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High-Throughput Screening to Identify Chemical Cardiotoxic Potential

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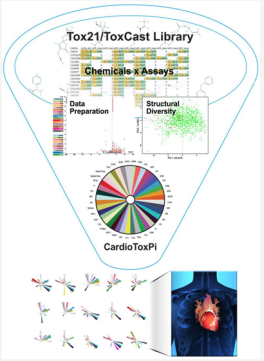
ABSTRACT: Cardiovascular (CV) disease is one of the most prevalent public health concerns, and mounting evidence supports the contribution of environmental chemicals to CV disease burden. In this study, we performed cardiotoxicity profiling for the Tox21 chemical library by focusing on high-throughput screening (HTS) assays whose targets are associated with adverse events related to CV failure modes. Our objective was to develop new hypotheses around environmental chemicals of potential interest for adverse CV outcomes using Tox21/ToxCast HTS data. Molecular and cellular events linked to six failure modes of CV toxicity were cross-referenced with 1399 Tox21/ToxCast assays to identify cardio-relevant bioactivity signatures. The resulting 40 targets, measured in 314 assays, were integrated via a ToxPi visualization tool and ranking system to prioritize 1138 chemicals based upon formal integration across multiple domains of information. Filtering was performed based on cytotoxicity and generalized cell stress endpoints to try and isolate chemicals with effects specific to CV biology, and bioactivity- and structure-based clustering identified subgroups of chemicals preferentially affecting targets such as ion channels and vascular tissue biology. Our approach identified drugs with known cardiotoxic effects, such as estrogenic modulators like clomiphene and raloxifene, anti-arrhythmic drugs like amiodarone and haloperidol, and antipsychotic drugs like chlorpromazine. Several classes of environmental chemicals such as organotins, bisphenol-like chemicals, pesticides, and quaternary ammonium compounds demonstrated strong bioactivity against CV targets; these were compared to existing data in the literature (e.g., from cardiomyocytes, animal data, or human epidemiological studies) and prioritized for further testing.



1. INTRODUCTION

Cardiovascular (CV) disease is a tremendous public health burden, being the leading cause of death for people of most ethnicities in the United States. Contributing factors have been relatively well-characterized and include lifestyle choices, genetic factors, and off-target effects from various pharmaceuticals,[1,2] where cardiotoxicity is a major cause of adverse drug reactions during late-stage preclinical and even clinical development.[3] Many cardiotoxic effects of pharmaceuticals, including heart arrhythmia (QT prolongation), change in blood pressure, altered cardiac contractility, or cardiomyocyte/myocardial injury, are identified preclinically, but some life-threatening effects such as stroke, myocardial infarction, and heart failure are more challenging, underscoring the need for better in vitro and in silico screening and testing programs.

A lesser studied but potentially substantial contributor to CV disease is exposure to various environmental pollutants and chemicals. There is a substantial body of literature supporting the link between industrial smog or vehicle exhaust and risk for cardiac events and heart attack, e.g., refs 4 and 5. Markers derived from epidemiological data support that these heart attacks primarily result from complications of atherosclerosis that may be exacerbated by exposure to air pollution, highlighting the importance of studying the impact of other environmental chemicals on cellular targets.[6]

Addressing the cardiotoxic effects of environmental chemicals is challenging due to the abundance of untested substances; the influence of chronic, low-dose exposure and mixed exposures; varying levels of susceptibility of hosts due to genetics, life stage, and co-morbidities; and the scarcity of robust testing approaches that are predictive of human CV effects.[7,8] The U.S. federal Tox21 research collaboration is focused on supporting the evolution of Toxicology in the 21st Century (Tox21) by developing methods to rapidly and efficiently evaluate the hazard identification of various commercial chemicals, pesticides, food additives/contaminants, and medical products.[9] Various predictive models have been developed leveraging the Tox21 high-throughput screening (HTS) data to define mechanistic bioactivity profiles relevant to toxicological endpoints such as developmental toxicity and carcinogenesis.[10−15] Previous work has shown that vascular targets are well represented in the Tox21 dataset, in the context of potential environmental chemical disruption of

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embryonic development via a vascular disruptive mechanism.[16−19] Here, we propose to leverage the Tox21 HTS data to characterize the potentially significant but underappreciated risk factor of environmental exposures contributing to the development and severity of CV disease more broadly.

The heart and vascular system have been shown to be vulnerable to several environmental agents: pesticides, flame retardants, polycyclic aromatic hydrocarbons (PAHs), plasticizers, ambient air pollution, and metals such as arsenic, cadmium, and lead. These exposures are widespread, with human, in vivo, and in vitro evidence of cardiotoxicity.[20−26] Like classical environmental risk factors, such as smoking and diabetes mellitus, these exposures may contribute to advancement of disease and mortality by augmentation or initiation of CV-associated pathophysiological processes, including changes in vascular function, carbohydrate and lipid metabolism, atherogenesis, and blood-pressure control.[27] It is well established that populations in highly polluted areas are at higher risk for CV diseases, but CV liability can also occur at exposure levels below current regulatory standards.[7] Considering the widespread prevalence of environmental exposure and CV disease, even modest contributions to CV risk can exert a significant effect on population health. Even though current conservative estimates relate at least 23% of all CV disease cases to environmental exposures, the identities and mechanisms of action of influential environmental agents remain uncharacterized to a great extent.[28] Most environmental hazard assessments are conducted in standardized rodent animal studies with evaluations (e.g., histopathology and clinical chemistry) that have little specificity for the CV system. These studies are limited by low throughput and potential significant functional disparities between animals and humans.[29] Those studies are also expensive and time-consuming, and they provide limited mechanistic insight or extrapolation to effects on co-morbidities or other susceptibilities to human toxicity and efficacy. Such experimental methods are not well suited for evaluation of many chemicals and drugs in early stages due to high expense and poor throughput. Therefore, there is an urgent need for the development of a cost- and time-effective, comprehensive, multi-parametric screening program to evolve the toxicological testing paradigm and identify environmental chemicals that might be contributing to CV disease.

Recent advances of in silico approaches and in vitro HTS tools hold promise for supporting rapid, human-relevant, and mechanistically informative hazard assessments. In order to evaluate the wide range of potential CV toxic effects, a translational paradigm with methods that have improved predictive performance and account for the multi-factorial determinants of cardiotoxicity needs to be developed. Initial sources for such bioactivity data that could serve as an initial step in a translational toxicology assessment pipeline are the Tox21 consortium and the associated United States Environmental Protection Agency (U.S. EPA) toxicity forecaster program (ToxCast), which has screened thousands of chemicals in more than 700 high-throughput assays.[30,31] We identified six fundamental ways the CV system manifests toxicity, termed "failure modes". Here, a predictive bioactivity profile of CV toxicity was used to characterize the Tox21/ToxCast chemical inventory based on a subset of in vitro HTS assays that measure largely human cellular and molecular targets which were associated with adverse events related to CV failure modes. We performed cardiotoxicity profiling for 1138 unique chemicals, based on results from 314 assays identified through a tiered approach. This application to screen chemicals for their bioactivity against CV-relevant targets has enabled us to identify environmental agents with putative CV hazard potential that could be further evaluated.

2. MATERIALS AND METHODS

2.1. Definition of Cardiovascular Failure Modes and Collection of Targets for Cardiotoxicity Profile. Much is known about the normal physiology of the CV system and many of its molecular mediators (e.g., ion channel-mediated transmembrane action potentials, calcium trafficking, excitation−contraction coupling, energy metabolism, autonomic modulation, etc.). Additionally, how we assess the CV system either clinically or in hazard assessment is predicated on our understanding of how it fails. We have focused on six fundamental failure modes in the CV system: (i) change in vasoactivity (vasodilation/vasoconstriction), (ii) change in inotropy (contractility), (iii) change in action potential (heart rate, rhythm), (iv) cardiomyocyte/myocardial necrosis/degeneration), (v) valvular injury/proliferation (valvulopathy), and (vi) endothelial injury/coagulation (hemorrhage, thrombosis). We used this framework to screen the unique 1399 Tox21 (including ToxCast) assay portfolio for molecular targets and cellular phenotypic responses known to be involved in mechanistic events linked to these manifestations of CV toxicity. We began with a panel of targets that are used in secondary pharmacology screening and play a role in adverse events linked to CV liabilities.[32] To include more information about various pathways involved in "cardiovascular disease" and "cardiotoxicity", we searched the NCATS BioPlanet database for those keyword categories and mapped the molecular targets found therein to the Tox21 assays.[33] The NCATS BioPlanet is a comprehensive, publicly accessible informatics resource that catalogues all pathways, their healthy and disease state annotations, and targets contained within and the relationships among them. The BioPlanet integrates pathway annotations from publicly available sources that have been subjected to thorough redundancy and consistency cross-evaluation via extensive manual curation. Finally, we identified clinical biomarkers from the literature from recent studies that characterize such targets as promising alternative solutions for the early detection of cardiotoxicity.[34]

2.2. CardioToxPi Generation. We began with the Tox21/ToxCast dataset of 9214 chemicals tested in various subsets of 1399 assays and cross-referenced the assay targets with the cellular and molecular events mapped to CV failure modes. To account for the sparsity of the data matrix, we analyzed the frequency distribution by chemical and by assay and applied a minimum 50% inclusion threshold for further consideration (i.e., a chemical had to be tested in at least 50% of the assays and an assay had to have data for at least 50% of the chemicals). Further, we applied a Z-score filter to exclude those chemical−assay concentrations that may be due to cell stress response or cytotoxicity.[35] The resulting assays and related chemicals were then processed using the ToxPi software (https://toxpi.org/), which is a flexible, decision-support visualization tool developed by Reif and co-workers to represent individual or cumulative key events for a possible toxicological effect.[36,37] The ToxPi tool enables integration of multiple sources of evidence by transforming data into scaled "slices" that make up a circle or pie and provide transparent, visual rankings. For each slice, the width represents the relative weight of the respective variable (here taken to be equivalent across targets), and the distance from the origin is directly proportional to the value of entered data, i.e., the normalized in vitro activity concentrations across all assays mapped to a target/slice.[37] The AC50 (activity concentration at 50% of maximal activity) values for each chemical across all the assays in one slice were summed and normalized by the slice maximum across all chemicals, providing a unitless number for rank ordering chemicals, and normalized scores for each slice were plotted as a radar chart.[37] The specific application of the ToxPi tool to the identified subset of CV-relevant assays is referred to here as "CardioToxPi". All the

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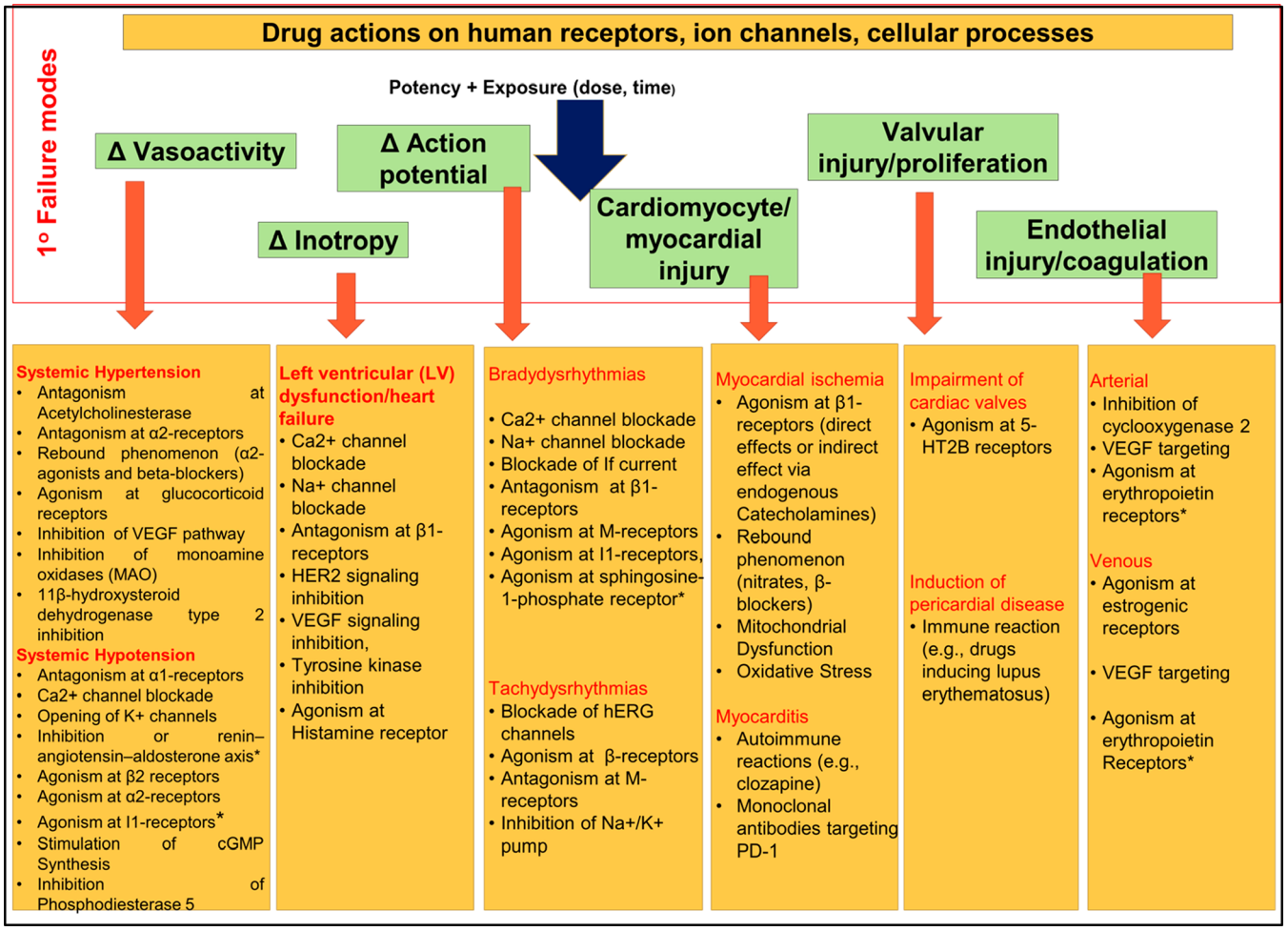

Figure 1. CV failure modes and associated molecular and cellular events. Targets not included in CardioToxPi are marked with asterisks.

unique chemicals in the Tox21 library have been evaluated by analytical chemistry quality control (QC) methods to provide detailed purity and identity information.[38] The chemical QC data accessible via the NTP Integrated Chemical Environment platform (https://ice.ntp.niehs.nih.gov/) was also mapped to the CardioToxPi results.

2.3. Chemical Clustering and Prioritization. We first determined the optimum number of clusters by the elbow method, which consists of plotting the within-cluster sums of squares (WCSS(x)) value on the y-axis according to the number of clusters (x) considered on the x-axis.[39] Chemicals were clustered using k-means and hierarchical clustering to identify chemicals with similar toxicity characteristics based on the CV-relevant in vitro biological space. In k-mean clustering, the clusters are plotted on a Principal Component Analysis (PCA) field, where each point represents the CardioToxPi for a single chemical. In hierarchical clustering, the cluster dendrograms are generated using one of six hierarchical clustering methods, with CardioToxPi profiles for individual entities (e.g., chemicals) at each leaf. The pairwise correlation between the various CardioToxPi targets was examined, and their respective contributions to the chemical clusters were assessed using PCA. Chemical clustering based on structure was performed using Kohonen self-organizing maps (SOMs) to identify regions of chemical space with higher CardioToxPi scores, indicating structural features that were potentially enriched for in vitro bioactivity related to cardiotoxicity.[40] Using OPERAv2.6, each chemical was generated into QSAR-ready structures followed by physiochemical and structural descriptor calculation.[41] Only informative and non-correlated descriptors were selected, as described in a recent study.[42] Briefly, descriptors were selected by removing those with a null variance and no discriminant, i.e., the same value for more than 90% of the chemicals. In the next step, the pairwise Pearson's correlation coefficient (ρ) was computed for each combination of descriptors, those with $\rho > 0.9$ were clustered, and one descriptor per cluster was selected randomly. Descriptor selection and SOM clustering were performed in R v3.4.

2.4. Comparison to Literature Data. We searched the literature and identified several previously studied compounds, including drugs and environmental chemicals, that are known to produce cardiotoxicity with varying degrees of in vitro or in vivo evidence available, referred to as CV-positive, as well as a set of chemicals that had been similarly tested but demonstrated no CV effects, referred to as CV-negative in subsequent sections. We cross-referenced this set of literature-derived compounds with the chemicals ranked by CardioToxPi. We also compared the CardioToxPi predictions to recently published data from human-induced pluripotent stem cell-derived functional cardiomyocytes (hiPSC-CMs) to determine the relative contribution of each screening approach and how they could potentially be combined to identify chemicals for additional testing.[43]

2.5. Computational Resources. The Tox21/ToxCast chemical–assay data was accessed using the invitroDB v3.2 database hosted on the U.S. EPA Web site (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data). Individual assay descriptions are available on the EPA CompTox Chemicals dashboard (https://comptox.epa.gov/dashboard). The CardioToxPi scores, profiles, and related images were generated using the ToxPi v2.3 software (https://toxpi.org/). Chemical structure processing and physicochemical property/structural descriptor predictions were performed using OPERA v2.6 (https://github.com/NIEHS/OPERA). All additional analyses, including clustering, PCA, SOM,

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correlation plots, and enrichment calculations, were performed using R v3.4 (packages "stats", "Kohonen", "ggplot2", and "tidyverse").

3. RESULTS AND DISCUSSION

3.1. Collection of Molecular and Cellular Events Linking to Cardiotoxicity. To structure our approach to defining relevant mechanistic bioactivity targets, we considered common forms of "failure" in the CV system, i.e., so-called failure modes (Figure 1). The CV system, like most biological systems, fails to perform its usual life-sustaining functions in a relatively finite number of ways. They include contractile or rhythmic dysfunction of the rhythmically beating myocardium; injury and death of the primary contractile cellular components (the cardiomyocytes); changes in the contractile state of major blood vessels (vasoactivity); activation, injury, or death of endothelial cells lining major and minor blood vessels; and structural alteration of heart valves that maintain unidirectional blood flow within the closed loop system. Injury or functional perturbation of any of these elements can affect the health and function of the others due to the interdependency of the individual components on overall function.[44] Accordingly, the phenotypic and functional manifestations of CV injury in vivo are far more complex and varied than the initiating failure modes.

The failure mode framework enabled a literature-based search to identify key molecular mediators of those failures. Figure 1 represents many of those mediators, many of which one could expect given their respective roles in normal CV physiology. These mediators represent a finite list of "screenable" bioactivity targets whose modulation would support a hypothetical hazard risk for the CV system.

The collected adverse events and molecular/cellular signaling pathways linked to various cardiac failure modes were used to identify Tox21/ToxCast assay targets that reflect bioactivities with the potential to be adverse to the CV system. This subset of human in vitro HTS assays whose endpoints map to CV adverse events comprise the CardioToxPi, which was used to visualize the relevant bioactivity of the Tox21/ToxCast chemical inventory.

Our initial focus was on a panel of mechanistic targets that are used in secondary pharmacological screening for predicting toxicity of drug candidates.[32] Of the 25 secondary pharmacological target families identified in Bowes et al., 19 target families were linked to the CV system as the main organ class, and all of these were available in the Tox21/ToxCast dataset: adenosine receptor, adrenergic receptor, dopamine receptor, endothelin receptor, histamine receptor, opioid receptor, muscarinic acetylcholine receptor, serotonin receptor, vasopressin receptor, acetylcholine receptor subunit, voltage-gated calcium channel, voltage-gated potassium channel, voltage-gated sodium channel, acetylcholinesterase, cyclooxygenase, phosphodiesterase, monoamine oxidase, glucocorticoid receptor, and serotonin transporter. To this core group of targets, we added 103 assays based on their biological relevance, e.g., phenotypic readouts in vascular endothelial cells or clinical biomarkers of vascular dysfunction. For example, low levels of oxidant species are required for endogenous signal transduction processes, but higher levels of oxidant species are associated with many pathological conditions, including oxidative stress, one of the major causes and effects of drug-induced cardiotoxicity.[45] Cardiomyocytes are involved in constant energy-utilizing contractility functions using enormous amounts of adenosine triphosphate (ATP) and necessitating dynamic formation and remodeling of mitochondrial networks. Mitochondria have an essential role in myocardial tissue homeostasis; thus, deterioration in mitochondrial function eventually leads to cardiomyocyte and endothelial cell death and consequent CV dysfunction.[46] Tissue factor (TF) is a protein that plays an important role in maintenance of blood coagulation cascade, and multiple lines of evidence support the significance of TF in clinical heart pathologies.[47] Similarly, drugs that inhibit tyrosine kinases have been associated with heart failure, left ventricular (LV) dysfunction, conduction abnormalities, QT prolongation, acute coronary syndromes, myocardial injury, arterial thromboses, and hypertension.[48,49] Intracellular mitogen-activated protein kinase (MAPK) signaling cascades likely play an important role in the pathogenesis of cardiac and vascular disease.[50] The aryl hydrocarbon receptor may exert adverse effects associated with endothelial dysfunction, and its activation leads to vascular inflammation and promotes atherosclerosis.[51] Therefore, we have included the assays associated with these targets from the Tox21/ToxCast inventory.

In an attempt to cover all pathways involved in CV disease and cardiotoxicity, we additionally searched the NCATS BioPlanet database (https://tripod.nih.gov/bioplanet/#).[52] Searching "Cardiovascular disease" and "Cardiotoxicity" yielded 197 pathways. Many of these pathways do not contain any Tox21/ToxCast assay targets, but we were able to identify 49 pathways and seven additional molecular targets with assays available in Tox21/ToxCast (activator protein-1, aromatase protein, estrogen receptor alpha, hypoxia inducible factor 1, nuclear factor-kappa B (NFKB), peroxisome proliferator activated receptor gamma, and TP53). Out of the 24 targets we had already identified, 11 of them were also mapped to CV pathways using BioPlanet, confirming their biological relevance. For example, activator protein 1 (AP-1) is a transcription factor controlling cellular differentiation and growth processes, and studies implicate that it is involved in pro-inflammatory pathways of atherosclerotic disease.[53] We also added assays that correspond to clinical biomarkers with literature supporting their utility as a tool for cardiotoxicity risk prediction and early detection of subclinical events.[54−56] We have assays associated with interleukin-6 (IL6), tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), intercellular adhesion molecule 1 (ICAM-1), natriuretic peptides, and serum amyloid A (SAA). Natriuretic peptides include the B-type natriuretic peptide (BNP) and its amino-terminal fragment (NT-pro-BNP) and are released in response to elevation in LV filling pressure and wall stress.[57] It has been reported that acute myocardial hypoxia results in a rapid increase in release of pro-BNP peptide from the ventricular myocytes, in both in vitro and in vivo systems.[54] Increases in IL6 and reactive oxygen species (ROS) were significantly correlated with the reduction of LV systolic function.[58] Endothelial activation can lead to vascular dysfunction and accelerated atherosclerosis. ICAM-1, PAI-1, and t-PA are known markers of atherosclerosis and cardiac fibrosis.[59−61] Activation of ICAM-1 can lead to reduction in cardiomyocyte contractility that can be measured in activated isolated cardiomyocytes.[62] A data mining-based approach identified increases in SAA, an acute phase protein marker of systemic inflammation, as an in vitro marker for CV toxicity measured in a coronary artery smooth muscle cell-based model of vascular inflammation (the BioMAP CASM3C system used here).[63]

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Table 1. CardioToxPi Legend, Including the Target Name Corresponding to Each Slice, Its Reported Effect on the CV System, the Supporting Reference, and the Slice Color (See Figure 2)

Slice	Target name	Effect	Reference	Slice Color
ADORA	Adenosine Receptor	Vasodialation, alterations in BP	Bowes et al., 2012	
ADR	Adrenergic Receptor	Arrhythmia, Alterations in BP	Bowes et al., 2012	
CHRM	Muscarinic Acetylcholine Receptor	Alterations in BP and HR, tachycardia	Bowes et al., 2012	
DRD	Dopamine Receptor	Alterations in BP and HR, Vascular relaxation	Bowes et al., 2012	
EDNR	Endothelin Receptor	Alterations in BP, Can exert adverse effects during	Bowes et al., 2012	
HTR	Serotonine Receptor	Alterations in BP, Potential cardiac valvulopathy	Bowes et al., 2012	
AVPR	Vasopressin Receptor	Alterations in BP and HR, Cardiac hypertrophy	Bowes et al., 2012	
HRH	Histamine Receptor	Positive inotropy	Bowes et al., 2012	
OPR	Opioid Receptor	Alterations in BP and Cardiac contritility	Bowes et al., 2013	
CHRNA	Cholinergic receptor	Alterations in BP and HR	Bowes et al., 2012	
SCN1A	Voltage-gated Sodium Channel	Slowed cardiac conduction; prolonged QRS interval	Bowes et al., 2012	
CACNA	Voltage-Gated Calcium Channel	Alterations in BP, QT prolongation, Arrhythmia	Bowes et al., 2012	
KCNH2	Potassium Voltage Gated Channel	QT prolongation	Bowes et al., 2012	
VEGF	Vascular Endothelial Growth Factor	Alterations in BP , Cardiac Ischemia	Touyz & Herrmann, 2018	
VascularTissue	Vascular Tissue	Myocardial ischemia, cardiac Arrhythmias		
OxidativeStress	Oxidative Stress	Cellular Hypertrophy; Cardiac Cell Death	Takimoto & Kass, 2007	
MtDysfunction	Mitochondrial Dysfunction	Cardiac dysfunction; Cardiomyopathy	Marin-Garcia, 2003	
TissueFactor	Tissue Factor	Alterations in BP and ventricular hypertrophy	Bode & Mackman, 2015	
PDE	Phosphodiesterase	Alterations in cardiac contractility, HR and BP	Bowes et al., 2012	
MAO	Monoamine Oxidase	Alterations in BP	Bowes et al., 2012	
JNK	c-Jun N-terminal kinase	Vascular injury, cardiac hypertrophy	Muslin, 2008	
TyrKinase	Tyrosine Kinase	Alterations in BP, LV dysfunction, conduction abnormalities, QT prolongation	Lamore, Kohnken, Peters, & Kolaja, 2020	
AroPro	Aromatase Protein	Ischemic heart disease	Khosrow-Khavar et al., 2017	
ACHE	Acetylcholinesterase	Alterations in BP and HR	Bowes et al., 2012	
COX	Cyclooxygenase	Myocardial infarction; Alteration in BP; Ischaemic stroke; Atherothrombosis	Bowes et al., 2012	
ERAlpha	Estrogen receptor Alpha	Abnormal cardiac contractility, cardiac hypertrophy	Pugach, Blenck, Dragavon, Langer, & Leinwand, 2016	
NR3C1	Glucocorticoid receptor	Alterations in BP; Arrhythmia	Bowes et al., 2012	
PPARG	Peroxisome Proliferator Activated Receptor Gamma	Cardiac hypertrophy , Atherosclerosis	Das & Chakrabarti, 2006	
AHR	Aryl hydrocarbon receptor	Endothelial dysfunction, Atherosclerosis	Wu et al.,2011	
AP1	Activator protein1	Atherosclerosis	Meijer et al., 2012	
HIF1A	Hypoxia Inducible Factor1Alpha	Ischaemia disease	Semenza, 2014	
NFKB	NF Kappa B	Atherosclerosis	Fiordelisi et al, 2019	
TP53	Tumor Protein p53	Alteration in cardiac function	Mercer & Bennett, 2006	
ICAM-1	Intercellular adhesion molecule-1	Markers of endothelial dysfunction	Boyd et al., 2008	
IL6	Interleukin 6	Markers of inflammation and oxidative stress	Chu et al., 2020	
t-PA	Tissue Type plasminogen activator	Markers of endothelial dysfunction	Mason, 2017	
PAI -1	Plasminogen activator inhibitor	Markers of endothelial dysfunction	Mason, 2017	
NPA	Natriuretic peptide A	Release in response to elevation in LV filling pressure and wall stress	Mason, 2017	
SAA1	Serum amyloid A1	Direct promotion of vascular dysfunction through SAA within vascular tissues	Berg, Polokoff, O'Mahony, Nguyen, & Li, 2015	
SLC6A	Serotonin transporter	Pulmonary Hypertension,Cardiac Arrhythmiasand Cardiac Valve Abnormalities	Bowes et al., 2012	

Table 1 lists the CardioToxPi assay endpoints, color-coded by target family: G protein-coupled receptors (GPCRs) (pale yellow), ion channels (blue), signal protein (pale orange), vascular tissue (VT) (pale green), cellular events (orange), cofactors (gray), enzymes (pink), nuclear receptor (NR) (green), transcription factors (cyan), biomarkers (yellow), and transporters (dark yellow). Specific assay names are available in Supplementary File 2 as Table S1, and brief descriptions are provided here, while further details may be accessed via the U.S. EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) and via the cited references. Most of the GPCRs, enzyme class targets, and ion channel assays are evaluated by the cell-free Novascreen (NVS) platform, as described in Sipes et al.[64] The signal protein (e.g., vascular endothelial growth factor, VEGF) and VT-related assays were performed by NVS and Bioseek (BSK). BSK assays are performed in co-cultured human primary cells under various stimulatory conditions.[13] Here, VT corresponds to phenotypic readouts for primary umbilical vein endothelium and coronary artery smooth muscle cells. Assays related to mitochondrial dysfunction (MtDys) and oxidative stress were performed using the Apredica (APR) platform and via the National Center for Advancing Translational Sciences (NCATS) Tox21 assays in various cell lines to evaluate the decreases in mitochondrial membrane potential (MMP) and changes in cellular stress response pathways.[65] The assays for nuclear receptors are performed in ACEA, Attagene (ATG), NVS, Odyssey Thera (OT), and Ceetox/OpAns (CEETOX) platforms in cell-based formats such as HEK293, HepG2, and H295R cells.[66,67] The transcription factor assays are performed in APR, ATG, and Tox21 platforms. The assays for CV-relevant biomarkers were measured in BSK system in primary human umbilical vein endothelium, bronchial epithelial cells, coronary artery smooth muscle cells, and foreskin fibroblast cells.[68] The roles of these targets and putative CV effects, based on evidence in the literature, are shown in Table 1.

3.2. CardioToxPi Generation. It was evident from the analysis of Tox21/ToxCast data that not all the chemicals were tested in all the assays and vice versa, requiring data filtering

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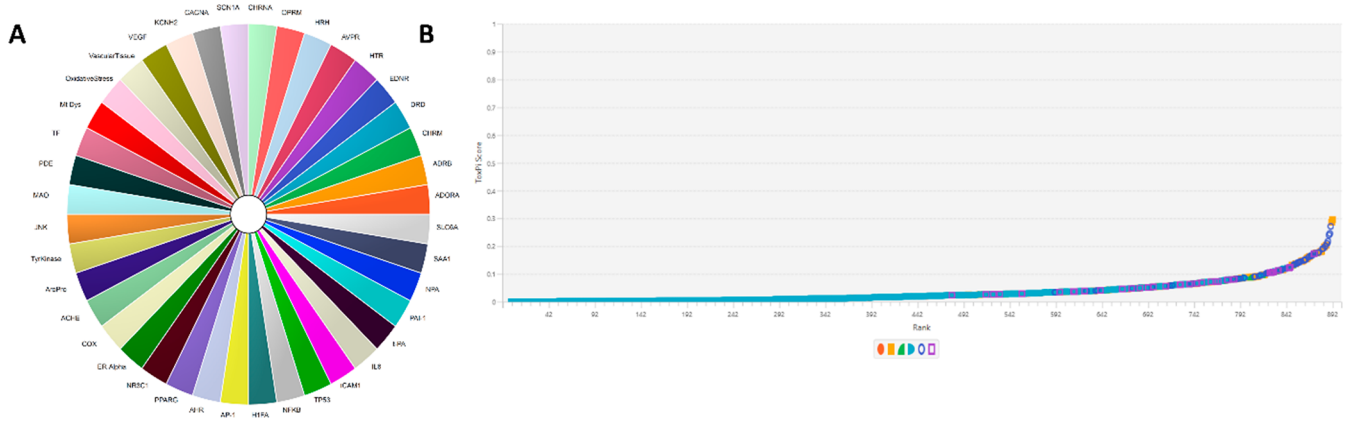

Figure 2. (A) CardioToxPi profile. (B) Graphical illustration of the prioritization profile showing chemicals from lowest to highest CardioToxPi score, and frequency histogram on the side.

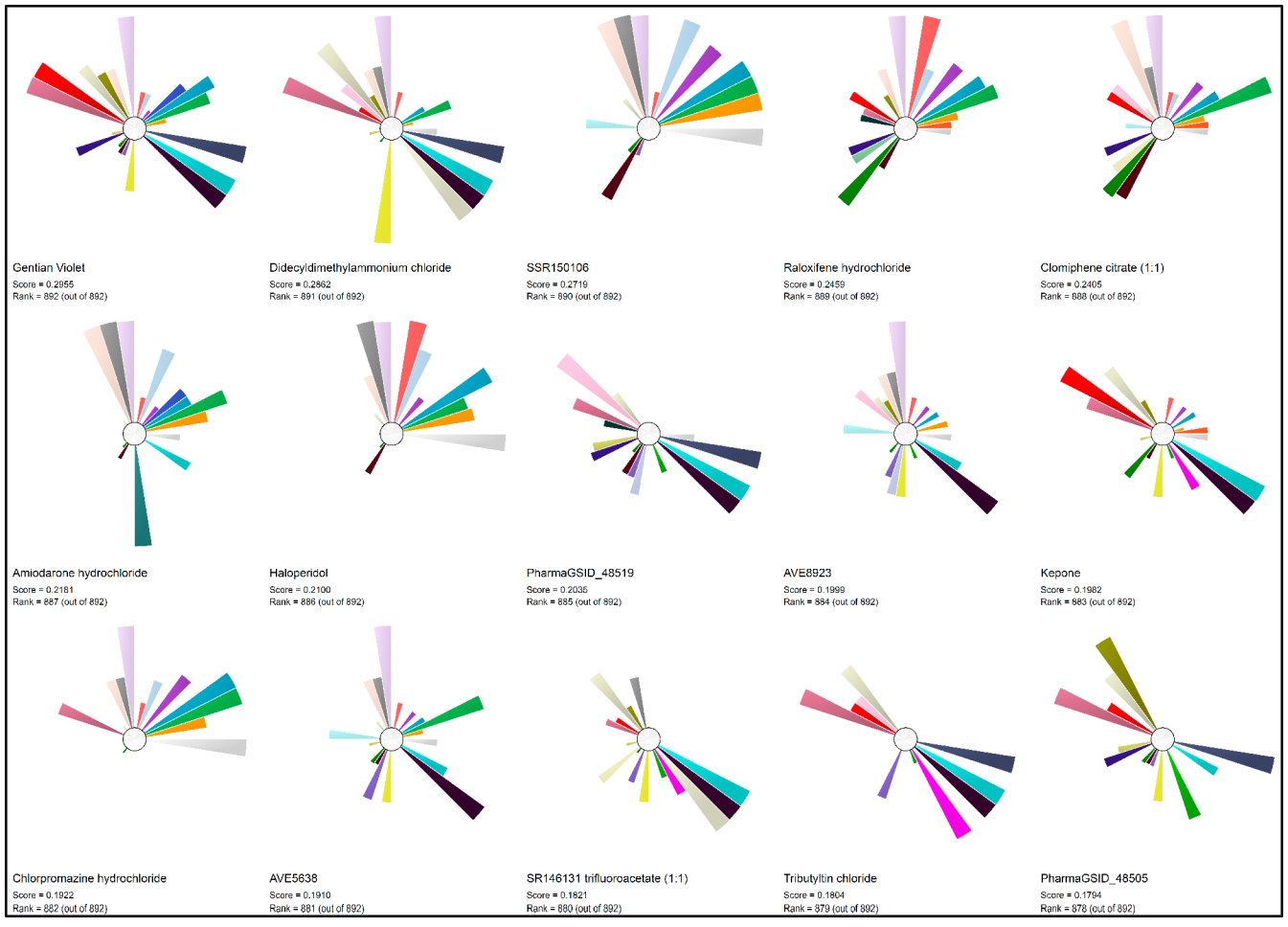

Figure 3. Individual CardioToxPi of the top ranked chemicals with their scores and ranks.

steps to account for sparseness. Initially, we found 434 assays mapped to the above-mentioned CardioToxPi targets and retained 1138 chemicals tested in >50% of the assays. Applying a similar frequency distribution of assays across chemicals yielded 314 assays with data on >50% of the chemicals. Judson et al. reported that chemical toxicity can be a result of interruption of specific biomolecular functions or a more generalized cell stress and cytotoxicity-mediated phenomenon.[35] Therefore, it may happen that a substantial amount of measured activity may be due to a false positive response caused by chemical−assay interference associated with cytotoxicity and other related "bursts" of activity. Z-score is a metric based on the concentration range at which cytotoxicity was observed. Hits with low Z-scores (deeper in the cytotoxicity region) are more likely to be associated with an interference process relative to hits with high Z-scores (active well below where cytotoxicity is seen).[35] Therefore, we applied a filter to retain activity with Z-score > 3 to remove those chemical−assay concentrations that may invoke cell stress responses or cytotoxicity and do not represent relevant

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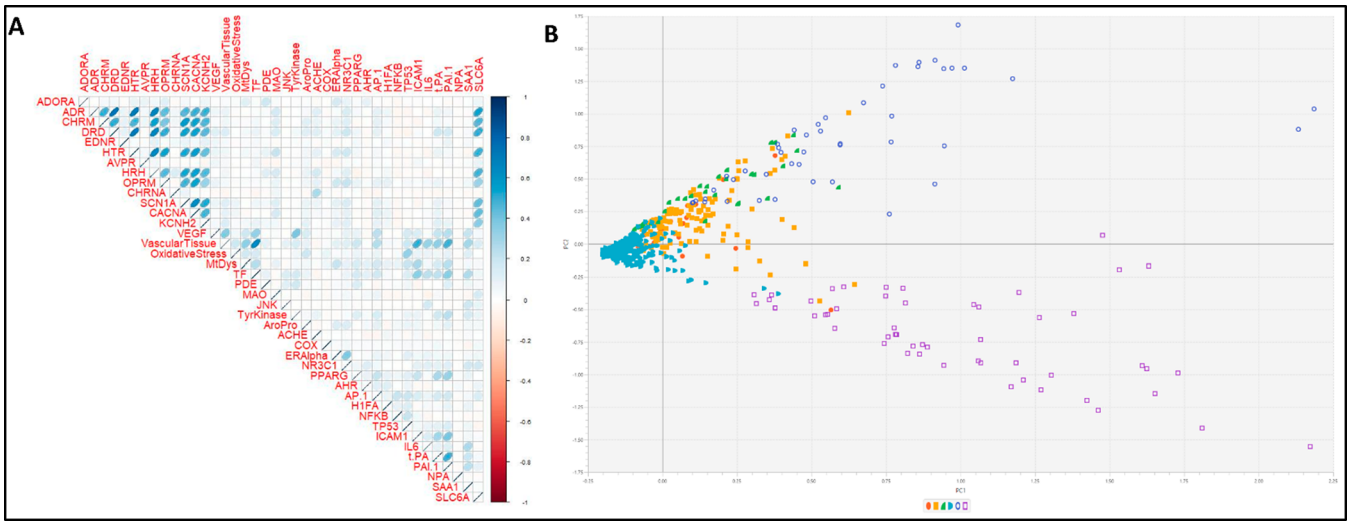

Figure 4. (A) Correlation matrix of the different variables in the CardioToxPi Profile; the magnitude of these values is visualized using circle size/color combinations. (B) Overview of the cluster structure obtained by clustering of the compounds into six clusters by *k*-means clustering. The clusters are plotted on a PCA coordinate field, where each point represents the CardioToxPi for a single chemical. The points are colored and shaped according the six clusters: Cluster 1, orange filled square; Cluster 2, blue empty circle; Cluster 3, red filled circle; Cluster 4, blue arrow; Cluster 5, purple empty square; Cluster 6, green quarter circle.

bioactivity. The full dataset of 434 assays and 9214 chemicals prior to filtering, and the CardioToxPi input dataset of 314 assays and 1138 chemicals post-filtering, are available in Tables S1 and S2, respectively. The mapping of these assays to the six CV failure modes is provided in Table S3.

3.3. CardioToxPi. The 1138 chemicals were ranked according to their activity across 314 assays and visualized using the ToxPi tool (Figure 2A). Briefly, the ToxPi score provides a dimensionless index that combines diverse HTS data sources and allows a formalized, rational integration of information from the different platforms. Visually, ToxPi is represented as component slices of a unit circle, with each slice representing information on a particular mechanistic target/phenotypic response/biomarker. The slice distance from the origin is proportional to the normalized value (e.g., assay potency) of the data points composing that slice, and all slices were set to have equal weight. To normalize the output, the slice weights were scaled to have a net sum of 1, producing ToxPi scores for every chemical between 0 and 1, where a score of 1 would indicate the chemical had the highest possible potency against every assay/target in every slice.[37] The CardioToxPi (application of ToxPi to the CV targets) scores and ranks are presented in Figure 2B. The highest CardioToxPi score for any ToxCast chemical was 0.295, and the lowest was 0. Compounds with a CardioToxPi score of 0 were predicted by this analysis to be negative for CV toxicity, based on the in vitro HTS bioactivity profile examined here. Chemical CardioToxPi scores, individual slice scores, and QC flags are given in Table S4.

The CardioToxPi profiles of the 15 highest ranked chemicals are shown in Figure 3. For many of the example chemicals with the highest CardioToxPi scores, these predictions are concordant with reports in the literature describing the potential of these chemicals to induce cardiotoxicity, e.g., didecyldimethylammonium chloride, tributyltin chloride, tributyltin methacrylate, kepone, 3-methyl-4,6-di-*tert*-butylphenol (4,6-di-*tert*-butyl-*m*-cresol), amiodarone, haloperidol, and clomiphene. It is worth noting that kepone had a Tox21 QC grade of "Fail", meaning that it did not achieve a sufficient analytical purity level, so the results should be interpreted with caution.

Didecyldimethylammonium chloride (CardioToxPi score: 0.286) is a quaternary ammonium compound (QAC) used as a germicide, antiseptic, and wood preservative which causes pulmonary inflammation and fibrosis in mice.[69] Many QACs have been identified as potent inhibitors of hERG potassium channels,[70] consistent with our data. Amiodarone (a class III anti-arrhythmic drug) and haloperidol and chlorpromazine (anti-psychotic drugs) were also found inhibit the potassium channel.[71−73] Clomiphene is a non-steroidal triphenylethylene anti-estrogen agent (CardioToxPi score: 0.240) shown to inhibit cardiac voltage-gated potassium current components and block the voltage-gated fast sodium current and the L-type calcium current, which may lead to QT prolongation.[71] Several highly ranked chemicals (e.g., tributyltin chloride, tributyltin methacrylate) are organotin compounds that are known to cause CV toxicity by triggering vascular reactivity abnormalities. It has been reported that acute exposure to tributyltin chemicals is responsible for an intense negative inotropic effect involving impairment in sarcoplasmic reticulum (SR) calcium handling.[74] The organochlorine insecticide kepone inhibits cardiac sodium pump activity and may reduce SR calcium transport mechanisms by altering phosphorylation of several proteins, including phospholamban in rat cardiac SR.[75] While these mechanisms from the literature are not specifically represented in the bioactivity screens examined here, these compounds were all identified as potassium channel inhibitors, and their profiles are suggestive of inflammation, oxidative stress, and mitochondrial dysfunction.

Other highly ranking compounds such as gentian violet and dinocap did not have existing literature support for CV toxicity that we could identify, and there were several failed pharmaceutical compounds (e.g., AVE8488, PharmaGSID_48519) lacking any additional detail. Despite having applied rigorous filters to the assay data informing CardioToxPi in an attempt to confine the activity to pre-cytotoxic concentrations, it is possible that some of the cardiotoxic potential identified here could be driven by

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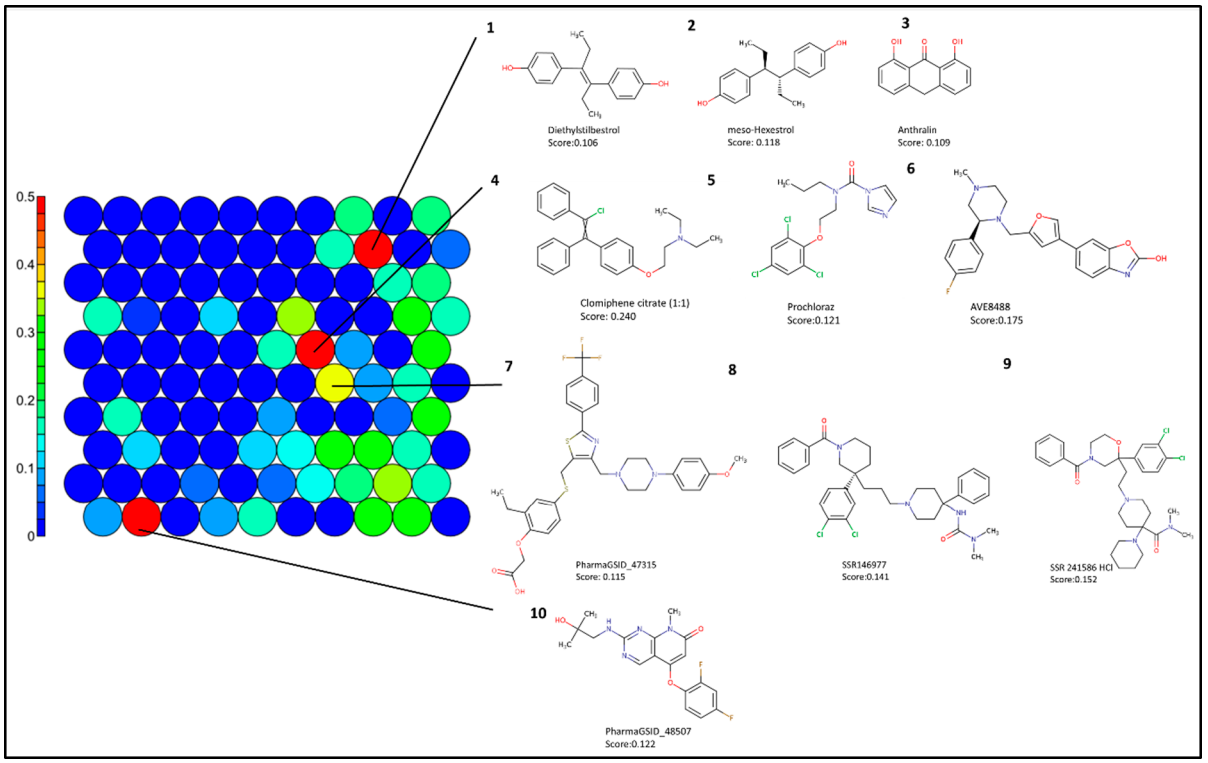

Figure 5. Structure-based SOM on the 1095 structure-curated chemicals included in CardioToxPi, distributed across 100 structural clusters and colored using percent active chemicals in CardioToxPi. Example chemical structures for clusters enriched for activity are displayed. Cluster 2, 1−3; Cluster 88, 4−6; Cluster 47, 7−9; Cluster 57, 10.

generalized cell stress mechanisms appearing just prior to overt cytotoxicity.

Differential correlation among these signatures/slices was examined by plotting the correlation of the variables with each other (Figure 4A). From these visualizations it is apparent that several variables are highly correlated with each other. Most of the GPCR targets (ADRB, CHRM, DRD, and HTR) are correlated with one another and with calcium-, sodium-, and potassium-gated ion channels, as well as with neurotransmitters SLC6A (subtypes 2 and 4), indicating that similar chemicals were active against both groups of targets. Another cluster consisted of VEGF, oxidative stress, MtDys, TF, and VT targets (as determined by cell type), where the last two were among the most highly correlated targets in the CardioToxPi. TF is an initiator of the extrinsic blood coagulation cascade and is a clinical risk factor for thrombosis.[47] This cluster was also correlated with another group of cytokines such as IL6 and molecular targets in the plasminogen activating system that signals extracellular matrix interaction. These correlations may also be driven in part by the fact that the same assay platforms (NVS in the case of GPCRs and ion channels, and BSK in the case of TF and cytokines measured in human primary cell cultures) were used to query these targets. To determine the relative contribution of each target to the overall ranking, the CardioToxPi slices were projected on a PCA defined using the individual ToxPi scores, where PC1 and PC2 captured ∼14.6% and 9.1% of the variance, respectively (see Supplementary File 1, Figure S1 for slice loadings). The results obtained are similar to those of the correlation plot, demonstrating that VT, TF, several GPCRs, and calcium channel activity are driving much of the variance.

Clustering. Cluster optimization (elbow method, Figure S2A) yielded six clusters of chemicals, visualized in Figure 4B, using the *k*-means approach. The clustering by hierarchical approach is presented in Figure S2B, and cluster membership was largely consistent across methods. Given the diverse number of targets in the CardioToxPi, clustering approaches are useful for identifying groups of compounds with similar bioactivity signatures, compounds which are atypical by any characteristic, and patterns potentially useful for identifying chemicals to test in subsequent qualifying assay systems like more complex in vitro and in vivo systems. Tables S4 and S5 contain information on cluster membership for each chemical (non-zero ToxPi score), and the activity patterns driving the clusters are described briefly here. Cluster 1 (N = 83) contains environmental chemicals with low CardioToxPi scores, and the cluster membership is largely driven by PAI-1 activity, which is involved in regulating coagulation. Cluster 2 is comprised of several high to moderate activity chemicals (N = 46). This cluster contains mainly environment chemicals, including gentian violet, didecyldimethylammonium chloride, kepone, organotins, and a few failed pharmaceuticals (Figure 3), and is characterized by strong activity in t-PA, PAI-1, and MtDys endpoints not seen in combination in other clusters. Cluster 3 is composed of 28 chemicals that demonstrate activity for HIF1A, which is an indicator of oxidative stress signaling, but with moderate to low overall CardioToxPi scores. The largest cluster (Cluster 4, N = 645) contains mostly chemicals which were either inactive or had very low CardioToxPi scores (weakly active in only one assay). Cluster 5 contains 55 chemicals with high to moderate CardioToxPi scores displaying activity across a more diverse range of targets, but with common activity across ion channels including KCNH2,

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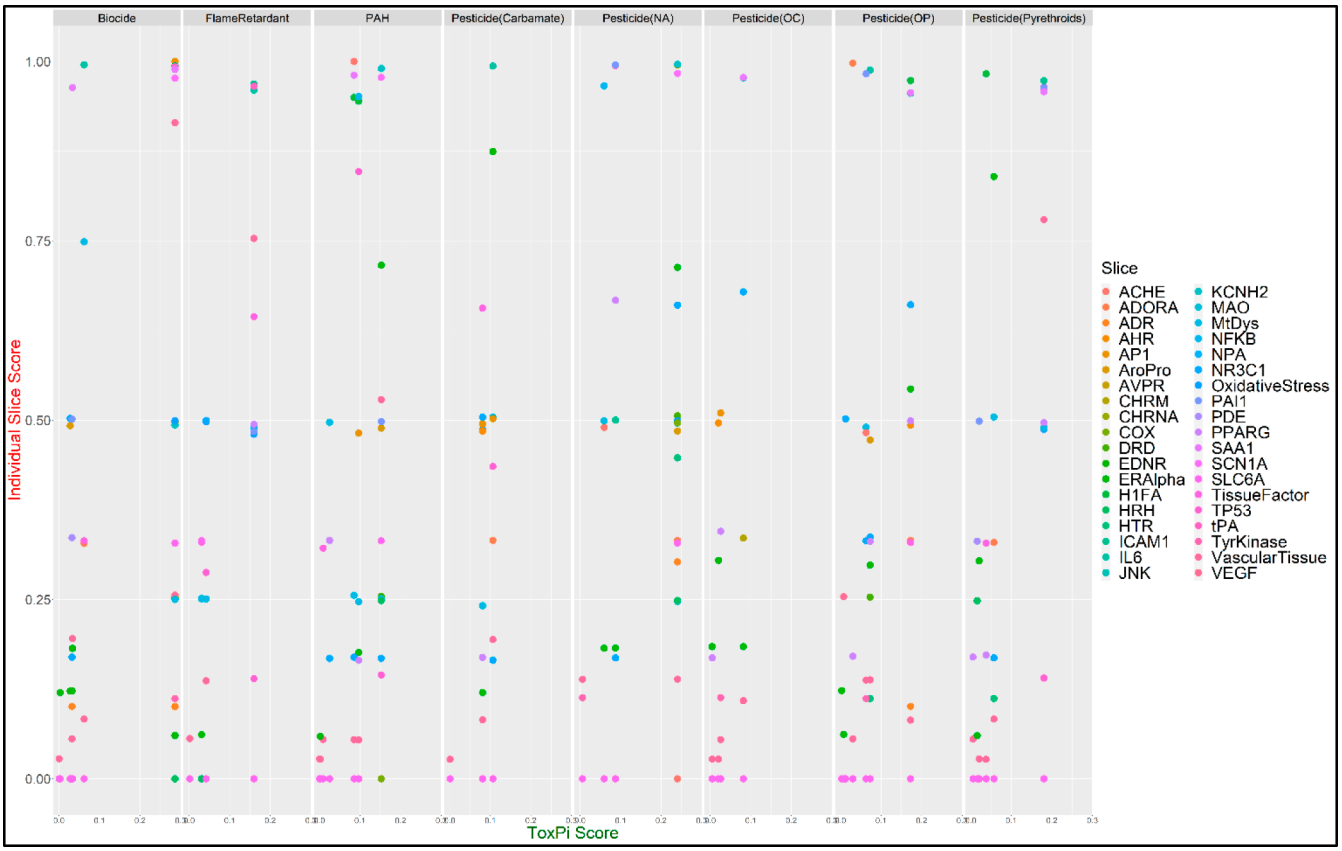

Figure 6. Scatter plot showing individual CardioToxPi slice scores for environmental chemicals by class. Slices are defined in Table S4.

SCN1A, and CACNA. Several chemicals with known QT-prolongation effects are found in this cluster, such as estrogenic modulators raloxifene and clomiphene, anti-psychotic drugs chlorpromazine and haloperidol, the anti-arrhythmic drug amiodarone, and other drugs such as tamoxifen, fabesetron, methadone, and failed pharmaceuticals,[71−73,76] lending support to the hypothesis that interference with ion channels contributes to the cardiotoxic arrhythmic effects caused by chemicals in this cluster. Cluster 6 is comprised of 35 chemicals with moderate to low scores, most of which show IL6 activity.

We next applied a SOM approach to identify structural clusters enriched for CV-relevant bioactivity profiles. From the CardioToxPi results, 1095 unique chemicals were extracted following structure curation and were clustered using SOM and a set of 62 non-correlated and informative 1D-2D molecular structure descriptors; see Methods. The values of calculated descriptors for these chemicals are provided in Table S6. The chemicals were clustered in a SOM fixed with 100 clusters, allowing segregation of the chemicals with no clusters empty, and two clusters with less than four chemicals (Figure S3). The SOM was colored using the percentage of CardioToxPi active chemicals found in each cluster. Chemicals with CardioToxPi score $\geq$ 0.1 were considered as active for generating the SOM (Figure 5). Active chemicals were distributed across 42 clusters, with 1.61 $\pm$ 0.76 active chemicals per cluster, an average percentage of active chemicals per cluster equal to 18.7%, and maximum equal to 50%. Four clusters that are enriched for chemicals active in terms of CardioToxPi score are highlighted, and example structures are shown in Figure 5. Cluster 2 (n = 6) and Cluster 88 (n = 6) both contain 50% active chemicals. Cluster 57 also contained the maximum percentage (50%) active chemicals, although total number of chemicals was only 2 in this cluster. Cluster 47 (n = 8) contained 37.5% active chemicals. However, no significant structure−activity relationship was indicated by the SOM analysis, confirming the difficulty in structurally separating active and inactive chemicals due to the large structural diversity of the chemicals and broad mechanistic space represented by the targets. Two-dimensional PCA (PC1 vs PC2) based on structural descriptors revealed broad chemical diversity among the selected chemicals without clearly segregated clusters based on activity (Figure S4).

Reference Data. As outlined in the Methods section, to compare CardioToxPi predictions with evidence from the literature, we identified 235 chemicals observed to cause adverse cardiac events or cardiotoxicity and 42 chemicals reported negative for CV effects. Initial literature-based reference chemical lists were categorized as positive or negative based entirely on the curated results from the available literature and did not contain any information from the Tox21/ToxCast assays. For the full list of 277 chemicals, the CV effects caused (or tested for and not observed) and the methods or lines of evidence available to support the categorizations are given in Table S7. It was observed that 75 out of the 277 collected chemicals with cardiotoxic effects reported in the literature were also found in our dataset and could be analyzed in the context of the CardioToxPi ranking. The analysis of the mapping between literature-based categorizations and CardioToxPi scores is presented in Figure S5, and details are provided in Table S8.

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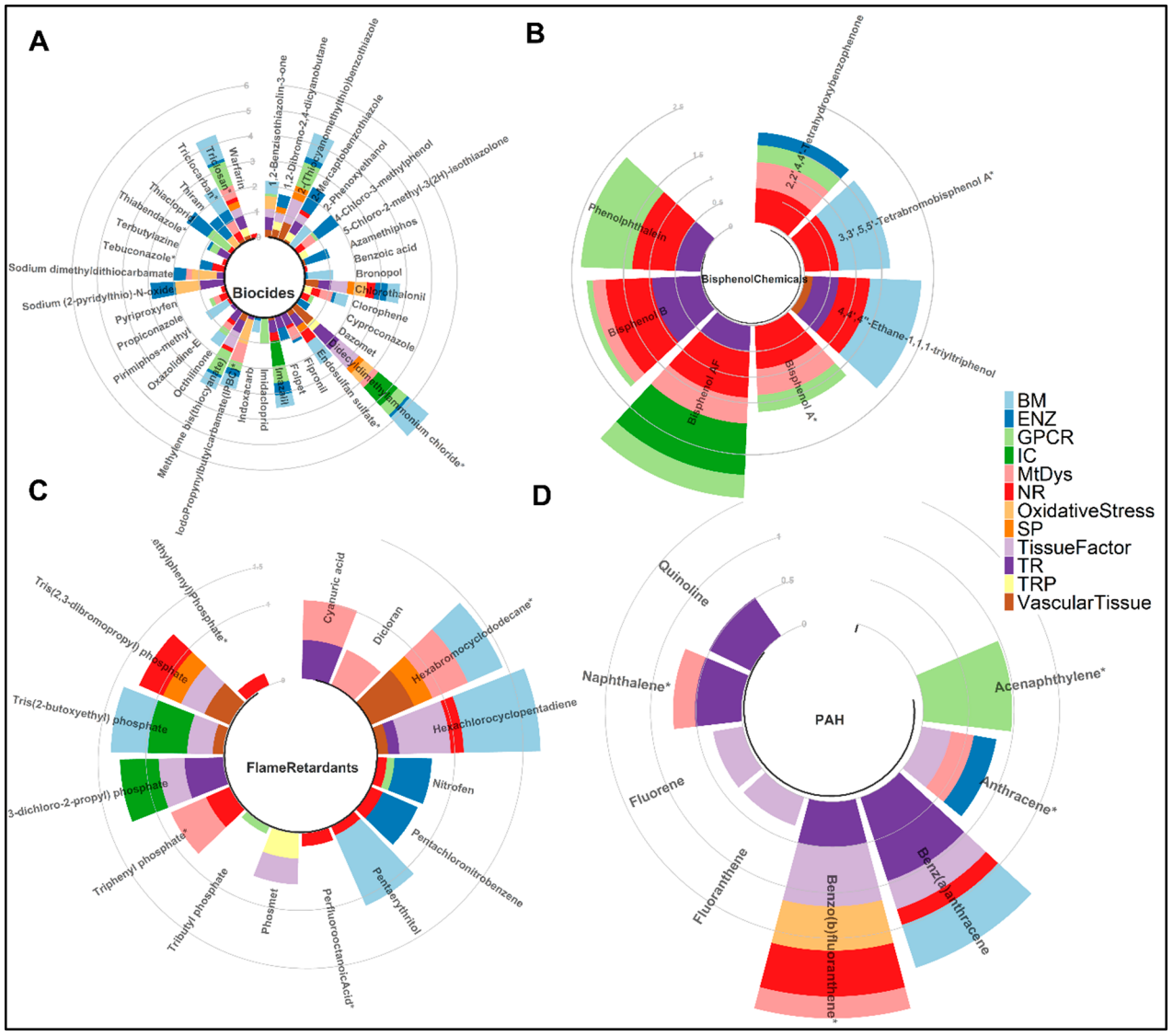

Figure 7. Activity across individual targets for different chemical classes: (A) biocides, (B) bisphenol-like chemicals, (C) flame retardants, and (D) PAHs. Chemicals with supporting evidence identified in the literature for cardiotoxicity are indicated with an asterisk. Targets defined as follows: GPCR protein (GPCR), ion channels (IC), signal protein (SP), vascular tissue (VT), cellular events (oxidative stress and mitochondria dysfunction, MtDys), cofactors (tissue factor, TF), enzymes (ENZ), nuclear receptor (NR), transcription factors (TR), biomarkers (BM), and transporter protein (TRP). Details given in Table 1.

Chemicals were broadly classified into drugs and environmental chemicals, which were further annotated as pesticides, biocides, flame retardants, bisphenols, and PAHs. Among these, pesticides were the most populated and were further subclassified into carbamates, organochlorines, organophosphorus, pyrethroids, triazines, and other. As shown in Figure 6, many environmental chemicals, e.g., bisphenol A, permethrin, endosulfan, dieldrin, and several PAHs, that have a substantial amount of cardiotoxic evidence available in the literature[77−84,21] were not highly active against the CV targets included in the CardioToxPi. Others, such as 3,3′,5,5′-tetrabromobisphenol A and the mitochondrial toxicant rotenone, showed significant scores. Notably, many biocides and organotin compounds with literature evidence of CV toxicity demonstrated significantly higher scores in CardioToxPi, the details of which are discussed in the previous section. The analysis also reveals that many positive drugs/pharmaceuticals from the literature have high CardioToxPi scores. Most of the "negative" chemicals based on lack of cardiotoxic effects in literature studies had either 0 or very low CardioToxPi scores, with the exception of mifepristone.

We analyzed the patterns observed among individual slice activity by chemical subclass, as depicted in Figure 6. Each chemical subclass identified in the literature associated with CV toxicity was expanded to include any additional compounds in the CardioToxPi dataset belonging to that category, based on the U.S. EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard). With the exception of didecyldimethylammonium chloride, all other environmental chemicals were inactive in assays associated with EDNR, CHRNA, JNK, and NPA. Multiple flame retardants were moderately active in assays associated with KCNH2 and MtDys, while exhibiting little or no activity in other slices. Most of the PAH chemicals exhibit moderate to low activity in assays associated with ERAlpha, NR3C1, MtDys, TF, and VT. Many chemicals in the pesticides class

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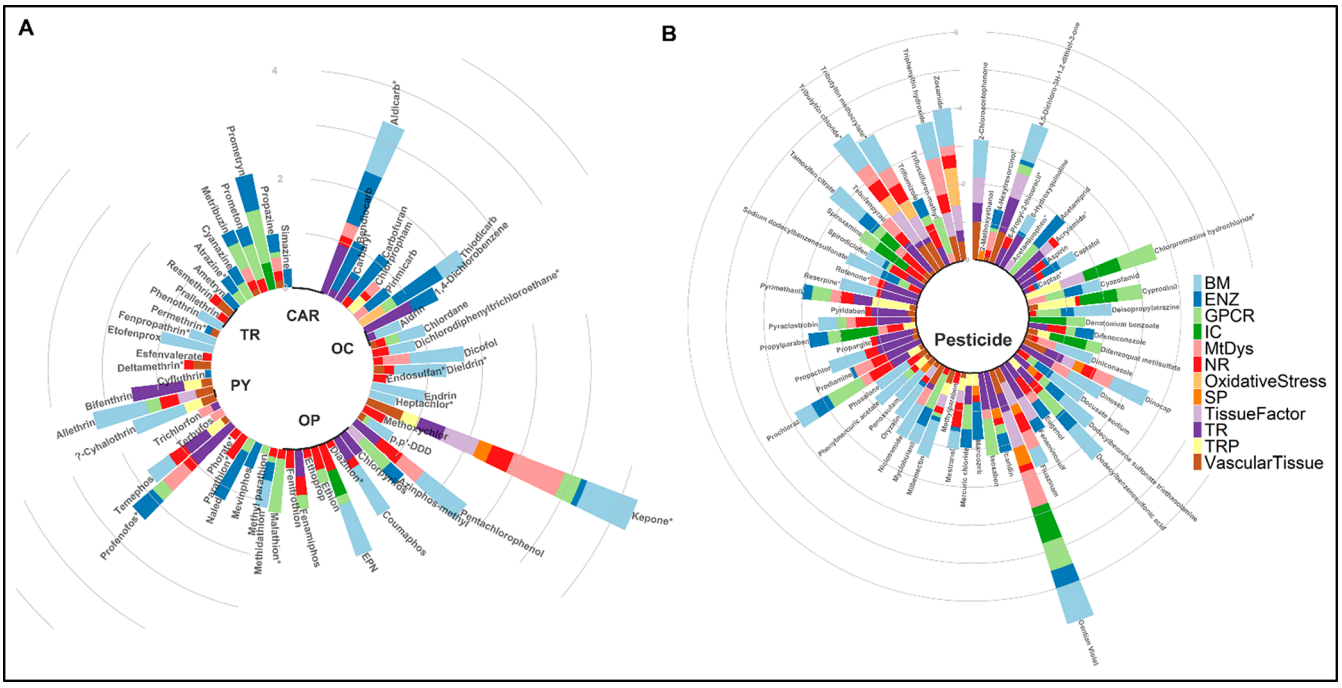

Figure 8. Activity of pesticides across individual target class. Chemicals with supporting evidence identified in the literature for cardiotoxicity are indicated with an asterisk. (A) Various subclasses of pesticides: carbamate (CAR), organochlorine (OC), organophosphate (OP), pyrethroids (PY), and triazine (TR). (B) Pesticides where no subclass is available. Targets are defined as follows: GPCR protein (GPCR), ion channels (IC), signal protein (SP), vascular tissue (VT), cellular events (oxidative stress and mitochondria dysfunction, MtDys), cofactors (tissue factor, TF), enzymes (ENZ), nuclear receptor (NR), transcription factors (TR), biomarkers (BM), and transporter protein (TRP). Details are given in Table 1.

exhibited activity in NR3C1, PPARG, and VT assays, while some also had moderate activity in KCNH2 and PAI1 assays, and others had high activity in assays associated with ADORA, AP, HIF1A, NFKB, SAA, and t-PA. None of the pesticides showed any activity in ACHE, AroPro, AVPR, MAO, NPA, and OPR assays.

We further analyzed the chemicals in each of the classes identified by the reference literature mapping to characterize the relationship between their reported mechanisms, phenotypic effects, and patterns of activity observed across our panel of mechanistic targets: GPCR protein (GPCR), ion channels (IC), signal protein (SP), vascular tissue (VT), cellular events (oxidative stress and MtDys), cofactors (TF), enzymes (ENZ), nuclear receptor (NR), transcription factors (TR), biomarkers (BM), and transporter protein (TRP); details are given in Table 1. Figure 7 displays these patterns for the classes of biocides, bisphenols, flame retardants, and PAHs, and Figure 8 displays pesticides, further subdivided into carbamates, triazines, pyrethroids, organochlorates, and organophosphates.

Many of the biocides (N = 38) show activity across at least one of the GPCR targets (Figure 7A). QACs are generally used as powerful disinfectants in the health care and food industries.[85,86] As mentioned, QACs have previously shown inhibition of potassium ion channels,[70] and didecyldimethylammonium chloride replicated that activity in the sodium and potassium channel assay here, in addition to hitting GPCRs (namely cholinergic receptor), VEGF, and MtDys assays with moderate potency and showing strong activity in TF, transcription factor (AP), and biomarker (tPA)-related assays. Endosulfan has been widely used in agriculture globally to control various insects and pests, and here it demonstrated significant activity in assays associated with NR (NFKB). A prior study suggested that myocardial cells of endosulfan-treated rats presented with cytoplasmic edema and mitochondrial vacuolization.[79] Tebuconazole (TEB) is a potent systemic broad-spectrum triazole largely used in agriculture and as an anti-fungal, and TEB exposure may affect myocardial cells' normal functioning and trigger apoptosis.[87] Here, TEB showed activity in NR and AroPro-related assays. Endosulfan and TEB were also active in potassium channel assays above subcytotoxic concentration. Triclosan and triclocarban act as antibacterial and antifungal agents widely used in cosmetic products such as soaps and shampoo, in other consumer products, including toothpaste, detergents, and toys, and in surgical cleaning treatments. A study has reported that triclosan and triclocarban induced an increase of the beating activity in hiPSC-CMs.[88] Triclosan shows higher activity in GPCR (AVPR) assays and moderate activity in ion channel (KCNH2) at higher concentration, while triclocarban demonstrates significant activity in assays related with BM (PAI-1). 3-Iodo-2-propynyl-*N*-butylcarbamate (IPBC) shows significant activity in oxidative stress and MtDys assays. IPBC is a water-soluble preservative and largely utilized in the production of the paints and wood preservatives and in the personal care and cosmetics industries. IPBC demonstrated alteration in beating parameters in hiPSC-CMs.[43] IPBC shows significant activity in oxidative stress and MtDys-associated assays. These chemicals show less activity across signaling proteins, enzymes, and NRs target classes, while some other biocides demonstrate high activity in TF and transcription factors.

Bisphenol-like chemicals (N = 7) do not show any activity in assays related to oxidative stress, signaling proteins, transporter protein, and TF but are highly active across NRs (Figure 7B). Bisphenol AF and bisphenol B show high activity across transcription factors (AP1 and HIF1A, respectively), while

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3,3′,5,5′-tetrabromobisphenol A is significantly active in assays related to biomarkers. Bisphenol AF shows high activity across ion channel (sodium channel) assay. Bisphenol A (BPA) shows significant activity in NR assay (ERAlpha), demonstrating little activity across other assays. BPA was shown to induce cardiac fibrosis in male Sprague−Dawley rats by activating the ERK1/2 pathway.[78]

Flame retardants (N = 13) had, in general, lower activity across the CardioToxPi targets, where most were inactive in oxidative stress and enzymes target classes (Figure 7C). Some of these chemicals, i.e., hexabromocyclododecane (HBCD), triphenyl phosphate, and cyanuric acid, showed moderate activity across MtDys assays. HBCD also demonstrated inhibitory activity in the potassium channel assay, but at supra-cytotoxic concentration. HBCD is a brominated aliphatic cyclic hydrocarbon and has applications in thermal insulation of building materials, upholstery textiles, and several electronics.[89] A study has reported that exposure to HBCD can cause disruption in normal calcium handling in immortalized rat cardiomyocyte.[26] Pentaerythritol had high activity against IL6, while tris(methylphenyl) phosphate was active in ion channel and NR protein assays.

PAH chemicals (N = 8) show activity across MtDys, NR, TF and transcription factors (Figure 7D). PAHs are omnipresent pollutants, formed naturally or by incomplete combustion of organic matter. PAH exposure can lead to severe ventricular arrhythmia in rats with acute myocardial infarction.[90] Acenaphthylene was broadly active against GPCR targets, benzo[b]fluoranthene and naphthalene showed significant activity in MtDys and transcription factor assays, and benzo[b]fluoranthene was also active in TF, oxidatives stress, MtDys, and NR assays.

The most populated class of chemicals is pesticides, several of which can be further subdivided into subclasses based on their chemical moieties [carbamates (n = 7), pyrethroids (n = 12), triazines (n = 8), organochlorines (n = 13), and organophosphates (n = 19)], while many of them cannot be categorized into a subclass (n = 317). The detailed analysis of these chemicals is presented in Figure 8. In Figure 8B, chemicals having very low score (CardioToxPi Score < 0.04) are not shown to increase the readability of the figure. However, the chemicals having evidence in literature for adverse CV effects are displayed and discussed.

It has been demonstrated that localized and systemic oxidative stress parameters tend to increase upon exposure to organophosphate and carbamate poisoning.[91] Organophosphate poisoning led to disturbed cardiac rhythms and arrhythmias, along with other implications such as QT prolongation, ST- and T-abnormalities, histopathological evidence of focal necrosis, and regeneration.[92] Here, we observed that EPN and coumaphos show moderate activity in ion channels. Profenofos is also moderately active in MtDys assays, which are closely associated with oxidative stress. However, organophosphates were not active in assays measuring oxidative stress at concentrations that were far enough below cytotoxicity cutoffs to be included in the CardioToxPi. In fact, five organophosphate pesticides (naled, coumaphos, dichlorvos, trichlorfon, and phorate) caused oxidative stress at concentrations approaching cytotoxicity, but this activity was filtered out by our workflow in order to try and identify more specific mechanisms not indicative of generalized cell stress. In a rare human case report, organochlorine (lindane) poisoning led to hypotension and electrocardiographic abnormalities, myocardial infarction, and LV myocardial dysfunction.[93] Interestingly, all the organochlorine chemicals demonstrate activity in potassium ion channel assays above sub-cytotoxic concentration but exhibit very little activity in other assays at sub-cytotoxic concentrations.

Tributyltin chloride and tributyltin methacrylate were broadly and potently active against CardioToxPi targets. As mentioned previously, tributyltin has been reported to affect myocardial contractility and intracellular calcium handling.[74] These chemicals show moderate activity in MtDys and oxidative stress-related assays at sub-cytotoxic concentration, and significant activity across BM-related assays (ICAM-1). However, at higher concentration, these chemicals show significant activity in ion channels, including calcium and potassium channels, oxidative stress, and MtDys assays. Many pesticides, such as captan, 2-methoxyethanol, 6-propyl-2-thiouracil, and acrylamide, have been reported in the literature to cause adverse CV events,[94] though they show very low scores in CardioToxPi.

Published work has revealed that pyrethroid insecticides affect ion channels present in both neuronal and cardiac cells, and it has been reported that cardiac arrhythmia could be a class effect, unique to certain pyrethroids.[95−97] As mentioned, none of the environmental chemicals other than biocides possess activity in CACNA, but it was observed that tetramethrin and permethrin exhibit moderate activity in KCNH2 above sub-cytotoxic concentration, which is a key player in cardiac arrhythmia. Permethrin-treated rats presented increased calcium influx level in hearts.[75]

All pyrethroids but tetramethrin demonstrated broad activity in VT assays, and many studies have reported that ROS generation in heart tissues has been implicated in pyrethroid toxicity.[98,99] None of the pyrethroids display activity in assays associated with oxidative stress or MtDys at sub-cytotoxic concentrations, but 13% and 80% of them were active against assays associated with oxidative stress and MtDys, respectively, at higher concentrations. Rotenone is a widely used pesticide and a well-established mitochondrial toxicant, which was recently shown to cause fatal arrhythmias in rats.[100] In CardioToxPi, it exhibited activity in assays associated with MtDys, TR, and NR.

Comparison with Cardiomyocyte Phenotypic Screening Data. The predicted cardiotoxic potential of chemicals based on HTS data included in this study was compared with various beating parameters and cell viability-related phenotypes obtained from hiPSC-CMs using a Ca^{2+} flux assay to examine the functional effects of chemicals on cardiomyocytes.[43] The study evaluated 139 test compounds in concentration−response (0.1, 1, 10, and 100 μM) and derived benchmark concentration POD values for different beating parameters, namely beats per minute, decay rise ratio, peak amplification, peak spacing to measure cardiotoxic potential, and total cardiomyocyte cell counts (cell viability-related parameter) to measure cytotoxic potential. We used the PODs given in the study by Burnett et al.,[43] followed by normalization of these PODs, and cross-referenced these cardiotoxicity screening phenotypes with our CardioToxPi results. A total of 61 Tox21/ToxCast chemicals mapped with the chemicals presented in this study, as provided in Table S9. We compared the overall CardioToxPi score as well as individual slice components of the chemical activity signatures with the cardiotoxicity screening phenotypes. The results of example

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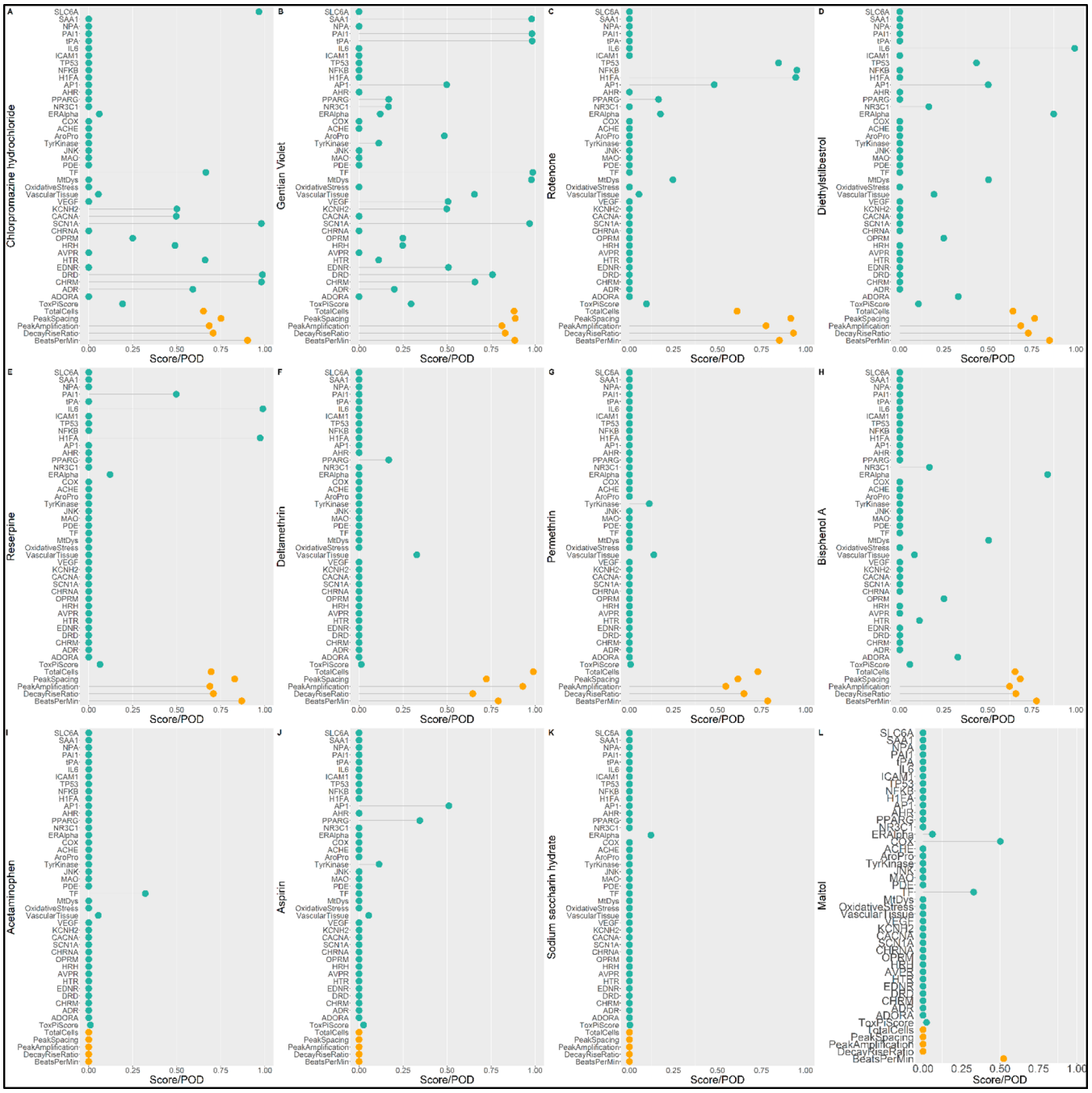

Figure 9. Comparison of phenotypic screening parameters in cardiomyocytes and CardioToxPi for example chemicals. The beating parameters are represented in orange color, while CardioToxPi assay scores are presented in green color.

chemicals which also had evidence returned in the literature search are displayed in Figure 9, covering those with positive effects in both HTS-based CardioToxPi and iPSC cardiomyocyte endpoints, or showing effects in only cardiomyocytes, with minimal ToxPi scores.

Figure 9A–D represents example chemicals (chlorpromazine hydrochloride, gentian violet, rotenone, and diethylstilbestrol) that exhibited high potency in affecting the beating and cytotoxic phenotypes and were also active in the selected HTS assay data. Chlorpromazine is considered a high-risk drug for QTc prolongation.[101] Interestingly, it shows activity across all three ion channel assays (sodium-, potassium-, and calcium-gated channels). Similarly, exposure to rotenone also leads to pro-arrhythmic effect due to the prolonged QTc.[100] Exposure to a high dose of diethylstilbestrol induced an eccentric hypertrophy in adult mice.[102] Notable, with respect to gentian violet, no studies have explored its potential effects on the CV system in vivo, although it shows significant effect in cardiomyocyte beating parameters as well as in CardioToxPi.

Figure 9E–H shows example chemicals (reserpine, permethrin, deltamethrin, bisphenol A) having significant activity in cardiomyocyte system, though very low activity in CardioToxPi. Reserpine, an alkaloid, decreases sympathetic tone and leads to hypotension and bradycardia by blocking uptake of monoamines into synaptic vesicles.[103] Permethrin and deltamethrin are pyrethroid-based pesticides. Early-life

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exposure to low doses of permethrin has long-term consequences leading to cardiac hypotrophy,[77] while exposure to deltamethrin manifested prolongation of Q-T interval in ventricular myocytes of adult cats.[104] Bisphenol A rapidly promoted arrhythmias in female rodent hearts by manipulating myocyte calcium handling.[105] However, these chemicals exhibit low overall ToxPi score as well individual slice scores.

As expected, acetaminophen and aspirin, drugs without known cardiotoxicity liability,[106,107] and sodium saccharin hydrate and maltol, food constituents, were largely without effect in both CardioToxPi and the in vitro cardiomyocyte system (Figure 9I–L). This is consistent with literature data on these compounds, which also suggested that they are non-cardiotoxic, and thus could be used as negative controls.

CONCLUSIONS

Here we present a detailed analysis of molecular targets mapped to CV failure modes and prioritize chemicals based on their bioactivity profiles against these CV-relevant targets using Tox21/ToxCast in vitro HTS data. Among the highly ranked chemicals were pharmaceuticals that have demonstrated CV effects in humans, such as estrogenic modulators associated with arrythmia and increased stroke incidence, psychotropic drugs with known QT prolongation effects, and intentionally vasoactive compounds such as anti-arrhythmic drugs, providing confirmation of the utility of the approach. Polycyclic aromatic hydrocarbons are environmental compounds with known impacts on the CV system which were also broadly active against the targets screened here. We identified several classes of environmental chemicals such as organotins, bisphenol-like chemicals, pyrethroid pesticides, and QACs with strong bioactivity against CV targets that we compared to existing data in the literature (e.g., from cardiomyocytes, animal data, or human epidemiological studies) and prioritized for further testing. Many of the predicted cardiotoxicants were substantiated based on results from the literature, and specific chemical classes such as QACs and organotin compounds were identified for future study. Although our study has immediate applications in prioritizing chemicals for CV toxicity evaluation, there are several shortcomings associated with relying upon HTS data. The multi-criteria-based CardioToxPi signature provides relative ranks for the Tox21/ToxCast chemicals using in vitro bioactivity profiles but does not account for potential environmental and human exposure. The lack of parameters including absorption, distribution, metabolism, and elimination that collectively influence toxicokinetics and systemic factors is a major limitation with almost all in vitro-based screening platforms. Some of the Tox21/ToxCast HTS data are derived from cell types not particularly relevant to cell specification in the CV system, and there are certainly many CV-relevant targets that are not represented in the dataset. Depending on the assessment and specific concerns, other cardiotoxicity mechanism/pathways of interest may need to be considered.

On the other hand, our work presents improvements in a prioritization approach based on detailed toxicity scores for cardiotoxic potential of several environmental chemicals as well as pharmaceuticals, especially indicating their potential mechanism of action and CV failure mode. It is evident from the study that several environmental chemicals demonstrate bioactivity at sub-cytotoxic concentrations against key mechanistic targets potentially involved in CV toxicity. CardioToxPi can assist in prioritizing and identifying compounds for additional testing as part of a translational toxicology pipeline to support more targeted decision-making, risk assessments, and monitoring steps. The methodology applied in this study is highly versatile, permitting inclusion of additional data systems, toxicity mechanisms and pathways, and thresholds that could be tailored to fit various decision contexts. This study also emphasizes the importance of hybrid in vitro–in silico models as an alternative to the lack of in vivo data for CV toxicity potential of environmental chemicals and drugs, and supports the continuing shift in reliance upon novel, more human-relevant alternative approaches. In the next steps, the characterization of the chemicals identified in the current study would likely include in-depth investigations of the putative mechanisms of action (e.g., effects on ionic permeation/selectivity and/or activation, deactivation, or inactivation kinetics of hERG channels, effects on contractility). We conclude that the approach used in this study will allow for the efficient identification and profiling of various environmental chemicals as potential cardiotoxicants in anticipated future screenings.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00382.

Supplementary File 1, with all the supplementary figures: Figure S1, vectors on the PCA plot indicating the relative influence of each slice on the plot of PC1 vs PC2; Figure S2, optimum number of clusters as obtained by the elbow method and overview of the cluster structure obtained by hierarchical clustering of the compounds; Figure S3, structure-based SOM on 1095 chemicals obtained from CardioToxPi analysis, with 100 clusters included in total; Figure S4, PCA of the 1111 chemicals obtained from CardioToxPi results, using the set of selected structural descriptors; Figure S5, drugs and environmental chemicals with reference literature data for positive and negative cardiotoxic effects, mapped to the CardioToxPi dataset (PDF)

Supplementary File 2, an Excel file with all the supplementary tables: Table S1, 9215 chemical and 434 assay pair; Table S2, input for generation of CardioToxPi, 1138 chemical and 314 assay pair; Table S3, mapping of assays to the six CV failure modes; Table S4, CardioToxPi results with score; Table S5, cluster description; Table S6, list of 1D-2D descriptors; Table S7, reference chemical information; Table S8, mapping of reference chemicals with CardioToxPi chemicals; Table S9, mapping of cardiomyocyte data with CardioToxPi chemicals (XLSX)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CV, cardiovascular; HTS, high-throughput screening; Tox21, Toxicology in the 21st Century; PAHs, polycyclic aromatic hydrocarbons; SOM, self-organizing map; hiPSC-CMs, human-induced pluripotent stem cell-derived functional cardiomyocytes; TF, tissue factor; LV, left ventricular; MAPK, mitogen-activated protein kinase; NFKB, nuclear factor-kappa B; AP-1, activator protein 1; IL6, interleukin-6; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor; ICAM-1, intercellular adhesion molecule 1; SAA, serum amyloid A; ROS, reactive oxygen species; GPCR, G protein-coupled receptor; VT, vascular tissue; VEGF, vascular endothelial growth factor; NVS, Novascreen; BSK, Bioseek; APR, Apredica; ATG, Attagene; MtDys, mitochondrial dysfunction; MMP, mitochondrial membrane potential; QAC, quaternary ammonium compound; SR, sarcoplasmic reticulum; NR, nuclear receptor; TEB, tebuconazole; IPBC, 3-iodo-2-propynyl-*N*-butylcarbamate; HBCD, hexabromocyclododecane; POD, points of departure

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