The Tox21 Strategy for Detecting Genotoxicants

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ILSI-HESI Workshop
Genetic Toxicology:
Opportunities to Integrate New Approaches

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And many more
Formation of the U.S. Tox21 Community

• Original 5-year Memorandum of Understanding (MoU) on “High-Throughput Screening, Toxicity Pathway Profiling and Biological Interpretation of Findings” released on Feb 14, 2008 signed by NHGRI (F.S. Collins), NIEHS/NTP (S.H. Wilson), and EPA (G.M. Gray).


• A “community resource” project
Tox21 Goals

• Identify patterns of compound-induced biological response in order to:
  — characterize toxicity/disease pathways
  — facilitate cross-species extrapolation
  — model low-dose extrapolation

• Prioritize compounds for more extensive toxicological evaluation

• Develop predictive models for biological response in humans
Tox21 Phase I – Proof of Principle

• NCGC screened 1408 compounds (1353 unique) from NTP and 1462 compounds (1384 unique) from EPA in >100 qHTS at 14 conc (5 nM to 92 µM typical).

• EPA via ToxCast™ screened 320 compounds (309 unique, primarily pesticide actives and some endocrine active compounds) in ~550 assays.

• Data released to the scientific community via:
  — EPA ACToR (Aggregated Computational Toxicology Resource; [http://epa.gov/actor](http://epa.gov/actor))
Phenotypic readouts
- Cytotoxicity
- Apoptosis: caspase 3/7, 8, 9
- Membrane integrity: LDH, protease release
- Mitochondrial toxicity

Genetox
- ATAD5
- p53
- DT40 DNA repair deficient cell lines

Cell Signaling
- Stress response: ARE, ESRE, HSP, HIF, AP-1
- Immune response: IL-8, TNFα, TTP
- Other: AP-1, CRE, ERK, HRE, JNK3, NFkB, LDR

Epigenetics: Locus DeRepression (LDR)

Drug metabolism
- CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4

Target specific assays
- Nuclear receptors: AR, AhR, ERα, FXR, GR, LXR, PPARα, PPARδ, PPARγ, PXR, RXR, TRβ, VDR, RORα, RORγ
- hERG channel
- Isolated molecular targets: 12hLO, 15hLO1, 15hLO2, ALDH1A1, HADH560, HPGD, HSD17b4, APE1, TDP1, DNA polymerase III, RECQ1 helicase, RGS4, BRCA, IMPase, O-Glc NAc Transferase, Caspase-1/7, CBFβ-RUNX1, PK, Tau, Cruzain, β-Lactamase, PRX, YjeE, NPS, Proteasome, SF1, SMN2, beta-globin splicing, Anthrax Lethal Factor, TSHR

Genetic variation: 87 HapMap CEPH Panel
Quantitative High Throughput Screening (qHTS)

• 1536-well plate format
• 14-point concentration-response curve
• DMSO soluble
• 5 nM to 92 µM typical
• ~5 µL assay volume
• ~1000 cells/well
• Assay must be homogeneous
• Cell assay duration = 1 to 48 hrs
Phase I qHTS Compound Activity Profile

Compounds screened: NTP-1408 and EPA-1462
Fox et al., High-throughput genotoxicity assay identifies antioxidants as inducers of DNA damage response and cell death. www.pnas.org/cgi/doi/10.1073/pnas.1114278109.

<table>
<thead>
<tr>
<th>Effect on Luc-ATAD5</th>
<th>Number of Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase</td>
<td>719</td>
</tr>
<tr>
<td>Decrease</td>
<td>482</td>
</tr>
<tr>
<td>None</td>
<td>2955</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4156</strong></td>
</tr>
</tbody>
</table>

99 Compounds with activity > 40% MMS
Polyphenols & Antioxidants
(22/99 Positive Hits)

**Polyphenols**
- 1-Amino-2-methylantraquinone (NTP)
- 2-Aminoantraquinone (NTP)

**Flavone (NTP)**
- Biacalein (TB)
- Primuletin (TB)
- 6-methylflavone (TB)
- 5-Methoxyflavone (TB)
- Sophoricoside (TB)
- Resveratrol (TB, NTP)
- Genistein (TB, NTP)
- Ipriflavone (TB)
- Daidzein (NTP, TB)
- Formononetin (TB)
- Piceatannol (TB)
- Tectochrysin (TB)
- Biochanin A (TB)
- Acacetin (TB)

**Antioxidants**
- Yagonin (NTP)
- Alizarin Yellow R (NTP)
- Tranilast (TB)
- N-Phenyl-2-naphthylamine (NTP)
- Norbixin (NTP)

1) act as pro-oxidants
2) cleave DNA
3) intercalate into DNA
4) inhibit topoisomerase
5) inhibit DNA polymerase
6) inhibit ribonucleotide reductase

Because of effective gene targeting, isogenic mutant clones of all known DNA damage response pathways have been developed.

The phenotype and karyotype of these cells are very stable.

The cells are maintained in suspension culture and are easy to culture (cell cycle ~ 8 hrs).
## List of genes deleted in DT40 cells and their functions

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Names</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>653</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>915</td>
<td>pol β (-/-) clone#1</td>
<td>DNA polymerase β repairs base damage and single-strand break</td>
</tr>
<tr>
<td>916</td>
<td>pol β (-/-) clone#2</td>
<td></td>
</tr>
<tr>
<td>1384</td>
<td>FancC(-/-)</td>
<td>FANCC is required for eliminating inter-strand crosslinks</td>
</tr>
<tr>
<td>100</td>
<td>ku70/rad54 (-/-)</td>
<td>Ku70 is required for non-homologous end-joining, Rad54 are involved in homologous recombination repair when double strand breaks occur.</td>
</tr>
<tr>
<td>657</td>
<td>rev3 (-/-)</td>
<td>Rev3(pol zeta) work as a TLS polymerase, might be involved in homologous recombination ,and also proposed to work epistatic with FancC in their cross-linking tolerance.</td>
</tr>
<tr>
<td>1782</td>
<td>ubc13 (-/-)</td>
<td>UBC13 have multiple functions in cellular tolerance to a variety of DNA damage.</td>
</tr>
<tr>
<td>245</td>
<td>ATM(-/-)</td>
<td>ATM arrests cell cycle when chromosomal breaks are present, makes cells tolerant to reactive O2 species</td>
</tr>
</tbody>
</table>
AC50 Profiles

Activation

Inhibition

≤10 nM
1 μM
100 μM
Inactive
100 μM
1 μM
≤10 nM

chicken-1384
chicken-653
chicken-245
chicken-1782
chicken-916
chicken-100
chicken-657
chicken-915

AC50 Profiles
actives=ACTIVE*[-1] or INCONCLUSIVE*[-3]

% OF TOTAL HITS (468)

CHICKEN CLONE

915p2  657p2  245p1  100p1  1782p2  1384p1
Tox21 Phase II

• EPA’s ToxCast™ Phase II: ~1000 compounds in ~600 assays.

• NCGC qHTS Phase II: >10K compounds 3x at 14 conc for:
  – nuclear receptor activation or inhibition (hAR, hAhR, hERα, hFXR, hGR, hLXR, hPPARα, hPPARδ, rPXR, hROR, hRXR, hTR, hVDR)
  – induction of stress response pathways (e.g., ATAD5, p53, DT40 repair deficient cell lines, Nrf2/ARE, HIF1α, HSPA6, IL-6, IL-8, TNFα, hERG, ESRE, Wnt signaling, Hedgehog signaling)

• Assay selection based on
  – Information from in vivo toxicological investigations
  – Phase I experience, advice of basic researchers, and nominated assays
  – Maps of disease-associated cellular pathways

• Future focus on disease-associated pathways (e.g., obesity/diabetes, autism) using stem cells/differentiated cells and high throughput gene array assays
Tox21 Compound Library

Library tested 3x in each assay

<table>
<thead>
<tr>
<th>Unique</th>
<th>EPA</th>
<th>NTP</th>
<th>NCGC</th>
<th>Total</th>
<th>Total Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIDs</td>
<td>3726</td>
<td>3076</td>
<td>3526</td>
<td>10328</td>
<td>8193</td>
</tr>
<tr>
<td>Tox21 IDs</td>
<td>3729</td>
<td>3210</td>
<td>3733</td>
<td>10672</td>
<td>10496</td>
</tr>
<tr>
<td>wells</td>
<td>4224</td>
<td>3726</td>
<td>3826</td>
<td>11776</td>
<td>11776</td>
</tr>
</tbody>
</table>

- unique substances in entire inventory (no dupes)
- unique solution IDs in entire inventory (remove 2 of 3 sets of 88 dupes)
- total number of test cmpd wells

2135 replicate substances (GSIDs) across 3 inventories
Tox21 Phase II qHTS 10K Library

- Drugs
- Drug-like compounds
- Active pharmaceutical ingredients
- FDA Drug Induced Liver Injury Project
- Failed Drugs
- ToxCast I and II compounds
- Antimicrobial Registration Program
- Endocrine Disruptor Screening Program
- OECD Molecular Screening Working Group List
- NTP-studied compounds
- NTP nominations and related compounds
- NICEATM/ICCVAM validation reference compounds for regulatory tests
- External collaborators (e.g., Silent Spring Institute, U.S. Army Public Health Command)
- Formulated mixtures
Preliminary Overlapping Calls between ATAD5 and p53

- Dichlone
- Retinal
- 1-Nitropyrene
- Daunomycin
- Melphalan
- 611
- 105
- 122
Follow up Primary Screening with High Content Screening

Cellular and Organelle Health

DNA Damage
- pATM
- p53
- MDM2
- pChk2
- Ku70/80
- pH2AX
- Micronucleus
- Rad51

Mitochondrial Membrane Potential Depolarization
- Cytotoxicity
- Apoptosis
- Mitox
- Oxidative Stress
- MnSOD
- JC10

Cytochrome C Release & Oxidation
- Caspase 9
- Caspase 3
- Cleaved PARP
- HSPs
- Cell Death
- Heme Oxygenase

NR Signaling
- ERalpha/beta Redistribution
- AR Redistribution
- Protein Expression
- Cell Signaling

From B. Goodwin (NCGC)
The Tox21 Genomes Project (with I. Rusyn, UNC)

- Assessment of variation within and between populations
- Mapping of genomic regions associated with variation of responses to individual chemicals or classes
- In a cell-based system, with carefully controlled growth and environmental conditions, the assay may serve as an endo-phenotype, with a greater proportion of variation explained by genomic variation than for a typical complex trait

- **Phase I** – 87 CEPH panel x 240 cmpds x 12 conc x 2 assays (cytotoxicity & caspase 3/7)*
- **Phase II** – 1090 lines (9 racial groups) x 180 cmpds x 8 conc x 1 assay (cytotoxicity)

*Lock et al., Tox Sci 126, 578 (2012)
“Individual” response vs “population” response:
Concentration responses (EC\textsubscript{10}) modeled for individuals (grey lines and histogram) and population (red line) (from F. Wright/I. Rusyn – UNC)

Range of inter-individual variability (5\textsuperscript{th}-95\textsuperscript{th} %ile of EC\textsubscript{10}) for all 179 compounds tested

Inter-individual range in EC\textsubscript{10} (5\%-95\%): ~10-fold

Inter-individual range in EC\textsubscript{10} (5\%-95\%): ~3-fold

Inter-individual range in EC\textsubscript{10} (5\%-95\%): ~100-fold

the 10-fold default
The Collaborative Cross
(8-way advanced recombinant inbred lines)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Letter</th>
<th>Color</th>
</tr>
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<tbody>
<tr>
<td>A/J</td>
<td>A</td>
<td><img src="#" alt="Yellow" /></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>B</td>
<td><img src="#" alt="Gray" /></td>
</tr>
<tr>
<td>129S1/SvImJ</td>
<td>C</td>
<td><img src="#" alt="Pink" /></td>
</tr>
<tr>
<td>NOD/ShiLtJ</td>
<td>D</td>
<td><img src="#" alt="Blue" /></td>
</tr>
<tr>
<td>NZO/LtJ</td>
<td>E</td>
<td><img src="#" alt="Green" /></td>
</tr>
<tr>
<td>CAST/EiJ</td>
<td>F</td>
<td><img src="#" alt="Green" /></td>
</tr>
<tr>
<td>PWK/PhJ</td>
<td>G</td>
<td><img src="#" alt="Red" /></td>
</tr>
<tr>
<td>WSB/EiJ</td>
<td>H</td>
<td><img src="#" alt="Purple" /></td>
</tr>
</tbody>
</table>

From J. French (NIEHS/NTP)
Collaborative Cross (CC) & Diversity Outbred (J:DO) Models

≈ 45 million segregating SNPs

≥10% minor allele frequency

From J. French (NIEHS/NTP)
28-Dy Inhaled Benzene: Each mouse is genetically different…

Peripheral Blood %MN-RET

From J. French (NIEHS/NTP)
Benzene, 100 ppm – Bone Marrow %MN-RET

From J. French (NIEHS/NTP)
Other Assays/Approaches Being Considered

• HepaRG cells
• Targeted HTS Gene Arrays (100-1000 genes)
  - L1000, qNPA, bDNA, RT-PCR
• ES/iPS cells (human and mouse)
• Differentiated ES/iPS cells (cardiomyocytes, neural,…)
• 3D culture systems (liver, skin, lung)
The NTP DrugMatrix Rat Toxicogenomics Database

- Integrated Collection of Data
  - 637 unique chemicals (mostly drugs)
  - 5600 drug-treatment transcript profiles in rat organs
  - 127,000 histopathology measurements
  - 100,000 blood chemistry measurements
  - 60,000 literature facts

- Over 500 validated signatures
  - Mode of action and pathology

- Comprehensive data mining
  - Formulate 100,000’s questions (phenotypes)
  - Test for ability to classify using transcript data only

- ~122,000 frozen tissues

- Automated genomics analysis

- Drugmatrix website: https://ntp.niehs.nih.gov/drugmatrix
- ToxFx website: https://ntp.niehs.nih.gov/toxfx/
Mining the NTP Archives for Disease Gene Signatures

- >2,000 studies
- >7.5 million histology slides
- >4.6 million paraffin blocks
- >230,000 bags formalin tissue
- >54,000 frozen specimens
- Supporting histopathology images/data
Critical In Vitro Issues

- Cells don’t get disease
- Not all compounds can be screened in vitro
- Essential need for xenobiotic metabolism
- Need to consider interactions between different cell types
- Need to extrapolate from acute to chronic exposure conditions
- How to measure human variability in sensitivity
- Need to be extrapolate from *in vitro* concentration to *in vivo* dose
- Need to identify human disease-associated pathways and useful assays for those pathways
- Need to integrate multiple data sources (e.g., *in vitro*, animal, human) and endpoints (e.g., HTS, ‘omics, disease) into publicly accessible databases with appropriate tools for mining