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Using High Throughput Screening to Identify Predictive In Vitro Biomarkers for Acute Systemic Toxicity

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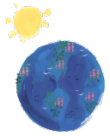
To meet the challenges of 21st century toxicology, the NTP Roadmap includes a major initiative to develop a high throughput screening (HTS) program with 3 main goals:

- Prioritize chemicals for further in-depth toxicological evaluation
- Identify mechanisms of action
- Develop predictive models for *in vivo* biological response



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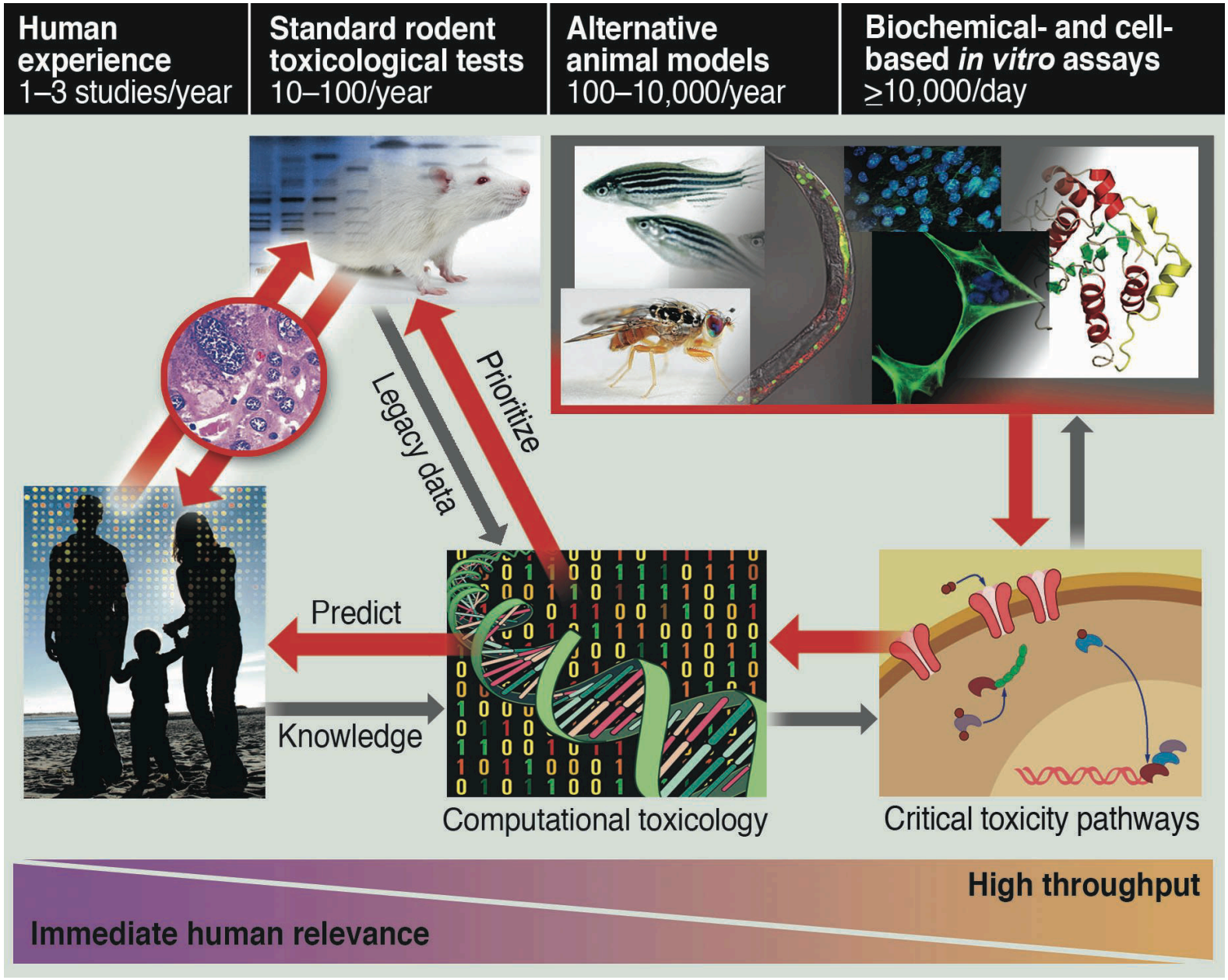
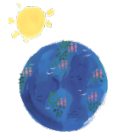


NIH Molecular Libraries Initiative

http://nihroadmap.nih.gov/molecularlibraries/

- HTS methods are being used to identify small molecules that can be optimized as chemical probes to study the functions of genes, cells, & biochemical pathways.
- In mid-2005, NTP became a formal participant in the MLI by establishing a collaboration with the NIH Chemical Genomics Center (NCGC).
- As a result, the NTP gained the opportunity to link data generated from HTS assays for biological activity to toxicity data produced by the NTP's testing program.



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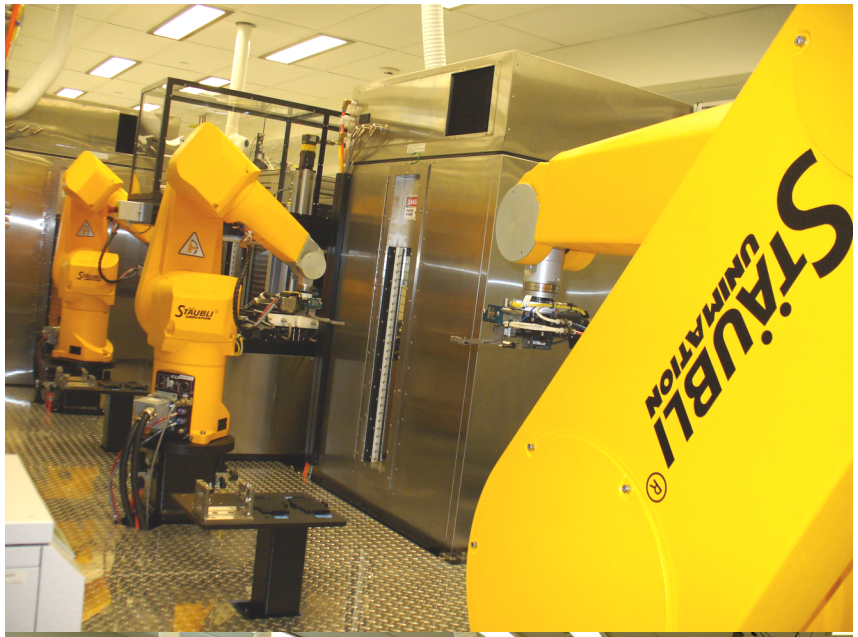
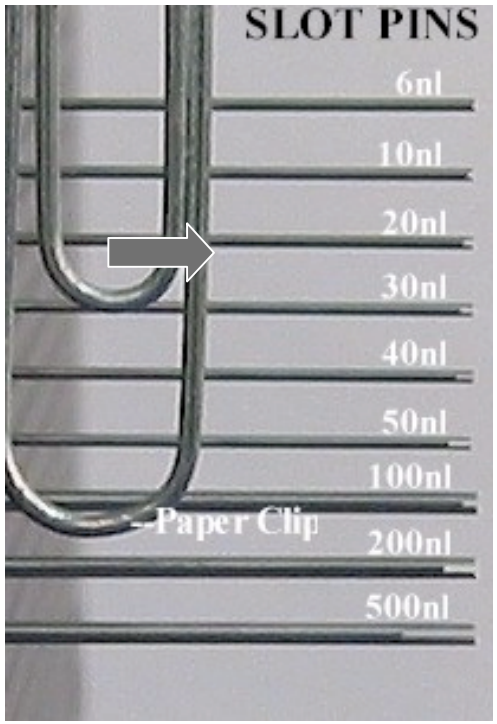
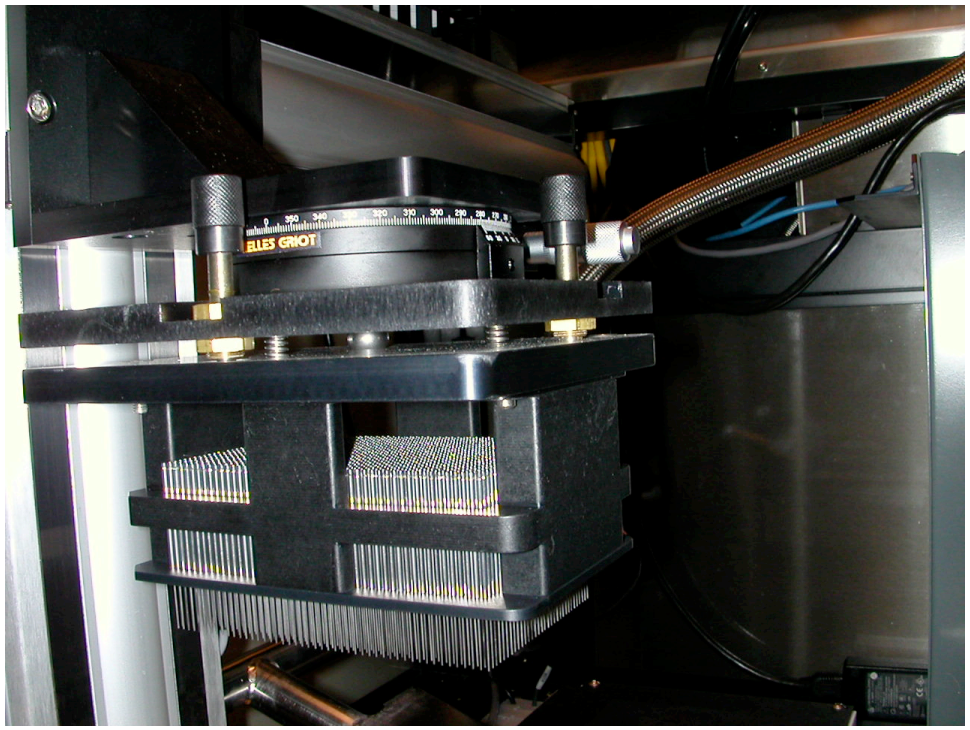
NCGC
NIH Chemical Genomics Center



Pin-tool for compound delivery from inter-plate titrations series

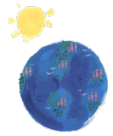




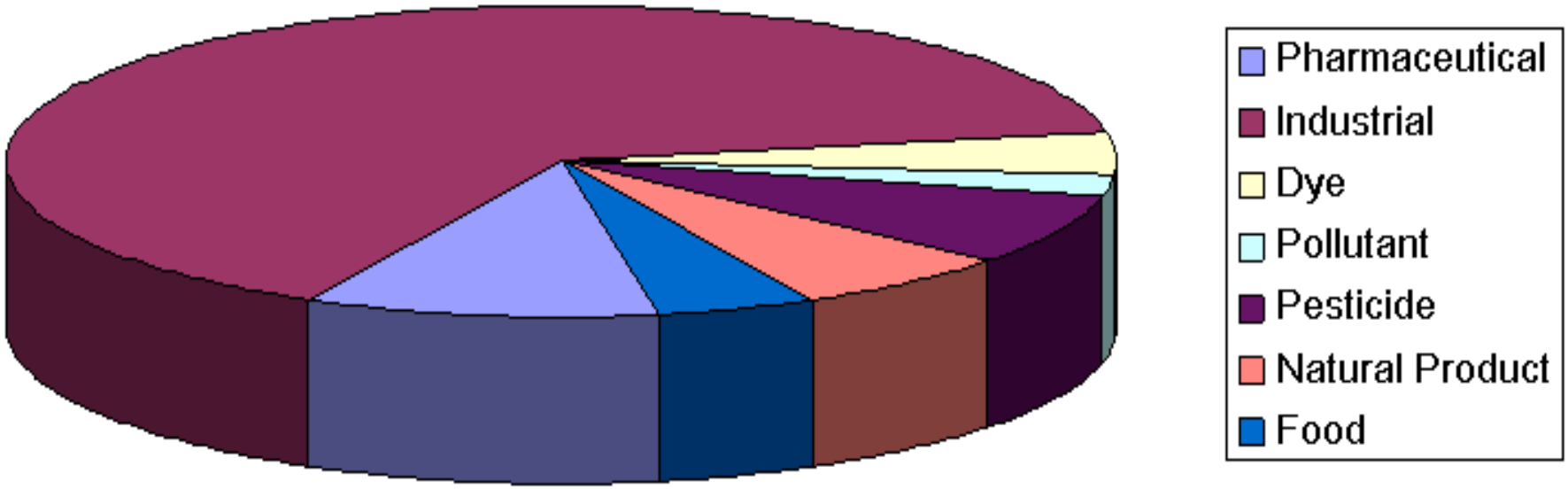
- 1536 compound -to- 1536 assay plate transfer
- Volume range for Pin-based transfers: 10 nL to 0.5 μL
- Transfer time ~ 1-2 min per plate (includes wash cycle)
- No intermediate dilutions of compounds required

The first NTP "1408" compound set

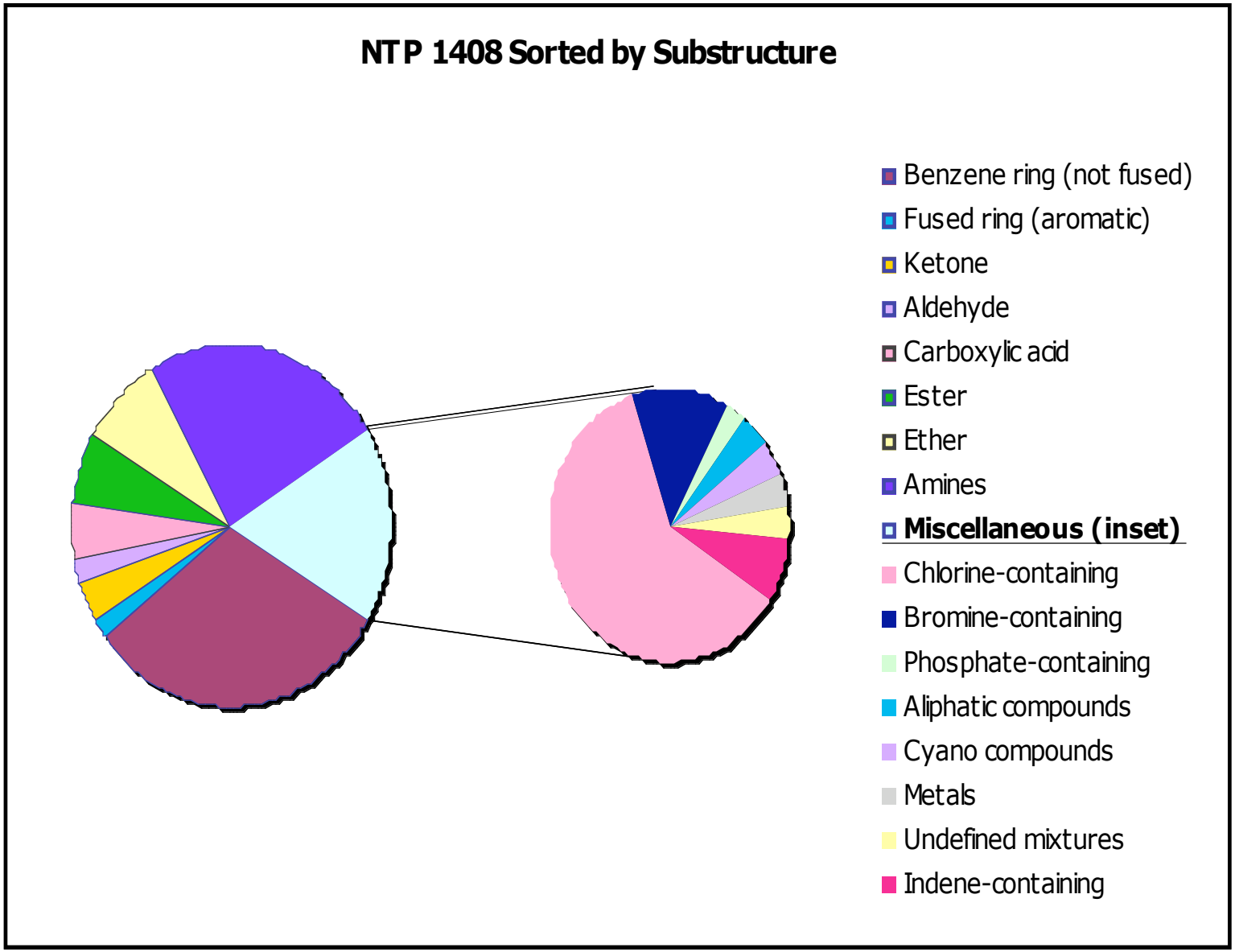
- All have been evaluated in one or more toxicological tests
  - 1353 unique compounds, 55 in duplicate to evaluate assay reproducibility
  - 1206 with NTP test data
  - 147 are ICCVAM reference substances recommended for the validation of alternative *in vitro* test methods (e.g., dermal corrosion, acute toxicity, endocrine activity).
- Selection was based on availability and solubility in DMSO at 10 mM, while avoiding excessive volatility.
- In addition to providing these compounds to the NCGC, we are providing the NTP compound library to the MLSCN repository so that other Centers, exploiting different HTS technologies, can have access to them.



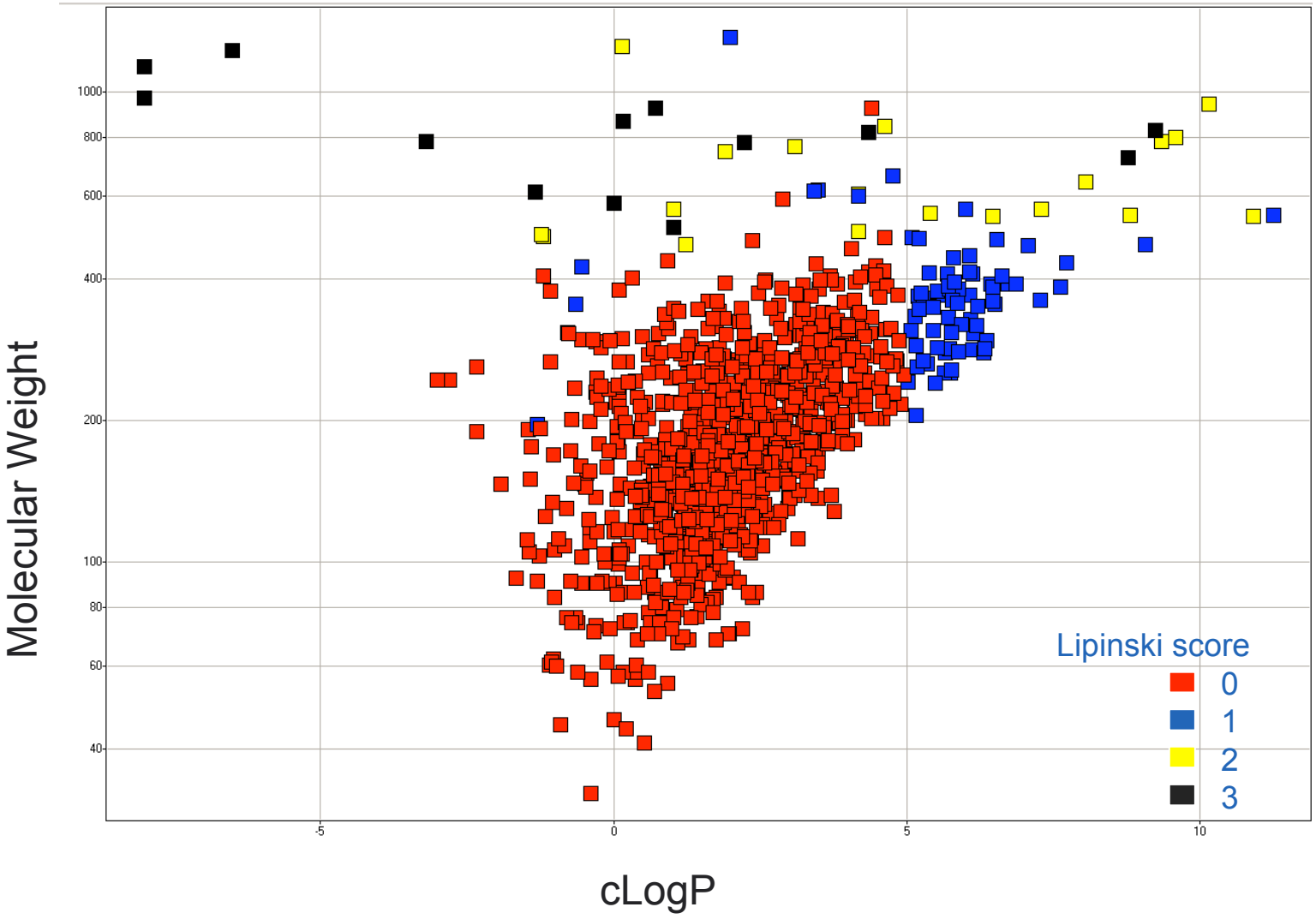
The 1353 Compounds - Product Classes



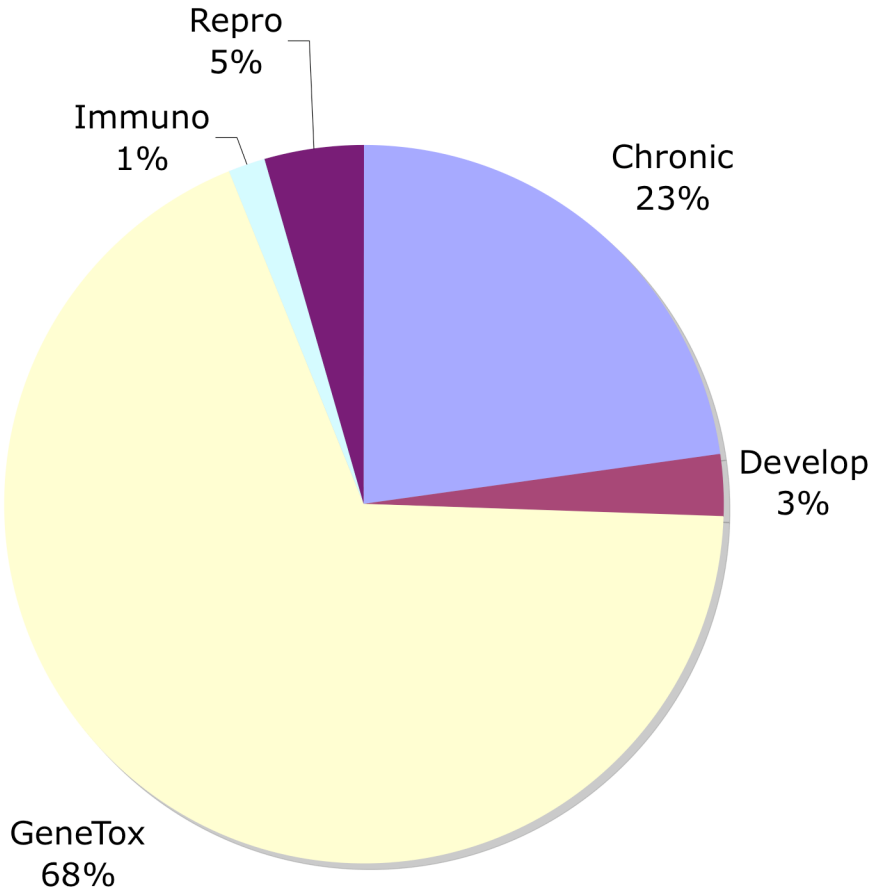
Substructural components of the 1353 compounds



Some characteristics of the 1353 compounds

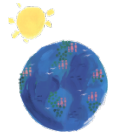


Distribution of the NTP "1206" by Assay



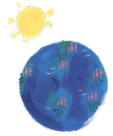
NCGC HTS Criteria

Criteria	Biochemical	Cell-based
Plate Format *	96-well or higher density plate NCGC: 1536-well format Assay volume 2 -6 ul	96-well or higher density plate NCGC: 1536-well format Assay volume 4 -6 ul
Assay Steps	≤10 steps with 96 -well plate. Steps include, reagent additions, timed incubations, plate transfers to incubator, reading, etc.	≤10 steps with 96 -well plate. Steps include, reagent additions, timed incubations, plate transfers to incubator, reading, etc.
Minimum time increments and maximum assay duration	Minimum assay window is 5 min. (i.e., earliest time point after last reagent addition)	< 24 hr is ideal; max 48 hrs. Minimum assay window is 5 min.
Reagent Addition Steps	4 maximum	4 maximum
Reagent removal steps *	No plate coating steps	No aspiration steps
Temperature	Between RT and 37°C	Between RT and 37°C
Demonstrated DMSO Tolerance *	0.5 – 1% DMSO	0.5-1% DMSO
Signal : Background Ratio	≥ 3-fold	≥ 3-fold
Day-to-Day variation of control (e.g., IC_{50}, EC_{50})	< 3-fold	< 3-fold
Reagent stability @ final working concentration	≥ 8 hrs @ RT or on ice bath; No on-line thawing	≥ 8 hrs @ RT or on ice bath; No on-line thawing
Validation run reagent supply	10 – 96-well plate equivalents	10 – 96-well plate equivalents
Protocol	Complete detailed protocol. Data from 96 -well or high density plate tests.	Complete detailed protocol. Detailed cell culture procedure, passage # .Data from 96 -well or high density plate tests.
Detectors	**PE ViewLux** (Top reading only: FI, TRF, FP, Abs, Luminescence) **MD Analyst** (bottom reading FI) **Acumen Explorer** (laser scanning imager)	**PE ViewLux** (Top reading only: FI, TRF, FP, Abs, Luminescence) **MD Analyst** (bottom reading FI) **Acumen Explorer** (laser scanning imager)



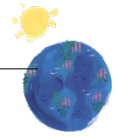
HTS Assays Supplied to the NCGC

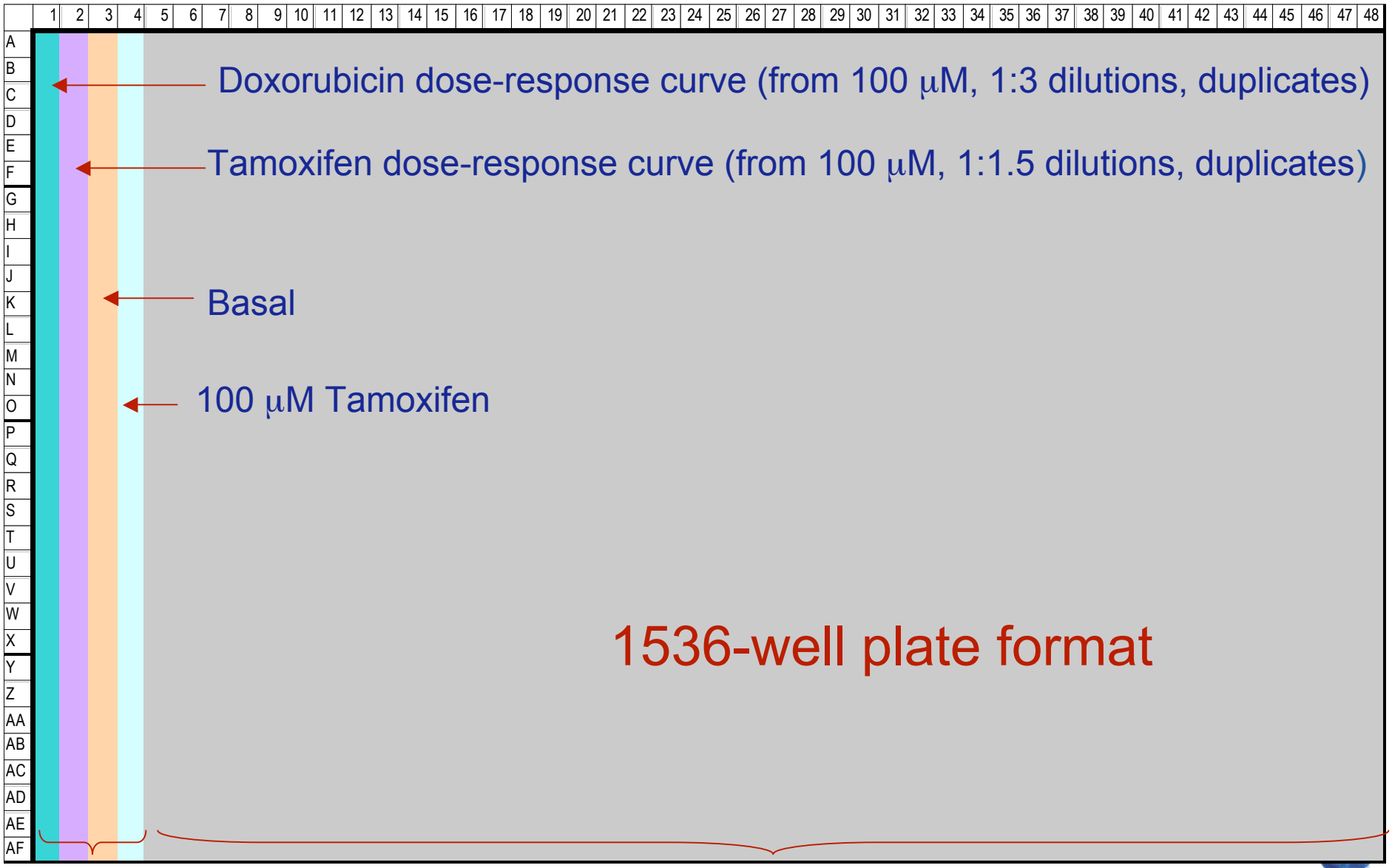
- **Cytotoxicity Assays** (selected because a measure of cytotoxicity is needed in virtually all cell-based HTS assays)
  - CellTiter-Glo® Luminescent Cell Viability Assay (measures ATP levels)
  - Cytotox-ONE™ Homogeneous Membrane Integrity Assay (measures release of lactate dehydrogenase from membrane-damaged cells)
- **Apoptosis Assays** (selected because a common pathway for many types of toxicity and diseases)
  - Caspase-Glo® 3/7 Assay
  - Caspase-Glo® 9 Assay
  - Caspase-Glo® 8 Assay
- **P-glycoprotein (Pgp-Glo™ Assay) ATPase Assay (aka MDR1 or ABCB1)** (involved in drug resistance)



NCGC: Human and Rodent Cell Types

Species	Cell names	Sources	
Human	Hek 293	Embryonic kidney cells	Transformed
Human	HepG2	Hepatocellular carcinoma	Transformed
Human	SH-SY5Y	Neuroblastoma	Transformed
Human	SK-N-SH	Neuroblastoma	Transformed
Human	Jurkat	T cell leukemia	Transformed
Human	BJ	Normal forskin fibroblasts	Non-Transformed
Human	HUV-EC-C	Normal vascular endothelial cells	Non-Transformed
Human	MRC-5	Normal lung fibroblasts	Non-Transformed
Human	Mesangial cell	Normal cells from renal glomeruli	Non-Transformed
Rat	Proximal tubules	Normal cells from kidney	Primary
Rat	H-4-II-E	Hepatoma	Transformed
Mouse	N2a	Neuroblastoma	Transformed
Mouse	NIH 3T3	Fibroblasts from mouse embryo	Non-transformed




1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
A B C D E F G H I J K L M N O P Q R S T U V W X Y Z AA AB AC AD AE AF
Doxorubicin dose-response curve (from 100 μM, 1:3 dilutions, duplicates)
Tamoxifen dose-response curve (from 100 μM, 1:1.5 dilutions, duplicates)
Basal
100 μM Tamoxifen
1536-well plate format
Controls
Test compounds (1408 wells)

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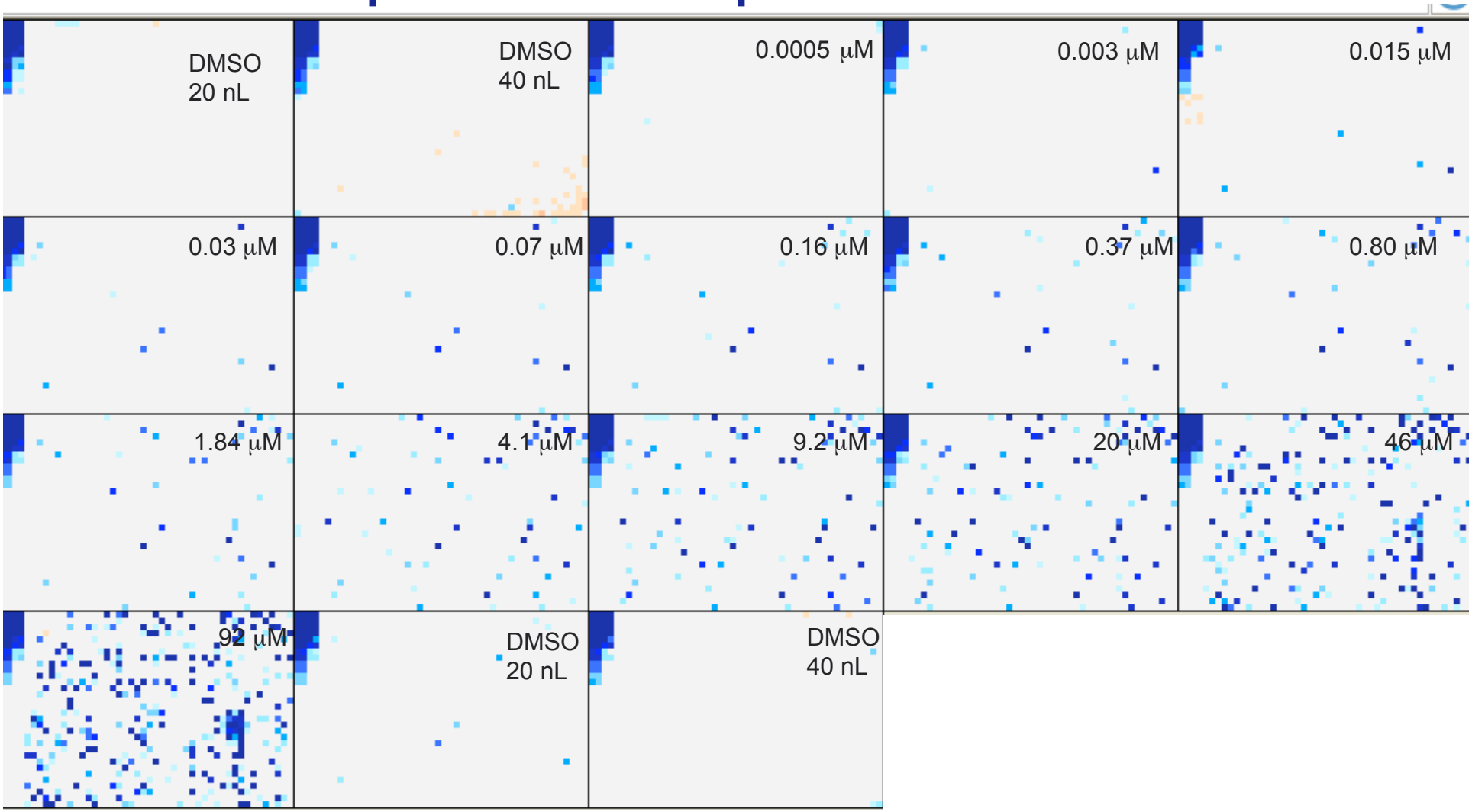
NCGC qHTS results map for Jurkat cell screen



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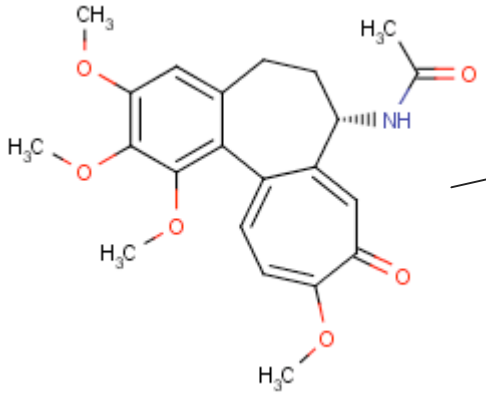
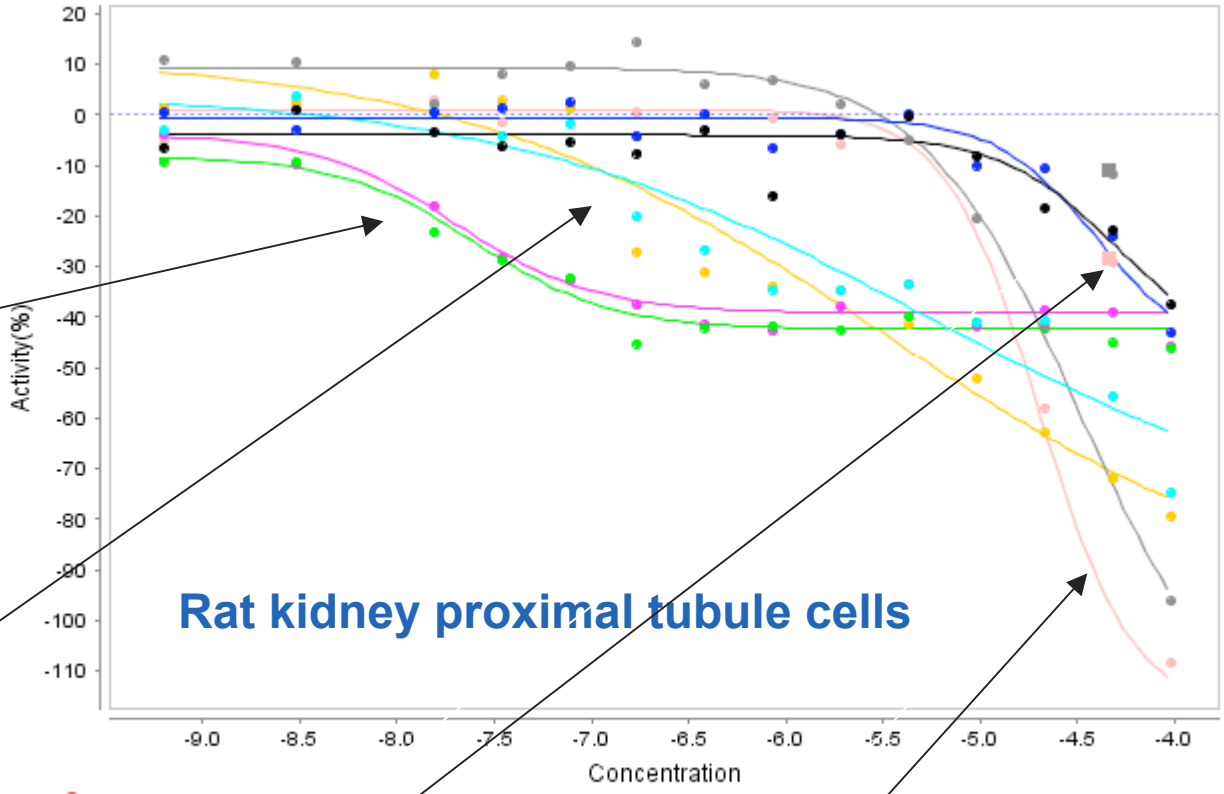
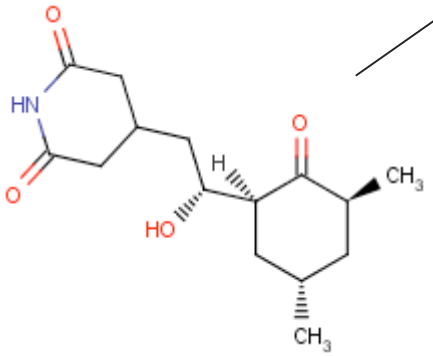
Cytotoxicity Concentration Response Curves of Duplicate Compounds



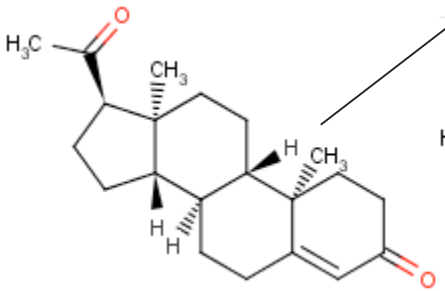
Colchicine



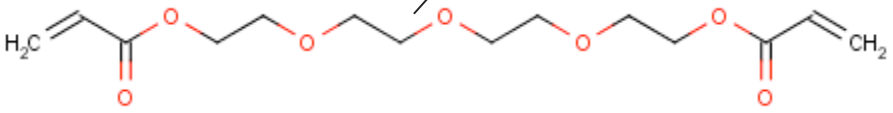
Rat kidney proximal tubule cells



Cycloheximide

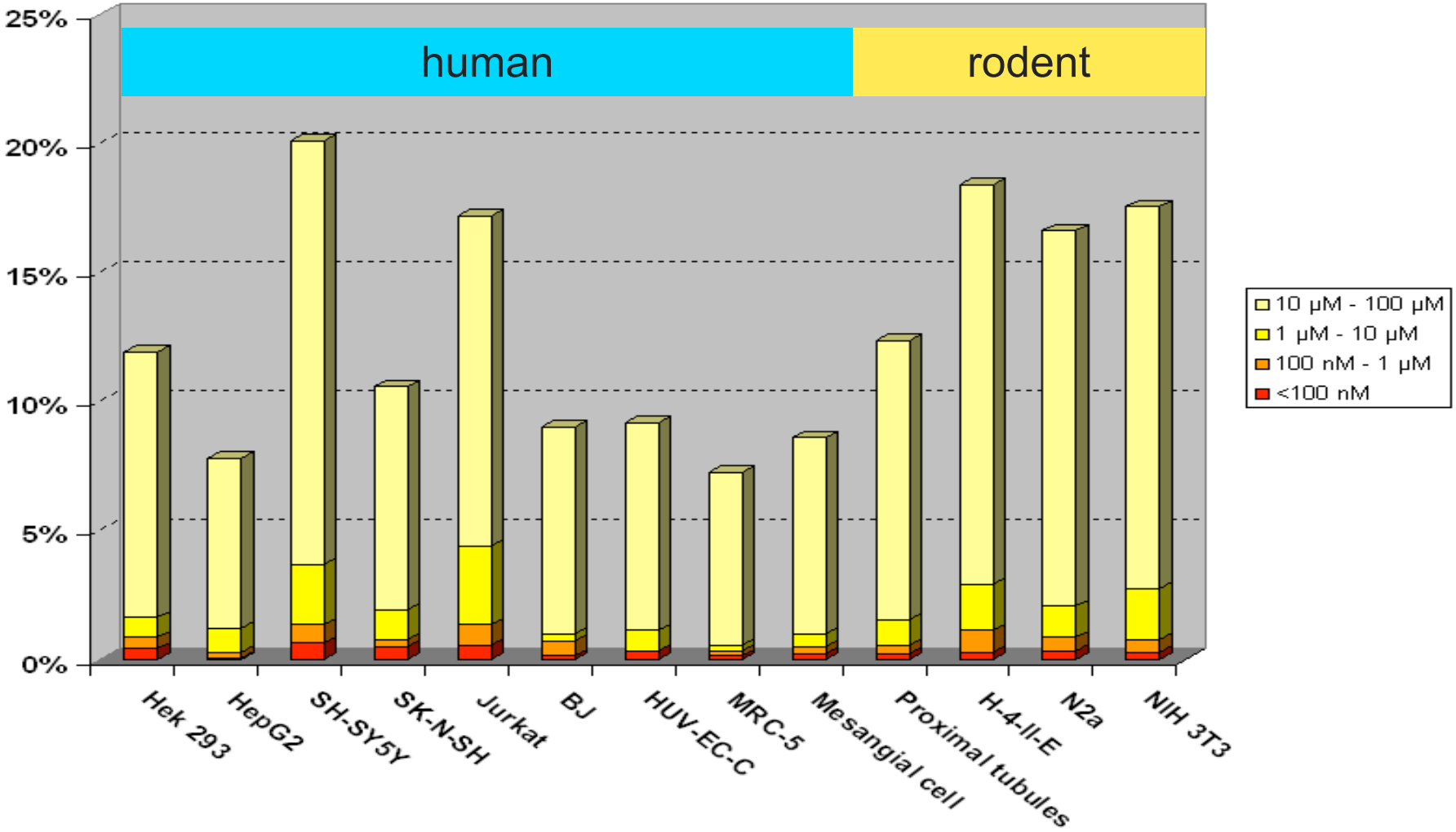


Progesterone



Tetraethylene Glycol Diacrylate

Cytotoxicity potency distribution of the NTP 1408 compounds in 13 cell types



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Structure-Toxicity Relationships Across Assays

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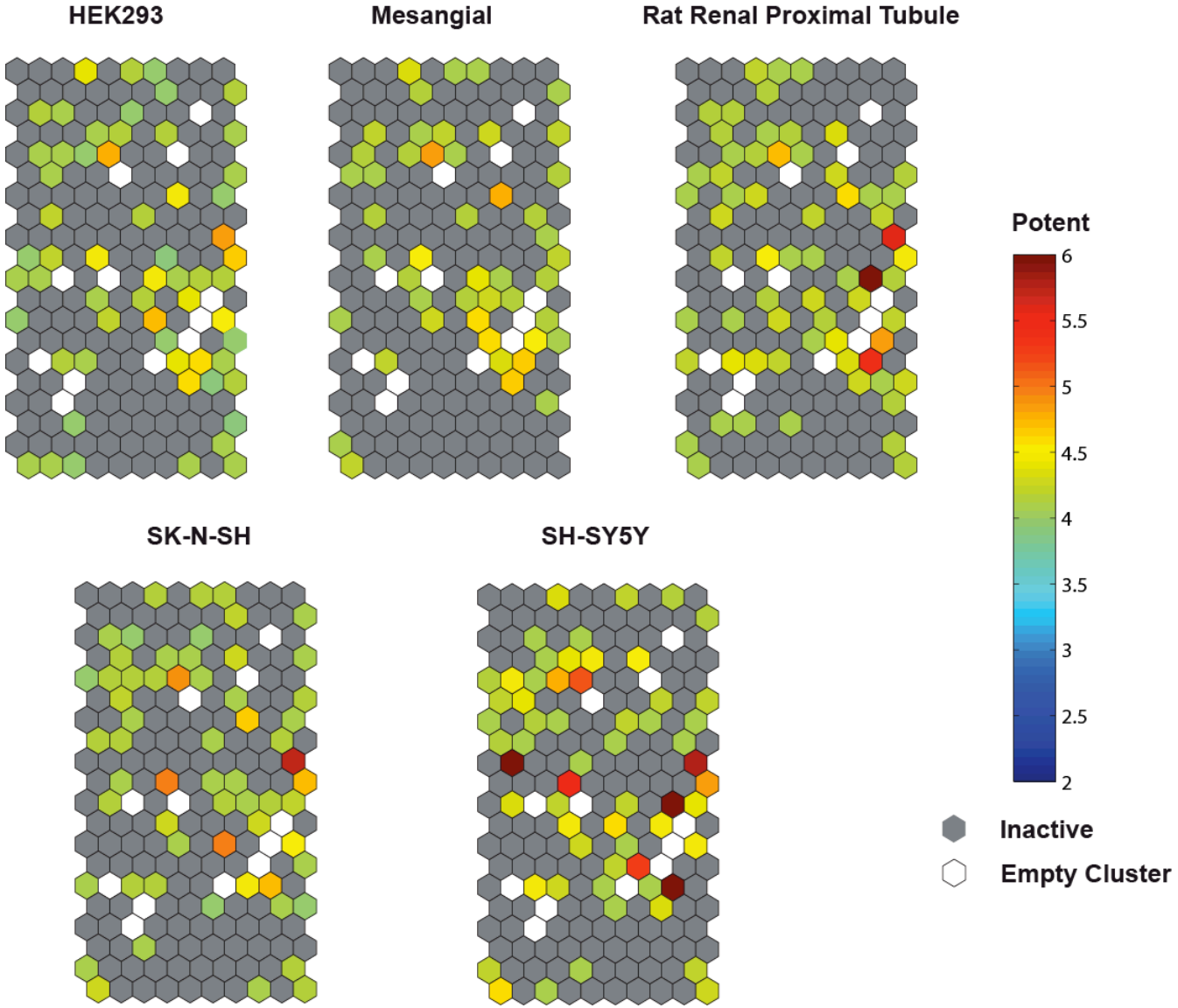
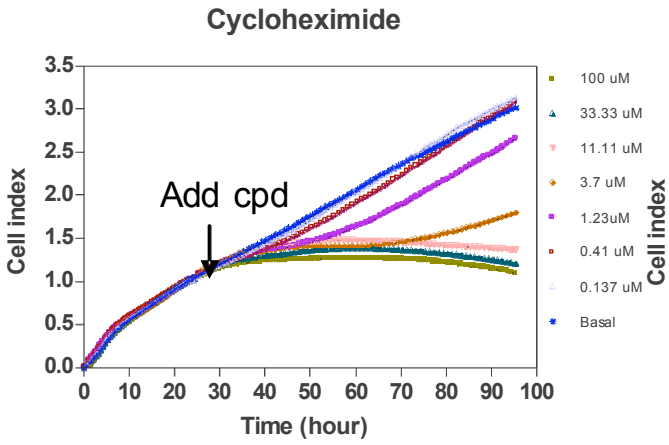
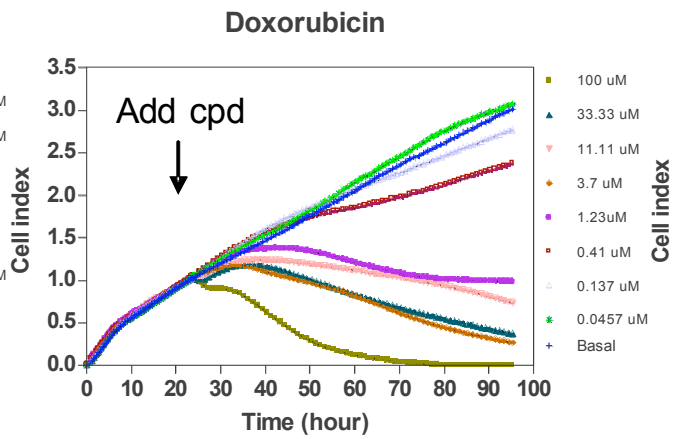


1353 compounds are clustered based on chemical signatures/fingerprints

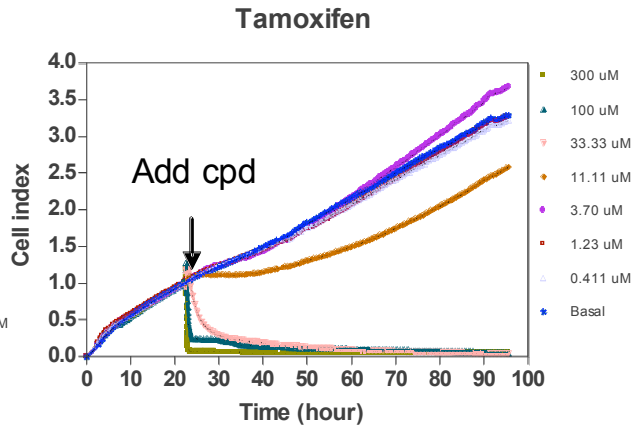
Toxicant compound signatures determined by RT-CES system in HepG2 cells



Protein synthesis inhibition



DNA damage



Apoptosis, Ion channel, Kinases

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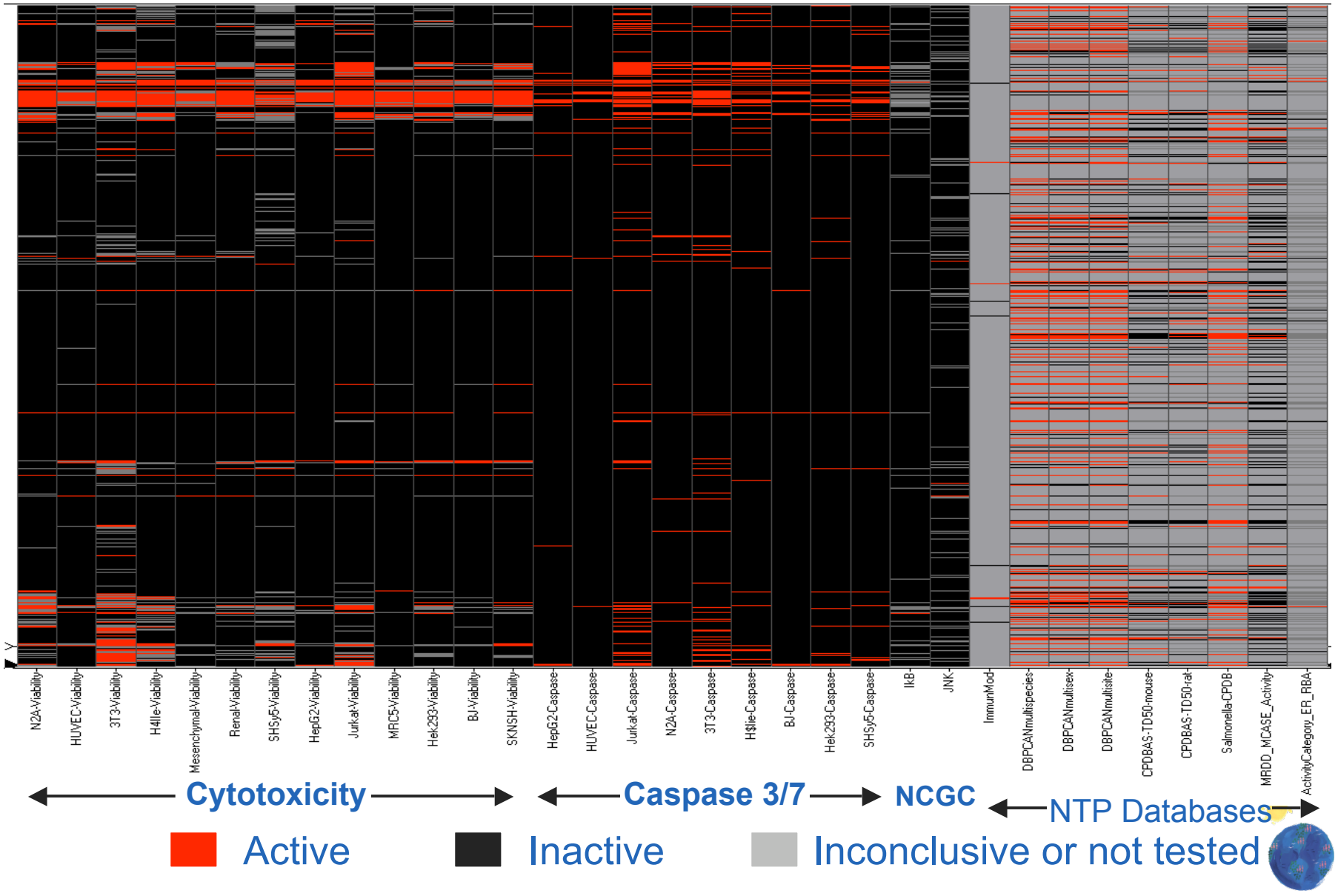
The NTP 1353 unique compounds



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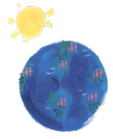
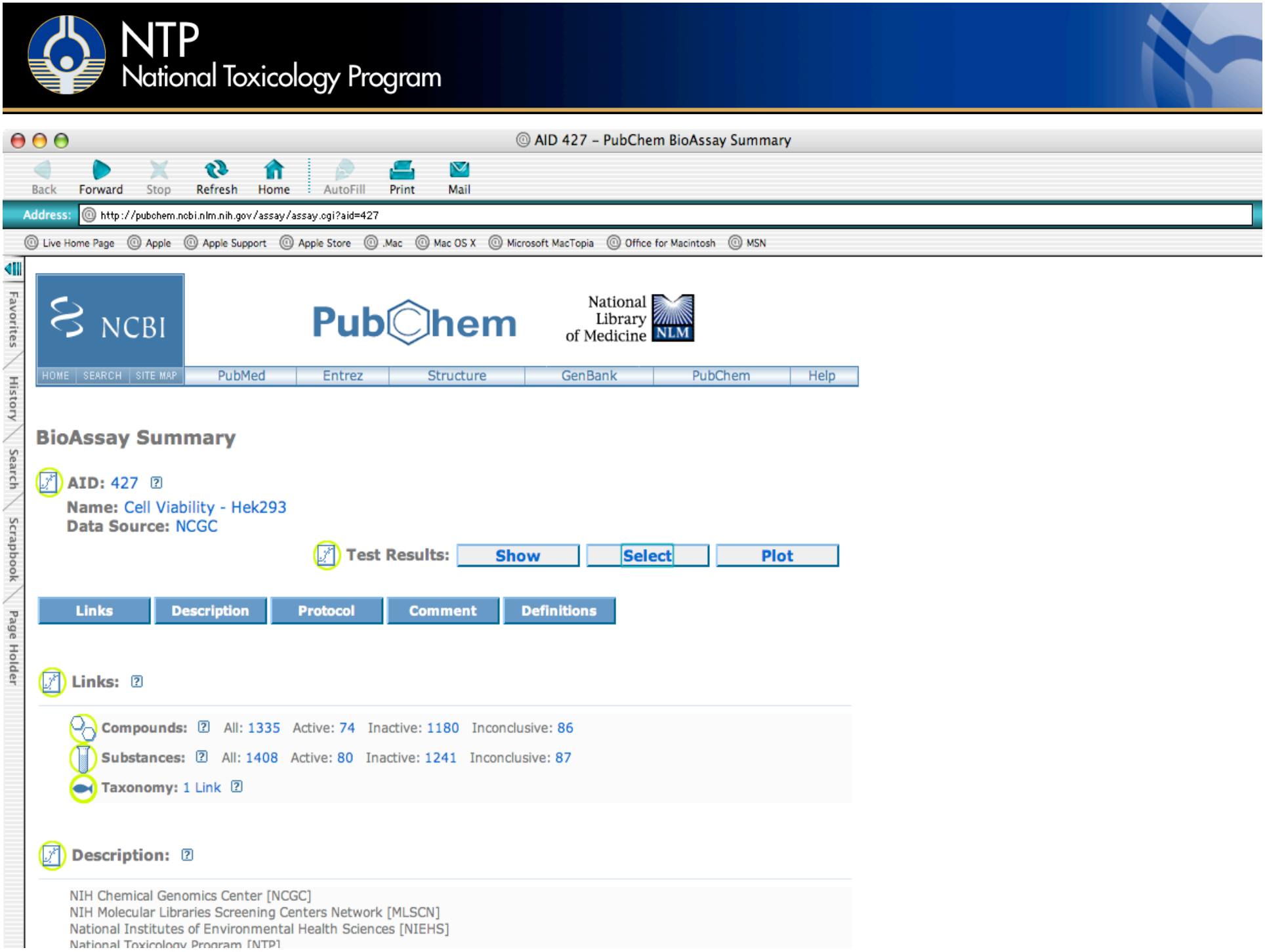
Target (s) / Biology	Assay Category	PubChem AID	# Compounds Screened	# Samples Screened	Data Points Measured
O-Glc NAc Transferase	Isolated Molecular Target	447	70,308	548,402	1,645,207
Schistosoma Peroxiredoxins	Isolated Molecular Target	448	70,308	548,402	21,135,360
Hsp90 co-chaperone interaction	Isolated Molecular Target	595	73,422	572,692	1,434,624
Tau polymerization	Isolated Molecular Target	596	71,982	561,460	811,008
Multi-protein DNA Replication System	Isolated Molecular Target	603	73,892	576,358	1,373,184
Caspase-1	Isolated Molecular Target	pending	80,124	624,967	7,848,960
Caspase-7	Isolated Molecular Target	pending	80,037	624,289	7,818,240
beta-lactamase	Isolated Molecular Target	584	71,982	561,460	12,026,880
BRCT-pSXXF (GREEN)	Isolated Molecular Target	pending	78,845	614,991	2,179,584
BRCT-pSXXF (RED)	Isolated Molecular Target	pending	78,845	614,991	2,179,584
YjeE:ADP binding	Isolated Molecular Target	605	71,977	561,421	1,387,008
Oxidoreductase HADH2	Isolated Molecular Target	pending	85,316	665,465	12,951,552
Oxidoreductase HSD17b4	Isolated Molecular Target	pending	78,845	614,991	3,111,936
15-Lipoxygenase 1 (15hLO1)	Isolated Molecular Target	pending	78,081	609,032	1,428,480
Pyruvate Kinase, Leishmania	Isolated Molecular Target	pending			
Cruzain, Trypanosoma cruzi	Isolated Molecular Target	pending			
12-Lipoxygenase (12hLO)	Isolated Molecular Target	pending			
15-Lipoxygenase 2 (15hLO2)	Isolated Molecular Target	pending			
JNK3 activation	Cellular Pathway, ALPHA	530	11,210	87,438	1,032,192
Potentiators of CRE signaling	Cellular Signaling, Reporter gene	662	93,601	730,088	2,981,376
Cell signaling AP-1 BLA	Cellular Signaling, Reporter gene	357	76,644	597,823	3,337,728
Cell signaling HRE BLA	Cellular Signaling, Reporter gene	pending	81,956	639,257	4,087,296
IkB stabilization	Cellular Signaling, Sensor	445	137,522	1,072,672	6,807,552
Anthrax intoxication pathway	Cellular Signaling, Sensor	pending	40,513	316,001	8,002,560
TSH R	Cellular Signaling, Sensor	pending	84,122	656,152	2,783,232
Neuropeptide S receptors (NPS)	Cellular Signaling, Sensor	pending	10,877	84,841	1,523,712
mRNA Splicing thalassemia	Cellular Signaling, Splicing reporter	pending	71,683	559,127	3,247,104
p53 two temp., synthetic lethal	Cellular viablity	pending	124,570	971,646	4,475,904
Huntington polyglutamine expansion-GFP/ATP	Cellular viablity	pending	56,494	427,094	854,188
ERK Phosphorlyation	Cellular Pathway, ALPHA	pending			
Imprinting	Cellular Signaling, Reporter gene	pending			
Profiling for detergent-sensitive inhibitors	Profiling, Chemical Library	585	71,982	561,460	18,278,400
P450 (CYP 3A4, Luc)	Profiling, Chemical Library	pending	19,727	153,871	405,504
TRF Sample Profiling	Profiling, Chemical Library	pending			

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AID 427 – PubChem BioAssay Summary
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Live Home Page
Apple
Apple Support
Apple Store
.Mac
Mac OS X
Microsoft MacTopia
Office for Macintosh
MSN
Favorites
History
Search
Scrapbook
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Structure
GenBank
PubChem
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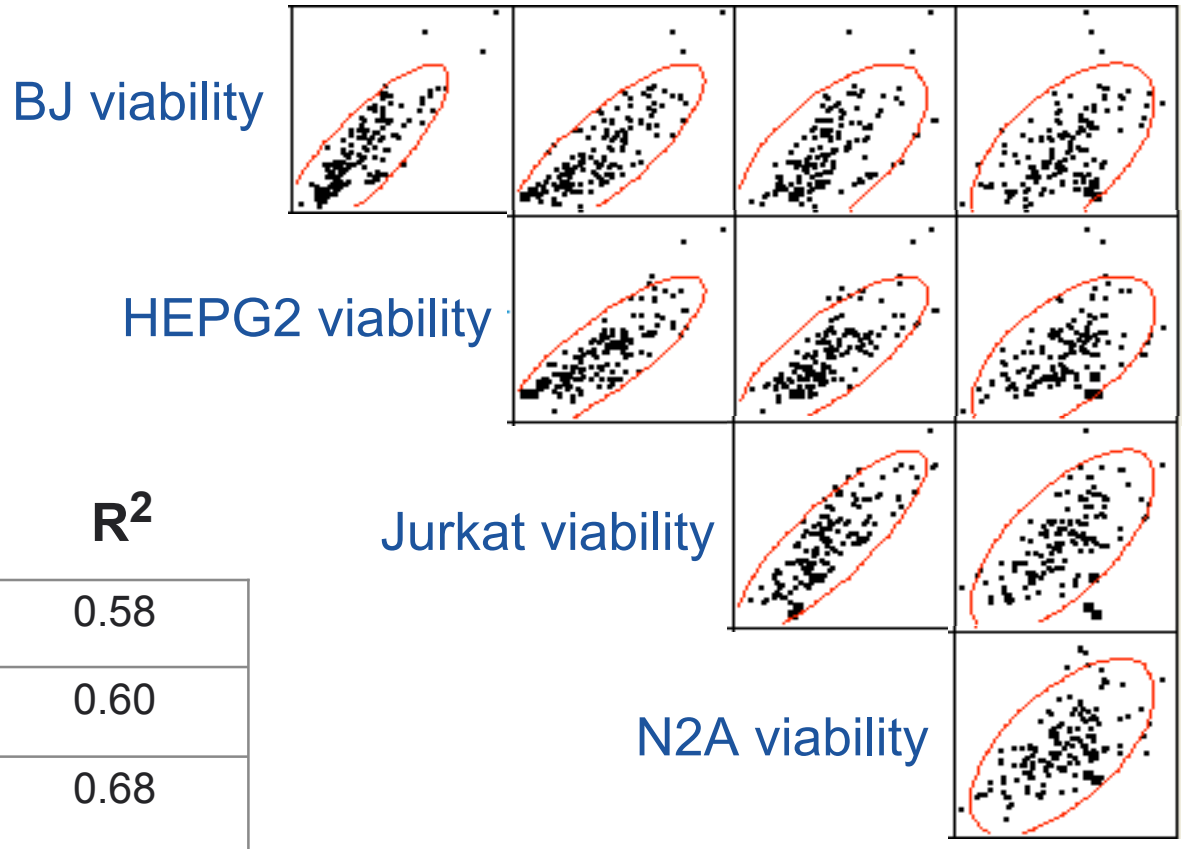
BioAssay Summary
AID: 427
Name: Cell Viability - Hek293
Data Source: NCGC
Test Results:
Show
Select
Plot
Links
Description
Protocol
Comment
Definitions
Links:
Compounds: All: 1335 Active: 74 Inactive: 1180 Inconclusive: 86
Substances: All: 1408 Active: 80 Inactive: 1241 Inconclusive: 87
Taxonomy: 1 Link
Description:
NIH Chemical Genomics Center [NCGC]
NIH Molecular Libraries Screening Centers Network [MLSCN]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]

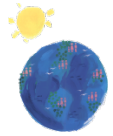
Understanding toxicity from biological and chemical fingerprints (Chihae Yang, Leadscope)



	R^2
BJ	0.58
HEPG2	0.60
Jurkat	0.68
N2A viability	0.63

EPA ToxCast™

- Formulated to address chemical screening and prioritization
- Testing contracts (*ACEA, Attagene, BioSeek, Cellumen, Expression Analysis, IVAL, NovaScreen, Phylonix*) with >400 endpoints explored
- Phased approach - Phase 1 = 320 well studied compounds
- Committed to stake holder involvement and release of data to public domain

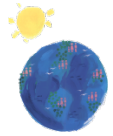


NIEHS/NTP

- **CEBS:** Integrates study design, clinical pathology, and histopathology data with microarray data, and enables discrimination of critical study factors.

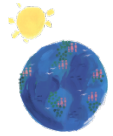
EPA

- **ACToR**: Centralizes many types and sources of data on environmental chemicals derived from more than 150 sources.
- **ToxRefDB:** Compiles *in vivo* toxicology data for ToxCast with current focus on all relevant data from data evaluation records on 280 food-use pesticides from OPPTS.
- **DSSTox:** Curates chemical structure and related assay data with its web site providing a publicly available forum for publishing downloadable chemical structure files.
- **Genomics Data Management:** Relies on Array Track to house genomics data from ORD labs.
- **BDSM:** Reference collection of gene-expression data for modeling animal development.



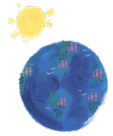
Current Activities (1)

- In the next set of 1408 compounds, focus on:
  - compounds of specific interest for cancer and immunotoxicity
  - structurally-related compounds that have a range of activities
  - compounds that require metabolic activation
  - include duplicates
- Focus on assays that are representative of key steps in pathways important to cancer and immunotoxicity.
- Expand the use of human primary cells (metabolically competent).
- Establish protocols for water-soluble compounds.
- Consider current concentration limits.



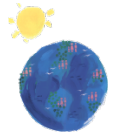
Current Activities (2)

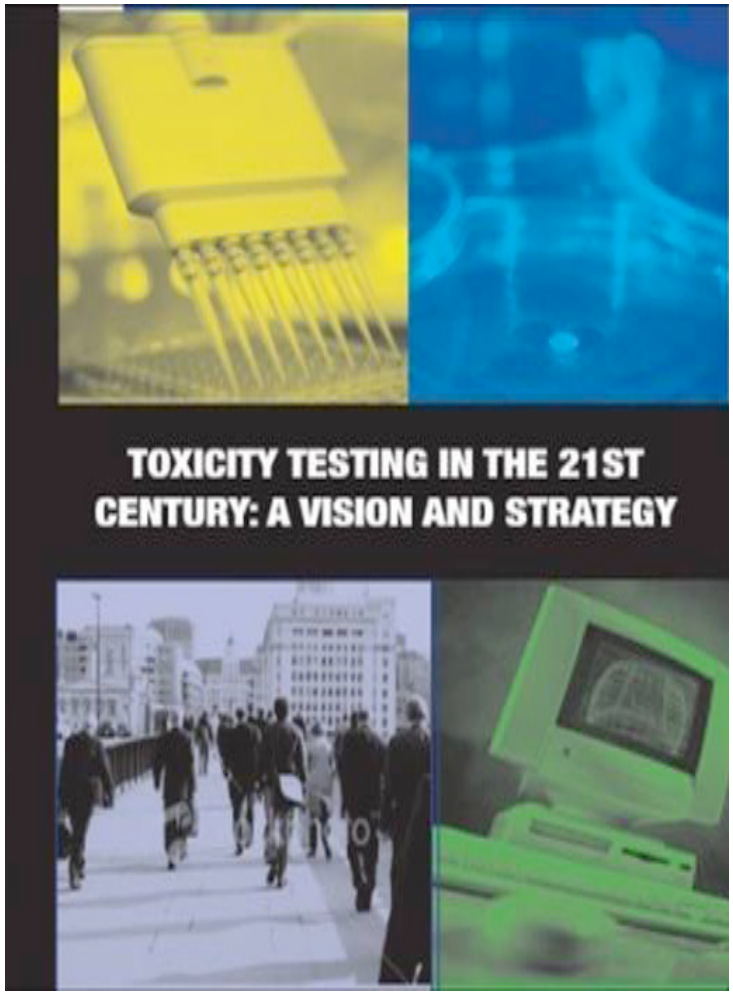
- Evaluate differential responses among cell types.
- Evaluate relationship between HTS and mid-throughput screening assay data (*C. elegans*, zebrafish embryos) and *in vivo* adverse health responses (e.g., acute toxicity, immunotoxicity, cancer).
- Incorporate various measures of chemical space (log p, molecular weight, number of rotatable bonds, number of hydrogen acceptors and donors) into the analysis.
- Evaluate genetic differences in sensitivity using human hapmap cell lines and mouse strain cell lines
- Evaluate assay calls, reliability, and relevance.



Genetics, Genomics, and Bioinformatics (C. Portier, NIEHS)

- Use knowledge about genes associated with disease
- Find the pathways linked to the genes and link them to disease
- Evaluate pathways most likely to be relevant targets
  - "Disease Pathways"
- Use toxicogenomics/proteomics databases on chemicals already studied to link chemicals to diseases through pathways
  - "Toxicity Pathways"
- Analyze the "Toxicity Pathways" to find best points for screening
  - Critical proteins/genes
  - Connection points between pathways
- Use "omics" and other molecular tools to validate choices





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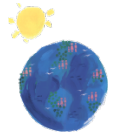
- Assessment of key exposures (life stages) and toxicity outcomes (neurotoxicity)
- State-of-the-science testing and assessment procedures (genomics, bioinformatics, pharmacokinetics)
- Efficient experimental design and reduced use of laboratory animals
- New and alternative test methods
- Computational and molecular techniques in risk assessment

Interagency cooperation

- Memorandum of understanding on “High-Throughput Screening, Toxicity Pathway Profiling and Biological Interpretation of Findings”
  - National Toxicology Program/ National Institute of Environmental Health Sciences
  - NIH Chemical Genomics Center/ National Human Genome Research Institute
  - Office of Research and Development/ US Environmental Protection Agency
- Purpose is to coordinate assays, compounds, data analysis (within and across assays, interpretation, and outreach.



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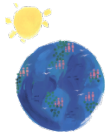
Huang R, Southall N, Cho M, Xia M, Inglese J, Austin CP (2008) Characterization of diversity in toxicity mechanism using in vitro cytotoxicity assays in quantitative high throughput screening format. *Chem. Res. Toxicol.* (In press).



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