VALIDATION OF ZEBRAFISH EMBRYOTOXICITY TEST (ZET) AS A QUALIFIED ALTERNATIVE ASSAY FOR ITS REGULATORY USE

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OBJECTIVE

Validation of zebrafish embryotoxicity test (ZET) as potential alternative for developmental toxicity evaluation

INTRODUCTION

The new ICH S5 (R3) guideline on reproductive toxicology that applies to all pharmaceuticals for which reproductive and/or developmental toxicity studies are appropriate, proposes the use of alternative testing assays as part of an integrated testing strategy to minimize the use of animals. The guide does not recommend specific assays, but provides a Reference Compound List that contains 29 compounds that have been shown to induce specific malformation or embryo-fetal lethality plus 3 negative compounds that can be used to support the qualification of an alternative assay.

Our research focused on the predictivity of **the zebrafish developmental toxicity assay**. Zebrafish embryo model is highly popular in toxicology and provides an ethically acceptable small-scale analysis system with the complexity of a complete organism. This model enables continuous developmental monitoring and has been widely used for the generation of relevant answers on mammalian developmental hazard. Our goal is to further validate this model for its regulatory use by testing the 32 (29 + 3) compounds indicated in the new ICH S5 guideline. To determine the teratogenic risk of these chemicals, the presence of morphological alterations was analyzed at two different stages and a Teratogenic Index (TI) was established as the ratio between LC50 and EC50.

Results presented in this poster correspond to the first replicate of a total of three replicates that are been conducted to study the reproducibility of the assay. Evaluation of the the internal concentration of these chemicals in zebrafish embryos to compare results obtained in this model with the mammalian exposures provided in the guide, will be performed too. This would allow to determine the ability of this assay to predict mammalian embryotoxic exposure.

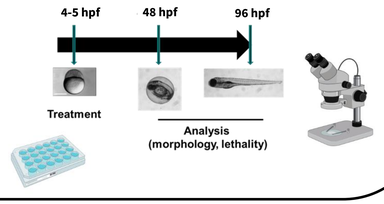
METHODS

Developmental toxicity assay:

1. Dose Range Finding (DRF): 4-5 hours post fertilization (hpf) embryos (10 embryos per experimental condition) were initially treated with a wide range of concentrations (from 0.1 to 1000 µM for compounds with LogP > 1 or from 1 to 10000 µM for compounds with LogP ≤ 1) to determine the relevant concentrations for developmental toxicity assessment.

2. Evaluation of developmental toxicity: embryos from the same stage (15 embryos per experimental condition) were treated with 8 concentrations per compound selected based on the previous DRF experiment. Detailed analysis of embryo morphology and lethality was performed at 2 and 4 dpf. EC50 (Half Maximal Effective Concentration) and LC50 (Lethal Concentration 50) were calculated with the percentage of affected and dead embryos applying a nonlinear regression test (sigmoidal dose-response curve). A teratogenic Index (TI) was estimated as the ratio between LC50/EC50 and compounds were classified as follow:

- Compounds with a TI>2: *Likely teratogenic*
- Compounds with a TI<2: *Toxic but likely teratogenic*
- Compounds that did not induce any toxicity manifestation: *Not toxic*
- Concentrations evaluated limited by compound precipitation or due to significant change of embryo medium pH: *Uncertain*



RESULTS

Table 1: Summary results.

POSITIVE CONTROLS		XLogP3	NOEL (µM)		EC50 (µM)		LC50 (µM)		TI		Classification
COMPOUND NAME	CAS NUMBER		2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Acitretin	55079-83-9	6.1	0,0003	0,0001	0.0009 (Interrupted)	0.0003 (Interrupted)	>0.100	0.0063 (Interrupted)	>111.1	21.00	Teratogenic
Aspirin	50-78-2	1.2	2000	2000	-	-	-	-	-	-	Not toxic
Bosentan	147536-97-8	3.8	700	150	2448 (916.7 to 6537)	280.8 (250.4 to 315.0)	>2000	1435 (1210 to 1701)	-	5.11	Teratogenic
Busulfan	55-98-1	-0.5	500	200	1068 (1018 to 1121)	526.9 (453.0 to 612.9)	>2000	2055 (1927 to 2191)	>1.87	3.90	Teratogenic
Carbamazepine	298-46-4	2,5	300	100	608.7 (565.1 to 655.7)	229.3 (195.0 to 269.8)	>500	591.6 (565.9 to 618.4)	-	2.58	Teratogenic
Cisplatin	15663-27-1	NR	100	2	320.9 (Very wide)	3.16 (2.68 to 3.71)	549.2 (0 to +infinity)	157.8 (114.8 to 217.0)	1,71	49.94	Teratogenic
Cyclophosphamide	50-18-0	0,6	400	400	898.0 (488.3 to 1652)	467.5 (230.5 to 948.3)	14381 (10723 to 19287)	2731 (2236 to 3335)	16,01	5.84	Teratogenic
Cytarabine	147-94-4	-2,1	>50000	>50000	-	-	-	-	-	-	Not toxic
Dabrafenib	1195765-45-7	4,8	2	4	6.88 (5.55 to 8.51)	4.48 (3.97 to 5.05)	>30.00	39.68 (35.01 to 44.94)	>4.36	8.86	Teratogenic
Dasatinib	302962-49-8	3,6	1	0,03	9.88 (Interrupted)	0.060 (0.047 to 0.078)	>100	24.22 (19.61 to 29.92)	>10.12	403.7	Teratogenic
Fluconazol	86386-73-4	0,4	10000	7000	15755 (12634 to 19648)	8586 (7365 to 10010)	>20000	20112 (Very wide)	>1.27	2.34	Teratogenic
5-Fluorouracil	51-21-8	-0,9	30000	5000	48419 (44166 to 53083)	9294 (8401 to 10281)	>50000	>50000	>1.03	>5.38	Teratogenic
Hydroxyurea	127-07-1	-1,8	20000	5000	29333 (27685 to 31080)	7355 (6477 to 8351)	54765 (50768 to 59076)	48759 (45149 to 52656)	1,87	6.63	Teratogenic
Ibrutinib	936563-96-1	3,6	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
Ibuprofen	15687-27-1	3,5	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
Imatinib	152459-95-5	3.5	600	10	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
Isotretinoin (13-cis-retinoic acid)	4759-48-2	6,3	0,015	0,006	0.047 (0.030 to 0.074)	0.0093 (0.0073 to 0.012)	>1.00	0.346 (Very wide)	>21.28	37.20	Teratogenic
Methotrexate	59-05-2	-1,8	300	40	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
Pazopanib	444731-52-6	3,1	0,6	0,1	1.34 (1.20 to 1.49)	0.391 (0.362 to 0.424)	>2.50	>2.50	>1.87	>6.39	Teratogenic
Phenytoin (Diphenylhydantoin)	57-41-0	2,5	>100	>100	-	-	-	-	-	-	Uncertain
Pomalidomide	19171-19-8	0,2	>200	>150	-	-	-	-	-	-	Uncertain
Ribavirin	36791-04-5	-1,8	20000	5000	29279 (23297 to 36797)	8670 (7501 to 10020)	>50000	>50000	>1.71	>5.77	Teratogenic
Tacrolimus	104987-11-3	2,7	0,3	0,03	1.31 (0.932 to 1.84)	0.080 (0.068 to 0.092)	>50.00	45.72 (Very wide)	38,17	571.5	Teratogenic
Thalidomide	50-35-1	0,3	>600	>600	-	-	-	-	-	-	Uncertain
Topiramate	97240-79-4	-0,8	100	20	319.0 (162.2 to 627.5)	50.58 (Interrupted)	>5000	497.9 (477.4 to 519.4)	>15.67	9.84	Teratogenic
Tretinoin (all-trans-retinoic acid)	302-79-4	6,3	0,0005	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
Trimethadione	127-48-0	0,3	10000	5000	14659 (13656 to 15736)	6715 (6079 to 7417)	47509 (Interrupted)	34330 (20502 to 57484)	3,24	5.11	Teratogenic
Sodium valproate	1069-66-5	3.4	400	100	663.8 (589.8 to 747.2)	148.9 (139.4 to 159.1)	2010 (Interrupted)	1016 (Very wide)	3,03	6.82	Teratogenic
Vismodegib	879085-55-9	3,8	>40	>40	-	-	-	-	-	-	Uncertain
NEGATIVE CONTROLS		**XLogP3**	**NOEL (µM)**		**EC50 (µM)**		**LC50 (µM)**		**TI**		**Classification**
COMPOUND NAME	CAS NUMBER		2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	
Cetirizine Dyhydrochloride	83881-52-1	1.7	2000	2000	-	-	-	-	-	-	Not toxic
Saxagliptin	361442-04-8	0,7	>20000	>20000	-	-	-	-	-	-	Not toxic
Vildagliptin	274901-16-5	0.9	>20000	>20000	-	-	-	-	-	-	Not toxic

Identity (including CAS number) and XLogP3 values for the 32 compounds of the ICH S5 (R3) guideline, plus results obtained including NOAEL (Non-Observable Adverse Effects Level), EC50, LC50, TI values (both at 2 and 4 dpf), and final classification are shown in this table. Numbers between brackets indicate the 95% confidence intervals, if intervals were very wide (data do not unambiguously define the parameters) or if fitting was interrupted.

- *Green: correctly classified*
- *Orange: no conclusive results (solubility or pH limitation)*
- *Red: not correctly classified*

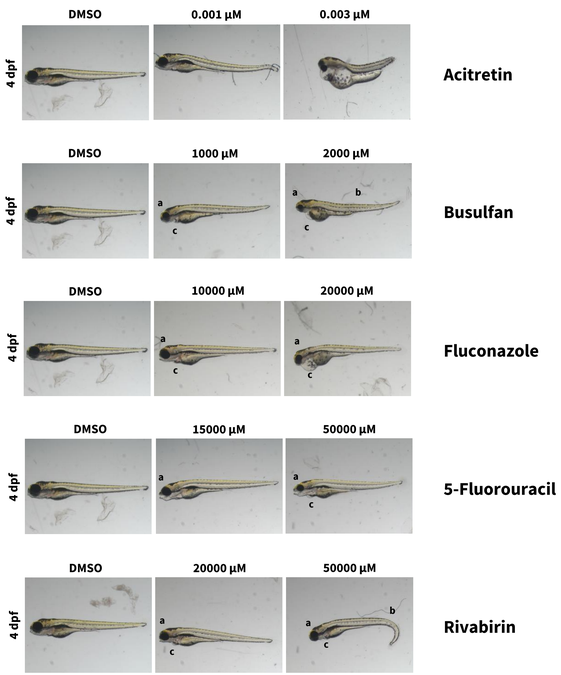


Figure 1. Examples of some of the toxicity manifestations displayed by 4 dpf embryos exposed to the indicated compounds at the specified concentrations. a: craniofacial malformations; b: trunk alterations; c: pericardial edema.

CONCLUSIONS

- **The 32 reference compounds indicated by the new ICH S5 (R3) guideline have been already assayed in the zebrafish developmental toxicity test: i) 23 out of the 29 positive compounds evaluated were correctly classified with this assay. For compounds not properly identified, results obtained for Aspirin and Cytarabine were conclusive. The number of concentrations that could be assayed for the other 4 compounds was restricted due to their limited solubility in embryo medium. ii) all negative compounds were correctly identified.**

SENSITIVITY= 80% **SPECIFICITY= 100%** **ACCURACY= 88%**

FUTURE EXPERIMENTS:

- **These promising results are been further confirmed by increasing the number of replicates for reproducibility estimation.**
- **Internal concentration of these chemicals in zebrafish embryos will be also analyzed to compare results with mammalian exposures.**