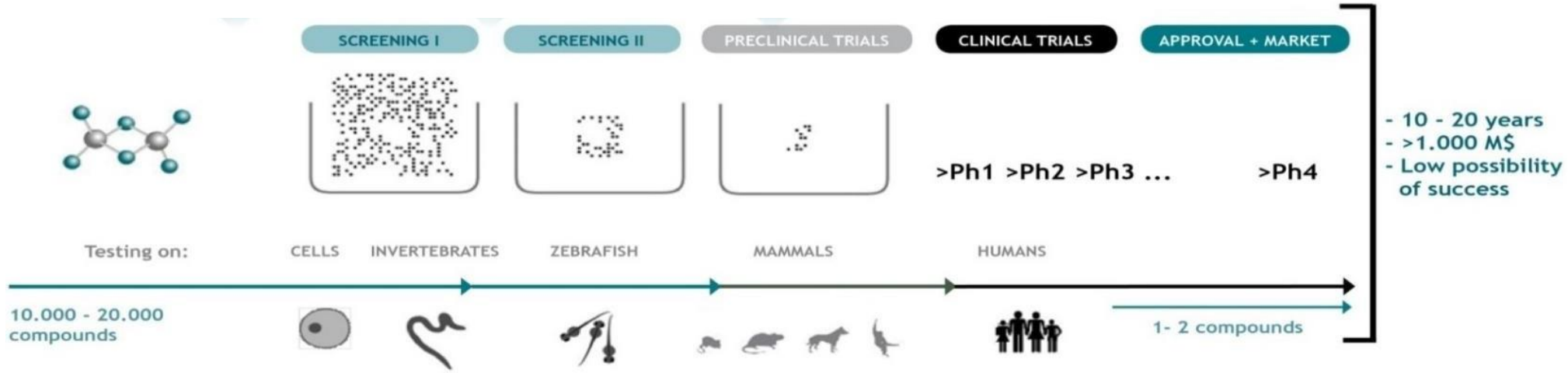
Reproductive and Developmental Toxicology Course

New Approaches and Methodologies in Safety assessment, especially in DART testing

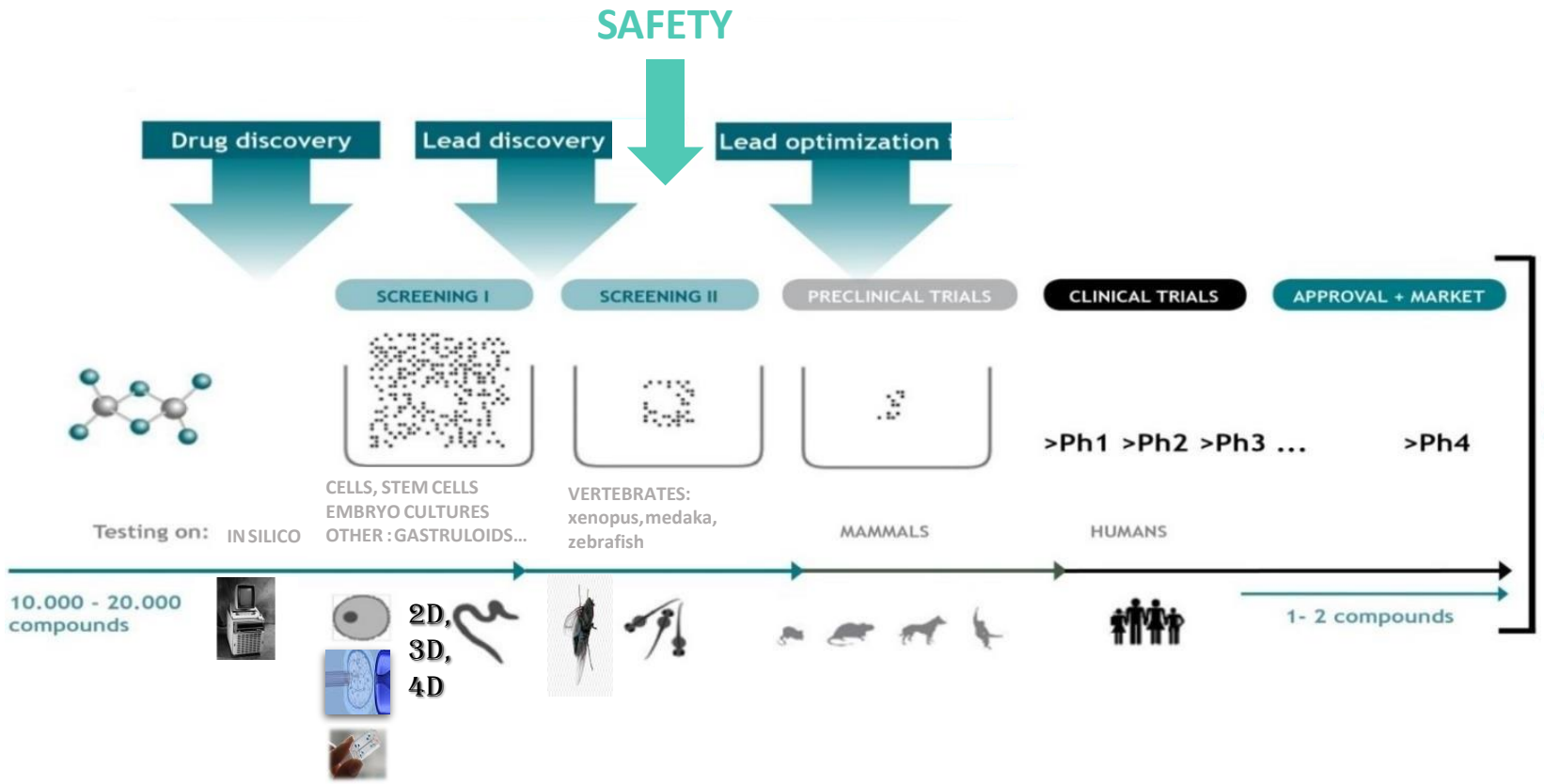
Zebrafish as a qualified NAM for DART

Arantza Muriana, BIOBIDE (Spain)

Traditional Drug Discovery & Development



Alternatives to the traditional R&D&i



- Decrease time & cost
- Increase success rate

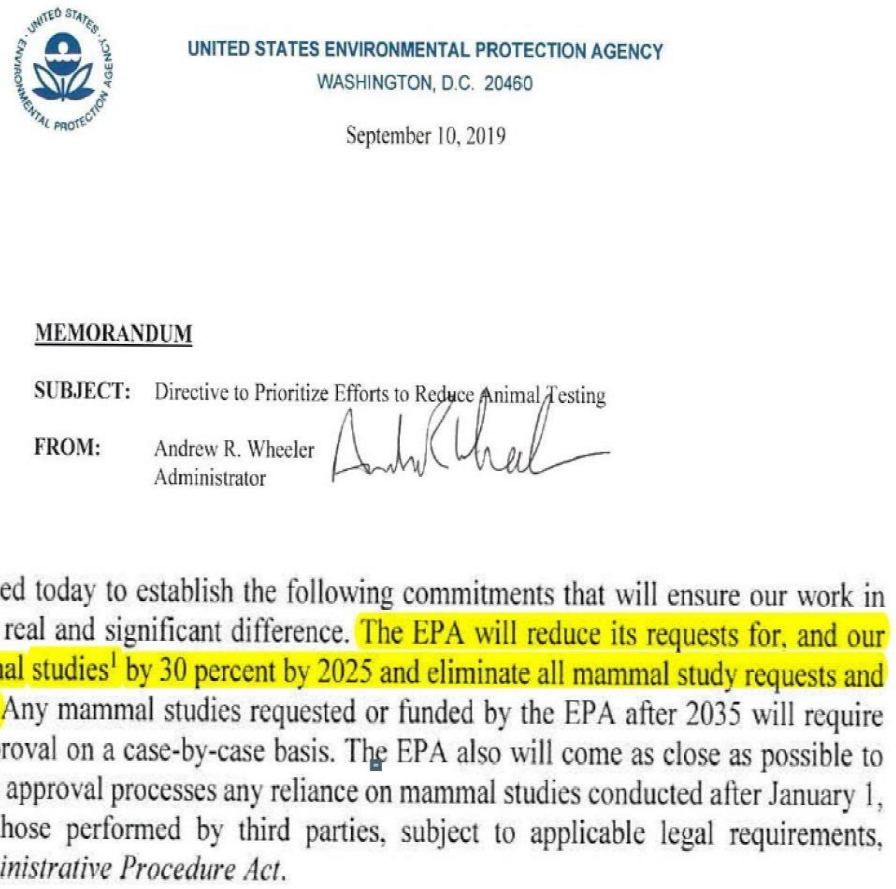
Regulation on 3Rs and Alternative Models

- **EURL-ECVAM** was established in 2011, due to the increasing need for new methods to be developed and proposed for validation in the European Union, hosted by the Joint Research Centre (Ispra, Italy) to evaluate **the validation of methods** which **Reduce, Refine or Replace the use of animals** for safety testing and efficacy/potency testing of **chemicals, biologicals, and vaccines submited by research laboratories.**

- Research laboratories can submit to EURL ECVAM for scientific validation the alternative methods to animal testing that they have developed and promotes the development and dissemination of alternative methods and approaches, for their application in industry and their acceptance by regulators.

- In 2016 EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches stated Replacement, Reduction and Refinement of animal testing was anchored in EU legislation:
  - Alternative non-animal approaches facilitate a shift away from animal testing.
  - Cell-based methods and computational technologies are integrated to translate molecular mechanistic understanding of toxicity into safety testing strategies.

Regulation on 3Rs and Alternative Models



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

September 10, 2019

MEMORANDUM

SUBJECT: Directive to Prioritize Efforts to Reduce Animal Testing

FROM: Andrew R. Wheeler
Administrator

I am pleased today to establish the following commitments that will ensure our work in this area makes a real and significant difference. The EPA will reduce its requests for, and our funding of, mammal studies[1] by 30 percent by 2025 and eliminate all mammal study requests and funding by 2035. Any mammal studies requested or funded by the EPA after 2035 will require Administrator approval on a case-by-case basis. The EPA also will come as close as possible to excluding from its approval processes any reliance on mammal studies conducted after January 1, 2035, including those performed by third parties, subject to applicable legal requirements, including the *Administrative Procedure Act*.

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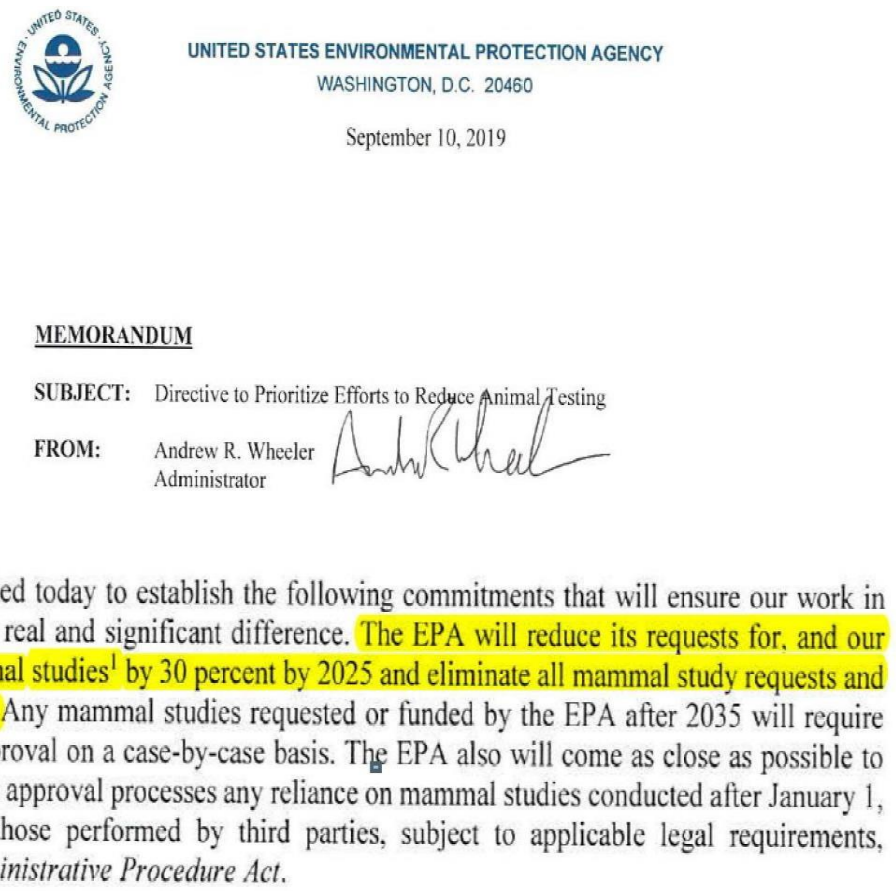
NAMs Work Plan: in December 2021, the agency released an update to its New Approach Methods (NAMs) Work Plan, outlining actions to reduce animal use in chemical testing through 2024.

Regulation on 3Rs and Alternative Models



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EPA has abandoned a controversial plan to phase out all use of mammals to test the safety of chemicals by 2035

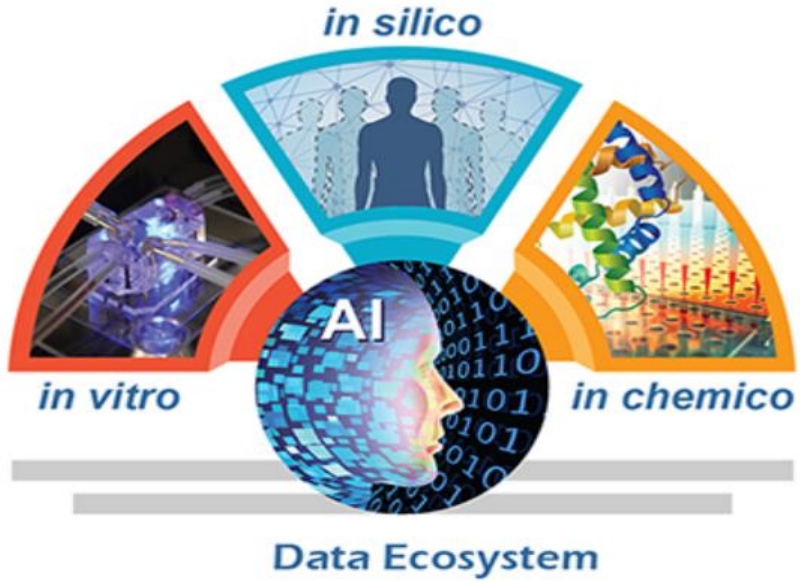
Regulation on 3Rs and Alternative Models

Complement Animal Research In Experimentation (Complement-ARIE) Program

NIH National Institutes of Health
Office of Strategic Coordination–The Common Fund

The NIH creates a Common Fund for speeding the development, standardization, validation, and use of human-based **New Approach Methodologies (NAMs),** to more accurately model human biology, and complement, or in some cases, replace traditional research models.

The Complement-ARIE program will build upon ongoing efforts related to NAMs, while identifying opportunities for innovation and coordination to advance understanding of human health and disease by providing a range of ready and standardized biomedical research models.



Regulation on 3Rs and Alternative Models

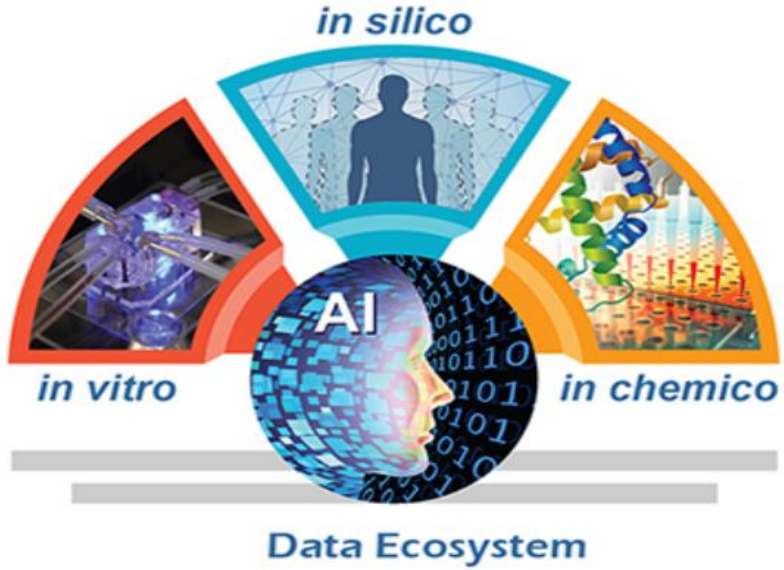
biobidel *Your Zebrafish Partner*

Complement-ARIE Program

The expertise required would be obtained through a **consortium of researchers** participating in various efforts:

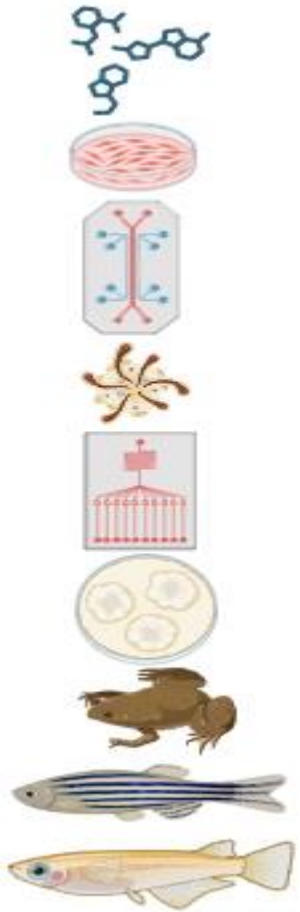
NIH National Institutes of Health
Office of Strategic Coordination–The Common Fund

- Technology development projects/centers to develop NAMs to fill in areas of greatest need, emphasizing biological complexity, high throughput techniques, combining approaches, and data sharing.
- A data & NAM resource *coordinating* center to create integrated data structures and a searchable NAMs repository.
- A validation network to accelerate deployment and regulatory approval of NAMs for biomedical research.
- Community engagement and training to promote the development of an inclusive, diverse, biomedical research workforce with the skills to build and use new NAMs.
- Strategic engagement with key partners to advance emerging opportunities in development and use of NAMs in basic, translational, and clinical research.



Alternative models in DART

1. In silico computer simulations
2. Cell cultures
    - *2D/3D/4D*
    - *Whole embryo cultures for DART*
    - *Organs on a chip*
    - *Heart, kidney, liver…*
3. Human-based cell models
    - Skin irritation
    - Embryoids, organoids and gastruloids
4. Microfluidic Chips
5. Fungal models
    - For drug metabolism
6. Alternative species
    - Xenopus
    - Medaka
    - Zebrafish



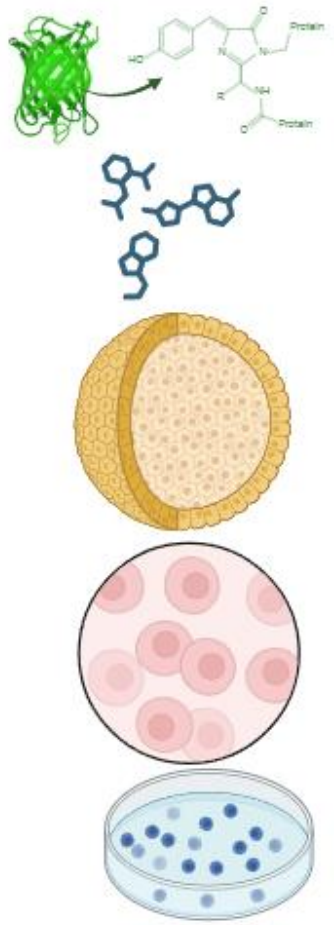
Alternative models in DART

1. In silico computer simulations

- *In silico* tools such as computer algorithms for searching chemical similarities and machine-learning models for activity prediction are used. Two different strategies can be pursued:
- Target-driven identification of potential molecules using these computational tools.
- Screening of compounds for the identification of DART activity for.

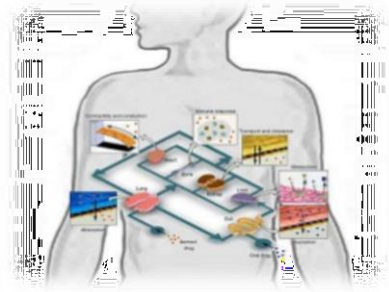
2. 2D/3D/4D Cell cultures

- 2D cultures are cells grown on plastic dishes and plates, widely used for understanding cell biology and drug responses. 3D cultures are grown in tridimensional structures mimicking more accurate cell interactions taking place at *in vivo* structures. 4D cultures are 3D cultures that take into account the time variable.
- Whole Embryo Cultures for DART (WEC): morphology-based whole embryo cultures of mammals are the most common alternative *in vitro* embryotoxicity method to evaluate the early developmental toxicity of compounds. With the disadvantage of lack of pharmacokinetics, but advantage of throughput, for massive screenings.



Alternative models in DART

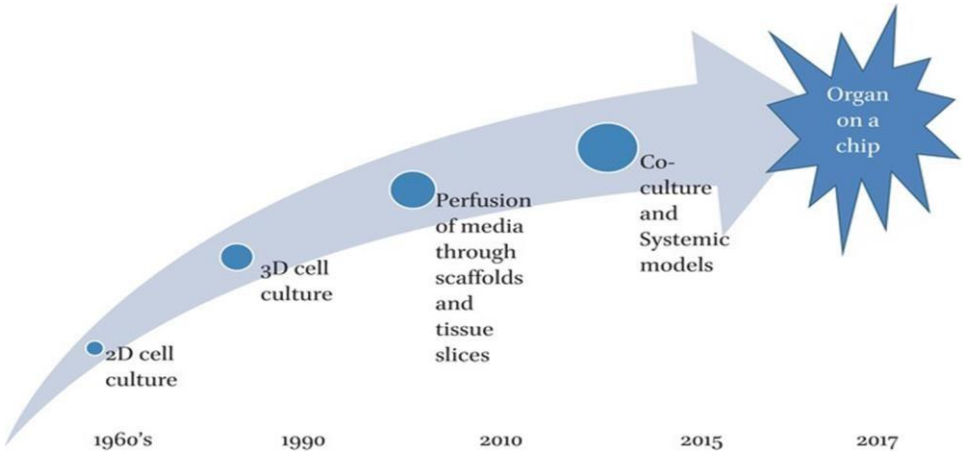
- Organs on a chip: *In vitro* recreation of artificial organs simulating the mechanisms, activities, or physiological response of entire organ´s systems. They use computer microchips to engineer microfluidic culture devices using several cell types that recapitulate the microarchitecture and functions of living human organs.



3. Human-based cell models

Specific models that help in screening potential drugs, also for safety. Examples include cell-based *in vitro* enteric systems (Caco-2 cell line), human stem cell-derived models, skin irritation models, …

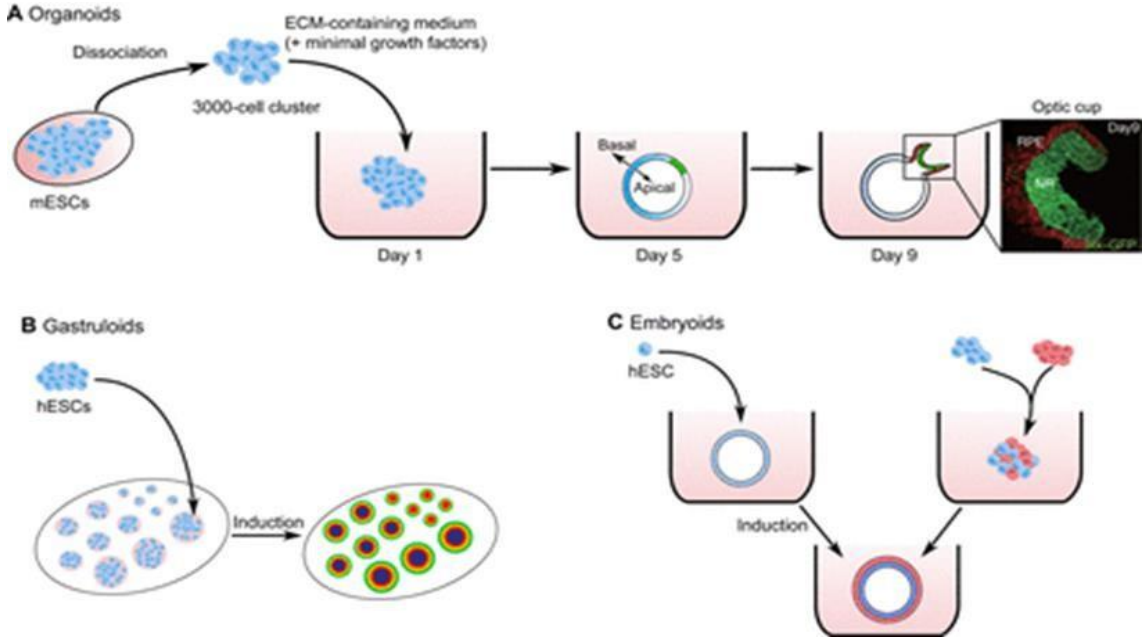
- Human Stem Cells (EST): it uses two permanent cell lines, murine 3T3 fibroblasts and murine Embryonic Stem Cells (ESCs). To establish developmental toxicity, the difference in sensitivity towards the cytotoxic potential of a given test compound between the adult and the embryonic cells is compared with an MTT assay.



The potential of organ on a Chip Technology for replacing Animal Testing. M Wilkinson. 2019

Alternative models in DART

- Organoids: a multicellular structure containing many of the cell types and tissue layers present in an adult organ, typically derived from stem cells *in vitro.*
- Gastruloid: a multicellular *in vitro* model of a gastrulating embryo.
- Embryoids: a more organized embryoid body, such as a cavitating or a multilayered cluster of differentiating ESCs that resembles an embryo at certain stages of early development. One example is a blastoid, a structure that contains the same cell types and tissue topology as a blastocyst.

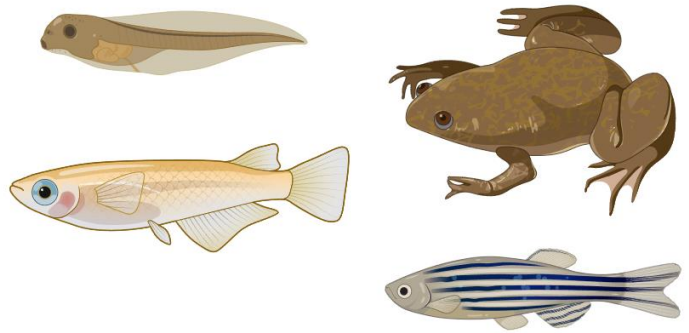


Self-organization into organoids, gastruloids, and embryoids. (A) A cluster of dissociated mouse embryonic stem cells (mESCs) cultured in a medium containing extracellular matrix (ECM) proteins and minimal growth factors spontaneously Self-organizes, first into a polarized quasi-spherical epithelial tissue, then to a structure resembling an optic cup. Microscopy image adapted from Eiraku et al. 2011

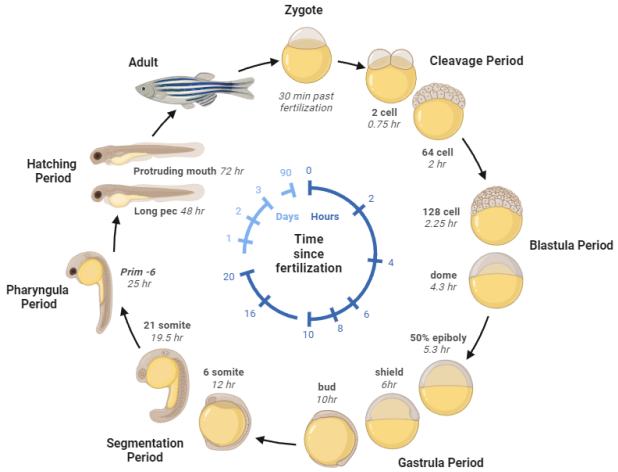
Alternative models in DART

6. Alternative species

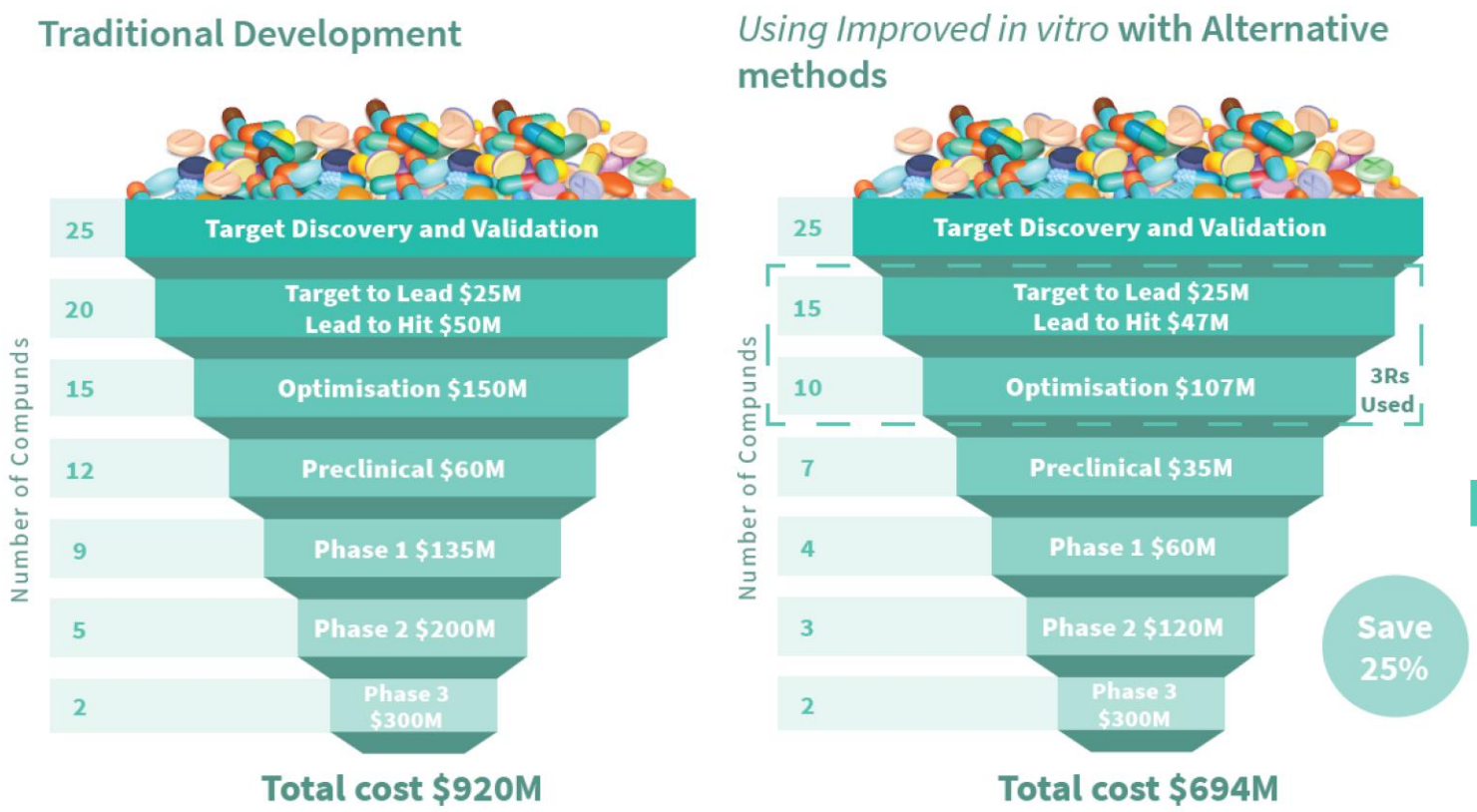
- Frog, Xenopus, Medaka, and Zebrafish are model organisms for studying vertebrate development of many organ systems.
    - **Zebrafish larvae:** especially used in toxicity and efficacy studies, because of the advantages of :
        - High genetic homology with humans (> 70%).
        - Fast development /organogenesis.
        - Small size-like cells: screening in well-plates.
        - High productivity: 100-300 eggs/couple per week.
        - Transparent embryos.
        - Under 5-6 days post fertilization are considered as *in vitro.* models, complying with the 3Rs.
        - Lower maintenance cost.



Zebrafish Developmental Timeline



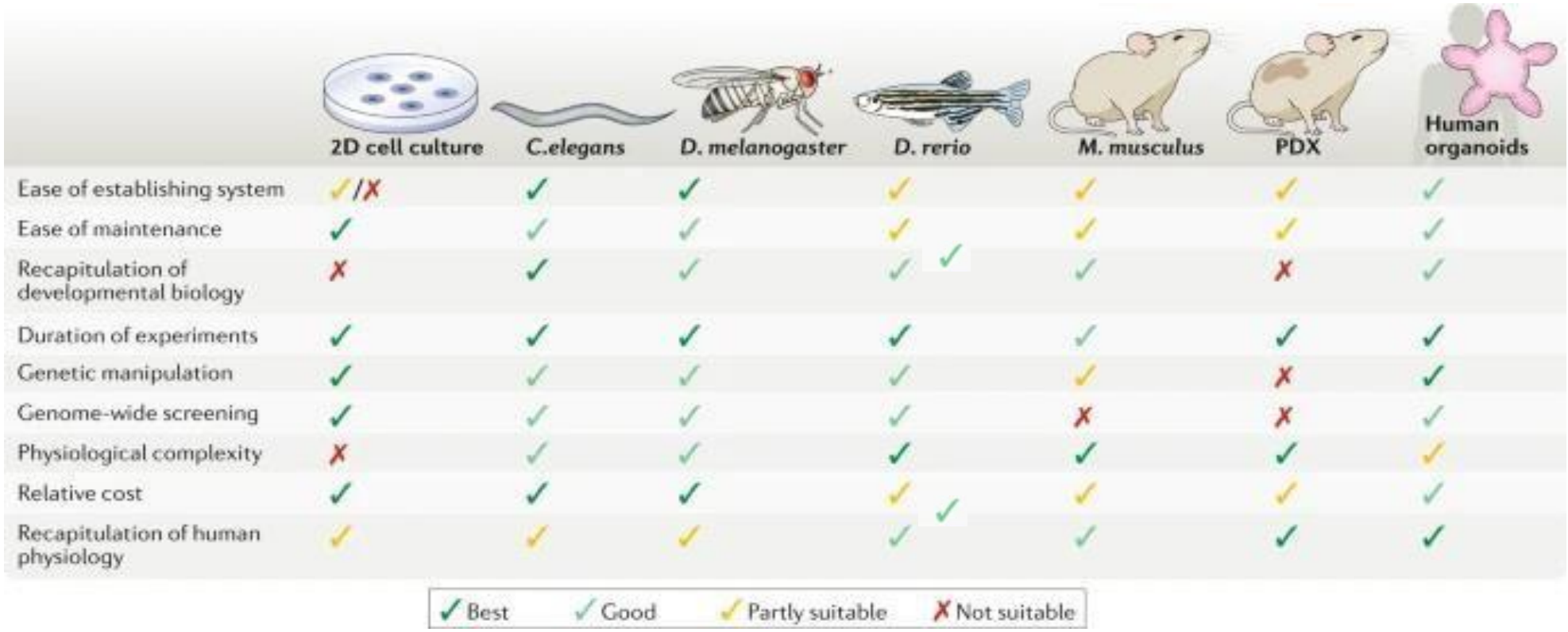
Alternatives to the traditional R&D&i



- 20-30% of cost
- ↓↓ 20% years of development
- ↑↑ success rate in 50-65%

Adapted from Animal Experimentation: Working Towards a Paradigm Change; Human-animal studies, Volume22; Kathrin Herrmann and Kimberley Jayne, 2019.

Alternative models'pros & cons



	2D cell culture	C.elegans	D. melanogaster	D. rerio	M. musculus	PDX	Human organoids
Ease of establishing system	✓/✗	✓	✓	✓	✓	✓	✓
Ease of maintenance	✓	✓	✓	✓	✓	✓	✓
Recapitulation of developmental biology	✗	✓	✓	✓	✓	✗	✓
Duration of experiments	✓	✓	✓	✓	✓	✓	✓
Genetic manipulation	✓	✓	✓	✓	✓	✗	✓
Genome-wide screening	✓	✓	✓	✓	✗	✗	✓
Physiological complexity	✗	✓	✓	✓	✓	✓	✓
Relative cost	✓	✓	✓	✓	✓	✓	✓
Recapitulation of human physiology	✓	✓	✓	✓	✓	✓	✓

✓ Best ✓ Good ✓ Partly suitable ✗ Not suitable

Some of the most common model organisms used in biomedical research including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* and *Mus musculus*, xenografts PDX, 2D cell cultures and human organoids, assessed for their relative benefits and limitations. Relative scores are represented as being the best (dark green tick), good (light green tick), partly suitable (yellow tick) and not suitable (red cross). Adapted from *Human organoids: model systems for human biology and medicine*. Nat Rev Mol Cell Biol 21, 571–584 (2020). Kim, J., Koo, BK. & Knoblich, J.A.

Regulation: 3Rs and Alternatives

3Rs: Replacement, Reduction, & Refinement

EURLS-ECVAM European Union Reference Laboratory for Alternatives to Animal Testing:

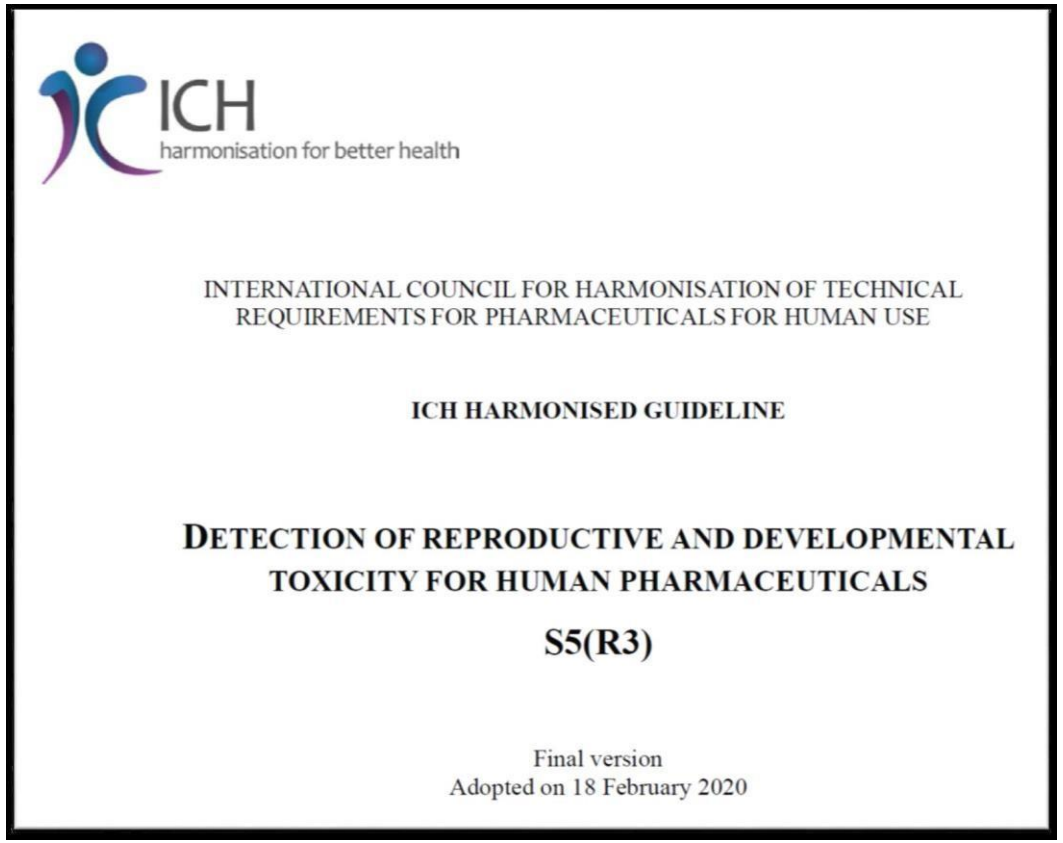
Recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Aquatic Toxicity Testing in Jul 2014.

Several OECD guidelines include zebrafish as a recommended animal models to assess Safety of chemical compounds, such as OECD 203, 210, 212, 229, 236.

EC Regulation 1223/2009 for Cosmetic testing: animal experimentation forbidden--- Alternative models, such as zebrafish, should be used.

Ex.: Zebrafish embryos are not considered animals until 5 dpf --- EU Directive 2010/63/EU.

Regulation on 3Rs and Alternative Models


ICH
harmonisation for better health

INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED GUIDELINE

DETECTION OF REPRODUCTIVE AND DEVELOPMENTAL TOXICITY FOR HUMAN PHARMACEUTICALS

S5(R3)

Final version
Adopted on 18 February 2020

ICH
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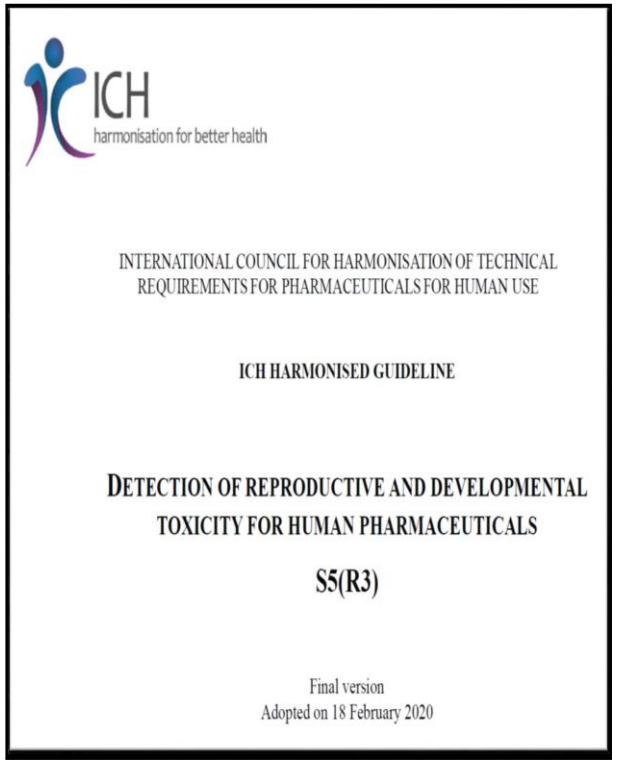
ICH S5 (R3) 2020

Data generated from **qualified alternative assays** conducted alone or in conjunction with one or more *in vivo* studies can be utilized to support hazard identification and risk assessment under limited circumstances.

Regulation on NAMs in Developmental and Reproductive Toxicology

On February 18, 2020, the final version of ICH S5 (R3) was adopted; underlying data generated from qualified alternative assays conducted alone or in conjunction with one or more *in vivo* studies can be utilized to support hazard identification and risk assessment under limited circumstances: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S5(R3)

Final version
Adopted on 18 February 2020

- Circumstances where there is evidence suggesting an adverse effect on EFD (e.g., a mechanism of action affecting fundamental pathways in developmental biology).
- Toxicity in animal species precludes attaining systemic exposures relevant to human exposures under conditions of use.
- As support for a weight of evidence assessment when there are equivocal findings in animal studies.
- As partial support for clinical trials including up to 150 WOCBP for up to 3 months duration.
- Pharmaceuticals being developed for certain severely debilitating for life

Goal: **to qualify** the zebrafish model for its regulatory use by testing the **reference compound list** indicated **by ICH S5 (R3) 29 compounds** that induce specific malformation or embryo-fetal lethality and **3 negative compounds** that can be used to support the qualification of an alternative assay).

First Qualification in NAMS for DART

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Reproductive Toxicology 123 (2024) 108513

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Validation of a zebrafish developmental defects assay as a qualified alternative test for its regulatory use following the ICH S5(R3) guideline

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ARTICLE INFO

Handling Editor: Dr. Bal-Price Anna

Keywords:
Zebrafish
Developmental defects
Embryotoxicity
New alternative method (NAM)
ICH S5 (R3) Guideline
Bioavailability
Bench Marking Concentration (BMC)

ABSTRACT

Zebrafish is a popular toxicology model and provides an ethically acceptable small-scale analysis system with the complexity of a complete organism. Our goal is to further validate this model for its regulatory use for reproductive and developmental defects by testing the compounds indicated in the "Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals" (ICH S5(R3) guideline.) To determine the embryotoxic and developmental risk of the 32 reference compounds listed in the ICH S5(R3) guideline, the presence of morphological alterations in zebrafish embryos was analyzed at two different stages to calculateLC50 and EC50 values for each stage. Teratogenic Indexes were established as the ratio between LC50 and EC50 critical for the proper compound classification as teratogenic when it is ≥ 2. A total of three biological replicates have been conducted to study the reproducibility of the assay. The chemicals' concentration in the medium and internally in the zebrafish embryos was evaluated. In this study, the 3 negative compounds were properly categorized while 23 compounds out of the 29 reference ones (sensitivity of 79.31%) were classified as teratogenic in zebrafish. The 6 that had false-negative results were classified 4 as inconclusive, 1 as not toxic, and 1 compound resulted toxic for zebrafish embryos under testing conditions. After the bioavailability experiments, some of the obtained inconclusive results were refined. The developmental defects assay in zebrafish gives an accuracy of 89.66%, sensitivity of 88.46%, specificity and repeatability of 100% compared to mammals; therefore, this is a well-integrated strategy using New Alternative Methods, to minimize the use of animals in developmental toxicity studies.

OBJECTIVE: to analyze the teratogenic potential of the 32 test items indicated in the new ICH S5 (R3) guideline by determining their toxic effects in zebrafish embryos.

1. Dose Range Finding (DRF) Test: to evaluate the toxic profile of the test compounds and decide which concentrations will be tested in the assay.
2. Evaluation of Developmental Defects: The Teratogenic Index (TI) will be estimated as the ratio between LC50 and EC50 at the stages of 2 and 4 dpf. Then, TIs will be calculated, one per stage analyzed.
3. Bioavailability Analysis: All the embryos treated at the highest concentration without effect and at the lowest concentration at which malformations are induced, will be shipped in dry ice to Biobide's approved provider for the evaluation of the internal dosing.

Qualified Teratogenicity Assay in Zebrafish

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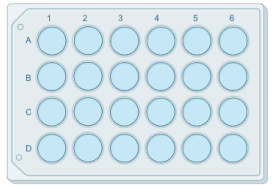
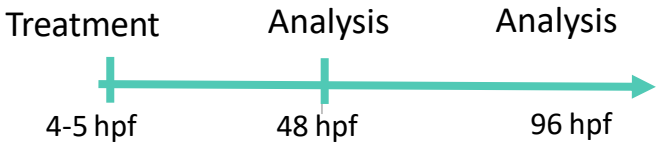
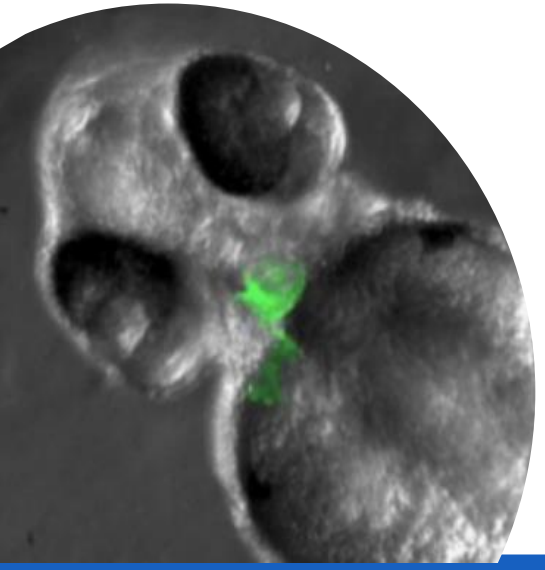
METHOD DESCRIPTION





1 Dose Range Finding (DRF): to evaluate the toxic profile of the test compounds and decide which concentrations will be tested in the assay.

- 5 concentrations/comp.: 0.1, 1, 10, 100, 1000 mM.
- 10 embryos/condition treated in 24 well plates (5 per well).
- Control group (vehicle-treated embryos).



2 Developmental Toxicity Assay: estimating the Teratogenic Index (TI) as the ratio between LC50 and EC50 at the stages of 2 and 4 dpf, one per stage analyzed.

- 8 concentrations/comp. based on the results obtained in the previous DRF.
- 15 embryos/condition treated in 24 well plates (5 per well).
- Control group (vehicle-treated embryos) and positive control (retinoic acid).

3 Bioavailability assay: evaluation of the internal dosing at the highest concentration without effect and at the lowest concentration at which malformations are induced by HPLC-Ms-Ms.

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Qualified Teratogenicity Assay in Zebrafish

1 DRF Results:

Day of analysis	2 dpf					4 dpf				
Concentration (µM)	1	10	100	1000	5000	1	10	100	1000	5000
Reference Compound										
Busulfan	10/0/0	10/0/0	9/0/1	8/2/0	9/1/0 **P**	10/0/0	9/1/0	5/4/1	0/10/0	0/0/10 **P**
Cisplatin	10/0/0	9/1/0	9/1/0	0/0/10	0/0/10	8/2/0	0/10/0	0/8/2	0/0/10	0/0/10
Methotrexate	10/0/0	10/0/0	10/0/0	0/7/3	0/0/10 **P**	10/0/0	10/0/0	7/3/0	0/0/10	0/0/10 **P**
Concentration (µM)	1	10	100	1000	10000	1	10	100	1000	10000
Cytarabine	10/0/0	8/2/0	9/1/0	10/0/0	10/0/0	10/0/0	8/2/0	9/1/0	9/1/0	8/2/0
Fluconazole	9/0/1	9/1/0	10/0/0	10/0/0	10/0/0	9/0/1	9/1/0	9/1/0	8/2/0	3/7/0
5-Fluorouracil	9/0/1	9/0/1	10/0/0	8/0/2	9/0/1	9/0/1	9/0/1	9/1/0	8/0/2	3/6/1
Hydroxyurea	10/0/0	10/0/0	9/0/1	8/1/1	7/1/2	10/0/0	9/1/0	9/0/1	8/1/1	3/5/2
Pomalidomide	10/0/0	10/0/0	10/0/0	10/0/0	10/0/0 **P**	10/0/0	10/0/0	10/0/0	7/3/0	9/1/0 **P**
Ribavirin	10/0/0	10/0/0	9/0/1	9/1/0	10/0/0	10/0/0	10/0/0	8/1/1	8/1/1	4/6/0
Saxagliptin	10/0/0	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0	10/0/0	10/0/0	9/0/1	9/1/0
Thalidomide	10/0/0	9/1/0	8/2/0	9/0/1	10/0/0	9/0/1	9/1/0	9/1/0	5/4/1	6/4/0
Topiramate	10/0/0	9/1/0	5/4/1	2/8/0	0/8/2 **P**	9/1/0	9/1/0	0/9/1	0/4/6	0/0/10 **P**
Vildagliptin	10/0/0	9/0/1	9/0/1	9/0/1	10/0/0	10/0/0	9/0/1	9/0/1	9/0/1	10/0/0
Concentration (µM)	0.001	0.003	0.01	0.03	0.1	0.001	0.003	0.01	0.03	0.1
Acitretin	10/0/0	10/0/0	7/3/0	0/10/0	0/0/10	8/1/1	9/1/0	0/10/0	0/5/5	0/0/10
Concentration (µM)	0.001	0.01	0.1	0.3	1	0.001	0.01	0.1	0.3	1
Isotretinoin	10/0/0	9/0/1	0/10/0	0/10/0	0/9/1	10/0/0	9/0/1	0/7/3	0/0/10	0/0/10

Green: not affected.
Orange: affected (more than 20% of the embryos showing developmental alterations and/or dead).
Red: 100% dead embryos.
P: precipitation.

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Qualified Teratogenicity Assay in Zebrafish

1 DRF Results:

Day of analysis	2 dpf					4 dpf				
Reference Compound										
Concentration (µM)	0.1	1	10	100	1000	0.1	1	10	100	1000
Aspirin-1	10/0/0	10/0/0	10/0/0	10/0/0	9/1/0	9/1/0	10/0/0	10/0/0	10/0/0	10/0/0
Bosentan Hydrate	10/0/0	10/0/0	9/1/0	10/0/0	9/1/0	8/2/0	9/1/0	9/1/0	8/2/0	0/5/5
Carbamazepine	10/0/0	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0	10/0/0	10/0/0	9/1/0	8/2/0
Cetirizine	10/0/0	8/0/2	10/0/0	9/1/0	8/0/2	10/0/0	8/0/2	10/0/0	10/0/0	7/1/2
Cyclophosphamide	10/0/0	10/0/0	10/0/0	4/6/0	0/7/3	9/1/0	10/0/0	9/1/0	2/8/0	0/0/10
Dabrafenib	10/0/0	10/0/0	2/8/0	0/6/4 **P**	0/3/7 **P**	10/0/0	9/1/0	0/10/0	0/0/10 **P**	0/0/10 **P**
Dasatinib	9/1/0	9/1/0	5/5/0	0/10/0	0/10/0 **P**	1/9/0	0/10/0	0/10/0	0/0/10	0/0/10 **P**
Ibrutinib	10/0/0	9/1/0	0/10/0	0/0/10 P	0/0/10 P	9/1/0	9/1/0	0/5/5	0/0/10 P	0/0/10 P
Ibuprofen	10/0/0	10/0/0	9/1/0	7/3/0	0/0/10	10/0/0	10/0/0	8/2/0	0/10/0	0/0/10
Imatinib	8/1/1	8/1/1	9/0/1	9/1/0	0/0/10 **P**	7/2/1	8/1/1	8/1/1	2/7/3	0/0/10 **P**
Pazopanib	10/0/0	5/5/0	0/10/0 **P**	0/10/0 **P**	0/10/0 **P**	9/1/0	0/10/0	0/7/3 **P**	0/9/1 **P**	0/9/1 **P**
Phenytoin	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0 **P**	9/1/0	10/0/0	10/0/0	9/0/1	9/1/0 **P**
Tretinoin	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0	0/0/10	0/0/10	0/0/10	0/0/10	0/0/10
Tacrolimus	9/0/1	2/8/0	0/10/0	0/10/0	0/10/0 **P**	6/4/0	0/10/0	0/10/0	0/0/10	0/0/10 **P**
Trimethadione	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0	9/1/0	10/0/0	10/0/0	10/0/0	10/0/0
Valproic acid	10/0/0	10/0/0	10/0/0	9/0/1	0/9/1	10/0/0	8/2/0	10/0/0	7/2/1	0/0/10
Vismodegib	10/0/0	7/2/1	10/0/0	9/1/0 **P**	9/0/1 **P**	10/0/0	7/2/1	10/0/0	7/3/0 **P**	10/0/0 **P**

Green: not affected.
Orange: affected (more than 20% of the embryos showing developmental alterations and/or dead).
Red: 100% dead embryos.
P: precipitation.

Qualified Teratogenicity Assay in Zebrafish

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2 Developmental Toxicity Assay:

Results Evaluation: based on LC50 (mortality) and EC50 values (number of abnormal embryos per endpoint), a Teratogenic Index (TI) was calculated as the ratio LC50/EC50 for each time point.
TI cut-off value of 2 b**ased on Selderslaghs et al. 2012 and Biobide's internal validation gives** high specificity to the assay in comparison to animal data.

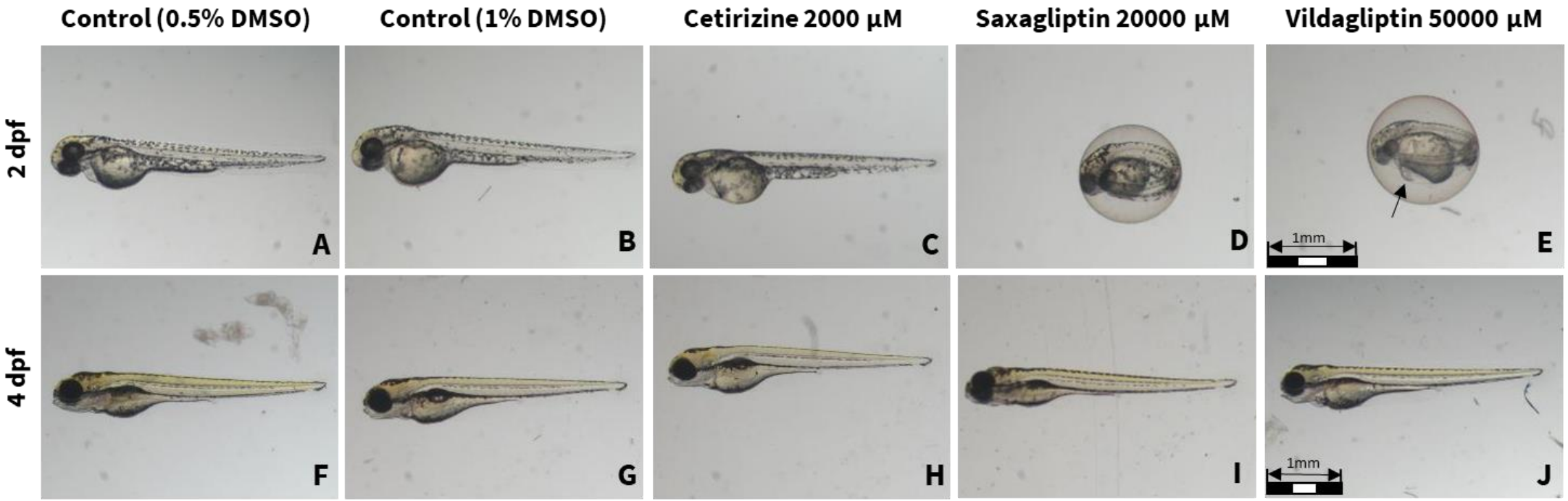
Classification:

- TI ≥ 2: Teratogenic
- TI < 2: Toxic but not teratogenic
- Not toxic for zebrafish embryos

		2 dpf	4 dpf
Craneofacial malformation	Altered jaw morphology		X
	Smaller/abnormal head shape	X	X
	Smaller/abnormal eye shape	X	X
	Presence of Edema	X	X
Malformation of the otic vesicle	Malformed or missing otic vesicle		X
Malformation of the heart	Presence of Edema/irregular shape	X	X
	Abnormal heartbeat	X	X
	Absence heartbeat	X	X
Deformed body shape	Abnormal length	X	X
	Curved axis	X	X
	Malformed or missing trunk	X	X
	Notochord malformation	X	X
	Malformed, disorganized or missing somites	X	X
Malformation of the caudal fin	Malformed or missing caudal fin	X	X
Yolk deformation	Presence of Edema	X	X
	Presence of opacity	X	X
Necrotic tissues	Presence of necrosis	X	X
Hatching	Delayed Hatching		X
Other		X	X

Qualified Teratogenicity Assay in Zebrafish

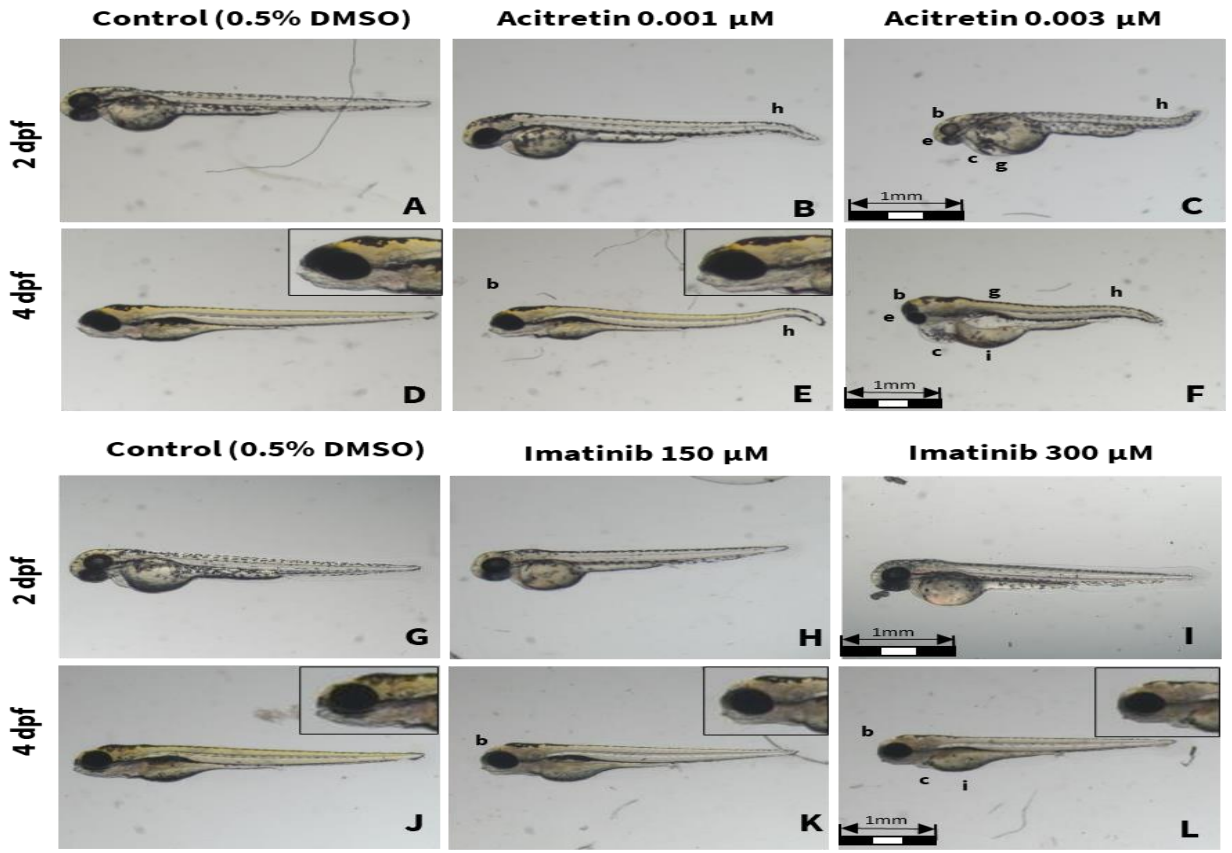
2 Developmental Toxicity Assay results:



Representative pictures from embryos at 2 dpf (A-E) and 4 dpf (F-J) dpf treated with **vehicle control** (A and F at 0.5% DMSO, while B and G at 1% DMSO) and **negative reference compounds** at the highest tested concentrations (Cetirizine C and H; Saxagliptin D and I; Vildagliptin E and J). The black arrow showed heart edema in E.

Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:



Representative pictures from embryos at 2 dpf (A-C and H-I) and 4 dpf (D-F and J-L) dpf treated with **vehicle control** (A, D, G, and J at 0.5% DMSO)**, Acitretin** (B-C and E-F)**,** and **Imatinib** (H-I and K-L) at the indicated concentrations. Defects were highlighted with different letters coding for: microcephaly (b), pericardial edema (c), microphthalmia (e), yolk sac edema (g), trunk alteration (h), and yolk opacity (i). In pictures D, E, J, K, and L, insets of magnified cephalic regions were included for better visualization of microcephaly defects.

General Picture Legends

a) Hydrocephaly
b) Microcephaly
c) Pericardial edema
d) Necrotic tissue
e) Microphthalmia
f) Jaw/facial alterations
g) Trunk alterations
h) Yolk opacity
i) Others

Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Acitretin	6.1	R1	0.0003	0.0003	0.0009 (Interrupted)	0.0003 (Interrupted)	> 0.100	0.0063 (Interrupted)	>100	21.00	Teratogenic
		R2	0.0003	0.0003	~ 0.0010 (Very wide)	0.0003 (Interrupted)	> 0.100	0.0032 (Interrupted)	>100	10.66	
		R3	0.0003	0.0003	0.00063 (Interrupted)	0.00089 (Interrupted)	> 0.100	0.0031 (Interrupted)	>100	3.48	
Aspirin	1.2	R1	2000	2000	> 2000	~2094 (Very wide)	> 2000	>2000	-	-	Toxic
		R2	1000	1000	~2060 (Very wide)	1484 (1386 to 1588)	2083 (Interrupted)	2047 (interrupted)	1.01	1.38	
		R3	2000	1000	> 2000	1963 (Interrupted)	> 2000	2083 (Interrupted)	-	1.06	
Bosentan	3.8	R1	700	150	> 2000	280.8 (250.4 to 315.0)	> 2000	1435 (1210 to 1701)	-	5.11	Teratogenic
		R2	1000	250	> 2000	506.4 (292.1 to 878.0)	> 2000	1402 (Very wide)	-	2.77	
		R3	150	150	> 2000	262.3 (235.3 to 292.5)	-	1143 (1037 to 1261)	-	4.36	
Busulfan	-0.5	R1	500	200	1068 (1018 to 1121)	526.9 (453.0 to 612.9)	-	2055 (1927 to 2191)	-	3.90	Teratogenic
		R2	50	50	899.7 (372.4 to 2173)	1754 (38.57 to 79736)	-	> 5000	-	> 2.85	
		R3	200	200	3158 (Very wide)	~ 444.4 (Very wide)	-	~ 4971 (Very wide)	-	~11.19	
Carbamazepine	2.5	R1	300	100	608.7 (565.1 to 655.7)	229.3 (195.0 to 269.8)	-	591.6 (565.9 to 618.4)	-	2.58	Teratogenic
		R2	500	200	723.2 (Interrupted)	257.1 (Interrupted)	-	>1000	-	-	
		R3	200	150	302.3 (276.1 to 331.0)	213.3 (189.7 to 239.8)	> 1000	847.7 (704.1 to 1020)	> 3.31	3.97	

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Orange: Inconclusive
Dash (-): parameter could not be calculated

Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Cisplatin	-2.35	R1	100	2	320.9 (Very wide)	3.16 (2.68 to 3.71)	549.2 (Very wide)	157.8 (114.8 to 217.0)	1.71	49.94	Teratogenic
		R2	300	2	1079 (776.3 to 1499)	29.76 (16.48 to 53.75)	-	548.2 (Very wide)	-	18.42	
		R3	30	2	409.2 (217.7 to 769.1)	~ 4.895 (Very wide)	~ 884.4 (Very wide)	359.8 (249.4 to 519.1)	2.16	73.5	
Cyclophosphamide	0.6	R1	400	400	898.0 (488.3 to 1652)	467.5 (230.5 to 948.3)	>10000	2731 (2236 to 3335)	-	5.84	Teratogenic
		R2	400	200	2119 (Very wide)	365.4 (264.8 to 504.3)	>10000	~ 3161	-	8.65	
		R3	800	400	~ 1542 (Very wide)	565.6 (528.6 to 605.2)	-	~ 2915 (Very wide)	-	5.15	
Cytarabine	-2.1	R1	> 50000	> 50000	-	-	-	-	-	-	Not toxic
		R2	> 50000	> 50000	-	-	-	-	-	-	
		R3	> 50000	> 50000	-	-	-	-	-	-	
Dabrafenib	4.8	R1	2	4	6.88 (5.55 to 8.51)	4.48 (3.97 to 5.05)	>30	39.68 (35.01 to 44.96)	> 4.36	8.86	Teratogenic
		R2	15	4	18.31 (3.070 to 109.2)	7.91 (6.739 to 9.290)	>100	31.16 (26.27 to 36.96)	> 5.46	3.94	
		R3	8	4	15.05 (Interrupted)	6.31 (5.450 to 7.301)	>100	30.59 (Interrupted)	> 6.74	4.85	
Dasatinib	3.6	R1	1	0.03	9.88 (Interrupted)	0.060 (0.047 to 0.078)	>100	24.22 (19.61 to 29.92)	-	>100	Teratogenic
		R2	1	0.03	~ 10.12 (Ambiguous)	0.040 (0.018 to 0.088)	>100	319.9 (135.0 to 758.3)	-	>100	
		R3	1	0.03	10.11 (Interrupted)	0.033 (Interrupted)	>100	57.29 (7.599 to 432.0)	-	>100	
Fluconazole	0.4	R1	10000	7000	15755 (12634 to 19648)	8586 (7365 to 10010)	>20000	20112 (Very wide)	-	2.34	Teratogenic
		R2	7000	4000	-	8261 (6798 to 10038)	>20000	>20000	-	> 2.42	
		R3	10000	4000	>30000	2001 (1226 to 3266)	>30000	28973 (22025 to 38114)	-	14.48	

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Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
5-Fluorouracil	-0.9	R1	30000	5000	48419 (44166 to 53083)	9294 (8401 to 10281)	>50000	>50000	-	> 5.38	Teratogenic
		R2	1000	5000	-	8261 (6798 to 10038)	-	>50000	-	> 6.05	
		R3	>50000	5000	>50000	9374 (8642 to 10169)	>50000	>50000	-	> 5.33	
Hydroxyurea	-1.8	R1	20000	5000	29333 (27685 to 31080)	7355 (6477 to 8351)	-	48759 (45149 to 52656)	-	6.63	Teratogenic
		R2	15000	2000	20691 (18928 to 22619)	6756 (6132 to 7444)	~ 48283 (Very wide)	46171 (Very wide)	~2.33	6.83	
		R3	1000	5000	15276 (13515 to 17266)	7661 (Interrupted)	34516 (31906 to 37339)	34516 (31906 to 37339)	2.26	4.50	
Ibrutinib	3.6	R1	8	2	9.22 (Very wide)	2.67 (2.48 to 2.87)	22.68 (Very wide)	8.15 (Interrupted)	2.46	3.05	Teratogenic
		R2	4	2	9.08 (5.79 to 14.20)	2.98 (2.061 to 4.300)	~ 30.89 (Very wide)	8.98 (3.425 to 23.54)	~3.40	3.02	
		R3	4	2	7.97 (Interrupted)	3.80 (Interrupted)	~ 22.68 (Very wide)	7.85 (Interrupted)	2.84	2.07	
Ibuprofen	3.5	R1	40	20	119.1 (106.0 to 133.7)	37.78 (36.56 to 39.04)	321.6 (Interrupted)	235.2 (220.0 to 251.5)	2.70	6.23	Teratogenic
		R2	80	40	172.9 (169.4 to 176.4)	88.11 (79.99 to 97.05)	315.9 (Interrupted)	296.5 (Interrupted)	1.83	3.37	
		R3	40	20	96.05 (86.71 to 106.4)	48.49 (40.49 to 58.08)	350.6 (129.0 to 953.4)	~ 160.9 (Very wide)	3.65	3.32	
Imatinib	3.5	R1	600	20	647.8 (Very wide)	31.98 (25.97 to 39.39)	631.4 (Very wide)	290.1 (271.9 to 309.5)	~1.00	9.07	Teratogenic
		R2	300	20	584.9 (553.4 to 618.3)	36.44 (34.35 to 38.65)	601.6 (Interrupted)	315.6 (187.2 to 532.0)	1.03	8.66	
		R3	300	20	562.2 (Interrupted)	48.03 (46.56 to 49.54)	605.1 (Interrupted)	~ 301.3 (Very wide)	1.07	6.27	

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Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Isotretinoin	6.3	R1	0.015	0.006	0.047 (0.030 to 0.074)	0.009 (0.007 to 0.012)	>1.00	0.346 (Very wide)	-	37.20	Teratogenic
		R2	0.040	0.006	0.063 (0.053 to 0.075)	0.017 (0.013 to 0.023)	>1.00	~ 0.339 (Very wide)	-	19.93	
		R3	0.015	0.006	0.059 (0.057 to 0.061)	0.021 (0.020 to 0.022)	>1.00	0.549 (Very wide)	-	25.94	
Methotrexate	-1.8	R1	300	80	321.8 (Very wide)	87.48 (79.53 to 96.22)	934.2 (895.8 to 974.3)	472.8 (439.8 to 508.3)	2.90	5.40	Teratogenic
		R2	150	80	~ 226.8 (Very wide)	184.9 (172.3 to 198.4)	1081 (Interrupted)	~ 580.6 (Very wide)	~4.77	~3.14	
		R3	300	40	444.6 (397.8 to 497.0)	70.71 (57.74 to 86.58)	868.0 (827.1 to 910.9)	423.6 (Very wide)	1.95	5.99	
Pazopanib	3.1	R1	0.6	0.1	1.34 (1.20 to 1.49)	0.39 (0.36 to 0.42)	-	> 20	-	> 51.28	Teratogenic
		R2	1.2	0.3	1.27 (Interrupted)	0.46 (Interrupted)	-	> 20	-	> 43.48	
		R3	0.6	0.1	3.54 (Very wide)	0.44 (0.37 to 0.51)	-	> 20	-	> 45.45	
Phenytoin	2.5	R1	100	100	-	-	-	-	-	-	Inconclussive
		R2	>150	75	-	-	-	-	-	-	
		R3	>150	75	-	135.1 (99.63 to 183.3)	-	-	-	-	
Pomalidomide	0.2	R1	>200	>150	-	-	-	-	-	-	Inconclussive
		R2	>500	>150	-	-	-	-	-	-	
		R3	200	150	422.5 (280.2 to 636.8)	188.4 (187.9 to 188.9)	-	-	-	-	
Ribavirin	-1.8	R1	20000	5000	29279 (23297 to 36797)	8670 (7501 to 10020)	-	>50000	-	> 5.77	Teratogenic
		R2	10000	10000	34566 (24740 to 48294)	12248 (Very wide)	49275 (Interrupted)	~ 48672 (Very wide)	1.42	3.97	
		R3	5000	5000	38678 (Very wide)	8319 (6883 to 10054)	38695 (Very wide)	38678 (Very wide)	1.00	4.65	

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Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Tacrolimus	2.7	R1	0.3	0.03	1.31 (0.932 to 1.84)	0.080 (0.068 to 0.092)	-	45.72 (Very wide)	-	>100	Teratogenic
		R2	0.1	0.1	0.188 (0.172 to 0.207)	0.173 (0.142 to 0.211)	-	35.21 (Interrupted)	-	>100	
		R3	0.1	0.03	-	0.089 (0.077 to 0.102)	0.461 (0.214 to 0.992)	31.63 (Very wide)	-	>100	
Thalidomide	0.3	R1	600	600	-	-	-	-	-	-	Inconclussive
		R2	600	600	-	874.3 (459.1 to 1665)	-	-	-	-	
		R3	600	600	-	782.0 (503.5 to 1214)	-	-	-	-	
Topiramate	-0.8	R1	100	20	319.0 (162.2 to 627.5)	50.58 (Interrupted)	>5000	497.9 (477.4 to 519.4)	-	9.84	Teratogenic
		R2	20	100	37.52 (21.17 to 66.47)	172.8 (Very wide)	-	279.6 (209.3 to 373.59)	-	1.62	
		R3	100	20	603.6 (435.3 to 836.9)	45.53 (39.47 to 52.52)	-	521.7 (303.0 to 898.5)	-	11.46	
Tretinoin	6.3	R1	0.0002	0.0002	0.00071 (0.00062 to 0.00081)	0.00046 (0.00040 to 0.00051)	0.051 (Very wide)	0.0097 (0.0087 to 0.0109)	71.83	21.09	Teratogenic
		R2	0.0002	0.0002	0.000811 (0.000724 to 0.000909)	0.000214 (Interrupted)	78.98 (Very wide)	0.02294 (0.01863 to 0.02825)	>100	>100	
		R3	0.0002	0.0002	0.0003521 (Interrupted)	0.0000346 (Interrupted)	0.03524 (Interrupted)	0.002936 (0.002550 to 0.003381)	>100	84.86	

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Qualified Teratogenicity Assay in Zebrafish

2 Developmental Toxicity Assay results:

Reference Compounds	XLogP3	R	NOAEL (µM)		EC50 (µM)		LC50 (µM)		TIs		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Trimethadione	0.3	R1	10000	5000	14659 (13656 to 15736)	6715 (6079 to 7417)	47509 (Interupted)	34330 (20502 to 57484)	3.24	5.11	Teratogenic
		R2	20000	5000	24506 (23545 to 25505)	~ 9930 (Very wide)	34849 (19631 to 61864)	35208 (14281 to 86796)	1.42	3.55	
		R3	20000	5000	24474 (Very wide)	8784 (7925 to 9736)	38781 (Very wide)	29795 (24118 to 36809)	1.58	3.39	
Valproic acid	3.4	R1	400	100	663.8 (589.8 to 747.2)	148.9 (139.4 to 159.1)	-	1016 (Very wide)	-	6.82	Teratogenic
		R2	100	100	331.4 (273.4 to 401.7)	203.7 (174.4 to 237.9)	-	~ 1480 (Very wide)	-	~7.27	
		R3	200	100	734.0 (429.4 to 1255)	175.7 (166.8 to 185.0)	-	1529 (1353 to 1729)	-	8.70	
Vismodegib	3.8	R1	>100	>100	-	-	-	-	-	-	Inconclusive
		R2	70	25	-	85.01 (71.19 to 101.5)	-	-	-	-	
		R3	>100	15	-	31.72 (25.70 to 39.16)	-	-	-	-	

NEGATIVE CONTROLS COMPOUND NAME	XLogP3	R	NOEL (µM)		EC50 (µM)		LC50 (µM)		TI		Classification
			2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Cetirizine Dyhydrochloride	1.7	R1	2000	2000	-	-	-	-	-	-	Not toxic
		R2	2000	2000	-	-	-	-	-	-	
		R3	> 2000	800	-	-	-	-	-	-	
Saxagliptin	0.7	R1	> 20000	> 20000	-	-	-	-	-	-	Not toxic
		R2	> 20000	> 20000	-	-	-	-	-	-	
		R3	> 20000	> 20000	-	-	-	-	-	-	
Vildagliptin	0.9	R1	> 50000	> 50000	-	-	-	-	-	-	Not toxic
		R2	> 50000	> 50000	-	-	-	-	-	-	
		R3	> 50000	> 50000	-	-	-	-	-	-	

Green: Correctly Classified
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Orange: Inconclusive
Dash (-): parameter could not be calculated

Qualified Teratogenicity Assay in Zebrafish

3 Bioavailability Analysis:

REFERENCE COMPOUNDS	XLogP3	R	Absorption (%)		Solubility (%)		Classification
			NOEL	LEL	NOEL	LEL	
Acitretin	6.1	R1	> 100	> 100	~ 100	57	Teratogenic
		R2	> 100	> 100	~ 100	52	
		R3	> 100	> 100	~ 100	56	
Aspirin	1.2	R1	-	< 1	-	28	Toxic (low pH)
		R2	-	< 1	-	25	
		R3	-	< 1	-	25	
Bosentan	3.8	R1	31	17	~ 100	~ 100	Teratogenic
		R2	12	34	~ 100	~ 100	
		R3	43	34	~ 100	~ 100	
Busulfan	-0.5	R1	18	27	~ 100	~ 100	Teratogenic
		R2	49	61	~ 100	~ 100	
		R3	6	16	~ 100	~ 100	
Carbamazepine	2.5	R1	~ 100	~ 100	78	74	Teratogenic
		R2	~ 100	~ 100	82	75	
		R3	~ 100	~ 100	70	64	
Cisplatin	-2.35	R1	> 100	> 100	95	98	Teratogenic
		R2	> 100	> 100	98	98	
		R3	> 100	> 100	99	99	
Cyclophosphamide	0.6	R1	13	10	62	-	Teratogenic
		R2	12	10	49	-	
		R3	14	5	44	-	
Cytarabine	-2.1	R1	-	2	-	~ 100	Not toxic (low penetration)
		R2	-	5	-	~ 100	
		R3	-	22	-	~ 100	
Dabrafenib	4.8	R1	26	28	59	98	Teratogenic
		R2	29	55	66	89	
		R3	42	28	56	79	
Dasatinib	3.6	R1	97	91	88	~ 100	Teratogenic
		R2	97	98	-	-	
		R3	~ 100	~ 100	14	84	

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Qualified Teratogenicity Assay in Zebrafish

3 Bioavailability Analysis:

REFERENCE COMPOUNDS	XLogP3	R	Absorption (%)		Solubility (%)		Classification
			NOEL	LEL	NOEL	LEL	
Fluconazole	0.4	R1	13	8	~ 100	86	Teratogenic
		R2	7	10	~ 100	83	
		R3	26	12	~ 100	90	
5-Fluorouracil	-0.9	R1	< 1	1	6	7	Teratogenic
		R2	2	1	6	7	
		R3	2	4	5	8	
Hydroxyurea	-1.8	R1	< 1	< 1	2	8	Teratogenic
		R2	-	< 1	-	4	
		R3	< 1	< 1	3	4	
Ibrutinib	3.6	R1	~ 100	> 100	~ 100	~ 100	Teratogenic
		R2	82	> 100	~ 100	~ 100	
		R3	~ 100	> 100	~ 100	~ 100	
Ibuprofen	3.5	R1	> 100	> 100	~ 100	~ 100	Teratogenic
		R2	> 100	> 100	~ 100	~ 100	
		R3	> 100	> 100	~ 100	~ 100	
Imatinib	3.5	R1	> 100	> 100	~ 100	~ 100	Teratogenic
		R2	~ 100	> 100	~ 100	~ 100	
		R3	> 100	~ 100	~ 100	~ 100	
Isotretinoin	6.3	R1	> 100	~ 100	-	-	Teratogenic
		R2	> 100	93	-	-	
		R3	> 100	~ 100	-	-	
Methotrexate	-1.8	R1	< 1	< 1	94	83	Teratogenic
		R2	< 1	< 1	95	99	
		R3	1	2	61	91	
Pazopanib	3.1	R1	> 100	> 100	7	7	Teratogenic
		R2	> 100	> 100	3	2	
		R3	> 100	53	8	1	

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Qualified Teratogenicity Assay in Zebrafish

3 Bioavailability Analysis:

REFERENCE COMPOUNDS	XLogP3	R	Absorption (%)		Solubility (%)		Classification
			NOEL	LEL	NOEL	LEL	
Phenytoin	2.5	R1	-	87	-	55	Inconclusive
		R2	-	48	-	42	
		R3	-	22	-	46	
Pomalidomide	0.2	R1	-	33	-	22	Inconclusive
		R2	-	17	-	16	
		R3	-	31	-	15	
Ribavirin	-1.8	R1	< 1	< 1	47	47	Teratogenic
		R2	9	1	30	43	
		R3	< 1	< 1	32	73	
Tacrolimus	2.7	R1	> 100	> 100	-	-	Teratogenic
		R2	> 100	> 100	-	-	
		R3	> 100	> 100	-	-	
Thalidomide	0.3	R1	-	4	-	10	Inconclusive (low penetration)
		R2	-	6	-	10	
		R3	-	5	-	10	
Topiramate	-0.8	R1	11	10	~ 100	~ 100	Teratogenic
		R2	7	3	~ 100	~ 100	
		R3	3	6	~ 100	~ 100	
Tretinoin	6.3	R1	> 100	> 100	~ 100	88	Teratogenic
		R2	> 100	> 100	~ 100	84	
		R3	> 100	> 100	~ 100	90	
Trimethadione	0.3	R1	6	3	68	85	Teratogenic
		R2	2	3	72	83	
		R3	3	13	69	68	
Valproic acid	3.4	R1	> 100	> 100	~ 100	~ 100	Teratogenic
		R2	> 100	> 100	~ 100	~ 100	
		R3	> 100	> 100	~ 100	~ 100	

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Qualified Teratogenicity Assay in Zebrafish

3 Bioavailability Analysis:

REFERENCE COMPOUNDS	XLogP3	R	Absorption (%)		Solubility (%)		Classification
			NOEL	LEL	NOEL	LEL	
Vismodegib	3.8	R1	-	-	-	-	Inconclusive
		R2	-	14	-	58	
		R3	-	21	-	84	
NEGATIVE CONTROLS							
COMPOUND NAME	XLogP3	R	Absorption (%)		Solubility (%)		Classification
			NOEL	LEL	NOEL	LEL	
Cetirizine	1.7	R1	-	32	-	~ 100	Not toxic
		R2	-	8	-	~ 100	
		R3	-	18	-	~ 100	
Saxagliptin	0.7	R1	-	5	-	66	Not toxic
		R2	-	6	-	78	
		R3	-	4	-	87	
Vildagliptin	0.9	R1	-	6	-	86	Not toxic
		R2	-	6	-	80	
		R3	-	-	-	94	

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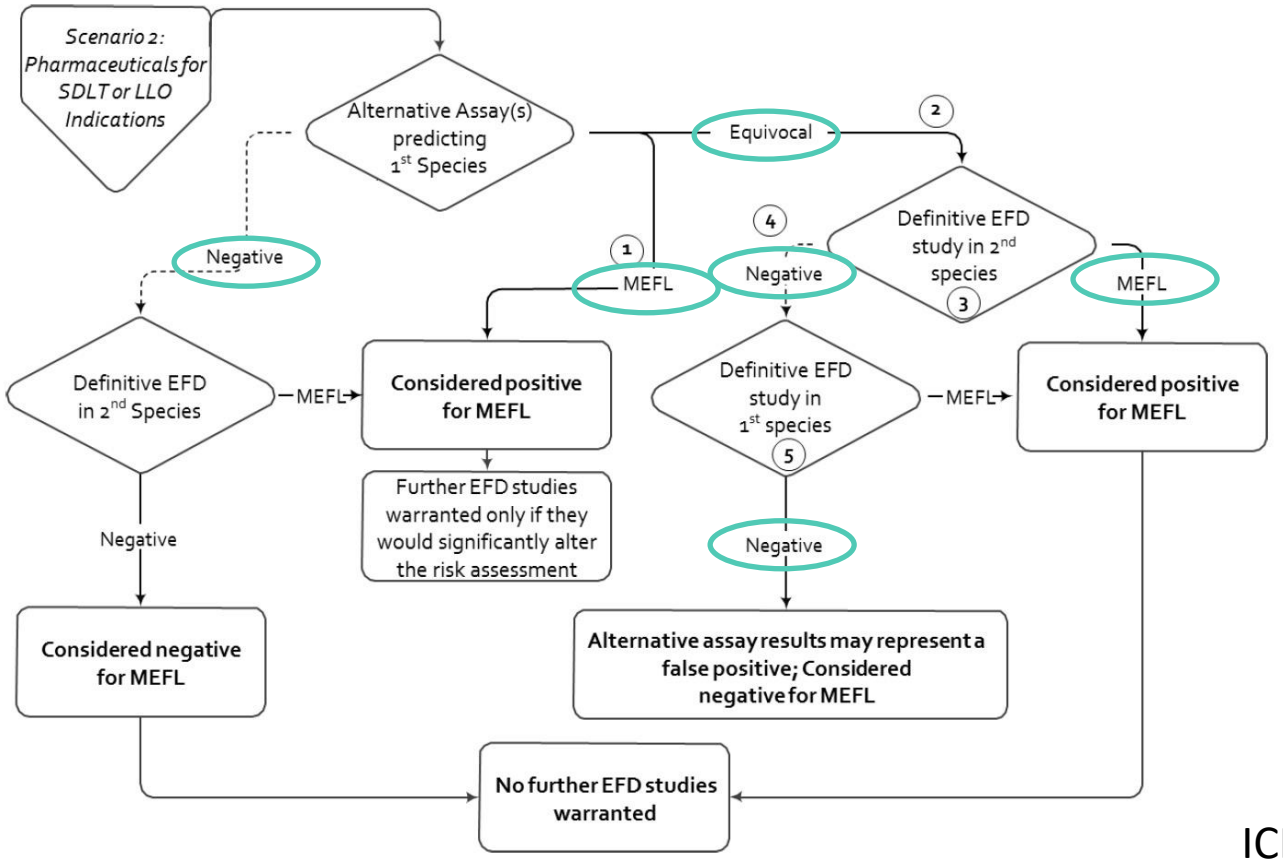
Qualified Teratogenicity Assay in Zebrafish for DART

Application of Zebrafish DART Assay Safety



ICHS5(R3) figure 2 Annex 2, p39.

*MEFL = malformations and embryo-fetal lehality.

Conclusions on the Qualification of a Developmental Toxicity Assay in NAMs

biobidel *Your Zebrafish Partner*

- The **32 reference compounds** indicated by the updated ICH S5 (R3) guideline were tested in the Zebrafish Developmental Toxicity Assay:
  - **23/29 positive compounds** evaluated were **correctly classified ----** True positives (TP)
  - **3/3 negative compounds** were **correctly identified ----** True negatives (TN)

- 6 /29 positive compounds not properly identified:
  - **Aspirin:** classified as **toxic** instead of teratogenic **----** False negative (FN)
  - **Cytarabine:** classified as **not toxic due to low penetration,** with **limited solubility** confirmed with the bioavailability assay, and coherent with the LogP – 2.1 **----** False Negative (FN)
  - **Phenytoin, Pomalidomide, Thalidomide, and Vismodegib gave inconclusive results:** the number of concentrations assayed was restricted due to limited solubility in the medium, as bioavailability results showed.

→ **Bioavailability analysis** (in zebrafish embryos and media) conducted in triplicates allowed the refinement of some of those **inconclusive results.**

- Zebrafish Developmental Toxicity Assay gave very good results in Accuracy, Sensitivity, Specificity, and Repeatability compared **to rodents and rabbit available data**, therefore, it is a well-integrated strategy to minimize the use of animals in reproductive and/or developmental toxicity studies, complementing and even substituting them:

SENSITIVITY = 88% **SPECIFICITY = 100%** **REPEATABILITY = 100%** **ACCURACY = 90%**

Questions & Answers

THANK YOU

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