Large-Scale Assessment of the Zebrafish Embryo as a Possible Predictive Model in Toxicity Testing

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Abstract

**Background:** In the drug discovery pipeline, safety pharmacology is a major issue. The zebrafish has been proposed as a model that can bridge the gap in this field between cell assays (which are cost-effective, but low in data content) and rodent assays (which are high in data content, but less cost-efficient). However, zebrafish assays are only likely to be useful if they can be shown to have high predictive power. We examined this issue by assaying 60 water-soluble compounds representing a range of chemical classes and toxicological mechanisms.

**Methodology/Principal Findings:** Over 20,000 wild-type zebrafish embryos (including controls) were cultured individually in defined buffer in 96-well plates. Embryos were exposed for a 96 hour period starting at 24 hours post fertilization. A logarithmic concentration series was used for range-finding, followed by a narrower geometric series for LC₅₀ determination. Zebrafish embryo LC₅₀ (log mmol/L), and published data on rodent LD₅₀ (log mmol/kg), were found to be strongly correlated (using Kendall’s rank correlation tau and Pearson’s product-moment correlation). The slope of the regression line for the full set of compounds was 0.73403. However, we found that the slope was strongly influenced by compound class. Thus, while most compounds had a similar toxicity level in both species, some compounds were markedly more toxic in zebrafish than in rodents, or vice versa.

**Conclusions:** For the substances examined here, in aggregate, the zebrafish embryo model has good predictivity for toxicity in rodents. However, the correlation between zebrafish and rodent toxicity varies considerably between individual compounds and species. We discuss the strengths and limitations of the zebrafish model in light of these findings.

Introduction

There is an unmet need for low-cost, high-throughput animal models in some fields of biomedical research such as drug screening and toxicity assessment [1,2]. The zebrafish embryo is emerging as one such model [1]. It has been proposed as a bridge between simple assays based on cell culture, and biological validation in whole animals such as rodents [1]. The zebrafish cannot replace rodent models but is complementary to them, being particularly useful for rapid, high-throughput, low-cost assays, as for example in the early (pre-regulatory) stages of the drug development pipeline [3].

Among the attractive features of the zebrafish embryo model are its small size, small volume of compound consumed and rapid development. The organogenesis of major organs is completed at 5 days post fertilization (dpf) [4]. Also, many fundamental cellular and molecular pathways involved in the response to chemicals or stress are conserved between the zebrafish and mammals [5]. Genomic sequencing has shown extensive homology between zebrafish and other vertebrate species (including humans), and some aspects of brain patterning, structure and function are also conserved [6–9]. We have shown for example that the glucocorticoid receptor of the zebrafish is functionally closer to that of the human than is its mouse cognate [10]. The availability of genomic tools in the zebrafish provides an advantage over other teleosts such as the fathead minnow (*Pimephales promelas*) used, for example, in environmental toxicity assessment in the United States [11]. Indeed, zebrafish embryos may be a suitable replacement for some of these adult fish toxicity tests [12].

The zebrafish is increasing being used in toxicological studies [reviewed by: 13,14]. Example include the use of adult zebrafish for the testing of lead and uranium [15], malathion [16], colchicine [17], anilines [18], and metronidazole [19]; and the use of juveniles for testing agricultural biocides [20]. Zebrafish embryos are also being used in toxicity studies [reviewed by: 21]. Examples include the use of zebrafish embryos for testing nanoparticles [22,23].

Although the body plans of zebrafish are in many aspects similar to those of mammals, there are important differences. The fish is ectothermic, and lacks cardiac septa, synovial joints, cancellous bone, limbs, lungs and other structures [24–26]. 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]. In pre-hatching embryos, therefore, the...
Table 1. Concentration-dependent mortality at 5 dpf after 96 h exposure.

| Compounds                          | logarthmic series (mg/L)       | geometric series* ± SEM |
|-----------------------------------|--------------------------------|-------------------------|
|                                   | C0  | C1  | C2  | C3  | C4  | C5  |
| 1 Aconitine                       | 0 0 | 0   | 63  | 100| 100| 0  |
| 2 Atropine                        | 0 0 | 0   | 100| 100| 100| 0  |
| 3 Berberine chloride              | 0 0 | 25  | 100| 100| 100| 0  |
| 4 Colchicine                      | 0 0 | 100| 100| 100| 0  |
| 5 Congo                            | 0 0 | 100| 100| 100| 0  |
| 6 α-Lobeline hydrochloride        | 0 0 | 100| 100| 100| 0  |
| 7 Morphine hydrochloride          | 0 0 | 0   | 0  |
| 8 Nicotine                        | 0 0 | 100| 100| 100| 0  |
| 9 Quinine sulfate                 | 0 0 | 88  | 94 | 0  |
| 10 (+-) Scopolamine hydrobromide  | 0 0 | 6   | 6  | 0  |
| 11 Strychnine hydrochloride       | 0 0 | 100| 100| 100| 0  |
| 12 Theobromine                    | 0 0 | 50  | 100| 100| 0  |
| 13 (+)-Tubocurarine chloride hydrate| 0 0 | 0   | 100| 100| 0  |
| 14 Yohimbine hydrochloride        | 0 0 | 75  | 100| 100| 0  |
| 15 Amygdalin                      | 0 0 | 25  | 94 | 100| 0  |
| 16 Arbutin                        | 0 0 | 100| 100| 100| 0  |
| 17 Convallatoxin                  | 0 0 | 78  | 100| 100| 0  |
| 18 Courmarin                      | 0 0 | 0   | 100| 100| 0  |
| 19 Digitoxin                      | 0 0 | 100| 100| 100| 0  |
| 20 Gentamycin sulfate             | 0 0 | 6   | 100| 0  |
| 21 Glycyrrhizin                   | 0 0 | 6   | 100| 100| 0  |
| 22 Hesperid                       | 0 0 | 69  | 100| 100| 0  |
| 23 Kanamycin monosulfate          | 0 6 | 38  | 38 | 0  |
| 24 Naringin                       | 0 0 | 63  | 94 | 0  |
| 25 Neohesperid                    | 0 0 | 100| 100| 100| 0  |
| 26 Ouabain octahydrate            | 0 0 | 19  | 100| 0  |
| 27 Phlorizin dihydrate            | 0 0 | 0   | 100| 0  |
| 28 Rutin hydride                  | 0 0 | 0   | 0  |
| 29 Streptomycin sulfate           | 0 0 | 6   | 31 | 0  |
| 30 Cadmium(II) chloride           | 0 38| 100| 100| 0  |
| 31 Copper(II) nitrate trihydrate  | 0 0 | 13  | 100| 100| 0  |
| 32 Lead acetate trihydrate        | 0 0 | 94  | 100| 0  |
| 33 Lithium chloride               | 0 0 | 0   | 0  |
| 34 Chloramphenicol                | 0 0 | 0   | 94 | 0  |
| 35 Ethanol                        | 0 0 | 0   | 0  |
| 36 Glycerol                       | 0 0 | 0   | 0  |
| 37 Tween 80                       | 0 0 | 0   | 100| 0  |
| 38 Acetic acid                    | 0 0 | 38  | 100| 0  |
| 39 Salicylic acid                 | 0 0 | 6   | 100| 100| 0  |
| 40 Sodium oxalate                 | 0 0 | 0   | 94 | 0  |
| 41 Trichloroacetic acid           | 0 0 | 6   | 56 | 100| 0  |
| 42 Ampicillin sodium              | 0 0 | 0   | 38 | 0  |
| 43 Cyclophosphamide monohydrate   | 0 0 | 0   | 0  |
| 44 Paracetamol                    | 0 0 | 0   | 100| 0  |
| 45 Phenacetin                     | 0 0 | 0   | 94 | 0  |
| 46 Benserazide hydrochloride      | 0 0 | 0   | 6  | 0  |
from the literature on rodent LD50. Compounds are added to the strength of correlation between zebrafish embryo LC50 and data compared, as a toxicology screen, with the aquatic crustacean Daphnia magna [34]. The zebrafish embryo system has also been known ‘Registry of Cytotoxicity’ which examines the predictive kind of comparative toxicity database represented by the well-known ‘Registra for validation of the model that we will necessarily share the same sensitivity to toxic substances. Therefore, there is a need for a more refined toxicology screen that uses cell assays [36].

Our aim here is to determine the toxicity of 60 compounds from diverse pharmacological and chemical classes, and examine the strength of correlation between zebrafish embryo LC50 and data from the literature on rodent LD50. Compounds are added to the water in which the embryos develop, and so we focus here on water soluble compounds to avoid any confounding 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 diertoezien (Article 9) of Dutch Law (National) and the same law administered by the Bureau of Animal Experiment Licensing, Leiden University (Local). The zebrafish was well-correlated with values reported from rodent studies around 445 million years ago [32] and so it is by no means certain that we will necessarily share the same sensitivity to toxic substances. Therefore, there is a need for validation of the model using compounds that have a known effect in other species [33]. One study has reported, using 18 toxic compounds, that toxicity in zebrafish was well-correlated with values reported from rodent studies [34]. The zebrafish embryo system has also been compared, as a toxicology screen, with the aquatic crustacean Daphnia magna [35]. Such studies are an important step towards the kind of comparative toxicity database represented by the well-known ‘Registry of Cytotoxicity’ which examines the predictive power of cell assays [36].

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Table 1. Cont.

| Compounds                        | Cumulative % mortality after 96 h exposure | logarithmic series (mg/L) | geometric series* ± SEM |
|----------------------------------|------------------------------------------|---------------------------|-------------------------|
|                                  |                                          |                           |                         |
| 47 Chlorpromazine hydrochloride  |                                           |                           |                         |
| 48 Isoniazid                     |                                           |                           |                         |
| 49 Phenelzine sulfate            |                                           |                           |                         |
| 50 Ethambutol dihydrochloride    |                                           |                           |                         |
| 51 Verapamil hydrochloride       |                                           |                           |                         |
| 52 Phenol                        |                                           |                           |                         |
| 53 Sodium azide                  |                                           |                           |                         |
| 54 Dimethyl sulfoxide            |                                           |                           |                         |
| 55 Formaldehyde                  |                                           |                           |                         |
| 56 Phenformin hydrochloride      |                                           |                           |                         |
| 57 Ropinirole hydrochloride      |                                           |                           |                         |
| 58 Amitriptyline hydrochloride   |                                           |                           |                         |
| 59 Sodium dodecyl sulfate        |                                           |                           |                         |
| 60 Barbital sodium               |                                           |                           |                         |

Key:

(*) a different geometric scale was used for different compounds because of the variations in toxicity found with the logarithmic range-finding. The values given are the mean percentage mortality from three replicates; the geometric series concentrations C0, C1, etc. are given for each compound in Table 2. For each concentration for each compound, N = 48 (3 replications x16 embryos); (% mortality was found but at these high concentrations, compounds were precipitated out of solution.

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chorion (a membrane perforated by channels of 0.5–0.7 μm in diameter), may provide a barrier to diffusion of compounds [28–31].

The evolutionary divergence of zebrafish and mammals is around 445 million years ago [32] and so it is by no means certain that we will necessarily share the same sensitivity to toxic substances. Therefore, there is a need for validation of the model using compounds that have a known effect in other species [33]. One study has reported, using 18 toxic compounds, that toxicity in zebrafish was well-correlated with values reported from rodent studies [34]. The zebrafish embryo system has also been compared, as a toxicology screen, with the aquatic crustacean Daphnia magna [35]. Such studies are an important step towards the kind of comparative toxicity database represented by the well-known ‘Registry of Cytotoxicity’ which examines the predictive power of cell assays [36].

Our aim here is to determine the toxicity of 60 compounds from diverse pharmacological and chemical classes, and examine the strength of correlation between zebrafish embryo LC50 and data from the literature on rodent LD50. Compounds are added to the water in which the embryos develop, and so we focus here on water soluble compounds to avoid any confounding effects of carrier solvents.

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). Fish were fed 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°C and 23°C, respectively). The fish were fed twice daily with ‘Spirulina’ brand flake food (O.S.L. Marine Lab., Inc., Burlingame, USA) and twice a week with frozen food (Dutch Select Food, Aquadistri BV, the Netherlands).

Defined embryo buffer

To produce a defined and standardized vehicle 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 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 [37–39].
Table 2. Zebrafish embryo LC50 values found in this study, and the corresponding rodent LD50 values based on the literature.

| Compounds                              | Zebrafish Embryo LC50 (mg/L ± SEM) | Zebrafish Embryo LC50 (mmol/L ± SEM) | Rodent LD50 (mg/kg) | Rodent LD50 (mmol/kg) |
|----------------------------------------|-------------------------------------|--------------------------------------|---------------------|----------------------|
| Aconitine                              | 34.3 ± 1.5                          | 0.05 ± 0.00                          | 1(7)                | 0.002                |
| Atropine                               | 607.8 ± 7.7                         | 2.10 ± 0.03                          | 500(7)              | 1.73                 |
| Berberine chloride                     | 129.2 ± 3.6                         | 0.35 ± 0.01                          | 60(*)               | 0.16                 |
| Colchicine                             | 41.5 ± 0.7                          | 0.10 ± 0.00                          | 5.9(*)              | 0.02                 |
| Conine                                 | 55.1 ± 0.2                          | 0.43 ± 0.00                          | 80(*)               | 0.63                 |
| N-lobeline hydrochloride               | 30.9 ± 0.9                          | 0.08 ± 0.00                          | 39.9(*)             | 0.11                 |
| Morphine hydrochloride                 | 9915.1 ± 0.8                        | 23.39 ± 0.00                         | 745(*)              | 1.76                 |
| Nicotine                               | 35.1 ± 0.5                          | 0.22 ± 0.00                          | 50(6)               | 0.31                 |
| Quinine sulfate                        | 562.4 ± 9.5                         | 1.44 ± 0.02                          | 800(*)              | 2.04                 |
| (-)-Scopolamine hydrobromide trihydrate| 11465.1 ± 166.1                     | 26.16 ± 0.38                         | 1413(*)             | 3.22                 |
| Strychnine hydrochloride               | 20.8 ± 0.58                         | 0.06 ± 0.00                          | 2.73(*)             | 0.01                 |
| Theobromine                            | 150.4 ± 1.8                         | 0.83 ± 0.01                          | 530(*)              | 2.94                 |
| (+)-Tubocurarine chloride hydrate      | 414.2 ± 3.6                         | 0.61 ± 0.01                          | 33(*)               | 0.05                 |
| Yohimbine hydrochloride                | 93.0 ± 1.5                          | 0.24 ± 0.00                          | 55(*)               | 0.14                 |
| Amygdalin                              | 268.5 ± 19.6                        | 0.59 ± 0.04                          | 250(*)              | 0.55                 |
| Arbutin                                | 120.9 ± 13.0                        | 0.44 ± 0.05                          | 500(*)              | 1.84                 |
| Convallatoxin                          | 36.6 ± 3.5                          | 0.07 ± 0.01                          | 15.2(*)             | 0.03                 |
| Coumarin                               | 241.2 ± 8.9                         | 1.65 ± 0.06                          | 293(*)              | 2.01                 |
| Digoxin                                | 0.5 ± 0.06                          | 0.001 ± 0.00                         | 4.1(*)              | 0.01                 |
| Gentamycin sulfate                     | 253.3 ± 6.5                         | 0.44 ± 0.01                          | 384(*)              | 0.67                 |
| Glycyrhizin                            | 55.8 ± 3.0                          | 0.07 ± 0.00                          | 589(*)              | 0.70                 |
| Hesperidin                             | 77.6 ± 3.2                          | 0.13 ± 0.01                          | 1000(*)             | 1.64                 |
| Kanamycin monosulfate                  | 1787.5 ± 16.8                       | 3.07 ± 0.03                          | 1700(*)             | 2.92                 |
| Naringin                               | 850.1 ± 78.5                        | 1.46 ± 0.14                          | 2000(*)             | 3.45                 |
| Neohesperidin                          | 199.5 ± 1.2                         | 0.33 ± 0.00                          | 1000(*)             | 1.64                 |
| Ouabain octahydrate                    | 184.1 ± 4.8                         | 0.25 ± 0.01                          | 3.75(*)             | 0.01                 |
| Phloridzin dihydrate                   | 793.2 ± 5.1                         | 1.68 ± 0.01                          | 500(*)              | 1.06                 |
| Rutin hydrate                          | 8722.9 ± 164.2                      | 14.29 ± 0.27                         | 2000(*)             | 3.28                 |
| Streptomycin sulfate                   | 3164.0 ± 35.4                       | 2.17 ± 0.02                          | 600(*)              | 0.41                 |
| Cadmium(II) chloride                   | 27.9 ± 0.1                          | 0.06 ± 0.00                          | 88(6)               | 0.18                 |
| Copper(II) nitrate trihydrate          | 58.7 ± 1.2                          | 0.24 ± 0.00                          | 940(*)              | 3.89                 |
| Lead acetate trihydrate                | 62.4 ± 1.1                          | 0.16 ± 0.00                          | 174(*)              | 0.46                 |
| Lithium chloride                      | 3324.2 ± 143.6                      | 78.42 ± 3.39                         | 1165(*)             | 27.48                 |
| Chloramphenicol                        | 525.0 ± 7.4                         | 1.62 ± 0.02                          | 400(*)              | 1.24                 |
| Ethanol                                | 36212.0 ± 501.8                     | 786.02 ± 10.89                       | 14008.3(6)          | 304.07                |
| Glycerol                               | 23357.4 ± 282.1                     | 253.58 ± 3.06                        | 12619(6)            | 137.00                |
| Tween 80                               | 323.4 ± 10.1                        | 0.25 ± 0.01                          | 25021(6)            | 19.10                 |
| Acetic acid                            | 186.3 ± 1.0                         | 3.10 ± 0.02                          | 3309.3(6)           | 55.11                 |
| Salicylic acid                         | 46.7 ± 1.2                          | 0.34 ± 0.01                          | 184(*)              | 1.33                 |
| Sodium oxalate                         | 372.2 ± 2.9                         | 2.78 ± 0.02                          | 155.4(*)            | 1.16                 |
| Trichloroacetic acid                   | 66.4 ± 4.7                          | 0.41 ± 0.03                          | 270(*)              | 1.65                 |
| Ampicillin sodium                      | 6068.5 ± 114.9                      | 16.34 ± 0.31                         | 5314(7)             | 14.31                 |
| Cyclophosphamide monohydrate           | 1777.4 ± 26.1                       | 6.37 ± 0.09                          | 1930.9(6)           | 6.92                 |
| Paracetamol                            | 535.8 ± 17.1                        | 3.54 ± 0.11                          | 367(*)              | 2.43                 |
| Phenacetin                             | 309.9 ± 8.4                         | 1.73 ± 0.05                          | 634(*)              | 3.54                 |
| Benserazide hydrochloride              | 4747.9 ± 28.7                       | 16.17 ± 0.10                         | 5000(*)             | 17.02                 |
| Chlorpromazine hydrochloride           | 7.0 ± 0.04                          | 0.02 ± 0.00                          | 20(*)               | 0.06                 |
| Isoniazid                              | 1297.5 ± 38.0                       | 9.46 ± 0.28                          | 1250(7)             | 9.12                 |
Table 2. Cont.

| Compounds                  | Zebrafish Embryo LC50 (mg/L ±SEM) | Zebrafish Embryo LC50 (mmol/L ±SEM) | Rodent LD50 (mg/kg) | Rodent LD50 (mmol/kg) |
|----------------------------|-----------------------------------|-------------------------------------|--------------------|-----------------------|
| 49 Phenelzine sulfate      | 11.5±0.13                         | 0.05±0.00                           | 125(*)             | 0.53                  |
| 50 Ethambutol dihydrochloride | 6325.9±197.2                    | 22.82±0.71                         | 6800(*)            | 24.53                 |
| 51 Verapamil hydrochloride  | 81.1±4.8                         | 0.17±0.01                           | 108(**)            | 0.22                  |
| 52 Phenol                  | 86.4±0.8                         | 0.92±0.01                           | 112(**)            | 1.19                  |
| 53 Sodium azide            | 1.4±0.04                         | 0.02±0.00                           | 19(+)              | 0.29                  |
| 54 Dimethyl sulfoxide      | 20964.6±158.1                    | 268.33±2.02                        | 19691.3(**)        | 252.03                |
| 55 Formaldehyde            | 12.7±0.1                         | 0.42±0.00                           | 42(**)             | 1.40                  |
| 56 Phenformin hydrochloride | 508.3±17.6                      | 2.10±0.07                           | 407(+)             | 1.69                  |
| 57 Ropinirole hydrochloride | 437.3±10.2                      | 1.47±0.03                           | 396(+)             | 1.33                  |
| 58 Amtriptyline hydrochloride | 8.0±0.1                      | 0.03±0.00                           | 21(+)              | 0.07                  |
| 59 Sodium dodecyl sulfate  | 3.6±0.3                         | 0.01±0.00                           | 118(+)             | 0.41                  |
| 60 Barbital sodium         | 3902.5±30.5                      | 18.93±0.15                          | 310(+)             | 15.04                 |

Key
(*) from Chemical Identification/Dictionary database at http://toxnet.nlm.nih.gov/cgibin/sis/search/;
(†) from [36].
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Egg water

Egg water was made from 0.21 g ‘Instant Ocean®’ salt in 1 L of Milli-Q water with resistivity of 18.2 MΩ·cm.

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 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°C (because preliminary studies indicated that all embryos die within sealed plates). The plates were kept at 28±0.5°C without refreshing the buffer (static non-replacement regime) and weighed at daily intervals on a digital balance. Results were calculated as mean from four different plates. Buffer volume from individual wells in different regions of the plate were also weighed at 4 days to determine the impact of well location on the evaporation rate.

Test compounds

We used water-soluble compounds representing a range of different chemical classes and biochemical activities (Table S1). The required dilution was always freshly prepared in buffer just prior to assay on zebrafish embryos.

Mortality scoring

Mortality rate (Table 1) was recorded at 48, 72, 96 and 120 hpf in both logarithmic series and geometric series using a dissecting stereomicroscope. Embryos were scored as dead if they were no longer moving, the heart was not beating and the tissues had changed from a transparent to an opaque appearance.

Range-finding

To determine a suitable range of concentrations for testing, we performed range-finding using a logarithmic series (0.0, 1.0, 10.0, 100.0 and 1000 mg/L) as recommended in standard protocols [11]. Zebrafish embryos of 24 hpf from Petri dish were gently transferred using a sterile plastic pipette into 96-well microtitre plates. 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) only as controls. We used 16 embryos for each concentration and 16 embryos as controls for each drug.

for all 96-well plate experiments reported in this study, the lids were in place but were not sealed with a sealing mat or film (because preliminary studies indicated that all embryos die within sealed plates). The plates were kept at 28±0.5°C without refreshing the buffer (static non-replacement regime) and weighed at daily intervals on a digital balance. Results were calculated as mean from four different plates. Buffer volume from individual wells in different regions of the plate were also weighed at 4 days to determine the impact of well location on the evaporation rate.

Table 2.

| Compounds                  | Zebrafish Embryo LC50 (mg/L ±SEM) | Zebrafish Embryo LC50 (mmol/L ±SEM) | Rodent LD50 (mg/kg) | Rodent LD50 (mmol/kg) |
|----------------------------|-----------------------------------|-------------------------------------|--------------------|-----------------------|
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| 52 Phenol                  | 86.4±0.8                         | 0.92±0.01                           | 112(**)            | 1.19                  |
| 53 Sodium azide            | 1.4±0.04                         | 0.02±0.00                           | 19(+)              | 0.29                  |
| 54 Dimethyl sulfoxide      | 20964.6±158.1                    | 268.33±2.02                        | 19691.3(**)        | 252.03                |
| 55 Formaldehyde            | 12.7±0.1                         | 0.42±0.00                           | 42(**)             | 1.40                  |
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| 59 Sodium dodecyl sulfate  | 3.6±0.3                         | 0.01±0.00                           | 118(+)             | 0.41                  |
| 60 Barbital sodium         | 3902.5±30.5                      | 18.93±0.15                          | 310(+)             | 15.04                 |
Geometric series and LC50 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 S2. The concentrations were in a geometric series in which each was 50% greater than the next lowest value [11]. 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 drug). LC50 (expressed in mg/L of buffer) was determined based on cumulative mortality obtained from three independent experiments at 120 hpf using Regression Probit analysis with SPSS Statistics for windows version 17.0 (SPSS Inc., Chicago, USA). Thus the embryos

Figure 1. Cumulative mortality and infertility of zebrafish in buffer or egg water. Embryos were kept in 92 mm Petri dishes with 40 ml of either buffer or egg water, 60 eggs per dish. Each error bar represents ±SEM of N = 420 embryos each for buffer and egg water. A, cumulative infertility and early mortality in buffer. B, the same, in egg water. There is no significant difference between the two media in terms of survival and fertilization percentage.
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are exposed to the drug for 96 h. The LC50 in mg/L was converted into LC50 mmol/L to make relative toxicity easier to examine.

Rodent data

The sources of LD50 data from rodents (rats and mice) are shown in Table 2.

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) or R (v. 2.12). One way ANOVA and Newman-Keuls Multiple Comparison test was employed for survival rate. Correlation and ANCOVA models were used to investigate the relationship between LC50 in zebrafish embryos and published LD50 values in rodents.

Results and Discussion

We have examined the toxicity, in zebrafish embryos, of a 96 h exposure (during the period 24 hpf to 5 dpf) to 60 compounds of differing biochemical classes. Our logarithmic and geometric
concentration series both showed concentration-dependent mortality. LC\textsubscript{50} values were determined, and compared with rodent LD\textsubscript{50} values from the literature.

Infertility and spontaneous early mortality of eggs/embryos

We found that, in controls (buffer only), 5% of eggs were unfertilised, and a further 9% represented embryos that died spontaneously in the first 24 hpf. This is similar to the spontaneous mortality of 5–25% reported elsewhere for early zebrafish development [40]. We find no significant difference between these values when Hank’s buffer was used as the medium, and when egg water was used (Figure 1A, B). In order to avoid this natural early mortality we began our assays at 24 hpf. This also makes our study consistent with a previous one, in which the zebrafish was exposed to different compounds at 24 hpf to find the correlation between zebrafish and rodent toxicities [34].

It could be argued that, by beginning exposure at 24 h, we are missing out on early developmental toxicity effects, such as the action of compounds on gastrula stages. However, this is likely to be a trade-off because other compounds mainly cause embryo death at these early stages. For example, a recent study [42] showed that exposure of zebrafish embryos at early stages (dome to 26-somite) to ethanol resulted in high mortality, while exposure at later stages (prim-6 and prim-16) led to a high incidence of abnormal embryos. Other examples of compounds which are more toxic to larval stages than to embryonic and adult stages of freshwater fish species are copper and cadmium [43–45]. Finally, it is known that presence of chorion at early stages acts as a possible barrier to diffusion of compounds [29,30,42].

Rate of evaporation from 96-well plates at 28.0°C

In our study, we did not replace the buffer. Therefore, we decided to check how much water would be lost during this period by evaporation from the 96-well plate (with its lid in place). We found that, by 96 h of incubation at 28.0°C, 9.46% of the buffer had evaporated (Figure 2A). Further investigation showed that the rate of evaporation was higher in the external rows and columns, and highest of all in the four corner wells (Figure 2B). In view of this evaporation pattern, we filled all the 96-wells with buffer, but did not plate embryos into wells A1-H1 and A12-H12. A way of mitigating the effects of this rate of evaporation would be to use dynamic replacement of buffer, as in a microfluidic chip [39], or static replacement (e.g. daily refreshing). Nonetheless, static non-replacement, as used here, is a popular technique for zebrafish embryo culture, and was used in a recent toxicity study [46].

Concentration response and LC\textsubscript{50} of compounds

For all compounds, mortality at 5 dpf was concentration-dependent (Table 1). This was true for both logarithmic and geometric series. By contrast, controls (vehicle only) showed 0% mortality. The LC\textsubscript{50} values are shown in Table 2.

Correlation between zebrafish embryo log LC\textsubscript{50} and rodent log LD\textsubscript{50}

To examine the ability of zebrafish assays to predict toxicity in rodents, we analysed a correlation between our zebrafish embryo concentration and LC\textsubscript{50} values with the corresponding LD\textsubscript{50} values from the literature. We found a significant correlation (Figure 3). The correlation coefficient was 0.73403 (Table 3). This indicates that the zebrafish embryo assay is a useful tool for predicting rodent toxicity.
log LC50 values, and rodent log LD50 from the literature. The comparison is shown graphically in Figure 3. A correlation test produced Spearman’s rank correlation of 0.7688 (p < 0.001) and a Pearson’s product-moment correlation 0.7832 (df = 178, p < 0.001) between zebrafish embryo LD50 and rodent log LD50 for the whole set of compounds. These values of correlation indicate that zebrafish LC50 and rodent LD50 values co-vary. This is consistent with a previous report [34] that the toxicity of 18 compounds in zebrafish embryos was well-correlated with values reported from rodent studies. It is also in line with another study [46] suggesting that zebrafish embryos could be used as a predictive model for the developmental toxicity of compounds.

Toxicity by compound class

We next developed a statistical model that examines the similarity between zebrafish and rodent toxicity values when the effect of the unknown error in the rodent LD50 values, the different compound classes seem to cluster in different regions in the graph.

Figure 4. Linear regression model: rodent log LD50 and zebrafish embryo log LC50. The effect of the different compounds on the slope and intercept of the ANCOVA model. Although we must consider the effect of the unknown error in the rodent LD50 values, the different compound classes seem to cluster in different regions in the graph.

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Table 3. Statistical analysis of regression per group of compound using the ANCOVA model described in the text.

| Coefficients   | Estimate | Std. error | t-value | p-value | Significance level |
|----------------|----------|------------|---------|---------|-------------------|
| Intercept: Others | -0.43    | 0.08704    | -4.930  | 1.96E-06 | #                  |
| Intercept: Alcohols | -0.64    | 0.35187    | -1.813  | 0.071546 |                    |
| Intercept: Alkaloids | 0.03     | 0.11947    | 0.24    | 0.810735 |                    |
| Intercept: Amides | -0.08    | 0.49548    | -0.168  | 0.866425 |                    |
| Intercept: Carboxylic acids | -0.17   | 0.23275    | -0.751  | 0.45351  |                    |
| Intercept: Glycosides | -0.2     | 0.10027    | -1.970  | 0.050426 |                    |
| Slope: Others | 1.27     | 0.08803    | 14.456  | 2.00E-16 | *                  |
| Slope: Alcohols | 1.24     | 0.21852    | -0.139  | 0.889249 | #                  |
| Slope: Alkaloids | 0.56     | 0.13171    | -5.427  | 1.97E-07 | *                  |
| Slope: Amides | 1.06     | 0.63408    | -0.326  | 0.744924 |                    |
| Slope: Carboxylic acids | 0.36   | 0.27869    | -3.279  | 0.001265 | *                  |
| Slope: Glycosides | 0.77     | 0.13576    | -3.684  | 0.000309 | *                  |

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alkaloids have a slope significantly less than 1.0 indicating that... than in rodents. The groups carboxylic acids, glycosides and contrasts, ‘others’ and alcohols have a slope significantly greater than 1.0, indicating a very similar toxicity in zebrafish and rodents. By type on intercept and slope.

ANCOVA model, while the dataset is displayed graphically in Figure 4. As can be seen, there is a significant effect of compound type on intercept and slope.

The slope for amides (Table 3) does not differ significantly from 1.0, indicating a very similar toxicity in zebrafish and rodents. By contrast, ‘others’ and alcohols have a slope significantly greater than 1.0, indicating that they are more toxic in zebrafish than in rodents. The groups carboxylic acids, glycosides and alkaloids have a slope significantly less than 1.0 indicating that they are more toxic in zebrafish than in rodents (Table 3).

If we look at the relative toxicity ([zebrafish LC50 mmol/L] + [rodent LD50 mmol/kg]) of individual compounds we see the following examples of compounds that have a similar toxicity in the two species: coumarin (0.95), henseraize hydrochloride (1.06), phenformin hydrochloride (1.11) and theobromine (1.11). Examples of compounds less toxic in zebrafish than in rodents are aconitine (0.01), ouabain octohydrate (0.02), tubocurarine hydrochloride (0.07), morphine hydrochloride (0.08) and colchicine (0.13). At the other extreme are compounds more toxic in zebrafish than in rodents including: Tween80 (103.01), sodium dodecyl sulfate (98.33), lead acetate trihydrate (29.49) and copper (II) nitrate trihydrate (19.40).

Among the alcohols, the general trend is a lower toxicity in zebrafish than in rodents. Tween 80 is an exception to this trend because it is much more toxic to zebrafish. This could be because of its surfactant properties, a support suggested by the comparably high relative toxicity to zebrafish (98.33) that we find for another surfactant tested, sodium dodecyl sulphate (SDS). Our LC50 for SDS in 96 h exposure to zebrafish embryos was 3.6 mg/L. This is similar to the dose of SDS that causes pathological changes in the gills of the teleost Thalassoma pavo [47]. Copper also appears to interfere with ion transport in the gills [reviewed in: 48] as does lead [49]. The lower relative toxicity of colchicine to zebrafish has been previously reported [17]. The suggestion is that teleosts may have some protection by virtue of being unable to oxidise colchicine to the much more toxic oxycolchicine [17]. It is also possible that experimental methodology underlies some of the species differences found here. The standard error for the rodent LD50 values were not available in Toxnet or the Registry of Cytotoxity. This is significant because error in the independent variable can have a significant effect on both slope and intercept. Other study-dependent influences on the data could include differences in exposure time, developmental stage, route of exposure between the zebrafish and rodent studies.

Conclusions

Our findings show that the zebrafish embryo is a tool that offers potential in the evaluation of drug safety. However, we show that the predictivity varies between the class of compound studied. More work is required to examine how the covariance of zebrafish and rodent toxicity is influenced by such factors as compound type, absorption, metabolism and mechanism of toxicity.

Supporting Information

Table S1 Summary of compounds used in this study for toxicity evaluation in zebrafish embryo.
(DOC)
Table S2 Concentrations used in geometric series.
(DOC)

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Author Contributions

Conceived and designed the experiments: SA MKR HGJvM. Performed the experiments: SA. Analyzed the data: SA MKR HGJvM. Contributed reagents/materials/analysis tools: MKR. Wrote the paper: SA MKR

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