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Research Paper

VeloceGenomics: An Accelerated *in Vivo* Drug Discovery Approach to Rapidly Predict the Biologic, Drug-Like Activity of Compounds, Proteins, or Genes

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Purpose. The aim of this study is to test the predictive power of *in vivo* multiorgan RNA expression profiling in identifying the biologic activity of molecules.
Methods. Animals were treated with compound A or B. At the end of the treatment period, *in vivo* multiorgan microarray-based gene expression data were collected. Investigators masked to the identity of the compounds analyzed the transcriptome signatures to define the molecular pathways affected by treatment and to hypothesize the biologic activity and potential therapeutic indications of the blinded compounds.
Results. For compound A, G-protein-coupled receptors and factors associated with cell growth were affected—growth hormone/insulin-like growth factor-1, glucagon/insulin axes, and general somatomedin-like activity. Deblinding showed the compound to be a somatostatin analog, SOM230, confirming the accuracy of the predicted biologic activity. For compound B, components of the inflammatory cascade potentially mediated by lipopolysaccharide, tumor necrosis factor, or proinflammatory cytokines were affected. The gene expression signatures were most consistent with an interleukin-6 family activity. Deblinding revealed that compound B was leukemia inhibitory factor.
Conclusions. VeloceGenomics is a strategy of coupling *in vivo* compound testing with genomic technologies. The process enables prediction of the mechanism of action and, coupled with other relevant data, prediction of the suitability of compounds for advancement in the drug development process.

KEY WORDS: *in vivo* compound profiling; *in vivo* RNA expression profiling; mechanism of action; microarray; pharmacogenomics.

INTRODUCTION

Because of the analytic power of gene expression studies, microarray technology has become an integral part of the drug discovery and drug development processes (1). This has been possible to a great extent because microarray experiments can measure simultaneously the expression of thousands of genes. Gene expression profiling in the pharmaceutical industry is applied in studies of toxicology, target identification, and validation and has become an integral component of drug discovery and the preclinical phase of drug development (2–4).

The traditional drug discovery process is based on screening chemicals or biologic products, in an *in vitro*, high-throughput mode, against a battery of defined, preselected drug targets (e.g., enzymatic reactions, ligand–receptor interactions) (5). The use of *in vitro* models for screening compound activity has proven to be successful because the complexity of the biologic response is reduced and focused onto specific cell types and molecular targets. However, the weakness of this approach is the "artificially disconnected" use of *in vitro* target models that do not reflect the tightly interconnected and interdependent relationships of the different targets that exist in a whole organism (5–7). Another drawback is that the biologic activity against all nonselected targets is missed. Therefore, the full biologic consequences—beneficial or detrimental—are not fully revealed. Drug discovery research strategies that introduce *in vivo* studies early in the process and truly validate the full pharmacologic activity of potentially interesting compounds can circumvent many of the pitfalls of early-stage research (4,8–11). An investigative approach that integrates molecular information

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ERK, extracellular signal-related kinase; GH, growth hormone; GPCR, G-protein-coupled receptor; IGF, insulin-like growth factor; IL, interleukin; JAK, Janus kinase; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; LRR, leucine-rich repeats; MAPK, mitogen-activated protein kinase; NF-κβ, nuclear factor kappa beta; OSM, oncostatin M; PI3K, phosphatidylinositol 3 kinase; SOCS, suppressor of cytokine signaling; SSTR, somatostatin receptors; STAT, signal transducer and activator of transcription; TIMP, tissue inhibitor of metalloproteinase; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor.

(e.g., gene expression profiling) on the actions of a compound throughout an organism may provide a more efficient and effective solution to the challenges of pharmacologic efficacy and safety evaluation in complex biologic systems.

We describe an approach to the drug discovery process that we have termed *VeloceGenomics*, an accelerated drug discovery strategy for predicting the biologic, drug-like function of a molecule using *in vivo* multiorgan whole transcriptome analyses (organism-wide gene expression profiling). This strategy may be used irrespective of the molecular target or the therapeutic indication. *In vivo* testing is the only accepted approach that can bring understanding of the full pharmacologic, toxicologic, and therapeutic potential of a compound under evaluation (10,11).

We tested the capability of this strategy to produce a compound-specific, multiorgan molecular signature through a series of blinded evaluations of two well-characterized proteins. The results demonstrated that through careful analysis of the gene expression profile, data miners working on the coded samples were able to predict the pharmacologic activities, most of the potential therapeutic indications, potential adverse effects, and protein family of the blinded compounds. This comprehensive approach, used early in the drug discovery process, should allow more accurate risk assessment of a compound's potential for drug development success.

MATERIALS AND METHODS

Outline of the VeloceGenomics Process

The VeloceGenomics process is outlined in Fig. 1. A gene (cDNA), protein, small molecule, or other type of compound is selected for analysis. The animals may be otherwise healthy, naive animals, or they may be specifically bred or engineered as disease models. Appropriate route of administration, dose, treatment schedule, and treatment duration are established. At the end of treatment, RNA is extracted from multiple organs, glands, and tissues. The primary data captured after treatment are the multiorgan RNA expression profiles. However, this information can be supplemented with a vast array of additional assays during and at the end of treatment (e.g., hematology or clinical biochemistry). Data mining and analysis are performed to enable prediction of the most plausible mode(s) of action, therapeutic indications, safety concerns, and issues that would affect the decision to promote a compound for further testing or commercial development.

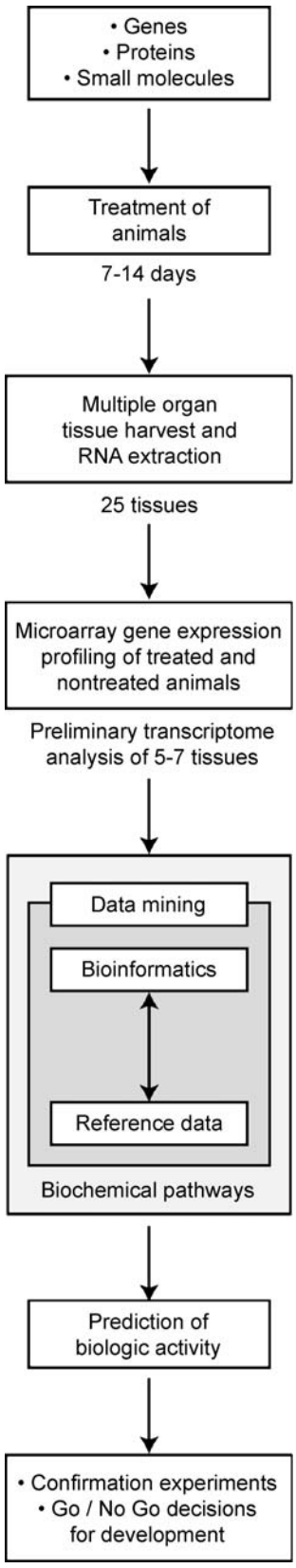

Fig. 1. Outline of the VeloceGenomics process.

Origin of Tissue, Treatments, and Tissue Processing

In this study, purpose-bred male and female cynomolgus monkeys (*Macaca fascicularis*), each approximately 24 months of age and weighing approximately 3 kg, were used. The monkeys were bred at the Centre de Recherches Primatologiques, Port Louis, Mauritius. At the breeder's facilities, the monkeys were treated against parasitic arthropods and helminths and subjected to a program of tuberculin testing.

The *in vivo* phase of this study was conducted at CIT (Evreux, France). On arrival at CIT, each animal was given a complete clinical examination under the supervision of a veterinarian. Anthelmintic treatment was administered. Coprologic examination and tuberculin testing were performed. The animals were held in quarantine for at least 3 weeks.

After quarantine, they were acclimated to the study conditions for at least 5 days before treatment began. The animals were individually identified by chest or thigh tattoo from the breeder's facility and by a unique CIT identity number given at the beginning of the study.

From their arrival at CIT, the animals were housed in a dedicated primate unit. The animal room conditions were set as follows: temperature, 24 ± 3°C; relative humidity, 50 ± 20%; light/dark cycle, 12 h each (0700–1900 h); ventilation, approximately 12 cycles/h of filtered, nonrecycled air. The corresponding instrumentation and equipment were checked and calibrated at regular intervals. Temperature and relative humidity were recorded continuously, and records were checked daily and filed.

All animals had free access to tap water (filtered with a 0.22-μm filter). Approximately 200 g of UAR 107 C pelleted diet (UAR; Villemoisson, Epinay-sur-Orge, France) was distributed daily to each animal. The food was given at least 1 h after dosing on the days of treatment. In addition, two fruits or vegetables were given daily to each animal.

The study was conducted in compliance with the following French Animal Health regulation: Council Directive No. 86/609/EEC of 24th November 1986 on the harmonization of laws, regulations, or administrative provisions relating to the protection of animals used for experimental or other scientific purposes.

For each treatment, two male and two female cynomolgus monkeys received compound A or B (100 μg/animal per day) or the vehicle (autologous serum) administered subcutaneously for 14 days. On day 15, all animals were humanely killed, and tissues for RNA extraction were immediately snap frozen and kept at −80°C until processing. Investigators were masked to treatment type.

RNA Expression Profiling

Total RNA was obtained from liver, kidney, spleen, thyroid gland, and pituitary by acid guanidinium isothiocyanate–phenol–chloroform extraction (12) using Trizol (Invitrogen Life Technologies, San Diego, CA, USA) and was purified on an affinity resin column (RNeasy; Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA microarray experiments were conducted as recommended by the manufacturer of the GeneChip system (Affymetrix Inc., Santa Clara, CA, USA) and as previously described (13). The human gene expression probe array used was HG-U95Av2 (Affymetrix), containing 12,627 probe sets interrogating approximately 10,000 full-length, annotated human genes. One GeneChip was used per tissue, per animal. The resultant image files (.dat files) were processed using the Microarray Analysis Suite 5 (MAS5) software (Affymetrix). Tab-delimited files containing data regarding signal intensity (Signal) and categorical expression level measurement (Absolute Call) were obtained. The data were quality checked and were transferred to GeneSpring software version 7.0 (Silicon Genetics, Redwood City, CA, USA) for analysis.

Data Analysis

Data were normalized by array to the 50th percentile of all measurements on that array, and each measurement for each gene in those arrays was normalized to the mean of that gene's signal intensity measurements in the corresponding control arrays. To determine which genes were differentially expressed between the treatment groups (i.e., treated *vs.* nontreated), averages of gene expression levels in the groups were calculated; low values were cut off using a lower threshold of 20 [based on PM − MM calculation where the mismatch (MM) value is deducted from the perfect match (PM) value], and one-way analysis of variance (ANOVA) was applied to genes that had been marked *present* by Absolute Call (see above) in at least one of the samples of a given tissue. No multiple testing correction was applied. Genes with $p < 0.05$ were considered statistically significant. Group fold changes were calculated by using the mean of the signal intensities of the treatment group compared with the control group. Data were combined for the treated male and female animals and for the control groups.

To rank genes based on their ability to differentiate among multiple groups of conditions (e.g., tissues), the hypergeometric Fisher exact test (from *K*-nearest neighbors' class prediction tool) was applied. Specificity ranks were calculated by taking the inverse of the sum of the p value from ANOVA (nonspecific) and the inverse value of the class predictor Fisher exact test prediction strength (1/prediction strength). The final number was the rank of the gene's specificity to multiple groups [specificity rank = 1/(ANOVA p value + 1/prediction strength)]. These filters can be applied to a dataset for individual tissues or can be used to identify genes with differential expression across tissues. The decision to keep or to reject a specific gene was based on the conjunction of numerical changes identified by comparative and statistical algorithms and on the relationship to other modulated genes that point to a common biologic theme. Data analysts assessed the weight of this relationship after a review of the relevant scientific literature. The annotation of the genes was determined by using NetAffx (http://www.affymetrix.com/analysis/netaffix/index.affix) (14). Information on probe sets was found in the literature or in the KEGG database (http://www.genome.ad.jp/kegg/kegg2.html).

RESULTS

At the end of the treatment period, more than 100 tissue samples per animal were dissected and snap frozen for storage. Total RNA was extracted from liver, kidney, spleen, thyroid gland, and pituitary and processed for gene expression profiling. Affymetrix microarray data were generated for each of the five tissue samples. The other stored tissues and organs may be evaluated if there was an indication from the analysis of the first five tissues that pointed to pathways better represented in specific tissues. The task of the investigators, who were masked to the treatment conditions, was to predict the identity or class of the peptide compounds with which the animals had been treated based solely on the gene expression signature from each compound.

To evaluate the effect of each compound on the transcriptome, genes were selected based on their expression level and on the statistical significance of the change in expression in all or a subset of the five tissues: kidney, liver, pituitary gland, spleen, and thyroid gland. This yielded a total

of 974 probe sets for compound A (ANOVA $p < 0.05$) and 1,074 probe sets for compound B (ANOVA $p < 0.01$).

Evaluation of Compound A

The bulk of changes in gene expression for compound A was related to signal transduction pathways through G-protein-coupled receptors (GPCRs) and glutamate receptors (Tables I and II). Downstream of these receptors, several pathways were affected, including the phosphatidylinositol 3′-kinase (PI3K), protein kinase C, phospholipase, calcium-calmodulin, Ras/mitogen-activated protein kinase/extracellular regulated kinase (Ras/MAPK/ERK), Janus kinase/signal transducer and activator of transcription (JAK/STAT), and adenylate/guanylate cyclase pathways (Tables I and II). A large number of gene expression changes with compound A also involved factors governing cell growth and apoptosis (Table II). In particular, the growth hormone/insulin-like growth factor-1 (GH/IGF-1) and the glucagon/insulin axes were altered in several organs (Tables I and II). Growth factors such as growth hormone (GH) and IGF-1, or an upstream regulator of their secretion such as somatomedin, can elicit this type of response. However, the change in expression level of receptors suggested that compound A was most likely a ligand rather than a downstream effector.

The primary molecular evidence of an endocrine hormonal effect was derived from the pituitary findings, including the alteration of the glucagon/insulin axes (Table II). For example, the involvement of a GH-like effect in the pituitary was clearly suggested by the compound's impact on IGFBPs, the leptin receptor, and the prostaglandins (15–17). Therefore, the alteration of the subsequent following pathways, adenylate cyclase, and phosphatidylinositol pointed to the transmission of the hormonal message through G-protein-coupled cell surface receptors (18–20). Other evidence, such as the effects on calcium, potassium channels, protein tyrosine phosphatases, and MAPKs, substantiated the hypothesis of the binding of a somatostatin receptor (SSTR) (21–25).

Based on these findings (i.e., signaling through G-protein-coupled receptors, effects on the entire GPCR downstream signaling cascade, changes in the GH/IGF-1 and insulin axes and on cell growth/apoptosis), we surmised that the effect of compound A was analogous to that of a somatomedin-like molecule (26). Binding to somatostatin receptors (SSTRs) results in activation of the PI3K signaling pathway, inhibition of adenylyl cyclase, activation of protein tyrosine phosphatases, modulation of MAPK, and coupling to inward-rectifying K^+ channels, voltage-dependent Ca^{2+} channels, Na^+/H^+ exchanger, AMPA/kainate glutamate channels, phospholipase C, and phospholipase A_2 (27,28). Somatostatin receptor activation blocks cell secretion by inhibiting intracellular cyclic adenosine monophosphate and Ca^{2+} and by a receptor-linked distal effect on exocytosis. SSTR1, SSTR2, SSTR4, and SSTR5 induce cell cycle arrest through the phosphotyrosine phosphatase-dependent modulation of MAPK, associated with the induction of the retinoblastoma tumor-suppressor protein and p21. SSTR3 triggers phosphotyrosine phosphatase-dependent apoptosis accompanied by the activation of p53 and Bax. Deblinding revealed that compound A was the somatostatin analog SOM230 (Sandostatin®).

Evaluation of Compound B

Treatment with compound B demonstrated an expression profile that included numerous genes involved in the inflammatory cascade, from acute-phase protein generation to immune response and repair (Tables III and IV). This type of response can be elicited by many agents, such as lipopolysaccharide (LPS), interleukin-1 (IL-1), and some of the IL-6 family cytokines (29–31). On specific binding to their cellular receptors, the proinflammatory cytokines of the IL-6 family generate downstream signaling events that include the JAK/STAT pathway, the suppressor of cytokine signaling (SOCS), and the Ras/MAPK pathway responsible for the physical manifestations of inflammation, from amplification to repair. In this study, gp130, the signal transducing subunit of IL-6 family receptors [oncostatin M (OSM) receptor or IL-6 receptor], was observed to be strongly up-regulated in each organ (Tables III and IV).

To determine whether compound B was an LPS-like molecule or a proinflammatory cytokine, we looked for a gene expression signature of LPS. Lipopolysaccharide has been shown to bind to the cell surface molecule CD14, which acts as a signal enhancer and associates with toll-like receptor 4 (TLR4) (32,33). Toll-like receptors, which recognize pathogen-specific molecules by their extracellular leucine-rich repeat (LRR) domain, then activate a signaling cascade involving MyD88, IL-1 receptor-associated kinase, TRAF6, IKKα and β, JNK1, and NF-κB through their cytoplasmic Toll/IL-1 receptor (TIR) domain and lead to the synthesis of inflammatory mediators, such as cytokines or nitric oxide. End products of this pathway are, for example, IL-1 and IL-6 family cytokines, such as OSM and leukemia inhibitory factor (LIF) (34–37). These factors can induce the expression of acute-phase proteins as an initial line of defense. None of the above-mentioned members of the Toll-receptor pathway were affected by treatment with compound

Table I. Data Analysts' Selection of Genes Deemed Most Significant in Guiding the Classification of Compound A

Analysis	Results
Kidney	$IGF2^-$, SLC9A1, urocortin$^+$, calmodulin 1 and 2^+, $VEGF^+$, $IGBP6^-$, EIF^+, $CACNB1^+$, $DAXX^+$, histone$^+$
Liver	$IGF2^-$, prostaglandin D2 synthase$^+$, SLC9A1, calmodulin 1 and 2^+, $VEGF^+$, EIF^+, $CACNB1^+$, adenylate cyclase 6^+, DAXX, histone$^+$, leptin R^+
Pituitary	prostaglandin D2 synthase$^+$, calmodulin 1 and 2^+, $VEGF^+$, EIF, adenylate cyclase 6, histone$^+$, leptin R^+
Spleen	$IGF2^-$, prostaglandin D2 synthase$^+$, calmodulin 1 and 2^+, $VEGF^+$, $IGBP6^+$, $CACNB1^+$, adenylate cyclase 6^+, $DAXX^+$, histone$^+$, leptin R
Thyroid	$IGF2^+$, $IGBP6^+$, EIF^+, $CACNB1^+$, $DAXX^+$, histone$^+$

+, Up-regulated expression in treated *vs.* nontreated animals; −, down-regulated expression in treated *vs.* nontreated animals.

Table II. Genes Affected by Treatment with Compound A

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Adenylate/guanylate cyclases and related pathways								
33134_at	Adenylate cyclase 3	1.3	1.3	1.4	1.2	1.4	*ADCY3*	AB011083
39383_at	Adenylate cyclase 6	1.1	1.9	1.3	1.4	1.0	*ADCY6*	AB007882
36754_at	Adenylate cyclase-activating polypeptide 1 (pituitary)	0.8	0.6	0.9	0.8	0.7	*ADCYAP1*	X60435
40788_at	Adenylate kinase 2	1.2	1.3	1.3	1.5	1.3	*AK2*	U84371
Cell surface receptors								
33470_at	Glutamate receptor-interacting protein 2	1.3	1.5	0.8	1.5	1.0	*GRIP2*	AF052177
37134_f_at	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1	1.0	2.6	1.4	1.4	1.4	*GRIN1*	L13266
37135_f_at	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1	1.0	5.4	1.9	1.9	0.7	*GRIN1*	L13268
37308_at	G-protein-coupled receptor 107	1.3	1.4	1.6	1.3	1.1	*GPR107*	AI888084
40299_at	G-protein-coupled receptor 161	1.4	1.5	1.2	1.5	0.9	*GPR161*	AF091890
40300_g_at	G-protein-coupled receptor 161	1.5	1.6	1.0	1.1	1.0	*GPR161*	AF091890
253_g_at	G-protein-coupled receptor 18	1.6	2.7	1.7	1.5	1.0	*GPR18*	L42324
31700_at	G-protein-coupled receptor 35	1.3	1.1	1.1	1.3	0.9	*GPR35*	AF027957
35769_at	G-protein-coupled receptor 56	1.4	1.1	1.2	1.1	1.2	*GPR56*	AJ011001
1392_at	G-protein-coupled receptor kinase 6	1.6	1.7	1.2	1.2	0.8	*GRK6*	L16862
40240_at	G-protein-coupled receptor, family C, group 5, member B	1.3	1.0	1.6	1.5	1.5	*GPRC5B*	AC004131
1457_at	Janus kinase 1 (a protein tyrosine kinase)	0.9	1.5	1.2	2.1	1.0	*JAK1*	M64174
Phosphatidyl inositol and related pathways/PKC, phospholipases								
33397_at	CDP-diacylglycerol-inositol 3-phosphatidyl transferase (phosphatidylinositol synthase)	1.1	1.9	1.2	1.1	1.1	*CDIPT*	AL050383
35755_at	Inositol 1,3,4-triphosphate 5/6 kinase	1.5	1.2	2.3	1.4	1.3	*ITPK1*	U51336
36154_at	Inositol hexaphosphate kinase 1	1.7	1.8	1.4	1.2	0.8	*IHPK1*	D87452
656_at	Inositol polyphosphate-1-phosphatase	1.8	1.4	1.7	1.6	1.2	*INPP1*	L08488
41285_at	Inositol polyphosphate-5-phosphatase, 40 kDa	1.8	2.4	0.9	2.2	1.0	*INPP5A*	X77567
38960_at	Phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	1.3	0.9	1.2	1.6	1.3	*PIB5PA*	U45975
37685_at	Phosphatidylinositol-binding clathrin assembly protein	0.8	1.0	1.5	1.3	1.5	*PICALM*	U45976
314_at	Phosphatidylinositol glycan, class B	1.2	1.2	1.7	1.7	1.2	*PIGB*	D42138
38297_at	Phosphatidylinositol transfer protein, membrane-associated 1	1.1	1.1	1.0	1.2	1.1	*PITPNM1*	X98654
31833_at	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	1.1	3.0	1.8	2.2	1.4	*PIP5K1A*	U78575
1269_at	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.6	0.6	1.1	1.0	0.9	*PIK3R1*	M61906
36151_at	Phospholipase D3	1.1	2.0	1.2	1.7	1.4	*PLD3*	U60644
39711_at	Protein kinase C substrate 80K-H	1.2	1.0	1.3	1.1	1.3	*PRKCSH*	J03075
35853_at	Protein kinase C, alpha-binding protein	1.5	1.1	1.0	1.3	1.5	*PRKCABP*	AL049654
Protein tyrosine phosphatases/other phosphatases								
922_at	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform	1.3	1.5	1.1	1.6	1.2	*PPP2R1A*	J02902
1094_g_at	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	1.4	1.8	0.9	1.1	1.7	*PPP2R1B*	M65254

Table II. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Protein tyrosine phosphatases/other phosphatases								
1383_at	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	1.4	1.2	1.3	1.4	1.1	*PPP2R2A*	M64929
903_at	Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	1.2	1.2	1.5	1.2	1.5	*PPP2R5A*	L42373
39068_at	Protein phosphatase 2, regulatory subunit B (B56), delta isoform	1.5	1.6	1.2	1.5	0.9	*PPP2R5D*	L76702
39127_f_at	Protein phosphatase 2A, regulatory subunit B′ (PR 53)	1.3	1.7	1.5	1.6	1.6	*PPP2R4*	X73478
38277_at	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)	1.5	1.8	1.3	1.3	1.0	*PPP3CB*	M29550
382_at	Protein phosphatase 4 (formerly X), catalytic subunit	1.5	1.3	1.4	1.1	0.8	*PPP4C*	X70218
392_g_at	Protein phosphatase 5, catalytic subunit	1.9	1.4	1.3	1.6	0.8	*PPP5C*	X89416
37581_at	Protein phosphatase 6, catalytic subunit	1.1	1.4	1.4	2.0	1.6	*PPP6C*	X92972
1241_at	Protein tyrosine phosphatase type IVA, member 2	1.3	0.9	1.3	1.1	1.3	*PTP4A2*	U14603
36008_at	Protein tyrosine phosphatase type IVA, member 3	1.3	2.1	1.0	1.2	1.0	*PTP4A3*	AF041434
588_at	Protein tyrosine phosphatase, nonreceptor type 1	1.8	1.4	2.1	2.1	1.3	*PTPN1*	M31724
38443_at	Protein tyrosine phosphatase, nonreceptor type 11 (Noonan syndrome 1)	1.3	1.1	1.6	1.3	0.9	*PTPN11*	U79291
1463_at	Protein tyrosine phosphatase, nonreceptor type 12	1.1	1.1	1.6	1.7	1.1	*PTPN12*	M93425
34198_at	Protein tyrosine phosphatase, nonreceptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	0.8	0.1	0.9	1.0	1.2	*PTPN13*	U12128
41782_g_at	Protein tyrosine phosphatase, receptor type, F polypeptide (PTPRF), interacting protein (liprin), alpha 1	1.9	1.4	1.5	1.6	0.9	*PPFIA1*	U22815
Ras/MAPK/ERK-related pathways and adaptor proteins								
36660_at	RAB11A, member RAS oncogene family	1.2	1.7	0.9	1.5	1.9	*RAB11A*	AF000231
40210_at	RAB13, member RAS oncogene family	1.3	3.0	4.9	1.2	1.5	*RAB13*	X75593
35325_at	RAB14, member RAS oncogene family	1.4	0.9	1.4	1.2	1.3	*RAB14*	AF052113
1074_at	RAB1A, member RAS oncogene family	1.3	1.2	1.5	1.9	1.0	*RAB1A*	M28209
623_s_at	RAB2, member RAS oncogene family	1.1	1.3	1.3	1.2	1.1	*RAB2*	M28213
39272_g_at	RAB4B, member RAS oncogene family	1.0	1.4	1.3	1.6	1.6	*RAB4B*	AA461365
37362_at	RAB5B, member RAS oncogene family	1.1	1.5	1.4	1.5	1.1	*RAB5B*	X54871
35304_at	RAB6A, member RAS oncogene family	0.9	1.3	1.2	1.7	1.1	*RAB6A*	AF052130
35339_at	RAB8A, member RAS oncogene family	1.3	0.9	1.1	1.6	1.0	*RAB8A*	AI743606
39628_at	RAB9A, member RAS oncogene family	1.1	1.0	1.2	1.2	1.3	*RAB9A*	AI671547

Table II. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Ras/MAPK/ERK-related pathways and adaptor proteins								
1394_at	Ras homolog gene family, member A	1.3	1.1	1.5	1.3	1.6	*RHOA*	L25080
1395_at	Ras homolog gene family, member C	1.5	1.6	1.3	1.5	1.3	*RHOC*	L25081
31846_at	Ras homolog gene family, member D	1.0	1.5	1.8	2.2	1.2	*RHOD*	AW003733
36902_at	Ras homolog gene family, member G (rho G)	1.4	1.3	1.2	1.2	1.0	*RHOG*	X61587
1675_at	RAS p21 protein activator (GTPase-activating protein) 1	1.0	1.2	1.5	1.7	1.0	*RASA1*	M23379
40864_at	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP-binding protein Rac1)	1.2	1.3	1.7	1.5	1.0	*RAC1*	D25274
39989_at	Ras-related GTP binding B	2.2	1.9	1.2	1.5	1.9	*RRAGB*	X90530
1000_at	Mitogen-activated protein kinase 3	1.5	1.0	1.2	1.3	1.1	*MAPK3*	X60188
35617_at	Mitogen-activated protein kinase 7	1.0	1.6	1.6	1.4	1.2	*MAPK7*	U29725
41279_f_at	Mitogen-activated protein kinase 8 interacting protein 1	1.5	1.4	1.3	1.2	1.1	*MAPK8IP1*	AF007134
1238_at	Mitogen-activated protein kinase 9	1.4	1.2	1.7	1.1	1.4	*MAPK9*	U09759
1844_s_at	Mitogen-activated protein kinase kinase 1	1.5	1.8	1.2	1.7	0.8	*MAP2K1*	L05624
1398_g_at	Mitogen-activated protein kinase kinase kinase 11	1.3	1.3	2.0	1.5	1.0	*MAP3K11*	L32976
520_at	Mitogen-activated protein kinase kinase kinase 12	1.2	1.3	0.9	1.9	1.1	*MAP3K12*	U07358
35651_at	Mitogen-activated protein kinase kinase kinase 4	1.0	1.4	1.6	1.4	1.2	*MAP3K4*	AF002715
1327_s_at	Mitogen-activated protein kinase kinase kinase 5	1.5	1.1	1.2	1.6	1.4	*MAP3K5*	U67156
36179_at	Mitogen-activated protein kinase-activated protein kinase 2	1.4	1.6	1.5	1.2	1.2	*MAPKAPK2*	U12779
GH/IGF/glucagon/insulin axis								
36782_s_at	Insulin-like growth factor 2 (somatomedin A)	0.5	0.5	1.0	0.3	1.3	*IGF2*	J03242
1741_s_at	Insulin-like growth factor binding protein 2, 36 kDa	1.8	2.1	2.0	2.0	1.2	*IGFBP2*	S37730
37319_at	Insulin-like growth factor binding protein 3	1.1	1.0	1.7	1.4	1.2	*IGFBP3*	M35878
39781_at	Insulin-like growth factor binding protein 4	0.9	1.2	1.5	1.3	1.4	*IGFBP4*	U20982
1736_at	Insulin-like growth factor binding protein 6	1.4	1.0	1.1	1.8	2.0	*IGFBP6*	M62402
41752_at	Growth hormone-inducible transmembrane protein	1.0	1.6	1.5	1.6	1.7	*GHITM*	W28190
33830_at	Leptin receptor	1.0	1.5	1.4	1.3	1.2	*LEPR*	AW026535
38406_f_at	Prostaglandin D2 synthase 21 kDa (brain)	1.2	1.9	1.3	3.8	1.0	*PTGDS*	AI207842
33772_at	Prostaglandin E receptor 4 (subtype EP4)	0.8	0.6	0.4	1.2	0.5	*PTGER4*	L25124
41246_at	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.2	1.2	2.5	1.0	1.3	*SERPINE2*	AI743134
Ion channels and related pathways								
38516_at	Sodium channel, voltage-gated, type I, beta	1.6	1.3	1.4	1.1	1.2	*SCN1B*	L10338
36558_at	Calcium channel, voltage-dependent, beta 1 subunit	1.0	1.3	1.1	1.9	1.4	*CACNB1*	M92302
38225_at	Potassium voltage-gated channel, subfamily H (eag-related), member 2	1.1	0.6	0.7	0.4	0.9	*KCNH2*	AF052728

Table II. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Ion channels and related pathways								
40527_at	Potassium voltage-gated channel, KQT-like subfamily, member 1	1.2	1.7	1.4	1.4	1.5	*KCNQ1*	AF000571
31608_g_at	Voltage-dependent anion channel 1	1.5	1.7	1.4	1.5	1.2	*VDAC1*	AJ002428
37697_s_at	Voltage-dependent anion channel 2	1.0	1.2	1.9	1.4	1.8	*VDAC2*	L08666
36102_at	Voltage-dependent anion channel 3	1.3	1.2	1.6	1.3	1.4	*VDAC3*	AF038962
38225_at	Potassium voltage-gated channel, subfamily H (eag-related), member 2	1.1	0.6	0.7	0.4	0.9	*KCNH2*	AF052728
Cell growth/proliferation								
39388_at	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	1.7	1.5	1.7	1.7	1.1	*CAMK2G*	AA902713
251_at	Calcium/calmodulin-dependent protein kinase I	1.7	2.0	1.4	1.3	1.0	*CAMK1*	L41816
33514_at	Calcium/calmodulin-dependent protein kinase IV	0.6	0.3	0.5	1.1	0.3	*CAMK4*	D30742
38716_at	Calcium/calmodulin-dependent protein kinase kinase 2, beta	1.7	2.7	1.8	1.7	1.0	*CAMKK2*	AB018330
41739_s_at	Caldesmon 1	1.3	1.0	1.6	1.7	1.2	*CALD1*	M83216
41288_at	Calmodulin 1 (phosphorylase kinase, delta)	1.1	1.4	1.5	1.1	1.9	*CALM1*	AL036744
33458_r_at	Calmodulin 2 (phosphorylase kinase, delta)	0.8	1.4	1.6	1.9	1.4	*CALM2*	AI688098
41421_at	Calmodulin-binding transcription activator 2	1.2	1.1	1.5	1.3	1.2	*CAMTA2*	AB020716
40887_g_at	Eukaryotic translation elongation factor 1 alpha 1	1.3	2.1	1.7	1.6	1.3	*EEF1A1*	L41498
41256_at	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	1.3	1.5	1.8	1.3	1.4	*EEF1D*	Z21507
40587_s_at	Eukaryotic translation elongation factor 1 epsilon 1	1.1	1.2	1.3	1.3	1.4	*EEF1E1*	AF054186
36587_at	Eukaryotic translation elongation factor 2	1.2	1.0	1.2	1.0	1.2	*EEF2*	Z11692
1154_at	Eukaryotic translation initiation factor 2, subunit 1 alpha, 35 kDa	1.3	1.4	1.1	1.2	1.1	*EIF2S1*	J02645
39368_at	Eukaryotic translation initiation factor 2, subunit 2 beta, 38 kDa	1.1	1.2	1.4	1.3	1.2	*EIF2S2*	AL031668
1272_at	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	1.7	1.8	1.1	2.1	1.4	*EIF2S3*	L19161
40515_at	Eukaryotic translation initiation factor 2B, subunit 2 beta, 39 kDa	1.3	1.4	1.0	1.5	1.0	*EIF2B2*	AF035280
32659_at	Eukaryotic translation initiation factor 2B, subunit 4 delta, 67 kDa	1.3	1.5	1.2	1.3	1.1	*EIF2B4*	AL050109
34758_at	Eukaryotic translation initiation factor 2B, subunit 5 epsilon, 82 kDa	1.5	1.7	1.1	1.4	1.3	*EIF2B5*	U23028
1644_at	Eukaryotic translation initiation factor 3, subunit 2 beta, 36 kDa	2.1	1.9	1.2	1.6	1.8	*EIF3S2*	U36764
35298_at	Eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67 kDa	1.6	1.5	1.2	1.3	1.6	*EIF3S7*	U54558
32844_at	Eukaryotic translation initiation factor 4 gamma, 1	1.4	1.4	1.1	1.2	1.5	*EIF4G1*	AF104913
41785_at	Eukaryotic translation initiation factor 4 gamma, 2	1.1	1.3	1.6	1.3	1.1	*EIF4G2*	U73824
1199_at	Eukaryotic translation initiation factor 4A, isoform 1	1.3	1.7	1.2	1.4	1.0	*EIF4A1*	D13748
1420_s_at	Eukaryotic translation initiation factor 4A, isoform 2	1.3	1.4	1.4	1.4	1.2	*EIF4A2*	D30655

Table II. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Cell growth/proliferation								
39110_at	Eukaryotic translation initiation factor 4B	1.3	2.7	1.4	2.4	2.2	*EIF4B*	X55733
31597_r_at	Eukaryotic translation initiation factor 4E binding protein 1	2.1	1.8	0.9	3.3	1.1	*EIF4EBP1*	L36055
32229_at	Eukaryotic translation initiation factor 4E-like 3	1.2	1.9	1.1	1.2	1.4	*EIF4EL3*	AF038957
40537_at	Eukaryotic translation initiation factor 5B	1.1	0.9	1.1	1.3	1.3	*EIF5B*	AB018284
37318_at	Eukaryotic translation termination factor 1	1.5	1.1	1.1	1.1	1.2	*ETF1*	X81625
38418_at	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	1.3	1.2	1.1	1.3	1.3	*CCND1*	X59798
41060_at	Cyclin E1	1.9	0.9	1.6	1.0	1.5	*CCNE1*	M74093
1836_at	Cyclin I	0.7	0.8	0.7	0.5	0.8	*CCNI*	D50310
39417_at	Cyclin-dependent kinase (CDC2-like) 11	1.4	1.4	3.2	1.2	1.5	*CDK11*	AB028951
1792_g_at	Cyclin-dependent kinase 2	1.2	1.2	1.1	1.3	1.1	*CDK2*	M68520
1942_s_at	Cyclin-dependent kinase 4	1.1	1.3	1.1	1.3	1.4	*CDK4*	U37022
40549_at	Cyclin-dependent kinase 5	2.3	1.8	1.2	3.5	1.9	*CDK5*	L04658
799_at	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	1.3	1.6	0.9	1.3	1.2	*CDK5R1*	X80343
795_s_at	Cyclin-dependent kinase-like 1 (CDC2-related kinase)	1.6	2.0	1.5	2.6	1.0	*CDKL1*	X66358
40404_s_at	CDC16 cell division cycle 16 homolog (*Saccharomyces cerevisiae*)	1.3	1.3	1.7	1.4	1.2	*CDC16*	U18291
505_at	CDC37 cell division cycle 37 homolog (*S. cerevisiae*)	1.7	1.4	1.4	1.8	1.2	*CDC37*	U43077
1005_at	Dual-specificity phosphatase 1	2.4	1.6	1.6	2.6	1.0	*DUSP1*	X68277
1597_at	Growth arrest-specific 6	1.8	4.6	1.6	1.8	1.0	*GAS6*	L13720
887_at	Growth differentiation factor 1	1.9	1.4	1.1	1.8	0.8	*GDF1*	M62302
1890_at	Growth differentiation factor 15	1.5	1.6	0.9	1.4	2.0	*GDF15*	AB000584
1680_at	Growth factor receptor-bound protein 7	1.7	2.1	1.3	1.5	0.8	*GRB7*	D43772
34308_at	Histone 1, H2AC	1.1	1.6	0.9	1.2	1.1	*HIST1H2AC*	U90551
34157_f_at	Histone 1, H2AL	1.6	2.1	1.7	1.8	1.4	*HIST1H2AL*	AI200373
31523_f_at	Histone 1, H2BE	1.7	1.3	1.1	1.9	1.7	*HIST1H2BE*	Z80780
31528_f_at	Histone 1, H2BM	2.7	1.5	1.2	2.4	1.4	*HIST1H2BM*	Z83738
36347_f_at	Histone 1, H2BN	1.8	1.2	1.4	1.5	1.6	*HIST1H2BN*	AA873858
762_f_at	Histone 1, H4I	1.6	2.2	1.2	1.3	2.0	*HIST1H4I*	AB000905
31521_f_at	Histone 1, H4J	1.2	1.8	1.2	1.6	2.8	*HIST1H4J*	X60484
286_at	Histone 2, H2AA	1.5	1.2	1.3	1.2	1.8	*HIST2H2AA*	L19779
476_s_at	Histone deacetylase 1	1.7	1.9	1.2	2.8	0.8	*HDAC1*	U50079
38810_at	Histone deacetylase 5	1.2	1.5	1.3	1.2	1.0	*HDAC5*	AF039241
1470_at	Polymerase (DNA directed), delta 2, regulatory subunit 50 kDa	1.1	1.1	1.7	0.9	1.6	*POLD2*	U21090
38397_at	Polymerase (DNA-directed), delta 4	1.3	1.4	1.9	1.1	1.2	*POLD4*	U09196
1594_at	Polymerase (RNA) II (DNA directed) polypeptide C, 33 kDa	1.1	1.3	1.1	1.0	1.6	*POLR2C*	J05448
39747_at	Polymerase (RNA) II (DNA directed) polypeptide G	1.5	1.2	0.9	1.3	1.3	*POLR2G*	U52427
1248_at	Polymerase (RNA) II (DNA directed) polypeptide H	1.5	1.2	1.4	1.2	1.0	*POLR2H*	U37689
1486_at	Polymerase (RNA) II (DNA directed) polypeptide J, 13.3 kDa	1.5	1.7	1.5	1.6	1.5	*POLR2J*	L37127
35841_at	Polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	1.2	1.2	1.1	1.1	1.2	*POLR2L*	N24355
782_at	Polymerase (RNA) III (DNA directed) polypeptide C (62 kDa)	1.5	0.9	1.0	1.2	1.8	*POLR3C*	U93867
34320_at	Polymerase I and transcript release factor	1.4	1.6	1.5	1.5	1.9	*PTRF*	AL050224

Table II. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Cell growth/proliferation								
2010_at	S-phase kinase-associated protein 1A (p19a)	1.3	1.8	1.5	1.8	1.4	*SKP1A*	U33760
1685_at	S-phase response (cyclin related)	0.9	1.1	1.8	1.9	0.8	*SPHAR*	X82554
Apoptosis								
36199_at	Death-associated protein	1.5	0.9	0.9	1.0	2.3		X76105
1754_at	Death-associated protein 6	1.4	2.6	1.2	1.9	1.1		AF006041
822_s_at	CASP2 and RIPK1 domain containing adaptor with death domain	1.7	1.4	1.1	1.7	1.2		U79115
1326_at	Caspase 10, apoptosis-related cysteine protease	0.9	0.9	0.8	0.9	0.7	*CASP10*	U60519
486_at	Caspase 9, apoptosis-related cysteine protease	1.3	1.7	1.4	2.4	1.0	*CASP9*	U60521
41816_at	Caspase recruitment domain family, member 10	1.4	1.3	2.0	2.6	0.7	*CARD10*	AL049851
1861_at	BCL2-antagonist of cell death	1.3	1.1	1.3	1.5	1.1	*BAD*	U66879
38050_at	BCL2-associated transcription factor 1	1.3	1.1	2.1	1.8	0.8	*BCLAF1*	D79986
2067_f_at	BCL2-associated X protein	1.2	2.0	1.2	2.0	1.8	*BAX*	L22475
1615_at	BCL2-like 1	1.2	1.1	1.3	1.6	0.9	*BCL2L1*	Z23115
36211_at	BCL2-like 2	1.4	1.4	1.2	1.4	1.1	*BCL2L2*	D87461
609_f_at	Metallothionein 1B (functional)	1.1	1.1	1.6	1.4	1.9	*MT1B*	M13485
36130_f_at	Metallothionein 1E (functional)	1.0	0.7	2.0	1.7	2.1	*MT1E*	R92331
41446_f_at	Metallothionein 1F (functional)	1.0	1.3	1.6	1.3	2.1	*MT1F*	H68340
39594_f_at	Metallothionein 1H	1.2	1.1	1.3	1.2	2.1	*MT1H*	R93527
855_at	Programmed cell death 2	1.3	0.9	1.7	0.9	1.2	*PDCD2*	S78085
37569_at	Programmed cell death 6	1.1	1.1	1.4	1.3	1.2	*PDCD6*	AF035606
32212_at	Programmed cell death 8 (apoptosis-inducing factor)	1.1	1.3	1.1	1.6	1.4	*PDCD8*	AL049703
1715_at	Tumor necrosis factor (ligand) superfamily, member 10	1.0	1.5	3.0	0.9	1.3	*TNFSF10*	U37518
1563_s_at	Tumor necrosis factor receptor superfamily, member 1A	1.2	1.3	1.2	1.8	1.2	*TNFRSF1A*	M58286

B. We concluded that the gene expression signature was not strongly related to the LPS signaling cascade.

We also considered whether a tumor necrosis factor (TNF)-like molecule may be compound B. Binding of TNF to one of its receptors can lead to induction of apoptosis through the involvement of death domain containing TNF-receptor binding proteins like TNF-receptor-associated death domain (TRADD) or Fas-associated death domain (FADD) or to the activation of NF-κB through IκB kinase (38,39). None of the TNF-receptor associated proteins were affected

Table III. Data Analysts' Selection of Genes Deemed Most Significant in Guiding the Classification of Compound B

Analysis	Results
Kidney	C/EBP delta$^+$, JunB$^+$, gp130$^+$, STAT3$^+$, SOCS2$^+$, TIMP-1^{++}, VCAM$^+$, osteopontin$^+$, collagen$^+$, annexin A3$^+$, CD83$^-$, CCL2$^+$, FGF1$^-$, haptoglobin$^+$, caspase 4$^+$, serpinA3$^+$, selectin P$^+$, phospholipase AII type II$^+$
Liver	C/EBP delta$^+$, JunB$^+$, gp130$^+$, STAT3$^+$, SOCS2$^+$, TIMP-1^{+++}, osteopontin$^+$, collagen$^+$, caspase 4$^+$, annexin A3$^+$, CD83$^-$, CCL2$^+$, FGF1$^-$, serpinA3$^+$, selectin P$^+$, phospholipase AII type II$^+$, VCAM$^+$
Pituitary	C/EBP delta$^+$, JunB$^+$, gp130$^+$, STAT3$^+$, SOCS2$^+$, TIMP-1, VCAM^{++}, osteopontin$^+$, collagen$^+$, caspase 4$^+$, annexin A3$^+$, CD83$^-$, FGF1$^-$, haptoglobin$^+$, serpinA3$^+$, selectin P$^+$
Spleen	C/EBP delta$^+$, JunB$^+$, gp130$^+$, STAT 3$^+$, SOCS2$^+$, TIMP-1$^+$, collagen$^+$, caspase 4$^+$, CD83$^-$, FGF1$^-$, haptoglobin$^+$, serpinA3$^+$, phospholipase AII type II$^+$
Thyroid	C/EBP delta$^+$, JunB$^+$, gp130$^+$, STAT3$^+$, SOCS2$^+$, TIMP-1$^+$, VCAM$^+$, osteopontin$^+$, collagen$^+$, caspase 4$^+$, annexin A3$^+$, CD83$^-$, CCL2$^+$, FGF1$^-$, haptoglobin$^+$, serpinA3$^+$, selectin P$^+$, phospholipase AII type II$^+$

+, Up-regulated expression in treated *vs.* nontreated animals; −, down-regulated expression in treated *vs.* nontreated animals.

Table IV. Genes Affected by Treatment with Compound B

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Acute-phase response								
36780_at	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	1.9	1.2	1.7	3.4	2.7	*CLU*	M25915
38052_at	Coagulation factor XIII, A1 polypeptide	1.1	1.4	1.6	1.9	1.2	*FI3A1*	M14539
40766_at	Complement component 4A	3.7	1.2	2.1	1.3	3.5	*C4A*	U24578
31444_s_at	Annexin A2	1.5	1.5	1.5	1.6	2.3	*ANXA2*	M62895
31792_at	Annexin A3	3.2	5.2	1.7	1.2	2.1	*ANXA3*	M20560
428_s_at	Beta-2-microglobulin	1.3	1.1	1.4	1.2	1.4	*B2M*	V00567
32550_r_at	CCAAT/enhancer-binding protein (C/EBP), alpha	0.8	0.5	0.7	0.9	0.8	*CEBPA*	Y11525
38354_at	CCAAT/enhancer-binding protein (C/EBP), beta	2.2	0.8	2.5	1.9	1.2	*CEBPB*	X52560
1052_s_at	CCAAT/enhancer-binding protein (C/EBP), delta	3.6	2.8	3.6	2.2	3.0	*CEBPD*	M83667
229_at	CCAAT/enhancer-binding protein zeta	1.2	1.2	1.1	1.3	2.1	*CEBPZ*	M37197
39008_at	Ceruloplasmin (ferroxidase)	1.2	1.6	5.0	1.6	1.4	*CP*	M13699
32363_at	Cholesterol 25-hydroxylase	3.2	3.6	2.1	2.3	2.1	*CH25H*	AF059214
38111_at	Chondroitin sulfate proteoglycan 2 (versican)	1.8	3.7	5.5	2.0	1.1	*CSPG2*	X15998
36984_f_at	Haptoglobin	2.2	1.2	3.4	1.8	2.8	*HP*	X89214
37621_at	Interleukin-6 signal transducer (gp130, oncostatin M receptor)	1.5	1.5	1.2	2.3	1.4	*IL6ST*	M57230
2049_s_at	Jun B proto-oncogene	4.7	8.6	1.8	3.8	5.5	*JUNB*	M29039
37025_at	Lipopolysaccharide-induced tumor necrosis factor	2.7	2.1	1.6	1.6	2.1	*LITAF*	AL120815
32855_at	Low-density lipoprotein receptor (familial hypercholesterolemia)	6.7	2.3	12.9	4.7	2.5	*LDLR*	L00352
39428_at	Lymphocyte adaptor protein	1.8	1.9	1.7	1.3	1.4	*LNK*	AF055581
1497_at	Lymphotoxin beta receptor (tumor necrosis factor receptor superfamily, member 3)	2.0	1.2	1.6	3.6	2.3	*LTBR*	L04270
1648_at	Oncostatin M receptor	1.8	4.6	2.6	1.6	3.7	*OSMR*	U60805
614_at	Phospholipase A2, group IIA (platelets, synovial fluid)	2.0	10.4	1.1	2.0	8.5	*PLA2G2A*	M22430
32569_at	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45 kDa	0.9	0.7	0.7	0.9	0.6	*PAFAH1B1*	L13385
32691_s_at	Prostaglandin E receptor 3 (subtype EP3)	1.0	1.4	1.1	1.1	1.7	*PTGER3*	D86096
718_at	Protease, serine, 11 (IGF binding)	1.1	1.2	1.0	2.8	1.7	*PRSS11*	D87258
39255_at	Protein C (inactivator of coagulation factors Va and VIIIa)	0.5	0.7	0.5	0.7	0.6	*PROC*	X02750
647_at	Protein C receptor, endothelial (EPCR)	2.7	1.7	1.4	2.1	3.1	*PROCR*	L35545
37432_g_at	Protein inhibitor of activated STAT, 2	1.2	1.9	1.7	2.4	2.0	*PIAS2*	AF077953
1810_s_at	Protein kinase C, delta	1.5	1.9	1.3	1.5	2.1	*PRKCD*	D10495
34342_s_at	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	13.9	1.8	2.2	1.4	5.6	*SPP1*	AF052124
32275_at	Secretory leukocyte protease inhibitor (antileukoproteinase)	1.2	2.1	2.9	2.2	1.8	*SLPI*	X04470
40366_at	Selectin P (granule membrane protein 140 kDa, antigen CD62)	1.7	5.5	3.7	1.1	4.4	*SELP*	M25322

Table IV. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Acute-phase response								
33825_at	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	3.6	2.0	3.3	2.0	2.4	*SERPINA3*	X68733
33305_at	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	4.4	1.5	4.6	1.2	12.7	*SERPINB1*	M93056
32103_at	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	1.2	1.1	1.5	2.3	1.1	*SERPINF2*	M20786
39775_at	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	1.0	1.1	1.5	1.0	1.1	*SERPING1*	X54486
40109_at	Serum response factor (c-*fos* serum response element-binding transcription factor)	1.6	1.6	1.2	1.2	1.8	*SRF*	J03161
1563_s_at	Tumor necrosis factor receptor superfamily, member 1A	1.6	1.8	1.6	2.2	1.7	*TNFRSF1A*	M58286
36988_at	Tumor necrosis factor, alpha-induced protein 1 (endothelial)	1.7	1.5	1.8	1.6	2.1	*TNFAIP1*	M80783
1519_at	v-*ets* erythroblastosis virus E26 oncogene homolog 2 (avian)	2.9	3.0	1.6	2.6	3.4	*ETS2*	J04102
1916_s_at	v-*fos* FBJ murine osteosarcoma viral oncogene homolog	2.0	6.3	2.1	1.6	3.7	*FOS*	V01512
37724_at	v-*myc* myelocytomatosis viral oncogene homolog (avian)	8.1	3.4	1.6	1.6	1.3	*MYC*	V00568
607_s_at	von Willebrand factor	1.4	1.0	3.4	2.9	1.4	*VWF*	M10321
Immune response								
1796_s_at	B-cell CLL/lymphoma 3	1.2	1.8	1.2	2.0	1.2	*BCL3*	U05681
40091_at	B-cell CLL/lymphoma 6 (zinc finger protein 51)	2.3	5.2	3.5	1.6	1.7	*BCL6*	U00115
2036_s_at	CD44 antigen (homing function and Indian blood group system)	2.6	1.7	1.9	1.2	1.1	*CD44*	M59040
37536_at	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	0.5	0.8	0.9	0.7	0.7	*CD83*	Z11697
875_g_at	Chemokine (C-C motif) ligand 2	2.1	1.6	1.0	1.1	2.9	*CCL2*	M26683
823_at	Chemokine (C-X3-C motif) ligand 1	1.2	1.1	1.1	1.4	1.4	*CX3CL1*	U84487
34344_at	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	0.8	0.6	0.6	0.8	0.8	*IKBKAP*	AF044195
120_at	Integrin, alpha 1	1.1	1.7	1.7	1.9	1.0	*ITGA1*	X68742
41005_at	Integrin, alpha 8	1.3	2.2	4.0	1.1	1.8	*ITGA8*	L36531
32808_at	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.2	1.3	1.1	2.1	1.3	*ITGB1*	X07979
37952_at	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	3.8	2.9	2.1	3.6	2.2	*ITGB3*	M35999
32640_at	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	3.7	3.5	5.0	1.9	3.6	*ICAM1*	M24283
38453_at	Intercellular adhesion molecule 2	1.2	1.1	2.6	1.1	1.2	*ICAM2*	X15606
676_g_at	Interferon-induced transmembrane protein 1 (9–27)	1.7	2.3	3.9	1.3	2.2	*IFITM1*	J04164

Table IV. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Immune response								
41745_at	Interferon-induced transmembrane protein 3 (1–8U)	2.5	2.7	2.1	1.6	3.7	*IFITM3*	X57352
669_s_at	Interferon regulatory factor 1	4.6	2.3	4.2	1.4	3.6	*IRF1*	L05072
36412_s_at	Interferon regulatory factor 7	2.2	2.7	1.7	1.4	2.8	*IRF7*	U53831
1358_s_at	Interferon, alpha-inducible protein (clone IFI-6-16)	2.2	6.4	1.1	5.9	1.6	*G1P3*	U22970
37641_at	Interferon-induced protein 44	1.1	1.8	1.4	1.8	1.8	*IFI44*	D28915
915_at	Interferon-induced protein with tetratricopeptide repeats 1	1.1	2.1	1.9	1.8	2.7	*IFIT1*	M24594
36472_at	N-myc (and STAT) interactor	1.9	1.4	1.8	1.0	1.2	*NMI*	U32849
40822_at	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.8	0.6	1.0	0.9	0.5	*NFATC3*	L41067
37397_at	Platelet/endothelial cell adhesion molecule (CD31 antigen)	1.1	2.4	1.6	1.5	1.0	*PECAM1*	L34657
33823_at	Scavenger receptor class B, member 2	1.2	1.1	1.1	1.3	1.4	*SCARB2*	D12676
39708_at	Signal transducer and activator of transcription 3 (acute-phase response factor)	1.6	1.6	2.3	1.8	2.2	*STAT3*	L29277
38994_at	Suppressor of cytokine signaling 2	2.6	1.3	1.4	1.6	1.3	*SOCS2*	AF037989
32669_at	Suppressor of cytokine signaling 5	1.3	1.4	1.9	1.1	1.2	*SOCS5*	AB014571
31557_at	Thymosin, beta 4, X-linked	1.2	1.2	1.5	1.0	1.2	*TMSB4X*	M17733
41433_at	Vascular cell adhesion molecule 1	2.5	1.5	6.4	1.2	2.0	*VCAM1*	M73255
Repair								
32307_s_at	Collagen, type I, alpha 2	1.5	2.2	1.1	1.7	1.6	COL1A2	V00503
32488_at	Collagen, type III, alpha 1 (Ehlers–Danlos syndrome type IV, autosomal dominant)	1.8	2.5	4.6	2.8	1.8	*COL3A1*	X14420
39333_at	Collagen, type IV, alpha 1	2.9	1.8	1.4	3.6	2.0	*COL4A1*	M26576
36659_at	Collagen, type IV, alpha 2	1.9	1.6	1.4	2.6	2.0	*COL4A2*	X05610
38722_at	Collagen, type VI, alpha 1	2.2	1.6	1.6	2.0	1.1	*COL6A1*	X15880
38077_at	Collagen, type VI, alpha 3	1.4	1.3	1.3	1.5	1.2	*COL6A3*	X52022
996_at	Fibroblast growth factor 1 (acidic)	0.6	0.6	0.6	0.8	0.4	*FGF1*	X59065
36184_at	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers–Danlos syndrome type VI)	1.5	1.2	1.5	2.1	1.9	*PLOD*	L06419
224_at	Transforming growth factor beta-inducible early growth response	1.0	1.1	1.8	1.1	1.4	*TIEG*	S81439
1693_s_at	Tissue inhibitor of metalloproteinase 1 (erythroid-potentiating activity, collagenase inhibitor)	8.9	21	1.5	7.2	6.2	*TIMP1*	D11139
1767_s_at	Transforming growth factor beta 3	1.2	2.4	1.8	1.7	2.5	*TGFB3*	X14885
1897_at	Transforming growth factor beta receptor III (beta glycan, 300 kDa)	2.3	1.3	1.1	1.4	1.2	*TGFBR3*	L07594
Programmed cell death								
1497_at	Lymphotoxin beta receptor (TNFR superfamily, member 3)	2.0	1.2	1.6	3.6	2.3	LTBR	L04270
1563_s_at	Tumor necrosis factor receptor superfamily, member 1A	1.6	1.8	1.6	2.2	1.7	TNFRSF1A	M58286
1911_s_at	Growth arrest and DNA-damage-inducible, alpha	2.6	1.2	1.3	2.3	2.0	GADD45A	M60974
195_s_at	Caspase 4, apoptosis-related cysteine protease	5.6	3.0	4.7	1.6	1.9	CASP4	U28014
38281_at	Caspase 7, apoptosis-related cysteine protease	1.8	1.2	2.1	1.1	1.8	CASP7	U67319

Table IV. Continued

Affymetrix ID	Gene title	Ratio of expression levels (treated/untreated)					Gene symbol	GenBank
		Kidney	Liver	Pituitary gland	Spleen	Thyroid gland		
Programmed cell death								
2092_s_at	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	11.0	1.6	2.8	1.6	2.0	SPP1	J04765
34342_s_at	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	13.9	1.8	2.2	1.4	5.6	SPP1	AF052124
31888_s_at	Pleckstrin homology-like domain, family A, member 2	1.2	1.7	2.4	0.9	1.5	PHLDA2	AF001294
34185_at	Poly(rC) binding protein 4	0.5	0.6	0.8	0.5	0.4	PCBP4	W22541
39020_at	CD27-binding (Siva) protein	0.8	0.7	0.7	0.8	0.8	SIVA	U82938
41349_at	Presenilin 2 (Alzheimer disease 4)	1.6	1.3	1.2	1.2	1.9	PSEN2	L43964
875_g_at	Chemokine (C–C motif) ligand 2	2.1	1.6	1.0	1.1	2.9	CCL2	M26683

by compound B treatment, nor did we detect significant changes in TNF-receptor-associated factor (TRAF) signaling through NF-κB. Some genes involved in programmed cell death were dysregulated in most tissues by compound B, but we did not see the hallmark caspases 3 and 8 (Table III). This effect could be attributed to an activation of TNF-signaling pathway through death domain bearing receptor-associated proteins. While IL-6 like cytokines have antiapoptotic activity (40,41), it could not be excluded that the observed changes may reflect direct or indirect effects of this cytokine family. Members of the IL-6 cytokine family bind to receptor complexes comprising combinations of specific signaling receptor subunits and a common subunit, gp130. Downstream signaling events involve, among others, the JAK/STAT pathway, SOCS, and the Ras/MAPK pathway, some of which were affected by treatment with compound B. Oncostatin M receptor (IL-6 receptor) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were up-regulated in all tissues. Tissue inhibitor of metalloproteinase-1, a target gene of LIF (42), was particularly overexpressed in the liver (Tables III and IV).

Because of the overall lack of upstream members of the TNF or LPS-stimulated signaling pathway in our gene list on one hand, and the presence of a number of members of the IL-6-family pathway, we came to the conclusion that the gene expression signature was not consistent with induction of the LPS or the TNF signaling cascades. Instead, we speculated that compound B could be a cytokine that may be downstream of those signaling pathways, with the ability to elicit acute phase response. Based on the components of the signaling pathways represented by gene expression signature, it was surmised that compound B was most likely a member of the IL-6 family of cytokines rather than an LPS-like or TNF-like molecule. In fact, deblinding revealed that compound B was LIF.

DISCUSSION

Microarray gene expression profiles provide a readout of the concerted regulation of the transcriptome and may be considered fingerprints of cellular physiology (43). The approach we describe here, VeloceGenomics, provides a detailed overview of the early transcriptional events induced by any potential drug-like compound, but it may also include the secondary responses that manifest in the form of metabolic and physiologic changes in the animal. An example of the usefulness of our strategy was demonstrated by showing that it was possible for data analysts masked to treatment conditions to determine the biologic function or activity of compounds based solely on multiorgan whole transcriptome signature analysis. The goal of the exercise was not to determine precisely the identity of the compound but rather to test whether the gene expression profiles allowed the positioning of the compounds into classes of activity, could suggest a mode of action, and could propose possible therapeutic indications.

The gene expression signatures exhibited by each group of animals allowed us to identify compound A as having a GH-like activity and compound B as having activity related to proinflammatory cytokines. Indeed, after deblinding, compound A was revealed to be a somatostatin analog (SOM230) and compound B to be LIF. In each case, the expression patterns provided clues as to what pathways and activities were being regulated. For SOM230, the nature of the changes suggested the involvement of the glucagon/insulin axes. The change in expression of various receptors suggested that compound A was acting along the lines of a ligand and stimulating many downstream pathways rather than acting as a downstream effector itself. For compound B, the gene expression signature clearly implicated the inflammatory response. More thorough evaluations ruled out certain possibilities (e.g., LPS) and suggested others as more plausible (i.e., IL-6-like cytokine activity).

The VeloceGenomics method may be applied to virtually any organism used in preclinical settings and is not limited to the analysis of gene expression data. Indeed, the process may be enhanced by collecting additional data—physiologic, biochemical, hematologic—as complementary information, such that an entire picture of the physiologic responses to a compound can be gained in a single appropriately designed experiment. The VeloceGenomics approach provides an early *in vivo* pharmacologic evaluation of efficacy and safety. Information garnered from these

experiments can lead to testable hypotheses of the mode of action and the biologic activity of the compound in focused experiments. The vast molecular readouts obtained from a single *in vivo* multiorgan transcriptome study can support a multitude of efforts in drug discovery research, including identifying and validating targets, selecting lead compounds, identifying new clinical indications for existing drugs, discovering biomarkers, and elucidating mechanisms of action. The VeloceGenomics approach combines the power of systems biology (physiology) and functional genomics (when applied to proteins or genes) to produce an innovative strategy to investigate the actions of compounds on an organism-wide level of complexity. Computational methods, such as structure-based design, have made significant contributions in the realm of synthetic chemical synthesis; however, such methods cannot replace empirical testing to establish the actual biologic activity of compounds (44). Our approach of *in vivo* global genomics is a strategy to capture quantitative data from such an empirical approach that has the potential to elucidate the entire spectrum of biologic activity of any compound.

The VeloceGenomics process may be used to derive the biologic function or action of any molecule or gene. Small molecules, proteins, and natural products—even genes through *in vivo* naked DNA injections—are all amenable to this strategy (45,46). Compounds can be applied to VeloceGenomics screening in the absence of any predetermined biologic selection criteria. The resultant *in vivo* organism-wide pattern of transcriptome expression data provides an overview of the activities affected at the molecular and organismal levels. The accumulation of information in different organs not only helps to elucidate the mode of action, but it can provide also valuable insight into the potential therapeutic usefulness or toxicologic consequences of any molecule by providing a comprehensive reconstruction of the compound-induced perturbations throughout the entire organism. *In vivo* gene expression analysis may reveal the extent to which a compound acts at more than one target and in more than one target organ (5); both factors may be critical in determining product safety.

Targets of many currently marketed drugs were identified from pharmacology (physiology) studies rather than from a direct molecular approach (2). Our *in vivo* global genomics approach combines a physiologic approach with a simultaneous effort to evaluate the molecular pathways impacted by treatment. The physiology of the whole organism affects how a compound acts *in vivo*, and the *in vivo* gene expression profile reveals the molecular targets and the metabolic and biochemical pathways involved in that compound's *in vivo* biologic activity (2). The VeloceGenomics process is suitable for several stages in the drug discovery and drug development process. The phase of the process at which our strategy is introduced may reflect the type of molecule under evaluation. For example, novel proteins or genes may be subjected to the VeloceGenomics approach early in the evaluation process. In the case of small molecule chemical screening, the VeloceGenomics process may be invoked once a limited set of lead compounds is identified based on high-throughput evaluations of compound libraries.

In the past three decades, drug discovery has shifted from the extensive use of animal studies (classical pharmacology) to high-throughput *in vitro* screening systems using a target-oriented approach (5). However, it has been repeatedly demonstrated that the *in vitro* testing of compounds leads to promising drug candidates that often fail to show the expected or predicted efficacy *in vivo* (44). A recent publication has elegantly demonstrated the limitations of *in vitro* models in which detection of the number of different proteins expressed *in vivo* by endothelial cells of the lung microvasculature was approximately 2.5-fold greater than the number of proteins expressed by the same cells *in vitro* (7). These results have profound implications for drug discovery using *in vitro* cell-based assays when testing for the biologic activity of compounds. If, as in this example, the *in vitro* system expresses far fewer proteins, the suggestion is that not all relevant pathways may be expressed *in vitro* compared with the same cells growing under *in vivo* conditions. Thus, the *in vitro* system may be an inaccurate representation of the biochemical and metabolic pathways active in the *in vivo* condition. However, at the *in vivo* level, the congruence between animal physiology and biochemistry is substantially valid when compared with human biochemistry and physiology (6). Furthermore, *in vitro* screening and tests for biologic activity lack aspects of functional kinetics. As such, *in vitro* screening lacks the aspects of systems biology in which the various molecular, biochemical, and physiologic pathways interact and interconnect kinetically (6). The VeloceGenomics approach addresses these concerns.

In addition, many compounds undergo metabolic conversion to generate the biologically active drug species (or, in some cases, toxic metabolites). The discovery of such a compound would be dependent on its interaction with a metabolically competent test system. Hence, the therapeutic effect of the drug would depend on its interaction with the organism; this interaction cannot take place in an *in vitro* system (11).

Attrition is one of the central issues confronting drug discovery and development. Most attrition that occurs late in drug development is attributed to lack of *in vivo* efficacy or to problems with absorption, distribution, metabolism, excretion, or toxicology (8). Predicting the biologic success or failure of a compound is critical in pharmaceutical discovery, where the guiding principle during research and development is to fail early and cheap. The ability to reconcile or unify genetics and physiology can play an important role in this process (48). Suggestions to improve the attrition rate include (a) strong evidence for mechanism (mode) of action, (b) elimination of compounds that have mechanism-based toxicity, (c) identification of biomarkers that signal correct dosing and on-target action, and (d) use of appropriate animal models for efficacy testing (8). The strategy presented by VeloceGenomics addresses each of these points and may be used to identify risk during the preclinical stages of drug discovery through an understanding of the mechanism of action and drug-like activity of a compound in a physiologically relevant setting (8,9). By refining or narrowing the scope of the *in vivo* global genomics approach—for instance, by using animal models of disease—the efficacy, potency, toxicity, and mechanism of action can be assessed in one study. Although this more focused approach may reduce the amount of information generated from an *in vivo* global genomics study, it does lend itself to focused drug discovery

(3). Rather, the value of the data would depend on the animal model having relevance to the human disease or to the question being addressed (6). In many instances, one can generate preliminary knowledge regarding these points in a well-designed *in vivo* study. Because the VeloceGenomics strategy has the potential to reveal a compound's mechanism of action, it can assist in the choice of the best animal models and disease models to assess efficacy and safety.

In vivo microarray experiments help in elucidating the underlying principles of *in vivo* drug action; however, there are limitations. Compounds with poor pharmacokinetics may be unable to generate reliable *in vivo* transcriptome profiles and, hence, may fail to reveal information hidden in RNA expression levels. Furthermore, biologic activities caused by translational activity and posttranslational modifications, such as protein phosphorylation, are not seen at the transcript level.

Rapid advances are being made in microarray-related technologies and information databases. The availability of more human tissue transcriptome profiles representing healthy and pathologic states will greatly improve concordance between animal and human tissue transcriptomes. Human pathologic tissue signatures will be used (and are already used, where available) to evaluate the effect of compounds in preclinical testing. For example, human cancer tissue transcript expression profiles may be compared before and after treatment with the transcript expression profile of an animal model counterpart. Establishing databases of treatment signatures based on real treatment samples will substantially improve the predicative power of *in vivo* preclinical testing.

It has been estimated that 12,000–14,000 genes encode secreted proteins in the human genome (3). If only 1–2% of these proteins become drugs, that would translate into 120–280 novel biopharmaceutics (3). Biologicals have a high success rate (approximately 24%) in passing through clinical development and registration (8). The growth of biotechnology-based drugs has been impressive. In 1982, recombinant insulin was registered. By 2000, 60 protein-based drugs were introduced to the market (47,49). The VeloceGenomics strategy outlined in this work would most quickly apply to the search for novel protein-based drugs.

CONCLUSION

In summary, VeloceGenomics *in vivo* whole transcriptome analysis attempts to answer in one study the following questions. (a) Is the compound worth further investigation? (b) What physiologic pathways are affected by treatment (i.e., mechanism of action)? (c) What are the potential therapeutic indications? (d) What are the potential safety concerns and adverse effects? Furthermore, through *in vivo* transcriptome analysis, additional targets for therapeutic intervention may be revealed. This information provides an important link back to disease research and discovery programs charged with the task of identifying valid, "drugable" targets for high-throughput screens. The drug discovery approach described as VeloceGenomics is a strategy to generate and analyze a vast amount of data from single *in vivo* studies to derive knowledge pertaining to a compound's mode of action, biologic activity, safety implications, and potential therapeutic indications. Use of an *in vivo* system maintains the integrity of all physiologic interactions among the different organs, tissues, and cells, and any cellular pathway or target can be analyzed under physiologically relevant conditions.

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