The National Toxicology Program (NTP) High Throughput Screening (HTS) Initiative: Current Status and Future Directions

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Introduction

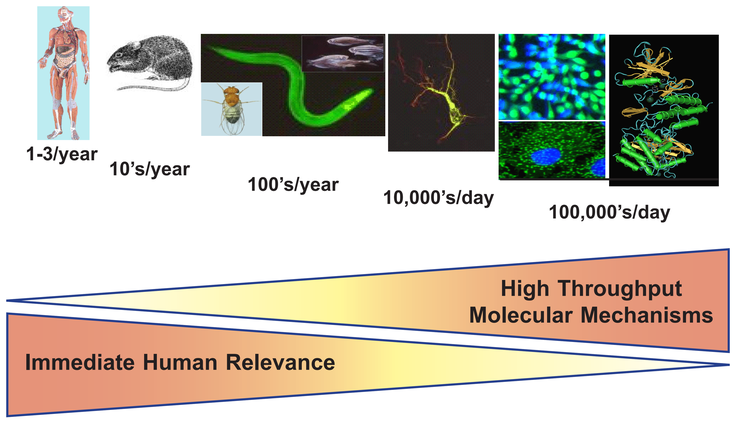
To meet the challenge of 21st century toxicology (**Figure 1**), the National Toxicology Program (NTP) Roadmap includes a major initiative to develop a high throughput screening (HTS) program with three main goals:

- Prioritize the very large number of untested or inadequately tested chemicals for further in-depth toxicological evaluation
- Identify mechanisms of action
- Develop predictive models for *in vivo* biological response, with the ultimate focus being able to predict human toxicity

Since mid-2005, the NTP has participated in the NIH Molecular Libraries Initiative (MLI) by collaborating with the NIH Chemical Genomics Center (NCGC) to use quantitative HTS (qHTS) assays to test compounds for activity against defined targets. qHTS, in contrast to traditional HTS employed by the pharmaceutical industry to identify potential new drug candidates, generates high confidence data over a broad dose range, providing a comprehensive activity profile for a compound (Inglese et al, 2006). The NTP-NCGC collaboration benefits both programs by adding toxicity testing capabilities to the MLI and by allowing rapid implementation of NTP's HTS program designed to screen large numbers of compounds for activity against targets and pathways believed to have toxicological relevance (e.g., oxidative stress, inflammation, apoptosis). The NTP plans to link HTS-produced toxicity data to data from traditional *in vivo* toxicological assays, with the goal of identifying mechanisms of action requiring additional investigation, developing predictive models for biological response, and prioritizing substances for further evaluation. In addition to the collaboration with NCGC, a medium throughput screen using *Caenorhabditis elegans* has been established to provide *in vivo* data on the same compounds screened at the NCGC (see **Poster 1184, Board 506**). Another aspect to this collaborative effort is a joint NTP/U.S. Environmental Protection Agency "faculty" that shares ideas and strategies and provides a broader venue for scientific debate on the utility of HTS in toxicity profiling.

Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. Proc Natl Acad Sci U S A. 2006 Aug 1;103(31):11473-8.

Figure 1. What Can Be Screened?



The Initial NTP Compound Library

To be compatible with the quantitative HTS testing paradigm used by the NCGC (**Figure 2**), the first set of compounds provided by the NTP for testing consisted of 1353 unique compounds and 55 duplicates (i.e., 1408 compounds total); the duplicates were included to assess assay reproducibility. Each compound had either been tested in one or more traditional NTP toxicity assays (e.g., for cancer, reproductive toxicity, immunotoxicity, bacterial mutagenicity) or had been selected by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a reference substance to be used in the development and/or validation of alternative test methods for acute oral toxicity, endocrine disruption, or ocular or dermal toxicity (see **Poster 1187, Board 506**). In addition to providing these compounds to the NCGC, we are in the process of preparing this initial NTP compound library for shipment to the Molecular Libraries Screening Centers Network compound repository so that other screening centers within the Molecular Libraries Screening Centers Network, exploiting different HTS technologies, can have access to them.

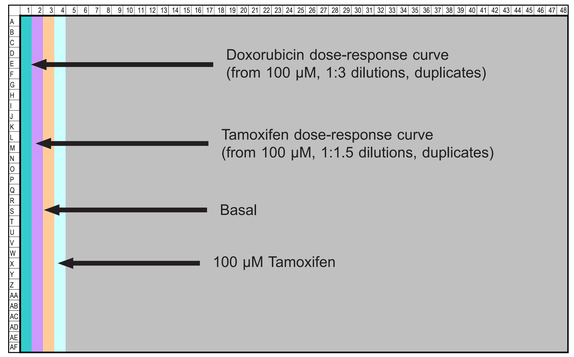
NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services
National Institutes of Health

Acknowledgements

The authors gratefully acknowledge the contributions of Dr. F. Parham for concentration response analysis and Dr. D. Germolec and Ms. R. Patterson, as well as the individual scientists (Drs. E. Corsini, R. Gallucci, V. Johnson, G. Ladics, B. Paige Lawrence, R. Pieters, S. Pruett, D. Sherr, H. van Loveren, and M. Woolhiser) they consulted, for an initial identification of immune dysfunction-related pathways. Similarly, the authors gratefully acknowledge the advice of Drs. R. Tennant, T. Archer, J. Dunnick, R. Paules, and E. Lobenhofer for a preliminary identification of cancer-related pathways. Special thanks go to our collaborators at the NCGC (Dr. C. Austin, J. Inglese, M. Xia, N. Southall; M. Cho; A. Jadhav; and R. Huang) for all they have contributed to this effort. J. Fostel is supported by the LMIT ITSS support contract at NIEHS. This work was supported in part by the Intramural Research Program of the NIH and NIEHS.

Figure 2. 1536-well Format Used At The NCGC



The Initial Set of NTP HTS Assays

Along with the first set of 1408 compounds provided to the NCGC, the NTP provided six commercially available, homogeneous cell-based assays to evaluate the ability to scale such assays for use in HTS testing protocols using a 1536-microwell format. Selection of the cell-based assays was based on criteria developed by the NCGC for identifying *in vitro* assays suitable for use in their robotics 1536-well format testing system (**Table 1**). The assays provided consisted of:

- Two 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)
- Three apoptosis assays (selected because apoptosis is a common pathway for many types of toxicity and diseases)
  - Caspase-Glo® 3/7 Assay
  - Caspase-Glo® 9 Assay
  - Caspase-Glo® 8 Assay
- A P-glycoprotein (Pgp) ATPase Assay (Pgp-Glo™ Assay; aka MDR1 or ABCB1) (selected because the P-glycoprotein is involved in multi-drug resistance)

Table 1 NCGC Criteria for HTS Assays

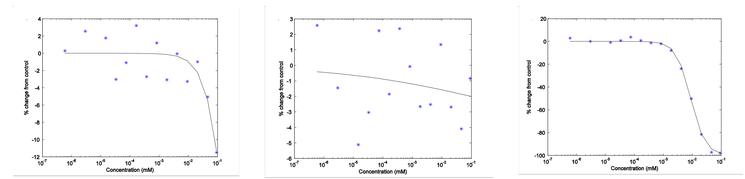
Criteria	Biochemical	Cell-based
*Plate Format**	96-well or higher density plate NCGC: 1536-well format Assay volume 2-6 µl	96-well or higher density plate NCGC: 1536-well format Assay volume 4-6 µl
Assay Steps	≤10 steps with 96-well plate. Steps include, reagent additions, timed incubations, plate transfers 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)

Results of Screening the NTP 1408 (Figure 3)

- All 1408 compounds have been tested for cytotoxicity in the CellTiter-Glo® assay over a 13-point concentration response curve (0 to 92 µM) in 7 human and 6 rodent cell types (see Poster 1185, Board 506). The resulting quantitative concentration-response data are publicly posted on PubChem (http://pubchem.ncbi.nlm.nih.gov/). An algorithm has been developed for analyzing the cytotoxicity concentration response curves that take into account intra- and inter-plate variability.
- All 1408 compounds have been tested for caspase 3/7 activation in 9 cell types. All quantitative concentration-response data will be publicly posted on PubChem. An algorithm is being developed for analyzing the cytotoxicity concentration response curves that take into account intra- and inter-plate variability.
- In addition to testing the NTP 1408 compounds in the 6 assays provided by the NTP, the NCGC is testing these compounds in the 50+ other HTS assays currently active within its testing program.
- As HTS data are being collected across different cell types for the same assay and across assays that evaluate different endpoints, the data are being evaluated internally by the NTP for reproducibility and for patterns of response potentially indicative of toxicological effects. These data will be available in the Biomedical Investigation Database (BID; see **Poster 1204, Board 534**).

Figure 3

Representative Normalized Concentration Response Data and Fitted Response Curves For NTP Compounds Tested in the HTS CellTiter-Glo® Cytotoxicity Assay (see Poster 1185, Board 506, for more information)



Captan | Carbon tetrachloride | Sodium nitrite

Future Activities

Disease-related Pathways and HTS Assays of Interest

The hypothesis supporting the NTP HTS Initiative is that *In vitro* assays that measure perturbations in cellular pathways involved in the etiology of specific diseases can be used to identify compounds capable of causing adverse health outcomes in humans.

The initial focus of the NTP will be on evaluating the ability of HTS assays to identify carcinogens and immunotoxicants. To achieve this goal, a preliminary assessment was made of pathways that were considered critical to the development of immune dysfunction and/or cancer. For example, general pathways with both immune and tumorigenicity significance include:

- Apoptosis
- NFKB
- MAPK
- Adenylate cyclase
- P13K
- Phospholipase C
- TNF/FAS
- TGFß

For more specific immunotoxicity-related processes, HTS assays that evaluate the ability of compounds to interact with various immune and inflammatory pathways involving cytokines and cytokine receptors, chemokines and chemokine receptors, and TOLL like receptors, and with specific immune cell types (Fc receptors, T-cell receptor signaling (I & II), B-cell receptor signaling) are needed. For more specific cancer-related processes, HTS assays that evaluate the ability of compounds to interact with p53, Ah receptors, the Wnt pathway via β–catenin or JNK, nuclear hormone receptors, G-protein coupled receptors, and cadherin are needed. In addition, the ability of compounds to induce gene or chromosomal mutations or cause epigenetic changes must be evaluated. To help identify the critical pathways and genes within those pathways for various diseases, the NTP plans to consider microarray results for compounds of interest, as well as pathways identified from the Genetic Association database (http://geneticassociationdb.nih.gov).

Once critical pathways have been identified and to a certain extent prioritized, the number of HTS assays in which NTP compounds are tested will be expanded by including assays for key steps in pathways considered critical to immune dysfunction and cancer.

The Next Set of 1408 Compounds

The composition of the next set of 1408 NTP compounds will focus on:

- Increasing the number of compounds for which there are immunotoxicity and cancer bioassay test results
- Including a greater number of structurally-related compounds that cover a broad range of activity levels (severe, moderate, mild, negative activity for the endpoint of interest)
- Including parent compounds that require metabolic activation and their metabolites of interest, in order to better evaluate issues relating to the need for metabolic activation in a HTS program

Analyses

When sufficient data from multiple assays have been collected, reiterative analyses will be conducted to evaluate the ability (and limitations) of HTS test data to identify immunotoxicants and carcinogens. These analyses will incorporate data from the additional assays on PubChem, and various measures of chemical space (log p, molecular weight, number of rotatable bonds, number of hydrogen acceptors and donors).

The NTP will also continue to:

- Evaluate the reproducibility of the results obtained with each HTS assay
- Evaluate whether it is better to use primary cells or immortalized cell lines in these screening assays
- Develop routine and high throughput methods for stock solution compound concentration determinations
- Develop protocols for testing compounds that are insoluble in DMSO
- Investigate the extent to which HTS and C. *elegans* test results, separately or together, aid in the prediction of toxicological outcomes of interest