Zebrafish embryos and Larvae : a new generation of disease model and drug screens

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Chapter 5: Behavioral profiling of zebrafish larvae exposed to a range of compounds

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This chapter has been accepted in *Behavioural Brain Research*.

Abstract

The zebrafish is a powerful whole animal model which is complementary to *in vitro* and mammalian models. It has been shown to be applicable to the high-throughput behavioral screening of compound libraries. We have analysed 60 water-soluble toxic compounds covering a range of common drugs, toxins and chemicals, and representing various pharmacological mechanisms. Wild-type zebrafish larvae were cultured individually in defined buffer in 96 well plates. They were exposed for a 96 h period starting at 24 hour post fertilization. A logarithmic concentration series was used for range-finding, followed by a narrower geometric series for LC_{50} determination. LC_{50} values were determined at 24 h intervals and behavioural testing was carried out on day 5. We used the visual motor response test, in which movement of individual larvae was analysed using automated video-tracking. For all compounds, LC_{50} values were found to decrease as the embryo developed. The majority of compounds (57/60) produced an effect in both the basal and challenge phases. These effects were either (i) suppression of locomotor activity (monotonic concentration-response); (ii) stimulation then suppression (biphasic response); (iii) stimulation (monotonic response). We conclude that behavioural assays with zebrafish embryos could be useful for pharmaceutical efficacy and toxicity screening. The precise readout obtained with behavioural assay varies with compound class.

Introduction

The discovery of new psychiatric drugs has the lowest success rate compared to other therapeutic arenas [76,336]. One primary reason causing many drugs to be withdrawn before reaching the clinic is the unforeseen neurotoxicity in clinical trials. This occurs because the cell assays used for target discovery and validation in the initial steps of the drug pipeline fail to predict how toxic a given drug will be when tested later in a whole organism. *In vitro* cell assays lack the complexity of whole organisms, and this complexity is necessary to model complex central nervous system (CNS) functions and neurotoxicity pathways. The larval zebrafish model represents a viable solution to these issues as it is becoming increasingly used in toxicology studies conducted in pre-clinical setups [6,11,35]. We recently demonstrated that larval zebrafish toxicology assays relying on mortality as an endpoint, offer high predictive value relative to traditional rodent assays [35].

1.1 The zebrafish model for toxicology research

In some fields of biomedical research such as drug screening, safety pharmacology and toxicity assessment, zebrafish embryo is emerging as a powerful and possible alternative model for the toxicity and teratogenicity of compounds in rodents [35,96,146,217,337,338]. This whole animal model offers a rapid, high-throughput, low-cost assay system in the early stages of the drug development pipeline [18]. There are several advantages of zebrafish and their embryos [refs. in [339]. Some of those are: the zebrafish embryo has a small size, small volume of test compound is required for testing and it has relatively rapid development. The major organ systems are developed at 5 days post fertilization (dpf) [4,27] and many basic cellular and molecular pathways implicated in the response to chemicals or stress are conserved between the zebrafish and mammals [322].

The zebrafish has been extensively used in acute toxicological studies, [reviewed by [13,196,324,339]. Examples include the use of adult zebrafish for the testing of lead and uranium [197], malathion [201], colchicine [198], anilines [199], and metronidazole [200]; and the use of juveniles for testing agricultural biocides [202]. Zebrafish embryos are also being used in toxicity studies (reviewed by [325]). Examples include the use of zebrafish embryos for testing nanoparticles [148,326], the teratogenic effects of ethanol [32,340] as well as other compounds on neurodevelopment [341]. We have recently characterized in the zebrafish embryo model, the toxicity of a selected panel of compounds from different pharmacological classes [35]. Overall, the zebrafish embryo model has been shown to offer a good predictive power for the identification of compounds known to be toxic in rodents [35,96,217,337].

1.2. The use of zebrafish behavioral-based assays for drug safety screening

Zebrafish larvae are emerging models for behavioral testing [32,342,343]. They have numerous qualities that make them complementary to the mammalian models currently used in the behavioral sciences, despite obvious differences between zebrafish and humans. This is because zebrafish have broad homologies to other vertebrate species (including rodents and humans) in terms of their genome, brain patterning, and the structure and function of several neural and physiological systems, including the stress-regulating axis [59,61,62,64–66,69,344–349]. Important systems associated for behavioral functionality such as the monoamines, dopamine, norepinephrine and serotonin are unambiguously present in larval zebrafish [350–354] complementary to adult zebrafish [353,355]. Neural nicotinic acetylcholine receptors (nAChRs) are known to be expressed in zebrafish embryos and mediate nicotine-induced alterations in embryonic morphology [356]. However, the identity of the fish dopamine system that has

functions similar to the mammalian mesolimbic (VTA-NAc) dopamine system is unclear. It is suggested that the posterior tubercle of teleosts may include cells functionally analogous to mesolimbic dopaminergic neurons in mammals [357]; however, this is controversial [354]. On the other hand, the habenula brain structure, which plays a key regulatory role in addiction response, is well interpreted and conserved between zebrafish and humans [358]. Several studies have reported that analysis of swimming activity of larval zebrafish could provide predictive mechanisms of action of unknown or less known compounds [73,343].

Although there are some fundamental similarities between zebrafish and mammals but there are also some importance differences. Some of these are: The fish is ectothermic so that physiology is not identical to humans, and lacks cardiac septa, synovial joints and other structures [1,23,327]. The evolutionary divergence of zebrafish and mammals is around 445 million years ago [204]. Therefore, some toxic effects seen in humans are difficult to model in the zebrafish. Furthermore, the zebrafish embryo remains inside the chorion at least up to 48 hpf [27]. Therefore, the chorion may propose a barrier to compounds diffusion [57,329]. The full list of advantages and disadvantages of the use of zebrafish in the biomedical research was reported [refs. in [339]. Therefore, there is a urgent need for additional validation of the zebrafish model [205].

In the present study, we reason that the inclusion of information related to behavioral phenotypes in addition to data gathered from traditional LC_{50} toxicity assays could greatly enhanced the ability to detect compounds at sub-lethal concentrations that have mechanism-based toxicity. The use of a physiology-based strategy is particularly important for screening of compounds that may exert effects on the nervous system. We also believe that behavioural phenotypes can predict, in some cases, efficacy of neuroactive or psychoactive compounds. We

use the 'visual motor response test' which was previously used in high-throughput studies identifying biological central nervous system (CNS) targets for compounds not previously assigned to these targets [73,74,96,343]. In order to expand on those findings, we tested the effects of a range of sub-lethal concentrations of 60 compounds whose toxicity (LC_{50}) we have previously characterised in some depth in the zebrafish embryo model [35]. The data reported here are unpublished sets from that same study. For our present study, compounds are added to the water in which the embryos develop, and so we focus here on water soluble compounds to avoid any confusing effects of carrier solvents.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. The local regulation is the *Wet op de dierproeven* (Article 9) of Dutch Law (National) and the same law administered by the Bureau of Animal Experiment Licensing, Leiden University (Local). This local regulation serves as the implementation of *Guidelines on the protection of experimental animals by the Council of Europe*, Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 days after fertilization). Because embryos used here were no more than 5 days old, no license is required by Council of Europe (1986), Directive 86/609/EEC or the Leiden University ethics committee.

Animals

Male and female adult zebrafish (Danio rerio) of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, the Netherlands) who obtain stock from Europet Bernina International BV (Gemert-Bakel, the Netherlands). Other strains include Wik, Tu, TL and India

show high interstrain genetic polymorphisms, and therefore are possibly to exert different behavior. Therefore we used AB strain as this strain is a laboratory strain that has been bred for many generations in many labs and a good choice for behavioral study. Fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm; W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08.00). Water and air were temperature controlled (25±0.5^{o}C and 23^{o}C, respectively). The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., Burlingame, USA) and twice a week with frozen food 'artemias' (Dutch Select Food, Aquadistri BV, the Netherlands).

Defined embryo buffer

To produce a defined and standardized vehicle (control) for these experiments, we used 10% Hank's balanced salt solution (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO) at a concentration of 0.98 g/L in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma Cat S5761), and adjusted to pH 7.46. A similar medium has been used previously [31–35].

Embryo care

Eggs were obtained by random pairwise mating of zebrafish. Three adult males and four females were placed together in small breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. The breeding tanks (L 26 cm; H 12.5 cm; W 20 cm) had mesh egg traps to prevent the eggs from being eaten. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml fresh embryo buffer. Eggs were washed four times to remove debris. Further, unfertilized, unhealthy and dead

embryos were identified under a dissecting microscope and removed by selective aspiration with a plastic Pasteur pipette. At 3.5 hpf, embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28±0.5^{o}C, either in the incubator or a climatised room under a light cycle of 14 h light: 10 h dark (lights on at 08.00).

Test compounds

We used water-soluble toxic compounds representing a range of different chemical classes and biochemical activities see Table 21. The required dilution was always freshly prepared in buffer just prior to assay on zebrafish embryos.

Table 21. **Summary of compounds used in this study for toxicity evaluation in zebrafish embryo.**

	Name (synonym)	**Supplier (City, Country)**	**Cat. number**	**Molecular weight [(a)] (g/L)**	**Water solubility (mg/L)**	**Chemical structure***
1	Aconitine	Sigma (Zwijndrecht, NL)	A8001	645.74	607[(a)]	Diterpene alkaloid
2	Atropine	BDH (Poole, UK)	27276	289.38	2200[(b)]	Tropane alkaloid
3	Berberine chloride	Sigma (Zwijndrecht, NL)	B3251	371.81	soluble[(a)]	Isoquinoline alkaloid
4	Colchicine	Sigma (Zwijndrecht, NL)	C3915	399.44	45000[(b)]	Alkaloid
5	Coniine	Sigma (Zwijndrecht, NL)	C9392	127.23	18000[(b)]	Piperidine alkaloid
6	α-Lobeline hydrochloride	Sigma (Zwijndrecht, NL)	62630	373.92	25000[(a)]	alkaloid
7	Morphine hydrochloride	Sigma (Zwijndrecht, NL)	67357	423.89	soluble[(a)]	Opiate alkaloid
8	Nicotine	Sigma (Zwijndrecht, NL)	N3876	162.26	1000000[(b)]	Solanaceous alkaloid
9	Quinine sulfate	Sigma (Zwijndrecht, NL)	Q0132	391.47	1430[(b)]	Cinchona alkaloid
10	(-)-Scopolamine hydrobromide trihydrate	Sigma (Zwijndrecht, NL)	S1875	438.31	666667[(a)]	Tropane alkaloid
11	Strychnine hydrochloride	Sigma (Zwijndrecht, NL)	S8753	370.87	28571[(a)]	Secologanin tryptamine alkaloid
12	Theobromine	Sigma (Zwijndrecht, NL)	T4500	180.16	500[(a)]	Xanthine alkaloid
13	(+)-Tubocurarine chloride hydrate	Sigma (Zwijndrecht, NL)	T2379	681.65	50000[(a)]	Isoquinoline alkaloid
14	Yohimbine hydrochloride	Sigma (Zwijndrecht, NL)	Y3125	390.90	7300[(b)]	Secologanin tryptamine alkaloid
15	Amygdalin	Sigma (Zwijndrecht, NL)	A6005	457.43	83333[(a)]	Glycoside
16	Arbutin	Sigma	A4256	272.25	50000[(c)]	Glycoside

		(Zwijndrecht, NL)				
17	Convallatoxin	Sigma (Zwijndrecht, NL)	C9140	550.64	500[(b)]	Cardiac glycoside
18	Coumarin	Sigma (Zwijndrecht, NL)	C4261	383644	1900[(b)]	Glycoside
19	Digitoxin	Sigma (Zwijndrecht, NL)	D5878	764.94	10[(a)]	Cardiac glycoside
20	Gentamycin sulfate	Duchefa (Haarlem, NL)	G0124	575.67	soluble[(a)]	Amino glycoside
21	Glycyrrhizin	Sigma (Zwijndrecht, NL)	50531	839.96	soluble[(a)]	Terpene glycoside
22	Hesperidin	Sigma (Zwijndrecht, NL)	H5254	610.56	57[(d)]	Flavanone glycoside
23	Kanamycin monosulfate	Duchefa (Haarlem, NL)	K0126.0005	582.6	soluble[(a)]	Amino glycoside
24	Naringin	Sigma (Zwijndrecht, NL)	N1376	580.53	1000[(b)]	Flavanone glycoside
25	Neohesperidin	Sigma (Zwijndrecht, NL)	N1887	610.56	61[(e)]	Flavanone glycoside
26	Ouabain octahydrate	Sigma (Zwijndrecht, NL)	O3125	728.77	13333[(a)]	Cardiac glycoside
27	Phloridzin dihydrate	Sigma (Zwijndrecht, NL)	P3449	472.44	1300[(a)]	Glycoside
28	Rutin hydrate	Sigma (Zwijndrecht, NL)	R5143	610.52	soluble[(c)]	Flavanone glycoside
29	Streptomycin sulfate	Sigma (Zwijndrecht, NL)	S6501	1457.38	>20000[(a)]	Amino glycoside
30	Cadmium(II) chloride	Sigma (Zwijndrecht, NL)	439800	479.67	soluble[(a)]	Metal salt
31	Copper(II) nitrate trihydrate	Merck KGaA, Darmstadt, Germany	A911353	241.60	1378000[(a)]	Metal salt
32	Lead acetate trihydrate	BDH (Poole, UK)	10142	379.33	625000[(a)]	Metal salt
33	Lithium chloride	JTB	0157	42.39	769000[(a)]	Metal salt
34	Chloramphenicol	Sigma (Zwijndrecht, NL)	C0378	323.15	2500[(a)]	Alcohol
35	Ethanol	Merck KGaA, Darmstadt, Germany	100971	46.07	1000000[(b)]	Alcohol
36	Glycerol	BDH (Poole, UK)	K33625960	92.11	1000000[(b)]	Sugar alcohol
37	Tween 80	Sigma (Zwijndrecht, NL)	P1754	1310	soluble[(c)]	Alcohol
38	Acetic acid	Merck KGaA, Darmstadt, Germany	K30123563	60.05	1000000[(b)]	Carboxylic acid
39	Salicylic acid	Sigma (Zwijndrecht, NL)	S0875	138.12	2240[(b)]	Carboxylic acid
40	Sodium oxalate	Sigma (Zwijndrecht, NL)	71800	134	37000[(a)]	Carboxylic acid
41	Trichloroacetic acid	BDH, VWR Leuven, Belgium	20741.290	163.38	10000000[(a)]	Carboxylic acid
42	Ampicillin sodium	Duchefa (Haarlem, NL)	A0104.0025	371.4	5000000[(a)]	Amide, penicillin G
43	Cyclophosphamide monohydrate, cytoxan	Sigma (Zwijndrecht, NL)	C0768	279.1	40000[(a)]	Phosphor amide mustard
44	Paracetamol (Acetaminophen)	Sigma (Zwijndrecht, NL)	A7085	151.17	14000[(b)]	Amide
45	Phenacetin	Sigma (Zwijndrecht, NL)	77440	179.22	766[(b)]	Amide
46	Benserazide hydrochloride	Sigma (Zwijndrecht, NL)	B7283	293.70	10000[(c)]	Hydrazine
47	Chlorpromazine hydrochloride	Sigma (Zwijndrecht, NL)	C8138	355.33	400000[(c)]	Phenothiazine

48	Isoniazid	Sigma (Zwijndrecht, NL)	I3377	137.14	140000[(a)]	Hydrazine
49	Phenelzine sulphate	Sigma (Zwijndrecht, NL)	P6777	234.27	soluble[(a)]	Hydrazine
50	Ethambutol dihydrochloride	Sigma (Zwijndrecht, NL)	E4630	277.2	50000[(a)]	Ethylenediamines
51	Verapamil hydrochloride	Sigma (Zwijndrecht, NL)	381175	491.1	83000[(a)]	Phenethylamine
52	Phenol	BDH (Poole, UK)	10188	94.11	15000[(a)]	Carbolic acid
53	Sodium azide	Sigma (Zwijndrecht, NL)	S8032	65.01	417000[(a)]	Inorganic azide
54	Dimethyl sulphoxide	Sigma (Zwijndrecht, NL)	60153	78.13	1000000[(b)]	Sulfoxide
55	Formaldehyde	Sigma (Zwijndrecht, NL)	25254-9	30.03	400000[(b)]	Aldehyde
56	Phenformin hydrochloride	Sigma (Zwijndrecht, NL)	P7045	241.72	soluble[(a)]	biguanide
57	Ropinirole hydrochloride	Sigma (Zwijndrecht, NL)	R2530	296.84	400000[(b)]	Indole
58	Amitriptyline hydrochloride	Sigma (Zwijndrecht, NL)	A8404	313.87	soluble[(a)]	Dibenzocycloheptene
59	Sodium dodecyl sulphate	LKB (Broma, Sweden)	1836	289.43	1000000[(b)]	Alkane sulfonate
60	Barbital sodium (Barbitalum natricum, Ph.Helv.)	BUFA (Ijsselstein, NL).	175310	206.2	200000[(a)]	Barbiturate

Key: (a), from [359]; (*), From the Pubchem database at http://pubchem.ncbi.nlm.nih.gov/; (b), from Chemical Identification/Dictionary database at http://toxnet.nlm.nih.gov/cgi-bin/sis/search/; (c), from http://www.sigmaaldrich.com/catalog/DisplayMSDSContent.do/ ; (d), from [360]; (e), from [361].

Test compounds exposure

We used a chronic exposure regime of 96 h, starting at 24 hpf and ending at 120 hpf, thus encompassing the major stages of organ development. This gives us the maximal chance of detecting an effect, in the case that a particular drug has a narrow time window or 'critical period' of effect. Recently, we found that in controls (buffer only), 5% of eggs were unfertilized, and a further 9% represented embryos that died spontaneously in the first 24 hpf [35]. Another study [314] also reported spontaneous mortality of 5-25 % for zebrafish development at 24 hpf. In order to keep away from this natural early mortality we began our assays at 24 hpf.

Range-finding

To determine a suitable range of concentrations for testing, we performed range-finding using a logarithmic series (0, 1, 10, 100 and 1000 mg/L) as recommended in standard protocols [203]. Zebrafish embryos of 24 hpf from Petri dish were gently transferred using a sterile plastic Pasteur pipette into 96-well microtitre plates (Costar 3599, Corning Inc., NY). A single embryo was plated per well, so that dead embryos would not affect others and also to allow individual embryos to be tracked for the whole duration of the experiment. A static non-replacement regime was used. Thus there was no replacement or refreshment of buffer after the addition of compound. Each well contained 250 µL of either freshly prepared test compound; or vehicle (buffer/0 mg/L) only as controls. All pipetting was done manually, with an 8-channel pipetter. We used 16 embryos for each concentration and 16 embryos as controls for each compound. The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates.

Geometric series and LC_{50} determination

After the range-finding experiments, a series of concentrations lying in the range between 0% and 100% mortality were selected. The actual concentrations used are shown in Table 22. The concentrations were in a geometric series in which each was 50% greater than the next lowest value [203]. Each geometric series of concentrations for each compound was repeated three times (in total 48 embryos per concentration and 48 embryos for vehicle for each compound). The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates in each independent experiment. LC_{50} (expressed in mg/L of culture buffer) was determined based on cumulative mortality obtained from three independent experiments at 48, 72, 96 and 120 hpf using Regression Probit analysis with SPSS Statistics for

windows version. 17.0 (SPSS Inc., Chicago, USA). The LC_{50} in mg/L was then converted into LC_{50} mmol/L.

Table 22. **Concentrations used in geometric series.**

		Concentrations in geometric series (mg/L)					
	Compounds	**C0**	**C1**	**C2**	**C3**	**C4**	**C5**
1	Aconitine	0	50	100	200	400	800
2	Atropine	0	100	200	400	800	1600
3	Berberine chloride form	0	50	100	200	400	800
4	Colchicine	0	10	20	40	80	160
5	Coniine	0	10	20	40	80	160
6	α-Lobeline hydrochloride	0	10	20	40	80	160
7	Morphine hydrochloride	0	1000	2000	4000	8000	16000
8	Nicotine	0	10	20	40	80	160
9	Quinine sulfate	0	30	60	120	240	480
10	(-)-Scopolamine hydrobromide trihydrate	0	1000	2000	4000	8000	16000
11	Strychnine hydrochloride	0	10	20	40	80	160
12	Theobromine	0	30	60	120	240	480
13	(+)-Tubocurarine chloride hydrate	0	100	200	400	800	1600
14	Yohimbine hydrochloride	0	10	20	40	80	160
15	Amygdalin	0	10	20	40	80	160
16	Arbutin	0	10	20	40	80	160
17	Convallatoxin	0	30	60	120	240	480
18	Coumarin	0	70	140	280	560	1120
19	Digitoxin	0	0.5	1	2	4	8
20	Gentamycin sulfate	0	100	200	400	800	1600
21	Glycyrrhizin	0	10	20	40	80	160
22	Hesperidin	0	10	20	40	80	160
23	Kanamycin monosulfate	0	250	500	1000	2000	4000
24	Naringin	0	50	100	200	400	800
25	Neohesperidin	0	10	20	40	80	160
26	Ouabain octahydrate	0	50	100	200	400	800
27	Phloridzin dihydrate	0	70	140	280	560	1120
28	Rutin hydrate	0	1000	2000	4000	8000	16000
29	Streptomycin sulfate	0	250	500	1000	2000	4000
30	Cadmium(II) chloride	0	10	20	40	80	160
31	Copper(II) nitrate trihydrate	0	6.25	12.5	25	50	100
32	Lead Acetate trihydrate	0	10	20	40	80	160
33	Lithium chloride	0	1000	2000	4000	8000	16000
34	Chloramphenicol	0	100	200	400	800	1600
35	Ethanol	0	1000	2000	4000	8000	16000
36	Glycerol	0	2000	4000	8000	16000	32000
37	Tween 80	0	100	200	400	800	1600
38	Acetic acid	0	50	100	200	400	800
39	Salicylic acid	0	7.5	15	30	60	120
40	Sodium oxalate	0	100	200	400	800	1600
41	Trichloroacetic acid	0	20	40	80	160	320
42	Ampicillin sodium	0	250	500	1000	2000	4000
43	Cyclophosphamide monohydrate	0	1000	2000	4000	8000	16000
44	Paracetamol	0	100	200	400	800	1600
45	Phenacetin	0	50	100	200	400	800
46	Benserazide hydrochloride	0	250	500	1000	2000	8000
47	Chlorpromazine hydrochloride	0	1	2	4	8	16
48	Isoniazid	0	200	400	800	1600	3200
49	Phenelzine sulphate salt	0	5	10	20	40	80

50	Ethambutol dihydrochloride	0	1000	2000	4000	8000	16000
51	Verapamil hydrochloride	0	10	20	40	80	160
52	Phenol	0	10	20	40	80	160
53	Sodium azide	0	0.5	1	2	4	8
54	Dimethyl sulphoxide	0	2000	4000	8000	16000	32000
55	Formaldehyde	0	2	4	8	16	32
56	Phenformin hydrochloride	0	100	200	400	800	1600
57	Ropinirole hydrochloride	0	100	200	400	800	1600
58	Amitriptyline hydrochloride	0	2	4	8	16	32
59	Sodium dodecyl sulphate	0	1	2	4	8	16
60	Barbital sodium	0	500	1000	2000	4000	8000

For each compound, a geometric series of concentrations (C0 – C5) was used, based on the results of the logarithmic range-finding series.

Behavioral analysis

The visual motor response test was performed at 5 dpf according to [32] on all living larvae of both range finding experiments and geometric series. The test was performed in the presence of original solutions added at 24 h. Thus, there was no replacement or refreshment of buffer before test. The temperature used for testing was 28±0.5^{o}C. The visual motor response test has been previously characterized and typically consists of brief (less than 10 min) frequently alternating periods of light and dark. A key feature of this test is the robust but transient behavioral activity that occurs in response to sudden transitions from light to dark [33,100,343,362]. Because such behavioral response has been shown to be highly sensitive to neuroactive chemical compounds, the visual motor response test has become a validated tool to assess the impact of a wider range of chemical agents on neuronal and physiological integrity of the developing zebrafish [33,100,343,362]. Here we used a modified version of this test consisting of a single transition from light to dark. The activity of each larva was automatically recorded and analyzed in the ZebraBox recording apparatus equipped with VideoTrack software (both from Viewpoint S.A., Lyon, France). The white light intensity of the ZebraBox was 500 lux. The experimental recording consists of two steps. First, larvae we acclimated to the behavioral setup with lights ON for 10 min. This period was necessary and sufficient to ensure low and

stable behavioral activity. Once basal levels of locomotor activity were stabilized following the acclimatizing period, basal swimming activity was recorded during 4 min with lights ON. This period is referred to as 'basal context'. Immediately following the basal activity recording, the lights were suddenly turned off for 4 min. Behavioral activity in the dark was also automatically recorded during this period. This period is referred to as the 'dark challenge context'. We chose four-minute session to prevent habituation, and also to favor more robust behavioral changes. Because of the robustness of the behavioral changes induced by varying illumination, this task can be used to reveal more readily than any other tasks, defective brain function, aberrant nervous system development and/or locomotor and visual defects caused by toxic compounds.

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs. To analyse the impact of compounds on embryo locomotion in the visual motor test challenge test, we used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance. LC_{50} was determined using Regression Probit analysis (Chi-Squares test, Pearson Goodness-of-fit test and 95% confidence interval) with SPSS Statistics for windows version. 17.0 (SPSS Inc., Chicago, USA).

Results

Relationship between LC_{50} and duration of exposure

For most of compounds, zebrafish embryo LC_{50} values were dependent on the duration of exposure, such that longer exposures were associated with lower LC_{50} values.

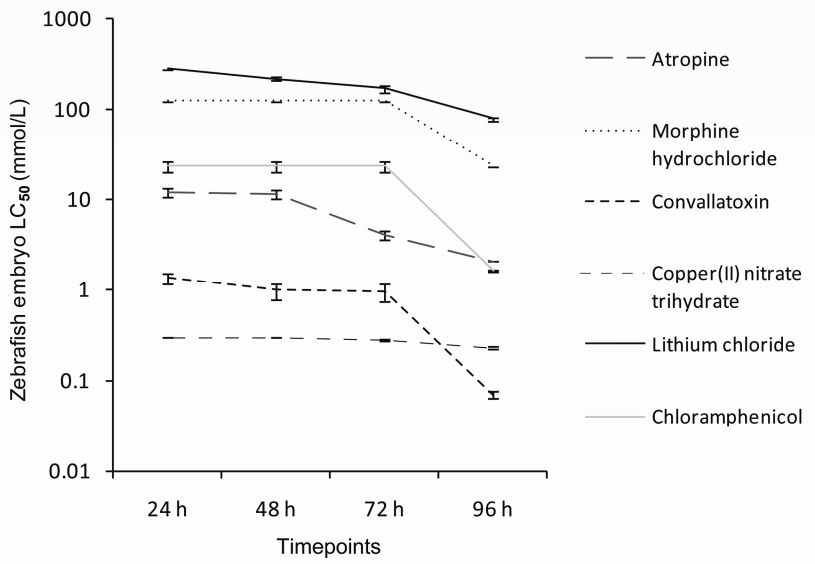

Figure 29. **Selected examples of cumulative LC_{50} (mmol/L) at different durations of exposure in zebrafish embryos.** The full dataset can be seen in Table 19. Note that longer exposures are associate with lower LC_{50} values and therefore greater cumulative toxicity. Each error bar represents ±SEM from three replications.

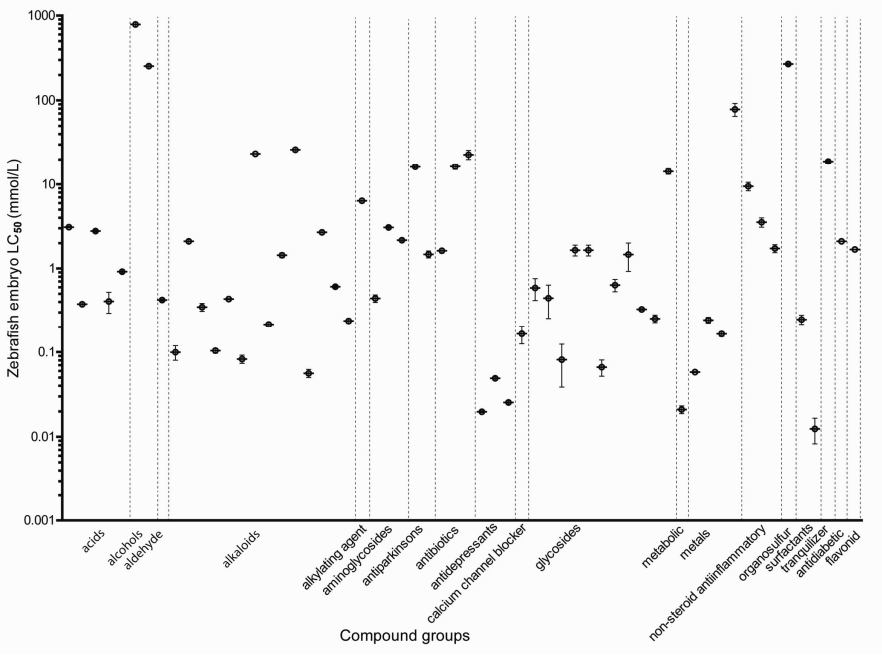

Figure 30. **Zebrafish embryo LC_{50} (mmol/L), sorted by compound type**. LC_{50} was determined based on cumulative mortality from three independent experiments after 96 h exposure of compounds. Each error bar represents ±SEM from three replications.

To give one example, the LC_{50} for convallatoxin is 1.35 mmol/L after 24 h exposure, 0.99 (48 h), 0.95 (72 h) and 0.07 mmol/L after 96 h exposure. Further, selected examples are shown in Figure 29 and the full dataset is in Table 19. The LC50 after 96 h exposure are shown in Figure 30.

Table 23. **Relationship between LC_{50} and duration of exposure in zebrafish embryos.**

		Zebrafish embryo LC_{50} at various timepoints							
		24 h		48 h		72 h		96 h*	
	Compounds	**(mg/L ±SEM)**	**(mmol/L ±SEM)**	**(mg/L ±SEM)**	**(mmol/L ±SEM)**	**(mg/L ±SEM)**	**(mmol/L ±SEM)**	**(mg/L ±SEM)**	**(mmol/L ±SEM)**
1	Aconitine	n/d	n/d	578.7 ±72.7	0.90±0.1	126.4±16.0	0.20±0.02	34.3±1.5	0.05±0.0
2	Atropine	3443.9 ±401.0	11.90± 1.4	3329.9 ±418.3	11.51± 1.5	1186.6± 118.7	4.10±0.41	607.8±7.7	2.10±0.03
3	Berberine chloride	n/d	n/d	n/d	n/d	287.3±35.9	0.77±0.10	129.2±3.6	0.35±0.01
4	Colchicine	n/d	n/d	245.2 ±41.2	0.61±0.10	63.9±9.0	0.16±0.02	41.5±0.7	0.10±0.0
5	Coniine	464.5 ±44.7	3.65±0.3 5	308.6 ±25.5	2.43±0.20	234.6±15.7	1.84±0.12	55.1±0.2	0.43±0.0
6	α-Lobeline hydrochloride	222.8 ±16.8	0.60±0.0 4	170.1 ±9.5	0.45±0.03	105.5±4.3	0.28±0.01	30.9±0.9	0.08±0.0
7	Morphine hydrochloride	52509. 0 ±0.0	123.87±0 .0	52509.0 ±0.0	123.87±0.0	52509.0±0.0	123.87±0. 00	9915.1±0.8	23.39±0.0 0
8	Nicotine	76.8 ±9.7	0.47±0.1	45.0 ±5.6	0.28±0.03	29.0±3.6	0.18±0.02	35.1±0.5	0.22±0.0
9	Quinine sulfate	n/d	n/d	n/d	n/d	n/d	n/d	562.4±9.5	1.44±0.02
10	(-)-Scopolamine hydrobromide trihydrate	n/d	n/d	n/d	n/d	n/d	n/d	11465.1±16 6.1	26.16±0.4
11	Strychnine hydrochloride	n/d	n/d	166.8 ±24.6	0.45±0.1	117.4±14.9	0.32±0.04	20.8±0.6	0.06±0.0
12	Theobromine	n/d	n/d	567.6 ±141.9	3.15±0.8	419.5±52.7	2.33±0.3	150.4±1.8	0.83±0.01
13	(+)-Tubocurarine chloride hydrate	3775.1 ±943.8	5.54±1.4	5986.5 ±821.3	8.78±1.20	771.0±96.9	1.13±0.14	414.2±3.6	0.61±0.01
14	Yohimbine hydrochloride	n/d	n/d	255.9±6 4.0	0.65±0.2	148.7±18.6	0.38±0.1	93.0±1.5	0.24±0.0
15	Amygdalin	n/d	n/d	n/d	n/d	n/d	n/d	268.5±19.6	0.59±0.04
16	Arbutin	n/d	n/d	n/d	n/d	255.9±64.0	0.94±0.2	120.9±13.0	0.44±0.1
17	Convallatoxin	745.0 ±85.3	1.35±0.3	543.2 ±110.3	0.99±0.2	523.8±112.7	0.95±0.2	36.6±3.5	0.07±0.01
18	Coumarin	525.6 ±29.9	3.60±0.2	493.2 ±34.7	3.38±0.2	473.8±36.5	3.24±0.3	241.2±8.9	1.65±0.1
19	Digitoxin	13.0± 3.3	0.02±0.0	13.0 ±3.3	0.02±0.0	13.0±3.3	0.02±0.0	0.5±0.1	0.001±0.0
20	Gentamycin sulfate	n/d	n/d	n/d	n/d	1608.7±402.2	2.79±0.7	253.3±6.5	0.44±0.01
21	Glycyrrhizin	83.3 ±20.8	0.10±0.0 2	422.5 ±43.2	0.50±0.1	152.6±12.3	0.18±0.01	55.8±3.0	0.07±0.0

22	Hesperidin	n/d	n/d	430.9 ±56.6	0.71±0.1	72.3±9.1	0.12±0.01	77.6±3.2	0.13±0.01
23	Kanamycin monosulfate	n/d	n/d	n/d	n/d	n/d	n/d	1787.5±16.8	3.07±0.03
24	Naringin	n/d	n/d	n/d	n/d	357.5±44.7	0.62±0.1	850.1±78.5	1.46±0.1
25	Neohesperidin	n/d	n/d	n/d	n/d	166.6±21.8	0.27±0.04	199.5±1.2	0.33±0.0
26	Ouabain octahydrate	n/d	n/d	1483.6 ±205.8	2.04±0.3	398.2±50.0	0.55±0.1	184.1±4.8	0.25±0.01
27	Phloridzin dihydrate	n/d	n/d	n/d	n/d	1811.2±320.2	3.83±0.7	793.2±5.1	1.68±0.01
28	Rutin hydrate	n/d	n/d	12739.7 ±3184.9	20.87±5.2	17954.3±2369.5	29.41±3.9	8722.9±164.2	14.29±0.3
29	Streptomycin sulfate	n/d	n/d	n/d	n/d	n/d	n/d	3164.0±35.4	2.17±0.02
30	Cadmium(II) chloride	255.9 ±64.0	0.53±0.1	407.3± 45.2	0.85±0.1	327.0±55.1	0.68±0.1	27.9±0.1	0.06±0.0
31	Copper(II) nitrate trihydrate	73.2 ±1.3	0.30±0.01	73.2 ±1.3	0.30±0.01	68.5±2.1	0.28±0.01	58.7±1.2	0.24±0.0
32	Lead acetate trihydrate	38.5 ±9.6	0.10±0.03	38.4 ±9.6	0.10±0.03	162.0±6.3	0.43±0.02	62.4±1.1	0.16±0.0
33	Lithium chloride	11782.3 ±141.0	277.95±3.3	9329.0 ±447.7	220.08±10.6	7218.7±713.7	170.29±16.8	3324.2±143.6	78.42±3.4
34	Chloramphenicol	7550.1 ±943.8	23.36±2.9	7550.1 ±943.8	23.36±2.9	7550.1±943.8	23.36±2.9	525.0±7.4	1.62±0.02
35	Ethanol	n/d	n/d	n/d	n/d	n/d	n/d	36212.0±501.8	786.02±10.9
36	Glycerol	n/d	n/d	n/d	n/d	25366.1±31.0	275.39±0.3	23357.4±282.1	253.58±3.7
37	Tween 80	726.8 ±90.9	0.55±0.1	726.8 ±90.9	0.55±0.1	697.8±87.2	0.53±0.1	323.4±10.1	0.25±0.01
38	Acetic acid	377.1 ±21.3	6.28±0.4	360.7 ±23.6	6.01±0.4	328.9±27.3	5.48±0.5	186.3±1.0	3.10±0.02
39	Salicylic acid	47.5 ±1.1	0.34±0.01	47.5±1.1	0.34±0.01	46.7±1.2	0.34±0.01	46.7±1.2	0.34±0.01
40	Sodium oxalate	n/d	n/d	4422.9 ±552.9	33.01±4.1	459.4±57.7	3.43±0.4	372.2±2.9	2.78±0.02
41	Trichloroacetic acid	86.5 ±6.5	0.53±0.04	86.5 ±6.5	0.53±0.04	86.5±6.5	0.53±0.04	66.4±4.7	0.41±0.03
42	Ampicillin sodium	n/d	n/d	n/d	n/d	n/d	n/d	6068.5±114.9	16.34±0.31
43	Cyclophosphamide monohydrate	6957.6 ±174.9	24.93±0.6	4495.9 ±160.6	16.11±0.58	4248.4±213.7	15.22±0.8	1777.4±26.1	6.37±0.1
44	Paracetamol	n/d	n/d	888.5 ±111.1	5.88±0.7	815.7±103.2	5.40±0.7	535.8±17.1	3.54±0.1
45	Phenacetin	1518.9 ±379.7	8.47±2.1	2358.0 ±274.8	13.16±1.5	2057.8±319.1	11.48±1.8	309.9±8.4	1.73±0.1
46	Benserazide hydrochloride	n/d	n/d	n/d	n/d	1407.4±351.8	4.79±1.2	4747.9±28.7	16.17±0.1
47	Chlorpromazine hydrochloride	6.33± 0.8	0.02±0.0	5.9 ±0.7	0.02±0.0	5.7±0.7	0.02±0.0	7.0±0.04	0.02±0.0
48	Isoniazid	n/d	n/d	n/d	n/d	3499.3±874.8	25.52±6.4	1297.5±38.0	9.46±0.3
49	Phenelzine sulfate salt	87.5 ±21.9	0.37±0.1	20.7 ±2.7	0.09±0.01	9.4±1.2	0.04±0.01	11.5±0.1	0.05±0.0
50	Ethambutol dihydrochloride	4303.5 ±1075.9	15.53±3.9	4303.5 ±1075.9	15.53±3.9	36372.2±2932.7	131.21±10.6	6325.9±197.2	22.82±0.7
51	Verapamil hydrochloride	63.2± 15.8	0.13±0.03	123.8 ±15.7	0.25±0.03	86.0±10.8	0.18±0.02	81.1±4.8	0.17±0.01

52	Phenol	255.9 ±64.0	2.72±0.7	354.4 ±55.9	3.77±0.6	172.9±22.2	1.84±0.2	86.4±0.8	0.92±0.01
53	Sodium azide	1.8 ±0.5	0.03±0.0 1	5.1 ±0.1	0.08±0.0	4.0±0.2	0.06±0.0	1.4±0.04	0.02±0.0
54	Dimethyl sulfoxide	n/d	n/d	31187.2 ±3911.9	399.17±50. 1	18815.9±236 4.4	240.83±30 .3	20964.6±15 8.1	268.33±2. 02
55	Formaldehyde	n/d	n/d	12.9 ±1.6	0.43±0.1	11.1±1.4	0.37±0.1	12.7±0.1	0.42±0.0
56	Phenformin hydrochloride	n/d	n/d	1040.8 ±130.1	4.31±0.5	788.6±100.5	3.26±0.4	508.3±17.6	2.10±0.1
57	Ropinirole hydrochloride	879.3 ±112.3	2.96±0.4	533.5 ±66.7	1.80±0.2	455.4±58.7	1.53±0.2	437.3±10.2	1.47±0.03
58	Amitriptyline hydrochloride	88.9 ±16.8	0.28±0.1	83.2 ±17.6	0.26±0.1	80.9±17.8	0.26±0.1	8.0±0.1	0.03±0.0
59	Sodium dodecyl sulfate	4.8 ±0.5	0.02±0.0	3.9 ±0.3	0.01±0.0	3.6±0.3	0.01±0.0	3.6±0.3	0.01±0.0
60	Barbital sodium	n/d	n/d	n/d	n/d	n/d	n/d	3902.5±30.5	18.93±0.2

Key: (n/d), No embryos had died at these timepoints so, no values of LC_{50} could be determined. (*),The data for the 96 h timepoint has already been published by us elsewhere [35].

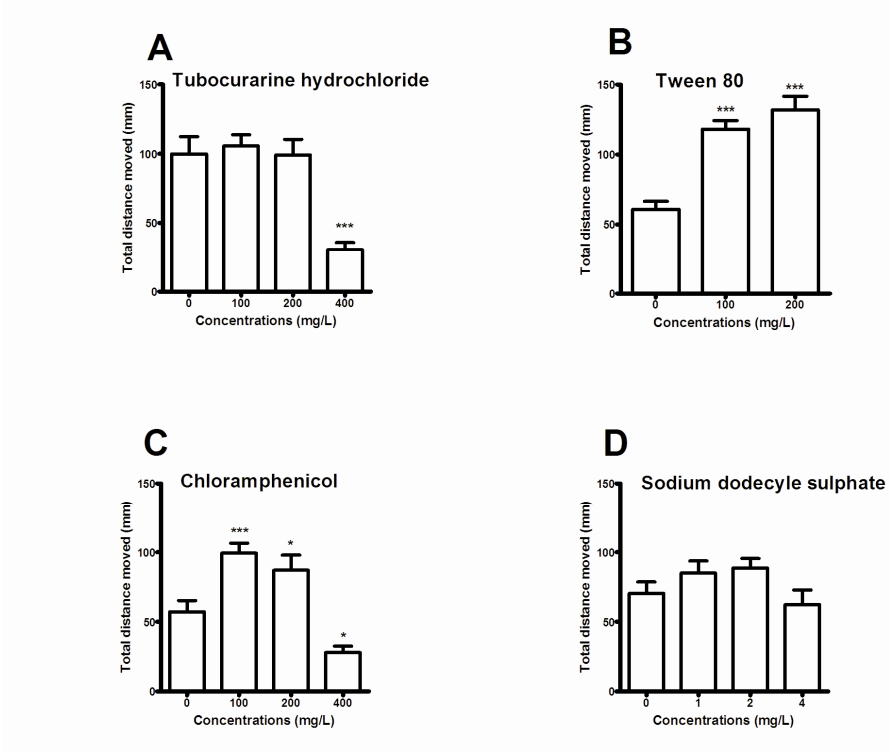

Figure 31. **Visual motor challenge test of live zebrafish embryos at 5 dpf during basal phase**. **A**, an example of a compound that produced, in basal phase, significant concentration-dependent locomotor suppression with no stimulation. **B**, a compound that produced significant stimulation only during basal phase. **C**, a compound that showed biphasic locomotor response in basal phase, i.e. significant stimulation at low concentrations and suppression at higher concentrations. **D**, a compound that showed no significant difference in locomotor response compared to vehicle in basal phase. Each error bar represents ±SEM of N=48 embryos

for vehicle and survived embryos for each concentration for each compound from three independent experiment. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

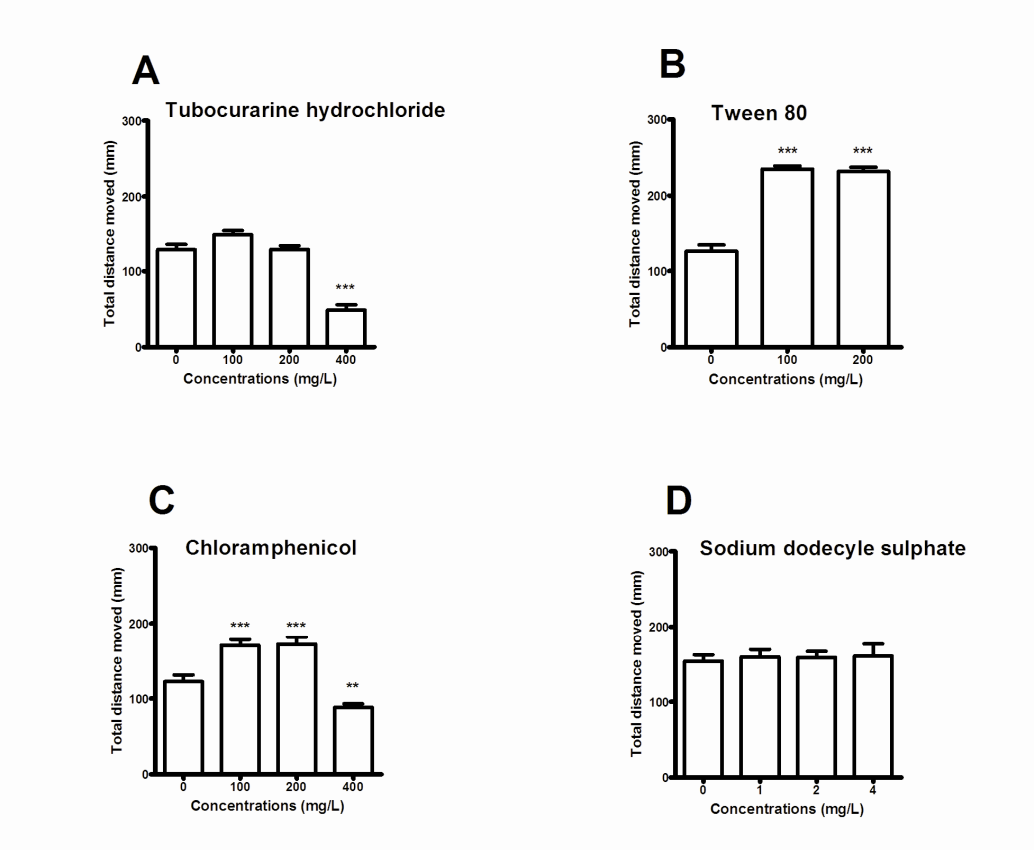

Figure 32. **Visual motor challenge test of live zebrafish embryos at 5 dpf during challenge phase**. **A**, an example of a compound that produced, in challenge phase, significant concentration-dependent locomotor suppression with no stimulation. **B**, a compound that produced significant stimulation only during challenge phase. **C**, a compound that showed biphasic locomotor response in challenge phase, i.e. significant stimulation at low concentrations and suppression at higher concentrations. **D**, a compound that showed no significant difference in locomotor response compared to vehicle in challenge phase. Each error bar represents ±SEM of N=48 embryos for vehicle and survived embryos for each concentration for each compound from three independent experiment. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

Functional impairment at concentrations used

We next sought to determine the degree of functional impairment caused by toxic compounds. We used a behavioral test, the visual motor response test, which relies on the integrity of the central and peripheral nervous system, including the visual system, and on normal locomotor and skeletal system development. The data are given in Table 24. For selected examples, see Figure 31 and Figure 32.

Table 24. **Concentration-dependent functional impairment (visual motor response test) caused during both range finding experiment and geometric series compared to vehicle.**

		Total distance moved during basal phase (light on) in treatment group			**Total distance moved during challenge phase (light off) in treatment group**		
		=	↓	↑	=	↓	↑
	compounds	**concentration (mg/L)**	**concentration (mg/L)**	**concentration (mg/L)**	**concentration (mg/L)**	**concentration (mg/L)**	**concentration (mg/L)**
1	Aconitine	1, 10, 50, 100	-	-	1, 10	50, 100	-
2	Atropine	200, 400, 800	-	1, 10, 100	-	800	1, 10, 100, 200, 400
3	Berberine chloride	1, 10, 50, 100	-	-	1, 10, 50	100	-
4	Colchicine	1, 20, 40	-	10	20	40	1, 10
5	Coniine	1, 10, 20, 40	-	-	1, 10, 20, 40	-	-
6	α-Lobeline hydrochloride	1, 10, 20	-	-	1, 10	20	-
7	Morphine hydrochloride	1, 10, 100, 1000, 2000	4000, 8000	-	1, 10, 100	8000	1000, 2000, 4000
8	Nicotine	1	10, 20, 40	-	1	10, 20, 40	-
9	Quinine sulfate	1, 10, 30, 60, 120, 240, 480	-	-	1, 10, 30, 60, 120, 240	480	-
10	(-)-Scopolamine hydrobromide trihydrate	1, 10, 100, 1000, 2000, 4000, 8000	16000	-	1, 10, 100, 1000, 2000, 4000	8000, 16000	-
11	Strychnine hydrochloride	1	10, 20, 40	-	-	10, 20, 40	1
12	Theobromine	1, 10	30, 60, 100, 120, 240	-	1, 10,30, 60, 100	120, 240	-
13	(+)-Tubocurarine chloride hydrate	1, 10, 100, 200	400	-	1, 10, 100, 200	400	-
14	Yohimbine hydrochloride	1, 10, 20, 40, 80	100	-	1	10, 20, 40, 80, 100	-
15	Amygdalin	1, 10, 20, 40, 80	160		1, 80	160	10, 20, 40
16	Arbutin	80, 160		1, 10, 20, 40	80	160	1, 10, 20, 40
17	Convallatoxin	1, 10, 30	60, 100	-	1, 10, 30	60, 100	-
18	Coumarin	1, 10, 70, 100, 140, 280	-	-	1, 10, 70	100, 140, 280	-
19	Digitoxin	0.5, 1	-	-	-	0.5, 1	-
20	Gentamycin sulfate	1, 10, 100, 200, 400	-	-	100, 200, 400	-	1, 10
21	Glycyrrhizin	1, 20, 40, 80	-	10	1, 10, 20, 40	80	-

22	Hesperidin	1, 10, 20, 40, 80, 100	-	-	1, 40, 80, 100	-	10, 20
23	Kanamycin monosulfate	1, 10, 100, 250, 500, 1000, 2000	-	-	1, 10, 100, 500, 1000, 2000	-	250
24	Naringin	1, 10, 50, 100, 400, 800	-	200	1, 10, 50, 100, 200, 400	800	-
25	Neohesperidin	1, 20, 40, 80, 160	-	10	1, 10	160	20, 40, 80
26	Ouabain octahydrate	1, 10, 50, 100	200	-	1, 10, 50, 100	200	-
27	Phloridzin dihydrate	1, 10, 70, 100, 140, 280, 560, 1120	-	-	1, 10, 70, 560	1120	100, 140, 280
28	Rutin hydrate	1, 10, 100, 4000	8000	1000, 2000	1, 10, 100, 1000, 2000	4000, 8000	
29	Streptomycin sulfate	1, 10, 100, 250, 1000, 2000, 4000	-	500	2000	4000	1, 10, 100, 250, 500, 1000
30	Cadmium (II) chloride	1	10, 20, 40, 80	-	-	1,10, 20, 40, 80	-
31	Copper (11) nitrate trihydrate	-	1, 6.25, 10, 12.5, 25	-	-	1, 6.25, 10, 12.5, 25	-
32	Lead acetate trihydrate	1, 10, 40	-	20	20, 40		1, 10
33	Lithium chloride	1, 10, 100, 1000, 2000, 4000	-	-	1, 10, 100,	1000, 2000, 4000	-
34	Chloramphenicol	1, 10	400	100, 200	1, 10	400	100, 200
35	Ethanol	1, 10, 100, 1000, 2000, 8000, 16000	-	4000	1, 10, 100, 1000, 8000	16000	2000, 4000
36	Glycerol	1, 10, 100, 1000, 2000, 4000, 8000, 16000	-	-	1, 10, 100, 1000, 2000, 4000, 8000, 16000	-	-
37	Tween 80	1, 10	-	100, 200	1, 10		100, 200
38	Acetic acid	1, 10, 50, 100, 200	-	-	1, 10,	100, 200	50
39	Salicylic acid	40	-	1, 10, 20	1, 10	-	20, 40
40	Sodium oxalate	1, 10, 100, 200, 400, 800	-	-	1, 10, 100, 400	800	200
41	Trichloroacetic acid	1, 10, 80	100	20, 40	1, 10, 20	80, 100	40
42	Ampicillin sodium	1, 10, 100, 4000	-	250, 500, 1000, 2000	-	4000	1, 10, 100, 250, 500, 1000, 2000
43	Cyclophosphamide monohydrate	1, 10	1000, 2000	100	1, 10, 100	1000, 2000	-
44	Paracetamol	1, 10, 100, 200	400, 800	-	1, 10, 100, 200	400, 800	-
45	Phenacetin	1, 10, 200	-	50, 100	1, 10, 50, 200	-	100
46	Benserazide hydrochloride	1, 10	-	-	-	-	1, 10
47	Chlorpromazine hydrochloride	1, 2, 4, 8	-	-	1, 2, 4	8	-
48	Isoniazid	1, 10, 100, 200, 400, 800, 1000, 1600	-	-	1, 10, 100, 200	800, 1000, 1600	400
49	Phenelzine sulphate salt	1	5, 10	-	1, 5, 10	-	-
50	Ethambutol dihydrochloride	100, 1000, 2000, 4000	8000	1, 10	-	8000	1, 10, 100, 1000, 2000, 4000
51	Verapamil hydrochloride	1, 10, 20, 80	-	40	1, 10, 20, 40	80	-
52	Phenol	1, 10, 20, 40, 80	-	-	40	80	1, 10, 20
53	Sodium azide	0.5, 1	-	-	0.5	1	-
54	Dimethyl sulphoxide	1, 10, 100, 1000, 2000, 4000	8000	-	1000, 2000, 4000	8000	1, 10, 100
55	Formaldehyde	1, 2, 4, 10, 16	-	8	1, 2, 4, 10, 16	-	8
56	Phenformin	1, 10, 100, 400	-	200	1, 10, 400	-	100, 200

	hydrochloride						
57	Ropinirole hydrochloride	1, 10	100, 200, 400	-	1, 10, 100, 200	400	-
58	Amitriptyline hydrochloride	1	2, 4, 8	-	1	2, 4, 8	-
59	Sodium dodecyl sulphate	1, 2, 4	-	-	1, 2, 4	-	-
60	Barbital sodium	1, 10, 100, 1000, 2000	4000	500	1, 10, 100, 500	1000, 2000, 4000	-

This table was intended to explain concentration-dependent functional impairment by combing behavior data from both logarithmic and geometric series. Number of survived larvae for vehicle and for each concentration for each compound on both logarithmic and geometric scales are given in Table 25.

Keys: '=', equal to vehicle; '↓', significantly lower than vehicle; '↑', significantly higher than vehicle.

Table 25**. Survivors at 5 dpf after 96 h exposure.**

		Survived larvae at 5 dpf (N)										
		range finding experiment (mg/L)					**geometric series***					
	Compounds	**0**	**1**	**10**	**100**	**100**	**C0**	**C1**	**C2**	**C3**	**C4**	**C5**
1	Aconitine	16	16	16	7	0	48	16	1	0	0	0
2	Atropine	16	16	16	16	0	48	48	48	40	13	0
3	Berberine chloride form	16	16	16	12	0	48	48	40	6	0	0
4	Colchicine	16	16	16	0	0	48	48	46	28	1	0
5	(±)-coniine	16	16	16	0	0	48	48	48	47	0	0
6	α-Lobeline hydrochloride	16	16	16	0	0	48	48	45	6	0	0
7	Morphine hydrochloride	16	16	16	16	16	48	48	48	48	44	3
8	Nicotine	16	16	16	0	0	48	47	44	22	0	0
9	Quinine sulfate	16	16	16	2	1	48	48	48	48	48	28
10	(-)-Scopolamine hydrobromide trihydrate	16	16	16	15	15	48	48	47	46	39	11
11	Strychnine hydrochloride	16	16	16	0	0	48	40	29	16	0	0
12	Theobromine	16	16	16	16	2	48	45	41	30	20	0
13	(+)-Tubocurarine chloride hydrate	16	16	16	16	0	48	48	45	31	0	0
14	Yohimbine hydrochloride	16	16	16	7	0	48	45	42	37	34	6
15	Amygdalin	16	16	12	1	0	48	48	47	44	40	29
16	Arbutin	16	16	16	0	0	48	48	44	25	24	16
17	Convallatoxin	16	16	16	6	0	48	29	11	2	0	0
18	Coumarin	16	16	16	16	0	48	44	37	29	1	0
19	Digitoxin	16	12	0	0	0	48	39	3	0	0	0
20	Gentamycin sulfate	16	16	16	15	0	48	38	32	16	4	4
21	Glycyrrhizin	16	16	15	0	0	48	47	42	31	15	3
22	Hesperidin	16	16	16	9	0	48	48	44	43	18	6
23	Kanamycin monosulfate	16	15	14	10	10	48	47	47	41	26	6
24	Naringin	16	16	16	7	1	48	48	47	45	43	11
25	Neohesperidin	16	16	16	0	0	48	48	48	48	48	32
26	Ouabain octahydrate	16	16	16	13	0	48	47	45	17	2	2
27	Phloridzin dihydrate	16	16	16	16	0	48	48	47	45	42	17
28	Rutin hydrate	16	16	16	16	16	48	46	44	44	43	6
29	Streptomycin sulfate	16	16	16	15	11	48	48	48	48	42	13
30	Cadmium(II) chloride	16	10	10	0	0	48	37	36	19	8	0
31	Copper(II) nitrate trihydrate	16	16	14	0	0	48	48	47	42	8	0
32	Lead Acetate trihydrate	16	16	16	1	0	48	36	32	31	3	3

33	Lithium chloride	16	16	16	16	16	48	48	41	19	0	0
34	Chloramphenicol	16	16	16	16	1	48	48	48	42	3	0
35	Ethanol	16	16	16	16	16	48	48	48	48	48	38
36	Glycerol	16	16	16	16	16	48	48	48	48	48	1
37	Tween 80	16	16	16	16	0	48	48	47	6	0	0
38	Acetic acid	16	16	16	10	0	48	48	46	16	0	0
39	Salicylic acid	16	16	15	0	0	48	48	48	43	0	0
40	Sodium oxalate	16	16	16	16	1	48	48	32	23	11	1
41	Trichloroacetic acid	16	16	15	8	0	48	48	32	19	0	0
42	Ampicillin sodium	16	16	16	16	10	48	48	48	39	39	31
43	Cyclophosphamide monohydrate	16	16	16	16	16	48	47	13	2	0	0
44	Paracetamol	16	16	16	16	0	48	48	48	44	5	0
45	Phenacetin	16	16	16	16	1	48	48	48	47	6	0
46	Benserazide hydrochloride	16	16	16	16	15	48	48	47	44	33	6
47	Chlorpromazine hydrochloride	16	16	1	0	0	48	48	48	46	16	0
48	Isoniazid	16	16	16	15	10	48	48	47	47	16	3
49	Phenelzine sulphate salt	16	16	13	0	0	48	47	37	0	0	0
50	Ethambutol dihydrochloride	16	16	16	16	16	48	48	48	40	13	0
51	Verapamil hydrochloride	16	16	16	0	0	48	48	47	43	28	0
52	Phenol	16	16	16	0	0	48	48	48	48	30	0
53	Sodium azide	16	0	0	0	0	48	48	43	5	0	0
54	Dimethyl sulphoxide	16	16	16	16	16	48	48	48	48	46	0
55	Formaldehyde	16	16	8	0	0	48	48	48	41	14	0
56	Phenformin hydrochloride	16	16	16	14	0	48	47	45	40	4	0
57	Ropinirole hydrochloride	16	16	16	16	0	48	44	44	38	2	0
58	Amitriptyline hydrochloride	16	16	6	0	0	48	47	45	29	0	0
59	Sodium dodecyl sulphate	16	16	1	0	0	48	44	32	20	4	0
60	Barbital sodium	16	16	16	16	15	48	48	48	42	24	5

Key: (*), geometric series of concentrations (C0 – C5) are given for each compound in Table 22.

Table 26. **Compounds according to their locomotor pattern in challenge phase.**

phases	**Effect on locomotor activity**	**compounds**	Proportion (%)
Basal (light on)	 no effect	aconitine, berberine chloride, coniine, α-lobeline hydrochloride, quinine sulfate, amygdalin, coumarin, digitoxin, gentamycin sulfate, hesperidin, kanamycin monosulfate, lithium chloride, glycerol, acetic acid, sodium oxalate, chlorpromazine hydrochloride, isoniazid, phenol, sodium azide and sodium dodecyl sulphate	33
	 monotonic suppression	tubocurarine hydrochloride, morphine hydrochloride, nicotine, scopolamine hydrobromide trihydrate, strychnine hydrochloride, theobromine, yohimbine hydrochloride, convallatoxin, ouabain octahydrate, phloridzin dihydrate, cadmium (II) chloride, copper (11) nitrate trihydrate, paracetamol, benserazide hydrochloride, phenelzine sulphate, dimethyl sulphoxide, ropinirole hydrochloride amitriptyline hydrochloride	30
	 monotonic stimulation	atropine, colchicine, arbutin, glycyrrhizin, naringin, neohesperidin, streptomycin sulfate, lead acetate trihydrate, ethanol, tween 80, salicylic acid, ampicillin sodium, phenacetin, verapamil hydrochloride, formaldehyde and phenformin hydrochloride	27
	 Biphasic (stimulation then suppression)	chloramphenicol, rutin hydrate, trichloroacetic acid, cyclophosphamide monohydrate, ethambutol dihydrochloride and barbital sodium	10
Challenge (light off)	 no effect	coniine, glycerol, phenelzine sulphate and sodium dodecyl sulphate	7
	 monotonic suppression	aconitine, berberine chloride, α-lobeline hydrochloride, nicotine, quinine sulfate, scopolamine hydrobromide trihydrate, theobromine, tubocurarine hydrochloride, yohimbine hydrochloride, convallatoxin, coumarin, digitoxin, glycyrrhizin, naringin, ouabain octahydrate, rutin hydrate, cadmium (II) chloride, copper (11) nitrate trihydrate, lithium chloride, cyclophosphamide monohydrate, paracetamol, chlorpromazine hydrochloride, verapamil hydrochloride, sodium azide, ropinirole hydrochloride, amitriptyline hydrochloride and barbital sodium	45
	 monotonic stimulation	gentamycin sulfate, hesperidin, kanamycin monosulfate, streptomycin sulfate, lead acetate trihydrate, tween 80, salicylic acid, phenacetin, formaldehyde, phenformin hydrochloride and benserazide hydrochloride	18
	 Biphasic (stimulation then suppression)	atropine, colchicine, morphine hydrochloride, strychnine hydrochloride, amygdalin, arbutin, neohesperidin, phloridzin dihydrate, chloramphenicol, ethanol, acetic acid, sodium oxalate, trichloroacetic acid, ampicillin sodium, isoniazid, ethambutol dihydrochloride, phenol and dimethyl sulphoxide	30

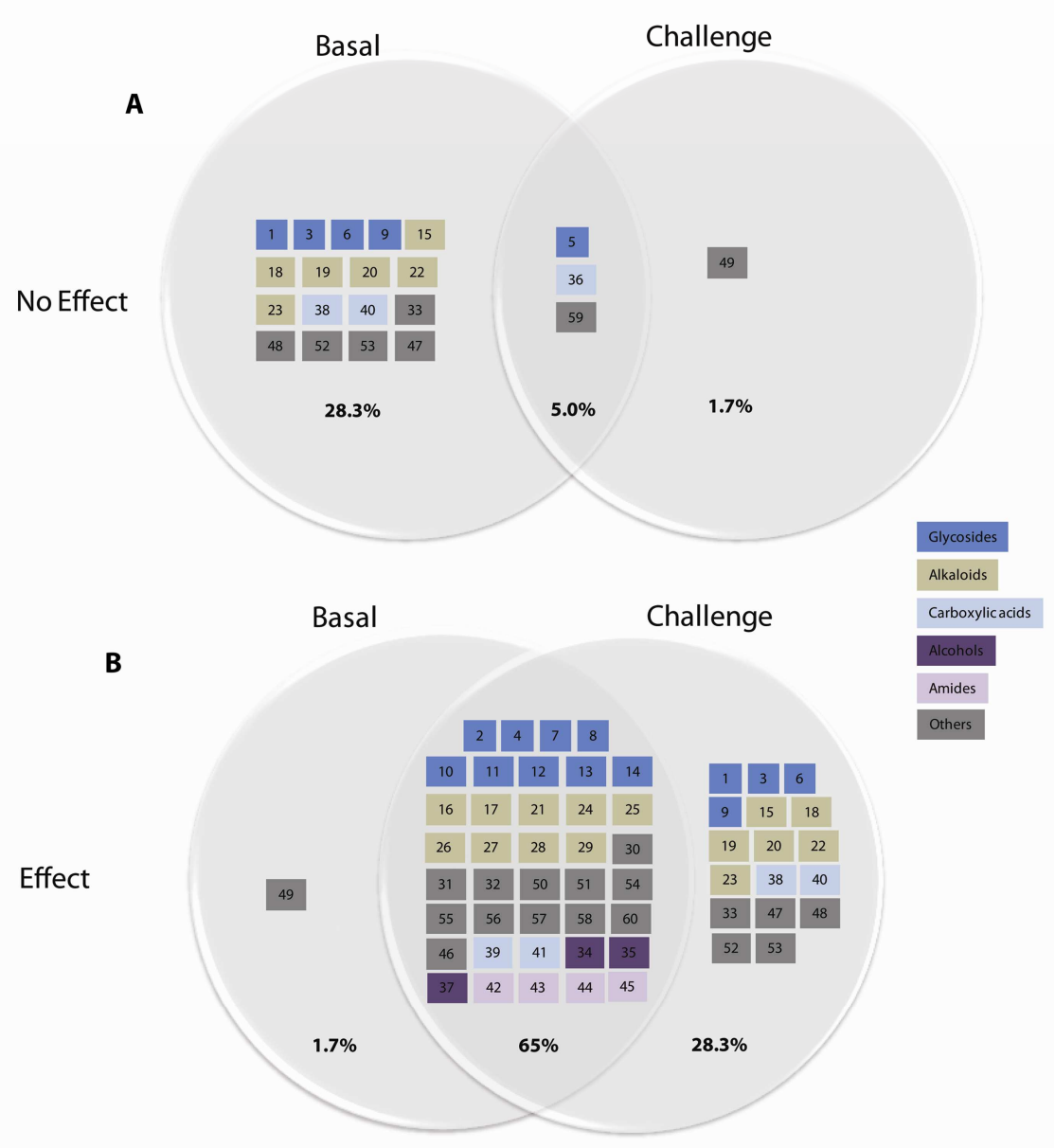

Figure 33. **Classification of compounds based on behavioral effects on zebrafish embryos during motor visual challenge test.** Effects of compounds were compared to vehicle. **A**, top left; compounds did not have any effects in basal; top middle, compound did not produce any effects in both basal and challenge phases; top right, compounds did not cause any effects in challenge phases. **B**, bottom left; compounds did not show any effects in basal phase; bottom middle, compound did not produce any effects in basal and challenge phases; bottom right, compounds did not cause any effects in challenge phases. Numbers 1, 2, 3,……..60 in small rectangular boxes represent the compound number as shown in Table 19.

As can be seen in Figure 31, Figure 32 and Table 26, the effects can be divided into (i)

suppression of locomotor activity with a monotonic concentration-response (Figure 31A); (ii) stimulation of locomotor activity, with a monotonic concentration response (Figure 31B); (iii) stimulation then suppression of locomotor activity (biphasic concentration-response, e.g. Figure 31C); and (iv) no significant effect (Figure 31D).

We found that the majority of compounds (57) tested at various sub-lethal dosages produced significant behavioral impairments. In addition, we observed distinct patterns of effects depending on whether the effects of compounds were assessed during the basal or dark-challenge context (Figure 33). In general, the possibility of detecting any effects on behavior was significantly greater (in total 93.3%) when compounds were tested under the dark challenge context as opposed to basal context (in total 66.7%). Only three compounds had no effect in the basal or challenge contexts, namely coniine, glycerol and sodium dodecyl sulphate.

For comparison with rodents, we found studies from the literature as given Table 28. Studies were selected regardless of the dose used, developmental stage of exposure, duration of exposure, route of administration. We were able to divide the tested compounds into three groups based on the effects seen in the zebrafish challenge phase: those that show similar locomotor effects in zebrafish compared to mammals (26 compounds); those that show dissimilar effects (12 compounds); and those for which we could not determine a corresponding rodent effect from the literature (22). These comparisons are summarized in Table 27.

Table 27. **Comparison of behavioral profiles in zebrafish embryos and mammals.**

Compound class	Compounds with similar behavioural profiles between zebrafish embryos and mammals	Compounds not similar between zebrafish embryos and mammals	* Proportion of compound class showing similar behavioural profiles in zebrafish embryos and mammals
alkaloids	aconitine, berberine chloride, coniine, α-lobeline hydrochloride, quinine sulfate, morphine hydrochloride, nicotine, yohimbine hydrochloride	atropine*, colchicine, (-)-Scopolamine hydrobromide trihydrate	8/11
glycosides	glycyrrhizin, naringin, ouabain octahydrate, rutin hydrate,	coumarin, hesperidin, neohesperidin	4/7
carboxylic acids	n/a	n/a	n/a
alcohols	ethanol	tween 80	1/2
amides	phenacetin, cyclophosphamide monohydrate	paracetamol	2/3
others	cadmium(II) chloride, lithium chloride, benserazide hydrochloride, chlorpromazine hydrochloride, phenelzine sulphate, verapamil hydrochloride, sodium azide, formaldehyde, phenformin hydrochloride, amitriptyline hydrochloride, barbital sodium	lead acetate trihydrate, isoniazid, dimethyl sulphoxide, ropinirole hydrochloride	11/15

Note: Behavioural profiles of zebrafish embryos analysed in challenge phase was compared to mammalian behavioural profiles.

*Atropine is placed here because in the rodent study cited, only low doses were used and so no suppression was reported [363]. For the following compounds, no comparable rodent values could be obtained from the literature: strychnine hydrochloride, theobromine, (+)-tubocurarine chloride hydrate, amygdalin, arbutin, convallatoxin, digitoxin, gentamycin sulfate, kanamycin monosulfate, phloridzin dihydrate, streptomycin sulfate, copper(ii)nitrate trihydrate, chloramphenicol, glycerol, acetic acid, salicylic acid, sodium oxalate, trichloroacetic acid, ampicillin sodium, ethambutol dihydrochloride, phenol, sodium dodecyl sulphate.

Key: *Percent proportion for each compounds was calculated by using total number of compounds for those behavioral profile was available; (n/a), not applicable.

Table 28. Locomotor activity pattern in mammals.

	Compounds	Effect in mammals	Reference
1	Aconitine*	mice: monotonic suppression	[364]
2	Atropine#	rats: monotonic stimulation	[363]
3	Berberine chloride#	mice: monotonic suppression	[365,366]
4	Colchicine*	rats g: monotonic stimulation	[367,368]
5	Coniine*	mice: no significant effect	[369]
6	α-Lobeline hydrochloride*	in rat: monotonic suppression	[370]
7	Morphine hydrochloride*	mice: triphaisc (excitement, sedation and coma)	[371]
8	Nicotine*	rats : monotonic suppression	[372]
9	Quinine sulfate*	mice: monotonic suppression	[373]
10	(-)-Scopolamine hydrobromide trihydrate*	rats: monotonic stimulation	[374]
14	Yohimbine hydrochloride*	mice: monotonic suppression	[375]
18	Coumarin*	mice: no significant effect	[376]
21	Glycyrrhizin*	rats: monotonic suppression	[377,378]
22	Hesperidin*	mice: monotonic suppression	[379,380]
24	Naringin#	mice: monotonic suppression	[380]
25	Neohesperidin#	mice: monotonic suppression	[380]
26	Ouabain octahydrate*	mice: monotonic suppression	[381]
28	Rutin hydrate#	mice: monotonic suppression	[380]
30	Cadmium(II) chloride*	mice: monotonic suppression	[382]
32	Lead acetate trihydrate*	mice: no significant effect	[383,384]
33	Lithium chloride*	rats: monotonic suppression	[385]
35	Ethanol*	mice and rats: biphasic stimulation at lower doses and suppression at high doses	[386,387]
37	Tween 80*	mice: monotonic suppression	[387]
43	Cyclophosphamide monohydrate*	mice: monotonic suppression	[388]
44	Paracetamol	rats: no significant effect	[389]
45	Phenacetin*	rats: monotonic stimulation	[390]
46	Benserazide hydrochloride*	locomotor activity in marmosets: monotonic stimulation	[391]
47	Chlorpromazine hydrochloride*	mice: monotonic suppression	[392]
48	Isoniazid*	rats: monotonic stimulation	[393]
49	Phenelzine sulphate*	mice: no significant effect	[394]
51	Verapamil hydrochloride*	mice: monotonic suppression	[395]
53	Sodium azide*	mice: monotonic suppression	[396,397]
54	Dimethyl sulphoxide*	mice: monotonic suppression	[387]
55	Formaldehyde*	rats: monotonic stimulation and locomotor activity in mice: monotonic suppression	[398,399]
56	Phenformin hydrochloride*	in rats: monotonic stimulation	[400]
57	Ropinirole hydrochloride#	mice 1-10 mg/kg: no significant effect. rats: biphasic suppression at low doses and stimulation at high doses.	[401–403]
58	Amitriptyline hydrochloride*	mice : monotonic suppression	[404]
60	Barbital sodium*	rats : monotonic suppression	[405]

Keys: (*), in these studies locomotor activity was recorded; (#), here ambulatory count/levels were scored.

Predictivity per compound class

In order to see whether the variation in predictivity of the zebrafish assay was due to compound class, or simply varied per compound regardless of class, we sorted the compounds by chemical class as previously described [35]. The classes were: alcohols, alkaloids, amides, carboxylic acids,

glycosides and the remaining compounds (others). As can be seen in Table 27, among all classes, the general trend is a similar behavioral profile between zebrafish embryos and mammals. The effects of compound classes alkaloids (8/11 compounds) and others (9/12 compounds) show similarity between zebrafish embryos and mammals. By contrast, alcohols (1/2 compound) has shown similarity of behavioral profiles.

Discussion

Our most significant finding here is that nearly all of a diverse panel of pharmacologically or toxicologically active compounds produced a change in zebrafish embryo behavior in both basal and challenge (dark context) phases compared to controls. Next in significance is our observation that some compounds produce similar locomotor responses in zebrafish as they do in rodents. Finally, we find that the toxicity of diverse compounds increases as the embryo gets older.

We recorded locomotor responses in a behavioural test (visual motor response test). The visual motor response test has been already proven to be highly effective in the assessment of drug effects on relatively simple locomotor behaviors, which provided the first proof-of-concept for high-throughput screening in zebrafish larvae [33,34,71,96]. We also recently showed that this test can be used to assess the integrity of the nervous system in a zebrafish model of fetal alcohol syndrome [32]. We chose 5 days as the cut-off point in order to conform to local ethical requirements. However, at 5 days, most of the organs are developed [4,27] and the larva already shows a complex behavioural repertoire [32,342,343].

A number of compounds that we tested showed a significant concentration-dependent suppression of locomotor activity in response to a sudden exposure to darkness. These include

agents that have a comparable effect in mammals. For example, aconitine produces locomotor impairment in humans by means of neuromuscular blockade following an interaction with voltage-sensitive sodium channels [406]. Tubocurarine is also a neuromuscular blocker in humans [407], and scopolamine is a tropane alkaloid that resembles atropine in action, but has a much more pronounced sedative effect while lacking the stimulant effects [408].

A second group caused a biphasic response in the zebrafish challenge phase, with locomotor stimulation at low concentrations, and suppression at high concentrations. This group also includes some compounds, which have comparable effects in mammals. Thus strychnine produces initially convulsions and tetanus followed ultimately by loss of consciousness [409] and morphine produces three phases: excitement, sedation then coma [371]. Also worthy of mention is the biphasic response to ethanol, which is also seen in humans [410] and adult zebrafish [38]. Brain dopamine system many be involved in stimulation caused by ethanol exposure because it is reported that stimulation caused by ethanol can be blocked by the addition of dopamine antagonist . Furthermore, similar domaminergic system responsible for hyper-locomotor activity has been observed in drosophila and rodents [411,412].

The third category produces locomotor stimulation only. Among these are several antibiotic substances (ethambutol, gentamycin). Lead acetate also stimulates locomotor activity in the challenge phase, in a concentration-dependent manner. This is in contrast to its effects in mice: acute intraperitoneal administration of lead acetate to adult mice causes a concentration-dependent suppression of locomotor activity [413].

Overall, twenty-six compounds showed a similar effect on movement as reported in the literature for rodents. Twelve compounds had effects on the zebrafish embryos that did not

match those seen in mammals. For 22, there was insufficient literature to make a determination.

In principle, the locomotor hypoactivity could be influenced by a general impairment/delay in locomotor system development and/or shorter body length incurred by toxic compounds treatment (as is seen for example with ethanol treatment [32]. In addition, it is also possible that visual impairment caused by the toxins could globally contribute to the behavioral defects both in dark and light. Again, ethanol exposure is known to cause abnormal eye development (microphthalmia) in zebrafish; see [32,48]. The fact that all larvae, regardless of treatment, responded to sudden changes in illumination argues against complete blindness, but it is however likely that visual sensitivity to varying illumination might be lower in toxic compounds-relative to vehicle-treated larvae.

One puzzling finding was that coniine, an alkaloid derived from the Hemlock plant (*Conium maculatum*) did not disturb the behavioural profile at used concentrations, either in the basal or challenge phases. In view of the fact that this alkaloid is highly toxic in humans, we are investigating possible explanations for this anomaly.

Possible explanations of locomotor hyperactivity include nervous system defects. Alterations in locomotor activity in response to a sudden transition to darkness was used here to provide an index of neuronal/physiological alterations. We have yet to still determine the impact of compounds on nervous system, eyes and locomotor system development in treated embryos.

Some genes (e.g., tyrosine hydroxylase gene) in zebrafish are present as two copies, creating extra work to determine functional role [reviewed in [414]. The development of zebrafish behavioral-based assays are quite new and thus literature history of these assays is not much

extensive as that found in the rodent literature, and also more work is required to study the drug absorption and metabolism rates in zebrafish. The neural anatomy of the zebrafish to make inclusive comparisons with mammalian structures is not fully defined, for example, nuclear accumbens (NAc) and ventral tegmental area (VTA), have not been clearly studied in the zebrafish [reviewed in [414]. Although it is reported that cholinergic system is conserved between humans and teleosts [415].

For all compounds examined, LC_{50} was found to decline as the embryo developed. Thus it seems that some developmental change takes place (particularly between 48 hpf and 72 hpf) [27] that renders the larva much more sensitive to toxins in general. Possible candidates could include changes in permeability of the gills or skin, or the rupturing of the chorion (hatching takes place at this time). Interestingly, the chorion is a substantial barrier to the entry of even small molecules such as ethanol [32].

Conclusions

Our findings show that behavioural recording on zebrafish embryo can provide a sensitive evaluation of toxicity of a wide range of compounds. It is also possible that this type of assay could be useful for assessing efficacy, that is, potential therapeutic effects. Thus it could, in principle, also provide aid in the discovery of new drugs for the human disease treatment. The methodology is complementary to traditional toxicity assays and offers a refinement of the traditional endpoint method relying on mortality at a discrete life stage. The addition of a physiology-based strategy increases the sensitivity of our toxicity assays by detecting potential neurotoxicity at dosage that could not be detected with traditional LC_{50} studies. This assay is amenable to high-throughput capacity and can be implemented early in the drug discovery pipeline for early assessment of drug safety.

Acknowledgements

We thank Merijn A. G. de Bakker and Peter J. Steenbergen for expert technical assistance and zebrafish breeding.