Predicting Toxic and Therapeutic Mechanisms of the ToxCast Chemical Library by Phenotypic Screening

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EPA United States Environmental Protection Agency

Abstract

Addressing safety aspects of drugs and environmental chemicals relies extensively on animal testing. However the quantity of chemicals needing assessment and challenges of species extrapolation require development of alternative approaches. Using 8 primary human cell systems (BioMAP), we screened in concentration-response format 776 chemicals from the ToxCast Phase II library (http://epa.gov/ncct/toxcast/chemicals.html) for perturbation of physiologically important pathways. Cell systems consisted of combinations of endothelial, peripheral blood mononuclear, bronchial epithelial and coronary artery smooth muscle cells; fibroblasts and keratinocytes. Chemical-response signatures from 87 endpoints covering molecular functions relevant to toxic and therapeutic pathways were generated. Assessment of profiling data by unsupervised clustering using Self Organizing Maps and supervised analysis using Support Vector Machine algorithms grouped chemical/concentration by potential mechanism class providing insight into polypharmacology and potential off-target effects of drugs. Clusters contained diverse mechanistic activity including kinase, TNFα, phosphodiesterase and Hsp90 inhibitors; Ah, estrogen and glucocorticoid receptor modulators; disruptors of mitochondrial and tubulin function; histamine antagonists; and statins. Novel associations identified included inhibition of tissue factor in endothelial cells by ER antagonists, AhR agonists and mTOR inhibitors, all chemical classes with susceptibility to venous thrombosis. Further, structure-based analysis demonstrated associations between chemical categories and mechanism class predictions. Our results yielded an extensive list of potential toxicological targets and biological pathways that we are incorporating into a chemical prioritization strategy for chemicals of concern to the Agency.

Objective

Use primary human cell phenotypic responses to classify and predict compound mechanisms of action and potential toxicities

Primary Cell Systems Used

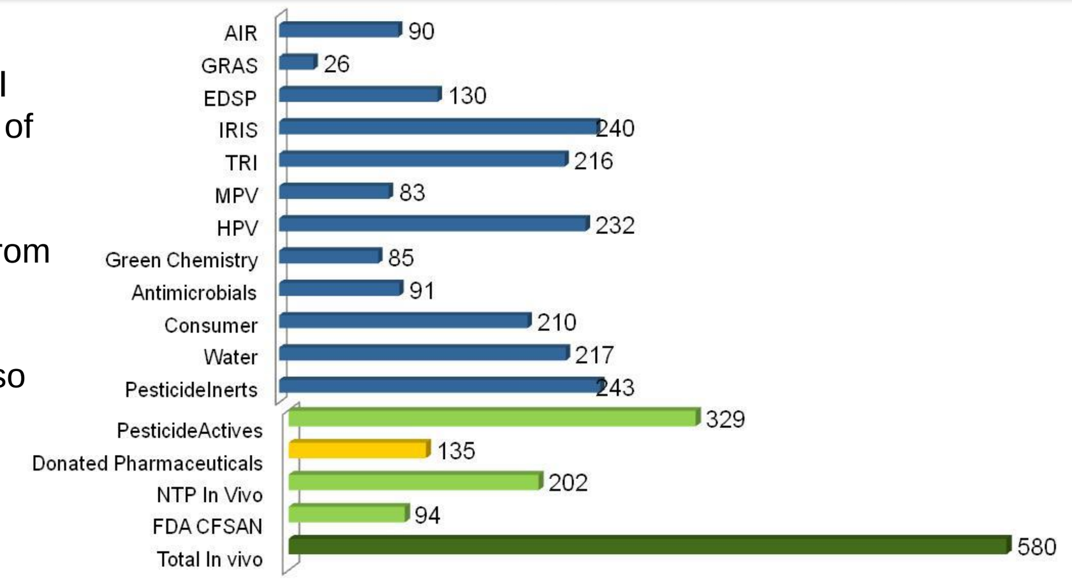
BioMAP System		3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
Primary Human Cell Types		Venular endothelial cells	Venular endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Bronchial epithelial cells	Coronary artery smooth muscle cells	Fibroblasts	Keratinocytes + Fibroblasts
Stimuli		IL-1β + TNF-α + IFN-γ	IL-4+Histamine	TLR4	TCR	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ + EGF + bFGF + PDGF-BB	IL-1β + TNF-α + IFN-γ + TGF-β
# of Endpoints		13	7	11	10	11	14	12	9
Endpoint Types	Acute Inflammation	E-selectin, IL-8		E-selectin, IL-1α, IL-8, TNF-α, PGE2	IL-8	IL-1α	IL-8, IL-6, SAA	IL-8	IL-1α
	Chronic Inflammation	VCAM-1, ICAM-1, MCP-1, MIG	VCAM-1, Eotaxin-3, MCP-1	VCAM-1, MCP-1	MCP-1, E-selectin, MIG	IP-10, MIG, HLA-DR	MCP-1, VCAM-1, MIG, HLA-DR	VCAM-1, IP-10, MIG	MCP-1, ICAM-1, IP-10
	Immune Response	HLA-DR		CD40, M-CSF	CD38, CD40, CD69, PBMC Cytotox., T cell Proliferation	HLA-DR	M-CSF	M-CSF	
	Tissue Remodeling					uPAR, MMP-1, PAI-1, TGFb1, SRB, tPA, uPA	uPAR,	Collagen III, EGFR, MMP-1, PAI-1, Fibroblast Proliferation, SRB, TIMP-1	MMP-9, SRB, TIMP-2, uPA, TGFb1
	Vascular Biology	TM, TF, uPAR, EC Proliferation, SRB, Vis	VEGFRII, uPAR, P-selectin, SRB	Tissue Factor, SRB	SRB		TM, TF, LDLR, SMC Proliferation, SRB		
Disease / Tissue Relevance		Cardiovascular Disease, Chronic Inflammation	Asthma, Allergy, Oncology, Vascular Biology	Cardiovascular Disease, Chronic Inflammation	Autoimmune Disease, Chronic Inflammation	COPD, Respiratory, Epithelial	Cardiovascular Inflammation, Restenosis	Fibrosis, Wound Healing	Psoriasis, Dermatitis, Skin

Methods

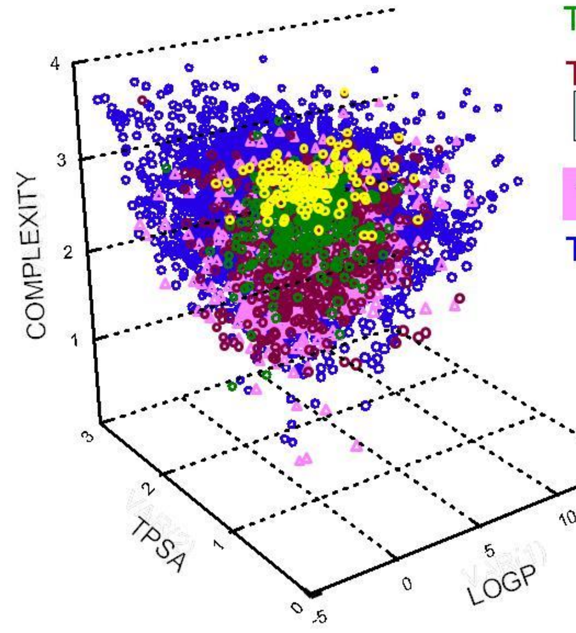
- Compounds were tested at 4 (or 8) concentrations in duplicate, 200 µM high concentration with half-log dilutions.
- Cells treated with compounds followed at one hr by stimulation of signaling pathways
- Cells harvested at 24 hr and endpoints measured by ELISA or staining (SRB)
- Data normalized to log_{10} Fold Change over DMSO controls
- AC50 values calculated using 4-parameter Hill model
- Compounds tested in blinded fashion and included internal replicates
- Predictive models for 28 mechanism classes were built using a two class approach with SVM using R SVM package e1071 (Berg et al., JBS 18:1260, 2013).
- Unsupervised clustering of all compounds at the individual concentration level was conducted in Partek Discover Suite using normalized rows (chemicals) and a 10X10 array.

Compound Library

The ToxCast Phase II chemical library was tested. It consisted of a total of 767 unique chemical structures. Tested compounds were designed predominately from EPA chemical inventories and included data-rich (green) and data-poor (blue) chemicals. Also included were 135 failed pharmaceuticals donated by industry partners.

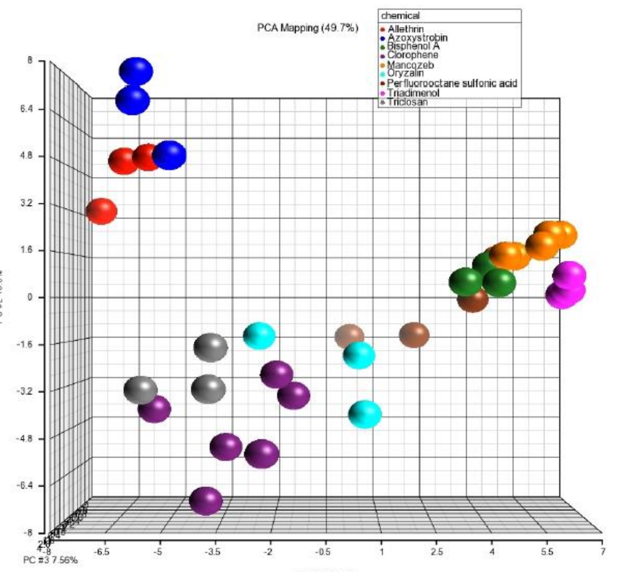


Analysis of diversity of compounds screened. ToxCast Phase I was primarily pesticide active ingredients (previously screened in these same assays). ToxCast Phase II was the library tested here (the donated failed drugs are indicated in yellow). ToxCast e1k and Tox 21 are additional libraries not tested in these assays but included for comparison. The Tox21 library includes more than human pharmaceuticals.



Analysis of Replicates

Analysis of replicates
Compound replicates (7 compounds, each present as 3 independent samples, and 2 compounds, each present as 6 independent samples) were analyzed by Principle Components Analysis using AC50 values for all endpoints. Replicates are indicated as same color symbols.



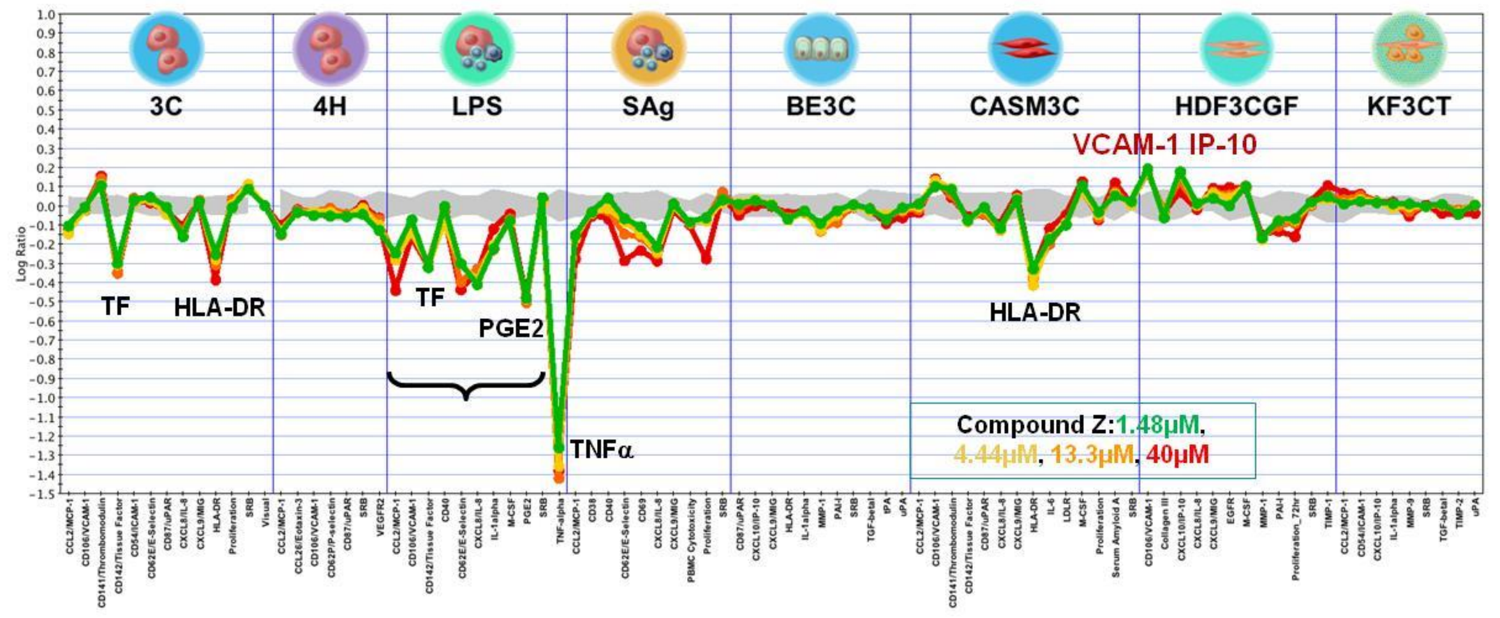
Results

SVM Models and Top Scoring Compounds

SVM Model	Chemical 1	Chemical 2	Chemical 3	Score 1	Score 2	Score 3
AhR Agonist	Benzo(b)fluoranthene, 1481 nM (2)	Benz[a]anthracene, 1481 nM	2-Naphthylamine, 13333 nM	0.667	0.591	0.581
Calcineurin Inhibitor	Triclocarban, 4444 nM	3-(2-Bromophenyl)-7-{[2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]amino}-1-methyl-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one, 2500 nM	Picoxystrobin, 40000 nM	0.387	0.335	0.311
EGFR Inhibitor	Perfluorooctane Sulfonic Acid, 40000 nM	5-(benzylsulfonyl)-2-{[2-(dimethylamino)ethyl](ethyl)amino}-N,N-diethyl-4-(4-phenylpiperidin-1-yl)benzamide, 13333 nM	N-[(1-{[2-(diethylamino)ethyl]amino}-7-methoxy-9-oxo-9H-thioxanthen-4-yl)methyl]formamide, 4444 nM	0.209	0.107	0.095
Enzyme Modulator:SR Ca++ ATPase Inhibitor	N-{trans-4-[(2R)-2-fluoro-2-(2-fluorophenyl)ethoxy]cyclohexyl}-5H-pyrazolo[3,4-d]pyrimidin-4-amine, 40000 nM (x2)	7,12-Dimethylbenz(a)anthracene, 40000 nM	Azoxystrobin, 40000 nM	0.459	0.372	0.242
EP Agonist	N-[(3R)-9-amino-4-oxo-1-phenyl-3,4,6,7-tetrahydro[1,4]diazepino[6,7,1-hi]indol-3-yl]pyridine-3-carboxamide, 40000 nM (3)	2-(4-fluorophenoxy)-N-[4-(2-hydroxypropan-2-yl)benzyl]pyridine-3-carboxamide, 4444 nM (2)	(2R)-2-{4-[({[2-(1,3-benzodioxol-5-yloxy)pyridin-3-yl]carbonyl}amino)methyl]-3-fluorophenoxy}propanoic acid, 40000 nM	0.858	0.828	0.825
ER Agonist	beta-Estradiol, 13333 nM	17alpha-Estradiol, 20000 nM	17alpha-Ethynylestradiol, 40000 nM	1.019	1.006	1.000
GR Agonist (Full)	Dexamethasone sodium phosphate, 18800 nM (7)	Triamcinolone, 40000 nM (4)	Prednisone, 1481 nM	0.944	0.866	0.716
H1 Antagonist	Diphenhydramine Hydrochloride, 40000 nM (x4)	Volinanserin, 40000 nM	Chlorpromazine Hydrochloride, 1481 nM	0.880	0.723	0.673
HDAC Inhibitor	Darbufelone mesylate, 40000 nM	4-[(9-Cyclopentyl-7,7-difluoro-5-methyl-6-oxo-6,7,8,9-tetrahydro-5H-pyrimido[4,5-b][1,4]diazepin-2-yl)amino]-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide, 18600 nM	Tris(2-ethylhexyl) phosphate, 13333 nM	0.718	0.677	0.610
HMG-CoA Reductase Inhibitor	Lovastatin, 1481 nM (3)	Simvastatin, 1481 nM (3)	Rosuvastatin, 3333 nM	1.120	1.069	0.792
Hsp90 Inhibitor	3-(2-Bromophenyl)-7-{[2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]amino}-1-methyl-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one, 19999 nM (2)	Cycloheximide, 35800 nM (3)	cis-4-cyano-4-(1-cyclohexyl-3-ethyl-1H-indazol-6-yl)cyclohexanecarboxylic acid,40000 nM	0.844	0.743	0.648
IKK2 Inhibitor	3-{[3,4-Bis(difluoromethoxy)phenyl](pyridin-3-ylmethyl)amino}benzoic acid, 19999 nM (3)	5-(2,4-Difluorophenoxy)-2-[(2-hydroxy-2-methylpropyl)amino]-8-methylpyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride (1:1), 20000 nM	6-[4-(2,5-difluorophenyl)-1,3-oxazol-5-yl]-3-(propan-2-yl)[1,2,4]triazolo[4,3-a]pyridine, 13333 nM	0.860	0.690	0.684
IL-17A Agonist	2,4,6-Trichlorophenol, 1481 nM	Perfluorooctanesulfonamide, 4444 nM	Phenolphthalin, 20000 nM	0.168	0.114	0.022
JAK Inhibitor	(1R)-1-[(ethoxycarbonyl)oxy]ethyl 1-{[5-(5-chlorothiophen-2-yl)-1,2-oxazol-3-yl]methyl}-2-{[1-(propan-2-yl)piperidin-4-yl]carbamoyl}-1H-indole-5-carboxylate hydrochloride, 37800 nM	{4-[5-(aminomethyl)-2-fluorophenyl]piperidin-1-yl}(4-bromo-3-methyl-5-propoxythiophen-2-yl)methanone hydrochloride, 40000 nM	2,4-Bis(1-methyl-1-phenylethyl)phenol, 40000 nM	0.094	0.040	0.013
MEK Inhibitor	3-(2-Bromophenyl)-7-{[2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]amino}-1-methyl-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one, 9999 nM (3)	3-(Difluoromethyl)-1-(4-methoxyphenyl)-5-[4-(methylsulfinyl)phenyl]-1H-pyrazole, 40000 nM	Genistein, 40000 nM	0.631	0.498	0.497
Microtubule Disruptor:Tubulin	Colchicine, 1481 nM (4)	5HPP-33, 13333 nM	7,12-Dimethylbenz(a)anthracene, 40000 nM	1.052	0.741	0.587
Microtubule Stabilizer:Tubulin	Cytarabine hydrochloride, 13333 nM (3)	4-[(9-Cyclopentyl-7,7-difluoro-5-methyl-6-oxo-6,7,8,9-tetrahydro-5H-pyrimido[4,5-b][1,4]diazepin-2-yl)amino]-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide, 1162 (2)nM	5-Fluorouracil, 40800 nM	0.845	0.838	0.741
Mitochondrial Inhibitor	Picoxystrobin, 4444nM (4)	Dinoseb, BSK-C018287, 40000 nM	Azoxystrobin, 14067 nM	1.004	0.996	0.919
mTOR Inhibitor	Cytarabine hydrochloride, 40000 nM	4-chloro-2-fluoro-5-{[4-(3-fluorophenyl)-4-{2-[3-(2-methyl-1H-benzimidazol-1-yl)-8-azabicyclo[3.2.1]oct-8-yl]ethyl}piperidin-1-yl]carbonyl}-N-methylbenzenesulfonamide, 40000 nM	Dodecyltrimethylammonium chloride, 13333 nM	0.691	0.676	0.672
p38 MAPK Inhibitor	6-[4-(2,5-difluorophenyl)-1,3-oxazol-5-yl]-3-(propan-2-yl)[1,2,4]triazolo[4,3-a]pyridine, 1481 nM (3)	5-(2,4-Difluorophenoxy)-2-[(2-hydroxy-2-methylpropyl)amino]-8-methylpyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride (1:1), BSK-C018858, 19999.913 nM (3)	2-[4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl)-1H-imidazol-1-yl]propane-1,3-diol, 13333 nM	1.011	0.991	0.908
PDE IV Inhibitor	1-ethyl-5-(4-hydroxyphenyl)-3-methyl-6,7-dihydropyrazolo[4,3-e][1,4]diazepin-8(1H)-one, 1481 nM (2)	Terbuthylazin, 40000 nM	N-[(3R)-9-amino-4-oxo-1-phenyl-3,4,6,7-tetrahydro[1,4]diazepino[6,7,1-hi]indol-3-yl]pyridine-3-carboxamide, 40000 nM	1.020	1.007	0.993
PI3K Inhibitor	1-[2-(3,4-dichlorophenoxy)-5-fluorophenyl]-N-methylmethanamine, 40000 nM	Elzasonan, 13333 nM	(2S,3S)-N-[2-methoxy-5-(trifluoromethoxy)benzyl]-2-phenylpiperidin-3-amine, 40000 nM	0.770	0.766	0.735
PKC (c+n) Inhibitor	3-(2-Bromophenyl)-7-{[2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]amino}-1-methyl-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one, 2500 nM (2)	Spironolactone, 20000 nM	Azathioprine, 40000 nM	0.818	0.498	0.441
Proteasome Modulator:20S Proteasome Inhibitor	Triglycidyl isocyanurate, 40000 nM (2)	Dicyclohexyl disulfide, 40000 nM	Retinol, 41800 nM	0.920	0.677	0.639
RAR/RXR Agonist	trans-Retinoic Acid, 4444 nM (2)	N-[(3R)-9-amino-4-oxo-1-phenyl-3,4,6,7-tetrahydro[1,4]diazepino[6,7,1-hi]indol-3-yl]pyridine-3-carboxamide, 40000 nM	Retinol, 1548 nM	1.006	0.809	0.778
Src Family Inhibitor	3-(2-Bromophenyl)-7-{[2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]amino}-1-methyl-3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one, 2500 nM (3)	Triclocarban, 4444 nM	Crystal violet, 1481 nM	0.708	0.351	0.293
TNF-alpha Antagonist	3-pyridinecarboxamide, 2-(2,1,3-benzoxadiazol-5-yloxy)-N-[[4-(1-hydroxy-1-methylethyl)phenyl]methyl]-, 13333 nM	3-{[3,4-Bis(difluoromethoxy)phenyl](pyridin-3-ylmethyl)amino}benzoic acid, 4500 nM	2-(4-fluorophenoxy)-N-[4-(2-hydroxypropan-2-yl)benzyl]pyridine-3-carboxamide, 4444 nM	0.769	0.724	0.706
Vitamin D Receptor Agonist	trans-Retinoic Acid, 4444 nM	({4-[(7-hydroxy-2,3-dihydro-1H-inden-4-yl)oxy]-3,5-dimethylphenyl}amino)(oxo)acetic acid, 13333 nM	N-[(3R)-9-amino-4-oxo-1-phenyl-3,4,6,7-tetrahydro[1,4]diazepino[6,7,1-hi]indol-3-yl]pyridine-3-carboxamide, 40000 nM	0.374	0.308	0.280

SVM models were built as indicated in Methods. For each model, the top three unique compounds are listed. If a compounds appeared more than once (at different concentrations), the number of times is indicated in parentheses after the compound name. The SVM model scores are shown to the right of the names.

Use of SVM to predict side effects

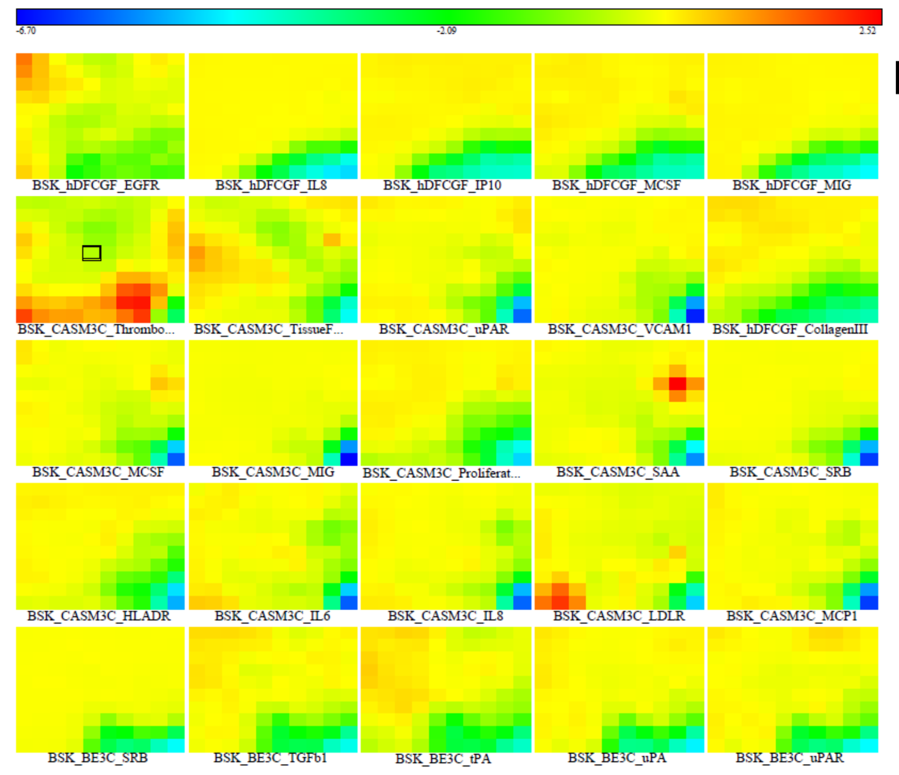


Phase II failed pharma compound. Highlighted endpoints are indicative of potential for skin rash. (Known class effect for p38 MAPK inhibitors). Key features listed below and label in graph.

- Inhibition of tissue factor in activated endothelial cells (3C)
- Strong inhibition of monocyte activation indicated by PGE2, CCL2, TF (LPS)
- Inhibition of HLA-DR in endothelial cells (3C) and smooth muscle cells (CASM3C)
- Upregulation of VCAM-1, IP-10 in human dermal fibroblasts (HDF3CGF)

Unsupervised Clustering using Self Organizing Maps

A.)



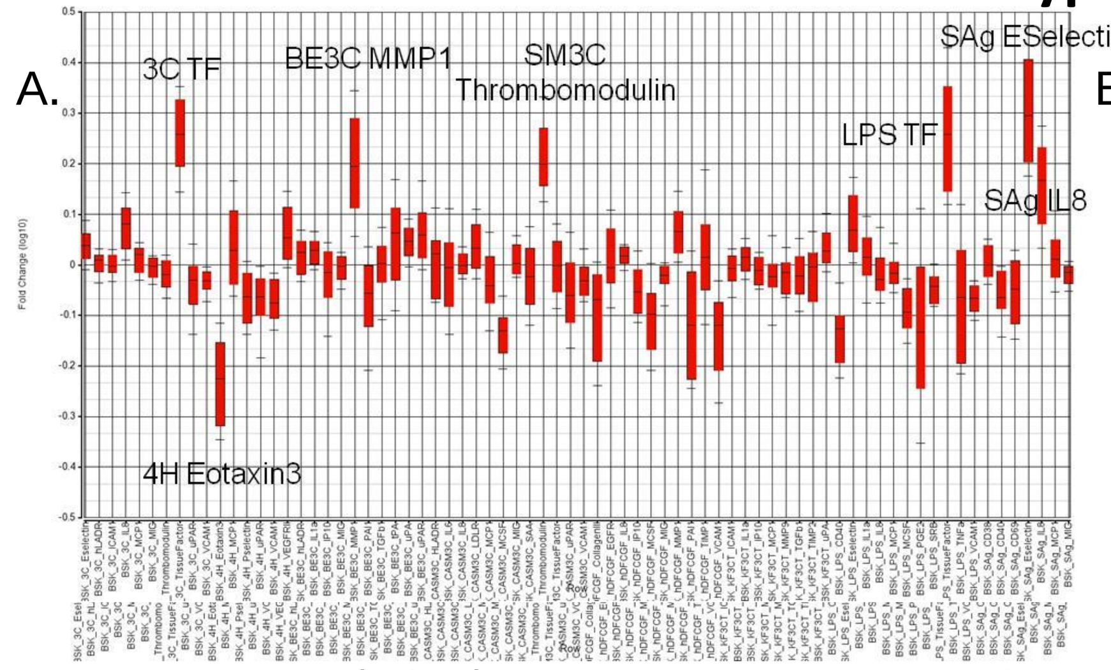
A visual representation of clustered activity across all tested concentrations of all chemicals for every assay endpoint shown as a trellis SOM plot, where each box on the 10x10 grid represents a heterogeneously sized cluster of chemicals/concentrations (A). Color scale represents direction of regulation (blue/green is downregulation, red is upregulation). Relative patterns of regulation for assay endpoint are shown in each plot. Some of the more interesting clusters are shown in the table on the right (B).

B.)

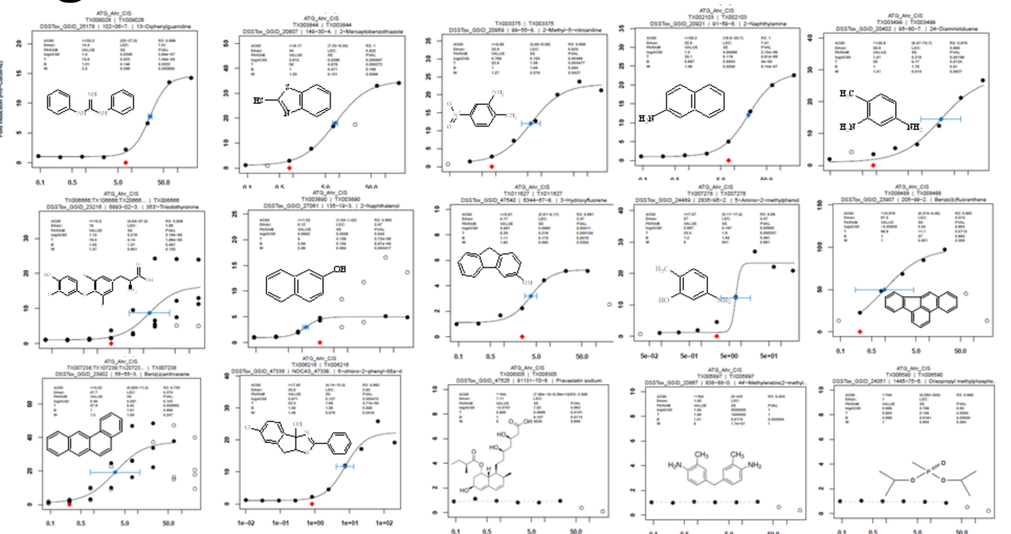
Norm. method {Cluster(s)}	Cluster Count	Common Activity	Example Compounds *known associations	Example Compounds *novel associations
Chemical {1}	78	Analgesics	Aspirin Indomethacin Celecoxib Diclofenec Darbufelone Clove leaf oil Eugenol Isoeugenol	Propyl gallate Fluridone
Chemical {65}	31	Steroid hormone receptor modulators	Cyproterone acetate Norgestrel Progesterone 17-hydroxyprogesterone Mifepristone	Mirex Donated pharma: PPAR pan agonist A3 adenosine receptor antagonist
Chemical {57, 67}	52	AhR ligands	Hydroquinone 4-Chloro-1,2-diaminobenzene 1,2-Phenylenediamine Fenaminosulf	C.I. Solvent yellow 14
Chemical {48}	27	ER antagonists	Clomiphene citrate Tamoxifen citrate Fulvestrant Raloxifene hydrochloride Tamoxifen 4-Hydroxytamoxifen	Cyclopamine Amiodarone hydrochloride Haloperidol Reserpine Donated pharma: NK1 receptor antagonist Bradykinin B1 receptor antagonist Lipid-lowering agent
Assay {46}	29	TNFα inhibition	All-trans retinoic acid Donated pharma: PDE inhibitors (8 cmpds)	Terbuthylazine Donated pharma: $GABA_A$1 receptor antagonist
Assay {39}	31	SAA upregulation	Prednisone Dexamethasone Corticosterone Triamcinolone	Coumarin 4-octylphenol Cyclohexanol Pentaerythritol
Assay {90,100}	58	Potent cytotoxicants	Tributyltin methacrylate Tributyltin chloride Gentian violet Didecyldimethylammonium chloride Triclosan Phenylmercuric acetate	Octyl gallate 4-Nonylphenol 9-Phenanthrol Donated pharma: Factor Xa inhibitor CCK1R agonist mast cell tryptase inhibitor

AhR Phenotypic Signature

A.



B.



Box and whisker plot for cluster 57 representing a signature for AhR activation (A). 85% of members of clusters 57, 67 (adjacent in the 10X10 SOM) were active in an AhR reporter gene assay (examples shown in (B)). Several up-regulated endpoints are associated with thrombosis (TF, tissue factor; E-Selectin). PAHs, which are AhR ligands, are major components of cigarette smoke and perhaps contribute to thrombosis associated with smoking.

Summary

- **The ToxCast Phase II library was screened in 8 complex cell culture systems measuring endpoints relevant to inflammatory signaling and vascular biology.**
- **Assays showed strong reproducibility across technical replicates and built-in test compounds.**
- **The BioMAP system identified potential targets, modes of action, and clinical side effects for compounds based on the reference database.**
- **Assays provided coverage of mechanisms/targets not directly represented in assay endpoints, e.g. AhR**
- **Phenotypic screening and computational analysis provides a unique opportunity to survey environmental chemicals for potential human bioactivity**