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基于专家判断的生殖发育毒性预测工具

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摘要: 生殖发育毒性终点涵盖了生物体整个发育阶段或生殖周期的毒性评估。用来预测生殖发育毒性的计算工具既可以为这个复杂毒性终点的评估提供方法,同时为中国筛选新污染物和管理新化学品提供有价值的决策支持。在我国,目前发表的生殖发育毒性预测模型比较少,但有很多相关的研发工作在进行中。2013 年,WU 等发表了基于专家判断和规则的生殖发育毒性预测决策树。该决策树运用已知的生殖发育毒性物质的相关信息,来预测目标化学物质的生殖发育毒性。在此过程中,准确的生殖发育毒性数据解读是构建和扩展决策树的重要基础。这篇文章我们通过案例研究,总结并解读了 4 个化学物质的生殖发育毒性数据,包括 8-羟基喹啉、3,5,6-三氯-2-吡啶醇、噻虫嗪、吡虫啉,从而得出它们的生殖发育毒性结论用来扩展已有的生殖发育决策树。我们首先从生态环境部固体废物与化学品管理技术中心的数据库中筛选出来若干化学品。通过核心结构特征、功能团、受体结合性质、代谢和可能的作用机制的分析,我们对这 4 个化学结构进行了初步分组。随后,通过收集化学信息,搜索、整合和解读生殖发育毒性数据,得出生殖发育毒性的结论。最后,结合化学结构,生殖发育毒性特征,以及生物活性,这 4 个化学物质分别被归入生殖发育毒性决策树现有的化学类别,或建立了一个新的类别。其中,8-羟基喹啉会影响发情周期、性器官质量和胚胎发育,而 3,5,6-三氯-2-吡啶醇则导致中枢神经系统畸形,这 2 个化学物质被纳入了决策树现有的子类别 8e(含多卤素和硝基的芳香化合物)。噻虫嗪导致难产和胎儿骨骼畸形,吡虫啉会干扰内分泌系统和男性生育能力,它们(含有 2-氯-5-甲基吡啶取代的咪唑烷环)有望被划分为新烟碱类农药类别。当前的研究阐述了一个透明的数据整理流程,用于解读生殖发育毒性数据,在有足够的相关结构及生殖发育毒性数据的情况下,通过在每个类别或子类别的适用范围内迭代地添加化学物质以达到扩展该决策树的目的。这个决策树有望作为我国新污染物筛选和新化学品评估的工具。

关键词: 生殖发育毒性;决策树;预测工具;专家判断;新化学品管理
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An Expert Judgment-based Prediction Tool for Developmental and Reproductive Toxicity (DART)

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Abstract: Developmental and reproductive toxicity (DART) endpoint entails a toxicological assessment of all developmental stages and reproductive cycles of an organism. *In silico* tools to predict DART will provide a method to assess this complex toxicity endpoint and will be valuable for screening emerging pollutants as well as for managing new chemicals in China. Currently, there are few published DART prediction models in China, but many related research and development projects are in progress. In 2013, WU et al. published an expert rule-based DART decision tree (DT). This DT relies on known chemical structures linked to DART to forecast DART potential of a given chemical. Within this procedure, an accurate DART data interpretation is the foundation of building and expanding the DT. This paper excerpted case studies demonstrating DART data curation and interpretation of four chemicals (including 8-hydroxyquinoline, 3,5,6-trichloro-2-pyridinol, thiacloprid, and imidacloprid) to expand the existing DART DT. Chemicals were first selected from the database of Solid Waste and Chemicals Management Center, Ministry of Ecology and Environment (MEESCC) in China. The structures of these 4 chemicals were analyzed and preliminarily grouped by chemists based on core structural features, functional groups, receptor binding property, metabolism, and possible mode of actions. Then, the DART conclusion was derived by collecting chemical information, searching, integrating, and interpreting DART data by the toxicologists. Finally, these chemicals were classified into either an existing category or a new category via integrating their chemical features, DART conclusions, and biological properties. The results showed that 8-hydroxyquinoline impacted estrous cyclicity, sexual organ weights, and embryonal development, and 3,5,6-trichloro-2-pyridinol caused central nervous system (CNS) malformations, which were added to an existing subcategory 8e (aromatic compounds with multi-halogen and nitro groups) of the DT. Thiacloprid caused dystocia and fetal skeletal malformation, and imidacloprid disrupted the endocrine system and male fertility. They both contain 2-chloro-5-methylpyridine substituted imidazolidine cyclic ring, which were expected to create a new category of neonicotinoids. The current work delineates a transparent process of curating toxicological data for the purpose of DART data interpretation. In the presence of sufficient related structures and DART data, the DT can be expanded by iteratively adding chemicals within the applicable domain of each category or subcategory. This DT can potentially serve as a tool for screening emerging pollutants and assessing new chemicals in China.

Keywords: developmental and reproductive toxicity; decision tree; prediction tool; expert judgment; new chemical management

0 Introduction

Developmental and reproductive toxicity (DART) occurs through many different mechanisms and involves a number of different target sites and developmental stages. Assessment of DART plays a crucial role in ensuring the safe use of new chemicals introduced into the market. DART testing uses a significant number of animals, making it resource-intensive in animal usage and cost. ROVIDA and HARTUNG made a conservative estimation that approximately 54 million vertebrate animals are needed for testing purposes under the European REACH legislation, with approximately 90% of these animals being utilized specifically for DART experiments[1]. As a result of the 3R principle (Replacement, Reduction, Refinement) and EU animal testing ban for cosmetic products and ingredients, the development of non-animal alternatives such as an *in silico* DART prediction model for supporting DART assessments holds substantial benefit for reducing animal usage while maintaining the safe use of new chemicals.

Computational and expert-rule-based models to predict DART have been developed. These models utilize various computational and experimental approaches to

evaluate the toxicity of chemicals, thereby aiding in decision-making regarding their registration and application. The Guideline for Environmental Management Registration of New Chemicals of China points out that in special cases where animal testing is not feasible, the registrants can submit non-test data from quantitative structure-activity relationship (QSAR) or read-across analyses. When submitting reproductive/developmental toxicity data for persistent or bio-accumulative new chemicals, an option is to submit data on developmental toxicity covering pregnancy plus a minimum of two types of DART prediction reports based on scientific and reasonable QSAR models. A domestic DART prediction model in China is under development. In 2022, China Emerging Pollutant Management Action Plan emphasized building a platform for computational toxicology and exposure prediction of chemicals. In line with the context of Action Plan and purpose for new chemical registration, a joint effort was made by P&G and Solid Waste and Chemicals Management Center of Ministry of Ecology and Environment (MEESCC) to explore an applicable DART prediction model in China.

Currently available *in silico* tools to predict DART endpoints include either statistical based models (e.g. US EPA T.E.S.T, VEGA CAESAR, Leadscope Model Applier, CASE Ultra) or empirical and rule-based decision tree (DT) models (e.g. VEGA PG, Procter & Gamble (P&G) DART DT Pipeline Pilot). The following paragraph provides a brief introduction and comparison of various *in silico* tools[2-3]. The US EPA Toxicity Estimation Software Tool (T.E.S.T) primarily utilizes QSAR models to predict DART. The accuracy depends on the availability and quality of training data. If data for specific chemicals or endpoints are missing, the predictive outcome may be compromised[4]. Virtual models for property Evaluation of chemicals within a Global Architecture (VEGA) Computer-Assisted Evaluation of industrial chemical Substances According to Regulations (CAESAR) is a QSAR-based computational model, which is available at the VEGA platform. The validation shows that this model has a good sensitivity (95%) but a relatively low specificity (59%)[5]. Leadscope Model Applier contains QSAR models to predict DART. The training set of the Leadscope model splits effects on Repro Female Rat (RFR) and Repro Male Rat (RMR). The RFR only comprises adverse effects of female reproductive system and fertility, but without effects of the fetus, gestation, or lactation. RMR only focuses on adverse effects of the reproductive system and fertility in male rats[6]. CASE Ultra platform is developed by Multi-CASE, classifying different DART endpoints based on *in vivo* animal data of mice, rats, and rabbits. The models in CASE Ultra are established according to statistical analysis on structural alerts, which are not validated through in-depth review of DART effects[7]. The DART DT was developed by P&G and automated in Pipeline Pilot software (Biovia version 2018). A total of 716 chemicals used to build the DT were analyzed according to their core structures (such as acyclic alkyl chain, cyclic/heterocyclic rings, aromatic/heteroaromatic rings, etc.), key functional groups (such as halocarbons, acids, alcohols, aldehydes, esters, amides, amines, urea, etc.), receptor binding activity (i.e. category 2, 3, 4, 5, 6 in the original DART DT), mode of actions (MOAs) (such as adverse outcome pathways), metabolites with known toxicological outcomes. The toxicological data sets of these chemicals were thoroughly reviewed by the toxicologists to confirm that the chemicals were correctly identified as exhibiting developmental and/or reproductive toxicity. In cases where clear evidence suggested that a category can be split or the chemicals were mis-classified, the rules used to define each category and subcategory were revised by the chemists. The 716 chemicals were eventually divided into 25 categories and approximately 129 subcategories (Table S1) in the DART DT. This rule-based model categorized chemicals not only relying on chemical classification system based on structural features but also considering the toxicological profile and bioactivity. Each step is designed to facilitate the expansion of categories or subcategories when there're sufficient related chemicals with DART data. Previous results showed very good predicting power as 629 in 635 DART toxicants (around 98%)

were identified using the DT[8]. The DT was re-applied in the VEGA tool, which can be used as a battery of tools to predict the developmental potential of a compound[9]. The DT is flexible and can easily accommodate the addition of categories, therefore, expanding the chemical coverage of the tree. New categories may be formed when there are DART data available for three or more chemicals of the new category. This flexibility allows for a more comprehensive representation of chemical diversity and enhances the applicability and predictive power of the DT.

DART involves the assessment of adverse effects of a full developmental and/or reproductive cycle such as gamete production, mating indexes, fertilization, implantation, embryogenesis, fetal development, parturition, and postnatal development, sexual maturation, and reproductive performance of the next generation. Therefore, a delineated and comprehensive interpretation of DART endpoint data is a fundamental and essential element for an expert judgment-based data curation and for building the DART prediction model. This paper excerpts a variety of types of DART case studies across rodent and non-rodent animal species to demonstrate how we interpreted DART data based on extensive data curation. Some critical and challenging points-to-consider for DART data interpretation are illustrated and discussed, including maternal toxicity, embryo lethality, morphological effects (variation and malformation), dose-responsiveness, statistical significance, biological relevance, and historical control data.

In line with exploring the DART prediction model and standardizing the modeling process and data interpretation, this paper aims to exemplify the use if expert judgment for DART data curation and demonstrates the procedure for adding chemicals to an existing category or for creating a new category in order to expand the chemical coverage of the DT. Our strategy will have profound and far-reaching impact on the application of emerging pollutants screening and new chemical registration management in China.

1 Materials and methods

1.1 Work process

The starting chemicals obtained from the MEE-SCC database were compared to the chemical list in the original publication[8]. Duplicated chemicals were removed, leaving 213 compounds for further evaluation. These 213 chemicals were screened using the automated tree written in Pipeline Pilot (DT v1.9) and added to existing categories of the tree if they satisfied the boundary conditions of a specific category or subcategory[10]. Finally, about 80 chemicals that include similar structural scaffolds as those of existing category members (DART precedent compounds) were prioritized for DART data curation, as these may be used to extend the current categories. The DART data were collected, reviewed and qualitatively agreed upon by toxicologists using expert judgment, by which the chemicals were grouped and added to the DART DT based on weight of evidence including DART effects, structural features, MOAs, etc. A schematic diagram of the work process is shown in Fig.1.

1.2 DART data curation

In the context of toxicological assessment, data curation refers to the process of collecting, integrating, and reviewing data related to the assessment objectives[11]. The DART data curation process presented in this paper included chemical identification, literature search, study quality evaluation, data summarization and integration, and data interpretation. The chemical identification required but was not limited to the confirmation of CAS RN, name, and structure. The DART data were searched through toxicological databases and authoritative publications such as eChemPortal, EPA Dashboard, ECHA, EFSA, AICIS, RIFM, SCCS, OECD, NTP, IPCS INCHEM, JECDB, and the scientific literature. The data were summarized to capture enough details for interpretation including but not limited to study year, test material information and purity, data source and quality, experimental design, animal species and number, route of exposure, examinations and effects, and point of departure. In accordance with the recommendations outlined in several OECD test guidelines such as OECD TG 414, 415, 416, 421, 422, 443, a highest dose level is selected that produces some maternal toxicity (e.g., a 10% decrease in weight gain over the treatment period) but not so high as to

cause severe suffering or mortality[12-13]. For innocuous compounds, a limit dose of 1 000 mg·kg^{-1}·d^{-1} is used. The DART studies reviewed in this paper were typically with a top dose up to 1 000 mg·kg^{-1}·d^{-1}. The dose-responsiveness, statistical significance, biological relevance, historical control data, and fetal effects (such as post-implantation loss, resorption, fetal death, skeletal malformation) in absence of maternal effects were comprehensively interpreted. For the scenario where one chemical has multiple sets of DART studies, the final conclusion was determined by weight of evidence analysis considering the most sensitive animal species, data quality, dose selection, route of exposure, data concordance and consistency, toxicological effects, etc. Toxicologists reviewed the data to determine whether a chemical has developmental and/or reproductive toxicity and derived a quantitative conclusion about NOAEL and LOAEL.

1.3　Chemical grouping

This paper adds chemicals into existing categories of the DT or creates new categories. All chemical structures were first analyzed by Pipeline Pilot (DT v1.9) and assigned to existing categories of the DT or to new categories based on chemical core structures, functional groups, metabolism, MOAs such as adverse outcome pathways, and receptor interactions as previously described[8]. Then, the toxicological data were curated by the toxicologists resulting in chemicals defined as DART positive, negative, or inconclusive (insufficient data to draw developmental or reproductive toxicity conclusions). For chemicals with DART positive or negative conclusions the possible MOAs were inferred considering structural characteristics, receptor binding property, and generation of toxic metabolites. Finally, core structures, MOAs, toxicological profiles, biological targets of the chemicals were considered together to determine if a chemical can be added to an existing category or belongs to a new category/subcategory as long as there are three or more chemicals with similar structure and toxicity characteristics.

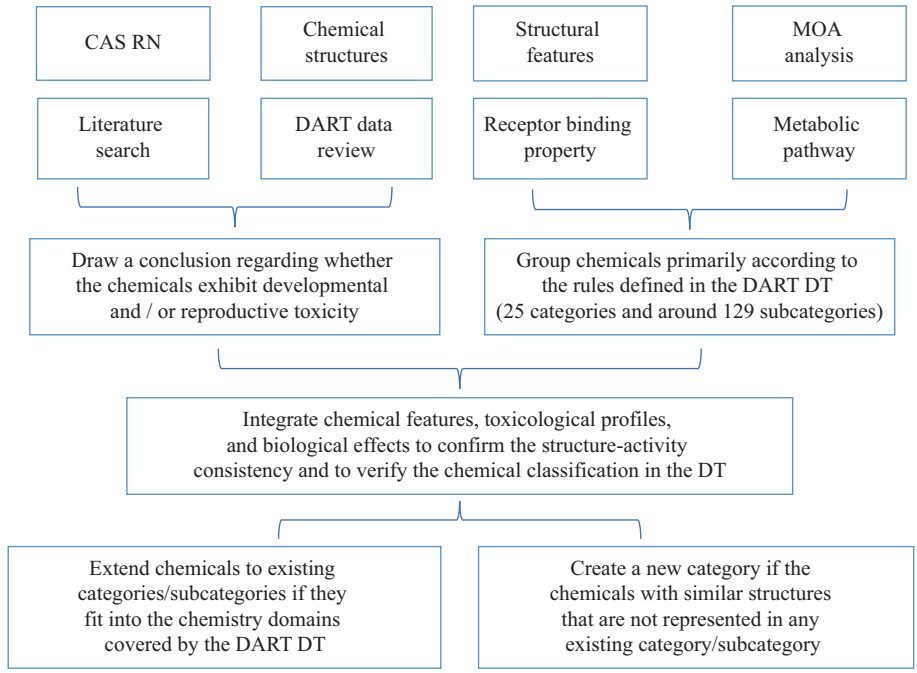


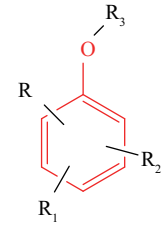
Fig. 1　DART DT expansion work process. The identity of chemicals (CAS RN and structures) was first confirmed by the chemists. Then, the chemicals were grouped based on structural features, MOAs, receptor binding property, and metabolites. In parallel, the DART data of the chemicals were searched from various toxicological data resources, summarized, and reviewed by the toxicologists to draw a developmental and/or reproductive toxicity conclusion. Finally, the chemical features, toxicological conclusions, and biological properties were integrated together to determine whether the chemicals can be added to the existing categories/subcategories or to a create new category.

2 Results

The initial set of 213 chemicals from the MEESCC database was processed using the automated DT, which was used to identify compounds consistent with those chemicals possessing a known precedence for DART potential. We screened 79 chemicals that contain the core structure of a category of chemicals in the DT. These 79 chemicals were prioritized for toxicological data curation. This paper illustrates the toxicological data curation for 4 chemicals that were grouped into 2 different categories.

2.1 Extension of subcategory 8e

In the original publication, category 8 contains aromatic compounds with alkyl, multi-halogen and nitro group substituents including subcategory 8e with structural requirements described in Fig.2. The predominant core structural features observed in subcategory 8e are 2,6-halogen, 2,4-nitro phenols, and their derived esters. The phenol ring can have various substituents, including hydrogen, alkyl, nitrile, or amine groups[8]. 8-hydroxyquinoline (CAS RN 148-24-3) and 3,5,6-trichloro-2-pyridinol (TCP) (CAS RN 6515-38-4) are two of the 79 MEESCC chemicals that may fit into this category. They both have the core structure of one or two aromatic rings with an electron-withdrawing group (e.g. nitro group or pyridine nitrogen atom). Due to the similarity between the nitro group and pyridine nitrogen atom, 8-hydroxyquinoline and TCP potentially belong to subcategory 8e. These 2 chemicals were identified to have structures in common with known DART precedent following the rules and sorting steps described in the DT (Fig.S1). These 2 chemicals were identified to have structures in common with known DART precedent following the rules and sorting steps described in the DT (Fig.S1). Step Ⅰ, organic compound; to Ⅱ, yes (contain a ring); to 2, no (are not an ER and AR binders); to 3, no (are not RAR/AhR binders or Prostaglandin); to 4, no (are not nAChRs binders or AChE inhibitors); to 5, no (are not ion channel/alpha/beta-adrenergic/ACE/ARA inhibitors or Shh signaling interference/Cholesterol synthesis inhibition); to 6, no (are not opioid/tubulin binders); to 7, no (are not nucleotide or nucleobase derivatives); to Ⅲ, yes, (contain aromatic or heteroaromatic ring); to 8, yes (contain poly-halogen or pyridine nitrogen group); to known precedent for DART. With this hypothesis, these two chemicals were comprehensively reviewed for all the available DART data to determine whether they show any developmental or reproductive toxicity potential.



R_3=H, -COR_4
R=R_2=-Br, I, R_1=CN

R_3=H, -COR_4
R=Cl, R_2=F, R_1=H

R_3=H, -COR_4
R=R_2=NO_2, R_1=alkyl, hydroxyethylamino

(R_4=satur./unsatur.alkyl)
(R, R_2 are normally at C-2 and C-6 positions)

Fig. 2 The structural scope of subcategory 8e encompasses halogenated and nitro-substituted phenols, and their ester derivatives (modified from Fig.S24 of WU et al, 2013[8]).

2.2 8-hydroxyquinoline affects estrous cyclicity, sexual organ weights, and embryonal development

8-hydroxyquinoline (CAS RN 148-24-3), also known as oxine, has a wide range of applications due to its chelating properties. It forms stable complexes with metal ions, such as copper, iron, and zinc. Certain derivatives of 8-hydroxyquinoline exhibit antimicrobial, antifungal, antiparasitic, antineurodegenerative, anticancer, and antioxidant properties[14]. There is a well-designed and good quality two-generation study in rats. This type of multigeneration study evaluates repeated exposure to a substance throughout all stages of the reproductive cycle and provides information on potential reproductive effects including impact on fertility, gestation, lactation, and the development of offspring across multiple generations[15]. There are two developmental toxicity studies with rats or rabbits to evaluate the developmental effects by 8-hydroxyquinoline. The rat developmental study indicates some fetotoxicity (reduced mean fetal body weight and in-

creased incidences of visceral and skeletal variations) but in the presence of maternal toxicity (reduced food consumption and reduced body weight and body weight gain). Due to limited data details, it cannot be clearly demonstrated that these fetal effects were solely attributable to 8-hydroxyquinoline or secondary to maternal toxicity. On the other hand, the developmental study conducted on rabbits provided a clear distinction between maternal toxicity and fetal toxicity, thus, this rabbit study is chosen to assess the developmental potential of 8-hydroxyquinoline.

In a two-generation study conducted in rats, the animals were administered 8-hydroxyquinoline in the diet at doses of 0, 1 000, 3 000, 8 000 ppm (equivalent to 119, 291, 855 $mg \cdot kg^{-1} \cdot d^{-1}$). In F1 generation, the number of complete estrous cycles per unit time were statistically significantly reduced (3.5 vs. 4.3 in control), whereas the duration of the estrous cycles was increased (5.3 days vs. 4.6 days in control) in the highest dose group. The historical control data in Sprague Dawley rats from Charles River Laboratories, Mattawan (from January 1st, 2011 to May 1st, 2019) show that the mean number of estrous cycles is 4.00 (minimum of 3.8 and maximum of 4.2) and the mean cycle length is 4.9 days (minimum of 4.4 days and maximum of 5.4 days) in F1 generation females. Thus, the estrous cycle duration is outside of the historical control range and even lower than that of the minimum cycle boundary after treatment of 8-hydroxyquinoline. Although the estrous cycle duration seems within the historical control range, it almost reaches to the maximum cycle length boundary[16]. Thus, we conclude that 8-hydroxyquinoline affected estrous cyclicity. Additionally, the weight of reproductive organs was changed after administration of 8-hydroxyquinoline, including a dose-dependent reduction of prostate weight in Parental (P) generation males at mid and high dose groups. Taken together, 8-hydroxyquinoline shows reproductive toxicity by affecting female estrous cycles and male prostate weights in rats. Our conclusion is consistent with the EFSA opinion, in which 8-hydroxyquinoline is reviewed to show reproductive toxicity[17]. Meanwhile, it's worth noticing that a dose-responsive reduction of pup survival (11.0%, 10.5%, 9.5% for 1 000, 3 000, 8 000 ppm group, equivalent to 119, 291, 855 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively) at day 0 in F1 generation was observed in all three dose groups compared with the control group (12.4%). This indicates that 8-hydroxyquinoline may also impair development[18].

In a developmental toxicity study, rabbits were orally gavaged with 8-hydroxyquinoline at doses of 0, 5, 15, and 60 $mg \cdot kg^{-1} \cdot d^{-1}$. The treatment-related developmental effects include an increased incidence of omphalocele observed in 5 fetuses of 3 litters at 15 $mg \cdot kg^{-1} \cdot d^{-1}$ group and 5 fetuses of 4 litters at 60 $mg \cdot kg^{-1} \cdot d^{-1}$ group. The incidence was 3.9% for fetuses and 16.7% for litters in the 15 $mg \cdot kg^{-1} \cdot d^{-1}$ group, and 4.3% and 23.5%, respectively for fetuses and litters in the 60 $mg \cdot kg^{-1} \cdot d^{-1}$ group. No omphalocele was detected in the low dose or control group. Omphalocele is regarded as a congenital developmental malformation caused by defects in the development of anterior abdominal wall[19-21]. The occurrence of omphalocele is uncommon based on historical control data of this lab. For example, the incidences of omphalocele are 0.8% (0% - 1.8%) for fetuses and 4% (0% - 8.3%) for litters. Moreover, there was no maternal toxicity such as alteration of body weight, body weight gain, or food consumption after administration of 8-hydroxyquinoline up to the highest dose tested. Administration of 8-hydroxyquinoline also caused dose-responsively increased incidences of visceral variations (periorbital hemorrhage and retinal fold) and skeletal retardations (unossified and rudimentary sternebrae) in absence of maternal toxicity. Additionally, a reduced pup viability was observed at the highest dose group (live kits/litter: 5.8) compared with the control group (live kits/litter: 7.3). This finding is consistent with reduced fetal survival starting from dose of 1 000 ppm, equivalent to 119 $mg \cdot kg^{-1} \cdot d^{-1}$ of F1 generation from the two-generation study with rats[18]. In summary, we consider that 8-hydroxyquinoline is a developmental toxicant based on the dose-responsive increase in omphalocele and reduced kits survival. This conclusion is in line with 2011 EFSA opinion, which reviewed 8-hydroxyquinoline as a developmental

toxicant[17].

2.3 3,5,6-trichloro-2-pyridinol (TCP) causes central nervous system (CNS) malformations

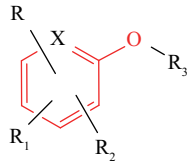
TCP is a major metabolite of chlorpyrifos (CPF). CPF is a widely used pesticide that inhibits acetylcholinesterase (AChE) activity[22]. It's interesting to note that whether CPF has developmental neurotoxicity (DNT) in animals, academic and industrial-supported toxicity studies have shown discrepant conclusions. MIE et al. re-evaluated a pesticide producer-sponsored DNT study with pregnant rats, which showed either no effects on brain development at 0.3 $mg \cdot kg^{-1} \cdot d^{-1}$ and 1 $mg \cdot kg^{-1} \cdot d^{-1}$ or some effects in the presence of maternal toxicity at 5 $mg \cdot kg^{-1} \cdot d^{-1}$. However, after regional analysis of the brain, they found that cerebellum height was dose-dependently decreased in all dose groups. Additionally, there was a statistically significant decrease in cerebellum height to brain weight ratio at mid- and low-dose groups in the absence of maternal toxicity. These data indicate that CPF has DNT on rat offspring[23]. Thus, although TCP (without organophosphate moiety) cannot inhibit AChE, the above studies reveal that the shared structure by TCP and CPF, i.e. the pyridinyl fused phenol, may be a developmental toxicity alert feature.

In a developmental toxicity study, groups of female New Zealand White Rabbits were treated with TCP (CAS RN 6515-38-4) once daily during gestation day (GD) 7−19 at doses of 0, 25, 100, or 250 $mg \cdot kg^{-1} \cdot d^{-1}$ via oral gavage. The study showed that there was a statistically significant reduction in maternal body weight gain at the highest dose group during the entire treatment period. Mean fetal body weight was comparable across all groups. There were 0 (0) total malformations in 25 $mg \cdot kg^{-1} \cdot d^{-1}$ group, 7 (5) in 100 $mg \cdot kg^{-1} \cdot d^{-1}$ group, 7 (6) in 250 $mg \cdot kg^{-1} \cdot d^{-1}$ group compared with 3 (3) in the control group regarding fetal alterations among litters (no. fetuses (no. litters)). This data showed a dose-dependent increase of total malformations at mid- and high-dose groups. Among the total malformations, the cases of total CNS anomalies were also increased with dose-responsive manner, 4 (2) and 5 (4) in mid- and high-dose groups, respectively compared with 1 (1) of the control group. The incidence of total CNS anomalies in each group (3.7% and 5.0%) is higher than that of the historical control incidence (2.2%) obtained from evaluation of 878 control fetus of 198 litters. Regarding total CNS anomalies, the hydrocephaly was not found in any fetus of the control group, whereas hydrocephaly cases were 3 (2) in 100 $mg \cdot kg^{-1} \cdot d^{-1}$ group and 3 (3) at 250 $mg \cdot kg^{-1} \cdot d^{-1}$ group[24]. TCP is reviewed as a developmental toxicant. Our conclusion is consistent with the EFSA opinion that the developmental NOAEL of TCP is 25 $mg \cdot kg^{-1} \cdot d^{-1}$ based on dose-responsive increase in the incidence of fetal and litter CNS malformations[25].

We added 8-hydroxyquinoline and TCP to subcategory 8e. 8-hydroxyquinoline has an impact on estrous cycle, prostate weight, and fetal development, and TCP causes CNS malformations, which confirms that they are DART positive chemicals and can be included in the tree. The compounds in this subcategory have core structural features that include phenol, pyridinyl fused phenol (e.g. 8-hydroxyquinoline), o-tBu hydroquinone and its 4-methoxyphenol derivatives (Fig.3). The developmental toxicity is associated with hydroquinone derivatives and phenols substituted with alkyl groups, although the exact mechanism is not clear. It has been reported that these chemicals generate redox mediators (RMs) (e.g. benzoquinone & etc.) and/or reactive oxygen species (ROS) to induce embryonic and fetal toxicity[26].

2.4 Expansion to include a possible new category: neonicotinoids

In order to define a new category, three or more chemicals with similar structures and toxicological profiles must be defined. Thiacloprid and imidacloprid, two neonicotinoid pesticides in the MEE of China data set, have similar structures that are not represented in an existing category of the DT. Thiacloprid acts on the nervous system of insects, disrupting nerve impulse transmission and leading to paralysis and death[26]. Imidacloprid is used in various food and feed crops, tobacco, ornamentals, buildings for termite control and on cats and dogs for flea control[27].



When X=C;

$R=R_2$=Br, I, R_1=CN
R_3=H, -COR_4

R=Cl, R_2=F, R_1=H
R_3=H, -COR_4

(R_4=satur./unsatur. alkyl)
(R, R_2 are normally at C-2 and C-6 positions)

$R=R_2=NO_2$, R_1=alkyl, hydroxyethylamino
R_3=H, -COR_4

R_3=H; R_1 and R_2=H
R=fused pyridine

When X=N;

R_3=H; R, R_1, R_2=Cl

Fig. 3　Extended subcategory 8e. The dashed line boxes represent substituent structures added to subcategory 8e.

2.5　Thiacloprid (CAS RN 111988-49-9) causes dystocia and fetal skeletal malformations

In a two-generation reproductive study following OECD TG 416, male and female Sprague-Dawley (SD) rats (30 rats/sex/dose) received chow containing thiacloprid at 0, 50, 300, and 600 ppm (equivalent to 0, 3.3, 20.4, and 41.0 $mg \cdot kg^{-1} \cdot d^{-1}$ for P generation (P0) females). The highest dose led to reduced parental body weight and body weight gain in both parental and F1 generations. The mid- and high-dose females of P0 generation also showed hepatocellular necrosis. There were no treatment-related effects on the reproductive function or performance, however, mid- and high-dose treatment caused an increased incidence of dystocia of P0 females. Considering that dystocia is not commonly found in historical control, this effect was considered to be a treatment-related adverse effect although it's not observed in F1 generation females[28]. The reproductive NOAEL is 50 ppm, equivalent to 3.3 $mg \cdot kg^{-1} \cdot d^{-1}$ for females. EFSA has reviewed the thiacloprid risk assessment and derived the same conclusion. EFSA also commented that aromatase activity in the ovaries was increased in both pregnant and lactating animals. The dystocia was proposed to be attributable to the alteration of sex hormone levels although a causal relationship cannot be confirmed[29]. In both F1 and F2 generations, thiacloprid resulted in reduced live birth index in the high-dose group and statistically significant reduction of fetal body weight in both mid- (in absence of maternal body weight change) and high-dose groups[28]. These data indicate that thiacloprid also shows developmental toxicity.

In two prenatal developmental toxicity studies both following OECD TG 414 with Wistar rats or Himalayan rabbits via oral gavage, thiacloprid caused maternal toxicity such as decreased body weight, body weight gain, and food consumption. It also induced post-implantation loss, skeletal variations and retarded ossification in the presence of maternal toxicity in both species. There was a marginally increased incidence of fetuses with supernumerary 13th ribs in the high dose group of the rabbit study, which is considered a variation. In rabbits, the incidence of supernumerary rib is 17%−19% and 32%−38% for short and full supernumerary rib, respectively, which are much higher than those in rats. No scientific evidence shows that an increased incidence of supernumerary ribs in animal studies is a reliable predictor of increased risk to human development[30−31]. However, it's worthy noticing that a treatment-related supernumerary 13th ribs with supernumerary lumbar vertebra, common malformation, was found in the high dose group of the rabbit study[28]. This malformation is considered adverse developmental effect. Taken together with the indication of developmental toxicity from two-generation reproductive study of SD rats, thiacloprid is reviewed as a developmental toxicant.

2.6　Imidacloprid (CAS RN 138261-41-3) disrupts the endocrine system and impairs male fertility

In a 10-week reproductive toxicity study by the Institute of Cancer Research (ICR), male mice were treated with imidacloprid via drinking water at concentrations of 3, 10, and 30 $mg \cdot L^{-1}$. Testicular morphology was severely damaged in the high dose group showing thinner seminiferous tubules, irregularly arranged and fewer layers of spermatogenic cells. The high dose of imidacloprid significantly reduced serum

testosterone (T) levels and mRNA levels of androgen receptor (AR) and decreased the activity of aromatase. Some genes responsible for synthesizing cholesterol and T were inhibited in a dose-responsive manner. These data revealed that imidacloprid disrupts androgen signaling of male mice[32].

In a 90-day repeated dose toxicity study, male Wistar rats were treated with imidacloprid at doses of 0, 0.5, 2, and 8 $mg \cdot kg^{-1} \cdot d^{-1}$. High dose exposure of imidacloprid significantly decreased sperm motility, increased abnormal sperm morphology, and decreased epididymal sperm concentration in the mid- and high-dose groups. High dose exposure of this chemical also significantly decreased the levels of T and glutathione (GSH), which indicates the impact of imidacloprid on the endocrine and antioxidant systems in testis[33].

Neonicotinoids, such as imidacloprid, thiacloprid, nitromethylene analog THPCHI and the N-unsubstituted imines (e.g. an imidacloprid metabolite, the descyano derivatives of thiacloprid, an olefin analogue (DCTHIA and DCTHIA-O)) as well as (−)-epibatidine, (−)- nicotine etc., act on the central nervous system of insects and mammals as inhibitors of acetylcholinesterase or agonists at the nicotinic acetylcholine receptor (nAChR)[34−35]. Structurally, these neonicotinoids have several features in common, containing 2-chloro-5-methylpyridine substituted imidazolidine cyclic ring and its isosteric moieties (thiazolidine, oxazolidine, tetrahydropyrimidine, or pyrrolidine) and nitroimino, cyanoimino, or nitromethylene substituents. The majority of neonicotinoids exhibit neurotoxic activity specifically targeting insects, as they bind more strongly to insect neuroreceptors than to those found in mammals. It's been reported that the loop C and loop D of insect nAChR contribute to the selective toxicity of neonicotinoids[36−38]. However, recent studies highlight that neonicotinoids also display agonist effects on mammalian nAChRs[39−40]. The key functional groups, NO_2, or CN substituted guanidine or imidazolidin-2-imine, thiazolidin-2-imine may play an important role in binding to nAChR (or by the active metabolites (e.g. formation of H_2CO, and N-CH_2OH) or via inhibition of induced nitric oxide synthase (Fig.4). Three or more structural-related chemicals with DART positive properties are preferred in order to create a more robust new category. If some DART negative chemicals within the scope of the new category are found, this could define the boundary of the category.

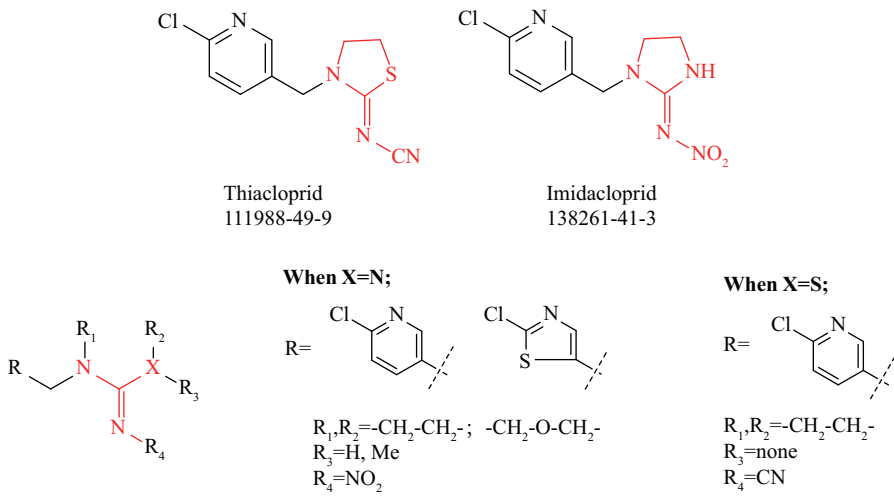


Fig. 4 Expansion of a possible new category: neonicotinoids

3 Discussion

In the past decade, P&G has introduced an empirical and rule-based DART DT that utilizes known chemical structures associated with DART to predict whether a chemical possesses the potential for developmental or reproductive toxicity[8]. This paper primarily demonstrates how DART data curation has been conducted to ensure accurate and transparent data interpretation and

ultimately to facilitate an appropriate expansion of the DT. The chemists initially grouped targeting chemicals into defined categories via Pipeline Pilot (DT v1.9). 8-hydroxyquinoline and TCP were added to subcategory 8e based on core structure analysis, while thiacloprid and imidacloprid were proposed to form a new category of neonicotinoids (Table S1).

A comprehensive assessment of DART data relies on the expertise and judgment of toxicologists due to the complexity of DART endpoints. When toxicologists assess DART, the dose-response, statistical significance and biological relevance, historical control data, fetal effects (including observed incidence across multiple litters), and maternal effects need to be taken into consideration collectively. Dose-responsiveness is the basic principle to interpret adverse toxicological effects, however, in specific cases where embryo lethality is observed at higher doses due to post-implantation loss or abortion, a clear dose-response for a fetal developmental effect may not be apparent. Instead, the developmental toxicity might be manifested based on trends observed at lower doses. In other cases, a dose-responsive fetal effect with low incidence (such as malformation) in the treated groups may not be statistically different compared with control group data, because statistical power is dependent on sample size and variability. This fetal effect might still be considered adverse or biologically relevant if the type of malformation is not or rarely seen in the historical control data of the same animal species or stains. In other scenarios, the occurrence of a malformation in the treated group, which falls within the historical control range, could be an incidental finding especially in absence of a dose-responsiveness. Therefore, it's important for toxicologists to interpret the data in a case-by-case manner and understand the difference between statistical significance and biological relevance and what they imply in DART studies. Another important aspect that needs expert judgment is to differentiate whether a fetal effect is directly attributable to the toxicant or secondary to the maternal toxicity. For example, delayed ossification and skeletal variation by thiacloprid may be secondary to maternal toxicity. Maternal toxicity is evidenced by maternal mortality, body weight loss or significant body weight gain reduction, depressed food consumption, quality or quantity of milk produced, and other clinical effects on dams such as coma, prostration, loss of righting reflex, hyperactivity, ataxia, etc.

The DT was originally developed based on a set of available DART data of 716 chemicals, chemical structure characteristics, receptor binding activities, metabolism, MOAs of different chemical categories. The tool has demonstrated its potential and feasibility for screening and prioritizing chemicals for testing by identifying structural groups with known DART effects. In the previous publication, the sensitivity of the DT was tested using four datasets including DT test set, CAESAR dataset, RIVM dataset, and P&G DART data. The DT showed good sensitivity with 74%, 89%, 88%, and 98%, respectively to identify DART positive chemicals using the four datasets (the chemicals from CAESAR, RIVM, and P&G DART datasets were also used for building up DT)[8]. In 2016, MARZOA et al. compared the predictivity of 6 developmental toxicity models (including P&G DT, SARpy, CAESAR, CASE Ultra, Derek Nexus, and Model Applier) and studied the outcome of integrating different models to increase the predictive performance. Considering sensitivity, specificity, concordance, and correlation of predicted binary classifications with actual outcomes, their analysis indicated that each model algorithm generally demonstrated superior performance in predicting chemicals that were part of its training set to build the model. When processing chemicals that are very dissimilar to the training set, each model might not differentiate well with these structures. Thus, the authors further evaluated the predictive performance of selected models if assessing chemicals within the applicability domain. For example, both CAESAR and P&G DT models showed increased sensitivity (0.87 to 1) across all data sets. But both models showed low specificity to identify true non-toxic chemicals when applied to the data sets other than their training data. This is because limited number of non-toxic chemicals were used to build the models. The authors also found

that when integrating two or three models, this will improve the performance of the models to predict the development toxicity via synergizing the advantages and filling in the data gaps across models[41]. Recently, a research group compared CAESAR and P&G DT models regarding their ability to predict the developmental toxicity of solvent chemicals. The P&G DT model demonstrated a much higher percentage of very reliable predictions than that of the CAESAR model. When further analyzing the chemicals that were predicted to have developmental toxicity by both models, the predictions for these chemicals were predominantly labeled as 'reliable'[42]. Our DT offers a qualitative prediction regarding the DART potential of a chemical; however, it does not provide a quantitative assessment of the DART potential due to lack of quantitative data (such as NOAEL/LOAEL). Another opportunity area of the DT is to set boundary or cutoff values within each category, which will require more negative chemicals. Future improvements can be made by increasing the number of both positive and negative chemicals within each subcategory. Recently, we published a structure-based search strategy to find chemical analogs via matched molecular pair (MMP) analysis considering physiochemical properties, reactivity, and metabolism of chemical pairs. This approach can be used to expand structural similar chemicals within a specific category and to predict the boundary of the category[43]. This will enhance the predicting power and robustness of the DT to predict the DART potential of chemicals. The next decade will witness the automation, customization, and adoption of DART DT across many applications such as in areas of chemical toxicity screening and potency prediction.

4 Conclusion

In recent years, the Ministry of Ecology and Environment of China has been working on several technical guidelines to support the computational methods in the application of emerging pollutants screening and chemical management when animal testing data is not available. We propose that the DT, in combination with other DART prediction tools, can be used as a screening tool to identify chemicals that have structural features consistent with known DART effects in order to prioritize chemicals of concern. Chemicals that exhibit structural features associated with known DART effects can be given higher priority for additional evaluation. Regulatory authorities can utilize the outputs of the DT to determine the level of scrutiny, data requirements, or risk mitigation measures necessary for the registration and safe use of the chemical. The other value of the DART DT is used as a weight of evidence to support the SAR-based read-across and to fill data gaps without generating additional test data. We expect this DART DT can play an important role in China emerging pollutant and new chemical domain for screening and assessment.

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Abbreviations

AChE, acetylcholinesterase; AR, androgen receptor; CAESAR, Computer-Assisted Evaluation of industrial chemical Substances According to Regulations; CNS, central nervous system; CPF, chlorpyrifos; DART, developmental and reproductive toxicity; DNT, developmental neurotoxicity; DT, decision tree; GD, gestation day; GSH, glutathione; ICR, Institute of Cancer Research; MEESCC, Solid Waste and Chemicals Management Center of Ministry of Ecology and Environment; MOA, mode of action; nAChR, nicotinic acetylcholine receptor; P&G, Procter & Gamble; QSAR, quantitative structure-activity relationship; RFR, repro female rat; RM, redox mediator; RMR, repro male rat; ROS, reactive oxygen species; SD, Sprague-Dawley; T, testosterone; TCP, 3,5,6-trichloro-2-pyridinol; T.E.S.T, Toxicity Estimation Software Tool; VEGA, Virtual models for property Evaluation of chemicals within a Global Architecture

Declaration of conflict of interest

The authors state that they have no identifiable competing financial interests or personal relationships that could have potentially influenced the findings presented in this paper.

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