwise interactions between the phosphoproteins we determined to be affected by either Hg²⁺ or pervanadate (Fig. 2). Strikingly different topologies between the Hg²⁺ graph and the pervanadate graph emerged from this analysis. This suggests that although the BCR signaling pathway is the primary pathway modulated by both Hg²⁺ and pervanadate, a deeper analysis of the data shows very unique phosphoproteomic profiles. The interaction maps in figure 2 dramatically demonstrate that with respect to mercury intoxication of B cells, altered phosphorylation of the non-receptor tyrosine-protein kinase Lyn is a central coordinating molecular feature. Since the same outcome is not observed after pervanadate exposures, we suggest that disruption of normal Lyn phosphorylation patterns might be useful as a bio-signature of mercury intoxication in B cells. Furthermore, as shown in figure 2, disruptions of Lyn phosphorylation by Hg²⁺ can potentially influence cell cycle control through Lyn's interaction with Cyclin Dependent Kinase 1 (CDK1), and indirectly affect RNA transcription through the effect that CDK1 has on the RNA polymerase II subunit POLR2A. These data reinforce the idea that Lyn may be a key mediator of mercury immunotoxicity in the B cell.

Lyn was recognized early on as having a pivotal role in the BCR signaling cascade, as it is the kinase primarily responsible for initiating the tyrosine phosphorylation signaling cascade initiated immediately after membrane immunoglobulin interaction with agonist [Dal Porto *et al.*, 2004]. Later discoveries which showed that Lyn^{-/-} knockout mice, as well as Lyn^{up/up} gain-of-function mice, both exhibit B cell antibody-mediated autoimmune phenotypes were initially confusing (Hibbs *et al.*, 1995; Hibbs *et al.*, 2002). However, it is now recognized that Lyn mediates negative as well as positive effects on BCR signaling, so that Lyn functions as a sort of “rheostat” for BCR signaling (Xu *et al.*, 2005; Lowell, 2004). This implies that maintaining a fine balance in Lyn activity is necessary for proper BCR signaling activity and, by extension, a healthy functioning immune system free from autoimmune disease (Ingley, 2012).

Targeted analysis of Lyn phosphorylation at 7 sites was evaluated using an MRM assay. These experiments validated the findings in the discovery work that identified Lyn as a Key Node in Hg²⁺ modulation of signaling and extended the findings to low concentrations of Hg that are more relevant to environmental Hg²⁺ exposures. Lyn is a Src family tyrosine protein kinase, and all members of this family are characterized by two well established canonical tyrosine control sites. The first is a C-terminal tyrosine (Y-508 for murine Lyn) whose phosphorylation negatively regulates kinase activity by allowing the C-terminal tail to fold over and block the active kinase site, while the second is a tyrosine in close proximity to the active site (Y-397 for murine Lyn) whose phosphorylation increases kinase activity (Gauld *et al.*, 2004). In our experiments, peptides containing Y-397 were sequenced, but we did not detect pY-397 in any treatment group.

We also found that exposures to Hg²⁺ affected the phosphorylation of several other conserved canonical tyrosine sites in Lyn (Figure 5). One of these sites is Y-501, whose phosphorylation is increased. Interestingly, in our MS/MS studies we only found pY-501 as a doubly phosphorylated peptide with pY-508, and this peptide was only found in mercury-treated cells. It is possible that pY-501 reinforces the inhibitory activity of pY-508, but the Y-501 site has not been studied to a great extent and at this point the significance of its phosphorylation remains an open question. As we have previously mentioned, decreases in Lyn activity are associated with a loss of negative control over the B cell antigen response, ultimately resulting in autoimmune sequelae (Lowell, 2004; Ingley, 2012).

In addition to the effects we have described on Y-501, we have found that Hg²⁺ exposures increase the phosphorylation of Y-194, located in the SH2 domain of Lyn (Figure 5). The Y-194 canonical site was first described with respect to Lck, the Src family kinase predominantly associated with T cells. It was shown that phosphorylation of Lck at Y-192 terminates the high affinity binding of Lck to ligands, so as to negatively regulate T Cell Receptor (TCR) signaling (Couture *et al.*, 1996). We subsequently found that transitory (within seconds after TCR engagement with agonist) phosphorylation of Lck Y-192 was positively associated with the initiation of TCR signaling (Ziemba *et al.*, 2009). However to best of our knowledge, Lyn pY-194 has not been discussed before now in the literature.

The third canonical tyrosine site in Lyn we find affected by Hg²⁺ is Y-117. This is a site that has not been extensively discussed in the literature and consequently its function remains unknown. It is located in the SH3 domain, proximal to the SH2/SH3 domain junction. Our prediction is that phosphorylation of Y-117, like phosphorylation of Y-192, will control Lyn/substrate interactions. In contrast to the other sites we have discussed, for which Hg²⁺ exposure increased phosphorylation, we found that Hg²⁺ decreases Y-117 phosphorylation, but to a somewhat less extent as does BCR stimulation with anti-Ig (Figure 4). Taking this into account, along with the fact that the Y-117 site is conserved among other Src kinases and across species as diverse as frog and human (Fig. 5), we suggest that while in absolute terms the effect may be small, it is likely to have biological significance.

In summary, we utilized systems biology tools to map the phosphoproteomic response of WEHI-231 B cells to mercury and found Lyn to be a key signaling protein. Lyn activity is known to be controlled through phosphorylation of tyrosine residues, particularly Y-508

(Gauld and Cambier, 2004). Our finding that Hg²⁺ alters the profile of Lyn tyrosine phosphorylation indicates that Lyn may be a pivotal mediator of mercury immunotoxicity. Future efforts will investigate the utility of Lyn phosphorylation status as a biomarker for mercury exposure. We postulate that mercury exposure during specific windows of B cell maturation may affect immunity and self-tolerance by altering the way the BCR pathway processes signal responses. This can contribute to the fate of B cells and may have downstream consequences later in life by impacting upon the incidence of autoimmune disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported through grants from the NIH: R21 ES019228 (AR); S10 RR020893 (PS) and P30 CA022453 (Wayne State University and Karmanos Cancer Center Proteomics Core).

ABBREVIATIONS

AD	Autoimmunue disease
FDR	False discovery rate
GVHD	Graft-versus-host disease
HgIA	Mercury induced autoimmune disease
HLA	Human leukocyte antigen
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MRM	Multiple Reaction Montoring
SLE	Systemic lupus erythematosus

Reference List

- Bagenstose LM, Salgame P, Monestier M. Murine mercury-induced autoimmunity: a model of chemically related autoimmunity in humans. *Immunol Res.* 1999; 20:67–78. [PubMed: 10467984]
- Brenden N, Rabbani H, Abedi-Valuggerdi M. Analysis of mercury-induced immune activation in nonobese diabetic (NOD) mice. *Clin.exp.Immunol.* 2001; 125:202–210. [PubMed: 11529910]
- Caruthers NJ, Stemmer PM, Shin N, Dombkowski A, Caruso JA, Gill R, Rosenspire A. Mercury Uniquely Alters B-Cell Protein Phosphorylation Profiles. *J.Proteome Res.* 2013 In Press.
- Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC.Bioinformatics.* 2013; 14:128. [PubMed: 23586463]
- Clarkson TW. The toxicology of mercury. *Crit.Rev.Clin.Lab.Sci.* 1997; 34:369–403. [PubMed: 9288445]
- Clarkson TW, Magos L. The toxicology of mercury and its chemical compounds. *Crit Rev.Toxicol.* 2006; 36:609–662. [PubMed: 16973445]
- Coggeshall KM, Cambier JC. B Cell Activation. VI. Effects of Exogenous Diglycerides and Modulators of Phospholipid Metabolism Suggest a Central Role for Diacylglycerol in Transmembrane Signaling by mIg. *J.Immunol.* 1985; 134:101–107. [PubMed: 2981089]

- Cooper GS, Parks CG, Treadwell EL, St Clair EW, Gilkeson GS, Dooley MA. Occupational risk factors for the development of systemic lupus erythematosus. *J.Rheumatol.* 2004; 31:1928–1933. [PubMed: 15468355]
- Couture C, Songyang Z, Jascur T, Williams S, Taylor P, Cantley LC, Mustelin T. Regulation of the Lck SH2 domain by tyrosine phosphorylation. *J.Biol.Chem.* 1996; 271:24880–24884. [PubMed: 8798764]
- Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol.Immunol.* 2004; 41:599–613. [PubMed: 15219998]
- Dantas DCM, Queiroz MLS. Immunoglobulin E and autoantibodies in mercury-exposed workers. *Immunopharmacol.Immunotoxicol.* 1997; 19:383–392. [PubMed: 9248865]
- Druet P. Metal-induced autoimmunity. *Hum.Exp.Toxicol.* 1995; 14:120–121. [PubMed: 7748597]
- Gauld SB, Cambier JC. Src-family kinases in B-cell development and signaling. *Oncogene.* 2004; 23:8001–8006. [PubMed: 15489917]
- Hansson M, Djerbi M, Rabbani H, Mellstedt H, Gharibdoost F, Hassan M, Depierre JW, Abedi-Valugerdi M. Exposure to mercuric chloride during the induction phase and after the onset of collagen-induced arthritis enhances immune/autoimmune responses and exacerbates the disease in DBA/1 mice. *Immunology.* 2005; 114:428–437. [PubMed: 15720444]
- Hasegawa K, Yajima H, Katagiri T, Ogimoto M, Arimura Y, Mitomo K, Mashima K, Mizuno K, Yakura H. Requirement of PEST domain tyrosine phosphatase PEP in B cell antigen receptor-induced growth arrest and apoptosis. *Eur J Immunol.* 1999; 29:887–896. [PubMed: 10092092]
- Havarinasab S, Haggqvist B, Bjorn E, Pollard KM, Hultman P. Immunosuppressive and autoimmune effects of thimerosal in mice. *Toxicol.Appl.Pharmacol.* 2005a; 204:109–121. [PubMed: 15808517]
- Havarinasab S, Hultman P. Organic mercury compounds and autoimmunity. *Autoimmun.Rev.* 2005b; 4:270–275. [PubMed: 15990073]
- Hibbs ML, Harder KW, Armes J, Kountouri N, Quilici C, Casagrande F, Dunn AR, Tarlinton DM. Sustained activation of Lyn tyrosine kinase in vivo leads to autoimmunity. *J.Exp.Med.* 2002; 196:1593–1604. [PubMed: 12486102]
- Hibbs ML, Tarlinton DM, Armes J, Grail D, Hodgson G, Maglitta R, Stacker SA, Dunn AR. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell.* 1995; 83:301–311. [PubMed: 7585947]
- Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res.* 2012; 40:D261–D270. [PubMed: 22135298]
- Hultman P, Taylor A, Yang JM, Pollard KM. The effect of xenobiotic exposure on spontaneous autoimmunity in (SWR x SJL)F1 hybrid mice. *J.Toxicol.Environ.Health A.* 2006; 69:505–523. [PubMed: 16574626]
- Ingle E. Functions of the Lyn tyrosine kinase in health and disease. *Cell Commun.Signal.* 2012; 10:21. [PubMed: 22805580]
- Jakway JP, Usinger WR, Gold MR, Mishell RI, DeFranco AL. Growth regulation of the B lymphoma cell line WEHI-231 by anti- immunoglobulin, lipopolysaccharide, and other bacterial products. *J.Immunol.* 1986; 137:2225–2231. [PubMed: 3489760]
- Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* 2012; 40:D109–D114. [PubMed: 22080510]
- Konigsberger S, Prodohl J, Stegner D, Weis V, Andreas M, Stehling M, Schumacher T, Bohmer R, Thielmann I, van Eeuwijk JM, Nieswandt B, Kiefer F. Altered BCR signalling quality predisposes to autoimmune disease and a pre-diabetic state. *EMBO J.* 2012; 31:3363–3374. [PubMed: 22728826]
- Liebler DC, Zimmerman LJ. Targeted quantitation of proteins by mass spectrometry. *Biochemistry.* 2013; 52:3797–3806. [PubMed: 23517332]
- Lowell CA. Src-family kinases: rheostats of immune cell signaling. *Mol.Immunol.* 2004; 41:631–643. [PubMed: 15220000]
- Marino E, Grey ST. B cells as effectors and regulators of autoimmunity. *Autoimmunity.* 2012; 45:377–387. [PubMed: 22432804]

- Mayes MD. Epidemiologic studies of environmental agents and systemic autoimmune diseases. *Environ.Health Perspect.* 1999; 107(Suppl 5):743–748. [PubMed: 10502540]
- McCabe MJ Jr, Laiosa MD, Li L, Menard SL, Mattingly RR, Rosenspire AJ. Low and Nontoxic Inorganic Mercury Burdens Attenuate BCR-Mediated Signal Transduction. *Toxicol.Sci.* 2007; 99:512–521. [PubMed: 17656488]
- Nakashima I, Pu M-Y, Nishizaki A, Rosila I, Ma L, Katano Y, Ohkusu K, Rahman SMJ, Isobe K, Hamaguchi M, Saga K. Redox mechanism as alternative to ligand binding for receptor activation delivering disregulated cellular signals. *J.Immunol.* 1994; 152:1064–1071. [PubMed: 7507957]
- Nyland JF, Fairweather D, Shirley DL, Davis SE, Rose NR, Silbergeld EK. Low-dose inorganic mercury increases severity and frequency of chronic coxsackievirus-induced autoimmune myocarditis in mice. *Toxicol.Sci.* 2012; 125:134–143. [PubMed: 21984480]
- Nyland JF, Fillion M, Barbosa F Jr, Shirley DL, Chine C, Lemire M, Mergler D, Silbergeld EK. Biomarkers of methylmercury exposure immunotoxicity among fish consumers in Amazonian Brazil. *Environ.Health Perspect.* 2011a; 119:1733–1738. [PubMed: 21868305]
- Nyland JF, Wang SB, Shirley DL, Santos EO, Ventura AM, de Souza JM, Silbergeld EK. Fetal and maternal immune responses to methylmercury exposure: a cross-sectional study. *Environ.Res.* 2011b; 111:584–589. [PubMed: 21396635]
- Park J, Liu AY. Pervanadate induces the hyperphosphorylation but not the activation of human heat shock factor 1. *J.Cell Physiol.* 2000; 185:348–357. [PubMed: 11056005]
- Percy AJ, Chambers AG, Smith DS, Borchers CH. Standardized protocols for quality control of MRM-based plasma proteomic workflows. *J.Proteome.Res.* 2013; 12:222–233. [PubMed: 23245390]
- Perlingeiro RCR, Queiroz MLS. Polymorphonuclear phagocytosis and killing in workers exposed to inorganic mercury. *International Journal of Immunopharmacology.* 1994; 16:1011–1017. [PubMed: 7705962]
- Pollard KM, Hultman P. Effects of mercury on the immune system. *Met.Ions.Biol.Syst.* 1997; 34:421–440. [PubMed: 9046578]
- Pollard KM, Pearson DL, Hultman P, Deane TN, Lindh U, Kono DH. Xenobiotic acceleration of idiopathic systemic autoimmunity in lupus-prone bxs mice. *Environ.Health Perspect.* 2001; 109:27–33. [PubMed: 11171521]
- Pollard KM, Pearson DL, Hultman P, Hildebrandt B, Kono DH. Lupus-Prone Mice as Models to Study Xenobiotic-Induced Acceleration of Systemic Autoimmunity. *Environ Health Perspect.* 1999; 107:729–735. [PubMed: 10502538]
- Queiroz MLS, Dantas DCM. B lymphocytes in mercury-exposed workers. *Pharmacology & Toxicology.* 1997a; 81:130–133. [PubMed: 9335070]
- Queiroz MLS, Dantas DCM. T lymphocytes in mercury-exposed workers. *Immunopharmacol.and.Immunotoxicol.* 1997b; 19:499–510.
- Rahman SM, Pu MY, Hamaguchi M, Iwamoto T, Isobe K, Nakashima I. Redox-linked ligand-independent cell surface triggering for extensive protein tyrosine phosphorylation. *FEBS Lett.* 1993; 317:35–38. [PubMed: 8381367]
- Rowley B, Monestier M. Mechanisms of heavy metal-induced autoimmunity. *Mol.Immunol.* 2005; 42:833–838. [PubMed: 15829271]
- Scott DW, Livnat D, Pennell CA, Keng P. Lymphoma models for B cell activation and tolerance. III. Cell cycle dependence for negative signalling of WEHI-231 B lymphoma cells by anti-m. *J.Exp.Med.* 1986; 164:156–164. [PubMed: 3487612]
- Silbergeld EK, Silva IA, Nyland JF. Mercury and autoimmunity: implications for occupational and environmental health. *Toxicol Appl Pharmacol.* 2005; 207:282–292. [PubMed: 16023690]
- Via CS, Nguyen P, Niculescu F, Papadimitriou J, Hoover D, Silbergeld EK. Low-dose exposure to inorganic mercury accelerates disease and mortality in acquired murine lupus. *Environ.Health Perspect.* 2003; 111:1273–1277. [PubMed: 12896845]
- Wright VP, Reiser PJ, Clanton TL. Redox modulation of global phosphatase activity and protein phosphorylation in intact skeletal muscle. *J.Physiol.* 2009; 587:5767–5781. [PubMed: 19841000]
- Xu Y, Harder KW, Huntington ND, Hibbs ML, Tarlinton DM. Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity.* 2005; 22:9–18. [PubMed: 15664155]

- Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW, Tedder TF. B-lymphocyte contributions to human autoimmune disease. *Immunol.Rev.* 2008; 223:284–299. [PubMed: 18613843]
- Ziemba SE, Menard SL, McCabe MJ Jr, Rosenspire AJ. T-cell receptor signaling is mediated by transient Lck activity, which is inhibited by inorganic mercury. *FASEB J.* 2009; 23:1663–1671. [PubMed: 19168706]

HIGHLIGHTS

- Inorganic mercury (Hg^{2+}) induces changes in the WEHI-231 B cell phosphoproteome
- The B Cell Receptor (BCR) signaling pathway was the pathway most affected by Hg^{2+}
- The Src family phosphoprotein kinase Lyn was the most interconnected node
- Lyn is likely central to the immunotoxic potential of Hg^{2+}
- Lyn phosphorylation profiles may be biomarkers for Hg^{2+} intoxication of B cells

Figure 1.

Mercury and pervanadate increase phosphoprotein abundance in a concentration-dependent manner. WEHI-231 cells were treated with the indicated concentrations of mercury (HgCl_2) or pervanadate for 10 min, washed, lysed and trypsinized.

Phosphopeptides were enriched by TiO_2 affinity then analyzed by LC-MS/MS [n=3; data represent phosphoproteins with significantly different spectral counts in comparison to untreated samples ($p < 0.05$) by Fisher's Exact Test]. Untreated controls for Hg-treated cells and PV-treated cells produced 1084 $\pm$ 56 and 1125 $\pm$ 97 protein identifications respectively, mean $\pm$ SEM.

Figure 2.

Protein-protein interaction network modeling identified Lyn as a key hub protein after mercury exposure. Phosphopeptides were enriched from control and treated cells, and the relative abundance of phosphoproteins in each sample was estimated using spectral counting. The sets of proteins significantly altered by treatment can be found in the Supplemental data. Proteins with the highest number of interacting proteins are colored yellow, their interaction partners are blue, the larger contiguous network proteins are pink, and other groups are shown in green. *A*, Protein networks altered by HgCl₂ treatment. Lyn was identified as the key hub protein. *B*, Protein networks significantly altered by pervanadate. Key hub proteins were identified as protein tyrosine phosphatase, non-receptor type 6 (PTPN6) and phospholipase C, gamma 2 phosphatidylinositol-specific (PLCG2).

Figure 3.

Lyn is phosphorylated on 7 tyrosine residues. Tryptic digests of murine Lyn were analyzed by LC-MS/MS from three sources: immunopurification and phosphopeptide enrichments from WEHI-231 cells, or from recombinant protein. **A**, Representative MS/MS spectrum of the doubly-charged Lyn phosphopeptide ⁴⁴⁸TNADVMSALSQG(pY)⁴⁶¹R. The phosphorylation site was unambiguously assigned to pY-460 based on the mass assignments of fragment ions y₃, b₁₂ and b₁₃. Not all peaks are annotated. More complete MS/MS peak assignments for this spectrum and for pY-117, pY-193, pY-194, pY-473, pY-501 and pY-508 phosphopeptide spectra can be found in the Supplemental data. **B**, Lyn sequence coverage. Identified sequences are enclosed, and phosphosites are shaded in black. Detailed peptide identification information can be found in the Supplemental data.

Figure 4.

Mercury alters the phosphorylation profile of Lyn. WEHI-231 cells were stimulated with HgCl_2 (A) or pervanadate (C) for 10 min, or with anti-Ig (in order to stimulate the B cell receptor pathway) for the indicated times (B). Relative quantitation of specific Lyn phosphosites was performed by multiple reaction monitoring (MRM) [n=3; error bars = standard error of the mean].

Figure 5.

Quantified murine Lyn phosphosites are conserved across Src kinase proteins in various species. A, The peptides containing mercury-sensitive Lyn phosphorylated residues were compared against other Lyn and Fyn protein sequences. Conserved residues are highlighted in black. B, Lyn protein diagram showing phosphosites in relation to functional domains.

Table 1

Pathway analysis. Phosphoproteins found to be significantly altered by exposure to either mercury or pervanadate were analyzed for KEGG pathway enrichment. The top 5 pathways, ranked by Combined Score, are shown for each treatment.

#	Term	p-value ¹	Z-Score ²	Combined Score ³	Genes
MERCURY					
1	HSA04662 B CELL RECEPTOR SIGNALING PATHWAY	2.13E-07	-2.0349	23.5571	SYK;LYN;CD72;BLNK;PTPN6;CD79B;CD79A
2	HSA04520 ADHERENS JUNCTION	0.0135	-1.8166	2.9467	WAS;PTPN6;PTPN1
3	HSA00271 METHIONINE METABOLISM	0.0072	-1.5915	2.9348	DNMT1;MARS
4	HSA05131 PATHOGENIC ESCHERICHIA COLI INFECTION EPEC	0.0489	-1.6444	1.9400	ARHGEF2;WAS
5	HSA04110 CELL CYCLE	0.0398	-1.6377	1.9321	CDC2;RBI;MCM3
PERVANADATE					
1	HSA04662 B CELL RECEPTOR SIGNALING PATHWAY	2.62E-09	-2.0349	31.9463	BTX;SYK;CD72;CD22;BLNK;PTPN6;PLCG2;CD79B;CD79A
2	HSA04664 FC EPSILON RI SIGNALING PATHWAY	3.29E-05	-1.9489	13.5561	BTX;SYK;PRKCD;MAPK1;MAPK3;PLCG2
3	HSA05223 NON SMALL CELL LUNG CANCER	0.0010	-1.9573	8.2299	STK4;MAPK1;MAPK3;PLCG2
4	HSA04650 NATURAL KILLER CELL MEDIATED CYTOTOXICITY	0.0006	-1.8108	7.9789	SYK;MAPK1;MAPK3;PTPN6;PTPN11;PLCG2
5	HSA05211 RENAL CELL CARCINOMA	0.0024	-1.6399	5.8535	PAK2;MAPK1;MAPK3;PTPN11

¹The p-value is computed from the Fisher's exact test which is a proportion test that assumes a binomial distribution and independence for probability of any gene belonging to any set.

²The rank-based ranking is derived from running the Fisher's exact test from many random gene sets in order to compute a mean rank and standard deviation from the expected rank for each term in the gene-set library and finally calculating a Z-score to assess the deviation from the expected rank.

³Combined score is computed by taking the log of the p-value from the Fisher's exact test and multiplying that by the Z-score of the deviation from the expected rank.