

PHARMACEUTICAL PROTEOMICS™

Protein Markers of Disease, Drug Efficacy and Toxicity, and their Use in Pharmaceutical Discovery and Development: A Review of Selected Published Applications

Large Scale Proteomics Corporation
(a subsidiary of Large Scale Biology Corporation)

November 1999

Proteomics offers an unbiased approach for monitoring biological mechanisms. Proteins whose quantitative expression is linked with a therapeutic effect, a toxic effect or a disease process are detected and identified to yield quantitative markers of these mechanisms *in vivo* and *in vitro*.

INTRODUCTION

Virtually all pharmaceutically-relevant biological mechanisms can be monitored quantitatively using specific protein markers in samples of serum, urine, or tissue. The value of effective biological markers is substantial, allowing early detection and classification of toxicities, ranking of potential lead compounds, acceleration of clinical trials, and stratification of patients into the most responsive groups. Nevertheless, few such markers are in current use - in fact few have so far been discovered. Pharmaceutical proteomics addresses this need through systematic discovery, characterization and validation of novel tissue and body fluid protein markers.

Proof of concept studies in pharmaceutical proteomics

LSB has led the advance of pharmaceutical proteomics through a wide-ranging series of proof-of-concept studies carried out in collaboration with numerous large pharma companies and academic laboratories. In addition to in-house projects on more than fifty pharmaceutical compounds, LSB has undertaken more than sixty commercial projects to date with a total of twenty-four clients, including twelve large pharma companies. Many of these studies have resulted in published data, while many others relating to proprietary development-stage compounds are currently unpublished and confidential. Here we summarize selected work published by LSB and collaborators illustrating the breadth of applications and scope of results.

PRE-CLINICAL APPLICATIONS

LSB has found protein markers of important mechanisms in a variety of animal sample types used in pre-clinical studies. Samples examined include rodent liver, brain and kidney tissue (toxicology), muscle (atrophy), adipose tissue, and dorsal root ganglia (pain models), as well as *in vitro* systems such as hepatocytes, kidney slices, Schwann cells and vascular endothelial cells. Published data from a subset of these studies provide informative examples of proteomics results.

Protein expression effects of drugs

Monitoring drug effects is one of the most productive applications of pharmaceutical proteomics. Binding of a drug to its protein target(s) typically causes measurable downstream effects on the abundance and/or properties of numerous other proteins involved in both the mechanism of action and potential toxicity [Figure 1]. The cell is equipped with numerous regulatory mechanisms that respond to the drug-induced perturbation by up- or down-regulation of the amount of specific proteins, in many cases through changes designed to restore cellular function to a pre-treatment state. Because all proteins are expected to have functions, these downstream regulatory effects can be functionally quite important, and in many cases they actually constitute the therapeutic or toxic mechanism activated by a drug. A central goal of pharmaceutical proteomics is to observe these changes, and create the experience base required to interpret them in functional terms.

Efficacy and toxicity markers for 'Statin'-class cholesterol lowering compounds

Agents acting to alter blood cholesterol (e.g., the statin HMG-CoA reductase inhibitors, cholestyramine, and high-cholesterol diets) alter the abundances of several proteins in rat liver. Most strongly affected is a protein we have identified as HMG-CoA synthase [1], a critical enzyme of the cholesterol synthesis pathway. Based on this data, HMG-CoA synthase can be used as an intracellular reporter of the pathway's performance [Figure 2]. The statins, but not cholestyramine, also induce a peroxisomal enoylhydratase-like protein, which we have identified as a sensitive marker for peroxisome proliferation (representing an undesirable side effect). The ratio between these efficacy and toxicity markers is different for different marketed statin drugs, indicating probable pharmacological inequivalence and providing an example of marker ratios useful in ranking lead compound candidates.

Signature patterns of peroxisome proliferators in mouse liver

A study in mouse liver of the effects of a set of strong peroxisome proliferators (PP; agonists of the nuclear receptor PPAR α) and a non-proliferator (an analog of one of the PP's tested and having similar pharmacological potency) demonstrated three distinct patterns of gene expression change. The patterns that could be distinguished corresponded to 1) PPAR α agonist activity [Figure 3], 2) animal age (30 days difference in age at sacrifice between 5 and 35 day treatment groups), and 3) action of the non-PP compound by a different mechanism [Figure 4] [2]. The results provided support for a unified receptor-based mechanism controlling the main PP response, but demonstrate that individual responsive genes can show quite different dose response curves. More than 100 proteins showed significant changes following PP treatment, and multiple sensitive markers were identified. Undertaken with Eli Lilly.

Efficacy marker of dithiolethione chemoprevention compounds in rat liver

A series of dithiolethiones that induce liver phase II enzymes was investigated in parallel with other cancer chemoprevention agents [3]. Oltipraz, the lead dithiolethione currently under clinical investigation for prevention of aflatoxin-induced liver cancer, was shown to affect a series of proteins in rat liver, the most prominent being the more than 7-fold increase in aflatoxin

B₁ aldehyde reductase (AFAR). AFAR is an enzyme known to be involved in detoxification of aflatoxin and thus its emergence as a marker for this class of therapeutic effect makes biochemical sense. Oltipraz analogs anethole trithione (ANTT), 1,2-dithiole-3-thione (1,2-DT-3-T) and 1,3-dithiole-2-thione (1,3-DT-2-T) were examined with respect to their ability to increase liver AFAR levels and the patterns of induction were found to be in line with their relative potencies. 1,3-DT-3-T, which did not significantly induce AFAR (and shows low potency in the liver chemoprevention application), was compared to an extended set of compounds. It was found to closely resemble the signature pattern of piroxicam at high dose, illustrating the detection in one structural class of a mechanism normally associated with another class. Undertaken with the U.S. National Cancer Institute.

Renal marker associated with kidney toxicity of immunosuppressants

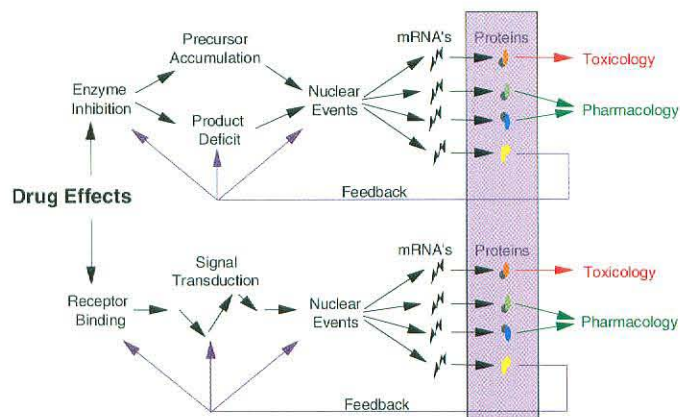
The use of cyclosporine A (CsA), a potent immunosuppressant, is limited by a kidney toxicity whose mechanism was unclear. Kidneys from CsA-treated rats showed profound down-regulation of the calcium binding protein calbindin d28 [4]. This protein is an intracellular calcium buffer and transport protein, and its near absence in the kidneys of CsA treated animals provides an explanation for the accumulation of calcium in the tubules of affected animals [Figure 5]. A subsequent SAR study showed that the down-regulation of calbindin was closely associated with immunosuppressant activity and that its down-regulation also occurred in humans showing CsA-related nephrotoxicity [5, 6]. These studies provide a prototypical example of the discovery of a marker via proteomics and its use in defining and analyzing a novel mechanism of toxicity. Undertaken with Novartis Pharma.

Markers of drug toxicity and efficacy to support lead compound prioritization

SDZ PGU 693 is a hypoglycemic agent that induces hepatocellular hypertrophy in the rat. In liver homogenates of treated rats several microsomal proteins including NADPH cytochrome P-450 reductase and cytochrome b5 were strongly increased, indicative of microsomal proliferation and induction of the P-450 enzyme system causing hepatocellular hypertrophy [7]. Decreases were evident in a series of mitochondrial proteins such as F₁ATPase- δ subunit and cytosolic liver fatty acid binding protein suggesting a down-regulation of the mitochondrial liver fatty acid metabolism, likely reflecting the pharmacological action of the compound. The availability of protein markers of toxicity

Drug Mechanisms Involve Changes in Gene Regulation Implemented at the Protein Layer

Fig. 1 A schematic representation of drug action through two types of target: enzyme inhibition and receptor binding. If an enzyme is inhibited, its substrates accumulate and its product is depleted. Cellular homeostatic mechanisms typically sense this departure from the normal state and trigger alterations in the levels of mRNA and proteins. Similarly, when an extracellular receptor is occupied, a signal transduction cascade is set in motion that then affects mRNA and protein levels. A similar set of events follows channel blockade. The proteins, as functional elements of the cell, are directly responsible for the pharmacologic and toxicologic effects of the drug.



Liver Cytosolic HMG-CoA Synthase is a Marker for Response to Cholesterol-Altering Treatments

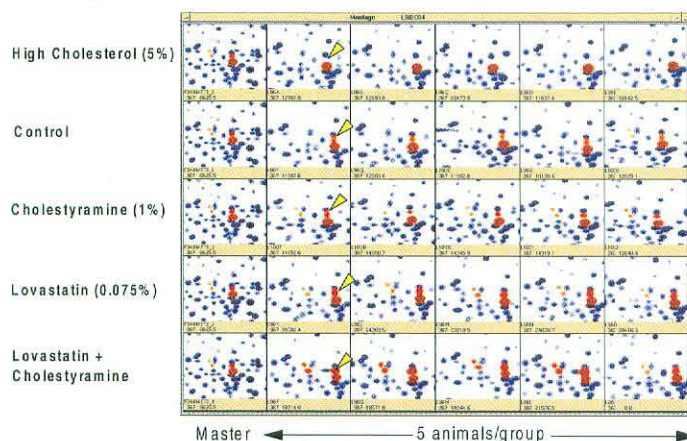
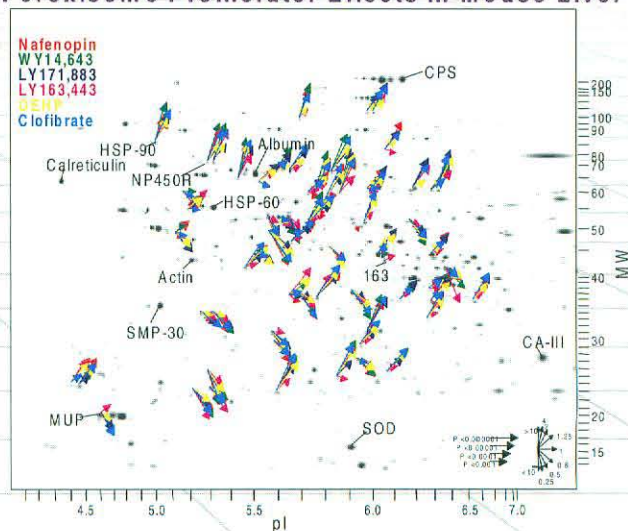


Fig. 2. The figure is a montage display generated by LSB's Kepler™ software showing a specific region from each of 25 rat liver 2-D gels, with a selected group of spots highlighted in red. These spots are the 'answer' to a user-originated statistical query aimed at finding spots (proteins) whose abundance varies in response to treatments affecting blood cholesterol. The montage has been arranged so that each of the 5 experimental groups occupies one row. Within each row, the first pattern is a copy of the rat liver master pattern (used to display group average results) and the succeeding 5 patterns are patterns of liver proteins from each of the 5 animals in the respective treatment group. This example illustrates the reproducibility achievable by 2-DE, as well as its power to reveal mechanism-related proteins in drug-effects studies.

Fig. 3. An "arrowplot" of comparative *in vivo* liver protein abundance changes caused by a series of six compounds, five of which are known peroxisome proliferators, and hence hepatocarcinogens, in rodents. The figure indicates the consistency of *in vivo* protein level effects caused by structurally-diverse compounds that act through a common pathway. Even small quantitative changes (increases or decreases of 20%) are consistently produced. The background picture is the master 2-D pattern of proteins from B6C3F1 mouse liver. Short arrows project from selected protein spots showing quantitative changes in treated animals versus controls: each arrow expresses the effect of one agent on one spot by summarizing the statistical difference between measurements on liver samples from treated animals (6 per group) versus measurements on a set of parallel control animals. Arrow angle indicates relative magnitude and direction of change (abundance ratio treated/control), and arrow length indicates t-test statistical significance of the difference (as shown in the legend at lower right).

Peroxisome Proliferator Effects in Mouse Liver



and efficacy allowed definition of a therapeutic window for each drug screened in the system and thus supported lead compound prioritization. Undertaken with Novartis Pharma.

Liver steatosis linked to protein expression considered to be adipocyte-specific

The liver toxicity of etomoxir, an inhibitor of carnitine palmitoyl transferase developed as a potential antidiabetic, was found to involve induction of the "adipocyte differentiation-related protein" (ADRP) [8]. This protein appears to be associated with lipid droplets which accumulate in the hepatocytes following treatment with etomoxir, and demonstrates that at least one gene product previously considered specific to the adipocyte is induced in hepatocytes by the drug. ADRP may represent an early marker for impaired lipid metabolism and potential lipid accumulation in liver. Undertaken with Novartis Pharma.

Adduct formation in hepatocyte mitochondria associated with hepatocarcinogenesis of methapyrilene

Methapyrilene (MPY) is an histamine H1 receptor antagonist which was withdrawn as a component of OTC medications when found to produce liver tumors in rats despite absence of genotoxicity. Liver protein patterns from MPY-treated rats showed widespread changes in protein expression, as well as extensive dose-dependent covalent modification of a series of mitochondrial proteins [9, 10]. These changes were absent following treatment with the non-tumorigenic MPY analog pyrillamine [11]. Both rat and mouse hepatocytes showed modifications paralleling those observed *in vivo*, while human hepatocytes showed no statistically significant effect [12], consistent with human epidemiological data suggesting a lack of tumors in man. MPY thus appears to be a rodent-specific liver carcinogen whose mechanism may involve adduction of mitochondrial DNA through a reactive metabolite produced in the mitochondrial matrix. The study illustrates the facility with which novel protein adducts and their effects can be analyzed via proteomics. Undertaken with Eli Lilly.

Protein modification pattern associated with hepatotoxicity of acetaminophen

Acetaminophen (APAP), a widely used analgesic drug, is hepatotoxic in large doses, whereas its m-hydroxy isomer, 3'-hydroxyacetanilide (AMAP), shows no serious liver toxicity. Comparative analysis of the effects of

APAP and AMAP on the mouse liver proteome showed specific effects of the former consistent with protein modification, against a background of other effects common to both agents [13]. Undertaken with the University of Washington.

Validation of *in vitro* systems used in drug screening and discovery

Analysis of 60 human tumor cell lines used by the NCI for screening candidate cancer chemotherapeutics showed that the protein expression patterns of lines from the same tumor type are generally not consistent (e.g., breast cancer lines can be less similar to one another than to melanomas). However, important associations were found between the expression of certain proteins in the 60 lines and the lines' relative susceptibility to various classes of chemotherapeutic drugs [14, 15], suggesting that these proteins were, or were co-expressed with, potential targets. This work represented the first time that cell lines used in a drug screening system had been subjected to comparative proteome analysis. Undertaken with the U.S. National Cancer Institute.

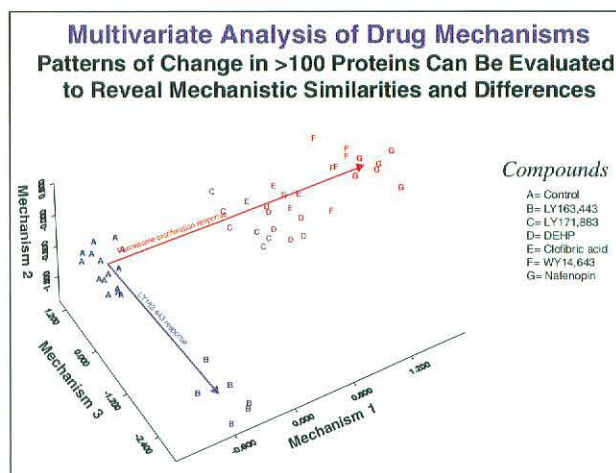
Early indications for linkage between methionine metabolism and apoptosis pathway

Early protein expression studies provided one of the first examples of what is now recognized as *apoptosis* (programmed cell death) [16]. In HL-60, a human monocytic leukemic line, a series of treatments including 5-azacytidine and methionine starvation were found to produce similar effects on protein expression correlated with nuclear morphological alterations which included specific proteolytic cleavages and translocation of some proteins to the nucleus. These effects led to an early linkage between the apoptosis pathway and methionine metabolism in differentiation-susceptible cell lines.

CLINICAL APPLICATIONS

LSB's principals have performed extensive proteomics studies on a variety of human samples. These include the first proteomics studies of human serum and plasma [Figure 6], identifying the major known proteins and characterizing glycosylation heterogeneity [17, 18, 19, 20, 21], as well as pioneering studies on the proteomics of urine [22, 23, 24], saliva [25], milk [26], seminal plasma [27], normal PBL [28, 29], platelets [30], RBC [31], lens [32], and skeletal muscle [33, 34, 35, 36]. In addition, LSB has done extensive unpublished work on proteomes of human liver, heart muscle and brain.

Fig 4. Results of a multivariate statistical analysis of the overall effects of 6 compounds in mouse liver, illustrating the resolution of separate mechanisms based on protein expression data. The "Mechanism 1" axis is the peroxisome proliferator effect - a complex, unified pattern of quantitative liver protein changes caused by PP treatment that distinguishes the liver protein patterns of control mice (A) from the patterns of livers affected by increasingly potent PP's (C-G). "Mechanism 3" separates a negative control compound (B, a non-proliferator) from the controls, and demonstrates that it acts by a separate mechanism (i.e., displaced along an orthogonal axis). Each letter designates a different treatment and each letter symbol represents the protein pattern of an individual mouse liver. More than 100 proteins showed significant abundance changes with these treatments and contributed to the mechanisms plotted here.



Histological Effects of CsA Treatment in Rat Kidney

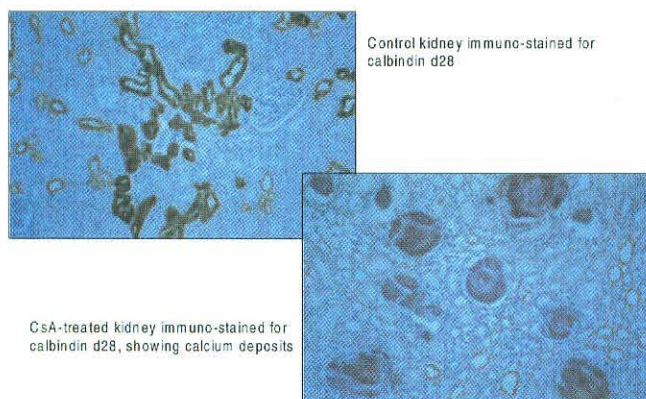
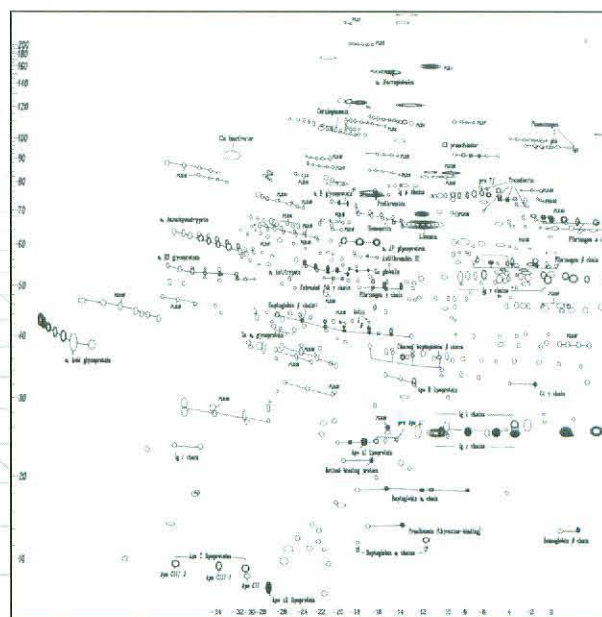


Fig. 5. Calbindin d28, discovered via proteomics to be a marker for cyclosporine's effect in rodent kidney, is confirmed by immunohistochemistry using an antibody to the protein. Protein abundance (staining intensity) is profoundly reduced in kidney sections from CsA-treated animals, and the protein is shown to be localized to the tubular epithelium. Calcium-containing inclusions are also observed within the tubules of treated kidneys, consistent with a failure to transport Ca across the tubules caused by lack of the calbindin d28 transport protein.

Figure 6. Diagram of the human plasma proteome, illustrating detection and identification of essentially all of the major, known human plasma proteins. LSB's founders published the first papers examining plasma via proteomics and contributed a majority of these identifications. In plasma and serum, most proteins are extensively glycosylated. The heterogeneity of this process leads to the detection of "trains" of spots for each glycoprotein, within which individual spots represent forms bearing different numbers of sialic acid groups. Important members of the classes of lipoproteins, coagulation proteins, transport proteins, immunoglobulins, and antiproteases are all represented and can be quantitated. By means of additional fractionation procedures, large numbers of additional low-abundance proteins can be detected.



Monitoring drug efficacy as ability to normalize a novel panel of acute-phase response markers

A series of studies on the acute phase response in human plasma demonstrated that numerous quantitative changes could be observed in plasma proteins in addition to the classical acute phase reactants. Analysis of a drug crossover trial comparing piroxicam and tenidap indicated that tenidap was more effective in normalizing the levels of the acute-phase responsive plasma proteins than piroxicam [37]. This study first demonstrated the use of multiple protein disease state markers to assess the ability of competing drugs to restore protein levels to normal values. Undertaken with Pfizer.

Disease markers of mental illness

Proteome analyses were carried out on frontal lobe tissue obtained postmortem from 89 individuals suffering from mental illness (schizophrenia, unipolar and bipolar depression) and from healthy controls. A panel of 19 proteins was found in which coordinated abundance differences could distinguish between normal and depression samples [Figure 7].

Using a more sophisticated multivariate analysis to correct for variables unrelated to disease, a number of individual proteins were identified that differed in level between mentally ill and unaffected brains [38]. Undertaken with the Stanley Laboratory, Johns Hopkins University.

Protein markers to classify cell types of origin

In collaboration with investigators at the Mayo Clinic, LSB principals undertook an extensive study of human leukemias [39], demonstrating that these tumors preserve major aspects of the gene expression patterns of their cell types of origin (e.g., T- and B-lymphocytes, monocytes and granulocytes), facilitating their detailed classification based on multiple quantitative protein markers.

Also in collaboration with the Mayo Clinic, LSB has compared bile duct epithelial cells (BDE, a minor liver cell type) with the dominant (~95%) liver hepatocytes. BDE cells produce numerous markers distinct from the hepatocyte, and among these are some unexpected proteins such as annexin V and the selenium binding protein [40] which suggest interesting functional properties attributable to these cells.

RELATIONSHIP OF PROTEOMICS AND GENOMICS IN THE SEARCH FOR USEFUL MARKERS

In marker studies using the primary clinical specimens (serum, plasma and urine), the most informative markers will be proteins because there is essentially no informative RNA present. Nevertheless in preclinical animal studies and in studies of human surgical or *in vitro* systems, where both protein and mRNA can practically be obtained, one must inquire into the relationship between protein and mRNA changes to determine where the best markers of biological effects may be found. In collaboration with Incyte Pharmaceuticals, LSB has compared [41] human liver protein abundances with the abundances of the corresponding mRNA's [Figure 8]. The result in this case, confirmed in other studies, is a correlation coefficient of less than 0.5 (closer to no correlation than to a perfect correlation of 1.0): the relationship between mRNA and protein is thus not directly predictable in the general case. Since proteins embody biochemical functions, while their mRNA's do not, there is a strong case that protein markers should be preferred as more reliable and functionally relevant.

PROTEOMICS TECHNOLOGY AND EXPERIENCE

As evidenced by the wealth of pharmaceutically-relevant data obtained to date, LSB's proteomics technology platform has proven to be a mature and stable research tool. As such it allows users to focus immediately on solving major problems in drug discovery and development, rather than beginning the long and time-consuming learning curve required to master complex analytical systems and informatics packages.

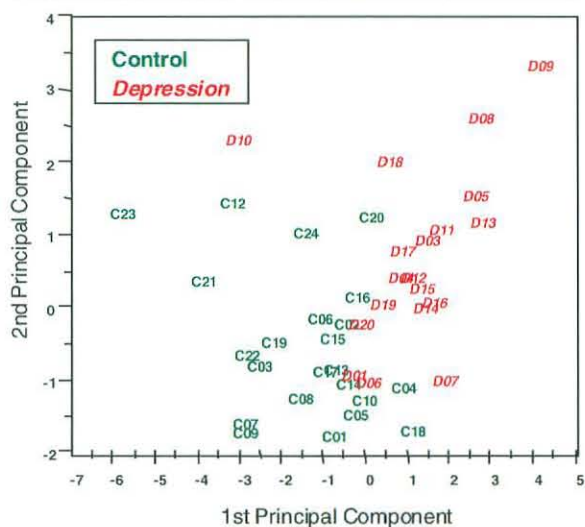
As with any sophisticated tool, experience in its use is critical. LSB has accumulated by far the broadest and deepest reservoir of results and expertise in pharmaceutical proteomics and uses advanced proprietary bioinformatics systems to accumulate these resources and apply them to current projects [Figure 9]. These systems comprise database architectures and analysis tools that allow statistically robust markers to be found in well-structured studies, and their specificity verified in comparisons across current and past studies of multiple species and organs.

FURTHER INFORMATION

References, abstracts and publications concerning many of the above-mentioned studies are available on LSB's website, accessible at <http://www.lsb.com>. To discuss business opportunities please contact Constance Seniff (connie@lsbc.com).

Figure 7. Multivariate statistical analysis (via principal components) allows scoring of samples based upon complex quantitative changes in a panel of selected proteins. This approach can provide disease discrimination under circumstances where no single marker protein or gene can distinguish disease or treatment from control groups. The figure shows such an example in which a panel of 19 selected protein spots was used to distinguish samples of human post-mortem brain from 1) normal individuals (C's), or 2) individuals diagnosed with depression without psychosis (D's). The proteins involved are unlikely to be known neurotransmitter receptors, and may instead represent a new class of intracellular disease-mechanism indicators and targets.

PCA Comparison of Human Brain Protein Patterns



Protein (2D) vs mRNA (RTI) Abundance in Human Liver

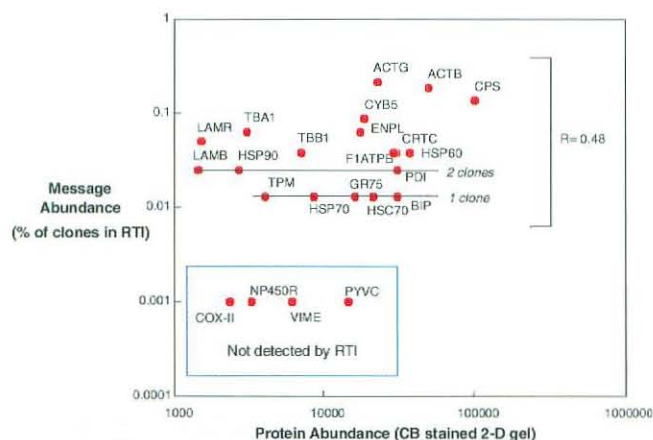
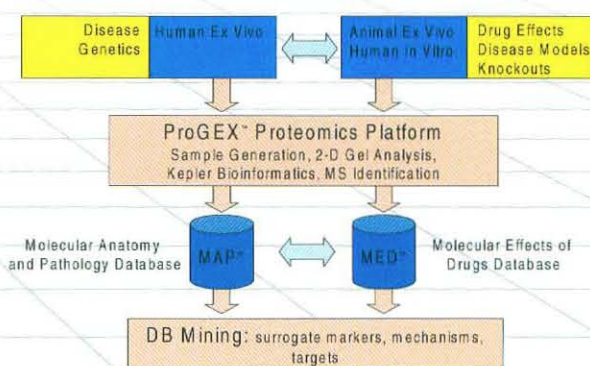


Fig 8. A log/log plot comparing the abundance of mRNA (vertical axis, determined via transcript imaging by Incyte Pharmaceuticals) and protein (determined by quantitative 2-D gel based proteomics by LSB) for a series of gene products in human liver. For those gene products measured in both systems, a correlation coefficient of 0.48 was obtained [41]. In this study, it emerged that almost all of the most abundant 50 messages in liver code for secreted proteins, while none of the most abundant 50 proteins in liver tissue were secretory. Thus in a tissue with a major secretory function, the spectrum of genes examined by protein and mRNA technologies can be quite different.

Figure 9. Diagram illustrating the relationships between LSB's MAP™ and MED™ proteomics databases. The Molecular Anatomy and Pathology™ (MAP™) database describes the baseline protein composition of all relevant normal tissues and separable cell types and the changes in protein composition associated with disease. The Molecular Effects of Drugs™ (MED™) database is focused on changes in protein composition associated with therapeutic drugs and other treatments (rodent *in vivo* and human *in vitro*). In order to "mine" the extensive information (millions of protein measurements) contained in the databases, LSB has developed a unique relational database architecture, advanced statistical analysis methodologies and a network-based access vehicle. These bio-informatics facilities allow an investigator to visualize the relationship of one complex biological effect to any others previously examined, and to inspect the response of selected proteins in all studies in the database.

Organization of Proteomics Data In Pharma Development: MAP™ and MED™ Databases



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