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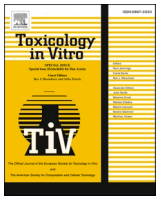

Review

Application of ToxCast/Tox21 data for toxicity mechanism-based evaluation and prioritization of environmental chemicals: Perspective and limitations

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ABSTRACT

In response to the need to minimize the use of experimental animals, new approach methodologies (NAMs) using advanced technology have emerged in the 21st century. ToxCast/Tox21 aims to evaluate the adverse effects of chemicals quickly and efficiently using a high-throughput screening and to transform the paradigm of toxicity assessment into mechanism-based toxicity prediction. The ToxCast/Tox21 database, which contains extensive data from over 1400 assays with numerous biological targets and activity data for over 9000 chemicals, can be used for various purposes in the field of chemical prioritization and toxicity prediction. In this study, an overview of the database was explored to aid mechanism-based chemical prioritization and toxicity prediction. Implications for the utilization of the ToxCast/Tox21 database in chemical prioritization and toxicity prediction were derived. The research trends in ToxCast/Tox21 assay data were reviewed in the context of toxicity mechanism identification, chemical priority, environmental monitoring, assay development, and toxicity prediction. Finally, the potential applications and limitations of using ToxCast/Tox21 assay data in chemical risk assessment were discussed. The analysis of the toxicity mechanism-based assays of ToxCast/Tox21 will help in chemical prioritization and regulatory applications without the use of laboratory animals.

1. Introduction

Under stringent chemical regulation regimes, toxicity information is required for many chemicals. New approach methodologies (NAMs) have been developed to efficiently and rapidly screen for the potential toxicity of chemicals. NAMs are a broad concept that includes *in silico*, *in chemico*, and *in vitro*, and aim to improve the understanding of the hazards of chemicals using new tools, including high-throughput screening (HTS) (ECHA, 2016). The data produced through NAMs can provide information on the mode of action of chemicals, allowing mechanism-based predictions of *in vivo* toxicity (Shukla et al., 2010). NAMs can also be used for toxicity screening or prioritizing chemicals in the regulatory context (Kavlock et al., 2018). For example, in Canada, a case study was conducted using various NAMs for regulatory purposes in prioritization and risk assessment of substituted phenols under the Chemicals Management Plan, and *in vitro* HTS data from the Toxicity Forecaster (ToxCast) and Tox21 programs were used to set priorities (OECD, 2018).

ToxCast was launched by the US Environmental Protection Agency (EPA) in 2007 to produce toxicity data on a large number of chemicals using high-throughput *in vitro* assays to develop improved toxicity prediction models (Richard et al., 2016). The EPA has analyzed and published biological data from hundreds of assays for thousands of environmental chemicals screened by the ToxCast and Tox21 collaboration using a standardized data analysis pipeline (Filer et al., 2017; Richard et al., 2021). Therefore, this database is particularly suitable and useful for research that identifies the mechanisms of toxicity of environmental chemicals and the development of adverse outcome pathways (AOPs). The AOP is a framework that can maximize the regulatory use of NAMs, integrate existing knowledge, and provide scientific evidence for mechanism-based toxicity assessments (Delrue et al., 2016; Perkins et al., 2015; Wittwehr et al., 2017).

This study analyzed ToxCast/Tox21 data and reviewed recent studies using ToxCast/Tox21 to aid mechanism-based chemical prioritization and toxicity prediction. Through data analysis, information on toxicity mechanism targets, the assay-specific activity of chemicals, and

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gene scores for chemical priorities were summarized. Through a literature review, the mechanisms and assays that could be utilized for toxicity prediction were suggested. Finally, the potential application and limitations of using of ToxCast/Tox21 assay data for chemical risk assessment were proposed.

2. Overview of ToxCast/Tox21 assays

The EPA's ToxCast program released version 3.2 data (INVITRODB_V3_2) in July 2019 (the data can be downloaded from https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data). The data include chemical information, bioassay information, and summary information about the model and generated data through its own analytical pipeline (Filer et al., 2017). AC50 or hitcall data are mainly used in the utilization of ToxCast, but information on the assay should be considered for the mechanism-based toxicity evaluation and classification of chemicals. The assay summary file provides various types of information for each assay, such as assay ID, assay source, used organism or cell model, assay component, detection technology, target information, citation information, and reagent information.

There were 13 assay sources (Table 1, the full list of assays in INVITRODB_V3_2 is provided in Table S1). As the assay platform, such as the species used and the exposure time is different for different assay sources, it is necessary to check which assay platform is used for each assay. Among the assay sources, NovaScreen (NVS) provided the most (441) assays but tested the fewest chemicals on average. NVS mainly measures binding and enzymatic activity, and uses not only human tissue, but also various experimental animal model-based platforms such as rat, mouse, guinea pig, sheep, rabbit, bovine, pig, and chimpanzee. Although the assay summary provides a variety of information, the use of ToxCast data for mechanism-based toxicity assessment and classification requires certain consideration of the target information and cytotoxicity. ToxCast provides target information, such as the targets of technological measurement and biologically intended targets. The technological target represents the specific target of each assay readout and is assigned solely based on the technological parameters. In contrast, the intended target represents the biological intention of the assay and considers the signal direction relative to the controls (US EPA, 2014). Therefore, the intended target information should be used for mechanism-based toxicity evaluation. In INVITRODB_V3_2, 949 assays had the intended target information, and 105 assays were associated with cytotoxicity (Fig. 1).

2.1. Assays with intended target

The intended targets belonged to 23 intended target families and nine intended target types (Table 2; the description of the intended target family and the number of assays by assay source are provided in Table S2). Nuclear receptors are the most common target family (192), followed by G protein-coupled receptors (GPCRs) (129), and kinases (122). Nuclear receptors play a role in mediating the activity of lipophilic substances, such as xenobiotics, endogenous hormones, and certain vitamins (US EPA, 2014) and attagene (ATG) provides 115 assays. GPCRs play a role in integrating extracellular signals such as hormones, growth factors, and neurotransmitters, into downstream responses (US EPA, 2014), and NVS provides 76 assays. As NVS provided most of the assays, it had assays corresponding to 14 of the 23 intended target families. Based on the intended target type, there are many assays in the order of protein, pathway, RNA, and molecular messenger. Among the protein types, there were 264 enzymes, 230 transcription factors, and 190 receptors. These target families include well-known toxic targets associated with genotoxicity, developmental biology, cell signal transduction pathways, and xenobiotic metabolism. As the understanding of toxic pathways increases, the use of ToxCast assays is expected to increase (Kavlock et al., 2012).

2.2. Assays for cytotoxicity

In the assay summary file, assays with "burst_assay" of "1", "cell_viability" of "1", or "intended target subfamily" of "cytotoxicity" were selected as cytotoxicity-related assays. A total of 105 cytotoxicity-related assays were included in the ToxCast INVITRODB_V3_2 assay data (Table 3, The full list of cytotoxicity-related assays is provided in Table S3). Based on the assay source, the cytotoxicity assay provided by Tox21 was the highest (77), followed by BSK (13). By criteria, assays

Table 1
Summary of assays by assay source in ToxCast INVITRODB_V3_2.

Assay Source	No. of assays	Model	Format (well plate)	Time point (h)	Readout (function)	Readout (detection)	Average no. of chemicals
ACEA	6	T47D, 22Rv1	384	80	Viability, signaling	Label Free Technology	2234
APR	160	HepG2, hepatocyte	384	1, 24, 48, 72	Viability, signaling	Fluorescence	398
ATG	265	HepG2	24	24	Reporter gene, background control, viability	Fluorescence	2375
BSK	174	Various cell lines	96	24	Background control, viability, signaling	Spectrophotometry, Fluorescence, Microscopy	1484
CEETOX	46	H295R	96	48	Detection of steroid hormone, viability	Spectrophotometry, Fluorescence	344
CLD	48	Hepatocyte	96	6, 24, 48	Reporter gene, background control	Luminescence	309
NCCT	5	HEK293T	384	0.5, 24	Viability, binding	Luminescence, Fluorescence, Spectrophotometry	424
NHEERL	8	J1 embryonic stem cells, HEK293T, zebrafish embryo	96	2, 3, 144, 192	Viability, signaling, enzymatic activity, viability, binding, developmental defect	Fluorescence, Spectrophotometry, Luminescence, Radioactivity, Microscopy	346
NVS	441	Various tissues	48, 96, 384	0.33–72	Binding, enzymatic activity	Spectrophotometry, Fluorescence, Radiometry	109
OT	17	HEK293T, CHO-K1, HeLa	384	2, 8, 16, 24	Reporter gene, binding	Luminescence, Fluorescence, Microscopy	1858
TANGUAY	19	Dechorionated zebrafish embryo	96	120	Developmental defect	Microscopy	1060
TOX21	280	Various cell lines	1536	0.5–48	Background control, reporter gene, signaling, viability	Luminescence, Fluorescence	6692
UPITT	4	NA	384	NA	NA	NA	1962

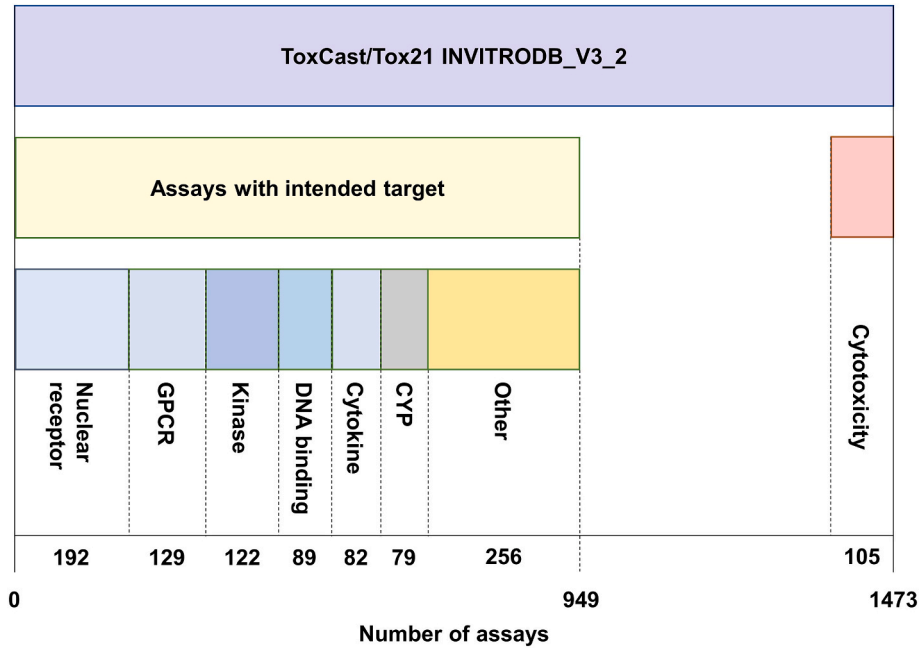

Fig. 1. ToxCast/Tox21 assay overview by endpoint category based on intended target family in INVITRODB_V3_2.

Table 2
Number of assays by intended target family and intended target type from ToxCast INVITRODB_V3_2.

Intended target type / Intended target family	Molecular messenger	Pathway	Protein						RNA	Total
			Enzyme	Protein-specified	Receptor	Transcription factor	Transporter	NA	mRNA	
Cell adhesion molecules	–	–	–	32	–	–	–	–	–	32
cyp	–	–	61	–	–	–	–	–	18	79
Cytokine	–	–	–	82	–	–	–	–	–	82
Deiodinase	–	–	1	–	–	–	–	–	–	1
dna binding	–	19	–	–	1	69	–	–	–	89
Esterase	–	–	12	–	–	–	–	–	–	12
gpcr	–	1	–	6	78	44	–	–	–	129
Growth factor	–	–	–	4	–	2	–	–	–	6
Histones	–	1	–	–	–	–	–	–	–	1
Hydrolase	–	1	11	–	–	–	–	–	–	12
Ion channel	–	–	–	–	20	–	–	–	–	20
kinase	–	–	86	–	36	–	–	–	–	122
Lyase	–	–	2	–	1	–	–	–	3	6
Methyltransferase	–	–	2	–	–	–	–	–	–	2
Misc protein	–	–	1	2	1	–	–	–	–	4
Nuclear receptor	–	25	–	–	51	115	–	1	–	192
Oxidoreductase	–	–	18	–	2	–	–	–	–	20
Phosphatase	–	–	39	–	–	–	–	–	–	39
protease	–	–	30	12	–	–	–	–	–	42
Protease inhibitor	–	–	–	4	–	–	–	–	–	4
Steroid hormone	22	–	–	–	–	–	–	–	–	22
Transferase	–	–	–	–	–	–	–	–	9	9
Transporter	–	–	1	–	–	–	11	–	12	24
Total	22	47	264	142	190	230	11	1	42	949

corresponding to “cell_viability” of “1” were 104 out of 105, except for the ‘CEETOX_H295R_MTT_cell_viability_up’ assay. This assay was analyzed in the positive fitting direction (looking to detect an increase in viability) relative to DMSO as the negative control and baseline of activity.

A study analyzing the ToxCast dataset by Judson et al. (2016) reported that cytotoxicity was observed in the tested concentration range for approximately half of the chemicals tested. Therefore, a significant proportion of the measured activities may be due to the assay interference process caused by cytotoxicity (cytotoxicity-associated “burst”). Therefore, cytotoxicity assays should be considered when using ToxCast data for toxicity mechanism research, as it is necessary to confirm whether assay activity is associated with specific biomolecular interactions (true positives) or false positives confounded by cytotoxicity. *Z*-scores have been proposed to allow for the alignment of cytotoxicity regions across chemicals, independent of specific AC50s (Judson et al., 2016). A high *Z*-score (> 3) indicated that the activity occurred at concentrations below the cytotoxic region.

When the *Z*-score was applied to the cytotoxicity assay, many chemicals showed activity at a Z-score of three or higher (Table 3). This may be because it is difficult to consider the concentration range of cytotoxicity for all chemicals and assays when calculating the *Z*-score. Therefore, when checking the activity of chemicals specific to an assay, it is necessary to check whether the *Z*-score is three or higher as well as

Table 3
Summary of cytotoxicity assays and their chemical activity from ToxCast INVITRODB_V3_2. The cytotoxicity assays are indicated by the "burst_assay", "cell_viability", and "intended_target_subfamily" fields in the assay summary file.

Assay source	No. of assays				Average no. of chemicals		
	Total	burst_assay (= 1)	cell_viability (= 1)	Intended target subfamily (cytotoxicity)	Total	Active	Active (*Z*-score > 3)
ACEA	3	3	3	3	2234	790	213
APR	6	2	6	6	560	149	13
ATG	1	0	1	1	3402	226	3
BSK	13	12	13	13	1484	384	78
CEETOX	2	0	1	1	655	59	25
NCCT	1	0	1	1	580	316	58
NHEERL	2	0	2	2	332	161	42
TOX21	77	69	77	72	7901	1003	119

the distribution of the cytotoxic concentration range of the assay of interest. Nevertheless, a high *Z*-score means that the chemical activity is more likely to be true positive (not associated with cytotoxicity) than a low *Z*-score, thus adding confidence to the assay-specific activity.

3. Overview of chemical-gene combinations in ToxCast/Tox21 data

The gene score was calculated based on potency (AC50) and specificity (Z-score) and can be used to set the priority of chemicals (Auerbach et al., 2016; Baker et al., 2020; Janesick et al., 2016; Leung et al., 2016). In this study, the gene score was calculated by removing chemicals with a cytotoxic Z-score of <3 and then integrating the *Z*-score with a negative log-molar transformed AC50 value [−log (AC50) + Z-score]. Chemical-gene combinations with a gene score of seven or higher were the most interesting, meaning that the Z-score was greater than three and the AC50 was <100 μM, because this was the widely tested concentration in most of the assays (Kleinstreuer et al., 2017).

The overall number of chemical-gene combinations of the 949 assays with the intended target information is summarized (Fig. 2). 1,796,348 chemical-gene combinations were tested (20.6% of a total of 8,739,341 combinations), and among them, 125,427 chemical-gene combinations were active (7.0% of tested combinations). This indicates that the data imbalance between active and inactive ToxCast/Tox21 is severe. The true positive chemical-gene combinations with *Z*-scores greater than three were 38,706 (30.9% of active combinations), and 12,669 combinations had gene scores greater than seven (32.7% of true positive combinations). A total of 698 genes (73.6%, out of 949) and 3346 chemicals (36.3%, out of 9209) were included.

In the analysis of the chemical-gene combination with the highest gene score, among the top 10 combinations, the estrogen receptor gene was the most common with five genes, followed by the androgen receptor and the p53 gene with two genes each (Table 4). The top-ranked chemical-gene combination was hydroxylamine sulfate (2:1)-TP53 with a gene score of 48.02, the second-ranked combination was hexabromobenzene-H2AFX with a gene score of 46.23, followed by the atracurium besylate-ESR1 combination with a gene score of 44.22. Because cytotoxicity may occur even when the *Z*-score is three or higher (Table 3), the higher the Z-score, the higher is the specificity of the activity. However, since the Z-score ranges from 3 to 41.81 and -log (AC50) ranges from −4.65 to 6.21, the specificity (Z-score) is considered more than the potential (AC50) in the gene score, which is simply the addition of the Z-score and -log (AC50). Therefore, attention should be paid to setting chemical priorities using gene scores, depending on the study purpose.

4. Recent studies using ToxCast/Tox21 assay data

The ToxCast/Tox21 dataset has been used in many studies on the toxicity mechanism of chemicals as a high-quality data generated through a consistent statistical process that includes the effects of thousands of chemicals on various protein targets (Auerbach et al., 2016; Sipes et al., 2017, 2013). By searching for "ToxCast" or "Tox21" as keywords in PubMed (https://pubmed.ncbi.nlm.nih.gov/), it was confirmed that the number of papers using each database continued to increase from 2006, when they first appeared (Fig. 3; accessed December 7, 2021). In total 542 papers were identified in PubMed database since 2006 (accessed May 30, 2022). Here, we have conducted a systematic review on 110 papers using ToxCast/Tox21 dataset published in the recent three years. These studies could be broadly classified

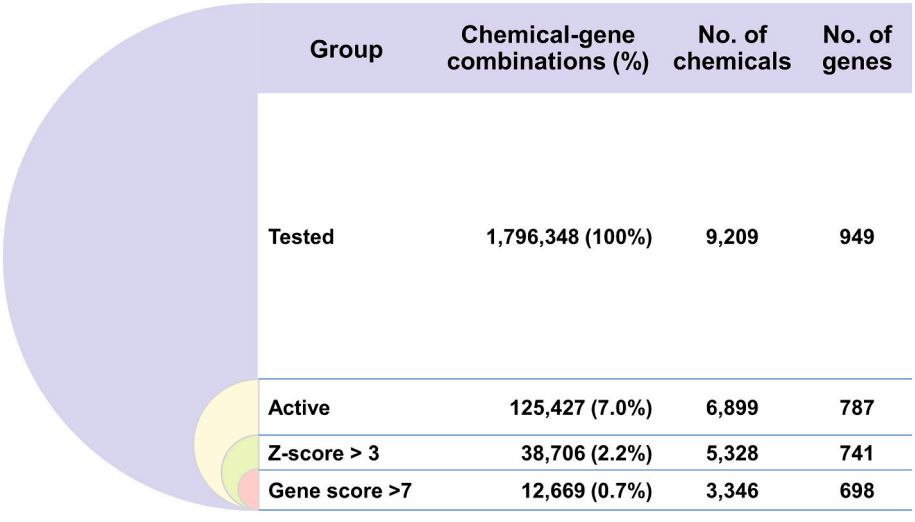

Fig. 2. Summary of chemical-gene combinations and number of chemicals and genes by groups in INVITRODB_V3_2.

Table 4
Top10 gene scores and their chemical-gene combinations.

Rank	Gene score	Assay name	Gene target	Chemical name	CAS No.
1	48.02	TOX21_p53_BLA_p1_ratio	TP53	Hydroxylamine sulfate (2:1)	10,039–54-0
2	46.23	TOX21_H2AX_HTRF_CHO_Agonist_ratio	H2AFX	Hexabromobenzene	87–82-1
3	44.22	TOX21_ERa_LUC_VM7_Agonist	ESR1	Atracurium besylate	64,228–81-5
4	44.19	TOX21_ERa_LUC_VM7_Agonist	ESR1	Iopamidol	60,166–93-0
5	44.17	TOX21_ERa_LUC_VM7_Agonist	ESR1	Tolazoline hydrochloride	59–97-2
6	43.85	TOX21_p53_BLA_p3_ratio	TP53	Lactobionic acid	96–82-2
7	43.59	TOX21_ERa_BLA_Antagonist_ratio	ESR1	Butirosin disulfate	51,022–98-1
8	43.31	TOX21_AR_BLA_Antagonist_ratio	AR	Butirosin disulfate	51,022–98-1
9	42.99	TOX21_AR_BLA_Antagonist_ratio	AR	Nithiamide	140–40-9
10	42.99	TOX21_ERa_BLA_Antagonist_ratio	ESR1	Gonadorelin hydrochloride	51,952–41-1

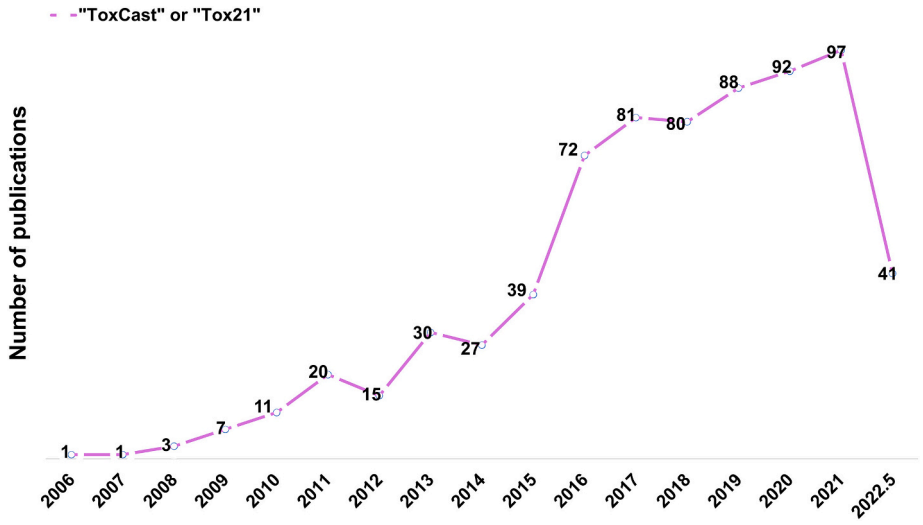

Fig. 3. Number of publications indexed by PubMed annually using search terms "ToxCast" and "Tox21".

into the following five categories according to their research purposes: Identification of toxicity mechanism of environmental chemicals, Prioritization of chemicals with their toxic potential, Identification of contaminants for environmental monitoring, Validation or development of novel assay, and AI-based toxicity prediction (Table S4, Fig. 4). Although there were some overlapping cases, they were classified as the most representative objectives. Among these, target toxicity endpoints, and target chemical groups were analyzed for 49 papers in 2021, excluding five review papers out of a total of 54 papers searched for with the keyword "ToxCast" in PubMed (a summary of the 49 papers is presented in Table S5).

When analyzing papers with target toxicity endpoints, the endpoints related to endocrine disruption were the most common (14 papers), followed by carcinogenicity (five papers), hepatic steatosis, immunotoxicity, and developmental toxicity with two papers each (Fig. 5A). Endocrine disruption is one of the toxicity endpoints that has been continuously studied since 2009 to the extent that the US EPA has been conducting the Endocrine Disruptor Screening Program (EDSP) using the ToxCast/Tox21 database since 2009 (Juberg et al., 2014). To this end, assays related to estrogen receptors (Klutzny et al., 2022; Kornhuber et al., 2021; Wang et al., 2021b) androgen receptors (Prichystalova et al., 2021), and thyroid hormone receptors (Garcia De Lomana

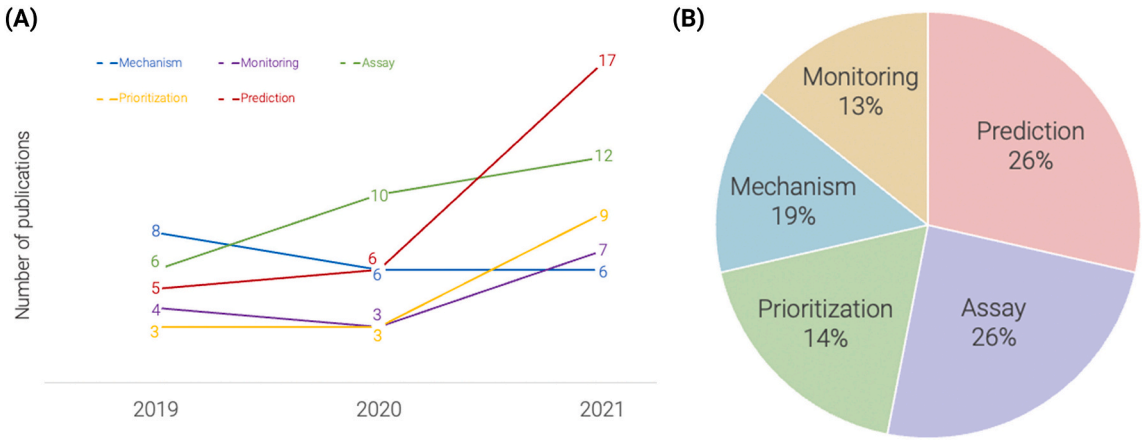

Fig. 4. Trend of the (A) study object using ToxCast/Tox21 in recent three years, and (B) its summary.

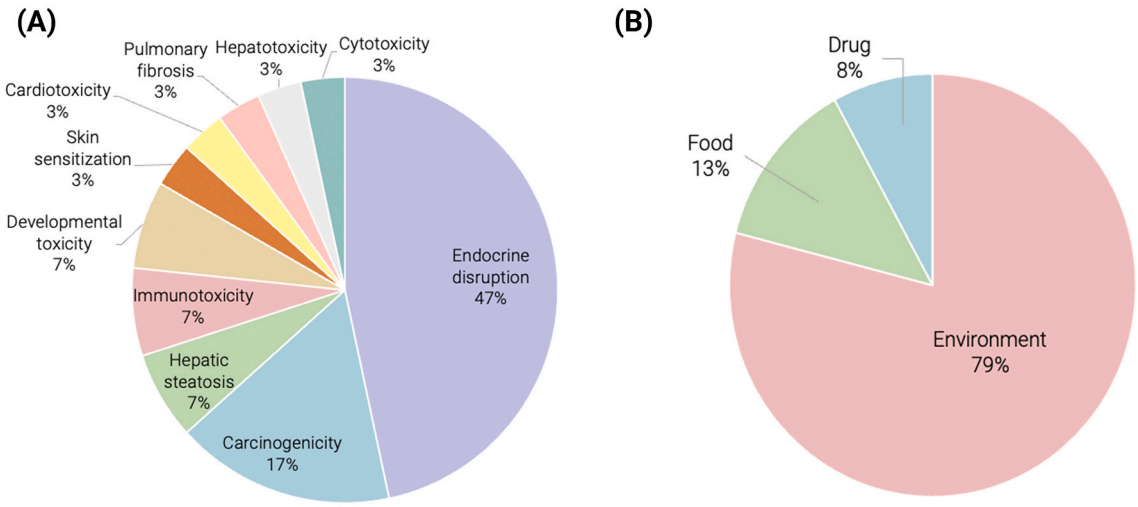

Fig. 5. Summary of the (A) target toxicity endpoints, and (B) target chemical groups used in studies using ToxCast/Tox21 datasets.

et al., 2021; Ramhøj et al., 2021) have been used. In the analysis of chemicals targeted in the papers, environmental chemicals were targeted in 41 papers, food-related chemicals in seven papers, and chemicals related to drugs in four papers (Fig. 5B). Although there have been many studies targeting environmental chemicals, about one-third of them did not target specific chemicals of interest, but instead analyzed ToxCast or Tox21 chemical libraries to screen toxicity endpoints (Feng et al., 2021; Mayasich et al., 2021; Wang et al., 2021a).

4.1. Study objectives: identification of toxicity mechanism of environmental chemicals

Seven studies were conducted to identify toxicity mechanism of environmental chemicals (Table 5). In these studies, there was an approach to obtain evidence of a toxic mechanism through the activity of various assays and an approach to determine the toxicity mechanism using assays related to specific toxicity endpoints. Of these, if the latter approach is used, not only can the toxicity mechanisms of the chemicals be identified, but the chemicals can also be prioritized for the corresponding endpoints. For example, in the study by Singh and Hsieh (2021), the coverage of 274 ToxCast/Tox21 assays for the key characteristics of carcinogens was evaluated, and the potential carcinogenic activity of 23 *per*- and polyfluorinated alkyl substances (PFAS) was confirmed. In a study by Ravichandran et al. (2021), 296 assays for skin sensitization were identified based on adverse outcome pathways in the AOP Wiki. Skin-sensitizing fragrance chemicals were identified among 153 fragrance chemicals. As the range of toxicity mechanisms that can be utilized in ToxCast/Tox21 is wide, it is necessary to identify assays related to various toxicity endpoints to study the toxicity mechanisms of a chemical of interest. However, the cytotoxicity range or *Z*-score was not considered in these seven studies. It is important to consider the specific activity in the study of toxicity mechanisms; therefore, it is recommended to be considered in future studies.

4.2. Study objectives: prioritization of chemicals with their toxic potential

Nine papers were conducted to prioritize chemicals with their toxic potential (Table 6). These studies used an approach of prioritizing the most hazardous chemicals for many chemicals using either the activity or AC50 values for the assay associated with the toxicity endpoint of interest. For example, Krishna et al. (2021) ranked 892 chemicals in the ToxCast chemical library using the CardioToxPi tool based on an assay related to cardiovascular toxicity. Based on the toxicity endpoint, the number of papers on endocrine disruption was the highest (five papers). A study by Zhao et al. (2021) used the ToxPi tool (Marvel et al., 2018; Reif et al., 2010) based on 97 assays targeting estrogen, androgen, and thyroid pathways and the glucocorticoid receptor, peroxisome proliferator-activated receptors (PPARs), and monoamine signaling. By integrating this and the hazard information obtained through toxicity prediction with the exposure information obtained from the US EPA Systematic Empirical Evaluation of Models (SEEM) (Wambaugh et al., 2014), 7770 chemicals (including the ToxCast/Tox21 chemical library) were prioritized based on the risk index. As the prioritization of chemicals is associated with a reduction in animal testing, many studies have linked ToxCast/Tox21 bioactivity data with *in vivo* toxicity or exposure information (Cardona and Rudel, 2021; Luo and Wu, 2021; Polemi et al.,

Table 5
Summary of studies for identification of toxicity mechanism.

No.	ToxCast/Tox21 data			Chemicals			Reference
	Assays	Endpoints	Parameter	Library	Category	Number	
1	94 Tox21 assays (invitrodb v3.2)	Carcinogenicity	Hitcall	Steviol glycosides	Food	–	(Chappell et al., 2021)
2	All assays (522) in which drugs were tested	–	AC50	Troglitazone, rosiglitazone	Drug	2	(Dirven et al., 2021)
3	ATG_PXRE_CIS_up ATG_PXR_TRANS_up ATG_PPARg_TRANS_up	Hepatic steatosis	AC50	Novel flame retardants	Environment	9	(Negi et al., 2021)
4	296 assays for skin sensitization	Skin sensitization	Hitcall	Fragrance chemicals	Environment	153	(Ravichandran et al., 2021)
5	All assays	–	AC50	Herbicide safeners	Environment	9	(Simonsen et al., 2021)
6	Assays on IARC key characteristics of carcinogens	Carcinogenicity	AC50	PFAS	Environment	23	(Singh and Hsieh, 2021)
7	47 Tox21 assays	–	AC50	Antiviral drugs	Drug	12	(Tarazona et al., 2021)

Table 6
Summary of studies for chemical prioritization.

No.	ToxCast/Tox21 data			Chemicals			Reference
	Assays	Endpoints	Parameter	Library	Category	Number	
1	Two HT-H295R steroidogenesis assay	Carcinogenicity	Hitcall, AC50, AC10	ToxCast libraries	Environment	654	(Cardona and Rudel, 2021)
2	314 assays	Cardiotoxicity	AC50	ToxCast libraries	Environment	1138	(Krishna et al., 2021)
3	76 assays, representing 11 nuclear receptors	Endocrine disruption	AC50	Pesticides in food	Food	79	(Luo and Wu, 2021)
4	39 immune-related assays, 16 ECM-related assays, 6 TF assays	Immunotoxicity	AC50	Food, PFAS	Food, Environment	63 22	(Naidenko et al., 2021)
5	All assays	Carcinogenicity	TP, ACC	Biomarker chemicals for breast cancer	Environment	43	(Polemi et al., 2021)
6	ER, AR assays	Endocrine disruption	Hitcall	Endocrine-disrupting chemicals (EDCs)	Environment	24	(Prichystalova et al., 2021)
7	TPO assay	Endocrine disruption	IC50	TPO inhibitors	Environment	320	(Ramhøj et al., 2021)
8	TOX21_Aromatase_Inhibition, NVS_ADME_hCYP19A1	Endocrine disruption	Hitcall, Ac50	Aromatase inhibitors	Environment	5	(Villeneuve et al., 2021)
9	97 assays for ER, AR, and TR pathways and GR, PPARs, and monoamine signaling	Endocrine disruption	AC50, Emax	ToxCast/Tox21 dataset	Environment	8845	(Zhao et al., 2021)

2021; Zhao et al., 2021). Recently, *in vitro*-to-*in vivo* extrapolation studies considering exposure and toxicokinetics have been being conducted (Honda et al., 2019; Paul et al., 2020; Ring et al., 2021). This is a desirable research direction for the use of HTS data in risk assessment in the future and will be discussed in more detail below.

4.3. Study objectives: identification of contaminants for environmental monitoring

Seven studies were conducted to identify contaminants for environmental monitoring (Table 7). These studies have used an approach to identify or prioritize the toxic mechanisms of chemicals in samples taken from the environment, such as surface water or soil, in the context of ecological risk assessment. Because these studies used environmental samples, exposure concentrations were considered using the exposure-activity ratio (EAR) approach (Becker et al., 2015; Schroeder et al., 2016) For example, in a study by Alvarez et al. (2021), 19 chemicals of concern and their toxic mechanisms were identified by calculating the EAR by comparing environmental concentrations and bioactivity data of ToxCast/Tox21 for samples from tributaries of the Great Lakes. In another study (Bradley et al., 2021b), EAR based on ToxCast data was used to confirm the cumulative effects of pesticides and pharmaceuticals on rivers. In this study, the activity concentration at cutoff (ACC), not the AC50, was mainly used to estimate the point of departure (PoD).

4.4. Study objectives: validation or development of novel assay

Twelve studies were conducted to validate or develop novel assay (Table 8). These studies did not focus on toxicity mechanisms, chemicals, or concerns, but rather on the use of ToxCast/Tox21 data or chemical libraries in the development of specific assays or novel approaches. For example, Kornhuber et al. (2021) used ToxCast data and reference chemicals in the process of developing the E-Morph assay, an image-based phenotypic screening assay, to evaluate endocrine disorders (Shah et al., 2021).

5. AI-based toxicity prediction using ToxCast/Tox21 assay data

The development of models for toxicity prediction is an emerging field in toxicology owing to the rapid development of computational technology since the introduction of big data-based AI technology to the toxicology field. Toxicity prediction in the field of environmental toxicology generally aims to obtain information for prioritization of chemicals for risk assessment by integrating information from chemical toxicity databases and computational techniques.

One of the most important aspects to consider in the development of a toxicity prediction model is that the performance of models relies heavily on the quantity and quality of data. The sparsity of data and data quality are the foremost problems in toxicity prediction. As ToxCast/Tox21 data are generated by automated robotic-technology-aided HTS technology for numerous environmental chemical substances, the data produced through this automated technology have high homogeneity. In

Table 7
Summary of studies for environmental monitoring.

No.	ToxCast/Tox21 data			Chemicals			Reference
	Assays	Endpoints	Parameter	Library	Category	Number	
1	321 assays after preprocessing	–	ACC	Waterborne contaminants in Great Lakes fields sample	Environment	143	(Alvarez et al., 2021)
2	848 assays (6th of November 2019)	–	AC50, ACC	Contaminants of emerging concern in North Sea	Environment	208	(Barbosa et al., 2021)
3	invitroDBv3.2	–	ACC	Pesticides and pharmaceuticals in water	Environment	328	(Bradley et al., 2021b)
4	invitroDBv3.2	–	ACC	Pharmaceutical, pesticide, organic wastewater indicators	Environment	389	(Bradley et al., 2021a)
5	–	–	Chemical library	ToxCast Phase I and Phase II chemicals	Environment	890	(Feng et al., 2021)
6	All assays	–	ACC	Organic contaminants in European rivers	Environment	476	(Malev et al., 2022)
7	All assays	–	Hitcall, AC50	Landfill leachate contaminants	Environment	322	(Rogers et al., 2021)

Table 8
Summary of studies for assay development or analysis.

No.	ToxCast/Tox21 data			Chemicals			Reference
	Assays	Endpoints	Parameter	Library	Category	Number	
1	–	–	Chemical library	Drugs, pesticides, industrial chemicals, food agents, etc	Drug, Environment, Food	1029	(Burnett et al., 2021)
2	Assays related to 93 genes	–	AC50	ToxCast Phase I and Phase II chemicals	Environment	1060	(Franzosa et al., 2021)
3	All assays	–	Hitcall	Herbicides	Environment	44	(Harrill et al., 2021)
4	–	Neurodevelopmental toxicity	ToxPi	Flame retardants and its metabolites	Environment	15	(Klose et al., 2021)
5	7 ER assays	Endocrine disruption	Hitcall	Industrial chemicals, biocides	Environment	430	(Klutzny et al., 2022)
6	18 ER assays	Endocrine disruption	ER Agonist score	ER chemicals	Environment	18	(Kornhuber et al., 2021)
7	–	Endocrine disruption	Chemical library	ToxCast libraries	Environment	356	(Mayasich et al., 2021)
8	–	Endocrine disruption	Chemical library	ToxCast libraries	Environment	1825	(Olker et al., 2021)
9	All assays	–	AC50	Food additives	Food	4	(Punt et al., 2021)
10	All assays	–	AC50	Chemicals with all data	Environment	51	(Shah et al., 2021)
11	–	–	–	–	–	–	(Sheffield et al., 2021)
12	–	Endocrine disruption	Chemical library	ToxCast e1k chemicals	Environment	804	(Wang et al., 2021a)

this context, ToxCast/Tox21 data, curated through rigorous quality assurance procedures for a large number of chemicals, are currently most suitable for the development of a toxicity prediction model for environmental chemicals.

In 2014, the National Institutes of Health (NIH) held the Tox21 Data Challenge. The Tox21 Data Challenge was the first worldwide competition for developing toxicity prediction models, aiming to predict the toxicity of chemicals using only chemical structure data and to utilize models with high performance in government agencies (Huang et al., 2016). Recently, the development of toxicity prediction models using ToxCast has accelerated.

When developing a toxicity prediction model, solving the problem of data imbalance between the major and minor classes is an important challenge for performance improvement. As with many toxicity databases, most assays in the ToxCast/Tox21 database are already highly imbalanced (Idakwo et al., 2020; Ring et al., 2021) and there are concerns about a decrease in performance when the number of active compounds is reduced in consideration of the *Z*-score (Kurosaki et al., 2020). Nevertheless, as mentioned above, it is essential to consider the cytotoxicity range when predicting specific toxicity endpoints. Most of studies used the resampling technique to solve this class-imbalance problem. The ToxCast/Tox21 are most frequently used for the development of toxicity prediction because of the higher quantity and quality of data along with the ease of interpretation of the prediction results compared to other apical endpoint-based models.

The scientific explanation of the predicted results is insufficient for regulatory applications in chemical management. This is the main reason why the use of the traditional QSAR models is limited to utilized in chemical regulation, as it is difficult to interpret the prediction results for the apical endpoint based on the chemical structure. In the field of toxicology, it is more valuable to develop a model with a reliable scientific basis rather than one with good performance. The mechanisms leading to the onset of apical toxicity are complex, and in the absence of evidence for the process, it is difficult to trust the results; in the worst case, it may be a mere coincidence. The AOP concept has emerged to solve this problem, which predicts toxicity through linkage with toxicity mechanisms. Unlike the traditional QSAR model, it is relatively easy to apply the AOP concept to toxicity prediction models using AI. Analyzing molecular endpoints with apical endpoints based on the AOP concept would lead to scientifically explainable toxicity predictions. These factors make the ToxCast/Tox21 database preferable over the conventional database for apical endpoints using *in vivo* models.

Among the studies using ToxCast/Tox21 assay data analyzed in this study, fourteen papers were classified as toxicity prediction models (Table 9). In these studies, mechanism-based toxicity prediction was developed, and endocrine disruption was the predominant toxicity endpoint. In a study by Jaladanki et al. (2021), a toxicity prediction model based on molecular docking was developed using the data of ToxCast/Tox21 for 12 nuclear receptors for potential endocrine disruption chemicals prediction. In addition, in a study by Garcia De Lomana et al. (2021), a classification model was developed using several machine learning algorithms trained on data from nine ToxCast assays to predict the interaction between chemical substances and molecular initiating events (MIEs) of thyroid hormone homeostasis. Four studies considered cytotoxicity range or pathway-specific assays (Firman et al., 2021; Garcia De Lomana et al., 2021; Rathman et al., 2021; Wu et al., 2021). In our group, we previously developed 25 artificial neural network models based on ToxCast/Tox21 bioassays for the selection of chemicals for validation of AOP (Jeong et al., 2019). We have also developed ToxCast bioassay-based models for the development of AOP relevant to microplastics (Jeong and Choi, 2020). ToxCast assays with intended gene targets were selected, and deep learning artificial neural network models were further developed based on the ToxCast assays for chemicals not tested in the ToxCast program. Collectively, these studies suggest the potential of ToxCast/Tox21 bioassay data in the development of models for the classification of bioactivity, which can be related to toxicity. They also suggested a combined approach using ToxCast/Tox21 bioassay, and models developed based on their data have potential in the prioritization of chemicals, as well as in the identification of the mechanism of toxicity of chemicals, whose mode of action is not understood.

6. Use of ToxCast/Tox21 assay data in chemical risk assessment: potential and limitations

The provision of highly curated data on abundant toxicological targets through strict quality assurance procedures has enabled ToxCast/Tox21 data to be widely used in research such as chemical prioritization, assay development, and toxicity prediction. These studies are a part of an effort to replace animal experiments using *in vitro* HTS data, which is one of the main goals of the ToxCast/Tox21 project. Although there have been valuable efforts at the international level to establish confidence in

Table 9
Summary of studies for toxicity prediction.

No.	ToxCast/Tox21 data			Chemicals			Reference
	Assays	Endpoints	Parameter	Library	Category	Number	
1	invitrodb_v3	–	AC50	Canada's Domestic Substance List	Environment	5801	(Beal, 2021)
2	600 assays	–	AC50	Food additives	Food	552	(Firman et al., 2021)
3	7 TR assays	Endocrine disruption	Hitcall	ToxCast dataset	Environment	802–6789	(Garcia De Lomana et al., 2021)
4	–	–	Chemical library	ToxCast Phase I and Phase II chemicals	Environment	1003	(Green et al., 2021)
5	NVS NR (AR, PR, GR, PPARα, PPARγ, RARα) assays	Endocrine disruption	Hitcall	Fatty acids and ToxCast chemicals	Food, Environment	252, ~3328	(Jaladanki et al., 2021)
6	24 assays related to AOP206	Pulmonary fibrosis	Hitcall	Chemicals in DPM	Environment	100	(Jeong et al., 2021)
7	ATG_ERE_CIS_up ATG_PXRE_CIS_up ATG_THRa1_TRANS_dn BSK_3C_uPAR_down	Carcinogenicity, Hepatic steatosis, Endocrine disruption, Immunotoxicity	Concentration-response data	PFOA	Environment	1	(Loizou et al., 2021)
8	18 Tanguay_ZF assays	–	Hitcall	ToxCast dataset	Environment	1018	(Lovrić et al., 2021)
9	–	Hepatotoxicity	–	Active pharmaceutical ingredients	Drug	98	(Rathman et al., 2021)
10	144 Tox21 assays	–	AC50	Chemicals with all data	Environment	221	(Ring et al., 2021)
11	Zebrafish embryo assay	Developmental toxicity	AC50	Pesticides and antimicrobials	Environment	188	(Saavedra and Duchowicz, 2021)
12	12 cytotoxicity- and proliferation-related assays	Cytotoxicity	Hitcall	Test chemicals in the assays	Environment	135	(Seal et al., 2021)
13	18 ER assays	Endocrine disruption	Hitcall	Test chemicals in the assays	Environment	1357	(Wang et al., 2021b)
14	All assays	–	AC50, Cytotoxicity limit	Phthalates and alternatives	Environment	5	(Wu et al., 2021)

the use of *in vitro* HTS data, such as ToxCast/Tox21, these have not been wholly successful for several reasons. The main drawbacks were the limited understanding of the mechanism and difficulties in defining how to meaningfully apply individual *in vitro* assays for risk assessment. To address this need, we explored several approaches as to what should be considered for identifying relevant assays and how they could be utilized (Fig. 6).

Among recent application studies using the ToxCast/Tox21 dataset, international collaborative regulatory agencies have conducted research to enhance the utilization of ToxCast/Tox21 data in chemical risk assessment (Barton-Maclaren et al., 2022; Bhuller et al., 2021), as a part of accelerating the pace of chemical risk assessment (APCRA) initiatives (Kavlock et al., 2018). In these studies, the authors suggested the possibility of using the toxicity values derived from the *in vitro* bioactivity data in risk assessment by comparing the PoD values calculated using ToxCast/Tox21 data with those from the traditional animal toxicity data. Paul et al. (2020) have compared PoD values for 448 substances

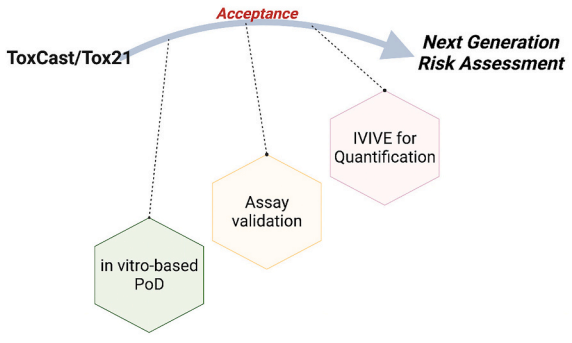

Fig. 6. Key considerations for increasing the use of ToxCast/Tox21 data in chemical risk assessment.

and derived 400 (89%) substances, of which PoD from bioactivity data are less than or equal to those from *in vivo* studies. Furthermore, they compared PoD based on the ToxCast/Tox21 dataset with exposure levels for specific chemical groups to identify the potential of ToxCast/Tox21 data as a risk screening tool. Another study was conducted by a research group in Health Canada (Beal, 2021), where they used ToxCast/Tox21 data to calculate *in vitro*-based PoD for applying NAMs-based chemical risk assessments to Canada's domestic substance list (DSL). They compared PoD values for 1042 substances and derived 990 (95%) substances in which PoD from bioactivity data was less than or equal to the PoD values from *in vivo* studies. Subsequently, they compared the PoD based on the ToxCast/Tox21 dataset with exposure levels for DSL chemicals to screen the risk.

In those studies, validation of bioactivity assay was not mainly addressed; nevertheless, we supposed these had been selected assays, of which PoD values were lower than those from traditional animal data. A more direct effort to validate of ToxCast/Tox21 bioassays for hazard identification was attempted in our previous study on the identification of AOP relevant to the additive chemicals in plastics (Jeong and Choi, 2020). In this study, we used ToxCast/Tox21 data to identify the molecular toxicity mechanisms of additive chemicals based on the AOP concept. We identified the most relevant mechanisms of toxicity to understand the mechanism of toxicity of plastic additives through correlation analysis between molecular targets from ToxCast bioassays and mammalian toxicity results from ChemIDplus. We hypothesized that ToxCast bioassays correlated with mammalian toxicity, as statistically validated.

The goal of using ToxCast data is to assess pathway-level and cell-based signatures that correlate with the observed *in vivo* toxicity *via* profiling the *in vitro* bioactivity of chemicals. Therefore, expert knowledge-based analyses of the relevant toxicity mechanisms cannot be excluded. Thus, it is necessary to select a toxicologically meaningful assay based on the target information, and to conduct advanced toxicity tests, mechanism-specific assays can be used either by themselves or to

provide the hypotheses needed to perform enrichment analysis of selected chemicals through data-driven and statistic-driven validations.

Finally, to maximize the applicability of the validated assays to chemical risk assessment, *in vitro-in vivo* extrapolation (IVIVE) can be applied to validated bioassays. Some studies have suggested approaches for IVIVE through high-throughput toxicokinetics (HTTK), which can predict *in vivo* exposure concentrations from *in vitro* HTS data. For example, in those studies, the authors converted the minimum *in vitro* PoD values to human oral equivalent doses through a simple toxicokinetic (TK) model or Physiologically based Toxicokinetic (PBTK) model (Shah et al., 2021; Luo and Wu, 2021; Punt et al., 2021).

To make better use of ToxCast bioassay in chemical risk assessment, efforts should be made on assay validation based on systemic association with animal toxicity data combined with expert-knowledge-based decisions. It is also important to conduct more case studies on the quantitative use of bioassays using substances with sufficient *in vivo*, toxicokinetic and exposure data. These efforts will aid in increasing the acceptance of new alternative methods and tools in the regulatory decision-making process. By combining the approaches used in this study with a sequentially structured framework, next generation risk assessments based on molecular toxicity mechanisms using NAMs, including ToxCast/Tox21 data, will be more successful.

7. Conclusion

With the paradigm shift in toxicity assessment, mechanism-based toxicity assessments have the potential to become mainstream in this field in the future. However, without a detailed analysis of the data obtained from toxicity studies, a misunderstanding of the mechanisms of toxicity can occur. The present work provides an overview of the factors to be considered for utilization of the ToxCast/Tox21 database. As the ToxCast/Tox21 database is widely used in toxicity prediction and chemical prioritization research, this study will contribute to the continuous efforts on how to use ToxCast/Tox21 for mechanism-based toxicity evaluation and risk screening of chemicals.

Declaration of Competing Interest

The authors declare no competing interest.

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Appendix A. Supplementary data

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