Advances in high-throughput screening technology for toxicology

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Abstract: The US Tox21 collaboration utilises a quantitative high-throughput screening (qHTS) platform to efficiently profile a large number of environmental chemicals. qHTS combined with informatics facilitates chemical prioritisation for further in-depth toxicity testing and development of computational models to predict chemical toxicity. The NIH Chemical Genomics Center (NCGC), now part of the National Center for Advancing Translational Sciences (NCATS), is a key contributor during all phases of the Tox21 collaboration, from assay development and compound screening to data analysis and model building. Since 2011, the Tox21/NCGC has been profiling the phase 2 Tox21 library of approximately 10,000 (10K) environmental chemicals and drugs. The advances in HTS assays and qHTS screens against the Tox21's and other chemical libraries are described in this review.

Keywords: cytotoxicity; environmental chemicals; drugs; high-throughput screening; HTS; nuclear receptors; pharmacology; quantitative high-throughput screening; qHTS; structure-activity relationship; SAR; stress-response pathways; Tox21 10K library; Tox21 datasets; toxicology.

Biographical notes: Chia-Wen Hsu is currently a Commissioner's Fellow working on Medical Countermeasures (MCM) and Comprehensive in Vitro Proarrhythmia Assay (CiPA) initiatives at the US Food and Drug Administration (FDA). As a Postdoctoral Fellow at the National Center for Advancing Translational Sciences, National Institutes of Health, she conducted several Tox21-related projects under the guidance of Menghang Xia, where she used various assay technologies and informatics approaches to identify environmental chemicals and drugs that potentially modulate nuclear receptors, epigenetics, or hypoxia signaling. Prior joining NCATS, she obtained her BS in Chemistry from National Taiwan University and PhD in Pharmaceutical Sciences from University of North Carolina at Chapel Hill.

Ruili Huang is the informatics group lead on the toxicity profiling team at the National Center for Advancing Translational Sciences, National Institutes of Health, and the Co-Chair of the Bioinformatics Working Group of the Toxicology in the 21st Century (Tox21) initiative. Her academic background includes a PhD in Chemistry and a post-doctoral training in computational biology and bioinformatics. Her research interests include high throughput screening assay evaluation and prioritisation, compound structure-activity relationships, drug-gene-pathway relationships, mechanisms of chemical toxicity and *in vitro* to *in vivo* toxicity modelling.

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Christopher P. Austin is the Director of the National Center for Advancing Translational Sciences (NCATS) at the US National Institutes of Health. NCATS' mission is to catalyse the generation of innovative methods and technologies that will enhance the development, testing and implementation of diagnostics and therapeutics across a wide range of human diseases and conditions. Before 2012, he was the Director of the NCATS Division of Preclinical Innovation, which focuses on translating basic science discoveries into new treatments and developing new technologies and paradigms to improve the efficiency of therapeutic and diagnostic development. He founded and directed numerous initiatives including the NIH Chemical Genomics Center (NCGC), the Therapeutics for Rare and Neglected Diseases program, and the Toxicology in the 21st Century (Tox21) program. Before joining NIH in 2002, he directed research programs genomics-based target discovery, pharmacogenomics, and neuropsychiatric drug development at Merck. He earned his BA in Biology from Princeton University and an MD from Harvard Medical School.

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Menghang Xia is a group leader of Systems Toxicology at National Center for Advancing Translational Sciences, National Institutes of Health. Her academic background includes a PhD in Pharmacology and Toxicology and a postdoctoral training in cellular and molecular immunology. She is serving as a Co-Chair in the assays/pathways working group of the Toxicology in the 21st Century (Tox21) program, she led the major effort to develop and validate a battery of *in vitro* toxicological assays in a quantitative high throughput screening platform. She is interested in studying the mechanism of action of drugs/chemicals in multiple cellular pathways and targets, such as hypoxia signalling, mitochondria function, and nuclear receptors.

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1   Introduction

As the pool of environmental chemicals and clinically-used drugs expands, it has been a challenge to quickly and efficiently profile the potential effects of such compounds on human health and the environment. Traditional toxicity testing, conducted *in vivo* on various animal models, provides valuable chemical safety information, but these testing methods are relatively expensive and low throughput, and sometimes it is difficult to extrapolate the test results to observations on human health due to species differences (Xia et al., 2008). The poor concordance of drug toxicities between humans and animals has also been reported (Greaves et al., 2004). Recently, high-throughput screening (HTS) has emerged as a complementary approach to probe compound actions and to prioritise chemicals for in-depth investigation. The HTS approach can be used to collect data on more than a thousand compounds per day at a much lower cost relative to *in vivo* experiments. More importantly, screens using human primary cells or cell lines might serve as useful indicators for compound toxicity in humans. With technological advances, human primary cells and stem cell-derived tissue specific cells are often used to screen chemicals in a HTS platform, which greatly reduces animal usage. To rapidly screen a large amount of environmental chemicals and facilitate compound prioritisation, the NCGC/NCATS focuses on implementation of assays suitable for quantitative high-throughput screening (qHTS) (Tice et al., 2013). Using qHTS to screen compounds at multiple concentrations greatly eliminates false positives and false negatives by scoring concentration-response curves of each tested compound. We have previously described and reviewed Tox21's efforts on assay development, robot implementation and the Tox21 screening process (Shukla et al., 2010; Attene-Ramos et al., 2013c; Tice et al., 2013). Here we report an update of available HTS assays at the NCGC/NCATS and the recent qHTS applications.

2   The Tox21 program at the National Center for Advancing Translational Sciences

The NIH Chemical Genomics Center (NCGC), founded in 2004, is currently part of the Division of Pre-clinical Innovation in the National Center for Advancing Translational Sciences (NCATS). NCATS aims to translate key findings from bench to new therapeutics, to improve understanding of disease biology and drug targets, and to facilitate drug safety assessment. The mission of the centre is "to catalyze the generation of innovative methods and technologies that will enhance the development, testing, and implementation of diagnostics and therapeutics across a wide range of human diseases and conditions" (http://www.ncats.nih.gov/). It includes the development of chemical probes for novel biological mechanisms, profiling of compound libraries for biological and physiochemical properties, and invention of technologies/paradigms for assay development, screening, informatics, and chemistry. Several scientific programs exist within this space, including Assay Development and Screening Technology, Chemistry Technology, RNA Interference (RNAi), and Toxicology in the 21st Century (Tox21).

The Tox21 program utilises expertise from multiple US federal agencies to rapidly profile a large number of environmental chemicals in multiple cellular toxicity pathways and to interpret the findings for chemical prioritisation and risk assessment. The Tox21 partners consist of the National Toxicology Program (NTP) at the National Institute of
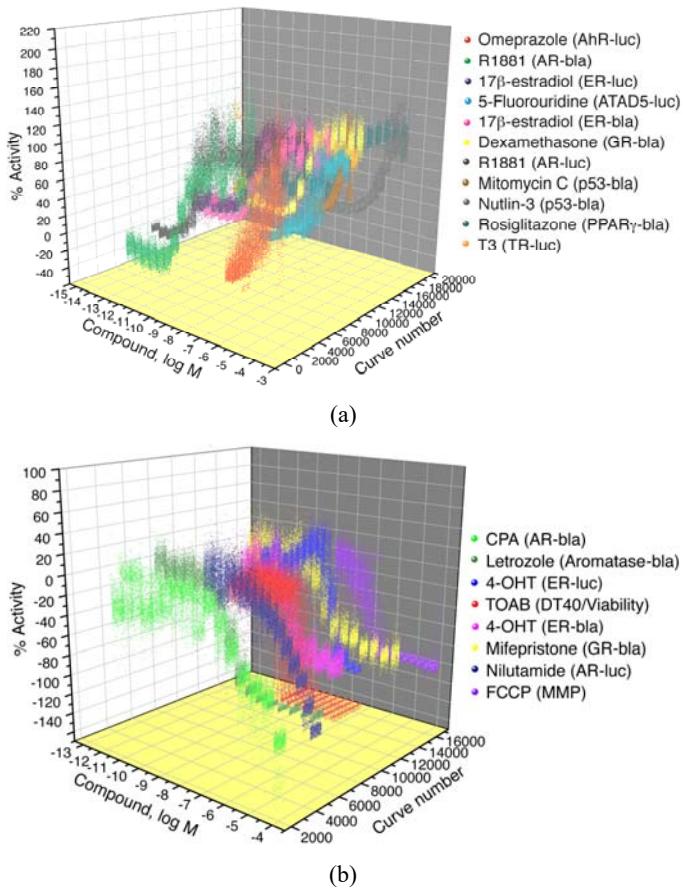
Environmental Health Sciences (NIEHS), the NCGC at the NCATS, the National Center for Computational Toxicology (NCCT) at the US Environmental Protection Agency (EPA), and the US Food and Drug Administration (FDA). The major roles of the NCGC are to provide cutting-edge assay technology, to conduct qHTS against the Tox21 compound collection, to perform data analysis, and to build predictive models of chemical toxicity. During the pilot phase, phase 1, of the Tox21 collaboration, screening methods were optimised and assessed for suitability for screening environmental chemicals in a 1,536-well plate format. A group of cell-based assays were screened against a collection of 2,816 environmental chemicals (i.e., 1,408 provided by the NTP and 1,408 provided by the EPA) and the performances of these assays were evaluated (Tice et al., 2013). In addition, various cell-based assays were also screened against the NCGC pharmaceutical collection (NPC) comprised of 2,816 approved and investigational drugs (Huang et al., 2011a). Phase 2 of the Tox21 program started in 2011 where the NPC compounds and additional environmental chemicals were incorporated to the Tox21 compound collection, forming a new library of approximately 10K chemicals (Tox21 10K library). The Tox21 phase 2 focused on the development and screening of a battery of cell-based assays with phenotypic, target-specific or mechanism-based readouts using a high-throughput or high-content screening (HCS) platform. Each assay is conducted three times on three separate days against the Tox21 10K library with the same compound contained in three different well locations. Three independent concentration-response curves are collected for each compound after primary screening (Attene-Ramos et al., 2013c). The qHTS data generated along with chemical and assay information are made publically accessible through the PubChem database (http://pubchem.ncbi.nlm.nih.gov) and other resources including CEBS (http://tools.niehs.nih.gov/cebs3/ui/) and ACToR (http://actor.epa.gov/actor/faces/ACToRHome.jsp) after internal evaluation by the Tox21 partners. The ultimate goal of the Tox21 program is to identify *in vitro* chemical signatures that could act as predictive surrogates for *in vivo* toxicity.

3 Quantitative high-throughput screening

qHTS is a powerful approach to generate reliable biological data from large chemical libraries. Unlike traditional HTS where a given compound is tested at a single concentration, qHTS tests compounds at multiple concentrations and generates concentration-response curves for hundreds and thousands of compounds in a single experiment, which greatly reduces the false positives and false negatives (Inglese et al., 2006; Attene-Ramos et al., 2013a). In order to perform automated (also referred to as online) screening successfully, selection and development of a suitable HTS assay is a key step. To meet screening requirements in terms of plate format, cell quality (i.e., mycoplasma-free certified) and assay robustness (i.e., steps, duration, temperature, format and performance), HTS assays are usually conducted in 96-well or higher density microtiter plates. Assay reagents including substrates are chemically and thermally stable in a final working condition for at least eight hours at room temperature or on an ice bath. To minimise well-to-well variation, the HTS assay steps including reagent additions, incubation time, plate transfer to incubators, and assay reading should be as short as possible and a homogenous assay format (i.e., addition only) is preferred. Cell-based assays are ideally completed within 24 hours and not more than 72 hours to limit evaporation and maintain cell health. Evaluation of the effect of dimethyl sulfoxide

(DMSO) concentration on cell-based assays is also a critical step in the assay optimisation because compound stock solutions are normally prepared in 100% DMSO. The cell-based assays usually tolerate DMSO up to concentrations of 1% (Xia et al., 2009b). Good assay performance is determined by signal-to-background ratios (S/B) $\geq$ 3-fold, coefficients of variation (CV) across DMSO plates without compounds $\leq$ 10%, and Z' factors (Zhang et al., 1999) $\geq$ 0.5. Changes of potency indicated by IC_{50}/EC_{50} (half maximal inhibitory concentration/half maximal effective concentration) values of positive controls should be less than three- to six-fold during day-to-day screening. In addition to assay selection and validation, automation and informatics are equally important in qHTS to ensure high quality results from a large number of samples.

Figure 1   A 3D scatter plot of the intra-plate concentration response curves of positive controls in each screening of the cell-based assays, (a) positive control curves in online agonist screens (b) positive control curves in online antagonist screens

(a)

(b)

Notes: Ahr: aryl hydrocarbon receptor: AR: androgen receptor. ATAD5: ATPase family, AAA domain containing 5. ER: estrogen receptor. GR: glucocorticoid receptor. PPARγ: peroxisome proliferator-activated receptor gamma. TR: thyroid hormone receptor. T3: triiodothyronine. CPA: cyproterone acetate. FCCP: trifluorocarbonylcyanide phenylhydrazone. MMP: mitochondrial membrane potential. 4-OHT: 4-hydroxy tamoxifen. TOAB: tetraoctyl ammonium bromide.

Tox21/NCGC uses qHTS assays in a 1,536-well plate format on a robotic system to enable the profiling of the Tox21 10K compound collection. The 1,536-well plates only require 3–6 µl of assay volume per well. The first four columns of the 1,536-well plate containing control wells, including concentration response curves of a positive control compound in each screening assay (Figure 1), are used to normalise the data and assess the assay performance. The remaining 1,408 wells are used to simultaneously measure biological responses of test compounds at a single concentration in the same plate. Importantly, compounds are placed in different locations in three sets of compound plates to minimise interference from factors including uneven illumination or unequal dispensing volumes. In this manner, a given compound is assayed as three independent dilution series. The qHTS screens are conducted on a robotic platform containing four components: cell incubators and compound storage, liquid handling equipment, detectors for assay readouts, and software-controlled robotic arms (Attene-Ramos et al., 2013c). Prior to each Tox21 10K qHTS screen, the candidate assay is validated with the online robotic system using a Library of 1,280 Pharmacologically Active Compounds (LOPAC[1,280]) plus 88 Tox21 compounds plated as duplicates in the 10K library in three independent repeats. The online screens of the Tox21 10K compound collection are assayed at 15 concentrations over 4 logarithmic units to yield more than 450 plates per screen.

Gross assay performance is assessed initially using quality metrics including CV, S/B, and Z' factor from each plate and also visually during the execution of the primary screen. 'Failed plates' identified by abnormally poor values are inspected visually and, if necessary, repeated as entire concentration-response curves. Assay data of good quality are subject to further processing, curation, and analysis. Compound concentration – response data are first normalised to positive and negative controls in every plate and corrected using the DMSO control plates as previously described (Huang et al., 2011b; Southall et al., 2009). Concentration-response titration points for each compound are fitted to a four-parameter Hill equation (Hill, 1910) yielding concentrations of half-maximal activity (AC_{50}) and maximal response (efficacy) values (Wang et al., 2010). Compounds are classified into different activity categories (curve class 1–4) according to the type of concentration-response curve observed (Huang et al., 2011b; Inglese et al., 2006). Curves are then manually curated to correct for any curve fitting anomalies. The 'clean' fitting results from the triplicate runs as well as the Tox21-88 duplicated compounds are assessed for activity reproducibility to determine the final assay performance. As soon as the initial data parsing and assessment at the NCGC/NCATS are complete, the concentration response data, the curve fitting results, the raw plate reads, assay conditions, and sample mapping information are shared with the Tox21 partners through a suite of databases and software tools custom built by the NCGC/NCATS for the Tox21 program (http://tripod.nih.gov/tox) in which the data are further scrutinised for quality and utility. The data are then released to the public domain in a number of public databases including PubChem (http://pubchem.ncbi.nlm.nih.gov/), CEBS (http://tools.niehs.nih.gov/cebs3/ui/; Waters et al., 2008) and ACToR (http://actor.epa.gov/actor/faces/ACToRHome.jsp; Judson et al., 2008).

4   Validated qHTS assays for compound profiling

A battery of cell-based assays has been developed and validated in a 1,536-well format at the NCGC/NCATS, some of which have been used to profile the Tox21 10K compound collection (Huang et al., 2014; Hsu et al., 2014; Attene-Ramos et al., 2015). The majority of assays were evaluated using various compound libraries including the LOPAC, EPA, NTP, or NPC, and summarised in several reviews regarding the Tox21 phase 1 screening (Shukla et al., 2010). These assays can be categorised into phenotypic, target-specific (e.g., nuclear receptors), or pathway-based assays (Table 1).

Table 1   List of NCGC toxicity-related assays

Assay name	Readout	Description	Reference
Cell viability	Bioluminescence	Intracellular ATP levels	Xia et al. (2008), Moeller et al. (2012), Lock et al. (2012) and Abdo et al. (2015)
Apoptosis	Chemiluminescence	Caspase 3/7 activity	Huang et al. (2008) and Lock et al. (2012)
Membrane integrity	Chemiluminescence	Protease release	Cho et al. (2008)
	Fluorescence intensity	LDH release	
DNA damage/ epigenetics	Fluorescence intensity	Micronucleus, locus derepression	Johnson et al. (2008), Yamamoto et al. (2011), Nishihara et al. (2016), Fox et al. (2012) and Hsu et al. (2016b)
	Chemiluminsecence	DNA damage, HDAC activity	
	Bioluminescence	ATAD5 production	
	HTRF	H2AX phosphorylation	
Mitochondrial toxicity	Fluorescence intensity (HTS and HCS formats)	Depolarisation of mitochondrial membrane potential	Sakamuru et al. (2012) and Attene-Ramos et al. (2013b, 2015)

Notes: FRET: fluorescence resonance energy transfer. HDAC: histone deacetylase. HTRF: homogeneous time resolved fluorescence. HCS: high content screening. HTS: high-throughput screening. TR-FRET: time resolved fluorescence resonance energy transfer. AhR: aryl hydrocarbon receptor. AP-1: activator protein 1. AR: androgen receptor. ARE: antioxidant response element. ATAD5: ATPase family AAA domain-containing protein 5. ATP: adenosine triphosphate. cAMP: cyclic adenosine monophosphate. CAR: constitutive androstane receptor. CRE: cAMP response element. CYP: cytochrome P450. ERa: estrogen receptor alpha. ESRE: endoplasmic reticulum stress response element. FXR: farnesoid X receptor. GPCR: G-protein coupled receptor. GR: glucocorticoid receptor. H2AX: H2A histone family, member X. hERG: human ether-à-go-go-related gene. HIF-1α: hypoxia inducible factor 1 alpha. HSE: heat shock element. Hsp70 and Hsp90: heat shock protein 70 and 90. IL-8: interleukin-8. LDH: lactate dehydrogenase. IP: inositol phosphate. LXR: liver X receptor. NF-kB: nuclear factor kappa-B. PDE: phosphodiesterase. PPARδ and PPARγ: peroxisome proliferator-activated receptor delta and gamma. PXR: pregnane X receptor. RAR: retinoic acid receptor. RORγ: RAR-related orphan receptor gamma. RXRα: retinoid X receptor alpha. SIE: sis-inducible element. TNF-α: tumour necrosis factor alpha. TRα and TRβ: thyroid hormone receptor alpha and beta. VDR: vitamin D receptor.

Table 1 List of NCGC toxicity-related assays (continued)

Assay name	Readout	Description	Reference
Phospholipidosis	Fluorescence intensity (HTS and HCS formats)	Intracellular phospholipids	Shahane et al. (2014)
Ion channel and GPCR signalling	Fluorescence intensity	hERG activity	Titus et al. (2009, 2008a, 2008b), Xia et al. (2011a, 2011b), Long et al. (2013) and Liu et al. (2010, 2008)
	TR-FRET	Calcium influx (GPCR or PDE activity)	
	HTRF, Fluorescence intensity	IP production (GPCR) cAMP production	
Cytokine secretion	HTRF	IL-8, TNF-α	Leister et al. (2011)
	AlphaLISA	TNF-α	
CYP induction	Chemiluminescence, bioluminescence	Activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP3A4	Veith et al. (2009) and Shukla et al. (2011)
Nuclear receptor signalling	β-lactamase coupled FRET	Transactivation of a β-lactamase reporter gene by AR, ERα, FXR, GR, LXR, PPARδ, PPARγ, RXRα, TRβ, VDR, or RORγ	Judson et al. (2010a, 2010b), Huang et al. (2011b, 2014), Teng et al. (2013), Rotroff et al. (2013) and Hsu et al. (2014)
	Bioluminescence	Transactivation of a luciferase reporter gene by PXR, CAR, AR, ERα, TR, AhR, or aromatase	Shukla et al. (2011), Lynch et al. (2013), Chen et al. (2014), Freitas et al. (2014) and Chen et al. (2015)
	TR-FRET	Ligand binding and coactivator recruitment to ERα, FXR, PPARγ, PXR, TRα, or TRβ	Shukla et al. (2009, 2011)

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Table 1   List of NCGC toxicity-related assays (continued)

Assay name	Readout	Description	Reference
Nuclear receptor signalling	Fluorescence polarisation	Ligand binding to TRβ	Johnson et al. (2011)
	Fluorescence intensity (HCS)	Nuclear translocation of AR or ER	Teng et al. (2013)
	Enzyme fragment complementation-coupled chemiluminescence	Nuclear translocation of GR	Zhu et al. (2008)
Stress response signalling	β-lactamase coupled FRET	Transactivation of a β-lactamase reporter gene by AP-1, ARE, CRE, HSE, HIF-1α, SIE, NF-κB, p53, or ESRE	Xia et al. (2009a, 2009b, 2009c, 2009d), Miller et al. (2010), Shukla et al. (2012), Hancock et al. (2009), Johnson et al. (2009), Bi et al. (2015) and Hsu et al. (2016a)
	Bioluminescence AlphaScreen	Transactivation of a luciferase reporter gene by ARE, CRE, HIF-1, IκBα, NF-κB, Hsp70, or Hsp90 Hsp90	Shukla et al. (2012), Xia et al. (2009c), Davis et al. (2007), Miller et al. (2010) and Hsu et al. (2016a)

Notes: FRET: fluorescence resonance energy transfer. HDAC: histone deacetylase. HTRF: homogeneous time resolved fluorescence. HCS: high content screening. HTS: high-throughput screening. TR-FRET: time resolved fluorescence resonance energy transfer. AhR: aryl hydrocarbon receptor. AP-1: activator protein 1. AR: androgen receptor. ARE: antioxidant response element. ATAD5: ATPase family AAA domain-containing protein 5. ATP: adenosine triphosphate. cAMP: cyclic adenosine monophosphate. CAR: constitutive androstane receptor. CRE: cAMP response element. CYP: cytochrome P450. ERa: estrogen receptor alpha. ESRE: endoplasmic reticulum stress response element. FXR: farnesoid X receptor. GPCR: G-protein coupled receptor. GR: glucocorticoid receptor. H2AX: H2A histone family, member X. hERG: human ether-à-go-go-related gene. HIF-1α: hypoxia inducible factor 1 alpha. HSE: heat shock element. Hsp70 and Hsp90: heat shock protein 70 and 90. IL-8: interleukin-8. LDH: lactate dehydrogenase. IP: inositol phosphate. LXR: liver X receptor. NF-kB: nuclear factor kappa-B. PDE: phosphodiesterase. PPARδ and PPARγ: peroxisome proliferator-activated receptor delta and gamma. PXR: pregnane X receptor. RAR: retinoic acid receptor. RORγ: RAR-related orphan receptor gamma. RXRα: retinoid X receptor alpha. SIE: sis-inducible element. TNF-α: tumour necrosis factor alpha. TRα and TRβ: thyroid hormone receptor alpha and beta. VDR: vitamin D receptor.
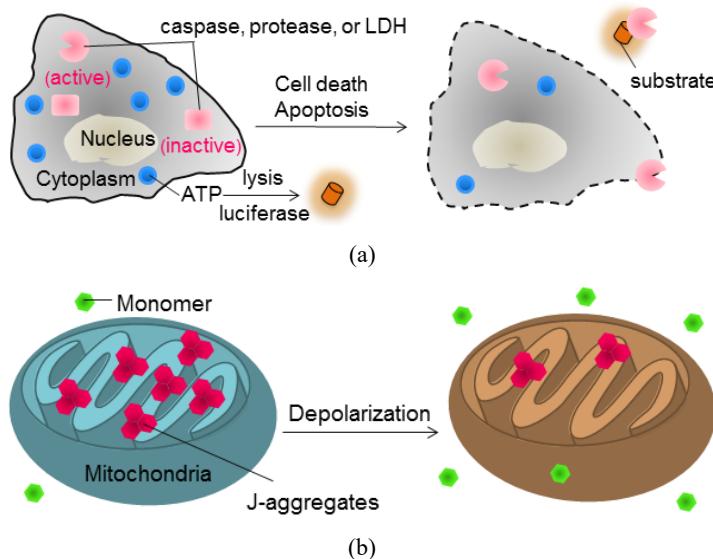
5   Phenotypic assays

Phenotypic assays measure phenotypic changes of cells upon compound treatment. Available phenotypic assays include cell death, DNA damage, membrane integrity, mitochondrial toxicity, and accumulation of biomolecules (Figure 2). The viability or cytotoxicity assays used in the Tox21/NCGC screening measure intracellular ATP production or membrane integrity by quantifying activity of protease or lactate

dehydrogenase (LDH) released from the damaged cells with fluorescence or luminescence readouts (Cho et al., 2008; Xia et al., 2008; Huang et al., 2008; Miller et al., 2010). These assays can be multiplexed with other assays including nuclear receptor- and pathway-based assays in the same well, which greatly reduce screening time and reagent usage, as well as provide a more information-rich dataset. Recent studies compared chemical-induced cytotoxicity on 81 and later 1,036 human lymphoblast cell lines of differential genetic backgrounds and populations (Lock et al., 2012; Abdo et al., 2015). The authors found a good correlation between toxicity phenotypes and genotypes, demonstrating the feasibility of using genomic data as predictors for compound toxicity. Another exciting breakthrough in hepatotoxicity testing is the use of human cryopreserved hepatocytes in 1,536-well plates (Moeller et al., 2012) because the majority of liver-derived cancer cell lines lack intracellular metabolic activity. For example, a human hepatocellular carcinoma cell line, HepG2, though commonly used to screen for cytotoxic compounds, lacks most phase 1 and phase 2 metabolic enzymes and transporters. Therefore, these human cryopreserved plateable hepatocytes could serve as a plausible alternative for hepatotoxicity testing that may generate data with better correlation with *in vivo* experiments. However, compared to HepG2 cells, human hepatocytes are much more expensive and difficult to get sufficient cell numbers for large scale screening. Recently, assay applications based on HepaRG, terminally differentiated hepatic cells derived from a human hepatocellular carcinoma cell line, have gained increasing interests due to its high metabolic activities (Kanebratt and Andersson, 2008). The utility of this cell line for HTS in a 1,536-well format is still under investigation. Cell viability assays have also been used to evaluate the effect of DNA-damage inducing compounds on a group of isogenic DNA repair-deficient chicken DT40 cell lines (Yamamoto et al., 2011; Nishihara et al., 2016). Differential cytotoxicity patterns were observed in these cell lines depending on the type of DNA-damage inducing compounds, making them good research tools for identifying genotoxic compounds and associated mechanisms. Another DNA damage assay, measuring ATAD5 induction, has been developed and validated (Fox et al., 2012). ATAD5 is the human homolog of yeast enhanced level of genome instability gene 1 (*ELG1*) that retains proliferating cell nuclear antigen to regulate the lifecycle of DNA replication. The authors took advantage of the fact that ATAD5 proteins become stabilised in response to DNA damage and designed an ATAD5-luciferase reporter gene system (ATAD5-*luc*) that measures changes of luminescence signal to reflect DNA damage. Several genotoxic and non-mutagenic antioxidants were identified as potential chemotherapeutic agents after the screening and follow up studies. One notable observation was that the current genotoxicity assays have low hit rates (Knight et al., 2009), most likely due to insufficient metabolic activity across the sensor cell lines.
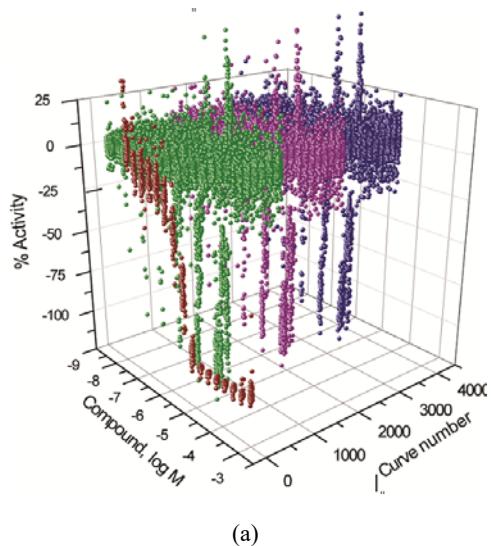
Cell-permeable fluorescent dyes not only report signalling events as whole-cell fluorescence changes but also capture spatio-temporal dynamics of a specific signalling event. For instance, a mitochondrial membrane potential (MMP) assay can detect mitochondrial dysfunction, an important cause of compound-induced heptatoxicity and cardiotoxicity. To identify environmental chemicals that disrupt MMP, assay optimisation, including dye selection, and robotic online validation of the MMP assay were conducted prior to the robotic online screen. Mito-MPS, a fluorescent lipophilic cationic dye, has been shown to sense MMP change in HepG2 cells with or without compound treatment and is superior to other cationic dyes such as JC-1, rhodamine 123, and tetramethylrhodamine (Sakamuru et al., 2012). In response to mitochondrial

depolarisation, the ratio of the mitochondrial red fluorescent aggregates to the cytosolic green fluorescent monomeric form decreases. The ratiometric (red/green) readout in fluorescence intensity can be measured by commonly used plate readers. The change of the red and green fluorescence in the assay can also be visualised and quantified by high-content imaging based readers including the ImageXpress Micro System. After screening the LOPAC and NTP collections, several compound clusters that contained tyrphostin, 3'-substituted indolone analogues and flavonoids were identified (Sakamuru et al., 2012; Attene-Ramos, et al., 2013b). This MMP assay using Mito-MPS dye has been validated with the online robotic system using the LOPAC plus 88 Tox21 compounds plated as replicates in triplicate runs [Figure 3(a)]. The MMP assay online validation performance satisfied the HTS assay requirements [Figure 3(b)] with a low mismatch rate under 1% [Figure 3(c)]. This assay has been applied to profile the Tox21 10K compound library for mitochondrial toxicity (Attene-Ramos et al., 2015; Potera, 2015). Another recently reported phenotypic assay workable in a high-throughput and high content format is a cell-based assay using the LipidTOX Red reagent to detect compound induced phospholipidosis (PLD), a phenotype of abnormal accumulation of intracellular phospholipids (Shahane et al., 2014). This cell-based PLD assay has been optimised in a 1,536-well format and validated by screening a LOPAC library in HepG2 cells. A group of known PLD inducers including propranolol and amiodarone and several novel PLD inducers, such as NAN-190, ebastine, GR127935, and cis-(Z)-fluphentixol, were identified and further confirmed to induce PLD using electron microscopy (EM). In general, the HTS capability of such assays allows rapid screening of large chemical libraries, and the HCS capability is particularly useful for compound confirmation as well as follow-up studies.

Figure 2   (a) Cytotoxicity related assays measure membrane integrity, ATP content, caspase 3/7 activity, and release of proteases or LDH (b) Mitochondrial toxicity is measured by membrane potential-sensing dyes that exist as green fluorescent monomers in the cytoplasm and red fluorescent J-aggregates at the electron-rich mitochondria
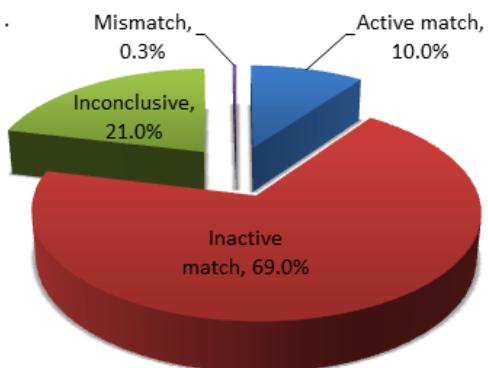

(a)

(b)

Figure 3 Online validation of a MMP assay, (a) concentration-response curves of the positive control carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and all tested LOPAC compounds in the MMP assay in triplicates (different colours) (b) screen statistics of the MMP assay (c) screening data reproducibility of the MMP assay

(a)

MMP Assay	Mean ± SD
S/B	9.4 ± 0.2
CV (%)	7.9 ± 0.4
Z' factor	0.77 ± 0.01

(b)

(c)

Notes: S/B: signal to background ratios. CV: coefficient of variation.

6   Target-specific assays

Abnormal secretion and transport of signalling molecules are indicators of potential toxicity of a given compound. For example, the cytokine tumour necrosis factor alpha (TNF-α) has been found to mediate effect of the environmental estrogens on apoptosis of MCF-7 breast cancer cells (Burow et al., 1999). Two homogeneous cell-based TNF-α assays have been developed and optimised in a 1,536-well plate format using homogeneous time resolved fluorescence (HTRF) and AlphaLISA immunoassay technologies. Leister et al. (2011) further validated these assays by conducting a LOPAC screen. The HTRF-based TNF-α assay requires labeling anti-TNF-α antibodies with HTRF donor or acceptor fluorophores. Newly secreted human TNF-α molecules bring the two types of dye-labelled antibodies in proximity, resulting in fluorescence resonance energy transfer (FRET) from the HTRF donor to the HTRF acceptor. The AlphaLISA-based TNF-α assay shares a similar assay design but with more complex components. The donor moiety consists of a biotinylated anti-TNF-α donor and a streptavidin-coated donor bead. The counterpart is a TNF-α antibody-coated acceptor bead. In the presence of TNF-α, the donor and the acceptor beads are at a close distance (< 200 nm), allowing laser-excited singlet oxygen released from the donor bead to travel to the nearby acceptor beads and produce signals. The two TNF-α assays yielded similar profiles of potency and efficacy values of cherry-picked compounds that inhibit TNF-α secretion. A similar cell-based HTRF assay used dye-labelled inositol-1-phosphate (IP1) tracers and IP1-specific antibodies to measures IP1 (Liu et al., 2008), a signalling molecule downstream of G-protein coupled receptors (GPCRs). GPCRs sense extracellular stimuli and transmit signals to the cyclic adenosine monophosphate (cAMP) signalling pathway or the phosphatidylinositol (PI) signalling pathway. GPCR profiling is important because many drugs raise safety concerns through off-target effects on GPCRs (Christopoulos et al., 2004). Several cell-based assays that measure GPCR signalling have been optimised in 1,536-well formats to probe cyclic nucleotide phosphodiesterases-mediated cAMP conversion (Titus et al., 2008b), G-protein coupled cAMP production (Xia et al., 2011a), Gq-protein coupled receptor-mediated calcium flux (Liu et al., 2010), and thyroid-stimulating hormone (TSH) receptor-mediated cAMP production (Titus et al., 2008a). A common cause of drug-induced cardiotoxicity is the inhibition of human ether-a-go-go-related gene (hERG) channel. To identify hERG inhibitors, Titus et al. (2009) developed a homogeneous cell-based hERG assay that measures the flux of fluorogenic indicators of thallium ions across the hERG channel. Quaternary ammonium salts such as tetra-n-octylammonium bromide (TOAB) and benzethonium chloride were later confirmed to be open-channel blockers of hERG that may induce long QT syndrome (LQTS) and cardiac arrhythmia (Xia et al., 2011b; Long et al., 2013).

Cytochrome P450 (CYP) enzymes are key players in xenobiotic metabolism that convert xenobiotic substances into metabolites with toxicity profiles different than the parent compounds (Coon, 2005). Veith et al. (2009) validated a group of enzyme-based bioluminescent CYP assays in a 1,536-well format and characterised compound effects on a panel of CYP enzymes comprising of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 using luminogenic substrates of individual CYP enzymes. The authors screened over 17K bioactive compounds and approved drugs and conducted structure-activity relationship (SAR) analysis of active compounds. They observed that greater extent of bioactive compounds than approved drugs inhibited all five CYP enzymes, probably
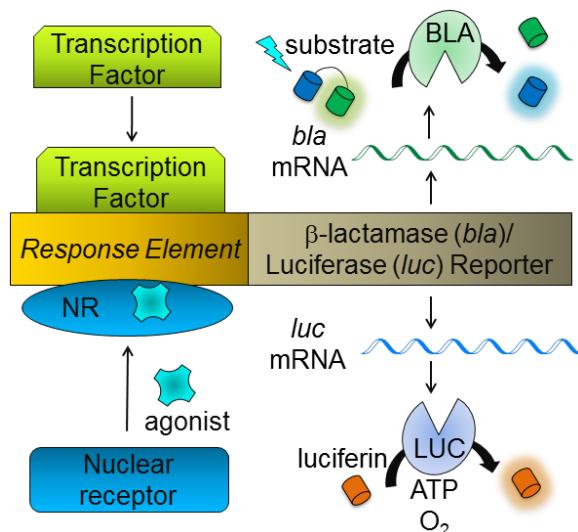
owing to the fact that most clinically-used drugs were optimised to avoid CYP activity during the drug development stage.

7 Nuclear receptor assays

Several nuclear receptor signalling pathways have been found to be the direct targets of highly hazardous substances such as endocrine disruptors (Casals-Casas and Desvergne, 2011). Endocrine signalling pathways including the androgen receptor (AR) and estrogen receptors (ERs) have been linked to reproductive and developmental defects. Abnormal activity of retinoic X receptors (RXRs) and thyroid hormone receptors (TRs) has diverse effects on the central nervous system (CNS), circadian rhythm, and metabolism (Yang, 2010). In addition to RXRs and TRs, the glucocorticoid receptor (GR) and the peroxisome proliferator-activated receptors (PPARs) govern energy homeostasis and lipid metabolism (Francis et al., 2003). The farnesoid X receptor (FXR), the pregnane X receptor (PXR), the liver X receptor (LXR), the constitutive androstane receptor (CAR), and the vitamin D receptor (VDR) are likely to mediate toxicity or protect cells from toxic substances due to their roles in homeostasis of bile acids and xenobiotic metabolism (Francis et al., 2003). Owing to the toxicological and pharmacological impacts of these nuclear receptors, several biochemical and cell-based assays have been developed by academia and industry to measure the effects of compounds on receptor binding, receptor translocation, or transactivation of target genes by the corresponding nuclear receptor (Table 1). Many of these assays are also commercially available for routine testing. The cell-based nuclear receptor transactivation assays are commonly constructed using β-lactamase-dependent FRET (NR-*bla*) or luciferase (NR-*luc*) reporter gene technologies (Figure 4). NR-*bla* assays usually use the ligand binding domain (LBD) of the nuclear receptor of interest and generate a ratiometric response in fluorescence intensity. Many of NR-*luc* assays are based on a full length nuclear receptor and luminescence readout. During the Tox21 phase 1 screening, a collection of approximately 2,800 compounds provided by both EPA and NTP were screened against a panel of NR-*bla* assays including AR, ERα, FXR, GR, LXRβ, PPARγ, PPARδ, RXRα, TRβ, and VDR in 1,536-well formats (Huang et al., 2011b). The agonist or antagonist response (activation/ inhibition phenotypes and EC_{50}/IC_{50}) of known positives generated by the NR-*bla* screens was consistent with literature, indicating the feasibility of these assays for biologically relevant studies. The study also revealed that many antagonists identified from the NR-*bla* screening had IC_{50} values similar to those found in the viability assays, suggesting potential false positives due to cytotoxicity. Freitas et al. (2014) identified the structural classes of TR agonists and TR antagonists by screening the LOPAC and NTP compounds in a luciferase reporter gene-based TR transactivation assay in the qHTS format. Four out of the five identified TR antagonist-like compounds were later found to be cytotoxic, conferring the importance of incorporating cytotoxicity testing in primary screens. Therefore, cell viability counter-screens multiplexed with pathway/target-based screens have been incorporated into the Tox21 phase 2 screening process. Some compounds such as polycyclic aromatic hydrocarbons (PAHs) and coumarin dyes are likely false positives in the NR-*bla* agonist screens owing to compound autofluorescence at the detection wavelengths of bla reporter signals. To overcome this, compounds from the Tox21 compound library have been tested for their autofluorescence potential at multiple wavelengths that are commonly used in screening assays (Simeonov et al., 2008). To

identify chemical classes specifically modulating FXR signalling, Hsu et al. (2014) developed a cross-comparison strategy that compares compound potency and efficacy in all available Tox21 NR-*bla* assays as well as an FXR coactivator assay. The results indicated that activation of FXR is highly ligand-selective and many FXR-environmental chemicals might indirectly inhibit FXR activity by altering intracellular trafficking or epigenetics regulation of FXR-related signalling molecules. Furthermore, orthogonal assays using different technologies are useful alternatives to rule out assay artifacts such as compound autofluorescence. For example, the cell-based ER-*luc* assay can be used to follow up on the positives identified from the ER-*bla* primary screen (Teng et al., 2013).

Figure 4   Both β-lactamase and luciferase reporter gene assays measure β-lactamase or luciferase production induced by nuclear receptors or transcription factors
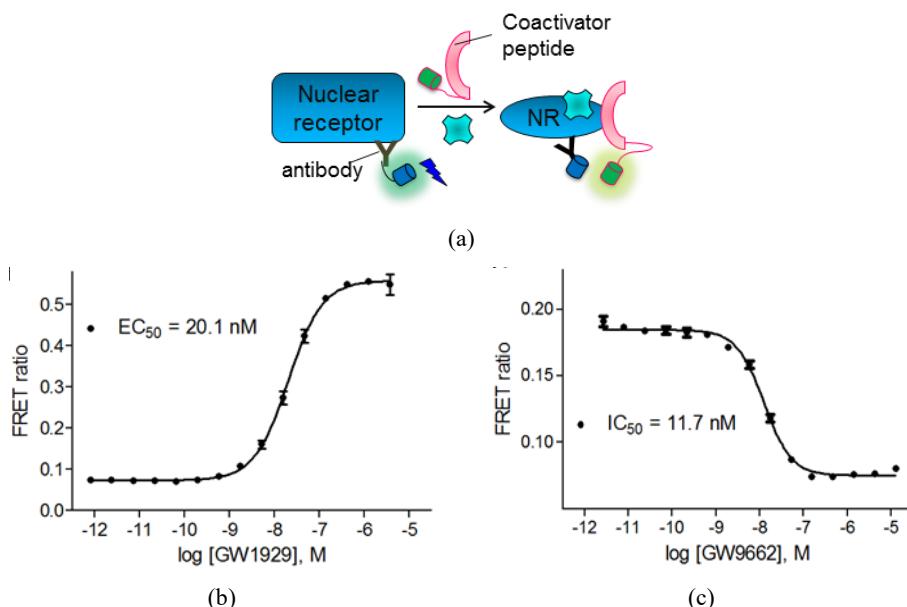

Notes: The β-lactamase activity is determined by a β-lactamase-sensitive FRET reaction, while the luciferase activity is measured by catalysing a reaction with luciferin to produce light.

Available assay technologies for nuclear receptor binding include AlphaScreen, fluorescence polarisation (FP), time-resolved fluorescence (TRF), and time-resolved FRET (TR-FRET). Johnson et al. (2011) identified inhibitors that block the binding between the human TRβ-LBD and a dye-labelled peptide derived from its coactivator proteins using a FP-based receptor binding assay. Comparing the assay performance of AlphaScreen, TR-FRET, and TRF technologies for FXR-ligand binding assays, Glickman et al. (2002) concluded that the FXR AlphaScreen assay was the most sensitive in terms of detection limit and dynamic range, and the FXR TR-FRET assay had the least well-to-well variation. However, the FXR TRF assay requires multiple washing steps, making it less favourable for HTS. Recently, homogeneous and versatile TR-FRET-based nuclear receptor co-activator assays have been designed for a wide selection of nuclear receptors (Table 1) in which fluorescein-labelled ligands/coregulator peptides and Tb-labelled antibodies are used [Figure 5(a)]. In the TR-FRET PPARγ assays shown in Figures 5(b) and 5(c), GW1929, PPARγ agonist (Brown et al., 1999), stimulated coactivator recruitment and GW9662, PPARγ antagonist (Davies et al., 2001), inhibited

coactivator recruitment in a dose-dependent manner. Shukla et al. (2009) screened over 80,000 compounds from various libraries for their ability to bind PXR using a homogeneous PXR TR-FRET assay containing fluorescein-labelled PXR ligands, GST-tagged PXR, and Tb-labelled anti-GST antibodies. The authors also took advantage of the qHTS in generating multiple concentration-response curves to probe for assay artefacts caused by compound autofluorescence, signal quenching, aggregate formation, or diffusion-enhanced FRET. In a related study, the PXR TR-FRET assay was applied to identify PXR agonists from the qHTS screen of human and rodent PXR-*luc* reporter gene assays (Shukla et al., 2011).

Figure 5 Nuclear receptor coactivator assay

Notes: (a) TR-FRET nuclear receptor coactivator assay reports agonist binding and recruitment of a coactivator peptide. A long-lived donor fluorophore is covalently attached to an antibody binding to the nuclear receptor. The pairing acceptor fluorophore is labelled on the coactivator peptide in which ligand binding brings two fluorophores in proximity and results in altered FRET. The concentration-response curves of the TR-FRET PPARγ coactivator assay were measured in (b) agonist mode and (c) antagonist mode in a 1,536-well format. GW1929 and GW9662 are known synthetic agonist and antagonist of PPARγ, respectively.

Nuclear receptor translocation is an important indicator of nuclear receptor function and can be measured in HTS and HCS platforms in order to study the mechanism of compound action. Environmental chemicals including polychlorinated biphenyls (PCBs), PAHs, and heavy metals have been shown to disrupt GR signalling and increase health risk (Odermatt et al., 2006). A cell-based β-galactosidase fragment complementation assay of GR translocation was validated in a 1,536-well format and applied to a qHTS screen (Zhu et al., 2008). The C-terminus of GR was fused to the enzyme donor (ED) fragment of β-galactosidase and the GR-ED fusion protein remains in the cytoplasm in the absence of agonist binding. Upon addition of a GR agonist, the activated GR-ED translocates to the nucleus and reconstitutes an active enzyme with the pairing enzyme

acceptor (EA) acceptor fragment of β-galactosidase. The reconstituted β-galactosidase enzyme is capable of converting a chemiluminescent substrate to report on GR activity. To explore the mechanism of the AR activity of bisphenol A (BPA) in live cells, Teng et al. (2013) monitored the redistribution of AR and ER after treatment of BPA in sensor cell lines stably expressing AR-EGFP (enhanced green fluorescent protein) or ERα-EGFP fusion protein. Upon activation, AR or ER translocates from the cytoplasm to the nucleus, thus nuclear receptor redistribution can be visualised and quantified on a high content imaging system. In the study, BPA was found to be an ERα agonist and an AR antagonist. The combination of nuclear receptor transcription, receptor binding and coactivator recruitment, and nuclear translocation assays provides informative insights to compound mechanism in a high-throughput manner.

8   Pathway-based assays

Pathway-based assays measure the activation or inhibition of compounds in a given signalling pathway. Selection of such assays requires prior knowledge of the toxicity outcomes as related to the pathway of interest. The β-lactamase (*bla*) reporter gene assay technology has been implemented to probe activity of several key intracellular signalling pathways. For example, the heat shock factor (HSF) pathway protects cells from proteostasis-related toxicity effects (Resenberger et al., 2012). A HeLa cell line was engineered to express the *bla* reporter gene under the control of HSF response elements (HSE) present in the promoter region of heat shock protein 70 (Hsp70). HSE-*bla* assays in 1,536-well formats have been developed and can be used to screen for modulators of the heat shock response signalling (Hancock et al., 2009). The HSE-*bla* assay exhibited robust assay performance including the response against known HSR inducers, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and bortezomib, and HSR-specific RNAi oligos, thus the HSE-*bla* is a promising platform for cell-based HTS of HSR modulators. Potential inhibitors that disrupt Hsp90-cochaperone interaction were identified by a bead-based proximity AlphaScreen Hsp90 assay after screening over 76,000 compounds (Yi et al., 2009). Hsp70 cooperates with the cAMP-response element-binding (CREB) pathway to ameliorate polyglutamine-caused toxicity in fruit flies (Iijima-Ando et al., 2005). To identify CREB signalling enhancers, Xia et al. (2009c) validated a β-lactamase reporter gene assay for CREB (CREB-*bla*) signalling activity in a 1,536-well format, screened over 73,000 compounds, and identified a group of compounds that potentiated the CREB signalling pathway. Hsp90 regulates the stabilisation of hypoxia-inducible factor 1 alpha (HIF-1α), another key cytoprotector during hypoxia (Isaacs et al., 2002). A β-lactamase-based cellular assay of HIF-1α transcription (HRE-*bla*) was optimised in a 1,536-well plate format and employed to identify compounds that induce HIF-1α activity (Xia et al., 2009d) or inhibit HIF-1α signalling pathway (Xia et al., 2009a).

The combined use of different assay platforms can provide additional insights into the mechanism of compound action. For example, the 1,408 compounds in the NTP collection were profiled for their ability to induce the antioxidant response element (ARE) signalling pathway using a cell-based ARE-driven β-lactamase reporter gene assay (ARE-*bla*) in HepG2 cells, followed by compound confirmation with a cell-based ARE-driven luciferase reporter gene assay (ARE-*luc*) (Shukla et al., 2012). A similar

approach was adapted to identify nuclear factor-kappa B (NF-κB) inhibitors from the NPC library (Miller et al., 2010). The NF-κB *bla* assay showed increased reporter activity when stimulated with tumor necrosis factor alpha (TNF-α), a known activator of the NF-κB pathway, and decreased TNF-α-induced NF-κB transactivation upon treatment of MG132 a known proteasome inhibitor. To deconvolute the compound action in the NF-κB signal transduction cascade, several follow-up assays including Iκ-Bα phosphorylation, LDH release, MMP, and caspase 3/7 activity were used to test the NF-κB inhibitors identified from the primary screening. The Iκ-Bα phosphorylation assay was based on TR-FRET between intracellular GFP-tagged Iκ-Bα and Tb-labelled antibody specific to the pS32 of Iκ-Bα. An alternative Iκ-Bα HTS assay developed by Davis et al measures Iκ-Bα stabilisation by quantifying emission ratios from a red luciferase and an Iκ-Bα-responsive green luciferase (Davis et al., 2007). In this study, the IK-Bα ratiometric sensor engineered into a patient-derived non-Hodgkin's lymphoma cell line was used to screen for NF-κB inhibitors. As HTS assays become applicable to high-density qHTS formats and automation, the rich datasets produced can be used to study the mechanism of compound action and SAR of a large number of compounds in a high-throughput manner.

9 Predictive modelling

The high quality concentration response data generated on a wide spectrum of pathways and phenotypic toxicity endpoints provide a valuable resource for predictive toxicity modelling. These data not only can serve as *in vitro* signatures to predict *in vivo* toxicity endpoints (Sipes et al., 2011; Martin et al., 2011) and to prioritise chemicals for more in depth toxicity testing (Judson et al., 2010a) that help to fulfil the goals of the Tox21 program, but also can provide rich training data sets for the QSAR (quantitative structure-activity relationship) modelling community to build more robust models (Huang et al., 2009; Sun et al., 2012c). QSAR models were developed for two of the Tox21 assays (Sun et al., 2012a, 2012b), with rigorous performance evaluation of all models using receiver operating characteristic curves (Schoonjans et al., 1996). Models were built based on the qHTS data generated on a library of drug-like molecules for their activity against five human cytochrome P450 (CYP) isozymes, CYP1A2, CYP2C9, CYP3A4, CYP2C19, CYP2D6 (Sun et al., 2011; Veith et al., 2009). These models were then applied to predict the CYP profiles of Tox21 phase 1 library of environmental chemicals and the predictions compared with the experimental results obtained for the same chemicals (Sun et al., 2012b). Within the test compound set, the environmental chemicals largely fall outside of the applicability domain (AD) of the models built with drug-like molecules. The predictions for three of the isozymes achieved >80% accuracies. The two models with lower predictive power were improved by rebalancing the training data. The atom typing-based structure descriptors (Sun, 2004) enhanced the coverage of the models' AD and enabled these models to make accurate predictions on the CYP activity of environmental chemicals that are dissimilar to drug-like molecules. This atom typing-based approach was also applied to build models for drug-induced PLD (Sun et al., 2012a). In this exercise, one-third of the qHTS data from screening a bioactive collection including the NPC, LOPAC and Tocris compound libraries was used as the training set to build the model and two-thirds of the data were held out to test the model performance.

One of the lessons learned from this exercise is that increasing the structure diversity of a training set is more important than increasing its size for improving the predictive power of a model. Using one-third of the data randomly selected to train the model to ensure structure diversity, the consensus model obtained 90% accuracy in predicting the remaining two-thirds of data. These results reassure the decision of expanding the Tox21 library to the 10K collection with more diverse chemicals that can serve as a solid foundation for better understanding of SARs and better models.

The prediction models built on HTS assay data may be useful for regulatory scientists to assess chemical toxicity. The EPA Endocrine Disruptor Screening Program (EDSP) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have been applying the Tox21 datasets to build QSAR prediction models and *in vitro-in vivo* correlation analysis (Zang et al., 2013; Chang et al., 2015). For instance, an ER Interaction Score model, generated based on 13 *in vitro* ER HTS assay data from 1,814 ToxCast/Tox21 chemicals, was utilised to predict *in vivo* uterotrophic activity of 45 chemicals, achieving 91% sensitivity and 65% specificity (Rotroff et al., 2013). Industrial toxicologists also started to use the ToxCast and Tox21 data as well as human exposure estimates to rank potential endocrine disruptors (Becker et al., 2015). Chang et al. (2015) at NICEATM conducted an *in vitro*-to-*in vivo* extrapolation (IVIVE) study of 230 potentially ER-active environmental chemicals tested in Tox21 10K screening, suggesting the need of compound pharmacokinetics in prioritising chemicals for further endocrine-related tests. Additionally, Politi et al. (2014) demonstrated the usefulness of HTS data in confirming results generated from virtual screening for TR ligands. To supplement current toxicity assessment methods with computational modelling, NCATS launched a crowdsource challenge in 2014 that asked the participants to build computational models predictive of chemical toxicity (Huang et al., 2015). Data generated from 12 assays, including a panel of nuclear receptor assays and a panel of stress response pathway assays, on Tox21 10K chemicals were provided as the training sets for the models. 378 models from 40 teams worldwide were received for final evaluation. All of the winning models displayed >80% accuracy, with some exceeding 90% accuracy (Huang et al., 2015). The high quality of models served as a validation of the Tox21 screening effort as the model quality is highly dependent on data quality. The winning models will become part of the Tox21 program arsenal of tools that help researchers identify the chemicals that have the most potential for toxicity and prioritise them for further toxicological evaluation.

10 Concluding remarks

The Tox21 program, a collaborative partnership of three US federal agencies, NIH, EPA, and FDA, integrates assay technology and informatics to enable rapid testing of hundreds of thousands of environmental chemicals and assessment of their potential adverse effect on human health. After the proof of principle study that validated and screened a battery of assays against approximately 2,800 environmental chemicals, the chemical collection was expanded to over 10K compounds and the screens were conducted on the Tox21 robotic system. During the Tox21 phase 2 screening, a group of nuclear receptor assays including AhR, AR, ERα, FXR, GR, PPARγ, PPARγ, RXRα, TR, and VDR (Table 1) has been used to profile the Tox21 10K compounds. Both β-lactamase-based (e.g., AR-*bla*, ER-*bla*) and luciferase-based (e.g., AR-luc, ER-luc) assays were employed in

primary screens to decipher mechanisms of newly identified AR and ER agonists and antagonists (Huang et al., 2014). Modulators of FXR identified from Tox21 10K screening were prioritised by comparing compound activity across all the nuclear receptor assays (Hsu et al., 2014). The second screening focus area was a group of stress response pathway assays including p53, NF-κB, HIF-1α, pH2AX, activator protein 1 (AP-1), endoplasmic reticulum (ER) stress, MMP (Attene-Ramos et al., 2015), ARE/Nrf-2, heat shock response, DNA damage, and reactive oxygen species (ROS). Cell viability or cytotoxicity readouts multiplexed with the nuclear receptor or stress response assays were incorporated into the online screening to rule out the positive responses due to compound cytotoxicity. The actives identified from primary screening were prioritised for further in-depth toxicological investigation based on considerations such as compound potency, efficacy, SAR and environment production volume. The Tox21 datasets generated from the screening were deposited into public databases and provide scientists with new resources for building predictive models for chemical toxicity.

To search for more biologically relevant assays for the Tox21 program, the future assay battery will focus on developmental toxicology, epigenetics, and human diseases including mitochondrial disorders. Development and validation of metabolically active sensor cells including human primary cells and stem cell derived tissue-specific cells for HTS assays may provide better extrapolation of chemical toxicity to humans. Expansion of the current HTS/HCS assay battery, development of organotypic cultures, and advances in informatics and robotic technology will enhance the capacity of secondary and follow-up studies for further compound prioritisation. Compound clustering based on structural similarity, biological functions, and assay response will improve the understanding of the interplay of toxicity pathways and identify useful biomarkers for safety assessment and disease biology. Ultimately, the aggregation of quality-controlled assay data could yield new hypotheses for toxicological studies and develop better methods to assess chemical safety in humans and environment.

Acknowledgements

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