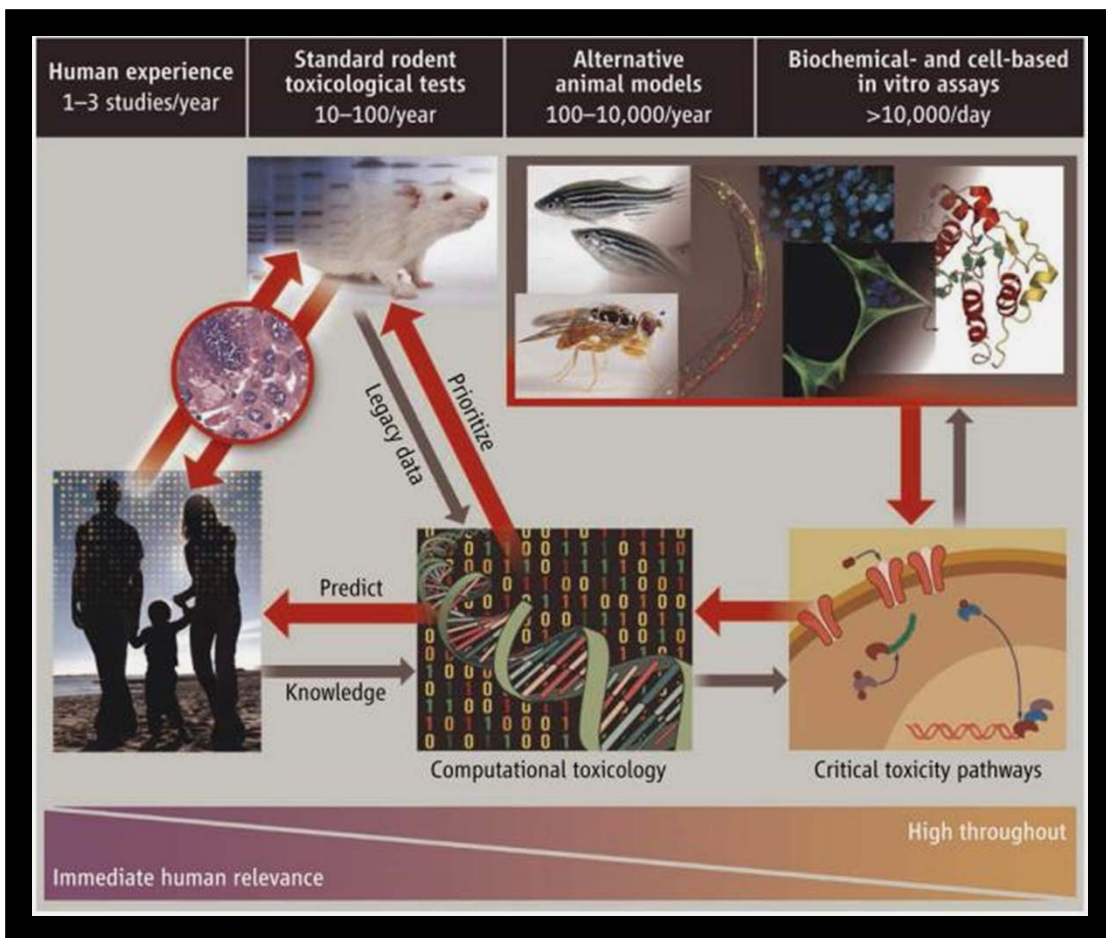# Bioactivity Profiling using Primary Human Cell Systems in Support of Predictive Toxicology

Keith Houck
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Office of Research and Development
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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

COMPUTATIONAL TOXICOLOGY

CPCP
23 October 2014

Tox21 Vision: Transforming Toxicity Testing

SOURCE: Collins, Gray and Bucher (2008) Toxicology. Transforming environmental health protection. Science 319: 906

National Center for Advancing Translational Sciences (NCATS)
http://www.ncats.nih.gov/

Office of Research and Development
National Center for Computational Toxicology

ToxCast /Tox21 Overall Strategy

- **Identify targets or pathways linked to toxicity (AOP focus)**

- **Identify/develop high-throughput assays for these targets or pathways**

- **Develop predictive systems models: *in silico/in vitro → in vivo***

- **Use predictive models (qualitative):**
    - **Prioritize chemicals for targeted testing**
    - **Suggest / distinguish possible AOP / MOA for chemicals**

- **High-throughput Exposure Predictions (ExpoCast)**
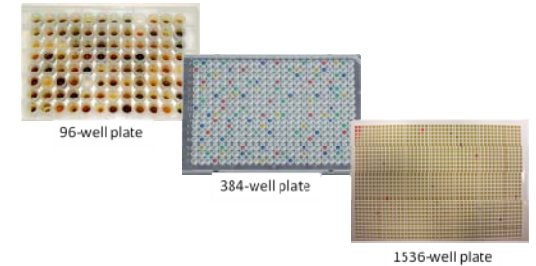
- **High-throughput Risk Assessments (quantitative)**

Toxicity Testing in the Twenty-first Century

"The committee envisions a future in which tests based on human cell systems can serve as better models of human biologic responses than apical studies in different species."

"The committee therefore believes that, given a sufficient research and development effort, human cell systems have the potential to largely supplant testing in animals."

TOXICITY TESTING IN THE 21ST CENTURY: A VISION AND A STRATEGY, NRC, 2007.

Office of Research and Development
National Center for Computational Toxicology

ToxCast Assays (>700 endpoints)

96-well plate

384-well plate

1536-well plate

Assay Provider
ACEA
Apredica
Attagene
BioReliance
BioSeek
CeeTox
CellzDirect
Tox21/NCATS
NHEERL MESC
NHEERL Zebrafish
NovaScreen (Perkin Elmer)
Odyssey Thera
Vala Sciences

Biological Response
cell proliferation and death
cell differentiation
Enzymatic activity
mitochondrial depolarization
protein stabilization
oxidative phosphorylation
reporter gene activation
gene expression (qNPA)
receptor binding
receptor activity
steroidogenesis

Target Family
response Element
transporter
cytokines
kinases
nuclear receptor
CYP450 / ADME
cholinesterase
phosphatases
proteases
XME metabolism
GPCRs
ion channels

Assay Design
viability reporter
morphology reporter
conformation reporter
enzyme reporter
membrane potential reporter
binding reporter
inducible reporter

Readout Type
single
multiplexed
multiparametric

Cell Format
cell free
cell lines
primary cells
complex cultures
free embryos

Species
human
rat
mouse
zebrafish
sheep
boar
rabbit
cattle
guinea pig

Tissue Source
Lung   Breast
Liver   Vascular
Skin   Kidney
Cervix   Testis
Uterus   Brain
Intestinal   Spleen
Bladder   Ovary
Pancreas   Prostate
Inflammatory Bone

Detection Technology
qNPA and ELISA
Fluorescence & Luminescence
Alamar Blue Reduction
Arrayscan / Microscopy
Reporter gene activation
Spectrophotometry
Radioactivity
HPLC and HPEC
ELISA

4

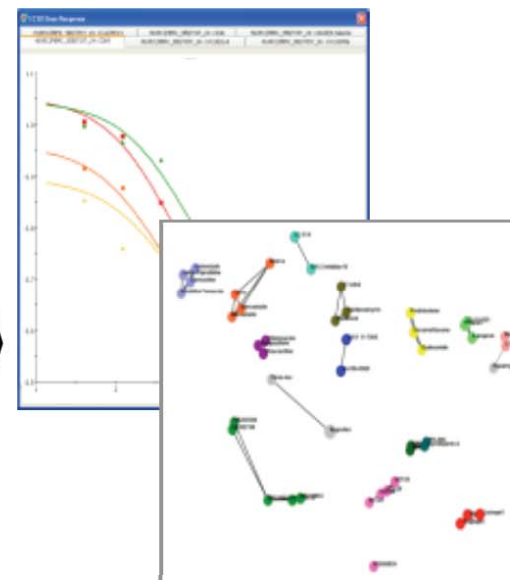*List of assays and related information at:* **http://www.epa.gov/ncct/**

BioMap Profiling Strategy
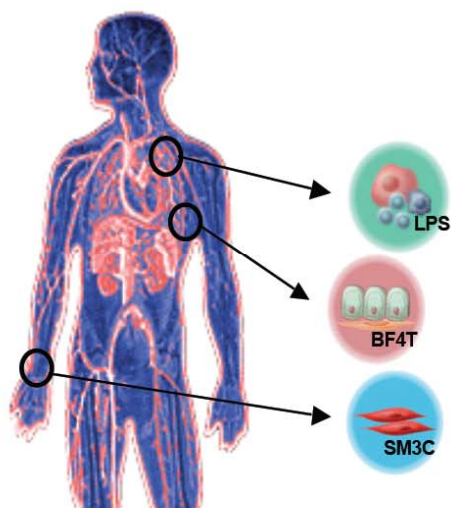
Human primary cells
Inflammatory stimuli

Biological profile
(BioMAP) database

Informatics and
data mining

Assay Systems

DiscoveRx BioMAP

High Throughput Human Biology

	3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
BioMAP System								
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

Total Endpoints
87

Endpoint Types	3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
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

Experimental Design

- Compounds were tested at 4 (or 8) concentrations in duplicate, 200 μM high concentration and half-log dilutions.
- Cells treated with compounds followed at 1 hr by stimulation of signaling pathways
- Cells harvested at 24 hr and endpoints measured by ELISA, staining (SRB), or microscopy
- 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
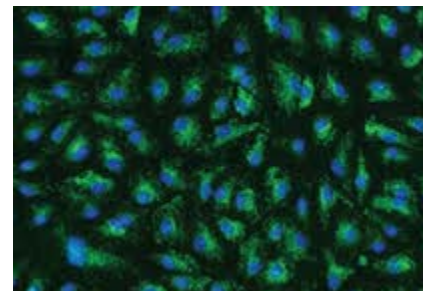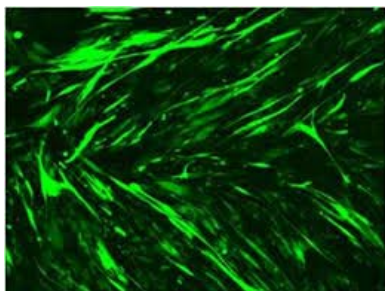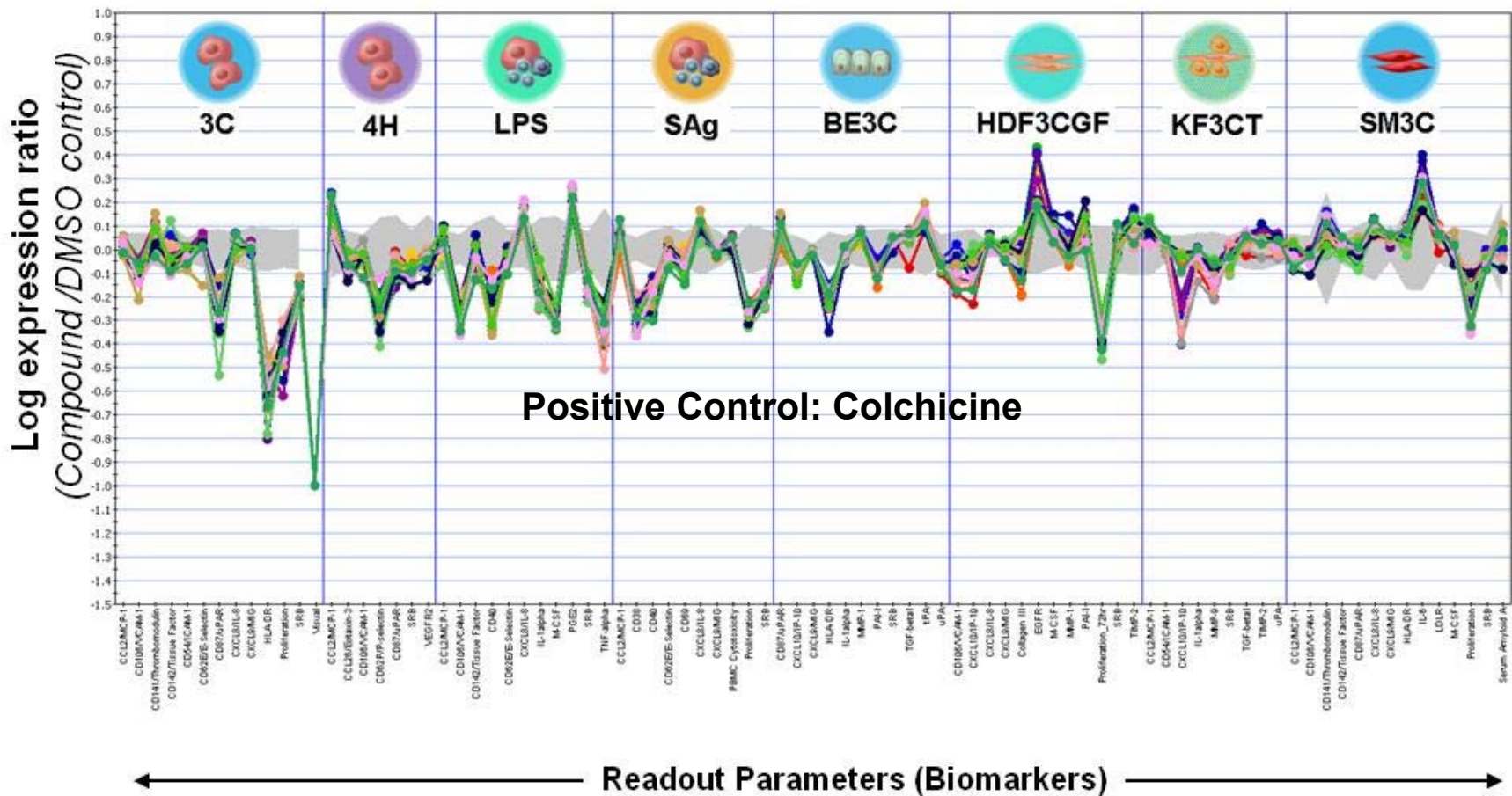
Phase I ToxCast Chemical Library Screening

- **320 chemicals, mostly pesticide actives**

- **8 assay systems**

- **Determined AC50 values for all active chemicals which were used in predictive models, e.g. vascular disruption**

- **Unsupervised analysis using best profile matches with compounds in BioSeek database**

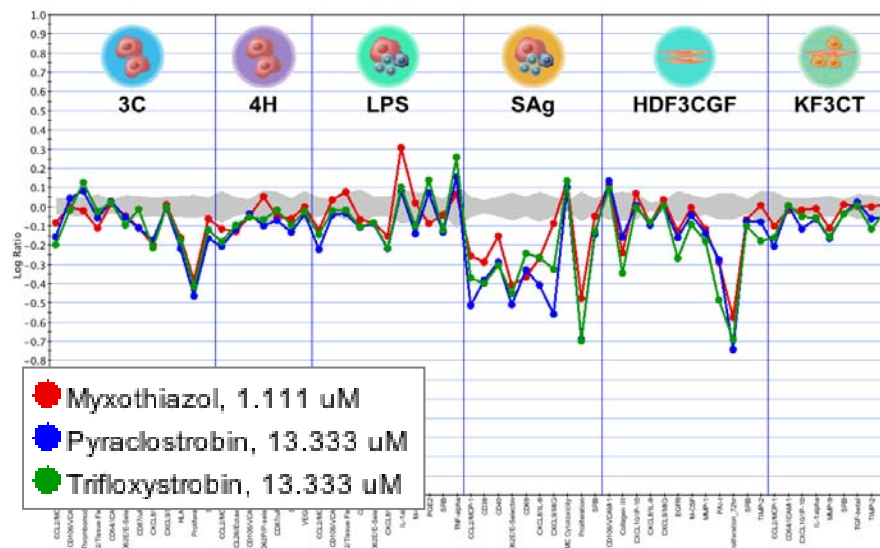# Example BioMap Profile: Colchicine Positive Control
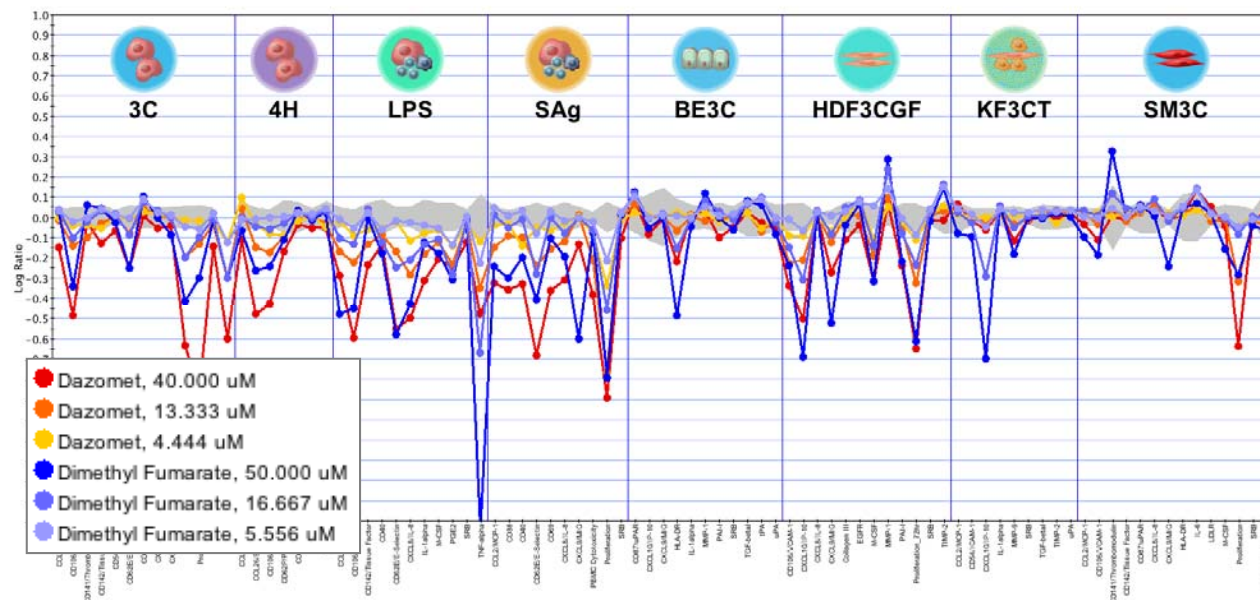
MOA by Correlation
Example Database Profile Matches

Methods
1. Remove overtly cytotoxic compound profiles
2. Correlation metric a combination of similarity metric + Pearson's correlation

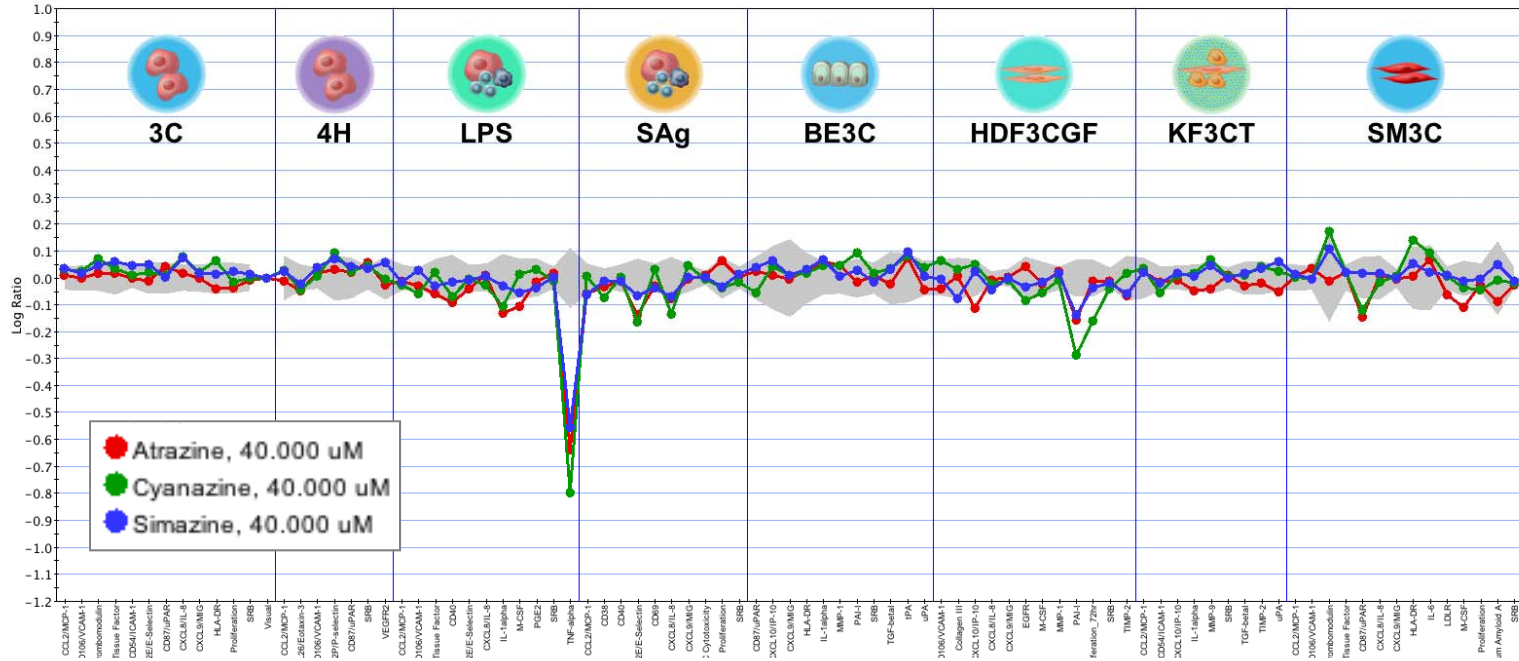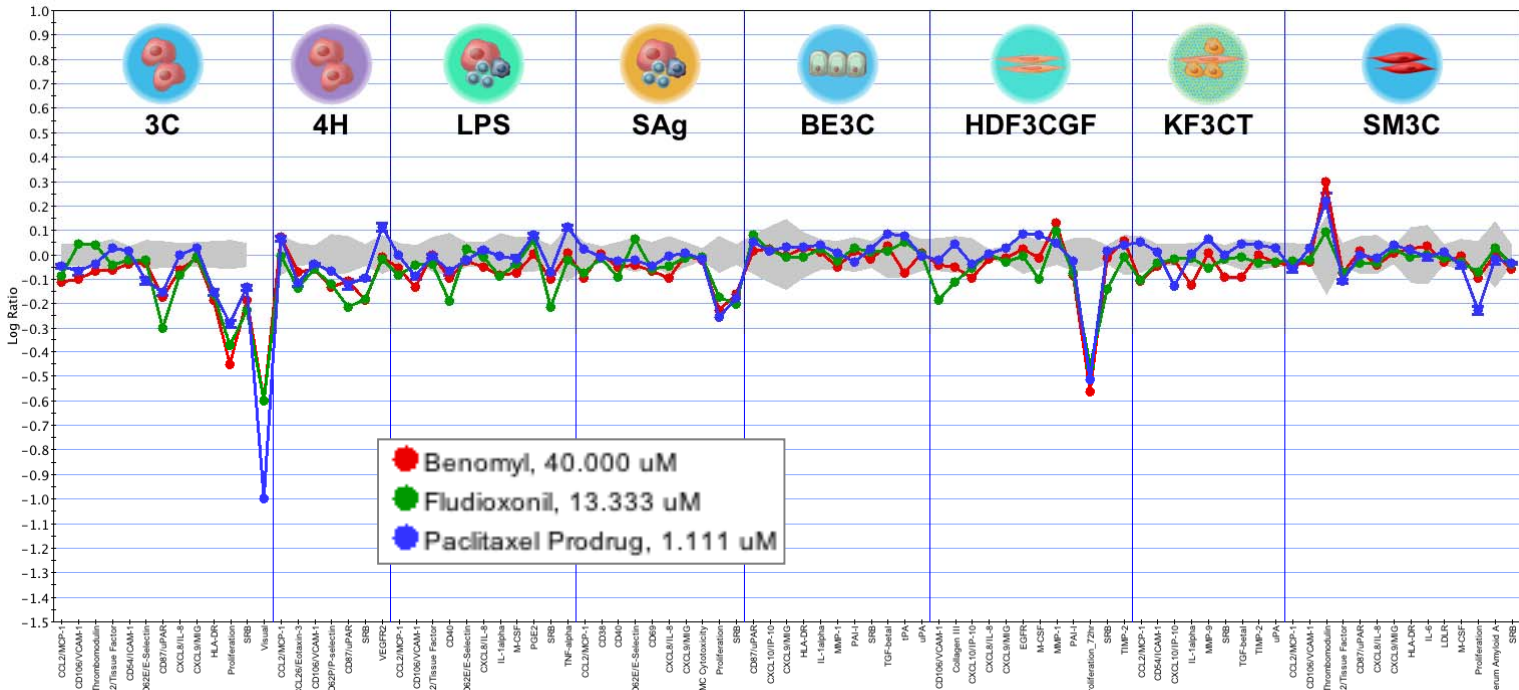mitochondrial respiratory chain uncouplers

Myxothiazol, 1.111 uM
Pyraclostrobin, 13.333 uM
Trifloxystrobin, 13.333 uM

NFkB inhibitors

Dazomet, 40.000 uM
Dazomet, 13.333 uM
Dazomet, 4.444 uM
Dimethyl Fumarate, 50.000 uM
Dimethyl Fumarate, 16.667 uM
Dimethyl Fumarate, 5.556 uM

Houck et al., JBS 14:1054, 2009

cAMP elevators

Legend:
- Atrazine, 40.000 uM
- Cyanazine, 40.000 uM
- Simazine, 40.000 uM

Microtubule stabilizers

Legend:
- Benomyl, 40.000 uM
- Fludioxonil, 13.333 uM
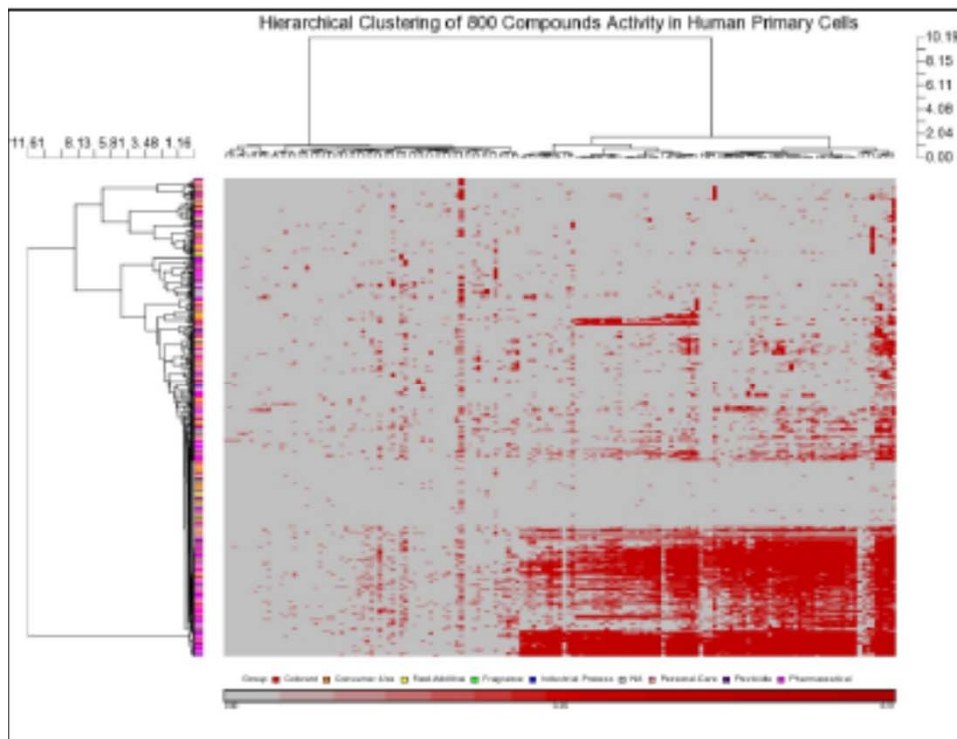- Paclitaxel Prodrug, 1.111 uM
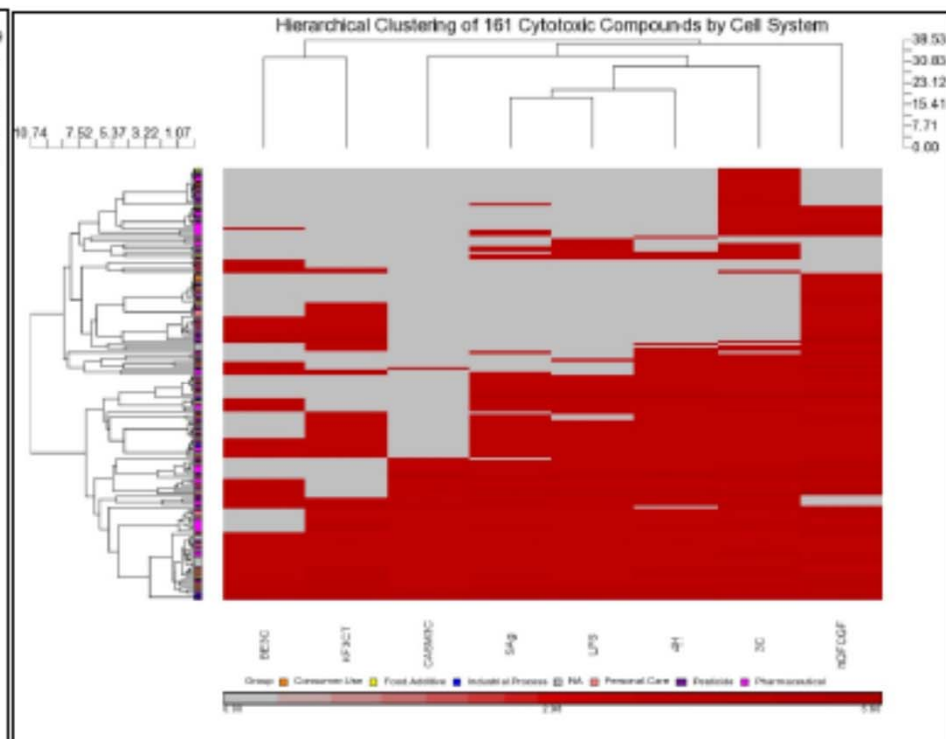
Phase II ToxCast Chemical Library Screening

- **800 chemicals**
- **8 cell systems**
- **Calculated AC50 for active chemicals**
- **Unsupervised profile matching**
- **Unsupervised clustering (SOM)**
- **Supervised analysis (SVM)**

Overall Summary of Phase II Chemical Activity

Endpoint AC50

Cytotoxicity AC50

- Most active chemical categories: pharmaceuticals and pesticides
- Least active chemical categories: food additives and consumer use products

Kleinstreuer et al. Nature Biotech. 2014

Activity by Chemical Category

| Chemical Class	#Samples Tested	% Active	% Cytotoxic	% Active and Non-Cytotoxic	% Inactive
ToxCast Phase I	321	94%	22%	72%	6%
ToxCast Phase II	800	73%	22%	51%	27%
Colorant	19	32%	0%	32%	68%
Consumer Use	64	67%	28%	39%	33%
Food Additive	92	60%	9%	51%	40%
Fragrance	3	33%	0%	33%	67%
Industrial Process	35	46%	9%	37%	54%
NA	113	80%	22%	58%	20%
Personal Care	136	68%	18%	49%	32%
Pesticide	88	82%	30%	52%	18%
Pharmaceutical	250	83%	29%	54%	17%

Analysis of Replicates
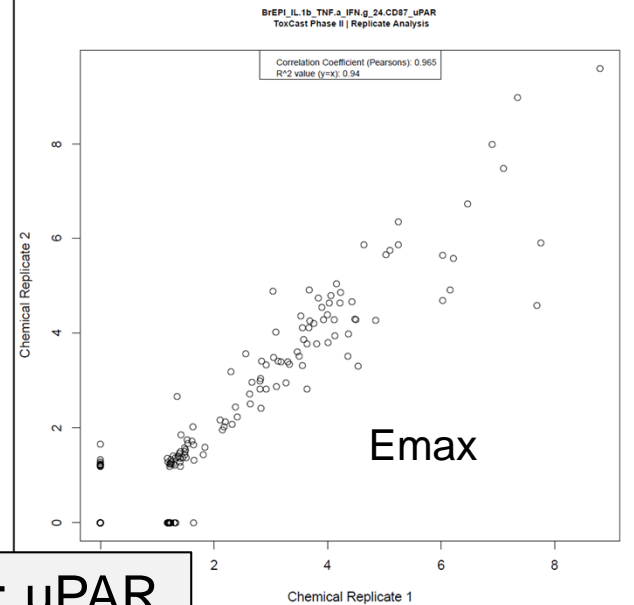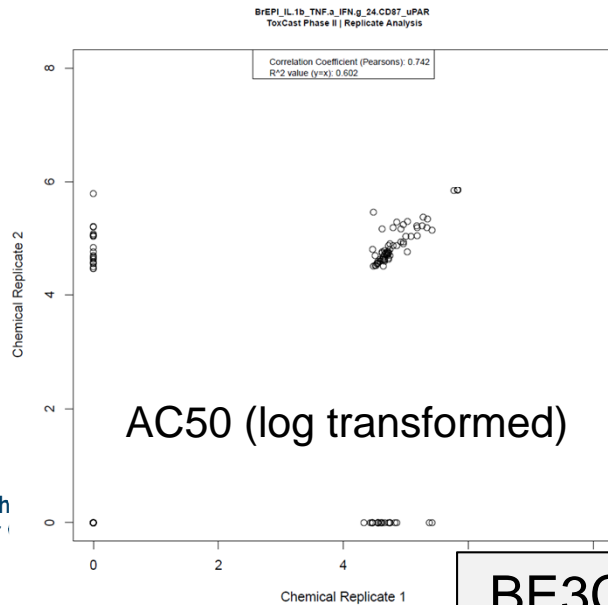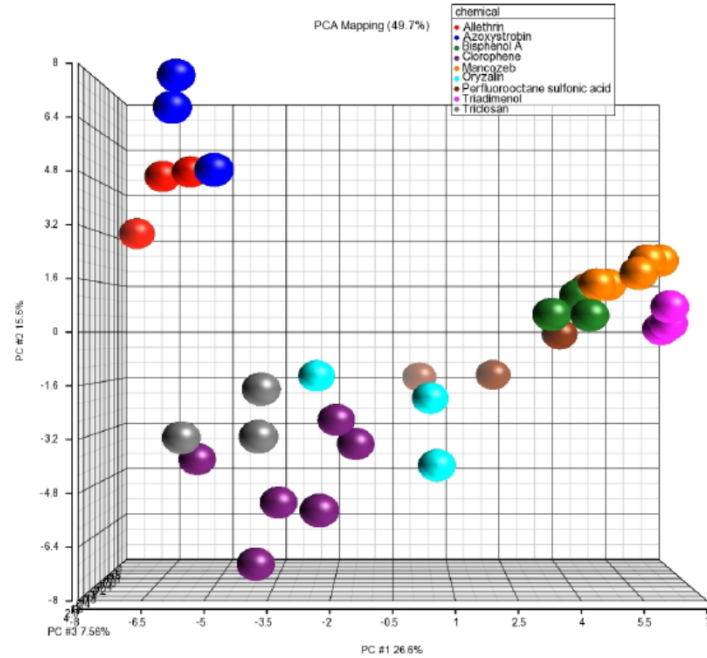
Example of Unsupervised Correlation Analysis:
BioMAP Profile TX011657
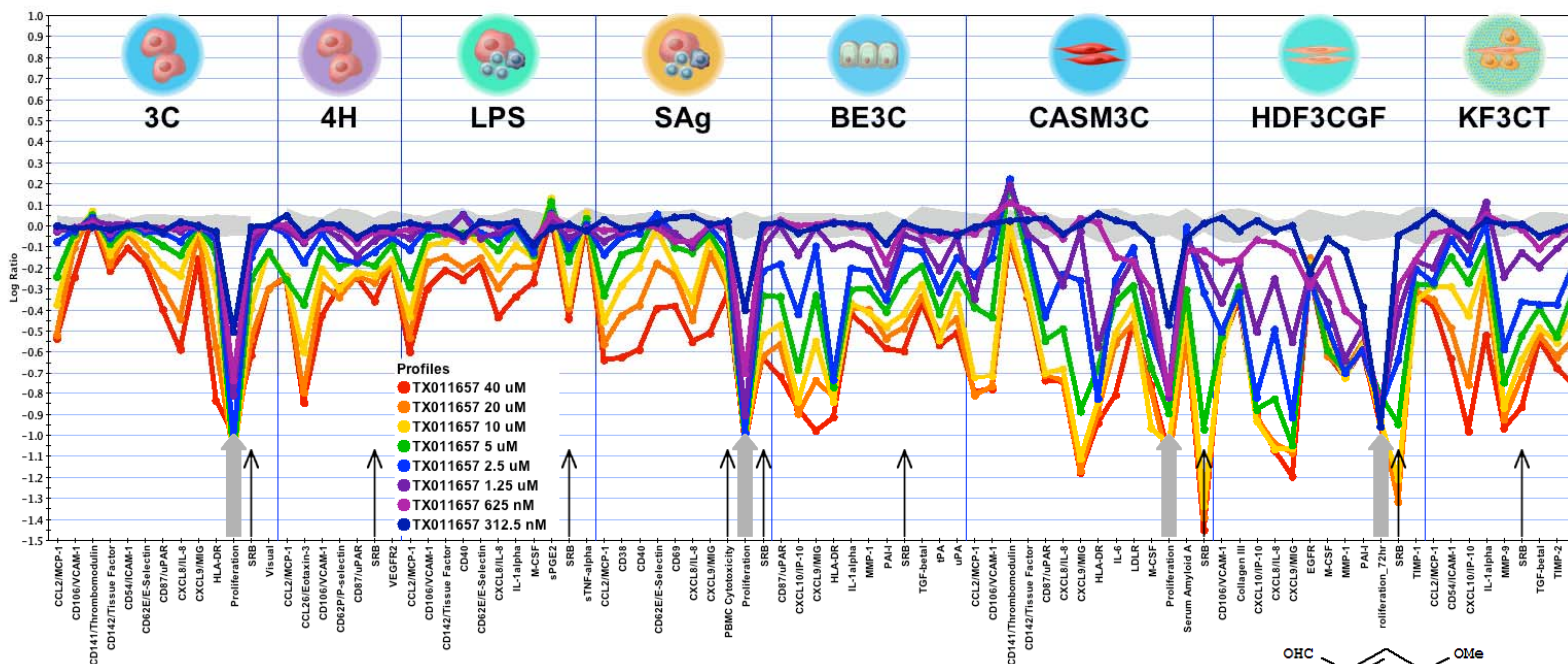
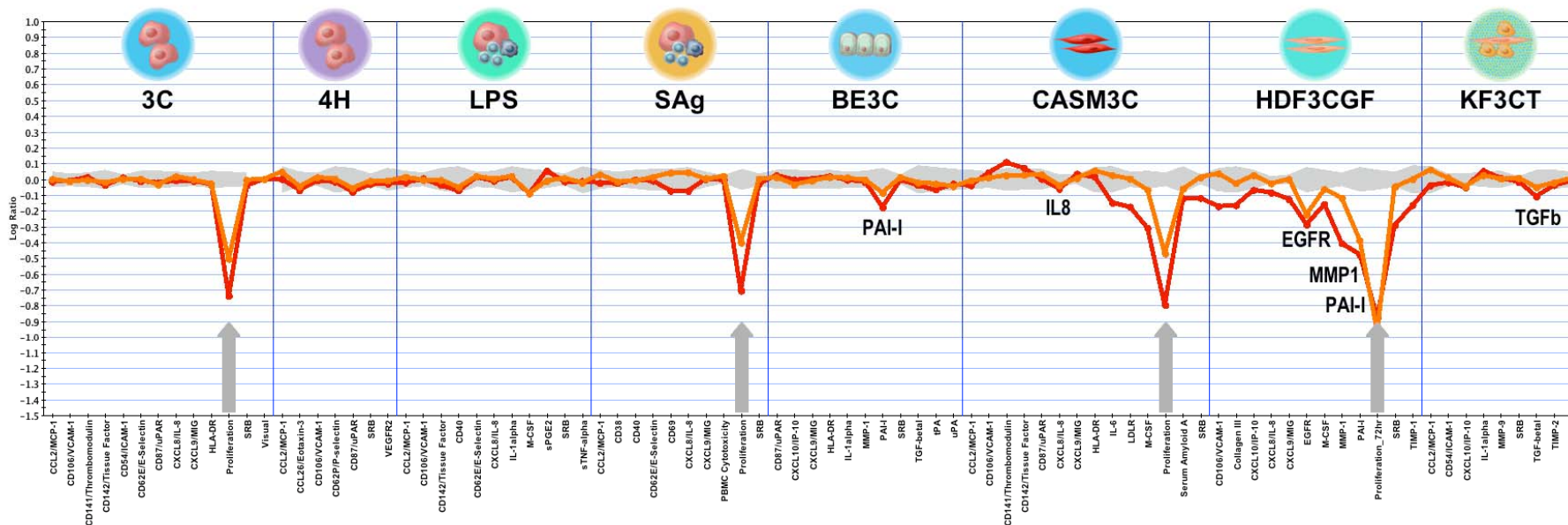**TX0011657**

- Mechanism: Unknown (failed pharma cmpd)

- Side effect: Fibroid lung in cancer patients

- Overtly cytotoxic to EC, T cells, epithelial cells, SMC and fibroblasts at ≥1.25 µM
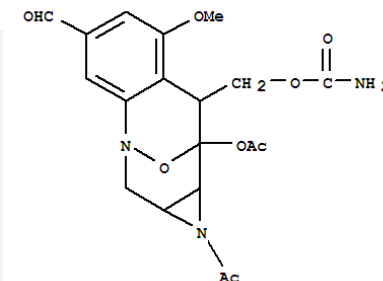
BioMAP Profile TX011657

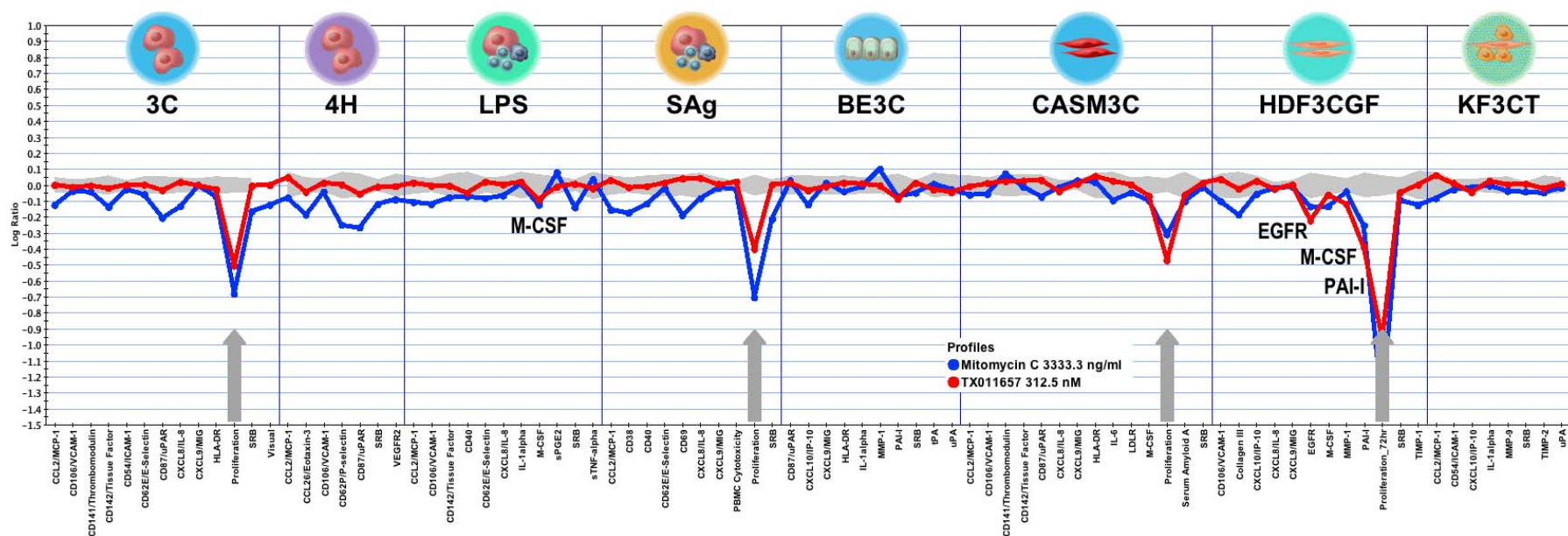**TX0011657 – non-cytotoxic doses**
- Anti-proliferative to EC, T cells, SMC and fibroblasts

Profiles
- TX011657 625 nM
- TX011657 312.5 nM

Database Match: TX011657
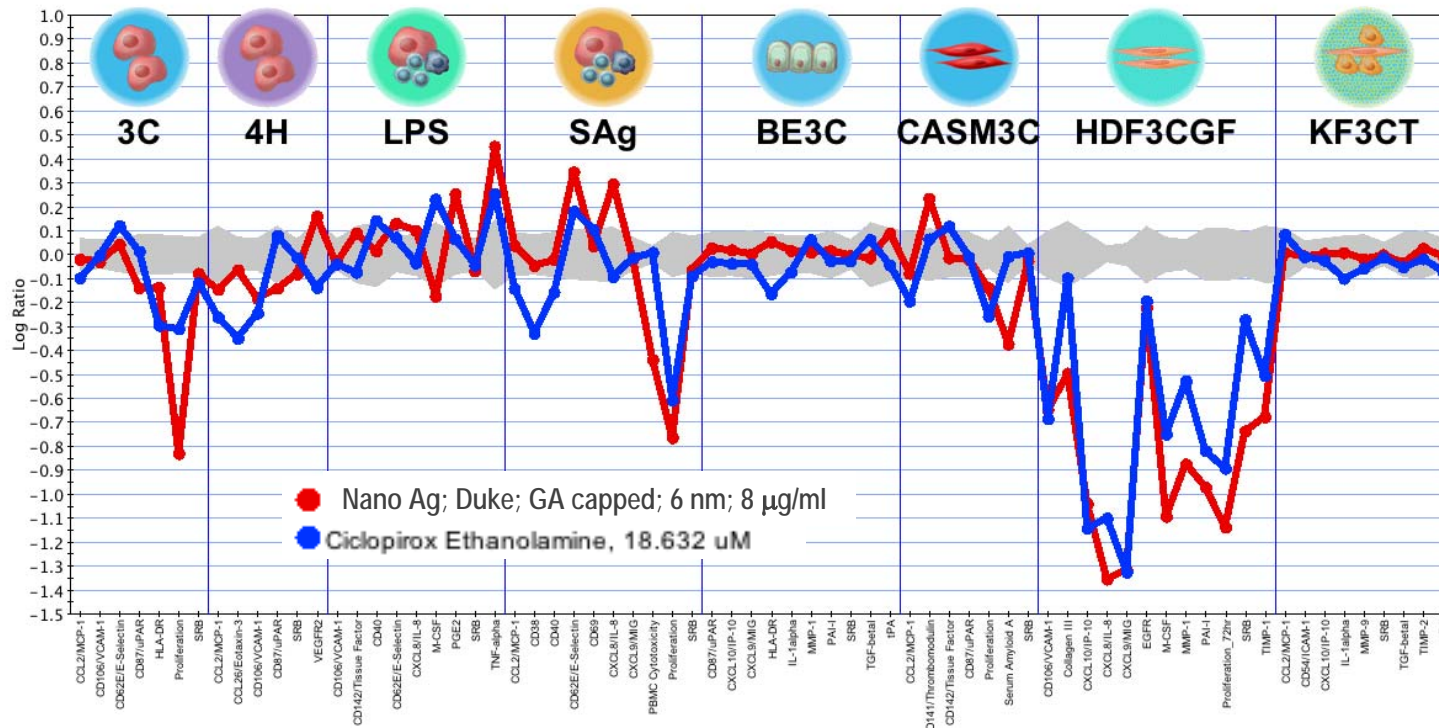
Overlay of TX011657 and Mitomycin C

- Pearson's correlation of r = 0.865

- Mitomycin C is a DNA crosslinker and chemotherapeutic agent. Side effects include bone marrow and renal damage, lung fibrosis

Inferred Mechanism of Toxicity: nano Silver

- **Ciclopirox – inhibitor of Na+ K+ ATPase**
- **Toxicity of silver is associated with inhibition of Na⁺K⁺ATPase (PMID: 6240533)**

Unsupervised Clustering using Self-Organizing Maps Yields Mechanistic Classes

- Chemicals analyzed at single conc level to minimize polypharmacology effect
- Self Organizing Maps (SOM): 10X10 Array/100 Clusters

SOM Analysis

- Self-Organizing Map (Kohonen map) is a type of artificial neural network trained by unsupervised learning and preserving the topological properties of input space
- Relies on knowledge of reference chemicals in classes
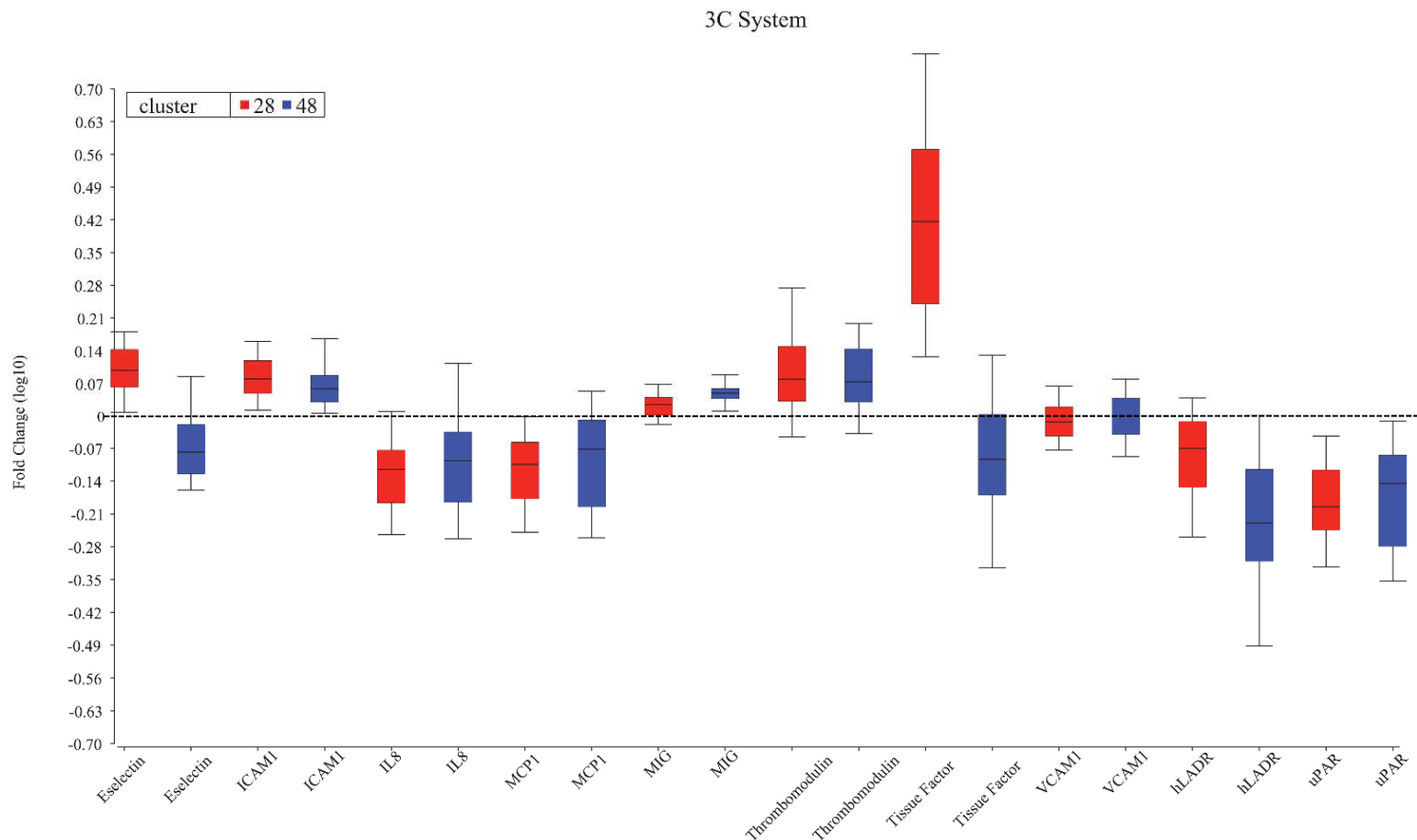- Can be used to classify new compounds

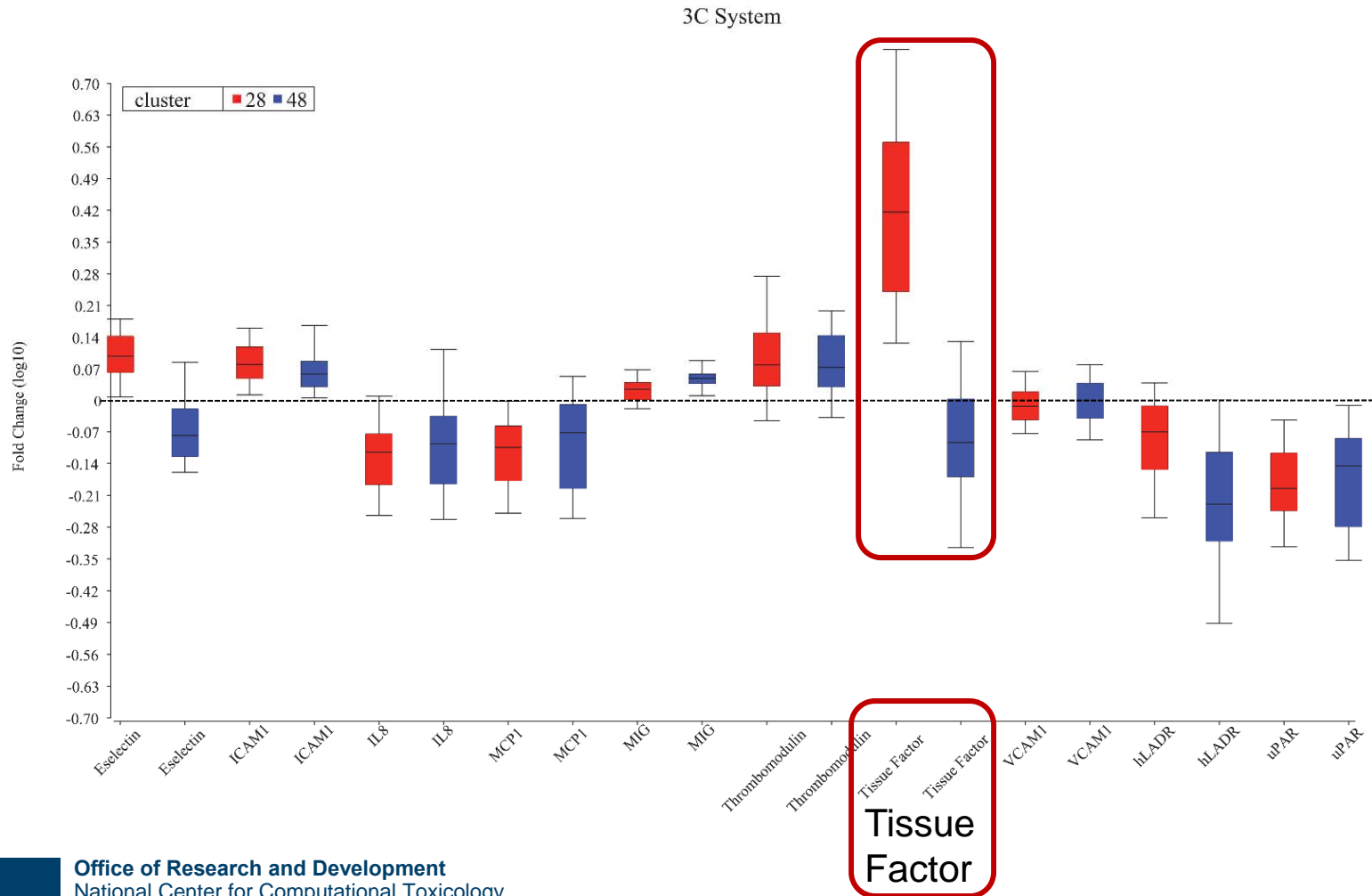# Unsupervised Clustering using Self-Organizing Maps Yields Mechanistic Classes
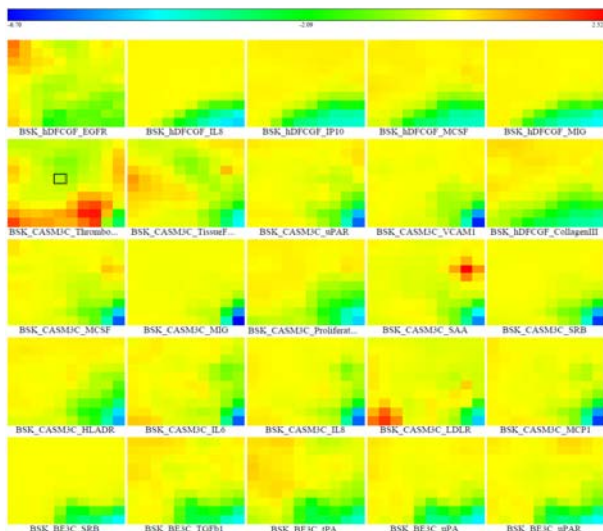
Office of Research and Development
National Center for Computational Toxicology

Examples of clusters that emerged from the SOM analysis				
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	Color Index. C.I. Solvent yellow 14
Chemical [48]	27	Estrogen receptor pathway modulators	Clomiphene citrate Tamoxifen citrate Fulvestrant Raloxifene hydrochloride Tamoxifen 4-hydroxytamoxifen	Cyclopamine Amiodarone hydrochloride Haloperidol Reserpine Donated pharma: NK1 receptor antagonist Bradykinin B1 receptorantagonist Lipid-lowering agent
Assay [46]	29	TNFα inhibition	All-trans retinoic acid Donated pharma: PDE inhibitors (8 compounds)	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

Comparison of Anti-estrogenic (28) and Estrogenic (48) Clusters in 3C Endothelial Cell System
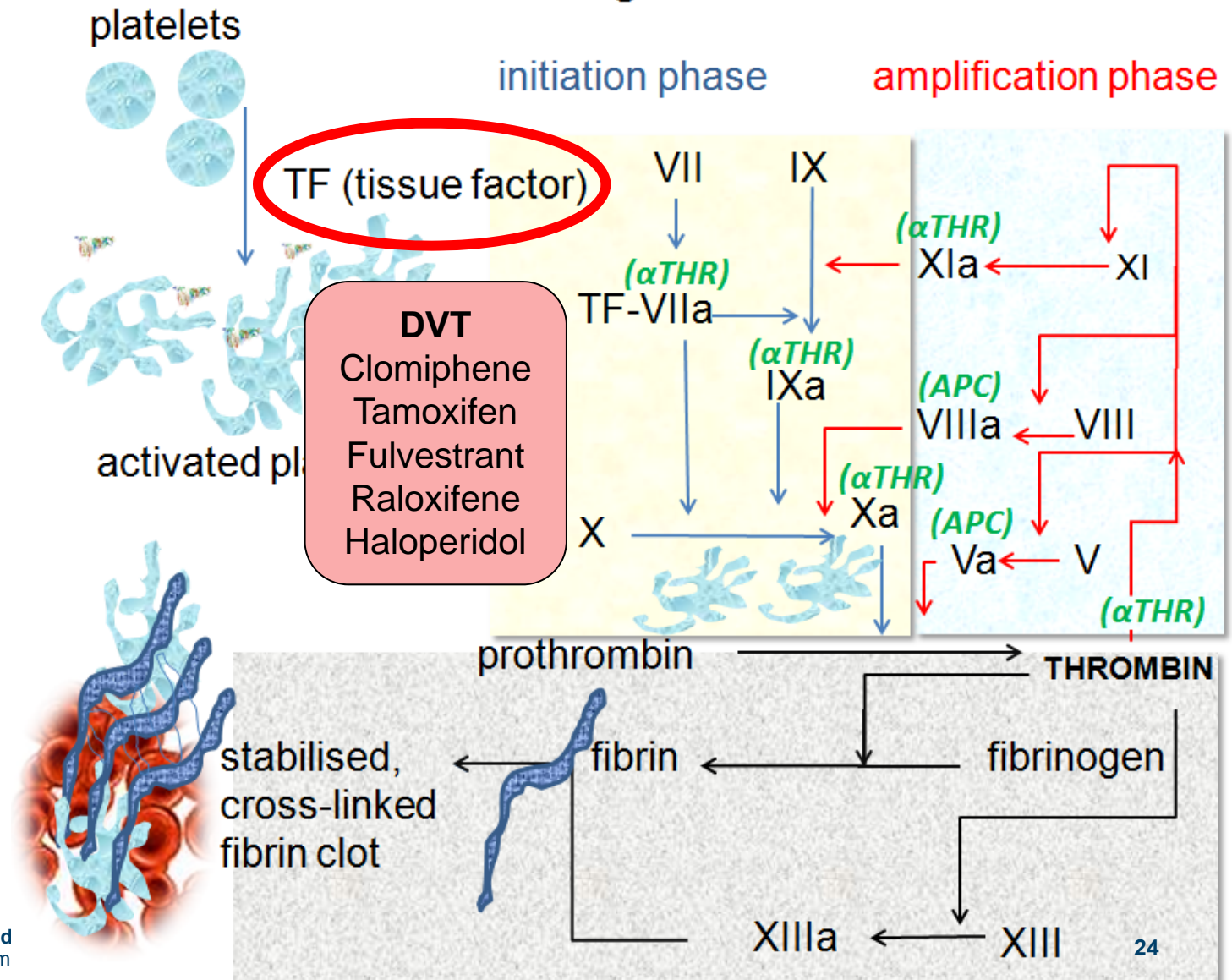

3C System

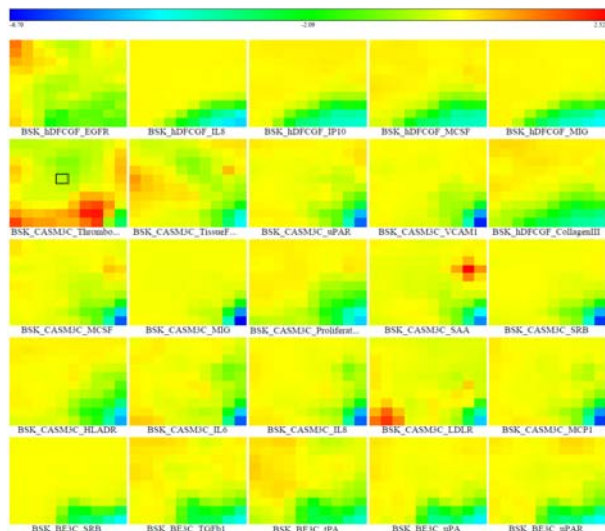# Comparison of Anti-estrogenic (28) and Estrogenic (48) Clusters in 3C Endothelial Cell System

Hypothesis Generation Example

Blood coagulation *in vivo*
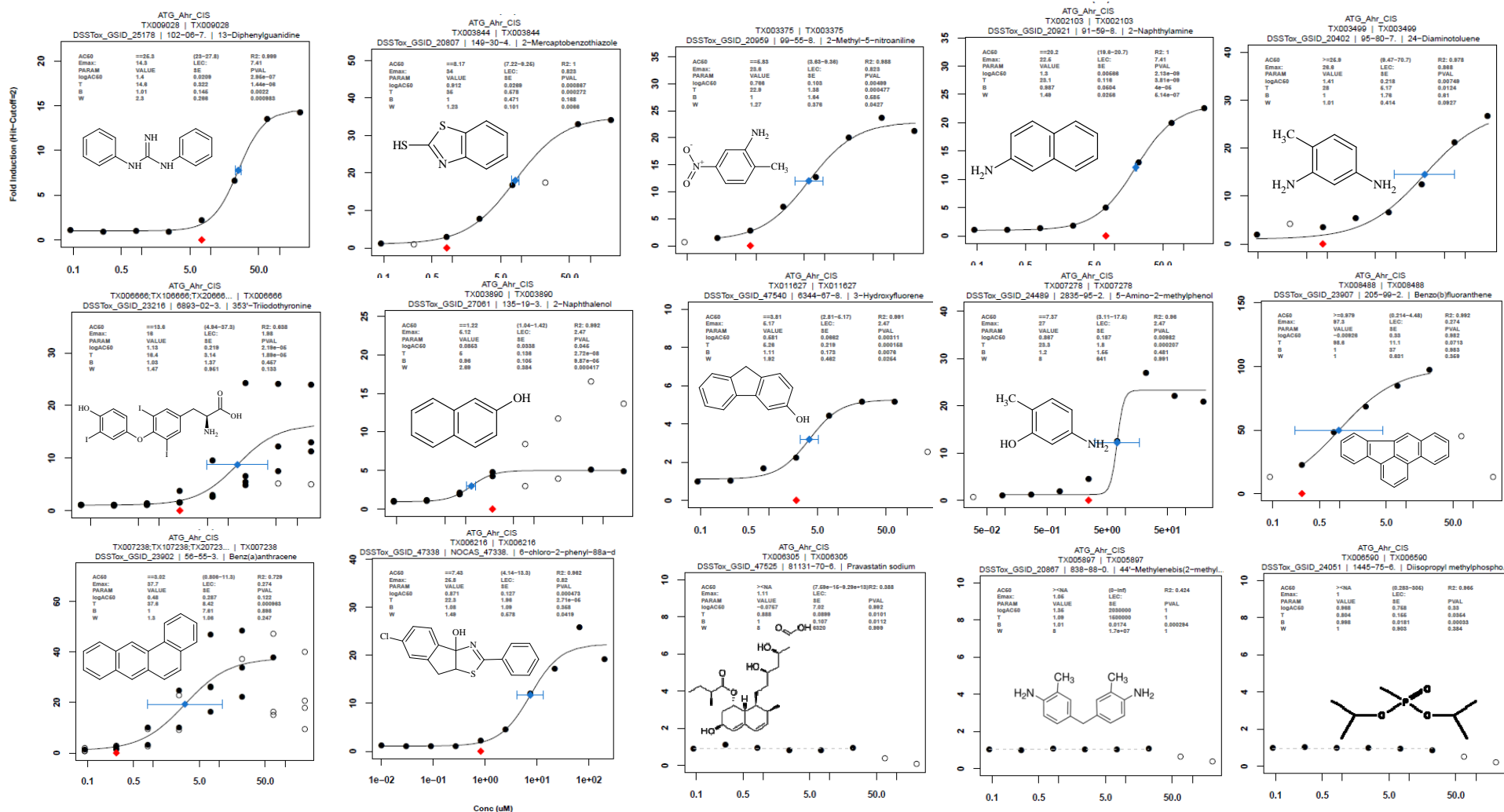
Dr Graham Beards, Wikipedia Commons

Unsupervised Clustering using Self Organizing Maps Yield Mechanistic Classes

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All 3 negatives were present at only one conc in the SOM cluster

Single Chemical Profile from AhR Cluster (57)

TX008488: **Benzo(b)fluoranthene**
SVM Score (AHR Agonist) = 0.67
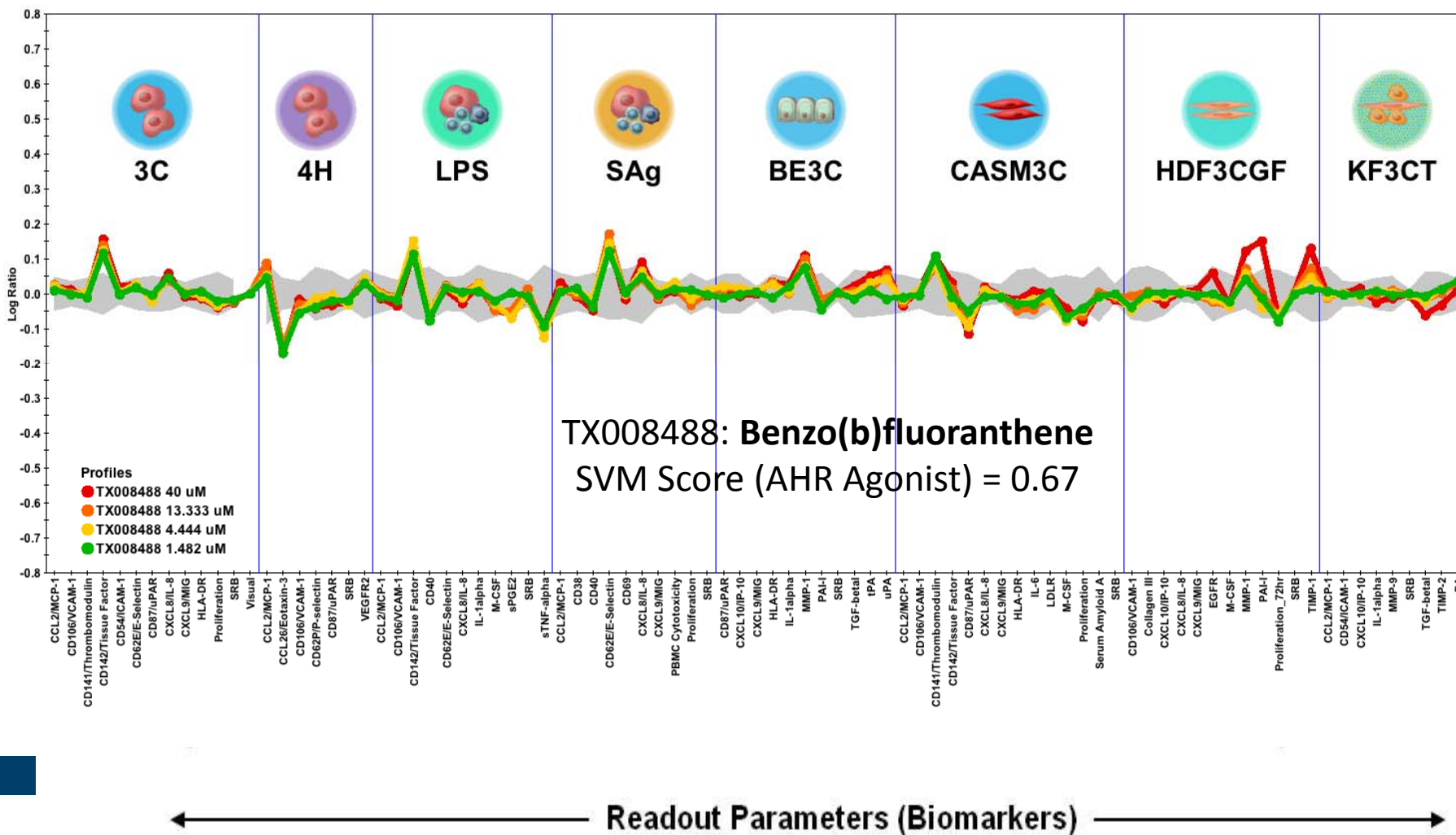
Bioactivity Signature Example: SOM Cluster #57: AHR Agonists
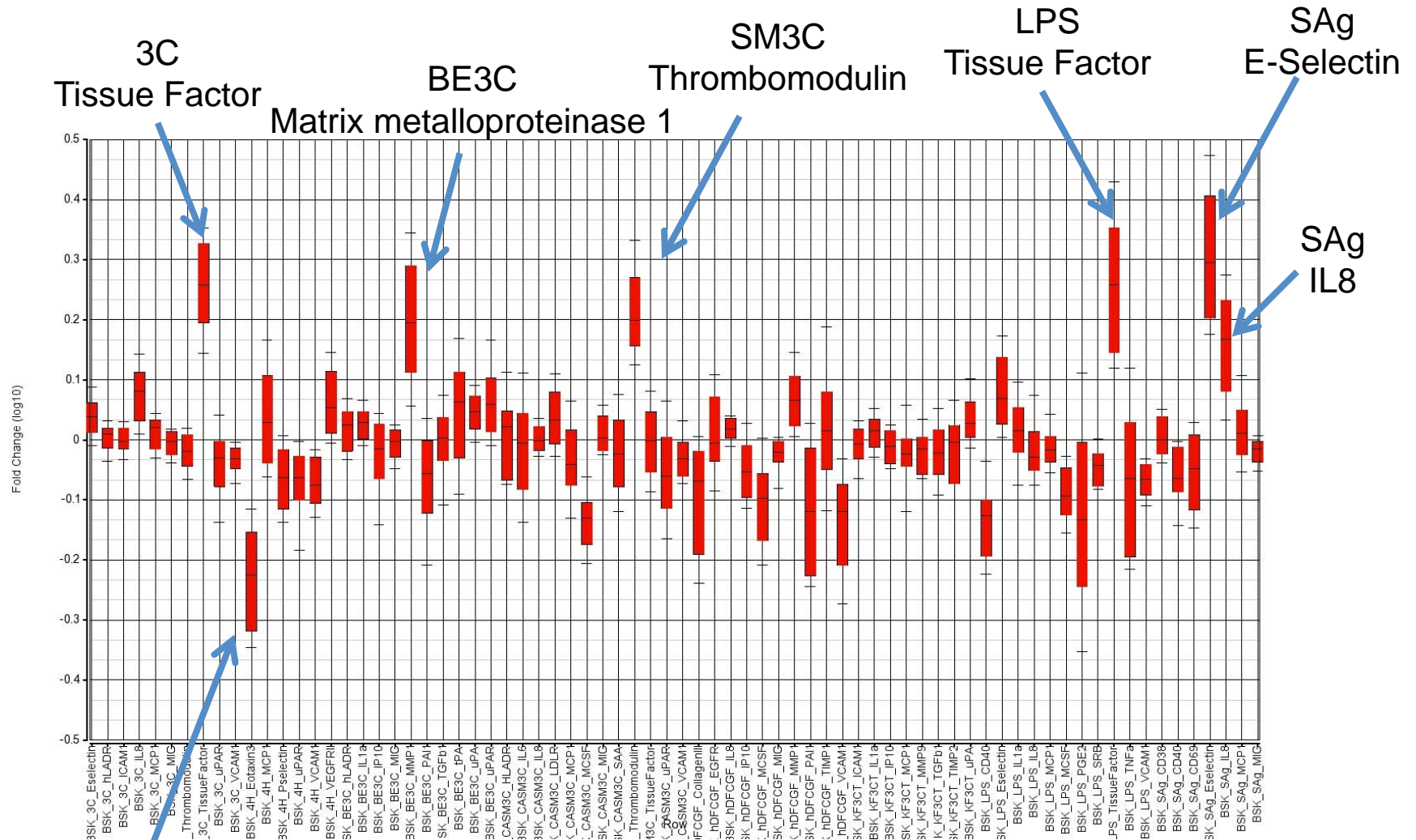
PAH's from cigarette smoke associated with atherogenesis/thrombosis

Kleinstreuer et al. Nature Biotech. 2014

Supervised Analysis: Support Vector Machines

- Supervised learning model for classification using a hyperplane in high-dimensional space

- Training on reference database compounds with clean activity for mechanism of interest

- 28 models built and applied to phase II chemicals

- Found chemicals with high Decision Values for specific mechanism classes (e.g. GR Agonists, PDE IV Inhibitors) and for multiple mechanism classes (PDE IV Inhibitors, RAR Agonists)

Office of Research and Development
National Center for Computational Toxicology
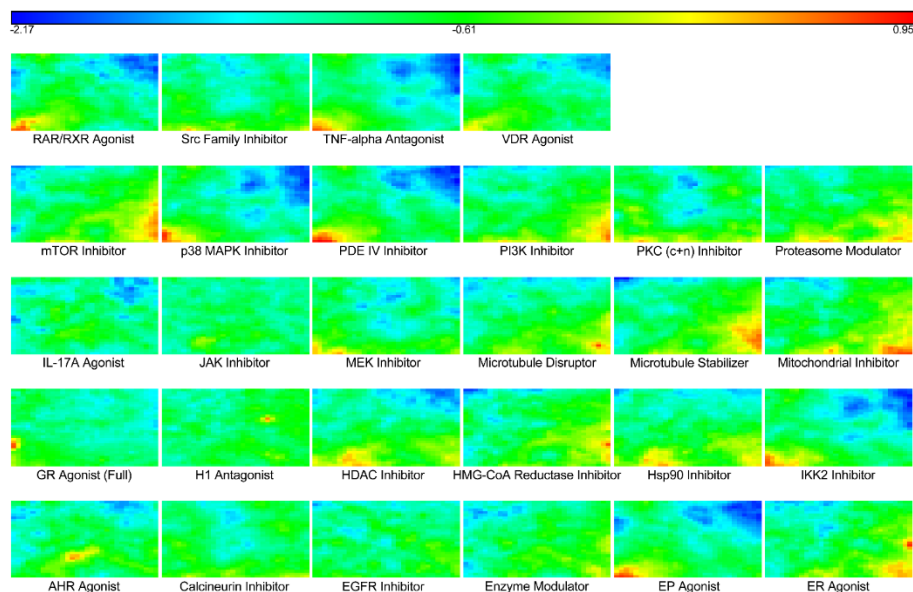
Application of SVM Predictions

ToxCast Phase II chemical library

Screen

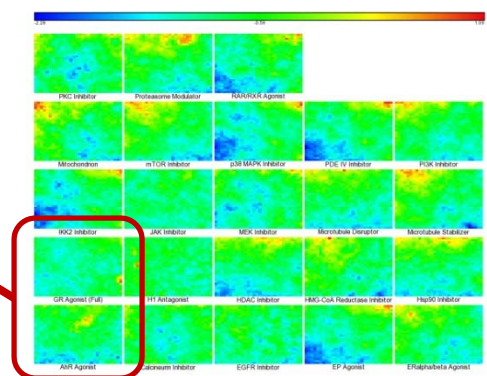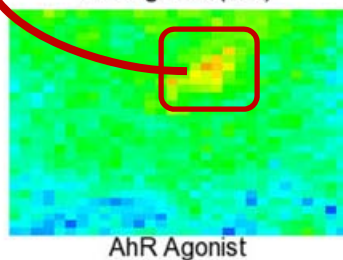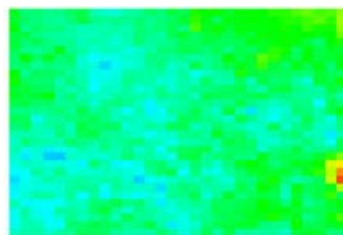| System		Cell Types	Environment	Readouts
3C		Endothelial cells	IL-1β+TNF-α+IFN-γ	MCP-1, VCAM-1, ICAM-1, Thrombomodulin, Tissue Factor, E-selectin, uPAR, IL-8, MIG, HLA-DR, Prolif., Vis., SRB (13)
4H		Endothelial cells	IL-4+histamine	VEGFRII, P-selectin, VCAM-1, uPAR, Eotaxin-3, MCP-1, SRB (7)
LPS		Peripheral Blood Mononuclear Cells + Endothelial cells	TLR4	CD40, VCAM-1,Tissue Factor, MCP-1, E-selectin, IL-1a, IL-8, M-CSF, TNF-a, PGE2, SRB (11)
SAg		Peripheral Blood Mononuclear Cells + Endothelial cells	TCR	MCP-1, CD38, CD40, CD69, E-selectin, IL-8, MIG, PBMC Cytotox., SRB, Proliferation (10)
BE3C		Bronchial epithelial cells	IL-1β+TNF-α+IFN-γ	uPAR, IP-10, MIG, HLA-DR, IL-1a, MMP-1, PAI-1, SRB, TGF-b1, tPA, uPA (11)
HDF3CGF		Fibroblasts	IL-1β+TNF-α+IFN-γ +bFGF+EGF+PDGF-BB	VCAM-1, IP-10, IL-8, MIG, Collagen III, M-CSF, MMP-1, PAI-1, Proliferation, TIMP-1, EGFR, SRB (12)
KF3CT		Keratinocytes + Fibroblasts	IL-1β+TNF-α+IFN-γ +TGF-β	MCP-1, ICAM-1, IP-10, IL-1a, MMP-9, TGF-b1, TIMP-2, uPA, SRB (9)
CASM3C		Coronary artery vascular smooth muscle cells	IL-1β+TNF-α+IFN-γ	MCP-1, VCAM-1, Thrombomodulin, Tissue Factor, IL-6, LDLR, SAA, uPAR, IL-8, MIG, HLA-DR, M-CSF, Prolif., SRB (14)

Use SVM to determine likelihood of belonging to 28 mechanism classes

GR Agonist (Full)

AhR Agonist

SOMs of SVM Clusters Conditioned by mechanism

Office of Research and Development
National Center for Computational Toxicology
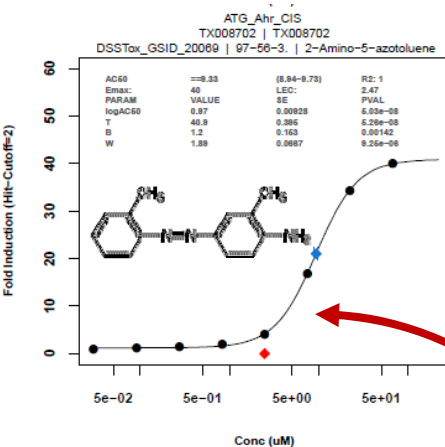
Compounds with High DV's

Mechanism Class	High DV Count		Example Compounds *known associations	Example Compounds *novel associations
	Conc.'s	Unique Cmpds		
AhR Agonist	14	7	Benzo(b)fluoranthene 4-Chloro-1,2-diaminobenzene Benz[a]anthracene 2-Naphthylamine 2-Methyl-5-nitroaniline 3,4-Dichloronitrobenzene	C.I Solvent yellow 14

ERalpha/beta Agonist	22	16	17alpha-Estradiol
			beta-Estradiol
			Norethindrone
			5alpha-Dihydrotestosterone
			Testosterone propionate
			17alpha-Ethynylestradiol
			Progesterone
			17-Methyltestosterone
				Dieldrin
				Azoxystrobin
				Triphenyl phosphate
				Donated pharma:
				    CCR5 antagonist
				    5-HT3 antagonist

GR Agonist	20	5	Dexamethasone Triamcinolone Prednisone Corticosterone	Pentaerythritol
H1 Antagonist	11	5	Diphenhydramine hydrochloride Chlorpromazine hydrochloride	Volinanserin Trelanserin Besonprodil
HDAC Inhibitor	23	15	Dicyclohexyl disulfide Donated pharma: cytotoxic DNA-interacting agent	1,2-Benzisothiazolin-3-one Darbufelone mesylate Tris(2-ethylhexyl) phosphate Hydroquinone
HMG-CoA Reductase Inhibitor	23	14	Lovastatin Simvastatin Pravastatin sodium	3,3,4,4,5,5,6,6,7,7,8,8,8- Tridecafluorooctyl methacrylate Diisopropyl methylphosphonate
Hsp90 Inhibitor	42	33	Cycloheximide Donated pharma: cytotoxic DNA-interacting agent	Didecyl dimethyl ammonium chloride 4,4',4''-Ethane-1,1,1-triyltriphenol Genistein Allethrin Triclocarban
IKK2 Inhibitor	57	21	Spironolactone Donated pharma: PDE IV inhibitors (5)	Pentachloropyridine

MEK Inhibitor	12	6	Donated pharma:
			    MAPK inhibitor
				Donated pharma:
				    unknown targets (3)

Microtubule Disruptor	15	11	Colchicine 17alpha-Estradiol beta-Estradiol	7,12-Dimethylbenz(a)anthracene Donated pharma: NMDA receptor (NR2B) antagonist
Microtubule Stabilizer	100	66	5HPP-33 5-Fluorouracil Rifampicin Clomiphene citrate	Bronopol Cytarabine hydrochloride Pentamidine isethionate 5-Chloro-2-methyl-3(2H)-isothiazolone p-Chloroaniline

Compounds with High DV's, cont.

Mitochondrion	102	74	Azoxystrobin Picoxystrobin Dinoseb Nitrofen Fenofibrate meso-Hexestrol Diethylstilbestrol Donated pharma: Na+ channel blocker	Triclocarban Triphenyl phosphate Bisphenol B 2,4,5-Trichlorophenol
mTOR Inhibitor	93	61	None in set	Dodecyltrimethylammonium chloride Heptachlor epoxide, isomer B Chlorthal-dimethyl Donated pharma: benzothiophene cell activator inhibitor

p38 MAPK Inhibitor	60	20	Donated pharma:
			  p38 MAPK inhibitor
			  MAPK inhibitor
				Donated pharma:
				  monoamine reuptake inhibitor
				  glycogen phosphorylase inhibitor
				  GABA$_A$1 receptor antagonist
				  HIV nucleocapsid protein zinc fingers inhibitor
				  IL-1 inhibitor
				  unknown targets (5)

PDE IV Inhibitor	61	23	Donated pharma: PDE IV inhibitors (8)	Terbuthylazine Isoproterenol hydrochloride

PI3K Inhibitor	35	27	None in set	Elzasonan
				Clomiphene citrate
				Donated pharma:
				  NK1 receptor antagonist
				  serotonin reuptake inhibitor
				  lipid lowering agent

Proteasome Modulator	66	51	Tributyltin chloride	2-Chloroacetophenone Triclosan Allethrin
RAR/RXR Agonist	43	14	Trans-Retinoic acid Retinol Donated pharma: thyroid receptor ligand	None in set

Mitochondrial Toxicants SVM

Chemical	Mitochrome
Pharma	0.988
Azoxystrobin	0.987
Picoxystrobin	0.987
Azoxystrobin	0.986
Picoxystrobin	0.986
2-Methyl-4,6-dinitrophenol	0.984
Azoxystrobin	0.982
Picoxystrobin	0.981
2,4,5-Trichlorophenol	0.98
Triphenyl phosphate	0.98
Picoxystrobin	0.979
Azoxystrobin	0.974
Azoxystrobin	0.969
Fenofibrate	0.969
Dinoseb	0.967
Pharma	0.964
Pharma	0.954
Triclocarban	0.953
Zamifenacin	0.951
Dinoseb	0.946
Azoxystrobin	0.939
Nitrofen	0.939
Azoxystrobin	0.938
Phenolphthalein	0.933
Sodium azide	0.925
Progesterone	0.923
Triclocarban	0.917
Pentachlorophenol	0.916
Pharma	0.914
Triclosan	0.913

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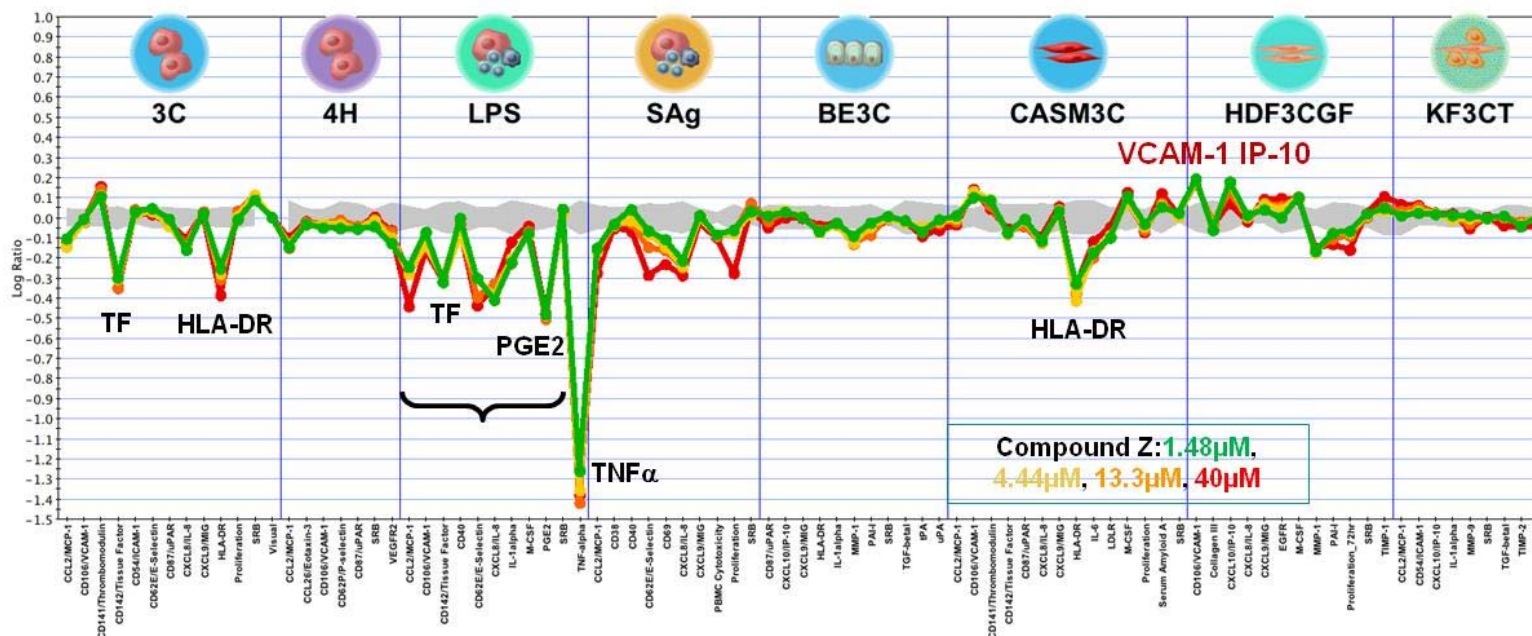
- Inhibition of tissue factor in activated endothelial cells (3C)
- Strong inhibition of monocyte activation indicated by PGE2, CCL2, TF (LPS)
- Inhibition of HLA-DR in endothelial cells (3C) and smooth muscle cells (CASM3C)
- Upregulation of VCAM-1, IP-10 in human dermal fibroblasts (HDF3CGF)

Use in Development of AOPs

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National Center
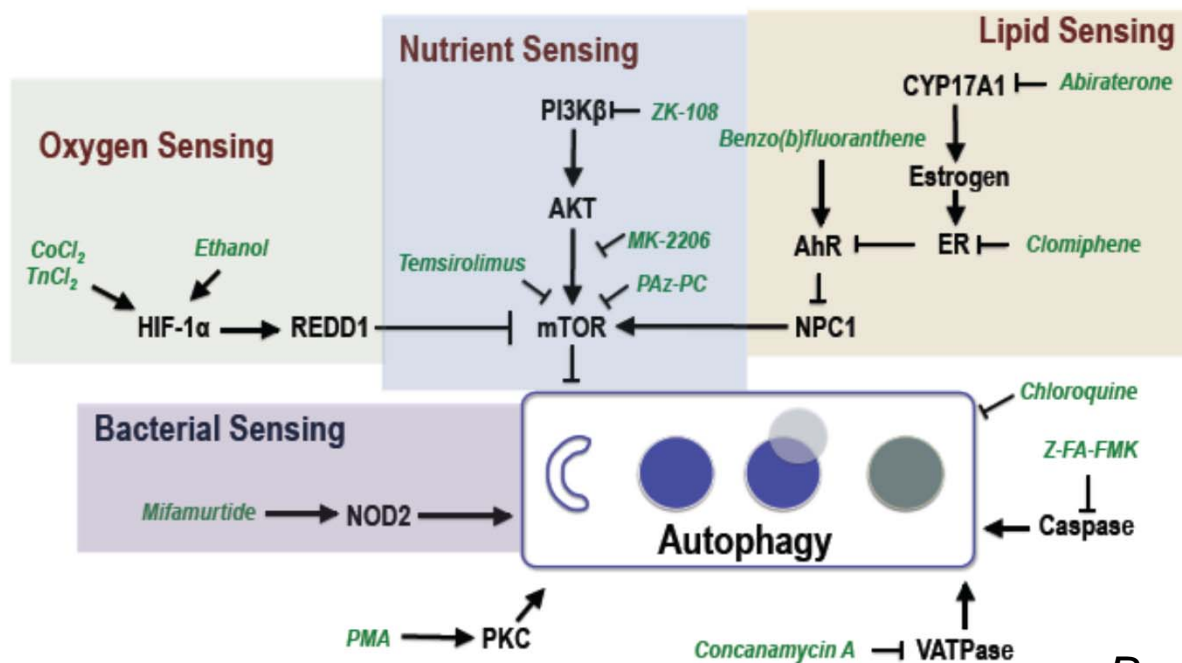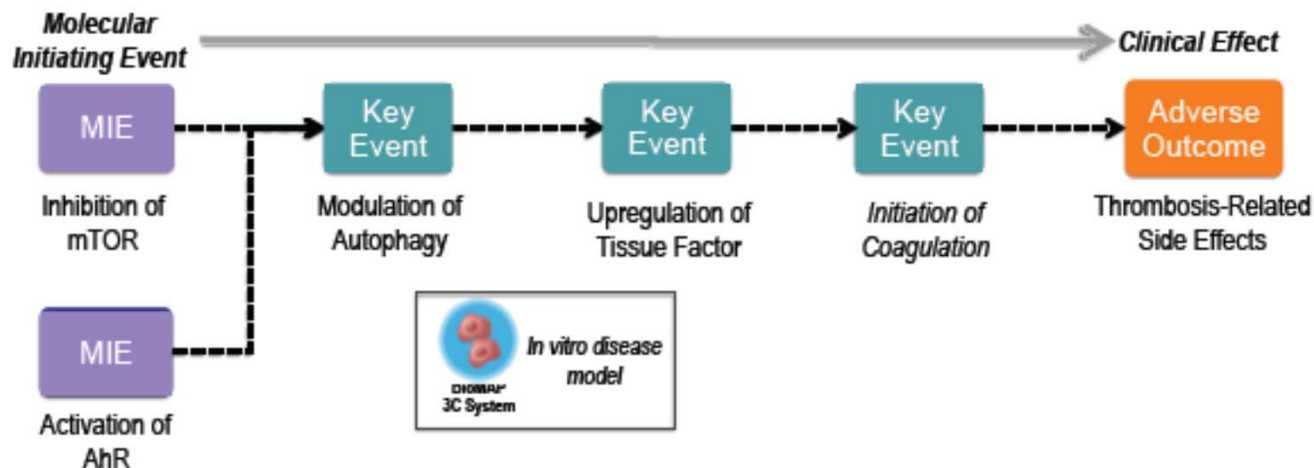
SUMMARY

- Complex primary human cell cultures can be used for robust chemical screening campaigns

- Primary human cells provide a rich diversity of biological activity useful for determining chemical mechanisms of action

- Reference chemicals with known MOA's and clean pharmacology are extremely useful in developing models for this type of approach

- Expanded assay diversity, e.g. other primary cell types, may be needed to more completely characterize chemicals of interest

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