Reproductive Toxicology 94 (2020) 55–64

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Computational approach for collection and prediction of molecular initiating events in developmental toxicity

Xabier Cendoya[a], Celia Quevedo[b], Maitane Ipiñazar[b], Francisco J. Planes[a,*]

[a] TECNUN, University of Navarra, San Sebastian, 20018, Spain
[b] BIOBIDE, San Sebastian, 20009, Spain

ARTICLE INFO

Keywords:
Developmental toxicity
Computational
Statistical model
Mechanism of action

ABSTRACT

Developmental toxicity is defined as the occurrence of adverse effects on the developing organism as a result from exposure to a toxic agent. These alterations can have long-term acute effects. Current *in vitro* models present important limitations and the evaluation of toxicity is not entirely objective. *In silico* methods have also shown limited success, in part due to complex and varied mechanisms of action that mediate developmental toxicity, which are sometimes poorly understood. In this article, we compiled a dataset of compounds with developmental toxicity categories and annotated mechanisms of action for both toxic and non-toxic compounds (DVTOX). With it, we selected a panel of protein targets that might be part of putative Molecular Initiating Events (MIEs) of Adverse Outcome Pathways of developmental toxicity. The validity of this list of candidate MIEs was studied through the evaluation of new drug-target relationships that include such proteins, but were not part of the original database. Finally, an orthology analysis of this protein panel was conducted to select an appropriate animal model to assess developmental toxicity. We tested our approach using the zebrafish embryo toxicity test, finding positive results.

1. Introduction

The Environmental Protection Agency (EPA) defines developmental toxicity as the occurrence of adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development or postnatally to the time of sexual maturation [1]. These effects may be detected at any point in the lifespan of the organism and major manifestations include death, structural abnormalities, altered growth and functional deficiency. Approximately 3% of new-borns present congenital anomalies and around 5–10 % of those are likely induced by exposure to developmentally toxic agents [2]. For this reason, regulatory organizations and the industry demand for effective methods to test the developmental toxicity of drugs, industry chemicals and waste products [3].

Toxicity studies are primarily carried out in rats, though testing in other species, such as rabbits, is recommended [4]. The European Centre for the Evaluation of Alternative Methods has funded validation studies for alternative models, such as the embryonic stem cell test, the mammalian micromass test and the whole embryo culture [5]. However, these models have limitations. Firstly, they usually do not cover the whole period of embryogenesis [5]. Moreover, while whole embryo culture assays tend to have a good predictability, morphologic evaluation remains subjective and subtle effects might go unnoticed [6]. Besides, a clear limitation of these models is that extrapolation of the results obtained from test animals does not always translate properly into other organisms [7,8]. Last but not least, there are ethical concerns related to the amount of animal testing that is necessary under the European REACH legislation [9] and a need has arisen to replace animal testing with alternative methods [10]. In fact, regulatory agencies like the EPA have recently called for a reduction of animal testing, with measures such as eliminating all mammal test funding by 2035 [11].

A number of published works used computational models to gain insights into developmental toxicity. Among them, we have strategies that use read-across [12], *in vitro* chemical high-throughput screenings, such as ToxCast experiment data-based models [13,14], and Quantitative Structure Activity Relationship [15–22]. However, despite these efforts, they have shown limited capacity to generalize their results with external datasets [17]. These unsatisfactory results might possibly be related with: i) limited sample size [23], ii) the use of unbalanced datasets, typically containing more active compounds that cause developmental toxicity than compounds that do not cause developmental toxicity, and iii) the underlying complexity of end-points in developmental toxicity and varied mechanisms of action [23].

* Corresponding author.
E-mail address: fplanes@tecnun.es (F.J. Planes).

https://doi.org/10.1016/j.reprotox.2020.03.010
Received 12 November 2019; Received in revised form 4 March 2020; Accepted 20 March 2020
Available online 26 April 2020

In order to address the underlying complexity of developmental toxicity, Adverse Outcome Pathways (AOPs) [10,14,18] constitute a promising concept. The model of the AOP, first developed by Ankley et al. [24], posits that toxicological effects can be viewed as a Molecular Initiating Event (MIE) that is related to an Adverse Outcome (AO) through a number of Key Events (KE) [24–26]. The strength of this model is related to its ability to make every step associated with an AO easily explainable and measurable. This abstraction has been successfully applied in a number of works studying developmental toxicity AOs [14,27,28]. However, we find that there is a disconnect between the mechanisms of action possibly linked to developmental toxicity by studies and the well-characterized AOPs that can be found in public knowledge bases. The AOPwiki [29], a database that contains the information about described and theoretical AOPs, only contains a handful of AOPs associated to developmental toxicity endpoints and several extensively studied MIEs, like folate antagonism [30], cannot be found among them.

In this direction, little work has been carried out in order to systematically establish which MIEs are associated with developmental toxicity. In Schachter and Kohane (2011) [18], the authors identified 140 protein targets that were annotated to Class X compounds (teratogenic effect has been clearly established in humans) and not to Class A compounds (non-teratogenic effect has been clearly established in animal models and humans). However, this study was based on 45 teratogenic compounds and relevant protein targets in teratogenesis were neglected. In this article, our aim is to extend this previous work to developmental toxicity using a more complete, updated and curated database, named DVTOX. Our database includes 430 compounds of diverse chemical categories with associated protein targets and a binary label for their developmental toxicity potential in humans. Out of these 430 compounds in DVTOX, 257 are labelled as toxic to development and 173 are labelled as not toxic to development, which allows for a more robust identification of protein targets associated with developmental toxicity.

With this dataset, we elucidated a list of human protein targets that are significantly associated to compounds that produce developmental toxicity effects and could be good candidates to act as MIEs in AOPs related to developmental toxicity. To illustrate the relevance of our list of proteins, we carried out a literature search to determine whether such targets had associations with developmental processes. We also searched other compounds that include these targets in their mechanisms of action, looking whether there was any evidence for their developmental toxicity. Importantly, we conducted an orthology analysis in various common animal models in order to assess the predictive power of our target-based approach to identify common paths of developmental toxicity across species when the target mechanism of action is conserved. Our approach was tested using the zebrafish embryo toxicity test with ketoprofen, a cyclooxygenase inhibitor, and captopril and enalapril, two angiotensin-converting enzyme inhibitors.

2. Methods

2.1. DVTOX

Our initial source of information on the indication of developmental toxicity was the database published as the supplementary data of Enoch et al. [12], which was taken from [31]. This database contained 290 compounds with annotated FDA categories for pregnancy risk. Because the publication is prior to the FDA's update of developmental risk labels [32], it still uses the old labelling categories: A, B, C, D and X. A and B category compounds were those in which a toxicity effect has not been established for humans. Category C included compounds in which there is a lack of studies to clearly determine whether the developmental effects that might have been observed in animals translate to effects in humans. Categories D and X were reserved for compounds in which a toxic effect in humans has been clearly established [33]. While there are further subtleties to these categories, for classification purposes we divided the 290 compounds in three categories and changed the label to -1 for categories A and B, 0 for category C and 1 for categories D and X. Previous works had also carried out this binarization process, but including uncertain molecules of category C in the same group as molecules for which toxicity has been established [19,21,34].

Next, we searched the EPA's Integrated Risk Information System (IRIS) [35] database for compounds with a known annotation for developmental toxicity. The mission of IRIS is to identify health hazards for compounds found in the environment. Excluding the compounds currently undergoing testing or flagged for testing in the future, we retrieved 32 unique compounds that had been assessed by the program and were registered as producing developmental effects through oral exposure and/or inhalation. All of the molecules that were included had No-Observed-Adverse-Effect-Levels (NOAEL) or Lowest-Observed-Adverse-Effect-Levels (LOAEL) well under 1000 mg/kg/day, which is the testing limit set by the OECD for developmental toxicity [36]. It was thus considered reasonable that developmental defects might happen at exposure levels that can be acquired by humans (a similar cut-off had been used for reproductive toxicity in a previous work [37]). In this database, no compounds were annotated which tested negative for developmental effects (Fig. 1).

We finally searched the TOXNET [38] Hazardous Substances Data Bank (HSDB) database for compounds with peer-reviewed research about developmental effects, with two objectives: 1) to find supporting research for the compound categories that we had retrieved from the other two sources, and 2) to add new agents into our database. This database contains peer-reviewed excerpts of published research on toxicity in animals and humans, summarizing the experimental methods and results. The texts relating to developmental and reproductive toxicity were reviewed for each compound.

The evidence from all sources was pooled and evaluated on compound-by-compound basis. Several levels of evidence were considered, ranked by their relevance to human developmental toxicity effects:

1 FDA pregnancy risk category: when this categorization was available, it was included, as the categorization involves thorough review of all the available literature for the developmental toxicity of a given compound [39].
2 Human epidemiological studies: these were the most relevant sources of information for categorization, as they establish whether the developmental toxicity effect is observed when the exposure happens on a population level.
3 Human case reports: these reports included single or a series of clinical observations of developmental toxicity that were not part of a controlled study.
4 Animal studies: these were studies carried out in experimental specimens of various species (most commonly rats, mice and rabbits) that reported developmental toxicity effects.

The categorization was carried out according to the amount of evidence and the agreement of that evidence. Unless there was disagreement in the data, FDA pregnancy risk categories and human epidemiological studies were considered the most relevant sources to categorizing the compounds. In cases in which only human case reports were found, we complemented with animal studies, requiring complete agreement between the sources and preferably a clear pattern of malformations. Moreover, in the case of animal studies, the reported exposure to the compound was taken into consideration to avoid flagging as toxic compounds that only produce developmental toxicity effects at doses way beyond normal human exposure levels. It is relevant to note that, because terminology that makes a difference between malformations and variations is still subject to debate [40–42], whenever this differentiation was relevant we considered reversible or harmless anomalies as variations (such as growth retardation) and irreversible and harmful anomalies as malformations. These evidences have been

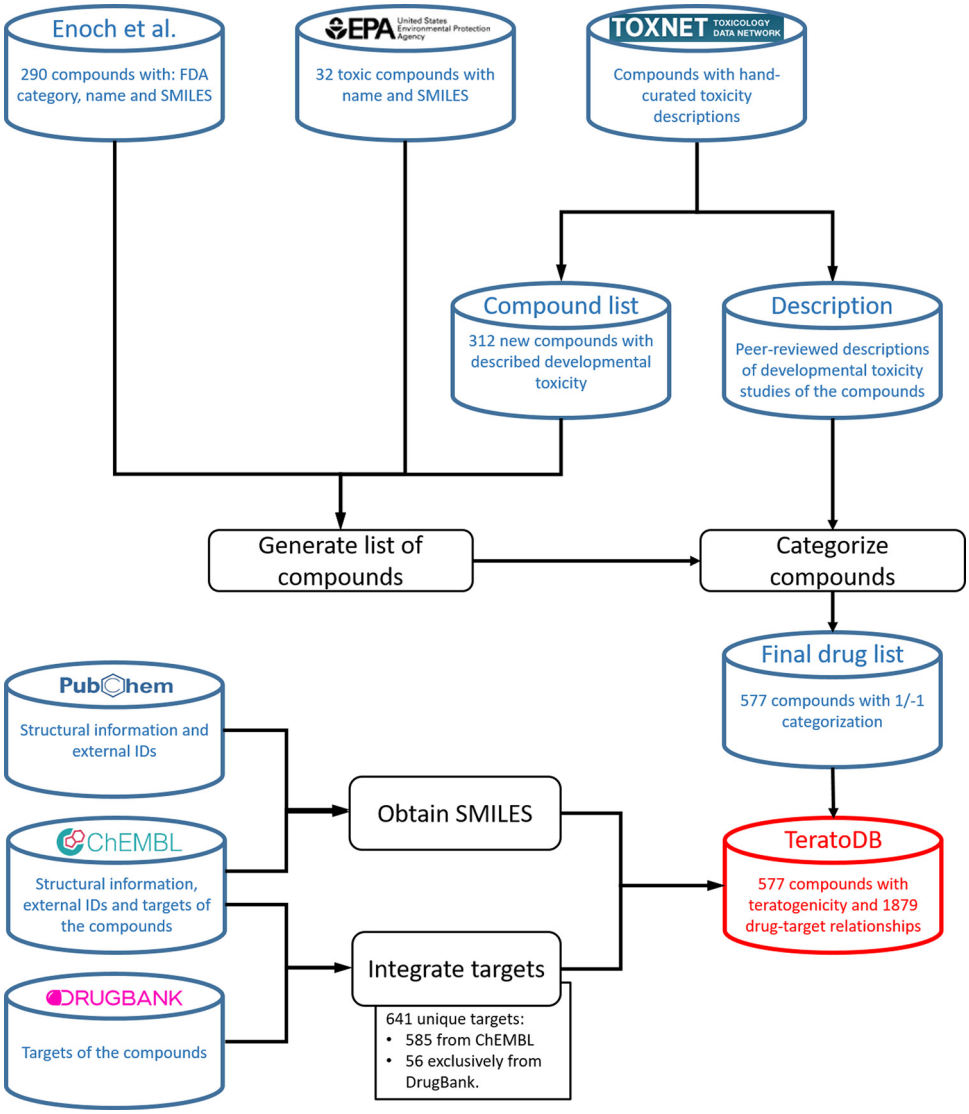

Fig. 1. Workflow followed for the generation of the DVTOX dataset (in red). The initial source of compounds was the dataset published by Enoch et al. [14]. To that, we added compounds contained in the EPA IRIS database and the TOXNET HSDB database. We also obtained published experiment excerpts from HSDB. With these, we obtained a curated list of compounds to which we added structural information from PubChem and ChEMBL and targets contained in both ChEMBL and DrugBank.

detailed in Supplementary Data 1.

Upon review, we included compounds that showed evidence of developmental toxicity with a 1 and those that did not show developmental toxicity with a -1. Compounds with conflicting evidence for toxicity were excluded from the database. In all cases, developmental toxicity effects were considered, while reproductive toxicity effects were not (see Supplementary Data 2). While the two are commonly presented together, the mechanisms by which the toxicity works are different and we were interested in capturing developmental toxicity effects in particular.

Depending on the availability of data that we had obtained from these sources, we used the name or CAS Number to search the PubChem [43] Compound database for structural information on each of the agents that had been selected in the previous step and saved the Canonical SMILES. Using the PubChem Compound database ensured that the obtained chemical structures were standardized [43,44]. In the case of agents that referred to mixtures, we searched for the most representative compound of the mixture. The remaining compounds were looked up in ChEMBL [45]. Compounds that could not be found in either database were deleted from this study.

In order to avoid annotation inconsistencies that have been noted in large compound databases like PubChem [46,47], we also obtained ChEMBL and DrugBank [48] identifiers for all molecules. Using these, we checked for consistency of the molecule identifiers (name, CAS Number, ChEMBL ID and DrugBank ID) to the reported structure. Inconsistencies were cleaned manually, either correcting the annotation that was wrong, or removing the compound from the database.

We also used the ChEMBL and DrugBank identifiers to retrieve the records of the compounds that were available in each of the databases. We extracted the targets annotated for each of the records that we obtained and created a table detailing drug-target relationships. We also created a target table summarizing the unique targets found between two databases. We used Uniprot [49] identifiers for the protein targets.

DVTOX, including drugs, targets and drug-target relationships, can be found in Supplementary Data 1. Note here that 147 out of the 577 compounds had no annotated drug-target interactions. For this reason, for the analysis of mechanisms of action presented below, we focused on the 430 compounds with target annotations.

2.2. Bioinformatics analysis

In order to identify the list of protein targets potentially involved in MIEs, we carried out a one-sided hypergeometric test for each of the proteins targets included in the database. In particular, we defined two event classes for each protein target: participation in the mechanism of action of molecules that have been associated to developmental toxicity endpoints (n) and participation in the mechanism of action of molecules that have not (m). In our database, the population size for each type of event is the number of compounds that have been categorized in each side, namely $N = 257$ and $M = 173$. We evaluated the statistical significance of n for each of the protein targets when the participation of mechanisms of action associated to developmental toxicity was random. For the calculation of the p-value, we used the Lancaster's mid p-value correction, which has better performance than standard procedure for small sample sizes [50]. The proteins with a p-value ≤ 0.05 were considered to be participating significantly more in mechanisms of action of toxic compounds than those of non-toxic compounds. The interactions with these proteins were selected as candidate MIEs of developmental toxicity and were taken into further validation.

In order to evaluate whether the obtained MIE candidates were significantly associated with developmental processes, we carried out a Gene Ontology category enrichment analysis. We introduced the Uniprot identifiers of the MIE candidates on the PANTHER overrepresentation test [51], choosing the Biological Processes subsystem in the Homo sapiens species. We carried out Fisher's exact test and corrected the p-values using the False Discovery Rate approach.

In addition to that, orthologues were obtained for the MIE candidates. We obtained Ensembl identifiers for the genes associated to the selected proteins and with those queried the Ensembl REST API [52], obtaining a list of orthologues based on sequence alignments. Ensembl contains calculated scores for homologues of several species, with percent identity and a confidence annotation. We selected the homologues of human genes found in several common experimental species in developmental toxicity: mouse (*Mus musculus*), rat (*Rattus norvegicus*), hen (*Gallus gallus*), rabbit (*Oryctolagus cunniculus*) and zebrafish (*Danio rerio*).

Furthermore, we carried out a SeqAPASS [53] level 1 and level 2 analysis for each of the proteins in the MIE candidate list. SeqAPASS is a tool developed by the EPA to predict the risk susceptibility of compounds with a certain mechanism of action across species. It contains three different levels of analysis: 1) full protein sequence alignment, 2) functional domain alignment and 3) key amino-acid alignment. We obtained full protein sequence alignments for each of the proteins contained in the MIE candidate list. Afterwards, we selected a number of relevant functional domains for each of the MIE candidates and carried out level 2 analyses. Level 3 analysis was not conducted due to lack of evidence of key amino acids in protein domains.

2.3. Zebrafish embryo toxicity test

The zebrafish (*Danio rerio*) has emerged as a promising alternative animal model for toxicity evaluation after chemical exposure. Assays performed with zebrafish embryos are cheaper and faster when compared with mammalian tests while compounds are evaluated in the context of an intact organism rather than under artificial in vitro conditions [54]. We carried out the zebrafish embryo toxicity test for three different compounds: ketoprofen, captopril and enalapril, as detailed in the Results section. The steps went as follows:

2.3.1. Fish husbandry and egg production

Adult zebrafish were housed and maintained in accordance with standard procedures. Briefly, fish were maintained under a photoperiod of 14:10 h light:dark at 28.5 °C in water continuously filtered at pH 7–7.8, conductivity 500–800 μS and O2 saturation at 60–90 %. Adults were fed with ground dry pellets (Gemma 300, Skretting) and artemia (Catvis) twice a day each. Healthy mature zebrafish pairs were used for egg production. Embryos were collected in E3 embryo media containing 0.0001 % methylene blue (Acros Organics, 96 + % purity) and 100 μg/mL ampicillin (Sigma-Aldrich) and kept in the incubator at 28.5 °C until they reached the stage specified below for each assay.

Zebrafish were maintained in accordance with the European Directive 2010/63 for the protection of animals used for scientific purposes and all experiments were approved by the ethical committee for animal experimentation of IIS Biodonostia (San Sebastián, Gipuzkoa, Spain).

2.3.2. Chemicals

Dimethyl sulfoxide (DMSO) (CAS 67-68-5, purity 99.9 %) (vehicle control) was obtained from Scharlau, Captopril (CAS 62571-86-2, purity ≥ 98 %) from Calbiochem and Ketoprofen (CAS 22071-15-4, purity ≥ 98 %), Enalapril maleate salt (CAS 76095-16-4, purity > 98 %) and 13-CIS-Retinoic acid (CAS 4759-48-2, purity > 98 %) (positive control of the assay) were obtained from Sigma-Aldrich. Tricaine (CAS 886-86-2) was obtained from Acros Organics. Stock solutions of each tested chemical were prepared in DMSO (for the 3 chemicals in the first dose-range finding study or only for Ketoprofen in the main study) or directly in E3 media containing 10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Sigma-Aldrich) (in the case of Enalapril maleate salt and Captopril in the main study).

2.3.3. Assessment of developmental toxicity

To determine the relevant concentrations, a dose-range finding (DRF) study was first conducted at concentrations ranging from 0.1–1000 μM. Fertilized embryos (from transgenic line expressing CopGFP under the myocardium specific promoter cmlc2 [55]) were placed in 24 well plates (5 embryos per well, 10 embryos per condition) and treated at 4 − 5 h post fertilization (hpf) with the corresponding chemical concentration. A group of embryos treated with 0.5 % DMSO was used as a vehicle control. Plates were incubated at 28.5 °C for 4 days and embryo media was replaced and test items added at 2 days post fertilization (dpf). Embryos were analyzed at 2 and 4 dpf (at this last stage embryos were anesthetized with 0.04 mg/mL Tricaine) and the incidence of lethality and the presence of gross developmental defects were recorded. After this, the main experiment was carried out and embryos were treated with the following concentrations of interest: 20, 50, 100, 300, 1000, 2000, 5000 and 10,000 μM for Ketoprofen; 1000, 1500, 2000, 3000, 5000, 7500, 10,000 and 20,000 for Enalapril maleate salt and Captopril. Embryos were treated in a similar manner as described for the DRF above with the exception that a total of 15 embryos (instead of 10) were tested per experimental condition. In addition to vehicle control (0.5 % DMSO for Ketoprofen treated embryos or just E3 media + 10 mM HEPES for embryos exposed to the other two compounds), a group of embryos were treated with 100 nM Retinoic acid (positive control). Detailed analysis of embryo morphology was performed at 2 and 4 dpf based on the teratogenic end points described by Beekhuijzen et al. (2015) [56]. Briefly, these included malformation the craniofacial structures, otic vesicle and tail, deformed body shape and edemas (recorded as presence or absence). The percentage of dead embryos was also calculated at both stages. The percentage of altered and dead embryos was used for Effective Concentration 50 % (EC50) and Lethal Concentration 50 % (LC50) calculations, respectively, applying a nonlinear regression test (sigmoidal dose-response curve) using the GraphPad Prism (GraphPad Software). A teratogenic Index (TI) was estimated as the ratio between LC50 and EC50. Two TIs were calculated, one per stage analyzed. Compounds with a TI higher than 2, at least in one of the stages analyzed, have been considered teratogenic [5] (Biobide's internal validation).

Table 1
List of proteins proposed as MIE candidates of developmental toxicity.

Uniprot ID	CHEMBL ID	Description	Toxic compounds	Non-toxic compounds	Described in Van Gelder et al, 2010
P10275	CHEMBL1871	Androgen Receptor	15	0	Yes
P12821	CHEMBL1808	Angiotensin-converting enzyme	9	1	Yes
P35354	CHEMBL2094253	Cyclooxygenase	14	3	Yes
P11511	CHEMBL1978	Cytochrome P450 19A1	4	0	Yes
P00374	CHEMBL202	Dihydrofolate reductase	5	0	Yes
P11388	CHEMBL1806	DNA topoisomerase II alpha	5	0	No
P25101	CHEMBL252	Endothelin receptor ET-A	4	0	Yes
P03372	CHEMBL206	Estrogen receptor alpha	15	2	Yes
Q92731	CHEMBL242	Estrogen receptor beta	7	0	
P14867	CHEMBL1962	GABA receptor alpha-1 subunit	24	1	Yes
P47869	CHEMBL4956	GABA receptor alpha-2 subunit	22	1	
P34903	CHEMBL3026	GABA receptor alpha-3 subunit	22	1	
P48169	CHEMBL2472	GABA receptor alpha-4 subunit	22	1	
P31644	CHEMBL5112	GABA receptor alpha-5 subunit	22	1	
Q16445	CHEMBL2579	GABA receptor alpha-6 subunit	22	1	
P28472	CHEMBL1847	GABA receptor beta-3 subunit	19	3	
O14764	CHEMBL3591	GABA receptor delta subunit	19	1	
P24046	CHEMBL3561	GABA receptor rho-1 subunit	6	0	
P28476	CHEMBL2375	GABA receptor rho-2 subunit	6	0	
P18505	CHEMBL2109244	GABA-A receptor; agonist GABA site	19	2	
P47870	CHEMBL2109244	GABA-A receptor; agonist GABA site	19	2	
P18507	CHEMBL2095190	GABA-A receptor; alpha-6/beta-3/gamma-2	18	1	
O00591	CHEMBL2093872	GABA-A receptor; anion channel	19	1	
P78334	CHEMBL2093872	GABA-A receptor; anion channel	19	1	
Q9UN88	CHEMBL2093872	GABA-A receptor; anion channel	19	1	
Q8N1C3	CHEMBL2109243	GABA-A receptor; benzodiazepine site	19	1	
Q99928	CHEMBL2109243	GABA-A receptor; benzodiazepine site	19	1	
A8MPY1	CHEMBL2109242	GABA-C receptor	6	0	
P04150	CHEMBL2034	Glucocorticoid receptor	9	1	No
Q13002	CHEMBL3683	Glutamate receptor ionotropic kainate 2	10	0	No
P42262	CHEMBL4016	Glutamate receptor ionotropic, AMPA 2	11	0	
P30968	CHEMBL1855	Gonadotropin-releasing hormone receptor	10	0	No
Q92769	CHEMBL2093865	Histone deacetylase	4	0	Yes
P04035	CHEMBL402	HMG-CoA reductase	8	0	Yes
P20839	CHEMBL1822	Inosine-5′-monophosphate dehydrogenase 1	4	0	No
P43681	CHEMBL1882	Neuronal acetylcholine receptor protein alpha-4 subunit	11	1	No
P36544	CHEMBL2492	Neuronal acetylcholine receptor protein alpha-7 subunit	11	1	
P09619	CHEMBL1913	Platelet-derived growth factor receptor beta	4	0	No
O75469	CHEMBL3401	Pregnane X receptor	6	0	Yes
P06401	CHEMBL208	Progesterone receptor	10	2	Yes
P10276	CHEMBL2055	Retinoic acid receptor alpha	5	0	Yes
P10826	CHEMBL2008	Retinoic acid receptor beta	5	0	
P13631	CHEMBL2003	Retinoic acid receptor gamma	5	0	
P19793	CHEMBL2061	Retinoid X receptor alpha	4	0	Yes
P28702	CHEMBL1870	Retinoid X receptor beta	4	0	
P48443	CHEMBL2004	Retinoid X receptor gamma	4	0	
P23921	CHEMBL1830	Ribonucleoside-diphosphate reductase M1 chain	4	0	No
P31350	CHEMBL1954	Ribonucleoside-diphosphate reductase M2 chain	4	0	
Q7LG56	CHEMBL2095215	Ribonucleoside-diphosphate reductase RR1	4	0	
P10721	CHEMBL1936	Stem cell growth factor receptor	5	0	No
P18405	CHEMBL1787	Steroid 5-alpha-reductase 1	4	0	Yes
P04818	CHEMBL1952	Thymidylate synthase	5	0	No
P30536	CHEMBL5742	Translocator protein	5	0	Yes
P68366	CHEMBL2095182	Tubulin	4	0	No
Q71U36	CHEMBL3661	Tubulin alpha-3 chain	4	0	
Q9H4B7	CHEMBL1915	Tubulin beta-1 chain	4	0	
P07437	CHEMBL5444	Tubulin beta-5 chain	6	0	
P30556	CHEMBL227	Type-1 angiotensin II receptor	7	0	Yes
P17948	CHEMBL1868	Vascular endothelial growth factor receptor 1	6	0	Yes
P35968	CHEMBL279	Vascular endothelial growth factor receptor 2	6	0	
P35916	CHEMBL1955	Vascular endothelial growth factor receptor 3	6	0	
Q9BQB6	CHEMBL1930	Vitamin k epoxide reductase complex subunit 1 isoform 1	4	0	No
O43497	CHEMBL4641	Voltage-gated T-type calcium channel alpha-1 G subunit	4	0	No
O95180	CHEMBL1859	Voltage-gated T-type calcium channel alpha-1H subunit	5	0	
Q9P0 × 4	CHEMBL5558	Voltage-gated T-type calcium channel alpha-1I subunit	4	0	

3. Results

3.1. Descriptive analysis of human developmental toxicity based on DVTOX

In Table 1, we present our list of proteins developmental toxicity MIE candidates, including their UniProt and ChEMBL IDs, name and annotated number of toxic and non-toxic compounds, respectively. Upon examination, it is clear that these proteins interact recurrently with molecules that are associated to developmental toxicity, rather than those that are not (one-sided Hypergeometric test: mid p-value ≤ 0.05, see Methods section). Out of the 181 compounds with which these 65 proteins interact, 168 (92 %) are with compounds that

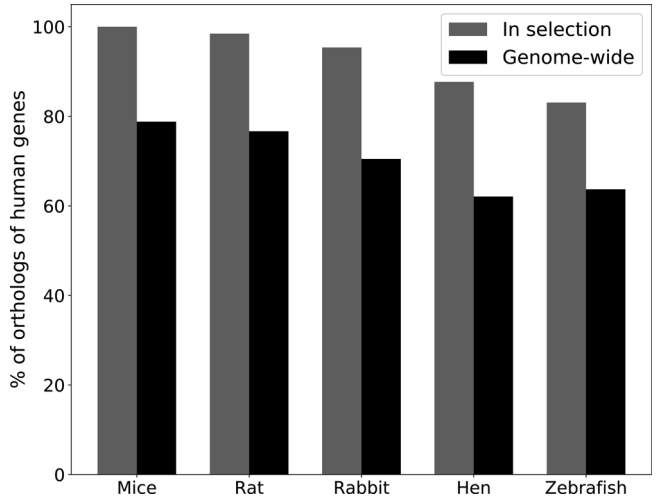

Fig. 2. Conservation of proteins associated with developmental toxicity across several model species. Most of the proteins in our selection have known orthologs in experimental species (in gray). This is especially true when compared with the expected amount of proteins that should have orthologs in each of the species (in black).

have been associated with developmental toxicity outcomes. The space of protein targets to be considered for MIEs is greatly reduced, as 65 candidates aggregate the toxicity of 65 % of the compounds. Given that, on average, the mechanism of action of any given compound contained in DVTOX has five annotated proteins, this is a considerable decrease.

In addition, we carried out an enrichment analysis based on Gene Ontology and found that the MIE candidates that had been selected are significantly enriched in pathways associated with growth and developmental processes (Fisher's exact test: p-value ≤ 0.05, see Methods section and Supplementary Data 2). We also compared our results with a highly cited review of mechanisms of action associated with teratogenesis [30,57]. For this analysis, we grouped proteins in Table 1 into 32 families. One of these families, for example, is that of the GABA receptors, which includes 19 proteins in Table 1. Out of 25 pathways/proteins mentioned in these reviews, we were able to recover 16, as noted in Table 1. This is more accurate than the previous work presented in Schachter and Kohane (2011), which only recovered 6 of these 25 mechanisms.

It is important to note that in Table 1 we also hypothesized mechanisms of action that were not mentioned in van Gelder et al. (2014). Ribonucleotide-diphosphate reductases and thymidylate synthase are typical targets for cellular proliferation in cancer and clear evidences exist for their importance for embryo development [58]. An analogue of thalidomide has been shown to interact with tubulins and, therefore, our prediction seems consistent. Inosine monophosphate dehydrogenase inhibitors were discovered to be teratogenic early on [59]. Similarly, the teratogenic effect of glucocorticoids has long been linked to their interaction with cytoplasmatic receptors [60], as is posed here. Our approach neglected NMDA receptors; however, we captured the other two ionotropic glutamate receptors: kainite and AMPA, whose role for organ formation and morphogenesis has been recently elucidated [61]. We also predicted the relevance of the neuronal acetylcholine receptor and DNA topoisomerase for embryo development, which is again supported by the literature [62,63]. Different tyrosine kinase receptors were identified in Table 1, particularly the platelet-derived growth factor and stem cell growth factor, which are associated with gonadal development [64]. This is also the case for the gonadotropin-releasing hormone receptor. Inhibitors of the vitamin k epoxide reductase complex subunit 1 isoform 1 have shown a clear developmental toxicity effects in different animal models such as the zebrafish [65]. The same conclusion was drawn for inhibitors of voltage-gated T-type calcium channels in mice [66].

We also found a number of these targets involved in previously described AOPs. AOPwiki, for instance, described vascular endothelial growth factor receptor disruption and estrogen receptor agonism as MIEs of AOPs describing developmental outcomes [29]. A review from 2016 introduced an AOP that started with tubulin binding as its MIE, with aneuploidy as its AO [67]. Retinoid imbalance has been associated to AOs for neural tube and axial defects [27]. The androgen receptor and cyclooxygenase had both been related to AOPs. However, the androgen receptor had been described in AOPs related to reproductive toxicity. Cyclooxygenase, on the other hand, had not been described as a MIE, but a KE in other AOPs. Based on this existing literature, we kept the list of proteins in Table 1 for further analysis.

3.2. Predictive analysis of developmental toxicity

Our central hypothesis here is that compounds that interact with proteins in Table 1 (set of *MIE candidates*) will have a developmental toxicity effect in humans. From ChEMBL, we extracted a list of compounds not included in DVTOX that had proteins in Table 1 as part of their mechanism of action (Supplementary Data 2). We obtained 440 compounds, out of which 241 were drugs related to 25 of the MIE candidates. In many cases, we found that the drugs in question had already been described to produce developmental toxicity effects in humans. Some of these drugs were antineoplastic agents, which are clearly toxic for embryo development. Others, however, were less clear, for example, different statins, whose target is HMG-CoA reductase, but their developmental toxicity has not been clearly delineated.

Considering that cross-species extrapolation has been one of the focuses of AOP research [10], we analysed whether the proteins of the candidate MIE list in Table 1 were conserved across five common experimental species. To that end, we extracted the orthologues of these proteins predicted by Ensembl. The rationale behind this was that orthologues tend to conserve high sequence similarities and that this translates into similar functions across organisms [7], which can be extended to mechanisms of toxicity. All five of the experimental animal models showed a high number of orthologues for the proteins selected in our panel (Fig. 2). In fact, the percentage of proteins that had identified orthologs in other species was higher for our selection than would be reasonably expected from the whole genome. This suggests that our target selection contains highly conserved mechanisms of action that are relevant to developmental processes across species.

In addition to that, we carried out SeqAPASS analyses for each protein. We studied the conservation of the proteins in our MIE candidate list in two different levels: full sequence alignment and functional group alignment. There were high similarity hits for all of the proteins in the MIE candidate list. The analysed functional domains showed even higher similarity in all cases (see Supplementary Data 2).

These results strongly suggest that these MIE candidates are conserved across several organisms. It also means that these experimental animals are most likely appropriate to study several common forms of developmental toxicity in humans. Thus, compounds that can participate in said MIEs should yield true positive results when tested for developmental toxicity. However, there will be a number of false negative results arising from the proteins that do not possess orthologues in the experimental species. This can be relevant for organisms like the hen or the zebrafish, which contain the lowest amount of orthologues.

We evaluated the available literature for experiments on the zebrafish embryo toxicity test that could serve as validation for our hypothesis. We found 22 molecules from DVTOX that had proteins in our MIE candidate list which had been tested on the zebrafish embryo [68–70]. We also found 8 of the 241 newly-found compounds that had been tested for the zebrafish embryo toxicity assay. We found positive toxicity effects for all of them. The reason for having so few hits in these large libraries of hundreds of compounds was the restrictiveness of our approach: we only picked compounds with known mechanisms of

action, and of those, only the ones that were known to include the protein targets in our list of MIE candidates. It is, thus, not surprising that we did not find many compounds, one of the reasons being that these libraries were quite enriched in pesticides (two of the studies were exclusively based on them [69,70]), whose modes of action are often poorly understood and still a relevant topic of research [71].

For further validation, we carried out additional experiments with the zebrafish embryotoxicity test in two different situations: compounds targeting cyclooxygenase (COX) and angiotensin-converting enzyme (ACE). In the first case, because Ensembl predicted that COX has an orthologue in the zebrafish, and the SeqAPASS analysis provided us with high similarity alignments in both the whole sequence and functional domain levels, the developmental toxicity MIE sould be conserved in the zebrafish embryo. In the second case, we found that angiotensin-converting enzyme (ACE) showed contradictory results: while SeqAPASS returned 66 % similarity for the whole sequence and 76 % similarity for the functional domains, the Ensembl database did not contain any orthologue of the human ACE in the zebrafish. With this information, we decided to evaluate whether the toxicity MIE of ACE was present in the zebrafish. Thus, we selected ketoprofen (CHEMBL571), a cyclooxygenase (COX1, COX2) inhibitor, and captopril (CHEMBL1560) and enalapril (CHEMBL578), two angiotensin-converting enzyme (ACE) inhibitors in the zebrafish. The zebrafish embryo should present developmental toxicity effects when exposed to ketoprofen, but when exposed to the ACE inhibitors there should only be developmental effects if the MIE (and, as an extension, the protein) is conserved.

Zebrafish embryos were exposed to the three studied compounds and morphological variations were registered (see Method section). Ketoprofen showed the expected results: teratogenic effects were established at 2 dpf (TI = 2.24) and 4 dpf (TI = 3.11). Enalapril and captopril, on the other hand, did not show teratogenic effects at 2 dpf ($TI_{Enalapril}$ = 1.04, $TI_{Captopril}$ = 1.10), but they both could have teratogenic effects at 4 dpf (TI higher than 2 for Enalapril and very close to 2 for Captopril) (Fig. 3).

Considering these results, we concluded that the mechanism of action by which ACE inhibitors act has to be conserved. Ensembl only had a zebrafish homologue of the human ACE2. While this is related to ACE, it is not relevant to the direct mechanism of action of the tested compounds. On the other hand, the Uniprot and NCBI Gene [72] databases both contained zebrafish orthologues for the human ACE, but with low experimental evidence for the former and provisional status for the latter. We also reviewed the compounds in question looking for possible off-target effects that might have triggered the toxicity. The annotated mechanisms of action of both captopril and enalapril only contained ACE, which would point towards a high selectivity. This was further confirmed when only one off target effect was found for captopril in bioactivity assays [73].

4. Discussion

In this paper, we have introduced DVTOX, a hand-curated drug-target relationship database containing developmental toxicity category information for approximately 600 compounds, and a methodology to identify compound mechanisms of action that could act as MIEs of developmental toxicity AOs. Through the usage of the hypergeometric test against the drug-target relationships contained in DVTOX, we have identified 65 proteins that are significantly associated to compounds that produce developmental toxicity effects in human embryos. A Gene Ontology analysis and the available literature have both supported our hypothesis that the selected targets were associated with development in humans, and that some of them are known to act as MIEs. Taking the analysis further, we have extracted new compounds from ChEMBL that contained such targets as part of their mechanisms of action, finding that many had already been annotated as developmental toxicants. This is indicative of the robustness of this methodology to identify mechanisms of action that initiate cascades associated to developmental toxicity AOs.

We have also studied the generalizability of the proposed MIEs to animal models. With that, we predicted that the zebrafish embryo toxicity test would yield a true positive for ketoprofen, and false negatives for enalapril and captopril if ACE did not have any homologues. While ketoprofen behaved as expected, enalapril and captopril did not. We had predicted that, because of the lack of a zebrafish orthologue of ACE, there would be no developmental toxicity effects in the zebrafish embryo when exposed to ACE inhibitors. However, both enalapril and captopril showed a pattern of embryonic toxicity similar to that which has been described in humans: low (if any) at early development, and higher toxicity towards the end of the developmental process [74,75]. The conclusion seems obvious: ACE is probably conserved in the zebrafish, acting in a similar way to the human one. Thus, the MIE that we had predicted in humans would also apply to the zebrafish.

Evaluating the available literature, although enalapril seemed to cause eye development defects in the zebrafish embryo [76], the knockdown of the zebrafish ACE gene did not result in developmental abnormalities in the fish. The authors proposed two options: that the knockdown was not complete or that enalapril mediated its toxicity through a different mechanism of action. When it came to captopril, while there seemed to be some teratogenic effects associated to the exposure of the compound in zebrafish embryos, these happened at high concentrations [77]. This might be related to problems of delivery of the compounds to the zebrafish embryos: compounds with high hydrophilicity tend to have worse uptake than those with high lipophilicity [78]. Both compounds have low LogP values, which means that higher concentrations in the medium will be necessary for the zebrafish embryos to start absorbing it.

We failed to predict that this experiment would actually yield a true positive result because of our starting data: the orthologues obtained from Ensembl, which contains curated relationships that have strong evidence for orthology and neglects putative orthologues for which there could be some lower level of evidence. While this is an evidence of the blindness that the algorithm can have, we believe that this in itself is not a major flaw of the methodology that we present here: all data analytics algorithms are dependent on the data they are fed. In this regard, the presented methodology can easily be applied to larger databases that are richer in annotations, or even other types of toxicity, which would overcome the limitations of the current study.

In summary, we propose that the 65 protein targets reported above are involved in Molecular Initiating Events (MIEs) for developmental toxicity. Note here that AOPs are a promising approach to identify critical biological events impacting human and ecological health. In fact, relevant regulatory programs have incorporated AOPs for chemical screening (Perkin et al., 2015) [79]. Although extensive work is required to complete AOPs in developmental toxicity, the systematic identification of MIE candidates constitutes a step forward. We expect this methodology to be a relevant part of MIE discovery, and to be integrated with other pathway-identification tools to make reliable predictions of AOPs, which will allow for better toxicity testing of compounds.

Funding

Research reported in this publication was supported by the Basque Government with the grant promoting doctoral theses to young predoctoral researchers [grant PRE_2017.1.0327 to X.C.], the Hazitek programme [grant ZL-2017/00170] and Fomento San Sebastian.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

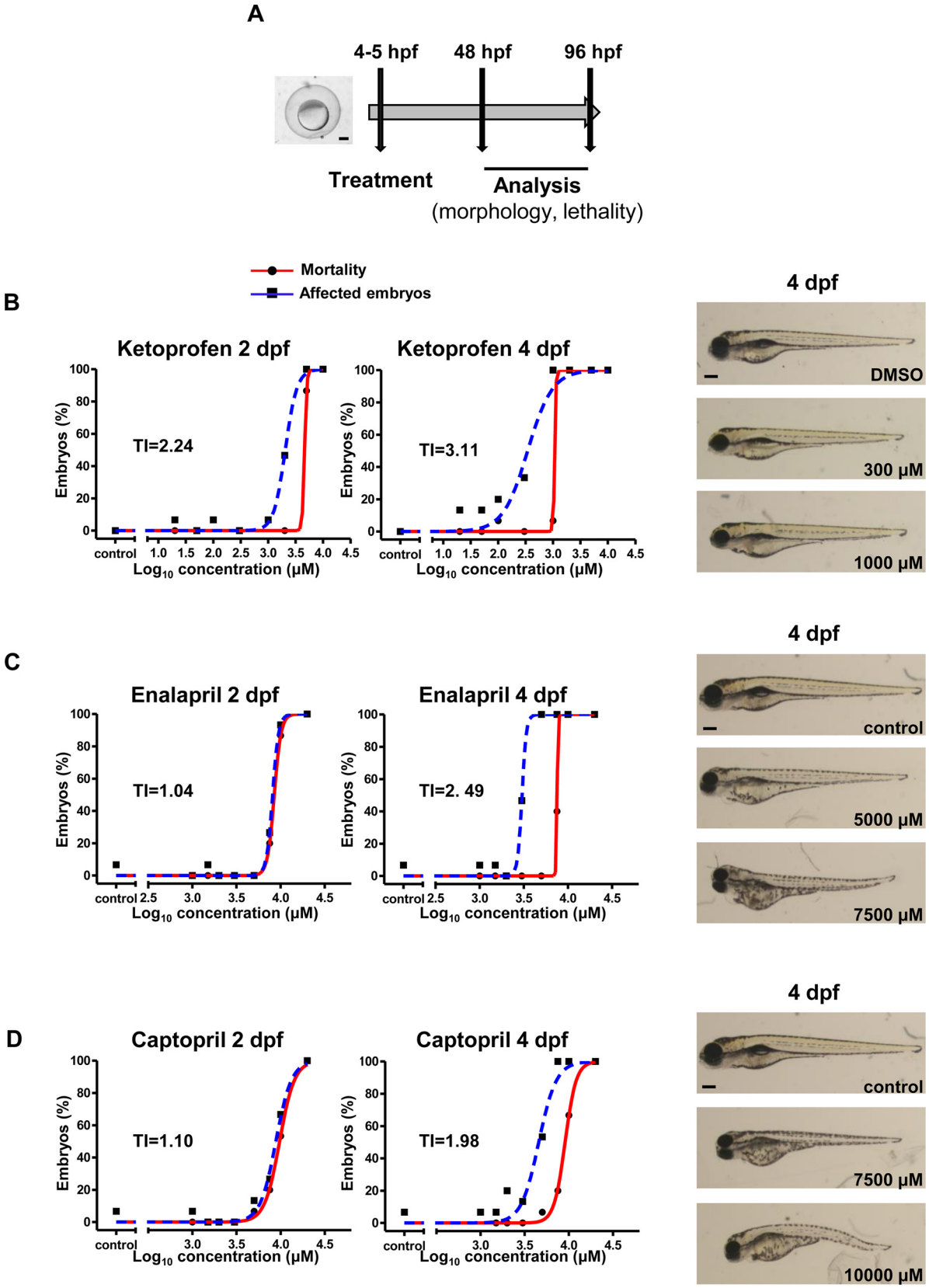

Fig. 3. Developmental toxicity assay of Ketoprofen, Enalapril maleate salt and Captopril. A) Schematic depicting a summary of the experimental design. B, C, D) Results obtained for Ketoprofen (B) and for the ACE inhibitors Enalapril maleate salt (C) and Captopril (D). Sigmoidal dose-response curves were calculated at 2 and 4 dpf for the percentage of affected and dead embryos. TI values (LC50/EC50) are shown. Representative bright field pictures of 4 dpf embryos at the concentrations indicated are also shown. Scale bar, 200 μM in all pictures.

influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2020.03.010.

References

[1] EPA, Guidelines for reproductive toxicity risk assessment, Fed. Regist. 61 (1996) 56274–56322 https://www.epa.gov/sites/production/files/2014-11/documents/guidelines_repro_toxicity.pdf.

[2] A. Seiler, P. Author, K. Fischer, U. Gruber, K. Hayess, J. Kaltenhäuser, M. Oelgeschläger, D. Sittner, B. Slawik, J. Tharmann, M. Liebsch, H. Spielmann, A. Luch, The use of embryonic stem cells for developmental toxicity testing, Reprod. Toxicol. (2009) 141–142.

[3] L. Yang, J.R. Kemadjou, C. Zinsmeister, M. Bauer, J. Legradi, F. Müller, M. Pankratz, J. Jäkel, U. Strähle, Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo, Genome Biol. 8 (2007) 1–17, https://doi.org/10.1186/gb-2007-8-10-r227.

[4] C. Rovida, T. Hartung, Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals – a report by the transatlantic think tank for toxicology, ALTEX 26 (2009) 187–208, https://doi.org/10.1080/10641197909379805.

[5] I.W.T. Selderslaghs, R. Blust, H.E. Witters, Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds, Reprod. Toxicol. 33 (2012) 142–154, https://doi.org/10.1016/j.reprotox.2011.08.003.

[6] S.A.B. Hermsen, T.E. Pronk, E.J. van den Brandhof, L.T.M. van der Ven, A.H. Piersma, Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation, Toxicol. Appl. Pharmacol. 272 (2013) 161–171, https://doi.org/10.1016/j.taap.2013.05.037.

[7] M.C. Celander, J.V. Goldstone, N.D. Denslow, T. Iguchi, P. Kille, R.D. Meyerhoff, B.A. Smith, T.H. Hutchinson, J.R. Wheeler, Species extrapolation for the 21st century, Environ. Toxicol. Chem. 30 (2011) 52–63, https://doi.org/10.1002/etc.382.

[8] S. Schneider, T. Hofmann, S. Stinchcombe, M.C.R. Moreno, I. Fegert, V. Strauss, S. Gröters, E. Fabian, J. Thiaener, K.C. Fussell, B. Van Ravenzwaay, Species differences in developmental toxicity of epoxiconazole and its relevance to humans, Birth Defects Res. Part B - Dev. Reprod. Toxicol. 98 (2013) 230–246, https://doi.org/10.1002/bdrb.21058.

[9] K. Van der Jagt, S. Munn, J. Torslov, J. de Bruijn, Alternative Approaches Can Reduce the Use of Test Animals Under REACH, (2004).

[10] K.E. Tollefsen, S. Scholz, M.T. Cronin, S.W. Edwards, J. de Knecht, K. Crofton, N. Garcia-Reyero, T. Hartung, A. Worth, G. Patlewicz, Applying adverse outcome pathways (AOPs) to support integrated approaches to testing and assessment (IATA), Regul. Toxicol. Pharmacol. 70 (2014) 629–640, https://doi.org/10.1016/j.yrtph.2014.09.009.

[11] A.R. Wheeler, US EPA Directive to Prioritize Efforts to Reduce Animal Testing, (2019), pp. 1–3.

[12] S.J. Enoch, M.T.D. Cronin, J.C. Madden, M. Hewitt, Formation of structural categories to allow for read-across for teratogenicity, QSAR Comb. Sci. 28 (2009) 696–708, https://doi.org/10.1002/qsar.200960011.

[13] N.S. Sipes, M.T. Martin, D.M. Reif, N.C. Kleinstreuer, R.S. Judson, A.V. Singh, K.J. Chandler, D.J. Dix, R.J. Kavlock, T.B. Knudsen, Predictive models of prenatal developmental toxicity from toxcast high-throughput screening data, Toxicol. Sci. 124 (2011) 109–127, https://doi.org/10.1093/toxsci/kfr220.

[14] N.C. Baker, N.S. Sipes, J. Franzosa, D.G. Belair, B.D. Abbott, R.S. Judson, T.B. Knudsen, Characterizing cleft palate toxicants using ToxCast data, chemical structure, and the biomedical literature, Birth Defects Res. 112 (2020) 19–39, https://doi.org/10.1002/bdr2.1581.

[15] A. Cassano, A. Manganaro, T. Martin, D. Young, N. Piclin, M. Pintore, D. Bigoni, E. Benfenati, CAESAR models for developmental toxicity, Chem. Cent. J. 4 (2010) 1–11, https://doi.org/10.1186/1752-153X-4-S1-S4.

[16] M. Ghorbanzadeh, J. Zhang, P.L. Andersson, Binary classification model to predict developmental toxicity of industrial chemicals in zebrafish, J. Chemom. 30 (2016) 298–307, https://doi.org/10.1002/cem.2791.

[17] M. Marzo, S. Kulkarni, A. Manganaro, A. Roncaglioni, S. Wu, T.S. Barton-Maclaren, C. Lester, E. Benfenati, Integrating in silico models to enhance predictivity for developmental toxicity, Toxicology 370 (2016) 127–137, https://doi.org/10.1016/j.tox.2016.09.015.

[18] A.D. Schachter, I.S. Kohane, Drug target-gene signatures that predict teratogenicity are enriched for developmentally related genes, Reprod. Toxicol. 31 (2011) 562–569, https://doi.org/10.1016/j.reprotox.2010.11.008.

[19] H. Zhang, J. Mao, H.Z. Qi, L. Ding, In silico prediction of drug-induced developmental toxicity by using machine learning approaches, Mol. Divers. (2019), https://doi.org/10.1007/s11030-019-09991-y.

[20] N.B. Sussman, V.C. Arena, S. Yu, S. Mazumdar, B.P. Thampatty, Decision tree SAR models for developmental toxicity based on an FDA/TERIS database, SAR QSAR Environ. Res. 14 (2003) 83–96, https://doi.org/10.1080/1062936031000073126.

[21] H. Zhang, J.X. Ren, Y.L. Kang, P. Bo, J.Y. Liang, L. Ding, W.B. Kong, J. Zhang, Development of novel in silico model for developmental toxicity assessment by using naïve Bayes classifier method, Reprod. Toxicol. 71 (2017) 8–15, https://doi.org/10.1016/j.reprotox.2017.04.005.

[22] N. Basant, S. Gupta, K.P. Singh, QSAR modeling for predicting reproductive toxicity of chemicals in rats for regulatory purposes, Toxicol. Res. (Camb) 5 (2016) 1029–1038, https://doi.org/10.1039/c6tx00083e.

[23] W. Muster, A. Breidenbach, H. Fischer, S. Kirchner, L. Müller, A. Pähler, Computational toxicology in drug development, Drug Discov. Today 13 (2008) 303–310, https://doi.org/10.1016/j.drudis.2007.12.007.

[24] G.T. Ankley, R.S. Bennett, R.J. Erickson, D.J. Hoff, M.W. Hornung, R.D. Johnson, D.R. Mount, J.W. Nichols, C.L. Russom, P.K. Schmieder, J.A. Serrrano, J.E. Tietge, D.L. Villeneuve, Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment, Environ. Toxicol. Chem. 29 (2010) 730–741, https://doi.org/10.1002/etc.34.

[25] M. Vinken, The adverse outcome pathway concept: a pragmatic tool in toxicology, Toxicology 312 (2013) 158–165, https://doi.org/10.1016/j.tox.2013.08.011.

[26] M. Vinken, D. Knapen, L. Vergauwen, J.G. Hengstler, M. Angrish, M. Whelan, Adverse outcome pathways: a concise introduction for toxicologists, Arch. Toxicol. 91 (2017) 3697–3707, https://doi.org/10.1007/s00204-017-2020-z.

[27] E.C.M. Tonk, J.L.A. Pennings, A.H. Piersma, An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis, Reprod. Toxicol. 55 (2015) 104–113, https://doi.org/10.1016/j.reprotox.2014.10.008.

[28] A. Bal-Price, M.E. (Bette) Meek, Adverse outcome pathways: application to enhance mechanistic understanding of neurotoxicity, Pharmacol. Ther. 179 (2017) 84–95, https://doi.org/10.1016/j.pharmthera.2017.05.006.

[29] Aopwiki, (n.d.). https://aopwiki.org/ (Accessed 14 February 2020).

[30] M.M.H.J. van Gelder, I.A.L.M. van Rooij, R.K. Miller, G.A. Zielhuis, L.T.W. de Jong-van den Berg, N. Roeleveld, Teratogenic mechanisms of medical drugs, Hum. Reprod. Update 16 (2010) 378–394, https://doi.org/10.1093/humupd/dmp052.

[31] G.G. Briggs, R.K. Freeman, S.J. Yaffe, Drugs in Pregnancy and Lactation: a Reference Guide to Fetal and Neonatal Risk, Lippincott Williams & Wilkins, 2012.

[32] Federal Register, Content and format of labeling for human prescription drug and biological products; requirements for pregnancy and lactation labeling. Final rule, Fed. Regist. 79 (2014) 72063–72103, https://doi.org/10.1017/CBO9781107415324.004.

[33] A. Addis, S. Sharabi, M. Bonati, Risk classification systems for drug use during pregnancy, Drug Saf. 23 (2000) 245–253, https://doi.org/10.2165/00002018-200023030-00006.

[34] M. Hewitt, C.M. Ellison, S.J. Enoch, J.C. Madden, M.T.D. Cronin, Integrating (Q) SAR models, expert systems and read-across approaches for the prediction of developmental toxicity, Reprod. Toxicol. 30 (2010) 147–160, https://doi.org/10.1016/j.reprotox.2009.12.003.

[35] N.R. Council, Review of EPA's Integrated Risk Information System (IRIS) Process, National Academies Press, 2014.

[36] OECD, OECD 414 Guideline for Testing of Chemicals - Prenatal Developmental Toxicity Study, (2018).

[37] M.T. Martin, T.B. Knudsen, D.M. Reif, K.A. Houck, R.S. Judson, R.J. Kavlock, D.J. Dix, Predictive model of rat reproductive toxicity from ToxCast high throughput Screening1, Biol. Reprod. 85 (2011) 327–339, https://doi.org/10.1095/biolreprod.111.090977.

[38] P. Wexler, TOXNET: an evolving web resource for toxicology and environmental health information, Toxicology 157 (2001) 3–10, https://doi.org/10.1016/S0300-483X(00)00337-1.

[39] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Evaluating the risks of drug exposure in human pregnancies, Rev. Guid. (2005) 20852–21448 https://www.fda.gov/downloads/Drugs/.../Guidances/ucm071645.pdf%5Cnhttp://www.fda.gov/cder/guidance/index.htm.

[40] I. Chahoud, J. Buschmann, R. Clark, A. Druga, H. Falke, A. Faqi, E. Hansen, B. Heinrich-Hirsch, J. Hellwig, W. Lingk, M. Parkinson, F.J.R. Paumgartten, R. Pfeil, T. Platzek, A.R. Scialli, J. Seed, R. Stahlmann, B. Ulbrich, X. Wu, M. Yasuda, M. Younes, R. Solecki, Classification terms in developmental toxicology: need for harmonisation, Reprod. Toxicol. 13 (1999) 77–82, https://doi.org/10.1016/s0890-6238(98)00060-4.

[41] S.L. Makris, H.M. Solomon, R. Clark, K. Shiota, S. Barbellion, J. Buschmann, M. Ema, M. Fujiwara, K. Grote, K.P. Hazelden, K.W. Hew, M. Horimoto, Y. Ooshima, M. Parkinson, L.D. Wise, Terminology of developmental abnormalities in common laboratory mammals (version 2), Reprod. Toxicol. 49 (2009) 487, https://doi.org/10.1016/j.reprotox.2012.06.005.

[42] R. Solecki, M. Rauch, A. Gall, J. Buschmann, R. Clark, A. Fuchs, H. Kan, V. Heinrich, R. Kellner, T.B. Knudsen, W. Li, S.L. Makris, Y. Ooshima, F. Paumgartten, A.H. Piersma, G. Schönfelder, M. Oelgeschläger, C. Schaefer, K. Shiota, B. Ulbrich, X. Ding, I. Chahoud, Continuing harmonization of terminology and innovations for methodologies in developmental toxicology: report of the 8th Berlin Workshop on Developmental Toxicity, 14–16 May 2014, Reprod. Toxicol. 57 (2015) 140–146, https://doi.org/10.1016/j.reprotox.2015.06.046.

[43] S. Kim, J. Chen, T. Cheng, A. Gindulyte, J. He, S. He, Q. Li, B.A. Shoemaker, P.A. Thiessen, B. Yu, L. Zaslavsky, J. Zhang, E.E. Bolton, PubChem 2019 update: improved access to chemical data, Nucleic Acids Res. 47 (2018) 1–8, https://doi.org/10.1093/nar/gky1033.

[44] V.D. Hähnke, S. Kim, E.E. Bolton, PubChem chemical structure standardization, J. Cheminform. 10 (2018) 36, https://doi.org/10.1186/s13321-018-0293-8.

[45] A. Gaulton, A. Hersey, M.L. Nowotka, A. Patricia Bento, J. Chambers, D. Mendez, P. Mutowo, F. Atkinson, L.J. Bellis, E. Cibrian-Uhalte, M. Davies, N. Dedman, A. Karlsson, M.P. Magarinos, J.P. Overington, G. Papadatos, I. Smit, A.R. Leach, The ChEMBL database in 2017, Nucleic Acids Res. 45 (2017) D945–D954, https://doi.org/10.1093/nar/gkw1074.

[46] K. Mansouri, C.M. Grulke, A.M. Richard, R.S. Judson, A.J. Williams, An automated curation procedure for addressing chemical errors and inconsistencies in public datasets used in QSAR modelling, SAR QSAR Environ. Res. 27 (2016) 939–965, https://doi.org/10.1080/1062936X.2016.1253611.
[47] C.M. Grulke, A.J. Williams, I. Thillanadarajah, A.M. Richard, EPA's DSSTox database: history of development of a curated chemistry resource supporting computational toxicology research, Comput. Toxicol. 12 (2019) 100096, , https://doi.org/10.1016/j.comtox.2019.100096.
[48] D.S. Wishart, Y.D. Feunang, A.C. Guo, E.J. Lo, A. Marcu, J.R. Grant, T. Sajed, D. Johnson, C. Li, Z. Sayeeda, N. Assempour, I. Iynkkaran, Y. Liu, A. MacIejewski, N. Gale, A. Wilson, L. Chin, R. Cummings, Di. Le, A. Pon, C. Knox, M. Wilson, DrugBank 5.0: a major update to the DrugBank database for 2018, Nucleic Acids Res. 46 (2018) D1074–D1082, https://doi.org/10.1093/nar/gkx1037.
[49] A. Bateman, M.J. Martin, C. O'Donovan, M. Magrane, E. Alpi, R. Antunes, B. Bely, M. Bingley, C. Bonilla, R. Britto, B. Bursteinas, H. Bye-AJee, A. Cowley, A. Da Silva, M. De Giorgi, T. Dogan, F. Fazzini, L.G. Castro, L. Figueira, P. Garmiri, G. Georghiou, D. Gonzalez, E. Hatton-Ellis, W. Li, W. Liu, R. Lopez, J. Luo, Y. Lussi, A. MacDougall, A. Nightingale, B. Palka, K. Pichler, D. Poggioli, S. Pundir, L. Pureza, G. Qi, S. Rosanoff, R. Saidi, T. Sawford, A. Shypitsyna, E. Speretta, E. Turner, N. Tyagi, V. Volynkin, T. Wardell, K. Warner, X. Watkins, R. Zaru, H. Zellner, I. Xenarios, L. Bougueleret, A. Bridge, S. Poux, N. Redaschi, L. Aimo, G. ArgoudPuy, A. Auchincloss, K. Axelsen, P. Bansal, D. Baratin, M.C. Blatter, B. Boeckmann, J. Bolleman, E. Boutet, L. Breuza, C. Casal-Casas, E. De Castro, E. Coudert, B. Cuche, M. Doche, D. Dornevil, S. Duvaud, A. Estreicher, L. Famiglietti, M. Feuermann, E. Gasteiger, S. Gehant, V. Gerritsen, A. Gos, N. Gruaz-Gumowski, U. Hinz, C. Hulo, F. Jungo, G. Keller, V. Lara, P. Lemercier, D. Lieberherr, T. Lombardot, X. Martin, P. Masson, A. Morgat, T. Neto, N. Nouspikel, S. Paesano, I. Pedruzzi, S. Pilbout, M. Pozzato, M. Pruess, C. Rivoire, B. Roechert, M. Schneider, C. Sigrist, K. Sonesson, S. Staehli, A. Stutz, S. Sundaram, M. Tognolli, L. Verbregue, A.L. Veuthey, C.H. Wu, C.N. Arighi, L. Arminski, C. Chen, Y. Chen, J.S. Garavelli, H. Huang, K. Laiho, P. McGarvey, D.A. Natale, K. Ross, C.R. Vinayaka, Q. Wang, Y. Wang, L.S. Yeh, J. Zhang, UniProt: the universal protein knowledgebase, Nucleic Acids Res. 45 (2017) D158–D169, https://doi.org/10.1093/nar/gkw1099.
[50] D.A. Biddle, S.B. Morris, Using Lancaster's mid-P correction to the Fisher's exact test for adverse impact analyses, J. Appl. Psychol. 96 (2011) 956–965, https://doi.org/10.1037/a0024223.
[51] H. Mi, X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, P.D. Thomas, PANTHER version 11: expanded annotation data from gene ontology and reactome pathways, and data analysis tool enhancements, Nucleic Acids Res. 45 (2017) D183–D189, https://doi.org/10.1093/nar/gkw1138.
[52] D.R. Zerbino, P. Achuthan, W. Akanni, M.R. Amode, D. Barrell, J. Bhai, K. Billis, C. Cummins, A. Gall, C.G. Girón, L. Gil, L. Gordon, L. Haggerty, E. Haskell, T. Hourlier, O.G. Izuogu, S.H. Janacek, T. Juettemann, J.K. To, M.R. Laird, I. Lavidas, Z. Liu, J.E. Loveland, T. Maurel, W. McLaren, B. Moore, J. Mudge, D.N. Murphy, V. Newman, M. Nuhn, D. Ogeh, C.K. Ong, A. Parker, M. Patricio, H.S. Riat, H. Schuilenburg, D. Sheppard, H. Sparrow, K. Taylor, A. Thormann, A. Vullo, B. Walts, A. Zadissa, A. Frankish, S.E. Hunt, M. Kostadima, N. Langridge, F.J. Martin, M. Muffato, E. Perry, M. Ruffier, D.M. Staines, S.J. Trevanion, B.L. Aken, F. Cunningham, A. Yates, P. Flicek, Ensembl 2018, Nucleic Acids Res. 46 (2018) D754–D761, https://doi.org/10.1093/nar/gkx1098.
[53] C.A. LaLone, D.L. Villeneuve, D. Lyons, H.W. Helgen, S.L. Robinson, J.A. Swintek, T.W. Saari, G.T. Ankley, Sequence alignment to predict across species susceptibility (seqapass): a web-based tool for addressing the challenges of cross-species extrapolation of chemical toxicity, Toxicol. Sci. 153 (2016) 228–245, https://doi.org/10.1093/toxsci/kfw119.
[54] C.K. Kaufman, R.M. White, L. Zon, Chemical genetic screening in the zebrafish embryo, Nat. Protoc. 4 (2009) 1422, https://doi.org/10.1038/nprot.2009.144.
[55] A. Letamendia, C. Quevedo, I. Ibarbia, J.M. Virto, O. Holgado, M. Diez, J.C. Izpisua Belmonte, C. Callol-Massot, Development and validation of an automated high-throughput system for zebrafish in vivo screenings, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0036690.
[56] M. Beekhuijzen, C. de Koning, M.E. Flores-Guillén, S. de Vries-Buitenweg, M. Tobor-Kaplon, B. van de Waart, H. Emmen, From cutting edge to guideline: a first step in harmonization of the zebrafish embryotoxicity test (ZET) by describing the most optimal test conditions and morphology scoring system, Reprod. Toxicol. 56 (2015) 64–76, https://doi.org/10.1016/j.reprotox.2015.06.050.
[57] M.M.H.J. van Gelder, I.A.L.M. van Rooij, L.T.W. de Jong-van den Berg, N. Roeleveld, Teratogenic mechanisms associated with prenatal medication exposure, Therapie 69 (2014) 13–24, https://doi.org/10.2515/therapie/2014003.
[58] F. Wang, Y. Wu, M.J. Quon, X. Li, P. Yang, ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development, Am. J. Physiol. - Endocrinol. Metab. 309 (2015) E487–E499, https://doi.org/10.1152/ajpendo.00121.2015.
[59] V.H. Ferm, C. Willhite, L. Kilham, Teratogenic effects of ribavirin on hamster and rat embryos, Teratology 17 (1978) 93–101, https://doi.org/10.1002/tera.1420170117.
[60] R. Jelínek, A. Pavlík, M. Peterka, Glucocorticoid receptor-mediated teratogenesis in the chick embryo, Teratog. Carcinog. Mutagen. 3 (1983) 1–7, https://doi.org/10.1002/1520-6866(1990)3:1<1::aid-tcm1770030102>3.0.co;2-q.
[61] S. Hirai, K. Hotta, Y. Kubo, A. Nishino, S. Okabe, Y. Okamura, H. Okado, AMPA glutamate receptors are required for sensory-organ formation and morphogenesis in the basal chordate, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 3939–3944, https://doi.org/10.1073/pnas.1612943114.
[62] B.T. Green, S.T. Lee, K.E. Panter, K.D. Welch, D. Cook, J.A. Pfister, W.R. Kem, Actions of piperidine alkaloid teratogens at fetal nicotinic acetylcholine receptors, Neurotoxicol. Teratol. 32 (2010) 383–390, https://doi.org/10.1016/j.ntt.2010.01.011.
[63] M. Isidori, C. Piscitelli, C. Russo, M. Smutná, L. Bláha, Teratogenic effects of five anticancer drugs on Xenopus laevis embryos, Ecotoxicol. Environ. Saf. 133 (2016) 90–96, https://doi.org/10.1016/j.ecoenv.2016.06.044.
[64] E. Lorenzi, M. Simonelli, A. Santoro, Infertility risk and teratogenicity of molecularly targeted anticancer therapy: a challenging issue, Crit. Rev. Oncol. Hematol. 107 (2016) 1–13, https://doi.org/10.1016/j.critrevonc.2016.08.005.
[65] S. Weigt, N. Huebler, R. Strecker, T. Braunbeck, T.H. Broschard, Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (Danio rerio) embryos, Reprod. Toxicol. 33 (2012) 133–141, https://doi.org/10.1016/j.reprotox.2011.07.001.
[66] F. Sullivan, P.R. McElhatton, A comparison of the teratogenic activity of the anti-epileptic drugs carbamazepine, clonazepam, ethosuximide, phenobarbital, phenytoin, and primidone in mice, Toxicol. Appl. Pharmacol. 378 (1977) 365–378.
[67] F. Marchetti, A. Massarotti, C.L. Yauk, F. Pacchierotti, A. Russo, The adverse outcome pathway (AOP) for chemical binding to tubulin Inoocytes leading to aneuploid offspring, Environ. Mol. Mutagen. 113 (2016) 57–87, https://doi.org/10.1002/em.
[68] L. Truong, D.M. Reif, L.S. Mary, M.C. Geier, H.D. Truong, R.L. Tanguay, Multidimensional in vivo hazard assessment using zebrafish, Toxicol. Sci. 137 (2014) 212–233, https://doi.org/10.1093/toxsci/kft235.
[69] S. Padilla, D. Corum, B. Padnos, D.L. Hunter, A. Beam, K.A. Houck, N. Sipes, N. Kleinstreuer, T. Knudsen, D.J. Dix, D.M. Reif, Zebrafish developmental screening of the ToxCast™ phase I chemical library, Reprod. Toxicol. 33 (2012) 174–187, https://doi.org/10.1016/j.reprotox.2011.10.018.
[70] S. Glaberman, S. Padilla, M.G. Barron, Evaluating the zebrafish embryo toxicity test for pesticide hazard screening, Environ. Toxicol. Chem. 36 (2017) 1221–1226, https://doi.org/10.1002/etc.3641.
[71] L.K. Rathnayake, S.H. Northrup, Structure and mode of action of organophosphate pesticides: a computational study, Comput. Theor. Chem. 1088 (2016) 9–23.
[72] N.C. for B.I. Bethesda (MD): National Library of Medicine (US), Gene, (2004) (Accessed 18 September 2019), https://www.ncbi.nlm.nih.gov/gene/.
[73] W. Yuan, Z. Zhong, A. Wetterholm, B. Samuelsson, Probing the inhibition of leukotriene A4 hydrolase based on its aminopeptidase activity, Bioorg. Med. Chem. Lett. 1 (1991) 551–556.
[74] W.Y. Lo, J.M. Friedman, Teratogenicity of recently introduced medications in human pregnancy, Obstet. Gynecol. 100 (2002) 465–473, https://doi.org/10.1016/S0029-7844(02)02122-1.
[75] A. Walfisch, A. Al-maawali, M.E. Moretti, C. Nickel, G. Koren, Teratogenicity of angiotensin converting enzyme inhibitors or receptor blockers, J. Obstet. Gynaecol. (Lahore) 31 (2011) 465–472, https://doi.org/10.3109/01443615.2011.579197.
[76] S.S. Kitambi, K.J. McCulloch, R.T. Peterson, J.J. Malicki, Small molecule screen for compounds that affect vascular development in the zebrafish retina, Mech. Dev. 126 (2009) 464–477, https://doi.org/10.1016/j.mod.2009.01.002.
[77] J.H. Westhoff, S. Giselbrecht, M. Schmidts, S. Schindler, P.L. Beales, B. Tönshoff, U. Liebel, J. Gehrig, Development of an automated imaging pipeline for the analysis of the zebrafish larval kidney, PLoS One 8 (2013) 1–13, https://doi.org/10.1371/journal.pone.0082137.
[78] C. de Koning, M. Beekhuijzen, M. Tobor-Kapłon, S. de Vries-Buitenweg, D. Schoutsen, N. Leeijen, B. van de Waart, H. Emmen, Visualizing compound distribution during zebrafish embryo development: the effects of lipophilicity and DMSO, Birth Defects Res. Part B - Dev. Reprod. Toxicol. 104 (2015) 253–272, https://doi.org/10.1002/bdrb.21166.
[79] E.J. Perkins, P. Antczak, L. Burgoon, F. Falciani, N. Garcia-Reyero, S. Gutsell, G. Hodges, A. Kienzler, D. Knapen, M. McBride, C. Willett, Adverse outcome pathways for regulatory applications: examination of four case studies with different degrees of completeness and scientific confidence, Toxicol. Sci. 148 (2015) 14–25, https://doi.org/10.1093/toxsci/kfv181.