Environment International 137 (2020) 105557

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Development of AOP relevant to microplastics based on toxicity mechanisms of chemical additives using ToxCast™ and deep learning models combined approach

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ARTICLE INFO

Handling editor: Martí Nadal

Keywords:
Microplastics
Additives
Adverse outcome pathway
Toxicity database
ToxCast
Deep learning

ABSTRACT

Various additives are used in plastic products to improve the properties and the durability of the plastics. Their possible elution from the plastics when plastics are fragmented into micro- and nano-size in the environment is suspected to one of the major contributors to environmental and human toxicity of microplastics. In this context, to better understand the hazardous effect of microplastics, the toxicity of chemical additives was investigated. Fifty most common chemicals presented in plastics were selected as target additives. Their toxicity was systematically identified using apical and molecular toxicity databases, such as ChemIDplus and ToxCast™. Among the vast ToxCast assays, those having intended gene targets were selected for identification of the mechanism of toxicity of plastic additives. Deep learning artificial neural network models were further developed based on the ToxCast assays for the chemicals not tested in the ToxCast program. Using both the ToxCast database and deep learning models, active chemicals on each ToxCast assays were identified. Through correlation analysis between molecular targets from ToxCast and mammalian toxicity results from ChemIDplus, we identified the fifteen most relevant mechanisms of toxicity for the understanding mechanism of toxicity of plastic additives. They are neurotoxicity, inflammation, lipid metabolism, and cancer pathways. Based on these, along with, previously conducted systemic review on the mechanism of toxicity of microplastics, here we have proposed potential adverse outcome pathways (AOPs) relevant to microplastics pollution. This study also suggests *in vivo* and *in vitro* toxicity database and deep learning model combined approach is appropriate to provide insight into the toxicity mechanism of the broad range of environmental chemicals, such as plastic additives.

1. Introduction

Microplastics, now widely recognized as environmental contaminants, are abundantly distributed throughout the environment (Barnes et al., 2009; Eriksen et al., 2014). Because of the small size (< 5 mm), microplastics are easily ingested by organisms (Botterell et al., 2019; Wright et al., 2013) and can be transferred to other organisms via trophic transfer (Farrell and Nelson, 2013; Nelms et al., 2018), raising concerns about human risk (Bouwmeester et al., 2015; Carbery et al., 2018). As a result, research on the toxicity effect of microplastics has been increasing recently (Burns and Boxall, 2018; de Sá et al., 2018). Recent studies of microplastics hazard warn that there may be toxic effects due to chemicals contained in plastics as additives, besides of microplastics themselves (Gallo et al., 2018; Karami et al., 2016). The chemicals contained in microplastics are divided into additive chemicals that originally present in the plastic products and externally adsorbed chemicals due to the hydrophobic properties of microplastics (Browne et al., 2013; Kwon et al., 2017). It has been suggested that chemical additives are more likely to contribute to the toxicity of microplastics than adsorbed chemicals from the environment (Koelmans et al., 2016; Kwon et al., 2017). Chemical additives can easily cause toxic effects as they can be diffused by the concentration gradient (Kwon et al., 2017). The stable plastic products may not exhibit toxicity by chemical additives, but when they are fragmented into micro-sized and/or nano-sized plastics in the environment, their surface area increases and additive chemicals can be eluted, resulting in toxicity of microplastics from their additives (Wright and Kelly, 2017).

Various additives, such as dipentyl phthalate (DPP), di(2-ethylhexyl) phthalate (DEHP), bisphenol A (BPA), 3.3′-5.5′-tetrabromobisphenol (TBBPA), and formaldehyde, are used in plastic products as plasticizers, antioxidants, and stabilizers to enhance the properties of plastics and increase durability (Hahladakis et al., 2018). Some

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https://doi.org/10.1016/j.envint.2020.105557
Received 27 November 2019; Received in revised form 21 January 2020; Accepted 5 February 2020

additives, such as BPA (Michałowicz, 2014) and DEHP (Tickner et al., 2001), are well studied for their toxicity mechanism, but most others are less studied. Recently, we conducted a systematic review of microplastic toxicity studies (Jeong and Choi, 2019), the main toxicity mechanism of microplastics was revealed as oxidative stress, neurotoxicity, inflammation, and lipid metabolism, but it will also depend on the additives included in the microplastics. Therefore, to have a complete understanding of the toxicity of microplastics, it is necessary to identify the toxicity of additives and their potential mechanisms.

As the 21st-century toxicity testing paradigm shifts to reduce animal testing, *in vitro* or *in silico* toxicity prediction alternative animal testing methods have been studied extensively (Doke and Dhawale, 2015; Lillicrap et al., 2016; Raies and Bajic, 2016). To encourage the development of alternative testing methods and to maximize regulatory applications, efforts are also being made to study the mechanism-based toxicity prediction approach. As part of this effort, the Toxicity Forecaster (ToxCast™) project was launched in 2006 by the US Environmental Protection Agency (EPA). ToxCast has conducted *in vitro* high-throughput screening (HTS) for numerous chemicals to support mechanism-based toxicity prediction techniques (Richard et al., 2016). However, coverage of ToxCast data is limited in terms of bioassays, as well as, chemicals. The use of missing data thus could lead to biased decisions in the decision-making process (To et al., 2018). Deep learning has attracted researchers' attention due to its efficient feature extraction and prediction properties. As a predictive modeling approach, this can provide a potential way to solve the missing data problem. Deep learning algorithms can learn from existing data and impute or predict missing data (Jerez et al., 2010; To et al., 2018). Particularly in the toxicology field, biological big data such as HTS, have been accumulated, and since many environmental chemicals do not have toxicity information, the use of the deep learning approach seems to be a promising tool for chemical toxicity prediction.

In our previous study, we have conducted a systematic review on the mechanism of toxicity of microplastics from the open literatures and used the Adverse Outcomes Pathway (AOP) framework to summarize them (Jeong and Choi, 2019). Through that study, we have identified some significant knowledge gaps for understanding hazardous potential from microplastic pollution. To fill this knowledge gap, here we focused on identifying the mechanism of human toxicity of chemical additives in plastics. To this end, we selected the 50 most commonly used chemical additives in plastics and explored their mechanism of toxicity by gathering information from the existing database and analyzing with mammalian toxicity data. To gain a systematic insight into the mechanism of toxicity, ToxCast assays having intended gene targets were selected. Based on these ToxCast assays, deep learning artificial neural network models were developed for the chemicals not tested in the ToxCast program. ToxCast and deep learning-based mechanisms of toxicity of chemical additives were proposed through correlation analysis with mammalian toxicity data. Finally, AOPs relevant to microplastic pollution have been proposed.

2. Materials and methods

2.1. Mammalian toxicity data collection

Mammalian toxicity data of the studied chemicals were collected from one of the TOXNET databases, ChemIDplus (https://chem.nlm.nih.gov/chemidplus) produced by National Institutes of Health, U.S. Library of Medicine. On the ChemIDplus database, median lethal doses (LD50) of the chemicals from the toxicity experiments conducted on rats or mice via oral exposure were collected as the reference toxicity level.

2.2. ToxCast assays data

We used hit call data (hitc_Matrix_151020.csv) and assay summary data (Assay_Summary_151020) from the US EPA ToxCast & Tox21 Summary Files in invitroDBv2 (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data). Among the assays, we selected 339 assays that have the biologically intended target information to identify the potential toxicity mechanism of chemicals that were active in the assays. The data includes the hit call results of 339 bioassays for a total of 8615 unique chemicals, but all bioassays are not performed on all chemicals.

2.3. Deep learning models

2.3.1. Data preparation

Using the ToxCast *in vitro* assays data, we built 339 artificial neural network models as described previously (Jeong et al., 2019). The canonical simplified molecular-input line-entry system (SMILES) strings describing the structure of the chemicals were collected from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The SMILES strings and ToxCast hit call data were then prepared as text files. Because each assay had a different number of experimental data, the number of input data to build the model was different.

2.3.2. Data imbalance

The ToxCast hit call dataset is highly imbalanced, in many assays, the ratio of inactive data far exceeds that of active data. A bias present between the classes can hinder the performance of the deep learning classification model (Wang et al., 2019), we used Synthetic Minority Oversampling Technique (SMOTE) to oversample the minor class (Kass and Raftery, 1995).

2.3.3. Multilayer perceptron modeling

For each ToxCast assay, we built 339 multi-layer perceptron (MLP) models. The MLP, deep neural network, is a widely used deep learning algorithm due to its simplicity (Hamadache et al., 2017). The structure of MLP consists of an input layer, a dense layer with a RELU activator, and a dense single neuron layer as the output with a sigmoidal activator. MLP was implemented by using Python 3.6, with Keras, the Python open-source neural network library, running on top of the TensorFlow library. The SMILES code was transformed into Morgan Fingerprints with a radius of two bonds using an RDKit (http://www.rdkit.org). Sci-kit Learn toolkit was used to perform the stratified 5-fold cross-validation. In 5-fold cross-validation, the dataset is randomly partitioned into 5 blocks of equal size, and the learning algorithm runs 5 times with each of the blocks is used as a test set. The average of the 5 results gives the test accuracy of the models (Diamantidis et al., 2000).

2.4. Correlation analysis

A point-biserial correlation is a method of determining the association between dichotomous variables and continuous variables, frequently used in the fields of toxicity, medicine, and epidemiology (Tate, 1954). The point-biserial correlation coefficient r_{pb} ranges from -1 to 1 depending on the distribution of continuous variables (McGrath and Meyer, 2006). In general, the correlation coefficient is interpreted as a small correlation from 0.1, intermediate correlation from 0.3, and large correlation from 0.5 (Babchishin and Helmus, 2016). We used the point-biserial correlation coefficient (r_{pb}) to investigate the associations between the activity of *in vitro* ToxCast assays and LD50 data from *in vivo* toxicity study. The hit-call data from each ToxCast assay was considered as the dichotomous variable (0 for inactive, 1 for active) while LD50 data from ChemIDplus as the continuous one. In the ToxCast assay, since 0 is inactive and 1 is active, the active chemicals have greater value, and in animal toxicity, the higher toxic chemicals have a lower LD50 value. Therefore, an assay having a negative correlation was identified. Statistical calculations of the correlation coefficients were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

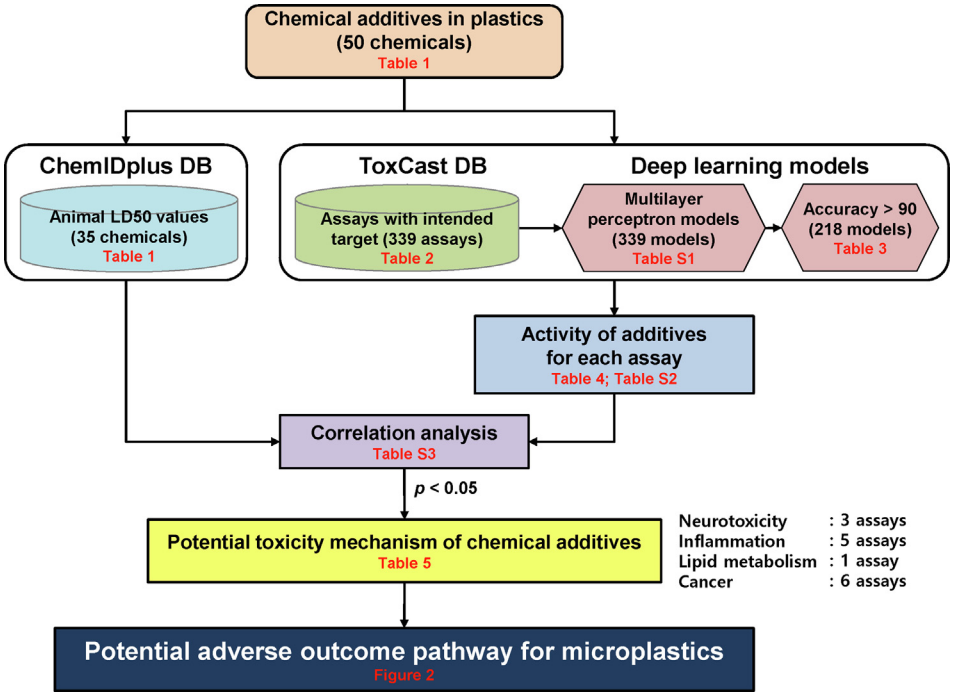

Fig. 1. Workflow for screening of mechanism of toxicity of plastic additives using ToxCast bioassay and their deep learning models.

3. Results and discussion

3.1. Workflow

A schematic workflow of this study is presented in Fig. 1. We screened the ToxCast database and developed deep learning models to classify the activity of the additives; analyzed the assays that associate with mammalian toxicity data using activity information; and identified potential toxicity mechanism; and proposed potential AOP.

3.2. Collection of chemical additives presented in plastics and mammalian toxicity data

Chemical substances used as additives in plastics are investigated to gain an understanding of the hazardous potential of microplastics. We collected a list of 50 chemicals most commonly applied as an additive in plastics from literatures (Table 1) (Hahladakis et al., 2018; Hansen et al., 2013; Hermabessiere et al., 2017; Kwon et al., 2017). The chemical additives were divided into four representative categories: 17 Plasticizers; 8 Biocides; 7 Flame retardants; and 18 Stabilizers, antioxidants, and organic pigments (Stabilizers). These additives are intentionally added to the plastics during the plastic manufacturing process and usually are not chemically bonded with the polymers (Hermabessiere et al., 2017). For this reason, chemical additives are recognized as one of the main contributors to the toxicity of plastics especially when plastics are fragmented into microplastics in the environment (Gallo et al., 2018).

The mammalian acute toxicity data were collected from the ChemIDplus database for those 50 additives. The main routes of human exposure to microplastics are suspected via ingestion or inhalation (Cox et al., 2019), but inhalation toxicity values are rarely provided in the database. Therefore, only oral acute toxicity values were collected. We were able to collect rat or mouse 24hr oral LD50 values for 35 additives (Table 1). The average LD50 values were 927.7 mg/L for the Biocides, 2080 mg/L for the Flame retardants, 2609.9 mg/L for the Stabilizers, and 8702.9 mg/L for the Plasticizers. Based on the individual chemical additives, the most toxic chemical was arsenic trioxide in Biocides, which have an LD50 value of 14.6 mg/kg, and the least toxic chemical was dicyclohexyl phthalate in Plasticizers with an LD50 value of 34,400 mg/kg.

3.3. Selection of ToxCast assays for analysis

To gain an insight into the mechanism of toxicity of chemical additives, ToxCast assays were investigated. We selected 339 assays that have intended biological target information to gain an insight into the mechanism of toxicity of plastic additives (Table 2). There were eight assay sources, mainly 121 NovaScreen (NVS) and 99 BioSeek (BSK). The number of test chemicals per assay source ranged from 214 to 7574. However, there is a large number of missing data in hit call data from ToxCast database. Of the 50 chemical additives, 11 were not tested in the ToxCast program at all. The number of test data for the rest 39 chemical additives, including active and inactive, is 6315 and it is 47.8% of all data to be tested (339 assays × 39 chemicals = 13,221). This high rate of missing data can impede the identification of chemical specific activity of the assays. Therefore, using the ToxCast database alone is limited to gain an overview of the toxicity mechanism of chemicals. Also, the average ratio of active chemicals in each ToxCast assay was about 24.7%, ranged from 0.5% (TOX21_TR_LUC_GH3_Agonist, AEID 803) to 83.1% (NVS_ADME_hCYP2C9, AEID 337). This indicates that the ToxCast assay data are highly imbalanced and not homogeneous by assay. This imbalance can reduce prediction accuracy in model development (Liu et al., 2015).

3.4. Development of deep learning models

To address this limitation, we built 339 MLP models for classify activities using the SMOTE to oversampling the minor class for balancing the data. The model accuracy obtained using stratified 5-fold cross-validation were ranged from 48.82 to 99.85 and average accuracy was 88.55 (Table S1). All assays with an accuracy of less than 90 were considered unreliable and excluded from further steps. Finally, 218 assays with model accuracy over 90 were used for the subsequent steps (Table 3; the full list is in Table S1). The intended targets belonged to 17 intended target families, mainly nuclear receptors (63 assays), cytokines (51 assays), DNA binding (40 assays), etc. Based on this

Table 1
List of chemical additives in plastics and animal toxicity data. The list of chemical additives was collected from the literature, and LD50 values were collected from ChemIDplus database.

Type	CAS No.	Name	Reference	LD50 (mg/kg)
Plasticizers	68515-42-4	1,2-benzenedicarboxylic acid, di-C7-11-branched and linear alkyl esters (DHNUP)	Hansen et al. (2013)	–
	101-14-4	2,2′-dichloro-4,4′-methylenedianiline (MOCA)	Hansen et al. (2013)	1140 (Rat)
	101-77-9	4,4′-methylenedianiline (MDA)	Hansen et al. (2013)	517 (Rat)
	85-68-7	Butyl benzyl phthalate (BBP)	Hansen et al. (2013)	2330 (Rat)
	NOCAS_24824	Chlorinated paraffins: C12, 60% chlorine	Hahladakis et al. (2018)	21.5 (Rat)
	103-23-1	Di(2-ethylhexyl) adipate (DEHA)	Kwon et al. (2017)	9100 (Rat)
	117-81-7	Di(2-ethylhexyl) phthalate (DEHP)	Hansen et al. (2013)	30,000 (Rat)
	117-82-8	Di(2-methoxyethyl) phthalate (DMEP)	Hansen et al. (2013)	3200 (Mouse)
	84-74-2	Dibutyl phthalate (DBP)	Hansen et al. (2013)	7499 (Rat)
	84-61-7	Dicyclohexyl phthalate (DCHP)	Hahladakis et al. (2018)	34,400 (Rat)
	84-66-2	Diethyl phthalate (DEP)	Hermabessiere et al. (2017)	8600 (Rat)
	14697-48-4	Diheptyl adipate (DHA)	Hahladakis et al. (2018)	–
	84-69-5	Diisobutyl phthalate (DiBP)	Hermabessiere et al. (2017)	15,000 (Rat)
	41451-28-9	Diisoheptylphthalate (DIHP)	Hansen et al. (2013)	–
	131-18-0	Dipentyl phthalate (DPP)	Hahladakis et al. (2018)	–
	50-00-0	Formaldehyde	Hansen et al. (2013)	100 (Rat)
	115-96-8	Tris(2-chloroethyl) phosphate (TCEP)	Hansen et al. (2013)	1230 (Rat)
Biocides	1327-53-3	Arsenic trioxide	Hansen et al. (2013)	14.6 (Rat)
	1118-46-3	Butyltin trichloride	Hahladakis et al. (2018)	2140 (Rat)
	683-18-1	Dibutyltin dichloride	Hahladakis et al. (2018)	50 (Rat)
	753-73-1	Dimethyltin dichloride	Hahladakis et al. (2018)	73.9 (Rat)
	1461-25-2	Tetrabutyltin	Hahladakis et al. (2018)	1268 (Rat)
	1461-22-9	Tributyltin chloride	Hansen et al. (2013)	129 (Rat)
	3380-34-5	Triclosan	Hansen et al. (2013)	3700 (Rat)
	76-87-9	Triphenyltin hydroxide	Hansen et al. (2013)	46 (Rat)
Flame retardants	1837-91-8	1,2,3,4,5,6-Hexabromocyclohexane	Hahladakis et al. (2018)	–
	68631-49-2	2,2′,4,4′,5,5′-Hexabromobiphenyl ether (BDE 153)	Hahladakis et al. (2018)	–
	5436-43-1	2,2′,4,4′-Tetrabromodiphenyl ether (BDE 47)	Hahladakis et al. (2018)	–
	79-94-7	3.3′-5.5′-Tetrabromobisphenol (TBBPA)	Hansen et al. (2013)	–
	10043-35-3	Boric acid	Hansen et al. (2013)	2660 (Rat)
	84852-53-9	Decabromodiphenylethane (DBDPE)	Hahladakis et al. (2018)	–
	13674-84-5	Tris(2-chlorisopropyl)phosphate (TCPP)	Hahladakis et al. (2018)	1500 (Rat)
Stabilizers, Antioxidants and Organic pigments	59653-74-6	1,3,5-tris[(2S and 2R)-2,3-epoxypropyl]-1,3,5-triazine-2,4,6-(1H,3H,5H)-trione (β-TGIC)	Hahladakis et al. (2018)	498 (Mouse)
	119-47-1	2,2′-Methylenebis(4-methyl-6-*tert*-butylphenol) (Cyanox 2246)	Hahladakis et al. (2018)	11,000 (Mouse)
	88-24-4	2,2′-Methylenebis(ethyl-6-*tert*-butylphenol) (Cyanox 425)	Hahladakis et al. (2018)	10,000 (Rat)
	88-32-4	2-t-butyl-4 hydroxyanisole (BHA)	Hahladakis et al. (2018)	–
	121-00-6	2-*tert*-Butyl-4-methoxyphenol	Hahladakis et al. (2018)	2910 (Rat)
	104-40-5	4-Nonylphenol	Hansen et al. (2013)	1620 (Rat)
	1806-26-4	4-Octylphenol	Hansen et al. (2013)	–
	80-05-7	Bisphenol A	Hansen et al. (2013)	3250 (Rat)
	128-37-0	Butylated hydroxytoluene (BHT)	Kwon et al. (2017)	890 (Rat)
	10108-64-2	Cadmium chloride	Hansen et al. (2013)	88 (Rat)
	10325-94-7	Cadmium dinitrate	Hahladakis et al. (2018)	300 (Rat)
	1306-19-0	Cadmium oxide	Hansen et al. (2013)	72 (Rat)
	71-48-7	Cobalt(II) diacetate	Hansen et al. (2013)	503 (Rat)
	67701-03-5	Fatty acid amides	Hahladakis et al. (2018)	–
	6683-19-8	Irganox 1010	Hahladakis et al. (2018)	–
	2451-62-9	Triglycidyl isocyanurate (TGIC)	Hansen et al. (2013)	188 (Rat)
	31570-04-4	Tris(2,4-di-*tert*-butylphenyl) phosphite	Hahladakis et al. (2018)	–
	26569-53-9	Tris-nonyl-phenyl phosphate (TNPP)	Hahladakis et al. (2018)	–

intended biological target information, the ToxCast database can be used for building an AOP or screening chemicals corresponding to the AOPs.

The assay that has the highest accuracy is BSK_4H_Pselectin_up, with a cross-validation accuracy of 99.85. The intended target of this assay is the SELP (selectin P) gene, a member of the cell adhesion molecules family, that encodes calcium-dependent membrane receptors in human platelets (Vestweber and Blanks, 1999). Of the more than 90 model accuracy, the accuracy of the model based on the ATG_PPRE_CIS_up assay was found to be 90.10. The intended targets of this assay were PPARA, PPARD, and PPARG belonging to the nuclear receptor family. These three subtypes of Peroxisome Proliferator-Activated Receptor (PPAR) affect genes associated with cell differentiation, immune and inflammatory responses (Berger and Moller, 2002). In addition to the model accuracy, other metrics that determine whether the model is well developed include true positive rate (sensitivity) and true negative rate (specificity). Depending on the assay sources, the average true positive rate ranged from 0.87 to 0.99 and the average true negative rate ranged from 0.87 to 0.96 (Table 3).

3.5. Classification of active additives

To identify active additives of the ToxCast bioassays, we screened selected 218 assays for 50 additives. For the missing data including 11 additives not tested at all in the ToxCast, deep learning models were used to predict their activity. ToxCast screening results and deep learning predictions were combined, and the activity of additives for each assay was finally analyzed (Table 4; the full list is in Table S2). In total, 50 chemical additives showed activity in an average of 43.7 assays (20%), and 34 assays showed no activity in any of the additives. The biocides were the most active category (37.3%), also in both ToxCast (39.6%) and deep learning models (34.2%). The stabilizers

Table 2
Summary of 339 ToxCast assays used in this study by assay source.

Assay		Average number of chemicals	Average number of active chemicals	Model	Format	Time point	Readout	
Source name	Number of assays						Function	Detection
ACEA	1	1737	299 (17.2%)	T47D	96-well plate	80 h	Signaling	Label Free Technology
APR	2	1038	203 (19.6%)	HepG2	384-well plate	24,72 h	Signaling	Fluorescence
ATG	67	3420	246.9 (7.2%)	HepG2	24-well plate	24 h	Reporter gene	Fluorescence
BSK	99	1444	241 (16.7%)	Umbilical vein endothelium, Bronchial epithelial cells, Coronary artery smooth muscle cells, Foreskin fibroblast, Keratinocytes and foreskin fibroblasts, Umbilical vein endothelium and peripheral blood mononuclear cells	96-well plate	24 h	Signaling	Fluorescence
NVS	121	162.8	66.5 (40.8%)	Cell-free, tissue-based cell-free	48,96,384-well plate	0 ~ 24 h	Enzymatic activity, Binding	Spectrophotometry, Fluorescence, Radiometry, Luminescence
OT	15	1723.7	187 (10.8%)	CHO-K1, HEK293T, HeLa	384-well plate	8,16,24 h	Reporter gene, Binding	Luminescence, Fluorescence, Microscopy
TOX21	32	7573.5	689.6 (9.1%)	HEK293T, MDA-kb2, MCF-7, BG1, HeLa, GH3, HepG2, HCT116, HEK293, ME-180	1536-well plate	24,48 h	Reporter gene	Fluorescence, Luminescence
NCCT	2	213.5	169.5 (79.4%)	Tissue-based cell-free	384-well plate	0.5 h	Binding	Fluorescence, Spectrophotometry

were the second active category, which was active in 21.6% of all assays (27.7% in ToxCast, and 17.3% in deep learning models). The flame retardants were the third active category being 20.1% active of all assays (16.7% in ToxCast, and 22.4% in deep learning models). The plasticizers were 11% active of all assays (10.9% in ToxCast, and 11.0% in deep learning models).

Representative intended target families of plasticizers were Oxidoreductase, CYP (Cytochromes P450), GPCR (G-protein-coupled receptors), biocides were Protease inhibitor, GPCR, Oxidoreductase, flame retardant were Oxidoreductase, CYP, GPCR, and stabilizers were Oxidoreductase, CYP, Protease inhibitor. Overall, it showed high activity in the oxidoreductase, CYP, and GPCR family assays, which is likely to be a potential toxicity pathway for chemical additives. Oxidoreductase is a target family that causes reactive oxygen species (ROS) in mitochondria and is a major cause of oxidative stress (Esterházy et al., 2008). CYP family involves the synthesis and metabolism of various molecules and chemicals and produces key enzymes in cancer formation (Rodriguez-Antona and Ingelman-Sundberg, 2006). GPCR family plays important roles in various physiological processes and diseases such as signal transduction and cancer pathways (Gamo et al., 2008; Lappano and Maggiolini, 2011).

3.6. Selection of assays and potential toxicity mechanism of chemical additives

To identify the relationship between *in vivo* apical toxicity data and *in vitro* molecular toxicity data, the point-biserial correlation analysis was performed with mammalian acute oral LD50 data of 35 additives (continuous variables) and the activity pattern (dichotomous variables, active = 1, inactive = 0) of ToxCast assay. Eighteen assays were found to have a statistically significant negative correlation, which tended to be active when the LD50 value of the chemical was small (Table S3). Among them, three assays, TOX21_AR_LUC_MDAKB2_Agonist ($r_{pb} = -0.424$, $p = .011$), ATG_LXRb_TRANS_up ($r_{pb} = -0.372$, $p = .028$), and ATG_p53_CIS_up ($r_{pb} = -0.355$, $p = .036$), are excluded because the number of active additives is too small (< 10%) (Table 5).

3.6.1. Neuronal toxicity

Analysis of the intended target gene showed that the NVS_GPCR_bDR_NonSelective ($r_{pb} = -0.509$, $p = .002$) targeted a Dopamine receptor D1 (DRD1), the NVS_GPCR_hAdra2A ($r_{pb} = -0.402$, $p = .017$) targeted an Adrenoceptor Alpha 2A (ADRA2A), and the NVS_GPCR_h5HT7 ($r_{pb} = -0.341$, $p = .045$) targeted 5-Hydroxytryptamine Receptor 7 (HTR7) are related to neuronal toxicity. All targets are members of the GPCR family. DRD1 is widely expressed in the brain and central nervous system (CNS) and plays an important role in locomotor activity and learning and memory (Cadet et al., 2010). ADRA2A plays a critical role in regulating neurotransmitters from adrenergic neurons in the central nervous system (Bücheler et al., 2002). HTR7 is a serotonin receptor expressed in the brain and CNS, regulate synaptic transmission, thermoregulation, and learning and memory (Ciranna and Catania, 2014). Statistically significant correlation on the GPCR family-related assays insinuates additives chemicals having neurotoxic potential.

3.6.2. Inflammation

The BSK_CASM3C_MCP1_down ($r_{pb} = -0.491$, $p = .003$) targeted cytokine C-C Motif Chemokine Ligand 2 (CCL2), which involved in immune and inflammatory response (Balkwill, 2003). Both the TOX21_GR_BLA_Antagonist_ratio ($r_{pb} = -0.382$, $p = .024$) and the TOX21_GR_BLA_Agonist_ratio ($r_{pb} = -0.364$, $p = .032$) targeted Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1), which encodes glucocorticoid receptor associated with stress and inflammatory responses (Yang et al., 2019). And the BSK_CASM3C_IL8_down ($r_{pb} = -0.377$, $p = .026$) and BSK_CASM3C_IL6_down ($r_{pb} = -0.359$,

Table 3
Summary of 218 deep learning models with accuracy over 90 by intended target families.

Potential event	Assay		Average model performance		
	Intended target family	Number of assays	Accuracy (S.D.)	True positive rate (S.D.)	True negative rate (S.D.)
Molecular Initiating Event	DNA binding	40	96.27 (2.59)	0.99 (0.01)	0.95 (0.05)
	GPCR	9	93.69 (3.52)	0.94 (0.07)	0.93 (0.07)
	Nuclear receptor	63	96.35 (2.49)	0.99 (0.03)	0.95 (0.04)
Key Event	Cell adhesion molecules	17	95.03 (1.92)	0.95 (0.04)	0.88 (0.08)
	CYP	6	94.72 (3.53)	0.9 (0.09)	0.94 (0.05)
	Cytokine	51	95.72 (2.33)	0.96 (0.04)	0.9 (0.07)
	Esterase	1	97.22 (NA)	0.87 (NA)	0.83 (NA)
	Growth factor	3	96.25 (1.42)	0.98 (0.02)	0.92 (0.05)
	Hydrolase	2	97.37 (2.61)	0.97 (0.04)	0.95 (0.06)
	Kinase	9	93.96 (2.05)	0.98 (0.03)	0.93 (0.04)
	Misc protein	2	97.28 (0.52)	0.99 (0)	0.95 (0.03)
	Oxidoreductase	2	92.78 (1.03)	0.87 (0.04)	0.87 (0.14)
	Phosphatase	2	90.52 (0.34)	0.88 (0.02)	0.78 (0)
	Protease	9	95.38 (2.43)	0.97 (0.03)	0.88 (0.09)
	Protease inhibitor	2	95.64 (0.03)	0.96 (0.00)	0.86 (0.02)

S.D.: standard deviation; NA: not available.

$p = .034$) targeted Interleukin 8 (IL8) and Interleukin 6 (IL6), respectively, which are chemotactic factors that mediate inflammatory response (Wang et al., 2009). Inflammation is therefore one of the mechanisms associated with the potential toxicity of chemical additives.

3.6.3. Lipid metabolism

The TOX21_PPARd_BLA_antagonist_ratio ($r_{pb} = -0.475$, $p = .004$) targeted Peroxisome Proliferator-Activated Receptor Delta (PPARD) in nuclear receptor family that plays an important role in the control of fatty acid oxidation and lipid metabolism (Luquet et al., 2005). Taken that some chemicals such as dibutyltin chloride, tributyltin chloride, and bisphenol A that are active in the assay are known as obesogens (Chamorro-García et al., 2018; Sena et al., 2017; Wang et al., 2013), this result also suggest the metabolic toxic potential of chemical additives.

3.6.4. Cancer

The TOX21_VDR_BLA_antagonist_ratio ($r_{pb} = -0.383$, $p = .023$) targeted Cytochrome P450 Family 24 Subfamily A Member 1 (CYP24A1), which is involved in the vitamin D metabolism pathway and plays an important role in the regulation of diseases such as cancer (Jeon and Shin, 2018). BSK_BE3C_uPA_down ($r_{pb} = -0.372$, $p = .028$) targeted urokinase plasminogen activator (PLAU), which are characteristic of cancer cells with functional Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling (Pavet et al., 2014). Both TOX21_HSE_BLA_agonist_ratio ($r_{pb} = -0.362$, $p = .032$) and ATG_HSE_CIS_up ($r_{pb} = -0.336$, $p = .049$) targeted Heat shock factor 1 (HSF1), which regulates stress response, inflammation, and cancer (Sharma and Seo, 2018; Vihervaara and Sistonen, 2014). And both TOX21_AR_BLA_Agonist_ratio ($r_{pb} = -0.356$, $p = .036$) and TOX21_AR_LUC_MDAKB2_Antagonist ($r_{pb} = -0.335$, $p = .049$) targeted Androgen Receptor (AR), which regulates gene expression and cellular proliferation and differentiation related to various diseases including neuronal disease and cancer (Heinlein and Chang, 2004; Jordan and DonCarlos, 2008; Parodi and Pennuto, 2011).

Overall, assays with significant correlations with mammalian toxicity were found to have targets that mostly follow neurotoxicity, inflammation, lipid metabolism, and cancer pathways.

3.7. Development of AOP based on mechanism of toxicity

Activity classification by the intended target family and *in vivo* mammalian toxicity correlation analysis showed that chemical additives mainly follow toxicity pathways such as oxidative stress, cancer, signaling pathways, inflammation, and lipid metabolism. These results are consistent with the main toxicity mechanism of the microplastics, oxidative stress, neurotoxicity, inflammation, and lipid metabolism, suggested in our previous study that reviewed the toxicity mechanism of microplastics (Jeong and Choi, 2019). By incorporating chemical additives toxicity pathways information, we complemented the AOP for human health proposed in the previous study (Fig. 2).

However, since these results are analyzed through correlation analysis, a direct relationship between the biological targets and the animal acute toxicity might be limited. Nevertheless, it may be considered as potential biological targets related to the toxicity. It is also important to note that the 15 assays derived from this study are the result of an integrated analysis of selected 50 additives, rather than confirming the toxicity mechanism of each additive. Therefore, to gain an insight into the hazardous potential of a wider range of chemicals, it is necessary to include those chemicals in the first step to perform the entire analysis process. Further research is also needed to confirm whether the potential assays are actually related to mammalian toxicity.

Table 4
Average number of assays and representative intended target families by additive type.

Type	ToxCast database		Deep learning models		Total		
	Average number of tested assays	Average number of active assays	Average number of predicted assays	Average number of active assays	Number of assays	Average number of active assays	Representative intended target families
Plasticizers	119.7	13.1 (10.9%)	98.3	10.8 (11.0%)	218	23.9 (11.0%)	Oxidoreductase, CYP, GPCR
Biocides	124.8	49.4 (39.6%)	93.3	31.9 (34.2%)	218	81.3 (37.3%)	Protease inhibitor, GPCR, Oxidoreductase
Flame retardants	91.3	15.3 (16.7%)	126.7	28.4 (22.4%)	218	43.7 (20.1%)	Oxidoreductase, CYP, GPCR
Stabilizers, Antioxidants and Organic pigments	89.5	24.8 (27.7%)	128.5	22.2 (17.3%)	218	47.0 (21.6%)	Oxidoreductase, CYP, Protease inhibitor
Total	106.2	23.2 (21.8%)	111.8	20.5 (18.4%)	218	43.7 (20.0%)	Oxidoreductase, CYP, GPCR

Table 5
Potential assays related to mammalian toxicity analyzed by point-biserial correlation.

Toxicity mechanism	Assay name	Assay AEID	Intended target family (subfamily)	Intended target	ToxCast HTS *in vitro* data		Deep learning prediction		Active additives (%)	Point-biserial correlation with mammalian LD50	
					Active/Total additives	Active (%)	Active/Total additives	Active (%)		Coefficient (r_{pb})	*p*-value
Neuronal toxicity	NVS_GPCR_bDR_NonSelective	611	gpcr (rhodopsin-like receptor)	DRD1	1/2	50.0	19/48	39.6	40.0	−0.509	0.002
	NVS_GPCR_hAdra2A	628	gpcr (rhodopsin-like receptor)	ADRA2A	2/2	100.0	32/48	66.7	68.0	−0.402	0.017
	NVS_GPCR_h5HT7	625	gpcr (rhodopsin-like receptor)	HTR7	4/5	80.0	32/45	71.1	72.0	−0.341	0.045
Inflammation	BSK_CASM3C_MCP1_down	215	cytokine (chemotactic factor)	CCL2	7/21	33.3	8/29	27.6	30.0	−0.491	0.003
	TOX21_GR_BLA_Antagonist_ratio	794	nuclear receptor (steroidal)	NR3C1	8/39	20.5	2/11	18.2	20.0	−0.382	0.024
	TOX21_GR_BLA_Agonist_ratio	793	nuclear receptor (steroidal)	NR3C1	10/39	25.6	0/11	0.0	20.0	−0.364	0.032
	BSK_CASM3C_IL8_down	211	cytokine (interleukins)	CXCL8	6/21	28.6	5/29	17.2	22.0	−0.377	0.026
	BSK_CASM3C_IL6_down	209	cytokine (interleukins)	IL6	6/21	28.6	10/29	34.5	32.0	−0.359	0.034
Lipid metabolism	TOX21_PPARd_BLA_antagonist_ratio	1125	nuclear receptor (non-steroidal)	PPARD	8/38	21.1	0/12	0.0	16.0	−0.475	0.004
Cancer	TOX21_VDR_BLA_antagonist_ratio	1132	cyp (xenobiotic metabolism)	CYP24A1	6/38	15.8	0/12	0.0	12.0	−0.383	0.023
	BSK_BE3C_uPA_down	203	protease (serine protease)	PLAU	6/21	28.6	7/29	24.1	26.0	−0.372	0.028
	TOX21_HSE_BLA_agonist_ratio	1113	dna binding (heat shock protein)	HSF1	12/38	31.6	1/12	8.3	26.0	−0.362	0.032
	ATG_HSE_CIS_up	84	dna binding (heat shock protein)	HSF1	7/31	22.6	2/19	10.5	18.0	−0.336	0.049
	TOX21_AR_BLA_Agonist_ratio	761	nuclear receptor (steroidal)	AR	6/39	15.4	0/11	0.0	12.0	−0.356	0.036
	TOX21_AR_LUC_MDAKB2_Antagonist	765	nuclear receptor (steroidal)	AR	9/39	23.1	2/11	18.2	22.0	−0.335	0.049

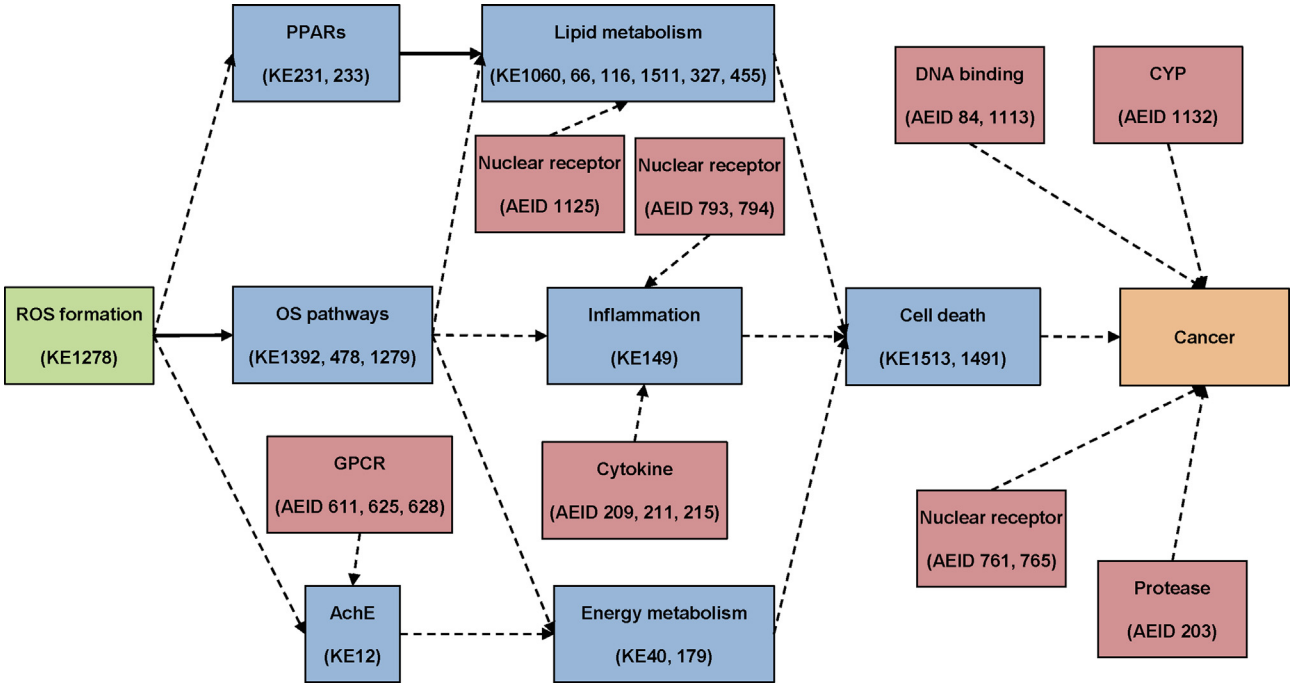

Fig. 2. Adverse outcome pathway relevant to microplastics based on toxicity mechanisms of chemical additives.

4. Conclusion

In this study, to gain a global overview of hazardous potential from microplastics pollution, the toxicity mechanism of plastic additives was investigated by analyzing the relationship between ToxCast *in vitro* bioassay activity data and *in vivo* animal acute toxicity data. We suggest that pathways corresponding to neurotoxicity, inflammation, lipid metabolism, and cancer pathways may be related to the toxic mechanism of plastic additives. Overall, the study showed that the *in silico-in vitro-in vivo* integration approach can provide toxicological information on the wide range of potential biological targets associated with apical toxic effects of the plastic additives, and the derived toxicity pathways could be used to fill the gaps in AOP for microplastics.

Funding

This work was supported by the Korean Ministry of Environment under the 'Environmental Health R&D Program' (2017001370001).

CRediT authorship contribution statement

Jaeseong Jeong: Writing - original draft, Methodology, Software, Investigation, Data curation, Writing - review & editing. **Jinhee Choi:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank Changheon Kim for contributions toward running deep learning python scripts.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105557.

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