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*A synopsis of the Tox21 initiative and a focus on the NIH Chemical Genomics Center's efforts within this program using **in vitro** methods and quantitative high-throughput screening.*

Foundation review: The future of toxicity testing: a focus on *in vitro* methods using a quantitative high-throughput screening platform

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The US Tox21 collaborative program represents a paradigm shift in toxicity testing of chemical compounds from traditional *in vivo* tests to less expensive and higher throughput *in vitro* methods to prioritize compounds for further study, identify mechanisms of action and ultimately develop predictive models for adverse health effects in humans. The NIH Chemical Genomics Center (NCGC) is an integral component of the Tox21 collaboration owing to its quantitative high-throughput screening (qHTS) paradigm, in which titration-based screening is used to profile hundreds of thousands of compounds per week. Here, we describe the Tox21 collaboration, qHTS-based compound testing and the various Tox21 screening assays that have been validated and tested at the NCGC to date.

Introduction

Traditionally, the toxicological evaluation of environmental chemicals has largely relied on animal models that have been used to extrapolate to potentially harmful events in humans. These models have been developed to evaluate specific toxicological endpoints, such as oral, dermal and ocular toxicity; immunotoxicity; genotoxicity; reproductive and developmental toxicity; and carcinogenicity. Although these animal models have provided useful information on the safety of chemicals, they are relatively expensive, low throughput and sometimes inconsistently predictive of human biology and pathophysiology. Recently, several major new initiatives have begun to utilize *in vitro* methods and a variety of new technologies to develop *in vitro* signatures and computational models predictive of *in vivo* response. These initiatives should enable researchers to identify a battery of *in vitro* assays that will detect perturbations in cellular pathways that are expected to contribute to or result in adverse health effects [1]. Furthermore, these initiatives represent a welcome movement away from traditional *in vivo* high-dose hazard studies [1]. To appreciate the scientific and technological advancements that are shaping toxicity testing today, it is important to appreciate where this new paradigm fits in the context of historical testing.

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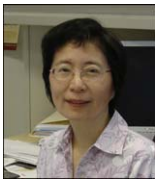
is currently a postdoctoral fellow at the NIH Chemical Genomics Center (NCGC). She is currently working under the guidance of Dr Menghang Xia in developing *in vitro* toxicity-based assay screening using a quantitative high-throughput screening platform. Furthermore, she is also working under the guidance of Dr Doug Auld in the area of assay development and high-content screening. Dr Shukla is the first recipient of the Humane Society/Procter and Gamble Fellowship honoring the advancement of alternatives to animal testing. Before joining NCGC, she received her Ph.D. in Human Genetics, with a focus on pharmacogenetics of anticancer agents, from the University of Chicago in the lab of Dr M. Eileen Dolan in 2007. Additionally, she received a Master of Public Health degree at Saint Louis University with a focus on Epidemiology and Environmental/Occupational Health in 2001. She has authored or co-authored 13 peer-reviewed publications.

Dr Christopher Austin

is director of the NIH Chemical Genomics Center (NCGC) and senior advisor to the Director for Translational Research at NHGRI. The NCGC is an ultra-high-throughput screening, informatics and chemistry center that develops novel compounds as probes of biology and starting points for development of new drugs for rare and neglected diseases, profiles small-molecule libraries for biological and toxicological activities and develops new paradigms to increase the efficiency and genome-wide reach of assay, screening, chemistry and informatics technologies. Dr Austin received his A.B. from Princeton and M.D. from Harvard, trained in neuroscience and genetics at Harvard, and came to NIH in 2002 from Merck.

Dr Menghang Xia



is group leader of cellular toxicity and signaling at the NIH Chemical Genomics Center (NCGC). Dr Xia and her research group are currently focused on the target-specific and mechanism-based toxicological studies, in collaboration with the Biomolecular Screening Branch at the National Toxicology Program (NTP) and National Center for Computational Toxicology at the US Environmental Protection Agency (EPA). Her group has developed and validated a battery of *in vitro* toxicological assays using a quantitative high through screening platform, and investigated the mechanism of action of chemicals in multiple cellular signaling pathways. Dr Xia received her Ph.D. from State University of New York at Buffalo, did postdoctoral training at University of California at San Francisco, and joined NCGC in 2005 from Merck.

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Traditional toxicity testing methods

Since its inception, toxicity testing has relied on animal models treated at maximum tolerated dose levels, with the results extrapolated to human health outcomes at lower doses. This approach dates back to the 1950s, when the use of more specific or mechanistic animal models, and knowledge of the underlying mechanisms for any particular toxicological response, were relatively unknown [2]. Such *in vivo* testing is costly, time consuming and low throughput [3]. The complete toxicological profiling of one chemical in standard *in vivo* assays consisted of the following toxicity tests: acute, subchronic and chronic toxicity; reproductive toxicity; developmental toxicity, ocular and skin irritation, hypersensitivity, phototoxicity and toxicokinetic studies [4]. Despite the disadvantages associated with testing in animals, the majority of the understanding regarding chemical toxicity has come from data obtained in such systems [5]. Even extensive animal testing does not provide a mechanistic understanding of toxicity and knowledge concerning adverse risks to humans is still inadequate [6]. Hence, a need for more mechanistic data and a 'theoretical framework for rational decision making' was noted in the early 1980s [6].

More recently, there have been numerous studies highlighting intra- and inter-species differences in mammals, including humans. Williams and Weisburger [7] pointed out that intra-species differences among different mouse strains affect the severity and incidence of neoplasms, making extrapolation of various cancers from mice to humans difficult. Inherent resistance to spontaneous and malignant tumors in nonhuman primate models has also led to variations in the manifestation of disease across these species [8]. In addition to inter- and intra-species differences in disease models, other species-specific differences that affect disease outcome and extrapolations include differences in basal metabolic rate, metabolic pathways, cancer type (sarcomas in mice versus carcinomas in humans), genetic aberrations associated with tumors, and telomere biology, especially with regard to humans and mice [9]. In addition to physiologic differences, the difference in observed high-dose toxicity in rodents and low-dose risks in humans will require knowledge of physiological differences with regard to mode, tissue of exposure, mechanism of action and knowledge of previous *in vitro* data regarding the agent in question.

Toxicity testing in the 21st century and the US Tox21 partnership

The advent of technological innovations in molecular and cellular biology prompted the National Toxicology Program (NTP) to propose a new Roadmap in 2004, 'A National Toxicology Program for the 21st Century' [10], focusing on three main areas: refining traditional toxicology assays, developing rapid mechanism-based predictive screens and improving the overall utility of data for making public health decisions. This Roadmap placed an increased emphasis on the use of alternative assays for identifying key pathways and molecular mechanisms linked to disease [10]. The US Environmental Protection Agency (EPA) started its ToxCast program in 2006 to address many of the same issues [11]. When these programs were in their early stages, a 2007 report from the National Research Council (NRC) entitled *Toxicity Testing in the 21st Century* (Tox21) enunciated what has become a widely accepted vision for future toxicology testing, calling for the development and use of *in vitro* models in human cells of toxicological response based on automated high-throughput screening (HTS) of pathway-based cellular assays related to toxicity and computational modeling [12]. The report envisioned that initially, such less expensive and higher throughput assays could be used to evaluate the modes of action of chemicals for more comprehensive testing programs and that eventually these data would enable the rapid and mechanism-based prediction of *in vivo* biological responses [2,13,14].

To move this research agenda forward, the NTP partnered with the NIH Chemical Genomics Center (NCGC) in 2005 to pilot the chemical, biological and informatics processes required for the transition from predominantly *in vivo* to *in vitro* toxicology. In 2006, this partnership was expanded to include the EPA. In 2008, in recognition of successful proof-of-principle studies [3,15] and galvanized by the NRC report, the 'Tox21' collaboration was formally established via a Memorandum of Understanding among the agencies (http://ntp.niehs.nih.gov/) and publication of a policy paper from the senior leadership of the three organizations [16].

The Tox21 collaboration takes advantage of the complementary strengths of the three partners (Fig. 1). The NTP, a trans-Department of Health and Human Services program headquartered at the NIH National Institute of Environmental Health Sciences, has enormous experience in experimental toxicology. The NCGC, a trans-NIH program administered by the National Human Genome Research Institute, has unparalleled capacity and expertise in *in vitro* assays, titration-based screening and informatics. The EPA National Center for Computational Toxicology, part of the EPA's Office of Research and Development, has deep computational

FIGURE 1

The Tox21 collaboration. The Tox21 collaboration brings together the experimental toxicology expertise of the NTP, high-throughput screening technology at the NCGC and computational toxicology expertise at the EPA.

toxicology expertise [14]. This combined expertise has enabled rapid implementation of the NRC vision, the application of novel methodologies to evaluate a large number of chemicals in a range of *in vitro* assays in a short period of time [17]. Although realization of the NRC vision might ultimately require a research effort on the scale of the Human Genome Project [18], success of this effort would be transformational for toxicology testing for environmental and pharmaceutical chemicals, providing cheaper, faster and more accurate assessment of the toxicological potential of new chemicals.

Role of the NCGC in the Tox21 collaboration

The NCGC was established in 2004 as the first assay development, screening, informatics and chemistry center of what was to become the NIH Roadmap Molecular Libraries Probe Production Center Network. The Molecular Libraries Initiative, a component of the NIH Roadmap for Medical Research, was born from the need for new approaches to determine function and therapeutic potential of human genes on the heels of the Human Genome Project and to accelerate the pace at which basic research is translated into small-molecule therapeutics [17] (http://nihroadmap.nih.gov/molecularlibraries/). As part of the NCGC's technology development program, a platform for automated testing of hundreds of thousands of compounds in titration-based format over a short period of time was developed [13,19], and this quantitative high-throughput screening (qHTS) platform has become a central aspect of the Tox21 program.

Traditional biological assays have been low throughput, employing animal models and labor-intensive testing of samples. Furthermore, the growth of small-molecule collections required the development of HTS technologies to test a large number of compounds in a timely manner [20]. Although HTS has successfully enabled the screening of large chemical libraries to generate hits for medicinal chemistry optimization in the setting of drug discovery, HTS as traditionally practiced is not suitable for toxicity testing because it assays each compound at only single concentration [19] and, thus, generates large numbers of false positives (FPs) and false negatives (FNs) [21]. By contrast, the qHTS paradigm tests each compound at multiple (7–15) concentrations across an approximately four-log concentration range, thus producing concentration–response-based activity profiles of all compounds from the primary screen with greatly reduced FN and FP rates. Miniaturized assay volumes (<10 μL/well) in a 1536-well-plate format provides the throughput to generate concentration–response curves (CRCs) for every compound library member tested [22]. Curve fitting and CRC classification characterizes each curve based on parameters, such as curve fit and efficacy after the primary screening in a qHTS format, enabling the identification of structure–activity relationships (SARs). The NCGC makes its screening data publicly available through PubChem (http://pubchem.ncbi.nlm.nih.gov) and its software available on its website (http://www.ncgc.nih.gov/pub/openhts/) to enable the scientific community to utilize the data in their own research [23].

The ability of qHTS to produce reliable activity profiles of chemicals has also enabled the NCGC to profile large libraries of chemicals for their propensity to produce assay artifacts, which would otherwise be interpreted as true biological effects [24]. NCGC has taken advantage of titration-based screening to identify compounds that produce a wide variety of different artifactual activities, including apparent enzyme inhibition through compound aggregation [25], compound autofluorescence [26] and firefly luciferase inhibition [27]. These profiling examples demonstrate the utility of qHTS in distinguishing true effects from artifacts for more reliable toxicity screening and efficient chemical probe development.

NCGC chemical library collection used for Tox21 assays

An essential component of NCGC's qHTS paradigm is the availability of large chemical libraries in a titration-based format. The availability of several concentrations across different plates gives the user flexibility to use concentrations relevant to the assays. In total, the NCGC has well over 400,000 compounds from the NIH Molecular Libraries Small Molecule Repository and NCGC-specific compound collections. The latter currently includes approximately 1400 compounds each from the NTP and EPA compound libraries and 2816 clinically used drugs in the NCGC Pharmaceutical Collection (NPC). Overall, compound selection is based on having a defined chemical structure and known purity, in addition to the extent of each compound's solubility in dimethylsulfoxide (DMSO) [13]. One limitation of compound storage in DMSO is precipitation (augmented by DMSO water content and number of freeze–thaw cycles) [28]. Compound integrity studies using P450 assays and qHTS at NCGC revealed decreased compound potency over time and lower efficacy of older samples stored in DMSO [29]. For this reason, compounds are used in screening collections at the NCGC for no longer than four to six months.

The current NTP compound collection consists of 1408 compounds, with more than 50 of the compounds represented twice to assess assay reproducibility. The NTP collection includes solvents, fire retardants, dyes, preservatives, plasticizers, therapeutic agents, inorganic and organic pollutants, drinking water disinfection by-products, pesticides and natural products [3]. Selection of the 1408 compounds was partly based on the availability of toxicological data from standard tests of carcinogenicity, genotoxicity, immunotoxicity and/or reproductive and developmental toxicity [3]. Compounds were prepared as 10 mM stock solutions in DMSO and 14 plates representing 2.23-fold dilutions in 1536-well compound plates from 384-well plates [30]. The current EPA collection consists of 1462 compounds prepared similarly to the NTP compound collection. Compounds were primarily selected based on the need to screen and prioritize environmental chemicals to which humans are exposed through the environment or food. These chemicals include those known to be bioactive, those manufactured or used in large quantities and those to which humans are exposed on a routine basis [31]. In the near future, approximately 1400 additional compounds will be added to each of the NTP and EPA libraries for testing as an integrated Tox21 library [14].

The NPC collection (R.H. *et al.*, unpublished) was prepared at the NCGC and currently contains 2816 small molecules, 52% of which are approved by the Food and Drug Administration for human or animal use in the United States. The remaining drugs are either approved for use in other countries, such as Europe, Canada or Japan, or are compounds that have been tested in clinical trials. The majority of the NPC collection is prepared as a 10 mM stock in DMSO and prepared as 15 2.23-fold dilution plates in 1536-well format [30]. Currently, an additional 1400 compounds are being

added to the NPC collection. The next phase of Tox21 testing will begin later this year, using the combined NTP, EPA and NPC compound sets, totaling more than 10,000 chemicals.

NCGC technology

The NCGC characterizes toxicity endpoints in cell-based assays utilizing integrated robotic systems combined with batteries of *in vitro* assays and computational analysis [32]. The NCGC robotic platform stores large compound collections, performs distinct assay steps and measures user-defined assay outputs in an integrated manner [23]. The compound storage unit is capable of storing approximately 300,000 compounds in seven-point titrations, which correlates to more than 2.2 million compound samples [23]. A pin transfer station performs the transfer of 23 nL of compound from a 1536-well compound plate to a 1536-well assay plate, with each plate holding up to 1408 compounds (located in columns 5–48). Assay-specific controls (located in columns 1–4) are located on an additional 1536-well compound plate and transferred simultaneously with the test compounds to the assay plate [23]. Solenoid dispensers, having the capability of dispensing volumes ranging from 200 nL to 20 μL, are used for reagent and cell dispensing. Furthermore, up to eight tips can be used for dispensing in either 90° direct dispense or 45° angled head dispense with regard to the well. This allows for the modification of straight head and angled dispense, depending on the reagent type and condition of cells (i.e. if they are grown in a delicate monolayer, then it might be suitable to use the angled head dispense). For the aspiration of liquid, each dispenser comes equipped with an aspiration head made of 32 stainless-steel tubes for column-wise removal of reagents or media from the plate. The aspirator head enables cell washing and fixing in 1536-well format [33] for cell-cycle protocols or protocols involving antibody steps. There are several factors that can be optimized with the dispenser and aspirators, such as dispense volumes, aspiration depth and aspiration speed [23].

There are four different types of detectors that are currently integrated into the robotic system. These detectors essentially enable a wide variety of assays to be performed at NCGC and accommodate various assay technologies. Furthermore, these readers cover the entire spectrum of speed and information content. Although charged-coupled-device-based camera imagers are capable of very fast read times per 1536-well plate (<1 min/plate), they provide the least information regarding characteristics of individual cells. The converse is true for confocal-based imaging readers, which can provide detailed information on subcellular structures with longer read times (~1 hour/plate). Specifically, the EnVision and ViewLux (PerkinElmer) are photomultiplier-tube- and charged-coupled-device-based instruments, respectively, which cover a wide range of fluorescence, absorbance and luminescence [3,15,34,35] bulk well readouts commonly used in high-throughput assays [23]. The ViewLux can be used for luminescence, fluorescence, absorbance, time-resolved fluorescence resonance energy transfer (TR-FRET) [36] and fluorescence polarization assays [23]. The EnVision is best suited for AlphaScreen assays and β-lactamase reporter assays [37,38] and can be customized for the detection of multiple wavelength regions [23] and TR-FRET assays [36]. Each assay format and readout has its own set of advantages. For example, the use of β-lactamase as a reporter has several advantages, such as ratiometric readouts from dual emissions (460 and 530 nm), which minimizes well-to-well and plate-to-plate variation caused by differences in plating density. In addition, the 530 nm fluorescent signal can be used as an indication of cell viability (and a proxy for compound cytotoxicity) and auto fluorescence [37].

For imaging assays, the user must sacrifice speed to obtain more information about individual cells and characterized cell populations. Two imaging platforms used at the NCGC are the Acumen Explorer (TTP Labtech) and the IN Cell Analyzer 1000 (GE Healthcare). The Acumen Explorer is a photomultiplier-tube-based laser-scanning microplate cytometer that is equipped with three excitation and four emission lasers for enumeration and characterization of fluorescent objects [39]. The Acumen is able to provide total well and individual cell fluorescence readings. Compared to the ViewLux, where the entire plate is read in less than 1 min, Acumen read times can vary between 10 and 20 min per plate, depending on the number of wavelengths required by the assay of interest. Thus far, the Acumen has been used in several assays performed at NCGC, such as GFP-based assays [33] and multiplexing of dual fluorescent drug-sensitive and drug-resistant cell lines [40]. For higher resolution automated fluorescent imaging, the IN Cell Analyzer is designed to collect data either at the single-cell or at the subcellular level. The data collected are often complementary to those collected from the Acumen because additional orthogonal phenotypes can be identified. Furthermore, the instrument comes with own algorithm to analyze the data acquired [41].

Once data have been obtained for each assay, the CRCs for each compound are analyzed and classified as previously described [3,19,36]. Briefly, raw plate reads for each titration point are normalized relative to an assay-specific positive control (100% or −100%) and DMSO-only wells (0%), then corrected by applying a pattern correction algorithm using DMSO-only plates at the beginning and end of each stack [3]. Half-maximal inhibition or activation concentration (AC_{50}) and efficacy values are obtained from fitting the concentration–response titration points to the Hill equation [42]. Compounds are classified as curve classes 1–4 according to the characteristics of the CRC, such as efficacy and quality of curve fit (R^2). Class 1 curves display two asymptotes, whereas class 2 curves display one asymptote. Class 1 and 2 curves are further subdivided into subclasses a (efficacy ≥ 6SD) and b (efficacy < 6SD). These curves have statistically significant curve fits and are usually selected for follow-up analyses. Compounds in curve class 3 only display activity at the highest concentration tested, and compounds with class 4 curves show no concentration response and are deemed inactive. The ability to decipher curves using qHTS for every compound tested is important because many responses, such as toxicity, are measured over broad concentration ranges (typically between 0.5 nM and 92 μM), which might greatly decrease the FP and FN rates.

Assay implementation for Tox21

Various assays (Table 1) in different cell types or lines (Table 2) have been successfully developed, miniaturized and validated for 1536-well plate format at NCGC and screened against the initial Tox21 compound collection. For the majority of assays, compound incubation durations were limited to 48 hours because

TABLE 1

Tox21 assays currently available at NCGC

Assay	Assay endpoint[a]	Cell type	Assay readout	Refs
Cell viability **Apoptosis**	Intracellular ATP content Caspase-3/7	Hek293; Jurkat; HepG2; SH-SY5Y; SK-N-SH; BJ; HUV-EC-C; MRC-5; mesangial; kidney proximal tubules; N2a; H-4-II-E; NIH3T3	Luminescence	[3,15]
Membrane integrity	LDH release Protease release	Hek293; mesangial	Fluorescence Luminescence	[34]
Mitochondrial toxicity	Membrane potential	HepG2	Fluorescence	
DNA damage	Micronucleus	CHO	Fluorescence	
Cytokine	IL-8; TNF-α	THP-1	Homogeneous time-resolved fluorescence	
Nuclear receptor	AR; ERα; FXR; PPARδ; PPARγ; RXR; TRβ; VDR	Hek293	β-Lactamase reporter	
	GR	HeLa		
	hPXR; AhR; rPXR	HepG2	Luciferase reporter	
Toxicity pathway	AP-1; HIF-1α; SIE; NFκB	ME-180	β-Lactamase reporter	[37,38,59,60]
	HSR; ESRE	HeLa		[61]
	ARE/Nrf2	HepG2		
	CREB	Hek293, CHO		[53]
	p53	HCT-116		
	ARE/Nrf2; HSR; ESRE	HepG2	Luciferase reporter	[58]
hERG channel	Thallium influx	U-2OS	Fluorescence	[45]

[a] AhR, aryl hydrocarbon receptor; AP-1, activator protein-1; AR, androgen receptor; ARE/Nrf2, antioxidant response element/NF-E2 related factor 2; CREB, cAMP response element binding; ERα, estrogen receptor α; ESRE, endoplasmic reticulum stress response element; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HIF-1α, hypoxia-inducible factor-1α; hPXR, human pregnane X receptor; HSE, heat shock response element; IL-8, interleukin-8; LDH, lactate dehydrogenase; NFκB, nuclear factor kappa B; PPARδ, peroxisome proliferator-activated receptor δ; PPARγ, peroxisome proliferator-activated receptor γ; rPXR, rat pregnane X receptor; RXR, retinoid X receptor; SIE, sis-inducible element; TNFα, tumor necrosis factor α; TRβ, thyroid hormone receptor β; VDR, vitamin D receptor.

of evaporation-induced edge effects observed in 1536-well plates [3]. Stainless-steel assay lids with rubber gaskets were also used to enable air exchange and minimize edge effects [23]. The assays described below demonstrate the adaptation of existing low-throughput assays to higher throughput, reliable 1536-well format assays. These studies show the ability to modify existing assays to profile larger numbers of compounds for toxicity-based safety studies.

TABLE 2

Cell lines tested

Cell type	Origin	Species
BJ	Foreskin fibroblasts	Human
CHO	Chinese hamster ovary	Hamster
HCT-116	Colorectal carcinoma	Human
Hek293	Embryonic kidney cells	Human
HeLa	Cervical carcinoma	Human
HepG2	Hepatocellular carcinoma	Human
HUV-EC-C	Vascular endothelial cells	Human
H-4-II-E	Hepatoma	Rat
Jurkat	T-cell leukemia	Human
Kidney proximal tubules	Freshly isolated from kidney	Rat
MRC-5	Lung fibroblasts	Human
Mesangial	Renal glomeruli	Human
ME-180	Cervical carcinoma	Human
N2a	Neuroblastoma	Mouse
NIH3T3	Embryonic fibroblasts	Mouse
SH-SY5Y	Neuroblastoma	Human
SK-N-SH	Neuroblastoma	Human
THP-1	Monocytic leukemia	Human
U-2OS	Osteosarcoma	Human

To assess chemical effect on cell membrane integrity, a newly developed cytotoxicity assay that measures released intracellular proteases upon membrane damage with a bioluminescent assay readout was evaluated [34]. Although there have been similar assays developed for lower density formats, few have been miniaturized and validated in a high-throughput format with a robust assay signal [34]. This protease-release assay for membrane damage detection was miniaturized in 1536-well format and was screened against the initial NTP compound collection in HEK 293 and human renal mesangial cells [34] (Tables 1 and 2). All compounds were tested in 14-point titration series to identify compounds that disrupt membrane integrity. The assay performed well in miniaturized format, with high reproducibility of the control compound across every plate in both cell lines, high signal-to-background ratios and high Z' values (a statistical measure of assay performance) [43]. In addition, replicate compounds within the NTP compound collection demonstrated high intra-experimental reproducibility, further indicating the reliability of the qHTS assay. The compounds identified from this assay were known membrane disrupters, including α-solanine and zinc pyrithione. The majority of compounds active in both cell lines were detergents such as digitonin, tetra-*N*-octylammonium bromide and *p*-*n*-nonylphenol, which are known to disrupt membrane integrity; additional non-detergent compounds known to be membrane disrupters were also identified in the assay. Furthermore, some compounds were shown to be uniquely active in one cell line or the other, thus revealing cell-line-specific membrane disruption potential.

Overall, this study demonstrated the successful miniaturization of an existing cytotoxicity assay using a luminescent readout. Furthermore, the application of qHTS to this assay format validated the need to characterize the biological activities of compounds over a broad concentration range [34].

Cardiotoxicity has been commonly examined in the human ether-a-go-go-related gene (hERG) potassium channel. The hERG channel is responsible for the repolarization of cardiac action potential, which is associated with certain forms of inherited and acquired long QT syndrome (LQTS) [44,45]. LQTS can lead to sudden death through a rare ventricular arrhythmia [45]. Some drugs have been removed from the market because of their potential to induce LQTS by inhibiting the hERG channel, which warrants the need for premarketing screening of drugs to minimize the risk of sudden death in the treatment of non-life-threatening diseases [45,46]. Patch-clamp electrophysiology technique is still the gold standard for hERG activity in drug development [47,48], but this assay is low throughput and costly and requires specialized training for personnel [45]. The radioligand-binding assay is also commonly used to test compound binding to the hERG channel, but this assay gives little or no information on the functional effect of the ligand on the channel (blocker, activator or no effect) and the allosteric effect of the ligands [49]. To overcome these limitations, a functional assay was developed for the hERG channel by measuring thallium influx into the cells and was validated in a 1536-well plate format. The assay principle is shown in Fig. 2, where thallium ions enter the cells through open hERG channels after stimulation and bind to the dye, yielding an increase in fluorescence. This fluorescence signal is inhibited in the presence of hERG channel blockers [45]. The qHTS screen identified a group of known hERG inhibitors, such as pimozide, amiodarone and verapamil, from a library of 1280 pharmacologically active compounds (LOPAC^{1280}). Furthermore, the activities of the hERG channel inhibitors in the thallium influx assay are well correlated with those obtained from automated patch-clamp experiments [45].

In vitro assays for cytotoxicity

One of the goals of the Tox21 collaboration is to establish *in vitro* signatures of *in vivo* human and rodent toxicity. To create *in vitro* signatures of compound cytotoxicity across species, 1408 compounds from the initial NTP collection were profiled for cytotoxicity across 13 different human and rodent cell types [3]. These human and rodent cell types were derived from six tissue types that are common targets of xenobiotic toxicity (Tables 1 and 2); thus, this study aimed to develop species- and cell-specific cytotoxicity profiles for each compound [3]. Each compound was tested at 14 concentrations (0.5 nM–92 μM) in a luminescent cell viability assay that measures adenosine triphosphate (ATP) levels of metabolically active cells. The luminescent ATP quantitation assay worked well in 1536-well format with robust Z', signal-to-background ratio and coefficient of variation values for the positive control compound (tamoxifen). Tamoxifen CRCs for each cell line were consistent across the plates; however, the response pattern was cell-line specific, with Jurkat cells being most sensitive and mesangial cells being least sensitive [3] (Fig. 3). Furthermore, the correlation of IC_{50} values for the 55 duplicate compounds present in the NTP compound collection across all 13 cell lines was significant (0.71, $P < 0.001$). There were 428 compounds that displayed cytotoxicity in at least one cell type, and clustering analysis was performed to decipher cytotoxicity profiles across cell types. Within the subgroup of active compounds, multiple effects from the compounds were identified within and across compound types, cell types and species. For example, human- and rodent-derived cells including SH-SY5Y, Jurkat, H-4-II-E, NIH 3T3, N2a, HEK293 and rat renal proximal tubule cells were most sensitive to compound-induced cytotoxicity, whereas human fibroblast and skin cells were least sensitive. Overall, the rodent cells used in

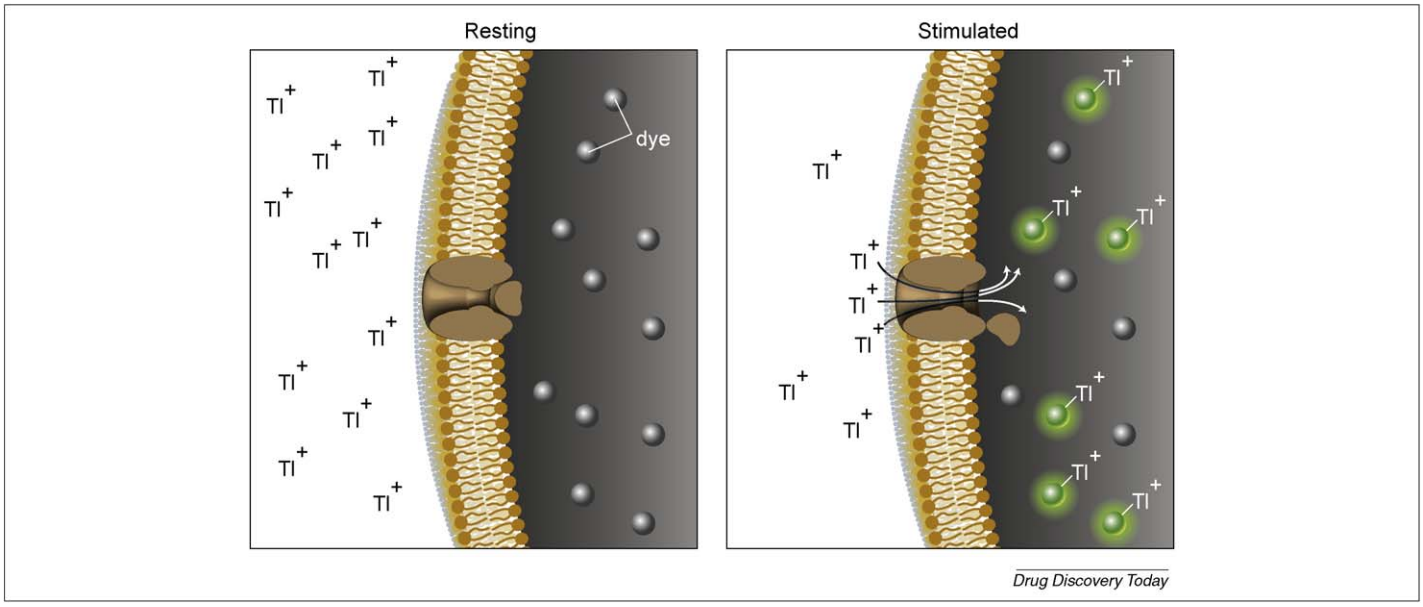

FIGURE 2

Principle of the thallium flux assay. At resting state, cells expressing hERG channels are loaded with dye from the assay kit. Upon stimulation, thallium ions enter the cells through open hERG channels and bind to the dye, yielding green fluorescence upon excitation, proportional to the bound dye.

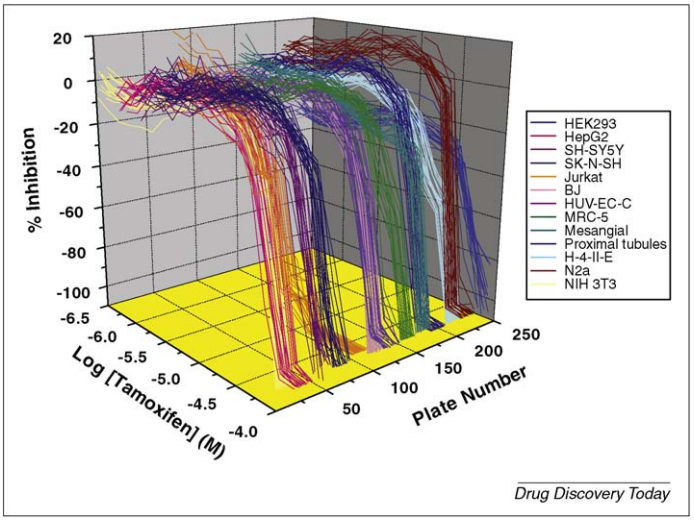

FIGURE 3

Positive control titration across human and rodent cell lines. The reproducibility of tamoxifen, used as a positive control in ATP-mediated cytotoxicity testing, is shown for each cell line for 234 plates tested. Jurkat cells were most sensitive to tamoxifen, and NIH3T3, HEK293, BJ and mesangial cells were least sensitive.

this study demonstrated more sensitivity to the compounds tested than the human cells. Compounds that showed activity in at least one cell type were clustered according to their IC_{50} values, revealing clusters and specific compounds that were selectively cytotoxic in a particular cell type and species. For example, digoxin was more cytotoxic in human HEK293 cells than rat renal proximal tubule cells. Actinomycin D, however, was much more cytotoxic in rat renal proximal tubules than in human HEK293 cells (Fig. 4). Overall, a striking finding was the lack of concordance in the patterns of compound activity in cells derived from the same tissue but from different species (there were also instances in which cells with similar tissue origin in the same species showed discordance in compound activity profiles), highlighting inter-species differences in response. Thus, an important finding from the study is that *in vitro* cytotoxicity in a particular cell type, even if from the same tissue and/or species, does not necessarily predict cytotoxicity in another cell type [3]. Thus, the combination of *in vitro* profiling with qHTS enables the generation of hypotheses related to mechanisms of toxicity and prioritization for more intense toxicological investigation related to *in vivo* toxicity.

The application of clustering to data with multiple endpoints can help uncover underlying mechanisms involved in broad phenotypes such as cytotoxicity. To examine the mechanism of compound-induced cytotoxicity in various cell types, two different endpoints (cytotoxicity and caspase-3/7 activation) were assessed by testing the 1408 NTP compounds for both endpoints across 13 different cell types [15]. The cytotoxicity and caspase-3/7 assays performed well in a 1536-well format and the quality of the data was suitable for use in computational efforts. The overall active rate for the 13 caspase assays (0.4–3.5%) was lower than the rate for the 13 cytotoxicity assays (4–11%). Hierarchical clustering

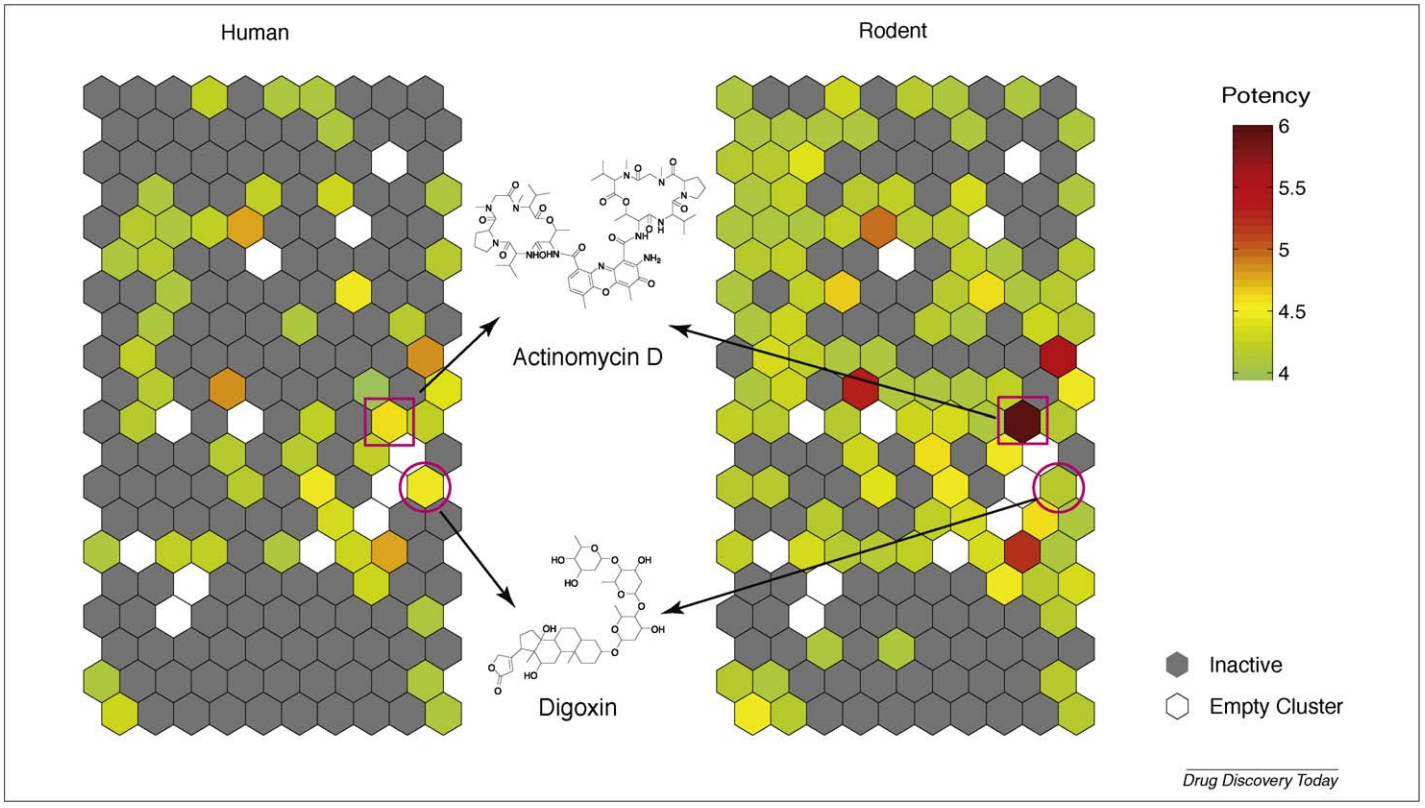

FIGURE 4

Species-selective compounds. Compound activity patterns were obtained through hierarchical clustering of compound IC_{50} values. Shown are compound activity patterns for human HEK 293 cells and rat renal proximal tubule cells. Two compounds, actinomycin D and digoxin, illustrate species-specific cytotoxicity for those particular compound clusters.

based on compound cytotoxicity and caspase EC_{50}/IC_{50} patterns revealed similar clustering based on endpoints rather than cell type, indicating that cytotoxicity and caspase activation assays provide distinct sets of information, and that most compounds induce cytotoxicity through mechanisms other than caspase-3/7 activation. The only exception was the Jurkat cell line, where the caspase and cytotoxicity assays clustered together. One explanation might be that the cell death induced by most compounds in Jurkat cells is dependent on caspase-3/7 activation [15]. The N2a cell line seemed to have the least number of active compound overlap between the cytotoxicity and caspase assays, indicating the contribution of mechanisms outside of caspase activation for cytotoxicity. This approach will be useful for generating hypotheses for compound mechanism of action; however, hypothesis generation will be strengthened by the inclusion of more compounds and endpoints to build stronger models predictive of *in vivo* toxicity.

Computational modeling

Because of the rapidly increasing number of environmental chemicals that need to be tested and the need for prioritization of those compounds for *in vivo* studies, more computational modeling is needed to complement experimental approaches to decrease the time associated with testing and accelerate prioritization of the data [50,51]. Some challenges associated with computational modeling of toxicity data include the diversity of compounds and structures that can produce the same outcome [50]. However, the production of CRCs for every compound tested provides a data-rich resource for SAR analysis, computational modeling and chemical prioritization for more extensive toxicological evaluation. The NCGC recently developed a weighted feature significance (WFS) algorithm, a fragment-based approach that does not rely on whole molecule similarity to model toxicity (Fig. 5), which is designed to achieve good prediction with structurally diverse sets of compounds [50]. Such approaches can be applied to generate testable hypotheses on mechanisms of compound toxicity. Starting with the structure of a compound, one could model and predict its toxicity in one assay or cell type and in multiple cell types, which essentially forms the activity pattern or signature that indicates the compound's mechanism of toxicity.

Models were developed for two aforementioned [3,15] assays, with rigorous performance evaluation of all models using receiver operating characteristic curves [52]. One advantage of the WFS approach is its ability to identify structural features responsible for toxicity [50]. For example, structural features significantly enriched in the pan-cytotoxic compounds include substituted benzenes, 1,3-dienes, heavy metals and imines [50]. Toxic features were also identified for caspase-3/7-activating compounds [15], such as cyclic alkyl ketones and alkyl halides. Thus, the significant toxic features present in compounds could be used to predict a particular mechanism of toxicity, such as caspase-3/7 activation [50]. Overall, the WFS approach can be applied to model other toxicity endpoints such as mutagenicity and hepatotoxicity and might be applicable to a larger array of compounds. Unlike other modeling methods, WFS can be used even when compound structures are highly diverse. In addition, WFS was shown to have comparable or better predictive power when compared to Native Bayesian clustering or a support vector machine approach in most

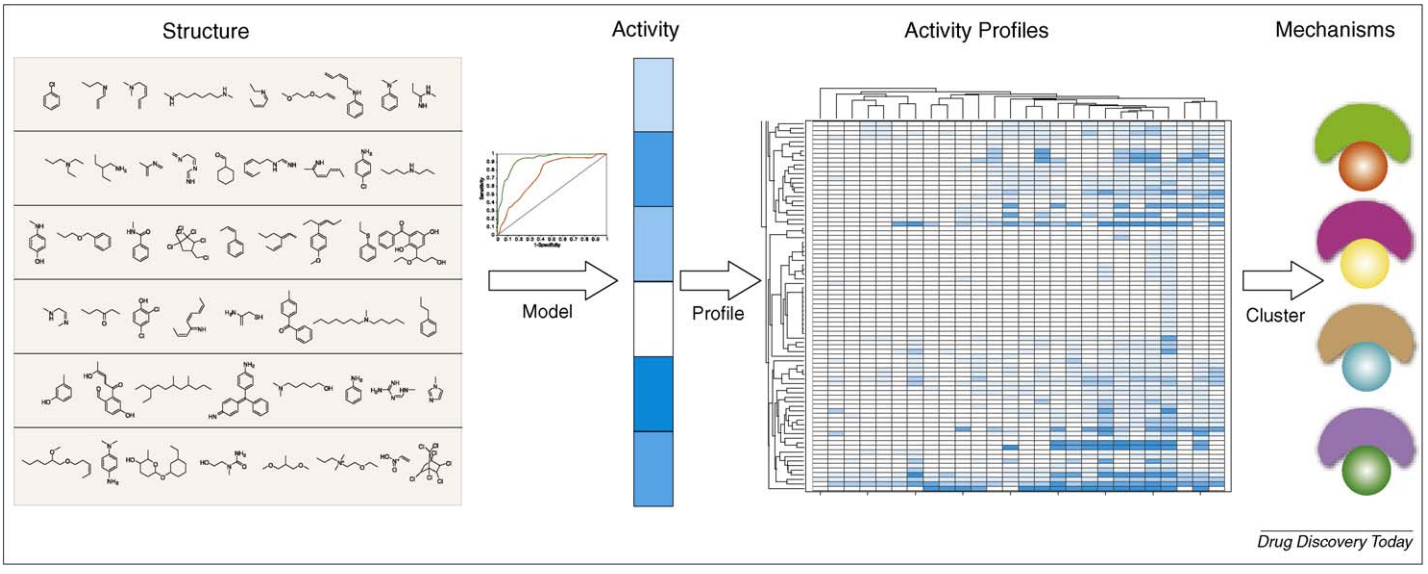

FIGURE 5

Weighted feature significance (WFS) algorithm. The algorithm has been developed to build fragment-based models to predict various toxicity endpoints, such as cell viability, caspase-3/7 activation, mutagenicity and hepatotoxicity, based on compound structure. The sensitivity and specificity of these models have been rigorously tested using receiver operating characteristic curves. Activity or toxicity profiles can be generated by testing compounds against a battery of cell lines or assays measuring the same toxicity endpoint. The activity pattern of a compound across such a battery of assays can be viewed as the compound signature, which can be used subsequently to group compounds into different activity clusters, each representing a distinct toxicity mechanism or mode of action. The underlying assumption is that compounds exhibiting similar activity patterns or signatures are likely to share the same biological target or mode of action. Such approaches can be applied to generate testable hypotheses on mechanisms of compound toxicity. Starting with the structure of a compound, one could then model and predict its toxicity in one assay or cell type and in multiple cell types, which essentially forms the activity pattern or signature that indicates the compound's mechanism of toxicity.

test cases [50]. An analysis of the initial Tox21 collection of 2800 compounds revealed that additional chemicals are required for enhancement of compound diversity in these collections to increase the number of robust structural predictors of the WFS, which validates the previously described initiative to expand the compound collection to more than 10,000 chemicals.

Cellular pathway assays

Although attractive, target-based screens can lead to the identification of active compounds that do not retain their activity in a physiological environment [53]. Thus, cell-based assays offer an alternative assay format in which the readout is dependent on specific components acting on a single signaling pathway. Furthermore, the combination of toxicity pathways associated with adverse health events with engineered cellular assays designed to measure the perturbation of these pathways in response to a chemical is a crucial implementation of the National Academy of Sciences Tox21 report [54]. As an example of such an approach, a β-lactamase reporter gene assay was employed to identify compounds that inhibit [37] or induce [38] hypoxia-inducible factor-1α (HIF-1α) activity (Fig. 6). Hypoxia, the reduction in the normal level of tissue oxygen tension within a tissue, is associated with several pathologies including cancer and inflammation [55]. Under hypoxic conditions, HIF-1 subunits heterodimerize and translocate into the nucleus before binding to a hypoxia-response element (HRE) upstream of target genes that activate angiogenesis and vascular endothelial growth factor (VEGF) [56]. Hypoxic conditions attenuate the degradation of HIF-1α, leading to the transcription of survival genes in many solid tumors and poor cancer prognosis [57]. Thus, compounds that inhibit HIF-1α responsive tumor hypoxia might be a valuable chemotherapeutic approach [37]. Furthermore, a separate screen for inducers of

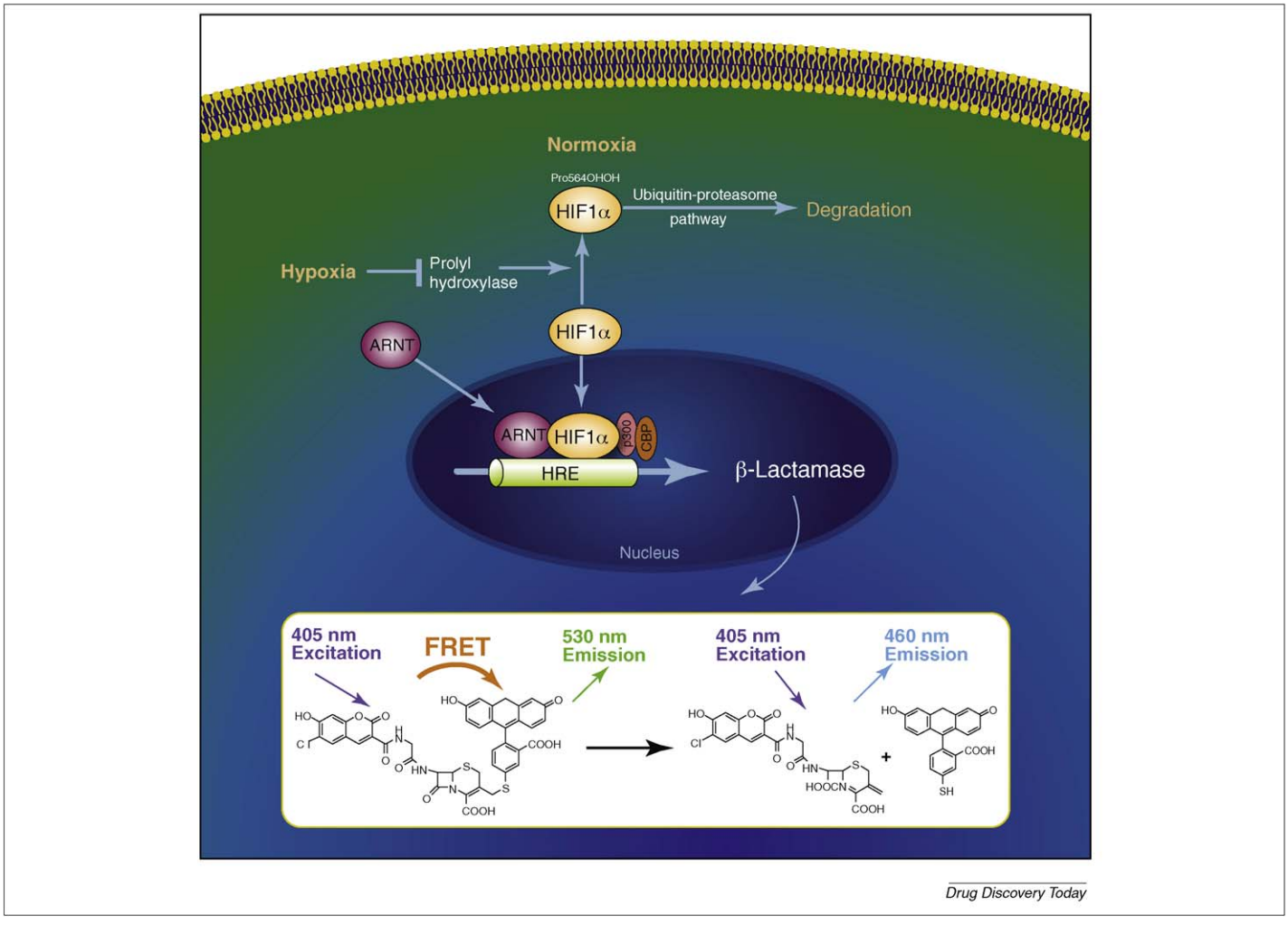

FIGURE 6

Reporter gene assay for HIF-1 activity. During normoxia, the HIF-1α subunit is degraded by the ubiquitin–proteosome pathway. In this case, the absence of β-lactamase expression leaves the fluorescent substrate molecule, which contains coumarin and fluorescein. Excitation of the coumarin results in fluorescence resonant energy transfer to the fluorescein moiety, resulting in the emission of green fluorescent signal (530 nm). Under hypoxic conditions, however, HIF-1α heterodimerizes with the HIF-1β subunit and translocates to the nucleus. Next, the HIF-1 complex binds to HRE regulatory sequences upstream of target genes. In this assay, stimulation under hypoxic conditions results in the transcription of β-lactamase, which cleaves the fluorescent substrate molecule, disrupting energy transfer. Excitation of the coumarin molecule in the presence of β-lactamase enzyme activity results in a blue fluorescence signal (460 nm). The ratio of the blue:green signals provides a normalized reporter response.

hypoxia is also valuable to identify those compounds that can serve as hypoxia mimetics [38].

To identify inhibitors of hypoxia, 73,000 compounds from the MLMSR compound collection [37] were screened between 7 and 15 concentrations in an HRE-*bla* assay performed in ME-180 cells (Tables 1 and 2). In addition, to identify HIF-1α inducers, 1408 compounds from the NTP collection were screened at 14 concentrations also utilizing the HRE-*bla* assay. The screening for both assays performed well and indicated the suitability for qHTS to identify inhibitors and activators of HIF-1α. Three hundred and fifty inhibitors with reliable curve classes were identified and SAR analysis of these compounds yielded 18 structural series sharing a common scaffold. Several follow-up studies, such as the evaluation of compound effects on low-oxygen-induced HIF-1 signaling and VEGF secretion, were employed to ensure the specificity of compound activity in the HIF-1α assay [37]. Overall, the primary qHTS and follow-up compounds identified from SAR analysis demonstrated specificity for inhibition of HIF-1α activity and little to no cytotoxicity and thus seem to be good candidates for further testing in other cancer cell lines or animal models [37]. Conversely, ten compounds were identified and confirmed as inducers of HIF-1α activity from the primary screening using the NTP compound collection [38]. In the follow-up studies, five of ten compounds notably induced VEGF secretion in human ME-180 cells in a concentration-dependent manner. These five compounds were further tested for their dependence on HIF-1 with regard to VEGF secretion by testing them in HIF-1α wild-type and knockout mouse embryonic fibroblast cell lines. Compounds involved in VEGF secretion dependently (such as phenathroline) or independently (such as 7,12-DMBA) of HIF-1α were identified. Finally, these five compounds were tested against a battery of reporter genes driven by hypoxia-responsive gene promoters [38,58] to establish the promoter activity profiles. For example, three compounds (including phenathroline) produced promoter activity profiles very similar to those produced by standard hypoxic conditions (1% O_2) used in cell-based studies [38,58]. Furthermore, this study highlights the use of biological profiling data with hierarchical clustering to group compounds that operate under a similar mode of action [58].

Concluding remarks and future directions

The Tox21 collaboration is combining technology, biology and computational methods to advance *in vitro* testing for toxicology [12]. The NCGC is working with its Tox21 partners to develop next-generation testing methods and alternative approaches to existing methods and to model *in vitro* and *in vivo* responses.

The examples given here are only a few of the assays that have been utilized to date to study specific endpoints or pathways in human and rodent *in vitro* assays; in total, the NCGC has generated more than six million data points and more than 400,000 CRCs for Tox21 chemicals in specific assays. The qHTS-driven production of CRCs for every compound tested provides a data-rich resource for SAR analysis, computational modeling and chemical prioritization for more extensive toxicological evaluation. This directly points to the advantages of using qHTS with regard to compound hazard identification; qHTS will enable a more accurate assessment of compound-induced toxicity using cell-based studies and an idea of starting doses to use in *in vivo* studies. SAR analysis will also enable the identification of toxic compounds with similar structures for follow-up testing. Thus, a basis of hazard characterization with regard to toxicity will emerge before *in vivo* studies. Furthermore, *in vitro* toxicity tests performed in human-derived cell lines might provide important biomarkers of exposure that can be directly tested in human populations [2]. Human risk assessment from some *in vitro* studies might prove difficult; thus, a step-wise approach starting with qHTS, computational modeling and carefully designed tissue- and species-specific cell-based assays will provide a stronger and mechanistically predictive approach for *in vivo* testing and human risk assessment [2].

Building on the solid foundation described here, future Tox21 goals include the inclusion of new platforms for qHTS (such as high-content screening and nanotechnology), assessment of genetic variation involved in human and rodent toxicity, incorporation of metabolism and biotransformation capability into the current and future assays, identification and prioritization of crucial cellular pathways and key targets for screening, expansion of the compound libraries including compounds that are DMSO or water insoluble, discerning the link between observed perturbations *in vitro* and pathologies in exposed humans and the creation of relational public databases and tools to interrogate the screening data. Table 1 comprises some of the current and future Tox21 assays at the NCGC, such as those for oxidative stress response [54]. Data production in Tox21 is now moving into its exponential growth phase, and over the next several years the interdisciplinary Tox21 collaboration will continue to innovate in assay biology, screening and computation, to usher in a new era of efficient, mechanistic and predictive chemical toxicology.

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