High-Throughput Screening and Hazard Testing Prioritization

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Abstract

Tox21 is a collaborative effort among the National Center for Advancing Translational Sciences, the Environmental Protection Agency, the National Toxicology Program, and the Food and Drug Administration to elucidate the toxic effects of compounds found in the environment and/or created by humans. Since 2008, this program has screened many different pathways, targets, or phenotypes (more than 70 assays) using an in vitro quantitative high-throughput screening approach. Endocrine disruption and stress-related signaling pathways have been the main focus of the Tox21 screening program. Nuclear receptors play an important role in endocrine disruption, modulating many different biological processes and metabolism. It is therefore important to classify endogenous and exogenous compounds for their ability to alter the function or quantity of these nuclear receptors. Stress-related signaling pathways are necessary for body homeostasis and are involved in many disease states as well. Identifying compounds which induce stress signaling pathways in the body is prudent to fully determine the safety of an environmental chemical. This book chapter describes an in-depth analysis of Tox21, a summary of select examples of their assays, and the future plan for the screening program.

Keywords

Acetylcholinesterase • Mitochondria • Nuclear receptor • Predictive toxicology • Quantitative high-throughput screening • Tox21

1 Introduction to Toxicology in the Twenty-First Century (Tox21)

An increasing amount of chemicals is released into the atmosphere each year, requiring more expedient and thorough screening techniques in order to effectively determine the toxicological effects on humans and the environment. Traditionally, the main source in identifying compound toxicity was in vivo animal models utilized to generate a detailed profile of each chemical (Greaves et al. 2004). These animal models were able to detect one specific toxicological endpoint (e.g., reproductive, oral, dermal, or developmental toxicity) per experiment (Shukla et al. 2010). However, these models may not fully represent the effects on humans, are performed at a low throughput, and/or are expensive to perform, leading to a lack in sufficient knowledge to evaluate safety concerns (NRC 1984). Regardless of these limitations, the preponderance of knowledge for a drug's toxicity and therapeutic window has mainly been founded based on these types of experiments, as a result of the lack of other robust in vitro options (Zurlo et al. 1994). Owing to these challenges, future chemical toxicity testing was suggested in a report by the National Research Council (NRC), which stated a predictive toxicology approach relying on identifying chemical modulators of cellular pathways using human cell-based in vitro assays and computational modeling was a novel method in tackling the identification of potential health risks (Gibb 2008). A collaborative effort, called Tox21, among the Environmental Protection Agency (EPA), the National Toxicology Program (NTP), and the National Chemical Genomics Center (NCGC) which is now a part of the National Center for Advancing Translational

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Sciences (NCATS) was generated to bring compound toxicity testing into the twenty-first century. In 2010, the Food and Drug Administration (FDA) joined this effort. Since the inception of this esteemed program, over 200 peer-reviewed scientific articles related to the Tox21 program have been published within about 56 journals (Thomas et al. 2018). All data acquired throughout this program is also available online for public viewing (e.g., https://tripod.nih.gov/tox21 and https://pubchem.ncbi.nlm.nih.gov), which is intended to progress toxicology and science beyond the scope that a single program could achieve.

Each of the collaborating entities plays a specific role throughout the Tox21 program. In 2004, NTP generated a vision for what science in the twenty-first century should look like, including the exploration of an alternative method to animal testing. The idea quickly led to establishing a high-throughput screening (HTS) program, which allowed for the testing of toxicity for thousands of environmental agents at a time (https://ntp.niehs.nih.gov/results/tox21/history-index.html). The EPA contributes to Tox21 by prioritizing toxic chemicals through the generation of data and predictive models using the Toxicity Forecaster (ToxCast, https://www.epa.gov/chemical-research/toxicity-forecasting). Building novel pathways to study the toxicity of the compounds, which the FDA regulates, fits into the overall theme of the Tox21 program. To fulfill this goal, the FDA has taken substantial strides toward the generation of comprehensive predictive toxic models (FDA's Predictive Toxicology Roadmap 2017). Lastly, NCATS' role for Tox21 includes performing the most advanced HTS models which the current technology has to offer so that more can be known about the hazards of commonly used chemicals in a quick and efficient manner (https://ncats.nih.gov/tox21/about/goals).

2 Tox21 Quantitative High-Throughput Assay Screening and Data Analysis

Quantitative HTS (qHTS) has become an innovative way to efficiently screen hundreds and thousands of chemical compounds at multiple concentrations in a short time. Each Tox21 qHTS assay is optimized into a 1536-well plate format so that each compound of the Tox21 10 K compound library, which includes ~ 8900 unique compounds, can be quickly tested at 15 different concentrations in triplicate. This compound collection, put together by NCATS, NTP, and the EPA, of environmental chemicals and clinically used drugs includes solvents, food additives, drinking water disinfection by-products, sunscreen additives, preservatives, industrial chemicals, flame retardants, synthesis by-products, natural product components, plasticizers, pesticide/herbicide additives and their metabolites, and therapeutic agents. Once an initial optimization is complete, the Tox21 10 K compound primary screen is run and potential active compounds are selected for further studies (Fig. 1). Any compound going through mechanism-based assays also go through compound quality control (QC) to verify the purity and specific molecular weight of each selected compound.

The assay types used for a primary screen are usually pathway-, target-, or phenotype-based assays. In this book chapter, we discuss certain target-specific assays (see Sects. 2.1 and 2.2); however, a more in-depth analysis and explanation of a few phenotypic assays can be found in a previous review paper (Hsu et al. 2017). Once the primary screen is complete, whether phenotypic, target, or pathway based, there is the potential ability to confirm certain compounds as agonists or antagonists for the specific endpoint being measured. Through a robust assay performance and a rigorous analysis of the data (see Sect. 2.3), Tox21 performs the initial step into profiling these potentially hazardous chemicals.

Owing to its ability to quickly and efficiently use a qHTS platform, Tox21 has performed more than 70 screens to identify the activity caused by environmental compounds in different signaling pathways and targets. Each screen was vigilantly optimized and tested to ensure robust performance data. A list of all current screens, performed by Tox21, is displayed in Table 1. The specific cell lines used for each assay target or pathway are shown alongside the endpoint readout followed by either a reference or where the cell line was acquired. For a few assay targets (androgen receptor, estrogen receptor α, and estrogen-related receptor α), multiple engineered stable cell lines were utilized to fully identify compounds, from the Tox21 collection, which were active for those respective assays.

2.1 Nuclear Receptors

Nuclear receptors play a pivotal role in development, homeostasis, and/or disease states (Giguere 1999). Modulation of some nuclear receptors, through environmental chemicals and/or exogenous compounds, can be hazardous or beneficial to the human body depending on the extent of the alteration. Classifying these compounds as inhibitors or inducers of certain nuclear receptors would expound upon the knowledge of their toxic or therapeutic effect on humans. Modifying the androgen receptor (AR), estrogen receptor (ER), or estrogen-related receptor (ERR) pathways can lead to endocrine disruption and potentially cause reproductive and developmental disorders, as well as cancer (Gonzalez et al. 2019; Park et al. 2016). Altering the activity of the constitutive androstane receptor (CAR) or pregnane X receptor (PXR) can potentially be utilized for therapeutic purposes in certain disease states (Gao and Xie 2010; Hedrich et al. 2016) or identify potentially hazardous drug–drug interactions. The

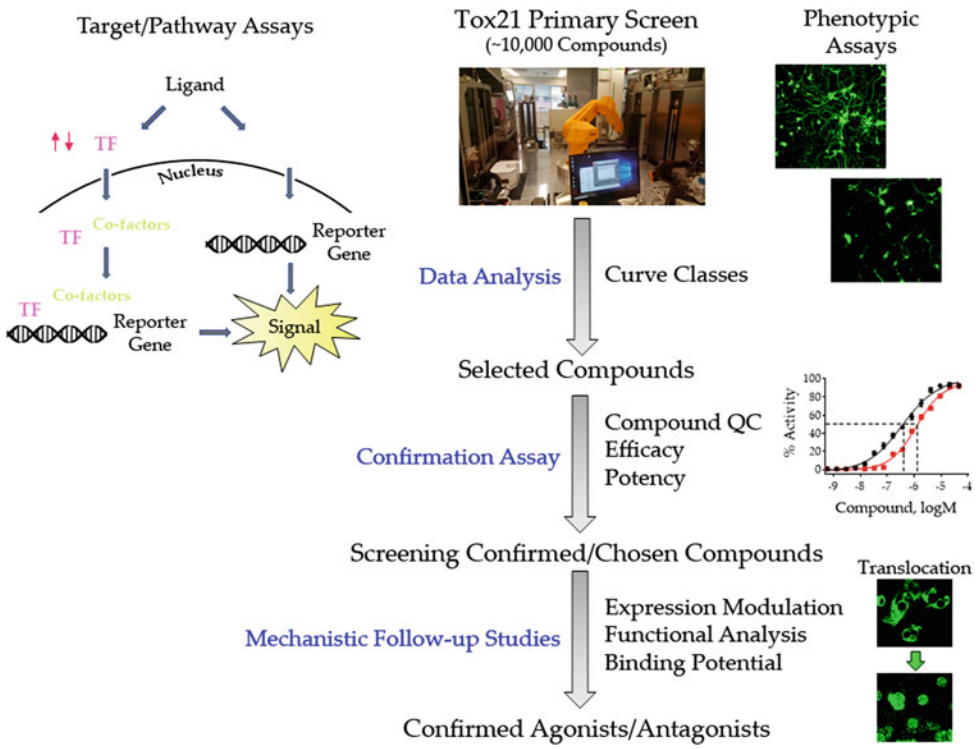

Fig. 1 Each screen starts with about 10,000 compounds from the Tox21 collection being assayed in a high-throughput screening manner, in either target/pathway or phenotypic-based platforms. The blue-colored words on the left side of the figure are the actions taken for each step to occur. The right side displays the endpoints used to generate a cutoff value for each future step, so that the collection can be further narrowed down in size based on significance. NR = nuclear receptor, TF = transcription factor

Tox21 program has screened different stably transfected cell lines for their activity on these nuclear receptors.

AR Agonist Identification The transcriptional factor AR regulates male sexual development, affects female fertility, and is involved in pathological processes which alter the state of certain diseases such as Kennedy's disease, Klinefelter's syndrome, and certain reproductive cancers (Culig et al. 2002; Pihlajamaa et al. 2015; Chang et al. 2014; Skakkebaek et al. 2014; Tanaka et al. 2012). Therefore, it is important to identify compounds, from the environment and elsewhere, which modify the activity of this important nuclear receptor. The Tox21 10 K chemical library was screened to categorize compounds as potential AR agonists if they generated AR activity in at least one of the two reporter gene cell lines utilized (Lynch et al. 2017). Through this endeavor, Tox21 scientists identified a potentially novel class of AR agonists—fluoroquinolone antibiotics. A binding assay was performed on the actives identified from the reporter gene assays to further define each compound as an AR agonist due to binding capability; an overall 72% concordance rate between binding and reporter assays demonstrated a high predictive ability of the cell-based primary screening results. Translocation of AR from the cytoplasm into the nucleus, the first step of activation, was also observed for 16 of the 17 most promising AR agonists, including GSK232420A, norethisterone enanthate, and prulifloxacin. This study was a first step in identifying certain compounds as potentially hazardous with respect to the AR pathway.

ERR Modulation Profiling Alongside its previously mentioned endocrine disruption involvement, ERR is also involved in energy homeostasis, as well as controlling mitochondrial oxidative respiration (Leone et al. 2005; Lin et al. 2004; Luo et al. 2003). A poor prognosis for breast, prostate, and endometrial cancer occurs when increases in ERRα gene expression levels are found within the respective tumors (Fradet et al. 2016; Matsushima et al. 2016; Park et al. 2016). With all the pathways and disease states that ERR regulates, it is important to identify agonists (toxicants) and antagonists (potential therapeutics) to determine the full scope of internalizing these compounds. One of the unique features of this nuclear receptor is its crosstalk with peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), which is sometimes a necessary component to ERRα activation (Teng et al. 2014). When the Tox21 10 K compound library was screened for ERR agonists, a class of novel compounds was identified—statins (Lynch et al. 2018). Interestingly, this group of compounds had no effect in the PGC/ERR cell line, which implies activation of ERRα independently of PGC-1α. A known ERRα inhibitor, XCT790, was co-treated with each statin and screened again using the ERR cell line. Each of the six statins showed concentration-dependent inhibition when co-treated with 0, 5, or 10 μM XCT790, indicating ERR dependence. Two of the statins, cerivastatin sodium and fluvastatin, were also used to treat ERR siRNA transfected cells which showed an inhibition in three ERRα-regulated genes, ERR, COX8, and

Table 1 Cell Line and Assay Readouts for Tox21 Screens

Cell lines	Assay target	Assay readout	Cell lines acquired/Reference
AChE-SH-SY5Y	Acetylcholinesterase	Absorbance Fluorescence	Li et al. (2017a)
AhR-HepG2	Aryl hydrocarbon receptor	Luminescence	He et al. (2011)
AP1-ME180	Activator protein 1	Fluorescence	Invitrogen (Carlsbad, CA)
AR-HEK293	Androgen receptor (LBD)	Fluorescence	Lynch et al. (2017)
AR-MDA	Androgen receptor (full)	Luminescence	Lynch et al. (2017)
Aromatase-MCF7	Aromatase	Luminescence	Chen et al. (2015)
CAR-HepG2	Constitutive androstane receptor	Luminescence	Lynch et al. (2013, 2014, 2015, 2016, 2019a)
HepG2	Caspase-3 and Caspase-7	Luminescence	Huang et al. (2008)
DT40	DNA repair	Luminescence	Nishihara et al. (2016)
ELG1-HEK293	Telomere length regulation protein ELG1	Luminescence	Fox et al. (2012)
ERα-HEK293	Estrogen receptor α	Fluorescence	Huang et al. (2011, 2014), Rotroff et al. (2014), Judson et al. (2015)
ER-MCF7	Estrogen receptor α	Luminescence	Huang et al. (2014), Judson et al. (2015), Rotroff et al. (2014)
ERβ-HEK293	Estrogen receptor β	Fluorescence	Invitrogen
ERR-HEK293	Estrogen-related receptor α	Luminescence	Lynch et al. (2018, 2019b), Teng et al. (2017)
ESRE-Hela	Endoplasmic reticulum stress response element	Fluorescence	Bi et al. (2015)
FXR-HEK293	Farnesoid X receptor	Fluorescence	Hsu et al. (2014, 2016a, 2016d)
GR-Hela	Glucocorticoid receptor	Fluorescence	Invitrogen
CHO	H2A histone family member X	Fluorescence	ATCC (Manassas, VA)
HCT116	HDAC I and HDAC II	Luminescence	Hsu et al. (2016c)
HRE-ME180	Hypoxia-inducible factors	Fluorescence Luminescence	Hsu et al. (2016b), Khuc et al. (2016)
HSE-Hela	Heat shock element	Fluorescence	Hancock et al. (2009)
MMP-HepG2-ME180	Mitochondrial membrane potential	Fluorescence	Li et al. (2017b), Sakamuru et al. (2012, 2016), Xia et al. (2018), Attene-Ramos et al. (2013, 2015)
NFκB	Nuclear factor-kappa B	Fluorescence	Miller et al. (2010)
Nrf2/ARE-HepG2	Antioxidant response element	Fluorescence	Shukla et al. (2012), Zhao et al. (2016)
p53-HCT-116	p53	Fluorescence	Witt et al. (2017)
PGC/ERR-HEK293	Estrogen-related receptor α	Luminescence	Lynch et al. (2018, 2019b), Teng et al. (2014)
PPARδ-HEK293	Peroxisome proliferator-activated receptor δ	Fluorescence	Invitrogen
PPARγ-HEK293	Peroxisome proliferator-activated receptor γ	Fluorescence	Invitrogen
PR-HEK293	Progesterone receptor	Fluorescence	Invitrogen
PXR-HepG2	Pregnane X receptor	Luminescence	Shukla et al. (2011), Dr. Taochen Chen
RAR-C3H10T1/2	Retinoic acid receptor	Luminescence	Chen et al. (2016)
RORγ-CHO	Retinoic acid-related orphan receptor γ	Luminescence	Dr. Anton M. Jetten
RXR-HEK293	Retinoid X receptor	Fluorescence	Invitrogen
ShhGli1-3T3	Sonic hedgehog pathway	Luminescence	Dr. Yanling Chen Dr. David H. Reese
SMAD-HEK293	Smad signaling pathway	Fluorescence	Invitrogen
TRE-GH3	Thyroid hormone receptor	Luminescence	Freitas et al. (2014)
TRHR-HEK293	Thyrotropin-releasing hormone receptor	Fluorescence	Codex Biosolutions (Gaithersburg, MD)
TSHR-HEK293	Thyroid stimulating hormone Receptor	Fluorescence	Codex Biosolutions
VDR-HEK293	Vitamin D receptor	Fluorescence	Invitrogen

IDH3. Owing to the newly identified connection of statins with ERRα agonism, it is plausible to assume that these compounds might potentiate a poor outcome or progression in different types of cancer. Further studies need to be performed to fully understand the implications of these initial studies performed by the Tox21 program.

A screen was performed on the Tox21 10 K compound collection to identify ERRα antagonists as well (Lynch et al. 2019b). Two major groups, antineoplastic agents and pesticides, were classified as antagonists of ERRα activity as well as some compounds inhibiting mRNA expression of five downstream genes (cytochrome c oxidase subunit 8A, COX8A; isocitrate dehydrogenase 3 (NAD(+)) alpha, IDH3α; peroxisome proliferator activated receptor alpha, PPARα; cytochrome c oxidase subunit 4I1, COX4I1; and cytochrome c). A heat map was also displayed showing the activity of each compound on multiple targets and pathways, including AR, nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE), CAR, ER, ERR, farnesoid X receptor, thyroid hormone receptor, mitochondrial membrane potential, p53, PPARγ, progesterone receptor, retinoic acid receptor, retinoic acid-related orphan receptor, and sonic hedgehog. Most of the antineoplastic agents (artemisinin, bortezomib, carfilzomib, decitabine, etoposide, topotecan, and suberoylanilide hydroxamic acid) activated the p53 pathway which is consistent with a previous study (Guo et al. 2019), while most of them (artemisinin, bortezomib, carfilzomib, etoposide, gimatecan, methodichlorophen, topotecan, and suberoylanilide hydroxamic acid) also had antagonistic activity in the sonic hedgehog assay, which is a pathway known for being associated with tumor development (Jiang and Hui 2008). Interestingly, many of the pesticides demonstrated an activation of the antioxidant responsive element (ARE) pathway (Lynch et al. 2019b) which is known to counter oxidative stress (Johnson et al. 2008). However, it is likely these pesticides are causing the formation of free radicals, which induce oxidative stress, and ultimately, the increase in the ARE pathway would then occur. The Tox21 10 K compound study, for both agonist and antagonist identification of ERRα modulation, was a major step into investigating the mechanism of action for many compounds, though future studies are certainly warranted and necessary to fully understand the scope of each specific compound and how they will interact in the body.

CAR Agonist Classification Classically, CAR had previously been known to regulate drug metabolizing enzymes and transporters which have an effect on all phases of drug metabolism (Qatanani and Moore 2005). It has recently been shown that CAR also plays an important role in energy homeostasis, as well as certain cancer progression and treatments (Gao and Xie 2010; Hedrich et al. 2016; Yamamoto et al. 2004). Owing to this novel function, it is important to identify any novel selective CAR agonists, which is what the Tox21 10 K compound collection was screened for in a previous publication (Lynch et al. 2019a). Four compounds (neticonazole, diphenamid, phenothrin, and rimcazole) were identified to be hCAR activators through a confirmation study, using human primary hepatocytes, examining mRNA and protein expression of cytochrome P450 (CYP) 2B6 and CYP3A4. A nuclear translocation assay was also performed to display these four compounds exhibiting the first step of hCAR activation—translocation from the cytoplasm into the nucleus. Future studies will need to be performed to truly understand the usage of these compounds in a therapeutic capacity, as well as to identify possible drug–drug interactions which may occur.

2.2 Stress-Related Pathways

Acetylcholinesterase Inhibitor Profiling Acetylcholinesterase (AChE EC 3.1.1.7), found primarily in neuromuscular junctions and cholinergic brain synapses, is an enzyme involved in the termination of impulse transmission with a highly specific catalytic activity for hydrolyzing acetylcholine (ACh) into choline (Quinn 1987; Taylor and Radic 1994). After this transformation, choline is taken up into the pre-synaptic nerve and combined with acetyl-CoA to produce acetylcholine through the action of choline-acetyltransferase (Soreq and Seidman 2001). The majority of AChE can be found in an amphiphilic globular tetramer (G4) form or a monomeric G1 form (Fernandez et al. 1996; Wang and Tang 2005), inside either motor neurons or sensory fibers (Massoulie et al. 1993). Within these two forms, there are two subsites of the active site, which are called the anionic subsite and the esteratic subsite (Nachmansohn and Wilson 1951). The anionic subsite binds ACh and quaternary ligands, acting as competitive inhibitors to assist in inhibiting AChE (Mooser and Sigman 1974; Wilson and Quan 1958). In addition, one or more peripheral anionic sites, distinct from the choline-binding pocket, were also identified to bind ACh and other quaternary ligands acting as uncompetitive inhibitors (Taylor and Lappi 1975). In the esteratic subsite, the basic function of hydrolyzing ACh into acetate and choline is performed (Nachmansohn and Wilson 1951). Inhibition of AChE can lead to acetylcholine accumulation in the synaptic space, enhanced nicotinic and muscarinic receptor stimulation, as well as disrupted neurotransmission (Colovic et al. 2013). Therefore, AChE inhibitors play an important role in both toxicology and pharmacology, and it is important to identify compounds which can be associated as such.

Depending on the mode of action, AChE inhibitors can be divided into two subcategories: reversible and irreversible inhibitors. Reversible inhibitors, competitive or noncompetitive, have therapeutic applications, while irreversible inhibitors are more commonly associated with having toxic

side effects. Tacrine, a noncompetitive reversible AChE inhibitor, was the first approved drug for the treatment of Alzheimer's disease; however, due to its hepatotoxic side effects, the use of this drug has since been eradicated (Watkins et al. 1994). Other AChE inhibitors which have been approved by the U.S. Food and Drug Administration (FDA), to be utilized as drugs, include donepezil, rivastigmine, and galantamine (Bond et al. 2012). Carbamates, a group of reversible AChE inhibitors, are organic compounds which can be used as therapeutic drugs (for treating Alzheimer's disease, glaucoma, and Parkinson's disease), pesticides, parasiticides (in veterinary medicine), or as a prophylaxis of organophosphorus compound poisoning (Giacobini 2000). Organophosphorus pesticides exert their pesticidal activity by inhibiting AChE activity irreversibly, causing toxic effects such as headaches, impaired memory and concentration, disorientation, severe depression, irritability, drowsiness, or insomnia (Colovic et al. 2013). Many environmental pollutants, such as heavy metals, other pesticides, polycyclic aromatic hydrocarbons, and dioxins also show inhibition of AChE activity (Ademuyiwa et al. 2007; Kang and Fang 1997; Reddy and Philip 1994; Xie et al. 2013). Therefore, measurement of AChE activity has been widely used as a biomarker of toxic effects on the nervous system following exposure to organophosphate and carbamate pesticides (Lionetto et al. 2013). Although AChE inhibitors have significant consequences to human health, there are still a large number of compounds which have not been identified as irreversibly inhibiting AChE activity, including synthesized drug candidates, food additives, and industrial chemicals.

Regarding the role of AChE in pharmacology and toxicology, many biochemical readouts, including spectrophotometric, colorimetric, radiometric, fluorometric, and electro-chemical, have been used to measure the activity of cholinesterase (Holas et al. 2012; Miao et al. 2010). The Ellman method is regarded as the golden method for determining AChE activity (Ellman et al. 1961). This highly regarded assay still has certain limitations, such as its reaction with AChE reactivators (e.g., oximes) as well as interference from hemoglobin in the blood (Sinko et al. 2007). Recently, a fluorescent assay has been developed using whole blood and cultured human neuroblastoma cells (SH-SY5Y) in which AChE activity was determined by measuring the fluorescence of resorufin, which is produced from coupled enzyme reactions involving acetylcholine, horseradish peroxisome, choline oxidase, and Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) (Santillo and Liu 2015). With the growing number of chemicals in the environment, as well as the need for novel therapeutics, developing AChE inhibition assays that are suitable to qHTS platforms will greatly add value to human health.

While the previously mentioned fluorescent method using Amplex Red was developed in a homogenous format using SH-SY5Y cells, an enzyme-based assay using eel AChE was also optimized into a 1536-well format. In the Tox21 program, both assays were used to screen 1368 compounds, which included a library of pharmacologically active compounds (Library of Pharmacological Active Compounds, LOPAC) and 88 additional compounds, at multiple concentrations in a qHTS format (Li et al. 2017a). Each assay exhibited exceptional performance characteristics, including assay signal window and reproducibility. A group of inhibitors were identified from this study, including known (e.g., physostigmine and neostigmine bromide) and novel AChE inhibitors (e.g., chelerythrine chloride and cilostazol). As a result, this screening method developed for AChE was determined to be a useful tool for profiling inhibitors of this enzyme.

Some organophosphorus pesticides are not active AChE inhibitors in their parent form and require bioactivation in order to be effective (Sultatos 1994). A high-throughput AChE assay, in a 1536-well format, using liver microsomes was developed to provide an accurate estimation of metabolism using an in vitro method. In order to validate this assay, a group of organophosphorus pesticide compounds, containing both parental compounds and their active metabolites, was screened for AChE inhibition activity (Li et al. 2019). The assay utilized recombinant human AChE protein with human or rat liver microsomes; the Ellman colorimetric or fluorescent method was then used to measure AChE activity. Once the assay was completed, the reproducibility was evaluated, and each compound was ranked in the order of potency. Large potency differences between some parent compounds and their metabolites were observed in the assay with microsome addition. Many parental organophosphorus pesticides, such as chlorpyrifos, tebupirimfos, and chlorethoxyfos, only showed the inhibitory effects on AChE after addition of the metabolic component into the reaction, signifying the need of bioactivation to occur in order to become potent AChE inhibitors. Together, these data demonstrated the promising ability to profile AChE inhibitors using metabolic simulation; further studies will be vital to acquire the full extent of safety assessment for each chemical. Cell- and enzyme-based AChE assays would increase the library of AChE inhibitors, having a significant impact on both the pharmaceutical and toxicology fields.

Mitochondrial Toxicant Identification Mitochondria, the intracellular powerhouse, generate 95% of cellular energy in the form of ATP through oxidative phosphorylation (Wallace et al. 1997). Mitochondrial membrane potential (MMP), the electric potential across the inner mitochondrial membrane, is generated by the mitochondrial electron transport chain through a series of redox reactions (Chen 1988). MMP is a key parameter for assessing mitochondrial function, cell health, and apoptosis. Several

cationic lipophilic fluorescent dyes are routinely used to evaluate MMP changes, including rhodamine 123 (R123) (Chen 1988), chloromethyl tetramethyl rosamine (Macho et al. 1996), tetramethylrhodamine methyl and ethyl esters (TMRM and TMRE) (Farkas et al. 1989), 3,3′-diehexiloxadicarbocyanine iodide (DiOC6(3)) (Farkas et al. 1989), and 5,5′,6,6′-tetracholoro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Salvioli et al. 1997).

Mitochondrial membrane potential indicator (m-MPI), a water-soluble derivative of JC-1 with improved signal to background performance, was developed by Codex Biosolutions to determine the MMP of certain chemicals. Using m-MPI, a homogenous cell-based assay was developed, optimized, and miniaturized into a 1536-well plate for assessing changes in MMP to determine mitochondrial toxicity (Sakamuru et al. 2012). In healthy cells, m-MPI accumulates in mitochondria as red fluorescent aggregates (emission at 590 nm); conversely, after mitochondrial toxicant treatment, the cells depolarize and become less healthy maintaining m-MPI in the cytoplasm as green fluorescent monomers (emission at 540 nm). The calculation of the ratio of red/green channel readings is then used to assess the mitochondrial function of the cells (Sakamuru et al. 2016). Using the m-MPI assay, the chemicals from the LOPAC, NTP, and Tox21 10 K compound collections were screened for mitochondrial toxicity by evaluating the effect of chemical compounds on changes of MMP in HepG2 cells (Attene-Ramos et al. 2015; Attene-Ramos et al. 2013; Sakamuru et al. 2012). The screening for mitochondrial toxicants from the NTP collection resulted in about 5% of the compounds having a potential decrease in MMP, while the selected active ones were further clustered based on structural similarity (Attene-Ramos et al. 2013). Some of these compounds were selected for confirmation and mechanistic studies based on potency, efficacy, and structural diversity by selecting at least one representative compound from each cluster. This study demonstrated the effectiveness of Tox21's strategy for evaluating the toxicological properties of a chemical collection.

In a separate study, the compounds identified as MMP inhibitors from the initial Tox21 10 K compound library screen were further profiled to identify the structural features associated with MMP changes (Attene-Ramos et al. 2015). For this approach, a multiplexed qHTS (measuring two endpoints: MMP and intracellular ATP) method was combined with structure-based clustering analysis. After the primary screening, about 11% of the compounds from the Tox21 10 K compound collection showed a decrease in MMP, among which several triarylmethane dyes and organotin compounds were identified to be potent. The cluster analysis from this study displayed that different categories of compounds, including flavonoids, chlorinated organic insecticides, parabens, and thiazolidinedione-based drugs, are capable of decreasing MMP. The most potent MMP toxicants from the Tox21 primary screen were further tested with a tier-based approach that evaluated the mechanistic characterization of chemicals affecting mitochondrial function, which can potentially reduce animal use for toxicological testing (Xia et al. 2018). Based on the follow-up m-MPI assay, performed in HepG2 cells and rat hepatocytes, a group of compounds were selected for further testing in assays which had an effect on reactive oxygen species (ROS) production, p53 signaling pathway modulation, Nrf2/ARE pathway modulation, cellular respiration (i.e., mitochondrial oxygen consumption), cellular Parkin translocation, as well as larval development and ATP content in the nematode Caenorhabditis elegans. From this study, a group of known mitochondrial complex inhibitors, uncouplers, and a few not well-characterized mitochondrial toxicants (e.g., lasalocid, picoxystrobin, pinacyanol, and triclocarban) were identified.

2.3 Tox21 Data Analysis

Tox21 data analysis for raw data processing as well as concentration–response curve fitting and classification follows a standardized qHTS data analysis strategy that has been developed at NCATS (Inglese et al. 2006). The raw plate reads for each concentration point are initially normalized to the positive control compound (agonist mode: 100%; antagonist mode: −100%) and negative control (DMSO; 0% for both agonist and antagonist modes). Percent activity is then calculated as equal to $((V_{compound} - V_{DMSO})/(V_{positive} - V_{DMSO})) \times 100$, where $V_{compound}$ denotes the compound well values, $V_{positive}$ denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO wells. The values are then corrected using two compound-free control plates (DMSO-only plates) placed before the compound plate stack. Concentration–response curves for each compound are fitted to a four-parameter Hill equation yielding concentrations of half-maximal activity (AC_{50}) and maximal response (efficacy) values (Wang et al. 2010). Concentration–response curves are then designated as classes 1–4 based on efficacy, quality of fit, and the number of data points observed above background activity. Each curve class is converted to a curve rank such that more potent and efficacious compounds with higher quality curves are assigned a higher rank (5–9) and inactive compounds are assigned curve rank 0 (Huang 2016). These curve ranks are numerical measures of each compound's activity. Since the Tox21 screens are run in triplicate, the assay performances from three independent runs are measured by reproducibility scores. Three types of reproducibility calls (match, mismatch, and inconclusive) are

made based on the concordance of the replicate assay runs (Huang et al. 2011). The active compounds are selected and consequently cherry-picked for secondary follow-up studies. Lastly, as previously mentioned, all Tox21 screening data is released to public domains such as PubChem, a database for chemical compounds which includes bioactivity data alongside their respective names and general information (http://pubchem.ncbi.nlm.nih.gov).

3 Usage of Tox21 Data and Future Directions

The vast amount of data generated from high-throughput screenings are of valuable resources for many scientific areas, including data mining and predictive modeling studies. The high-quality concentration–response data generated so far, as a part of the Tox21 collaboration, including a broad array of phenotypic-, target-, and pathway-specific assays, provide datasets which can be used in quantitative structure–activity relationship (QSAR) studies to build robust computer models. In 2014, the Tox21 data challenge utilized this immense amount of data by asking participants to predict the effect of compounds on cellular signaling pathways and targets using chemical structure information. The challenge generated several high-quality models, demonstrating that computational approaches can provide meaningful predictions in the toxicology field (Huang et al. 2016a). By combining the structural information of the compounds with the Tox21 screening data, predictive models for 72 in vivo toxicity endpoints were built with a cluster-based approach, which suggests that primary screening data not only serves as in vitro signatures for predicting in vivo toxicity but also helps to prioritize compounds for further toxicological evaluation (Huang et al. 2016b). Predictive models for human-adverse drug effects have also been built using the Tox21 screening data with or without compound structure data, as well as a combination of structure and screening data with or without drug target annotations and animal toxicity endpoints (Huang et al. 2018), which validated that further addition of drug-target annotations to the current dataset resulted in improved model performances. Therefore, these predictive computational models combining screening data alongside structural features will facilitate a faster approach for assessing interference of compounds on various targets and/or endpoints.

The Tox21 program has been an instrumental asset to prioritizing environmental chemicals as toxic or safe. However, throughout the process, new challenges have arisen due to the results from these previous methods. A main biological issue discovered was the lack of metabolically competent systems within the assays, meaning that only the parent compound of a chemical was being assessed, as well as an inability to determine if certain chemicals were still available once the initial metabolic process of the body was complete (Thomas et al. 2018). Due to these difficulties, Tox21 plans to use more physiologically relevant systems, including the use of liver microsomes and cells already comprising certain metabolizing enzymes. Another challenge that Tox21 is attempting to overcome is the issue of covering every pathway involved in a complex organism. Moving forward, new technologies will be used that can provide information that represents the global transcriptome, including global gene expression. Throughout the history of Tox21, it has become clear that this program is not only necessary for the identification of toxic chemicals but is a revolving, ever-changing entity which strives to improve and expand upon the knowledge of toxicity testing in the future.

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