Dose response from high throughput gene expression studies and the influence of time and cell line on inferred mode of action by ontologic enrichment.

Black, Michael B.[1], Karmaus, Agnes[2], Martin, Matthew T.[2], Naciff, Jorge M.[3], Daston, George P.[3], Thomas, Russell S.[2], Andersen, Melvin E.[1], Wetmore, Barbara A.[1]

[1] The Hamner Institutes, Research Triangle Park, NC, United States. [2] US EPA ORD NCCT, Research Triangle Park, NC, United States. [3] Procter and Gamble, Cincinnati, OH, United States.

Introduction

Gene expression with ontologic enrichment and connectivity mapping tools is widely used to infer modes of action (MOA) for therapeutic drugs. Despite progress in high-throughput (HT) genomic systems, strategies suitable to identify industrial chemical MOA are needed. The L1000 is a HT genomics platform that measures 1000 landmark genes to computationally predict expression across an equivalent whole human genome array. We used the L1000 system with visualization tools to assess predicted gene expression changes and ontologic enrichment for 9 agrochemicals at 9 concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM) across 5 cell lines (Table 1 & 2) at 6 and 24 hr after treatment. Genes significant for a monotonic dose response (log likelihood ratio test with permutations) were used for analyses.

Table 1. Cell lines selected for exposure screens

Cell Line	Replicates	Characteristics
HepaRG	5	Metabolically competent hepatic cells
A549	3	Lung cancer
A673	3	Bone cancer
HT29	3	Colorectal cancer
MCF7	3	Breast cancer

Table 2. Agrochemicals selected for gene expression analysis with the L1000 platforms. Each compound was tested across 9 treatment concentrations plus controls, and sampled at 6 hours and 24 hours.

Chemical	Agricultural Use	CAS No.
2,4-Dichlorophenoxyacetic acid (2,4-D)	Chlorophenoxy herbicide	94-75-7
Dimethenamid	Amide Herbicide	87674-68-8
Diphenylamine	Amine fungicide/antioxidant	122-39-4
Etoxazole	unclassified pesticide	153233-91-1
Fenbuconazole	Triazole Fungicide	114369-43-6
Imazalil	Azole Pesticide	35554-44-0
4-chloro-2-methylphenoxyacetic acid (MCPA)	Chlorophenoxy Herbicide	94-74-6
Rimsulfuron	Sulfonylurea, Herbicide	122931-48-0
Thidiazuron	Urea herbicide	51707-55-2

Methods

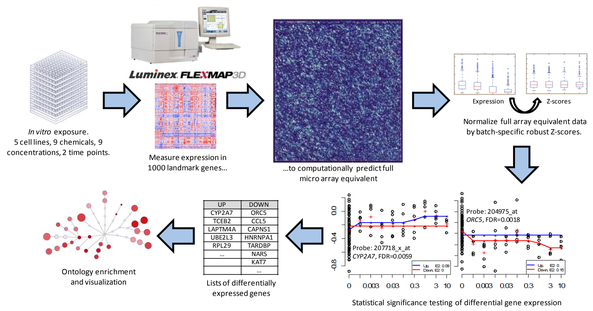

Figure 1. Cells were exposed in 384-well plates for scanning of landmark gene expression. The 1000 measured landmark genes were used to computationally predict the equivalent of an Affymetrix U133A full genome microarray (>20,000 probes). Each plate batch used independent vehicle controls, allowing for batch-specific normalization by Z-scores. Normalized whole array equivalent data was analyzed for differential expression by Likelihood ratio test of monotonic dose response, with 100 permutations using the R/Bioconductor package IsoGene.[1] Genes significantly up or down expressed relative to vehicle controls for a dose dependent monotonic response (FDR<0.05) were selected for ontology enrichment. Ontologic enrichment of genes from the predicted expression profiles was performed against the public Reactome ontology and patterns of functional category enrichment were compared using visualization software developed at the Hamner Institutes.[2]

Results

2,4-Dichlorophenoxyacetic acid (2,4-D)

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		0	670	5	23	1438
A549	1402		1	0	0	1
A673	41	1		1	35	2626
HT29	1448	44	455		0	16
MCF7	20	1	7	0		93
6 Hr.		124	1402	41	1448	20

Diphenylamine

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		1	465	57	20	1015
A549	2520		5	1	2	8
A673	0	0		250	131	3325
HT29	2248	44	981		50	520
MCF7	244	6	104	0		211
6 Hr.		183	2520	0	2248	244

Fenbuconazole

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		0	1879	128	112	7,856
A549	940		0	0	0	0
A673	0	0		126	105	3136
HT29	1203	123	260		15	253
MCF7	13	1	5	0		196
6 Hr.		965	940	0	1203	13

MCPA

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		35	0	0	0	382
A549	978		0	1	0	989
A673	0	0		0	0	1
HT29	55	0	16		0	1
MCF7	513	1	134	0		0
6 Hr.		51	978	0	55	513

Thidiazuron

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		6	169	6	10	484
A549	694		23	2	5	39
A673	6	0		28	44	1803
HT29	1829	10	294	1		67
MCF7	775	4	126	3	211	
6 Hr.		67	694	6	1829	775

Dimethenamid

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		0	280	54	4	787
A549	1132		0	0	0	0
A673	0	0		166	37	3098
HT29	2976	87	733	0		337
MCF7	347	8	138	0	221	
6 Hr.		277	1132	0	2976	347

Etoxazole

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		0	1288	124	71	3537
A549	4	0		0	0	0
A673	0	0		144	141	4279
HT29	1817	15	0	0		276
MCF7	287	3	1	0	91	
6 Hr.		48	4	0	1817	287

Imazalil

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		12	148	0	15	306
A549	92	0		143	32	34
A673	4	0	0		216	218
HT29	1251	0	11	0		58
MCF7	4644	0	54	2	471	
6 Hr.		0	92	4	1251	4644

Rimsulfuron

	HepaRG	A549	A673	HT29	MCF7	24 Hr.
HepaRG		0	1369	8	63	4616
A549	905	0		0	0	0
A673	0	0	0		6	75	2891
HT29	2512	1	443	0		1	18
MCF7	216	0	75	0	90		141
6 Hr.		4	905	0	2512	216

Table 3. Statistically significantly differentially expressed genes (Log likelihood ratio test with 100 permutations, FDR<0.05).
24 Hour results are shown in Green
6 Hour results are shown in Red

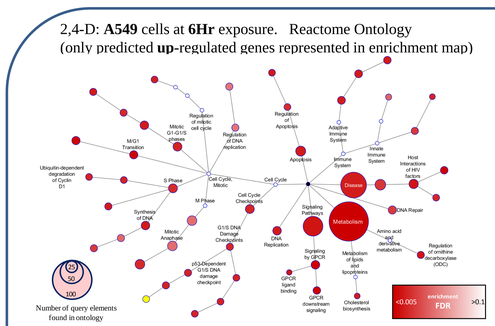

Figure 3a. Significant and up-regulated genes (643). There was no significant enrichment with down-regulated genes.

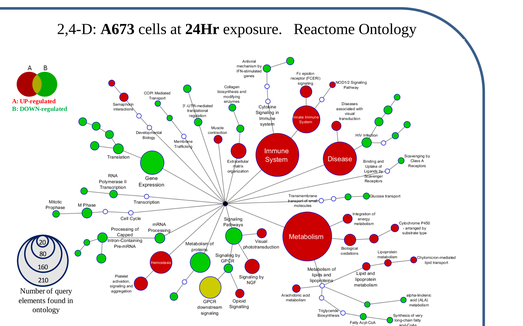

Figure 3b. Significant and up-regulated genes (1,845) and down-regulated genes (781).

Figure 3. Differences in ontologic enrichment across time: 2,4-D at 6Hr (A549 cells) and at 24Hrs (A673 cells)
All colored nodes significant at FDR<0.05 and with a minimum of 5 query elements found in category elements. White nodes are non-significant. Color coding in **3a** is relative to enrichment FDR. Color coding in **3b** is relative to the Venn diagram, with mustard indicating enrichment with both up- and down-regulated genes. Node size is proportional to the number of query elements found in the ontology category.

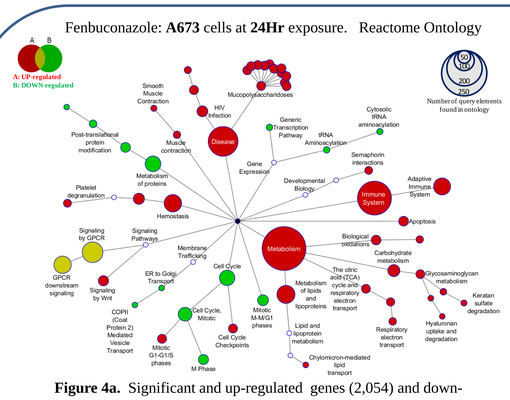

Figure 4a. Significant and up-regulated genes (2,054) and down-regulated genes (1,082).

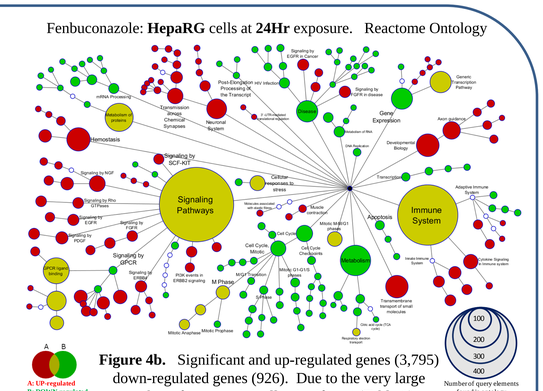

Figure 4b. Significant and up-regulated genes (3,795) down-regulated genes (926). Due to the very large number of genes originally significant (Table 1), HepaRG genes for enrichment were selected at a significance threshold of FDR<0.005

Figure 4. Differences in ontologic enrichment for Fenbuconazole at 24Hr with different cell lines.
All colored nodes significant at an enrichment FDR<0.05 for predicted gene expression and with a minimum of 5 query elements found in category elements. Color coding is relative to the Venn diagram, with mustard indicating enrichment with both up- and down-regulated genes. Size of nodes is proportional to the number of query elements found in the ontology category. White nodes are non-significant.

Differential gene expression levels varied significantly across cell lines and times, with no one cell line consistently responsive for any chemical at both times. A broad assessment of the 9 chemicals showed that A549 and HT29 cells (at 6 hr) and HepaRG and A673 cells (at 24hr) were typically the most responsive. At 6hr, A549 and HT29 cells tended to show significant gene enrichment of cell cycle mitotic processes. At 24hr, A673 cells tended to show gene enrichment of immune response, disease and cellular metabolic pathways.

The herbicide 2,4-D has been linked *in vitro* (HepG2 cells) to gene expression changes related to cell cycle processes, DNA repair mechanisms and immune response pathways[3]. At 6hr, the gene response observed with A549 cells showed ontologic enrichment for many of these same process categories. However, by 24hrs., the greatest number of gene expression changes were seen with A673 cells. These too demonstrated significant enrichment for immune processes but there was an absence of the cell cycle and mitotic process signals seen earlier.

Comparison of HepaRG and A673 cell responses (at 24hr) for Fenbuconazole, a fungicide associated with liver effects, showed enrichment of common parent pathways (DNA repair, cell signaling) in both cell types, but several child categories of these were identified only in HepaRGs. Fenbuconazole exposure is linked with increased liver weight, hepatocellular hypertrophy and proliferation, and clinical chemistry changes (changes in cholesterol and triglyceride levels, and increases in serum activity of hepatic enzymes) were observed in rat, dog and mouse exposures. This fungicide has also been seen to induce mouse liver cytochrome P450 activity in a manner similar to phenobarbital.[4] Active ToxCast assays for Fenbuconazole are associated with cell cycle pathways, cell proliferation and cell death.

Both A673 and HepaRG cells exposed to Fenbuconazole detected significant up-regulation in numerous cytochrome P450 genes (12 with A673 cells, and 25 with HepaRG cells), resulting in a strong enrichment signal for general metabolism processes. However, only HepaRG cells showed wide ranging enrichment in cell cycle and mitotic processes and cell signaling processes consistent with cell proliferation and hypertrophy.

Conclusions

- The L1000 platform has promise for HT genomic testing in human cells at reasonable costs after better validation efforts.
- Differential expression response varied widely across cell lines and time points. While L1000-based methods worked for predictions from expression responses, our sampling of compounds and time points in this poster remians limited.
- Where response was robust for multiple cell lines at a given time point, differences were seen between cell lines in ontologic enrichment. Future efforts with this platform might benefit from using compounds with more complete data bases on modes of action, including previous studies that include other gene expression data and toxicological studies.
- These findings suggest selection of appropriate time points and the use of multiple cell models should be considered in HT genomics strategies designed to inform chemical MOA.

References

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