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Article

Predicting Chemical Immunotoxicity through Data-Driven QSAR Modeling of Aryl Hydrocarbon Receptor Agonism and Related Toxicity Mechanisms

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Nada J. Daood, Daniel P. Russo, Elena Chung, Xuebin Qin, and Hao Zhu*

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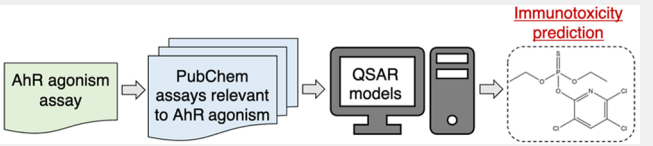
ABSTRACT: Computational modeling has emerged as a time-saving and cost-effective alternative to traditional animal testing for assessing chemicals for their potential hazards. However, few computational modeling studies for immunotoxicity were reported, with few models available for predicting toxicants due to the lack of training data and the complex mechanisms of immunotoxicity. In this study, we employed a data-driven quantitative structure−activity relationship (QSAR) modeling workflow to extensively enlarge the limited training data by revealing multiple targets involved in immunotoxicity. To this end, a probe data set of 6,341 chemicals was obtained from a high-throughput screening (HTS) assay testing for the activation of the aryl hydrocarbon receptor (AhR) signaling pathway, a key event leading to immunotoxicity. Searching this probe data set against PubChem yielded 3,183 assays with testing results for varying proportions of these 6,341 compounds. 100 assays were selected to develop QSAR models based on their correlations to AhR agonism. Twelve individual QSAR models were built for each assay using combinations of four machine-learning algorithms and three molecular fingerprints. 5-fold cross-validation of the resulting models showed good predictivity (average CCR = 0.73). A total of 20 assays were further selected based on QSAR model performance, and their resulting QSAR models showed good predictivity of potential immunotoxicants from external chemicals. This study provides a computational modeling strategy that can utilize large public toxicity data sets for modeling immunotoxicity and other toxicity endpoints, which have limited training data and complicated toxicity mechanisms.



KEYWORDS: *Immunotoxicity, QSAR, Machine learning, Aryl hydrocarbon receptor, Data mining*

INTRODUCTION

Immunotoxicity refers to the adverse effects on the immune system due to exposure to xenobiotic chemicals,[1] and the field of immunotoxicology studies the interactions between xenobiotics and the immune system that lead to toxicity. The field emerged in the 1970s as immune-related adverse events, such as cancer and reduced resistance to infections, began to rise with increased human exposure to pharmaceutical drugs and environmental chemicals.[2] Immunotoxicity was then categorized into four major types: hypersensitivity, immunosuppression, immunostimulation, and autoimmunity.[3] However, the various consequences of immunotoxicity and the complexity of the immune system make it difficult to determine the immunotoxic effects induced by a toxic chemical. Another challenge in assessing toxicity is the use of traditional animal testing, which can be expensive, time-consuming, and laborious, and it presents an issue of ethical concern.[4] This challenge signifies the need to develop alternative approaches to rapidly and efficiently predict immunotoxicity.

High-throughput screening (HTS) is a rapid *in vitro* testing method for screening thousands to millions of chemicals. Due to their cost-effectiveness and efficiency, HTS assays have become increasingly incorporated in toxicity evaluations.[5] Besides indicating specific toxicity mechanisms, the data from HTS assays can be used for computational modeling studies to prioritize chemicals that are highly likely to be toxic. For example, through machine learning (ML), quantitative structure−activity relationship (QSAR) modeling correlates the quantitative structural features of chemicals to their bioactivities/toxicities.[6] Employing these alternative approaches has shown promise in predicting some immunotoxicity results, such as skin sensitizations.[7] In this report, *in vitro* assays were developed to detect outcomes of key events that lead to skin sensitizations,[8] and ML models were trained using

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experimental results from these assays.[7] These efforts could successfully classify sensitizers and nonsensitizers of both animals and humans.[7] Aside from skin sensitivity, few studies used computational modeling for predicting immunotoxicity. For example, one study predicted immunotoxicity based on assays that tested for the cytotoxicity of B-cells and T-cells.[9] However, cytotoxicity is not the only indicator of immunotoxicity, as pollutants and drugs can cause adverse immune effects at noncytotoxic doses.[10] This lack of conclusive end points for immunotoxicity presents a challenge for computational modeling due to limited training data and the complex mechanisms of the immune system and associated toxicities.

Due to limited training data, QSAR models trained with small congeneric data sets have significant limitations, such as overfitting,[11] low chemical diversity,[12] and activity cliffs.[13] Furthermore, the activation or suppression of the immune system involves multiple steps comprising various immune cells and proteins. Proteins involved in the immune signaling pathway, like cytokines, are currently being studied as biomarkers for immune-related diseases.[14] However, some cytokines demonstrate proinflammatory and anti-inflammatory effects depending on the receptors that activate them, making it difficult to determine their specific roles in immunotoxicity.[15] Thus, current HTS assays testing for the release of cytokines cannot fully reflect or elucidate detailed pathways of immunotoxicity, and their dual role makes them unsuitable for training QSAR models.[16] On the other hand, the large amount of data generated from HTS assays can be used to expand the training data for QSAR modeling of immunotoxicity. Data from multiple key events (KEs) within immunotoxicity pathways can result in computational models that can predict immunotoxicity and cover broader toxicity mechanisms than testing against one or few assays. One KE that has been extensively studied as an immunotoxicity pathway is the activation of the aryl hydrocarbon receptor (AhR) signaling pathway.[17] AhR is a transcription factor that functions as a xenobiotic and environmental sensor. Upon activation, it regulates the transcription of multiple genes, including genes involved in immune response.[18] Some chemicals, including pesticides and environmental pollutants, exert their immunotoxic effects by activating AhR. For example, the activation of AhR by persistent organic pollutants in the environment, such as polychlorinated biphenyls (PCBs), dioxins, and furans, was associated with cancer and autoimmune diseases.[19] Previous QSAR modeling studies of AhR could predict toxic chemicals[20] and identify the chemical fragments that facilitate binding to AhR.[21] However, these modeling studies are only limited to predicting AhR binding and hepatotoxic outcomes, and currently, there are no existing models of AhR agonism that can lead to immunotoxicity. As a well-studied toxicity mechanism, we hypothesize that data from AhR agonism assays can help identify related KEs in immunotoxicity pathways, providing more data for QSAR modeling of immunotoxicity.

In this study, we compiled a probe data set of chemicals tested for AhR activation from the Toxicology in the 21st Century (Tox21) program. Tox21 is a collaborative effort by the National Toxicology Program, the National Center for Advancing Translational Sciences, the Environmental Protection Agency, and the Food and Drug Administration.[22] The Tox21 program has screened a library of around 10,000 known drugs and environmental chemicals against biomolecular targets using quantitative HTS assays.[22] We applied a data-driven QSAR modeling approach that leveraged the AhR activation assay from Tox21 as a probe data set to reveal KEs related to immunotoxicity pathways and thereby expand the available training data for predicting immunotoxicity. Similar data-driven modeling strategies were successfully applied for predicting multiple types of other complex toxicities, such as acute toxicity,[23] carcinogenicity,[24] developmental and reproductive toxicity,[25] endocrine disruption,[26] and hepatotoxicity.[27−29] To this end, an automatic in-house tool retrieved assays where chemicals from the AhR activation assay data set were present and shared similar responses to the AhR activation assay.[30] Statistically significant assays to AhR activation revealed likely KEs involved in immunotoxicity pathways, and toxicity data from these assays were employed to construct QSAR models.[31] Models with the best predictive performance were selected to predict multiple toxicity mechanisms in immunotoxic chemicals. The models also yielded reasonable predictions for external chemical data sets with immunotoxicity information. The efficiency and applicability of this strategy can advance QSAR modeling studies by enriching limited training data to model complex toxicities like immunotoxicity.

METHODS

Data Sets

The initial probe data set was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/bioassay/743122, accessed December 15, 2023), where 8,099 compounds were tested for activation of the AhR signaling pathway. The biological activities against AhR in this data set were summarized as active agonist, inactive, and inconclusive. Chemicals categorized as inconclusive were removed from the data set, and the data set was then curated and standardized using the CASE Ultra v1.9.0.4 DataKurator tool (MultiCASE Inc., Beachwood, OH). Duplicate and inorganic compounds were removed, and the largest organic components of mixtures were retained. This curation workflow was also applied to other selected assays and external data sets for modeling in this study. The resulting probe data set contained 6,341 unique compounds (Table S1), which served as the input for an in-house automatic profiling tool[30] to search PubChem (https://pubchem.ncbi.nlm.nih.gov/) for assays with *in vitro* data containing similar profiles to the probe data set. The profiling tool (code available on https://github.com/zhu-research-group/HTSProfiling) programmatically accessed and retrieved bioactivity information from the PubChem BioAssay database using PubChem's web service interface, the Power User Gateway Representational State Transfer (PUG-REST).[32]

Four external data sets were collected to validate the predictivity of the generated QSAR models. The first data set is another PubChem HTS assay (AID 2796) testing for activation of the AhR, deposited by the Scripps Research Institute Molecular Screening Center in PubChem (https://pubchem.ncbi.nlm.nih.gov/bioassay/2796, accessed February 24, 2022). QSAR models trained on the AhR probe data set were validated using this external data set. The second data set was compiled from the literature and consisted of 50 chemicals found to be immunotoxic in either humans or animals (Table S2). This data set was used as the primary validation set for predicting the toxicity activities of immunotoxic compounds across KEs. The third data set was obtained from the CosIng database provided by the European Commission, which contained information about cosmetic ingredients and substances (https://ec.europa.eu/growth/tools-databases/cosing/, accessed March 8, 2023). The CosIng database served as a negative external validation set in this study, as most of the compounds were likely to be inactive. This type of indirect validation was also used in our previous chemical toxicity modeling studies.[24,33] We hypothesize that the predictions for this data set would likely be inactive as most cosmetic ingredients are not

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toxic due to their extensive safety testing before their release into the market. The curation of this database resulted in a data set of 5,306 chemicals for the purpose of validating the QSAR models. The fourth data set was retrieved from the Toxin and Toxin-Target Database (T3DB) (http://www.t3db.ca/, accessed February 22, 2023), a project supported by the Metabolomics Innovation Center, the Canada Foundation for Innovation, and the Canadian Institutes of Health Research.[34] Aggregated from multiple resources, T3DB links information regarding 3,678 toxins, including drugs, pesticides, and pollutants, to their respective toxin target records. After curating this database, the 2,654 remaining chemicals were used for external validations.

Chemical Descriptors

Three types of binary chemical fingerprints were applied to quantify the chemical structures as features for modeling and predictions: molecular access system (MACCS) keys, extended-connectivity fingerprints (ECFP), and functional-class fingerprints (FCFP). These molecular fingerprints were implemented through the free cheminformatics package RDKit v2021.03.1 (https://www.rdkit.org/) using Python v3.9.4. The MACCS keys used in the QSAR modeling process are the publicly available set of 166 predefined binary keys that describe two-dimensional substructures. ECFPs and FCFPs are circular topological descriptors representing an unlimited number of unique molecular features within 1024-bit vectors.[35] Both fingerprints were generated through a variation of the Morgan algorithm by applying a bond radius of 3.

Feature Importance and Scaffold Analysis

Feature importance and Murcko scaffolds for the active AhR agonists in the curated probe data set were obtained to identify structural features that contribute to AhR agonism. The Murcko scaffolds were generated through the RDKit toolkit, while feature importance was evaluated using the random forest algorithm provided by the open-source ML library scikit-learn v0.24.1 using Python v3.9.4 (https://scikit-learn.org/). The algorithm determines which features (i.e., MACCS keys) contribute to the model's predictions of AhR agonism. Within the developed random forest model, the importance of MACCS keys was ranked by the gain in decreased impurity in each tree, with the most important MACCS fingerprint having the highest mean decrease in impurity.[36]

QSAR Model Development

Three classic machine learning algorithms and one artificial neural network were used to develop the QSAR models: random forest (RF), support vector machine (SVM), extreme gradient boosting (XGB), and multilayer perceptron (MLP). All algorithms were assigned the binary classification task of predicting a chemical as active (1) or inactive (0). The RF and SVM algorithms were applied using the scikit-learn library. RF is an ensemble learning algorithm made of multiple random, uncorrelated decision trees, where predictions are based on a majority vote across the trees.[37] SVM identifies the best line or hyperplane that separates and maximizes the distance between the active and inactive chemicals.[38] The XGB algorithm was implemented using the open-source gradient boosting library XGBoost v.1.7.3 with Python v3.9.4 (https://xgboost.readthedocs.io/en/). XGB is another ensemble method that combines "weak" predictor trees to form a stronger tree, with some of its advantages being its speed, high predictive performance, and efficiency in handling missing data.[39] Lastly, the MLP neural network, consisting of three hidden layers, was also implemented using the scikit-learn library. MLP is a feed-forward neural network trained through backpropagation using a nonlinear activation function.[40] In this study, the input layer contains information about the features (chemical fingerprints) of the chemicals in the training set, while the output layer represents the model's predicted probabilities for the target activity.

Hyperparameters for each ML algorithm were optimized using the grid-search algorithm provided by the scikit-learn library. As outlined in previous studies, each ML algorithm was fit to the training set with varying combinations of hyperparameters to identify the best-performing model.[41−43] For example, the maximum depth of the RF algorithm was set to 10, and the number of estimators for optimization were 5, 10, and 25. For SVM, the kernel was set to radial basis function, and the following parameters were optimized: the regularization parameter C (1, 10) and the kernel coefficient gamma (0.01, 0.001). For the MLP models, the following parameters were optimized: the number of nodes in the hidden layers (100, 1000), the optimization algorithm (stochastic gradient descent, Adam), the learning rate (constant, adaptive), and the L2 regularization term (0.0001, 0.001). The models with the optimal combination of hyperparameters were reserved for predicting the test set.

Twelve individual models were trained for each data set through a combination of one of the descriptors (MACCS, ECFP, FCFP) and one of the algorithms (RF, SVM, XGB, MLP). The models were built using an in-house modeling pipeline that was described in our previous publication,[31] and the relevant code is available on GitHub (https://github.com/zhu-research-group/auto_qsar). Averaging predictions from the resulting 12 individual models for each assay resulted in a consensus model. The performance of all the models was evaluated using a 5-fold cross-validation procedure. This process involved splitting the data set into five subsets, where four subsets (80% of the data set) were used to train the model, and the remaining subset (20% of the data set) was reserved to assess the predictivity of the model. This procedure was repeated five times to ensure that each compound in the original data set was used for assessment.

Statistical Analyses for Assay Selection and Model Performance

The assays extracted from PubChem were ranked based on their relevance to the AhR assay. To achieve this, the statistical significance (p-value) was calculated using Fisher's exact test.[23] This procedure requires building a 2 × 2 contingency table for each pair of the AhR probe data set and one of the PubChem assays, as shown below:

	Active chemicals in PubChem assay	*Inactive chemicals in PubChem assay*
Active chemicals in AhR data set	*A*	*B*
Inactive chemicals in AhR data set	*C*	*D*

(1)

where A is the sum of the number of chemicals shown as active in both the AhR data set and a PubChem assay, B is the sum of the number of chemicals shown as inactive in this PubChem assay but active in the AhR data set, C is the sum of the number of chemicals shown as active in this PubChem assay but inactive in the AhR data set, and D is the sum of chemicals shown as inactive in both the AhR data set and this PubChem assay. The p-value of each assay was then calculated using the values obtained from the contingency table (eq 2). Assays were selected using the standard threshold for statistical significance ($p < 0.05$).

$$p = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{(A+B+C+D)!A!B!C!D!} \quad (2)$$

The performance of the generated QSAR models was evaluated based on four metrics: sensitivity, specificity, correct classification rate (CCR), and positive predictive value (PPV). Sensitivity represents the proportion of active chemicals in the data set that were correctly predicted as active (eq 3), and specificity is the proportion of inactive chemicals that were correctly predicted as inactive (eq 4). CCR is the average of both the sensitivity and specificity (eq 5), reflecting the overall predictive performance of the resulting models, and PPV is the proportion of active predictions that were correctly predicted (eq 6). Two criteria were then applied to select consensus models for the prediction of external chemicals: 1) CCR > 0.7 across all individual models for the assay and 2) relevance of the assay to immunotoxicity.

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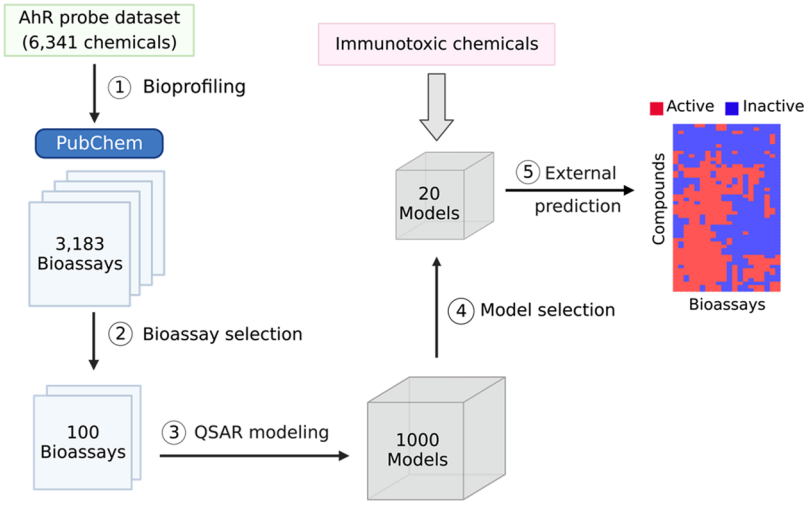

Figure 1. Computational modeling workflow of this study: (1) profiling the probe data set through PubChem, (2) bioassay selection, (3) QSAR modeling of selected bioassays, (4) model selection, (5) predictions of external chemicals.

$$Sensitivity = \frac{True\ positives}{True\ positives + False\ negatives} \quad (3)$$

$$Specificity = \frac{True\ negatives}{True\ negatives + False\ positives} \quad (4)$$

$$CCR = \frac{Sensitivity + Specificity}{2} \quad (5)$$

$$PPV = \frac{True\ positives}{True\ positives + False\ positives} \quad (6)$$

Applicability Domain (AD)

The QSAR model predictions yielded the probability of a chemical being active against a specific assay, ranging between 0 and 1. A standard single threshold was used to classify predictions as active (probability ≥0.5) and inactive (probability <0.5) for all compounds. As a measure of confidence prediction, an applicability domain (AD) was implemented across the QSAR model predictions. Predicted probabilities above 0.6 were considered active and probabilities below 0.4 were considered inactive. Predictions between 0.4 and 0.6 were considered inconclusive and were excluded from the assessment of QSAR model performance. By applying this domain to new chemicals, the closer the prediction probability to 0 or 1, the greater the confidence in the model's predictions. The implementation of similar ADs has been successfully applied in several of our previous studies.[24,28,44]

RESULTS AND DISCUSSION

Study Workflow

The steps for the workflow in this study are summarized in Figure 1. The bioactivity profile of the target chemicals in the curated AhR probe data set was first retrieved from PubChem. Assays in this profile were then ranked by statistical significance between their testing responses and the activities in the probe data set. The top-ranking assays, including the AhR assay data set, were then selected for QSAR modeling. From the resulting QSAR models, model performance and assay relevance to immunotoxicity were used as criteria for selecting the most suitable models for predicting new compounds. External chemicals from multiple data sets were then predicted by selected models against KEs, and their immunotoxicity potentials were assessed.

Probe Data Set and Bioprofile

The lack of curated data sets for chemical immunotoxicity limited the training of computational models that can predict chemical immunotoxicity. To overcome this limitation, known immunotoxicity mechanisms can be used to gather initial training data for modeling immunotoxicity. The AhR is a well-studied target that influences multiple pathways and mechanisms in the body. Crucial to regulating the immune response, AhR activation can affect the equilibrium of the immune response, acting as a switch that can turn on/off the immune signaling pathway.[18] Hence, excessive or potent activation of AhR can lead to forms of immunosuppression, such as increased susceptibility or reduced resistance to infection, or immunostimulation, such as autoimmune diseases.[18] All these toxicity mechanisms, especially the perturbation of pathways leading to immunotoxicity, make AhR a suitable target for computational modeling of immunotoxicity.

The probe data set in this study was collected from a Tox21 assay (PubChem AID 743122) that tested 10,486 substances for AhR activations, of which 8,099 were unique chemicals. After curation, a total of 6,341 unique chemicals remained in the probe data set, of which 746 were active agonists and 5,595 were inactive. The biological activity profiles for the chemicals in the resulting probe data set were retrieved from PubChem, where a threshold for the minimum number of actives for probe compounds in a PubChem assay was set as 10. The search yielded 3,183 assays with bioactivities for varying proportions of chemicals in the probe data set. This bioprofile revealed over 20 million data points consisting of active, inactive, and inconclusive or missing responses between the probe compounds and their respective assays. The responses in the bioprofile showed a bias toward inactive outcomes, as the ratio between active and inactive outcomes was approximately 1:15, including around 130,000 active and 2,000,000 inactive outcomes. This condition reflects the nature of HTS assays, as discussed in several previous and recent reviews.[45−47]

Within the initial bioprofile, most assays may have little to no relevance to AhR activation and immunotoxicity. Thus, the initial 3,183 assays within this bioprofile were ranked by their statistical significance to AhR agonism. The p-value was calculated using Fisher's exact test (eq 2), and the threshold for

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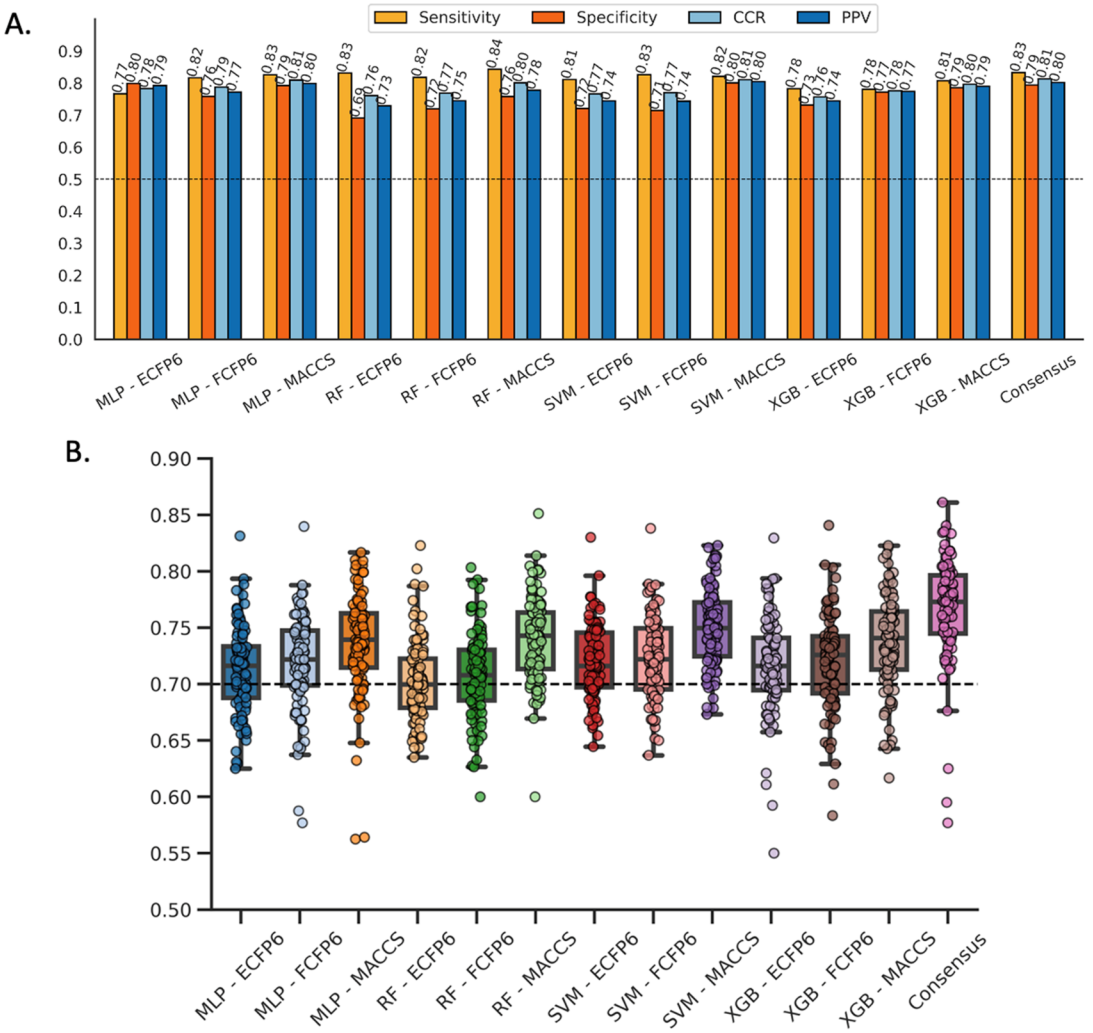

Figure 2. Performance of the generated individual and consensus QSAR models, where models were trained with different combinations of ML algorithms and molecular fingerprints. (A) 5-fold cross-validation results for the AhR probe data set, evaluated using sensitivity, specificity, CCR, and PPV. (B) 5-fold cross-validation results for the 100 selected bioassays. Each point represents a QSAR model for an assay trained using the respective algorithm and fingerprint. The dashed line highlights the assay selection criteria of CCR > 0.7.

statistical significance was set to the conventional $p < 0.05$. In the initial bioprofile, a total of 504 assays had a $p < 0.05$ (Table S3), indicating their potential association with AhR agonism. The top 100 statistically significant assays, including the AhR probe data set, were then selected for modeling. However, four assays from the 100 assays contained more than 200,000 chemicals and were thus excluded from the modeling process due to the biased nature of these HTS assays. The four assays were substituted with four successive assays containing less than 10,000 compounds. Thus, a total of 84 assays from the resulting 100 assays shared a large number of compounds with the probe data set, and many toxic chemicals showed similar active responses across these assays. The toxicity targets in these assays are expected to have significant contributions in multiple toxicity pathways and may also play essential roles in inducing immunotoxicity.

QSAR Modeling

Following a combinatorial QSAR modeling workflow, 12 individual models were trained for each of the 100 assays (Table S1) by varying combinations of binary fingerprints (ECFP6, FCFP6, and MACCS) and ML algorithms (SVM, RF, XGB, and MLP). Consensus models were also generated through averaging predictions for the 12 individual models for each assay, as consensus models were the closest in demonstrating the best predictive performance when compared to individual models, as shown by previous studies.[24,43,48−50] Since there was a disproportionate number of inactive to active responses across all 100 data sets, downsampling was applied to prevent the models from being biased to predicting external chemicals as inactive, which proved necessary in our previous studies.[24,25,27] All the data sets were balanced by randomly removing inactive compounds to equalize the ratio of active to inactive compounds. Moreover, chemicals found in both the training sets and the external validation sets were removed from the training sets to ensure that the external chemicals were new to the developed models. The performance of the models generated from the training sets was then evaluated through the 5-fold cross-validation procedure. This method assesses the performance of the models, as required by the Organization for Economic Co-operation and Development (OECD) guidance on validating QSAR models.[51] Appropriate and reliable methods for model validation, such as cross-validation, are one of the required

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Table 1. Details of the 20 Selected PubChem Bioassays

KE	PubChem AIDs	No. of compounds	No. of active compounds	Relevance to immunotoxicity
AhR agonism	743122	8099	875	Promotes inflammation through the NF-kB signaling pathway and the differentiation of T-helper 17 (Th17) cells; plays an immunosuppressive role in dendritic cells[54]
	651777	2327	81	
Androgen receptor (AR) antagonism	1259247	7671	935	Prevents binding of androgen, causing an increase in inflammation; associated with autoimmune disease[55]
	743063	8099	548	
CYP1A2 antagonism	1671199	7671	2442	Alters AhR-mediated immune response[56]
CYP2C9 antagonism	1671198	7671	2368	Slows metabolism of toxic chemicals, leading to the accumulation of such substances that can lead to immunotoxic outcomes[57]
	1645842	5095	1985	
CYP3A4 antagonism	884	13076	3439	Slows metabolism of toxic chemicals, leading to the accumulation of such substances that can lead to immunotoxic outcomes[57]
	1645841	5095	2105	
Estrogen-related receptor (ERR) agonism	1259404	7671	248	Regulates the generation and proliferation of protective effector T cells and suppressive regulator T cells[58]
	1259402	7671	320	
Mitochondrial membrane potential disruption	720637	8099	1006	Exacerbates inflammatory responses triggered by oxidative stress and the innate immune system[59]
	651755	1335	114	
Mutagenicity	1259407	7692	4671	Involves epigenetic factors that influence the development of autoimmune diseases and cancer[60,61]
Nuclear receptor binding SET domain protein 2 (NSD2) inhibition	1645876	8988	2409	Triggers inflammatory signaling pathways in response to cancers that exhibit immunosuppressive effects[62]
Pregnane X receptor (PXR) agonism	1347033	7671	1724	Induces the assembly of the NLRP3 inflammasome in macrophages and inhibits T lymphocyte response[63]
	651751	2327	129	
Progesterone receptor (PR) antagonism	1347031	7671	861	Prevents binding of progesterone, causing an increase in inflammation; associated with autoimmune disease[64]
Retinoic acid receptor (RAR) agonism	1159553	7331	364	Destabilizes the immune signaling pathway by preventing the binding of retinoic acid, an immunoprotective ligand[65]
Thyroid receptor (TR) antagonism	743065	8099	1536	Prevents binding of thyroid hormone, causing an increase in inflammation; associated with autoimmune disease[66,67]

elements for the successful integration of model predictions into regulatory risk assessments.[52]

Twelve individual QSAR models were built for the curated AhR probe data set. After curation and downsampling, the final training set for the probe data set included 1,474 chemicals (Table S4), where 737 chemicals were classified as active and 737 as inactive. Figure 2A illustrates the performance metrics for each individual model and the consensus model through the 5-fold cross-validation procedure. The overall model performance was acceptable, with CCRs above 0.74 across all models. The sensitivity, ranging from 0.77 to 0.84, and specificity, ranging from 0.69 to 0.80, showed a balanced prediction of actives and inactives. The PPV was also satisfactory, ranging from 0.73 to 0.80.

Additionally, the AhR probe data set models were externally validated using chemicals obtained from another PubChem assay (AID 2796). This assay also tested AhR activation for over 300,000 chemicals by the Scripps Research Institute Molecular Screening Center. Most bioactivity responses in AID 2796 were inactive outcomes, comprising 97.5% of the total responses. The data set was curated and randomly balanced, and inactive chemicals were selected if their PubChem activity score was zero. Model predictions for this data set yielded an average CCR of 0.77 (Table S5). The implementation of the AD improved predictions with an average CCR of 0.81. Sensitivity, specificity, and PPV were acceptable overall (>0.64); only specificity for the ECFP6 models was poor (<0.6) due to high bias. This result can be explained by the downsampling method used to balance the training data, as the resulting models were likely to emphasize active predictions. The details of model predictivities for the cross-validation procedure of the training set and the external set (AID 2796) are shown in Table S5.

For the remaining 99 assays, the training sets were curated and balanced using downsampling, where the number of training set compounds ranged from 78 (AID 1259390) to 6,444 (AID 884). The same workflow was applied for QSAR modeling, and a total of 1,300 models, including 12 individual models and a consensus prediction for each assay, were generated. Figure 2B shows the CCR values from the 5-fold cross-validation process for all the QSAR models developed for the 100 assays. 953 models achieved CCR values above 0.7, which can be considered good model performance.[53] Individual models also performed acceptably across the different combinations of fingerprints and algorithms, with a minimum of 50% of the ECFP6-RF models and a maximum of 91% of the MACCS-SVM models achieving CCR > 0.7. Thus, models with satisfactory performance can be used to predict new compounds of interest for their specific toxicity mechanism.

Model Selection

Models were selected based on model performance during 5-fold cross-validation and the relevance of their respective assays to immunotoxicity. From the 100 assays used for modeling, the 5-fold cross-validation procedure for 35 assays achieved CCR > 0.7 for all individual models. Of the 35 assays, four cell viability assays were removed as they mainly tested for target cytotoxicity through counter-screening. Three duplicate assays from Tox21 were also excluded, and only the summary assay was retained. Manual inspection of the remaining assays led to the exclusion of eight more assays, as their respective targets were not associated with immunotoxicity pathways. The resulting 20 assays, including the AhR probe data set (AID 743122), tested for key signaling pathways that showed relationships with immunotoxicity through impaired or abnormal immune responses. Table 1 provides an overview

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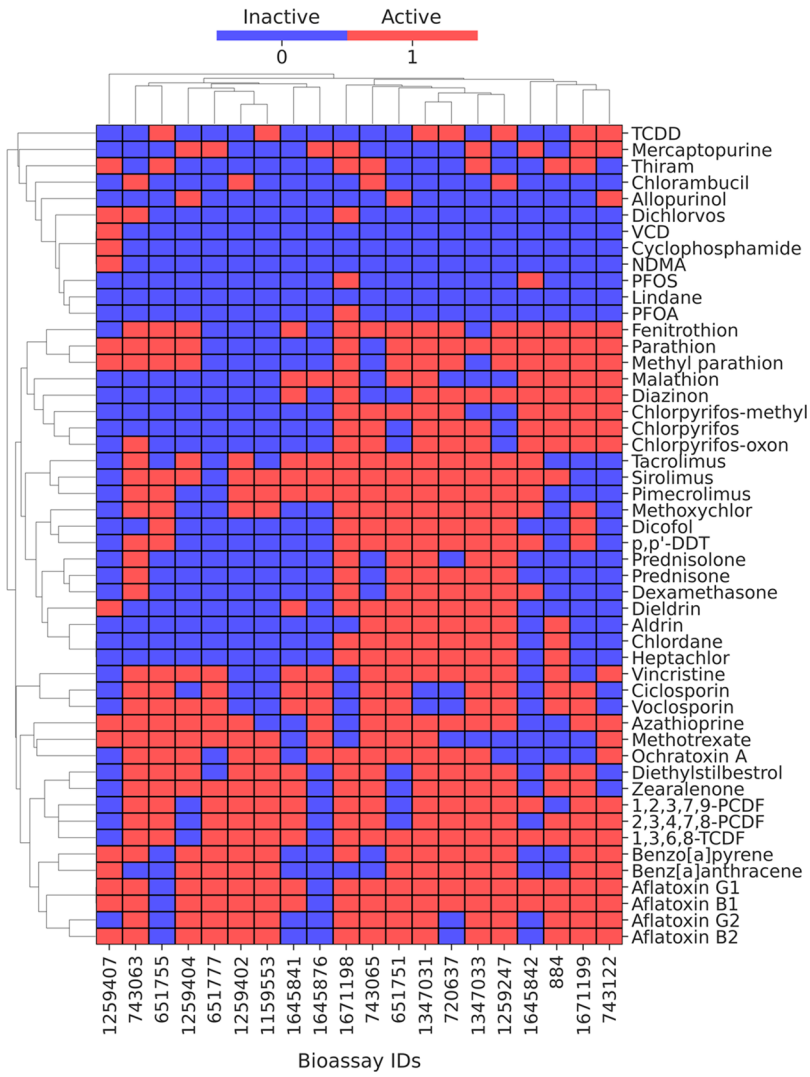

Figure 3. Predictions of 50 immunotoxicants by the consensus models of the 20 selected PubChem assays. PFOA - perfluorooctanoic acid; NDMA - N-nitrosodimethylamine; VCD - vinylcyclohexene dioxide; PFOS - perfluorooctanesulfonic acid; TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin; p,p′-DDT - dichlorodiphenyltrichloroethane; 2,3,4,7,8-PCDF - 2,3,4,7,8-pentachlorodibenzofuran; 1,2,3,7,9-PCDF - 1,2,3,7,9-pentachlorodibenzofuran; 1,3,6,8-TCDF - 1,3,6,8-tetrachlorodibenzofuran.

of these 20 assays and the mechanisms by which the immune response is suppressed or stimulated through their respective pathways. Models generated from these 20 assays were then selected for predicting new compounds.

External Predictions

The cross-validation procedure is a commonly used method to validate the performance of the resulting models and avoid overfitting. Furthermore, predictions of real unknown compounds by the resulting models are the ultimate proof of model predictivity. Based on our previous studies, consensus predictions have clear advantages over individual models for prediction purposes.[24,43,48−50] Thus, the consensus models for the 20 selected assays were employed for the prediction of bioactivities and potential immunotoxicity of the chemicals from the external data sets. A data set of 50 known immunotoxic chemicals was collected from various literature sources to evaluate predictions from the models (Table S2). Predicted immunotoxicity in this study was evaluated by averaging the predictions across the 20 models to obtain the mean likelihood of being toxic. A total of 49 out of the 50 chemicals showed active responses in at least one of the 20 assays, and 29 chemicals were confidently predicted as toxic because of active outcomes from over half of the assays (Figure 3). A total of 18 of these 29 toxic chemicals are known to promote inflammatory signaling pathways related to chronic inflammation and autoimmunity; these 18 chemicals included polyaromatic hydrocarbons (PAHs), aflatoxins, xenoestrogens, and organophosphate pesticides. The remaining 32 chemicals are immunosuppressants that include anticancer drugs, corticosteroids, organochlorine pesticides, anti-inflammatory drugs, and industrial chemicals. Models trained on assays that test for the same signaling pathway may yield different predictions for the same chemicals. This difference can be attributed to the variability in the size of the training sets and/or the experimental protocols of the assays, such as in the AhR activation assays AIDs 743122 and 651777.

The PAH benzo(a)pyrene (BaP, CID 2336) scored a high mean prediction of 0.654. BaP, a toxic environmental contaminant and a potent carcinogen, can mediate immunotoxic effects by activating AhR.[68] Consensus models for the AhR agonism assays AID 743122 and AID 651777 (Table 1) predicted BaP as active (Figure 3). Through binding to AhR

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and activating its signaling pathway, BaP is metabolized into epoxides and diols. These byproducts lead to the formation of reactive oxygen species (ROS) that can cause oxidative stress and high inflammation.[68] ROS generated from BaP metabolites can also disrupt the mitochondrial membrane integrity and potential. This damage results in the release of mitochondrial ligands that bind and activate pattern recognition receptors (PRRs) in the innate immune system, thereby triggering diverse inflammatory signaling pathways.[69] Reflecting this mechanism, the consensus model for the mitochondrial membrane potential disruption assay AID 720637 (Table 1) predicted BaP as active (Figure 3). Similarly, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, CID 15625) is another toxic environmental contaminant with strong AhR agonist activity.[70] TCDD was also predicted as active for AhR agonism and mitochondrial membrane potential disruption by the respective consensus models. TCDD is a well-known potent immunosuppressor[71,72] while BaP demonstrates both immunosuppressive and proinflammatory characteristics.[68] The difference in immune response between these two AhR ligands indicates a need to use multiple models, including animal toxicity studies, to identify different toxicity mechanisms underlying specific immunotoxicity effects.

The KEs indicated by the 20 assays demonstrate a relationship to immunotoxicity (Table 1), and chemicals predicted as active for some of these assays were found to induce immunotoxicity through these KEs. For example, consensus models for the AR antagonism assays AID 1259247 and AID 743063 (Table 1) predicted the toxic pesticide dichlorodiphenyltrichloroethane (p,p′-DDT, CID 3036) as active (Figure 3). p,p′-DDT, an androgen receptor antagonist, can mediate the reduced expression of the protein RACK1 (Receptor for Activated Kinase 1) that subsequently suppresses the immune response.[73] The mycotoxin aflatoxin B1 (CID 186907), scoring the highest mean prediction at 0.672, is a toxic agricultural contaminant that was predicted as active by the model for the mutagenicity assay AID 1259407 (Figure 3). One study analyzed the mutagenic activity of aflatoxin B1 in chick embryos and found that the mutagenic effects of aflatoxin B1 led to long-term immunosuppression.[74] Zearalenone (CID 5281576) is an estrogenic mycotoxin that binds to and activates PXR.[75] It was also predicted as active by the model of the PXR agonism assay (AID 1347033, Figure 3). Through PXR agonism, zearalenone suppressed NF-κB activation and reduced the release of the inflammatory cytokines IL-6, IL-1β, and TNF-α.[75]

Notably, the organochlorine insecticide lindane (CID 727), also known as gamma-hexachlorocyclohexane, was the only chemical not predicted to be active by any of the 20 consensus models (Figure 3). Compared to other chemicals, lindane has a special chemical structure that might induce toxicity by different mechanisms. Although its mechanism of immunotoxicity is not well-elucidated, lindane was shown to induce apoptosis in murine thymocytes by increasing the production of ROS, which may trigger immunotoxic outcomes.[76] This condition indicates the necessity of extending the current assay list by including more immunotoxicity assays. By adding immunotoxicants with new clearly defined mechanisms into the probe data set, we expect that the data mining process will result in more assays with more training data and eventually cover all potential immunotoxicity mechanisms.

Feature Importance and Scaffold Analysis

Figure S1 illustrates the top 10 Murcko scaffolds identified in the AhR agonists from the probe data set. The top four important MACCS keys that were found to contribute to AhR agonism represented aromaticity (MACCS 125 and 162) and nitrogen atoms within or attached to aromatic rings (MACCS 133 and 135). All of the top 10 scaffolds contain one or more of these MACCS fingerprints. Multiple studies indicate that aromaticity and hydrogen bond donors/acceptors are two key components for the binding of AhR agonists to the AhR ligand binding domain (LBD).[77−79] For example, bulky planar structures, majorly represented by aromatic rings, bind strongly to the bottom of the hydrophobic pocket within the AhR ligand binding domain.

Several AhR agonists, such as BaP, TCDD, and the aflatoxins, bind to nuclear receptors and affect their signaling pathways, as shown in Figure 3. The ligand-binding pockets (LBP) in nuclear receptors are lined with several hydrophobic residues and a few anchoring polar residues, which, in steroidal nuclear receptors like PR and PXR, tend to bind to ketone side groups.[80] Aromatic rings, including heterocycles, multiple carbon−carbon double bonds, and/or oxygen atoms represent some shared side groups in the 18 inflammatory highly predicted toxic chemicals that confer hydrophobicity and strengthen binding to the LBPs in nuclear receptors. Moreover, these side groups are also present in endogenous ligands like progesterone, testosterone, cholesterol, and thyroid hormone that bind to other nuclear receptors (PR, AR, ERR, TR). Thus, chemicals with the underlying scaffolds of AhR agonists (Figure S1) may behave as ligands of several nuclear receptors (Table 1), potentially affecting their signaling pathways and leading to immunotoxic outcomes.

External Validation

The 20 consensus models were also used to predict cosmetic ingredients from the CosIng database and toxins from T3DB. Figure 4 shows the probability distributions of mean predictions for compounds from these two data sets. The peaks in the distribution curves reflect the mean predictions of most compounds in a data set. The probability peak for the T3DB data set was 0.548, similar to the average value of the 50 immunotoxic compounds (0.570). In contrast, the peak of

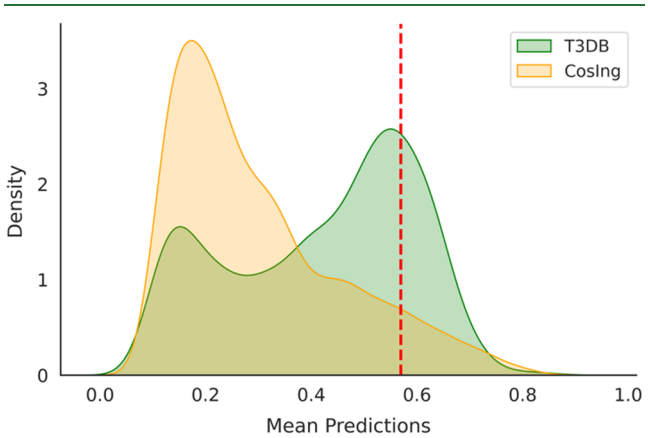

Figure 4. Kernel density estimate (KDE) plot of the probability distribution of mean predictions from the Cosmetic Ingredients (CosIng) database and the Toxin and Toxin-Target Database (T3DB). The red dashed line represents the peak of the predictions of the immunotoxic chemical data set.

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mean predictions for the CosIng data set was 0.170. This significant difference in probability distributions between compounds in T3DB and CosIng confirms that the chemicals in T3DB were more likely to induce immunotoxicity than cosmetic ingredients that were unlikely to be toxic. This indirect validation process also proves the utility of our resulting QSAR models and modeling strategy.

CONCLUSION

This study described a computational data-driven modeling framework that provided the first insight into comprehensive immunotoxicity-related KEs compared to classic modeling studies for single toxicity endpoints. Although the AhR signaling pathway encompasses a number of immune response mechanisms, which are the emphasis of this study, the data mining process resulted in training data for predictive modeling of several new immunotoxicity mechanisms. The expansion of limited training data tested by an AhR activation assay in PubChem provided ample data for generating hundreds of QSAR models through a combinatorial modeling approach. Consensus models were also generated through averaging predictions from individual models, and models were selected based on good predictive performance and relevance of the respective assay to immunotoxicity. This process revealed critical KEs related to immunotoxicity, such as PXR and RAR agonism, AR, PR, and TR antagonism, mutagenicity, and mitochondrial membrane potential disruption. Consensus models generated from 20 selected assays for these KEs were able to predict most immunotoxicants by covering various toxicity mechanisms. A variety of xenoestrogens, pesticides, and environmental pollutants with active predictions for specific KEs from the consensus models were validated to cause immunotoxicity through these KEs. Thus, predictions from this study can be used to assess potential immunotoxicants and illustrate their relevant immunotoxicity mechanisms. Our data-driven framework can also be applied to model similarly complex toxicity endpoints with limited training data to advance and accelerate chemical risk assessments and uncover underlying toxicity mechanisms.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00026.

Top 10 Murcko scaffolds in AhR agonists, with the number of AhR agonists in the probe data set sharing the same scaffold (Figure S1) (PDF)

Curated AhR probe data set from PubChem AID 743122 (Table S1). Details of the 50 immunotoxic chemicals used for external prediction (Table S2). Details of the 504 statistically significant bioassays (p-value <0.05) related to the curated AhR probe data set (Table S3). Randomly balanced AhR training set for QSAR modeling (Table S4). Statistical performance of the AhR consensus models with and without the applicability domain (AD) for the 5-fold cross-validation procedure and the prediction of the external validation set (Table S5) (XLSX)

AUTHOR INFORMATION

Corresponding Author

Hao Zhu − *Department of Chemistry and Biochemistry, Rowan University, Glassboro, New Jersey 08028, United States; Center for Biomedical Informatics and Genomics, Tulane University School of Medicine, New Orleans, Louisiana 70112, United States;* orcid.org/0000-0002-3559-6129; Email: hzhu10@tulane.edu

Authors

Nada J. Daood − *Department of Chemistry and Biochemistry, Rowan University, Glassboro, New Jersey 08028, United States*

Daniel P. Russo − *Department of Chemistry and Biochemistry, Rowan University, Glassboro, New Jersey 08028, United States*

Elena Chung − *Department of Chemistry and Biochemistry, Rowan University, Glassboro, New Jersey 08028, United States; Center for Biomedical Informatics and Genomics, Tulane University School of Medicine, New Orleans, Louisiana 70112, United States;* orcid.org/0000-0001-7577-9328

Xuebin Qin − *Tulane National Primate Research Center, Tulane University School of Medicine, Covington, Louisiana 70433, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/envhealth.4c00026

Author Contributions

Nada J. Daood: Formal Analysis, Data Curation, Methodology, Investigation, Validation, Visualization, Writing - Original Draft. Daniel P. Russo: Methodology, Visualization. Elena Chung: Data Curation, Visualization. Xuebin Qin: Writing − Review & Editing. Hao Zhu: Conceptualization, Supervision, Funding Acquisition, Writing − Review & Editing.

Notes

The authors declare no competing financial interest.

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