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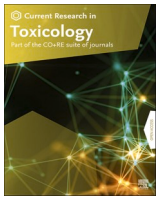

Toxicokinetics of a developmental toxicity test in zebrafish embryos and larvae: Relationship with drug exposure in humans and other mammals

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ABSTRACT

To study the effects of drugs on embryo/fetal development (EFD), developmental and reproductive toxicity studies in zebrafish (*Danio rerio*) embryos is expected to be an accepted alternative method to animal studies using mammals. However, there is a lack of clarity in the relationship between the concentration of developmental toxicity agents in whole embryos or larvae (Ce) and that in aqueous solution (Cw), and also between the amount of drug exposure required to cause developmental toxicity in zebrafish embryos or larvae and that required in mammals. Here, we measured Ce for developmental toxicity agents every 24 h starting at 24 h post fertilization (hpf). We found a high correlation (R^2: 0.87–0.96) between log [Ce/Cw] and the *n*-octanol–water distribution coefficient at pH 7 (logD) of each drug at all time points up to 120 hpf. We used this relationship to estimate the Ce values of the 21 positive-control reference drugs listed in ICH guidelines on reproductive and developmental toxicity studies (ICH S5). We then calculated the area under the Ce–time curve in zebrafish (zAUC) for each drug from the regression equation between log [Ce/Cw] and logD and compared it with the AUC at the no-observed-adverse-effect level in rats and rabbits and at the effective dose in humans described in ICH S5. The log of the calculated zAUC for the 14 drugs identified as positive in the zebrafish developmental toxicity test was relatively highly positively correlated with the log [AUC] for rats, rabbits, and humans. These findings provide important and positive information on the applicability of the zebrafish embryo developmental toxicity test as an alternative method of EFD testing. (267 words)

1. Introduction

Zebrafish (*Danio rerio*) embryos and larvae have been increasingly explored as alternative model organisms for *in vivo* toxicity screening in the early drug discovery process because of their low cost, the small amounts of drug required, and the high throughput (Chng et al., 2012; Eimon and Rubinstein, 2009; Gibert et al., 2013; MacRae and Peterson, 2015). They enable the continuous monitoring of developmental morphological alterations and exhibit rapid organogenesis within 72 h post fertilization (hpf), making them suitable for screens for developmental toxicity (Stallman Brown et al., 2012). Zebrafish have high genetic homology to humans (70 % of their genes have identifiable human orthologs) and also have important similarities in organogenesis and functional mechanisms (Howe et al., 2013; McGonnell and Fowkes, 2006). In addition, international momentum to eliminate animal testing in chemical risk assessment is growing every year. In particular, in the field of cosmetics, animal testing has been banned in the European Union (EU) since 2013 (EU, 2009), and this ban has spread to more and more countries each year (Burbank et al., 2023). the use of zebrafish embryos and larvae is in line with the 3Rs (reduction, refinement, and replacement) approach to animal use for scientific purposes, because in Europe they are considered non-protected animals until the stage of independent feeding at 120 hpf, on the basis of a directive on the protection of animals used for scientific purposes (EU Directive 2010/63/EU) (European Commission, 2010). Therefore, the use of zebrafish embryos is highly attractive from the perspective of animal welfare, which has become particularly important in recent years.

In 2020, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) revised the guideline for reproductive and developmental toxicity studies (S5) to allow the use of alternative methods to animal studies using mammals, such as rats and rabbits, in studies of the effects of drugs on embryo/fetal development (EFD) (International Conference on Harmonization, 2020). ICH S5 lists 29 positive-control reference drugs that have been

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Table 1
Drug name and nominal concentration.

Drug name	Supplier	LogD	Nominal concentration (μM)	Nominal concentration (mg/L)
ICH S5 positive control reference drugs				
Cytarabine	TCI	−2.24[a]	**10,000**	**2430**
Fluconazole	TCI	0.50[e]	1300, 2500, **5000**, 10,000	398, 766, **1530**, 3060
Imatinib	MedChemExpress	0.81[b]	25, **50**, 100	12.3, **24.7**, 49.4
Carbamazepine	FUJIFILM Wako	2.45[a]	50, 100, **200**, 400	11.8, 23.6, **47.3**, 94.5
Phenytoin	FUJIFILM Wako	2.47[a]	**75**, **150**	**18.9**, **37.8**
Aspirin	FUJIFILM Wako	−2.57[a]	200, 400, 800	36.0, 72.1, 144
Cisplatin	FUJIFILM Wako	−2.28[d]	0.80, 4.0, 20, 100	0.240, 1.20, 6.00, 30.0
Methotrexate	FUJIFILM Wako	−2.52[a]	63, 130, 250, 500	28.6, 59.1, 114, 227
Ribavirin	FUJIFILM Wako	−2.43[a]	1300, 2500, 5000, 10,000	317, 610, 1220, 2440
Hydroxyurea	FUJIFILM Wako	−1.27[h]	630, 1300, 2500, 5000, 10,000	98.9, 190, 380, 761
5-Fluorouracil	FUJIFILM Wako	−0.89[a]	80, 400, 2000, 10,000	10.4, 52.0, 260, 1300
Busulfan	Sigma-Aldrich	−0.52[a]	63, 130, 250, 500	25.5, 32.0, 61.6, 123
Thalidomide	FUJIFILM Wako	0.25[g]	400	103
Cyclophosphamide monohydrate	FUJIFILM Wako	0.63[a]	630, 1300, 2500, 5000	164, 339, 653, 1310 (as anhydride)
Dasatinib	FUJIFILM Wako	2.05[b]	0.013, 0.025, 0.050, 0.1000	0.00634, 0.0122, 0.0244, 0.0488
13-*cis*-Retinoic acid	FUJIFILM Wako	4.23[a]	0.0063, 0.013,	0.00189, 0.00391
Acitretin	Sigma-Aldrich	5.5[c]	0.00016, 0.00031, 0.00063, 0.0013, 0.0025	0.0000522, 0.000101, 0.000206, 0.000424, 0.000816
Topiramate	TCI	0.59[f]	31, 63, 130, 250, 500	10.5, 21.4, 44.1, 84.8, 170
Ibuprofen	FUJIFILM Wako	0.81[a]	31, 63, 130	6.39, 13.0, 26.8
All-*trans*-retinoic acid	FUJIFILM Wako	4.23[a]	0.00031, 0.00063, 0.0013, 0.0025	0.0000931, 0.000189, 0.000391, 0.000751
Trimethadione	Sigma-Aldrich	0*	5000, 10,000	716, 1430
Sodium valproate	FUJIFILM Wako	0.13[a]	31, 63, 130, 250	4.47, 9.09, 18.7, 36.1 (as anhydride)
Other developmental toxicity agents				
Sumatriptan succinate	TCI	−1.17[a]	1300, 2500, **5000**, 10,000	538, 1030, **2070**, 4130
Diclofenac sodium	Sigma-Aldrich	1.13[a]	**10.1**	**3.20**
Caffeine	FUJIFILM Wako	−0.07[a]	12.5, (**25.7**), 50, **51.5**, 200, 800, 3200	2.43, (**5.00**), 9.70, **10.0**, 38.8, 155, 621
Testosterone	FUJIFILM Wako	3.29[e]	5.2, **10.4**	1.50, **3.00**
Diethylstilbestrol	TCI	5.07[e]	0.25, 0.50, **1.0**, 2.0	0.0671, 0.134, **0.268**, 0.537

[a] Benet et al., 2011, [b] Giannoudis et al., 2007, [c] Hansen et al., 2010, [d] Jagodinsky et al., 2015, [e] Lombardo et al., 2001, [f] Kyowa Hakko Kirin Co., Ltd. (2022): Drug interview form of Topina®, [g] Fujimoto Pharmaceutical Corporation (2021): Drug interview form of THALED® CAPSULE, [h] Bristol-Myers Squibb Company (2022): Drug interview form of HYDREA® CAPSULE.
*Logarithm of the normal logarithm value for the *n*-octanol/water partition coefficient (logP) from DrugBank (https://go.drugbank.com).
The underlined values in bold indicate the concentrations that were also set in the experiment for measuring Ce.
Values in parentheses represent concentrations set only in the experiment for measuring Ce.

shown to induce morphological abnormalities or embryo–fetal lethality in nonclinical studies under conditions of no apparent maternal toxicity or in humans. The positive-control reference drugs are used for corroboration to confirm the eligibility of an EFD test alternative. Several validations for the detection of developmental toxicity in zebrafish embryos have been published and have shown high concordance rates (81 %–90 %) with the results from *in vivo* studies in mammals (Brannen et al., 2010; Selderslaghs et al., 2009, 2012; Song, et al., 2021; Weiner et al., 2024; Yamashita et al., 2014), so their use in drug discovery is accepted.

However, several problems remain in more accurately predicting the developmental toxicity of various drugs to humans and other mammals. Most developmental toxicity assessments that use phenotype-based methods are based on the drug concentration in aqueous solution (Cw) at a nominal concentration, without measurement of the actual concentration (Brannen et al., 2010; Selderslaghs et al., 2009, 2012; Yamashita et al., 2014). In general, the more lipophilicity a drug has—i.e., the higher the logarithm of the partition coefficient (logP)—the more it accumulates in fish (Arnot and Gobas, 2006). Therefore, Cw does not accurately reflect the drug concentrations directly related to the onset of developmental toxicity. To date, there few studies have measured the drug concentration in whole embryos or larvae (Ce) to explain the relationship between drug concentration and toxicity, and few reports have investigated the relationship between Ce and Cw for each timepoint according to the degree of lipophilicity (Weiner et al., 2024; Ball et al., 2014; Diekmann and Hill, 2013; Huang et al., 2010).

A further problem concerns bioaccumulation, which refers to the accumulation of chemicals in an organism via any route, including inhalation, ingestion, and direct contact (Sanz-Landaluze et al., 2015). The bioaccumulation of chemicals in typical model fish, including zebrafish, can be explained by two first-order kinetics processes—uptake and excretion—based on a one-compartment model (OECD, 2012), and the kinetics parameters and bioconcentration factors for zebrafish embryos or larvae calculated on the basis of a first-order model have been reported (Sanz-Landaluze et al., 2015). Although permeability and bioaccumulation and the temporal changes in Ce during zebrafish developmental toxicity testing may differ depending on the logP value of the drug, there are no reported experimental Ce data for each timepoint under varying logP values and under the same exposure conditions as in the test.

Another problem with using zebrafish embryo and larvae for alternative developmental toxicity testing is that the relationship between the amount of drug exposure required to cause developmental toxicity in zebrafish embryos or larvae and that required in mammals is unknown. The ICH S5 guideline states the need to clarify the relationship between the concentrations used in the alternative methods and the amount of drug exposure at which toxicity occurs in the animal species in which the predictions are made (ICH, 2020). Toxicokinetics studies in mammals for pharmaceuticals examine the dose and systemic exposure of the drug in animals and their relationship to the time course of exposure; they then relate the exposure information to the toxicity findings to help assess clinical safety in humans (Hood, 2012). Generally, in toxicokinetics studies in mammals or in human clinical studies, the blood concentrations of a drug are measured over time to determine the systemic exposure, and the maximum plasma concentration (C_{max}) or area under the curve (AUC) is calculated as a parameter that is related to systemic exposure and can be used to evaluate toxicity (Hood, 2012). However, zebrafish embryos and larvae are so small that blood concentration measurement is technically difficult and morphological observations must be made under the microscope. For these reasons, there

have been limited reports of drug exposure in zebrafish embryos and larvae and its relationship with exposure in humans and other mammals (Weiner et al., 2024).

Therefore, our aim here was to investigate the relationship between the Cw and Ce of developmental toxicity agents and between drug exposure in zebrafish and humans or other mammals to probe the applicability of the zebrafish developmental toxicity test as an alternative method of EFD testing. First, we used developmental toxicity studies to confirm the concentration–response relationships of various drugs in zebrafish embryos and larvae. After that, to examine the temporal changes in drug concentrations in zebrafish embryos and larvae, we measured Ce every 24 hpf and examined the relationship among Ce, Cw, and lipophilicity at each time point of exposure. On the basis of that relationship, the Ce values of the 21 ICH S5 positive-control reference drugs at no observed effect concentration (NOEC) were estimated, and the area under the Ce–time curve in zebrafish (zAUC) for each drug was calculated and compared with the AUC at the no observed adverse effect level (NOAEL) in rats and rabbits and at the effective dose in humans, as described in ICH S5.

2. Materials and methods

Test organisms and collection of fertilized eggs

Wild-type zebrafish (*Danio rerio*, NIES-R strain) were obtained from the National Institute for Environmental Studies (Tsukuba, Japan) and were bred at the breeding facility of the Chemicals Evaluation and Research Institute, Japan (Kurume, Japan). All experiments using the zebrafish were done according to the EU Directive 2010/63/EU for animal experiments (European Commission, 2010). Parent zebrafish were bred in dechlorinated tap water (tap water passed through a cylinder filled with activated carbon to remove chlorine and for aeration) at a temperature of 26 ± 1 °C with a 16-h light/8-h dark lighting cycle. The zebrafish were fed with recently hatched (<24 h old) brine shrimp (*Artemia*) from Great Salt Lake (EGGS-90, Kitamura, Kyoto, Japan) two or three times per day. For the experiment using fertilized eggs, adult male fish (two per container) were placed in glass containers, each of which contained an individual adult female fish, and mated to obtain fertilized eggs. After the fertilized eggs were collected, those at a normal developmental stage with no morphological abnormalities were selected under a stereomicroscope SMZ800 (Nikon, Tokyo, Japan) and used in the experiments.

2.2. Developmental toxicity test

We did not test all the 29 positive control reference drugs suggested by the ICH S5 guideline, only 22, plus other 5 well known teratogenic drugs in zebrafish. Table 1 lists the normal logarithm of the *n*-octanol–water distribution coefficient at pH 7 (logD) and the nominal concentrations of each drug. Preliminary studies were conducted to determine the nominal test concentrations under the same exposure conditions as those used in other tests in the study. The upper limit of concentration was set at 10,000 μM. The number of concentration levels and the geometric ratio of concentrations were not fixed, but the concentrations were set to obtain 100 % or 0 % of developmental toxicity as soon as possible. Aqueous drug solutions at each concentration were prepared by using reconstituted water (ISO 6341–1982) (OECD, 1992) to contain a final concentration of 0.5 % (v/v) dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan). The prepared aqueous drug solution was added to polystyrene 24-well plates (Sumitomo Bakelite, Tokyo, Japan) at 2 mL per well, and fertilized eggs of zebrafish were exposed in 12 replicates (1 egg/well) for between 5 and 120 hpf. During the exposure period, well plates were kept in an incubator (MIW-450 V, AS ONE CORPORATION, Osaka, Japan), without renewal of the aqueous solution, under a 14-h light/10-h dark lighting cycle and at 28 ± 1 °C. After exposure, tricaine methane sulfonic acid (MS-222, Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 0.03 %, and the anesthetized larvae were observed under an inverted microscope (CKX53, Olympus, Tokyo, Japan) to assess the items shown in Table 2, according to the criteria reported by Yamashita *et al.* (2014). The NOEC was defined as the maximum concentration at which no morphological or functional abnormality was observed in any individual organism. If no morphological or functional abnormalities were observed at the concentrations set near the upper limit concentration or water solubility, those concentrations were used as the NOEC.

Table 2
Developmental toxicity observation points.

Organ	Observation points
Heart	Heartbeat abnormalities[a]
	Size anomalies[a]
	Chamber anomalies[a]
Facial shape	Eye anomalies[a]
	Otic anomalies[a]
	Lower jaw anomalies[a]
Body shape	Notochord anomalies[a]
	Tail anomalies[a]
Blood circulation	Facial edema[a]
	Abdominal edema[a]
	Blood circulation anomalies[a]
Hatching	Hatching retardation[b]

[a] Based on criteria published by Yamashita et al. (2014).
[b] Judged as abnormal if not hatched after 72 hpf.

2.3. Evaluation of developmental toxicity in zebrafish

For the ICH S5 positive-control reference drugs listed in Table 1, the presence or absence of developmental toxicity was classified based on findings observed in developmental toxicity studies using zebrafish embryos and larvae. The methods of scoring the toxicity and determining a drug as positive or negative were the same as those reported by Yamashita et al. (2014). For each parameter in each individual, the morphology or function listed in Table 2 without abnormality was assigned a score of zero, and an observed abnormality was assigned a score of 10. The mean total morphological score at the highest concentration at which the survival rate was 50 % or higher was regarded as MS_{50}. When the MS_{50} value of a tested drug was 10 or higher, the drug was judged as "positive" for developmental toxicity in zebrafish. The results of the determination were confirmed to be consistent with the developmental toxicity in humans and mammals.

2.4. Exposure to test drugs and preparation of analytical samples for measuring drug concentration in zebrafish embryos and larvae

The drugs used for measuring drug concentration in zebrafish embryos and larvae and their nominal concentrations in aqueous solutions are shown in Table S1. Collection of fertilized zebrafish eggs and preparation of aqueous drug solutions were performed in the same manner as in the developmental toxicity test. The prepared aqueous drug solutions were added to 500-mL or 1000-mL glass containers, into which the required volume of 5-hpf fertilized zebrafish eggs (density: ≤1 egg/mL) was placed to start exposure. During the exposure period, the glass containers were kept in an incubator (MIW-450 V) under a 14-h light/10-h dark lighting cycle and at 28 ± 1 °C. To maintain the drug concentration in aqueous solution as closely as possible, the aqueous solution was freshly prepared and renewed at 48 hpf for testosterone and every 24 hpf for diethylstilbestrol. For the other drugs, the aqueous solutions were not renewed because no decrease in concentration was observed in our preliminary study.

Exposed embryos of larvae (three to 100 embryos or larvae per replication, three replicates per level per time point) were sampled and analyzed every 24 hpf up to a maximum of 120 hpf. The chorions of embryos were removed with forceps before measurement of the Ce. The

Table 3
Results of the developmental toxicity test.

Drug name	Nominal concentration (μM)	Nominal concentration (mg/L)	Total number	Developmental toxicity		Lethality		Developmental toxicity findings
				Number	Incidence (%)	Number	Incidence (%)	
Cytarabine	10,000	2430	12	0	0	0	0	–
Sumatriptan succinate	1300	538	12	0	0	0	0	HR, DHB, LJA, AE
	2500	1030	12	0	0	0	0	
	5000	2070	12	0	0	0	0	
	10,000	4130	12	6	50	0	0	
Fluconazole	1300	398	12	0	0	0	0	HR, DHB, HSR, LJA, AE
	2500	766	12	0	0	0	0	
	5000	1530	12	0	0	0	0	
	10,000	3060	12	11	92	1	8	
Imatinib	25	12.3	12	0	0	0	0	DHB, HSR
	50	24.7	12	0	0	0	0	
	100	49.4	12	6	50	0	0	
Diclofenac sodium	10.1	3.20	12	0	0	0	0	HR, DHB, HSR, AE, DBC
	20.1	6.40	12	11	92	0	0	
	40.2	12.8	12	–	–	12	100	
	80.5	25.6	12	–	–	12	100	
Carbamazepine	50	11.8	12	0	0	0	0	HR, HSR, NA, TA, FE, AE, DBC
	100	23.6	12	0	0	0	0	
	200	47.3	12	0	0	0	0	
	400	94.5	12	12	100	0	0	
Phenytoin	75	18.9	12	0	0	0	0	–
	150	37.8	12	0	0	0	0	
Testosterone	5.2	1.50	12	0	0	0	0	HR, DHB, HSR, NA, TA, FE, AE,
	10.4	3.00	12	0	0	0	0	DBC
	20.8	6.00	12	2	17	0	0	
	41.6	12.0	12	11	92	0	0	
	83.2	24.0	12	–	–	12	100	
Diethylstilbestrol	0.25	0.0671	12	0	0	0	0	HR, DHB, HSR, EA, LJA, TA, FE,
	0.5	0.134	12	0	0	0	0	AE, DBC
	1.0	0.268	12	0	0	0	0	
	2.0	0.537	12	12	100	0	0	
Aspirin	200	36.0	12	0	0	0	0	DBC
	400	72.1	12	1	8	0	0	
	800	144	12	–	–	12	100	
Cisplatin	0.8	0.240	12	0	0	0	0	HR, DHB, HSR, NA, TA, AE, DBC
	4.0	1.20	12	9	75	0	0	
	20	6.00	12	12	100	0	0	
	100	30.0	12	12	100	0	0	
Caffeine	12.5	2.43	12	0	0	0	0	DHB, HSR, NA, FE,
	50	9.70	12	0	0	0	0	AE, DBC
	51.5	10.0	12	0	0	0	0	
	200	38.8	12	4	33	0	0	
	800	155	12	12	100	0	0	
	3200	621	12	–	–	12	100	
Methotrexate	63	28.6	12	0	0	0	0	DHB, HSR, EA, LJA, NA,
	130	59.1	12	0	0	0	0	TA, FE, AE, DBC
	250	114	12	2	18	1	8	
	500	227	12	7	100	5	42	
Ribavirin	1300	317	12	1	8	0	0	DHB, HSR, LJA, NA,
	2500	610	12	0	0	0	0	TA, FE, AE
	5000	1220	12	2	17	0	0	
	10,000	2440	12	8	67	0	0	
Hydroxyurea	630	47.9	12	0	0	0	0	DHB, HSR, LJA, NA,
	1300	98.9	12	0	0	0	0	TA, FE, DBC
	2500	190	12	1	8	0	0	
	5000	380	12	0	0	0	0	
	10,000	761	12	8	100	4	33	
5-Fluorouracil	80	10.4	12	0	0	0	0	HR, DHB, HSR, EA,
	400	52.0	12	0	0	0	0	LJA, NA, TA, AE, DBC
	2000	260	12	1	8	0	0	
	10,000	1300	12	9	100	3	25	
Busulfan	63	15.5	12	0	0	0	0	DHB, HSR, LJA, AE, DBC
	130	32.0	12	1	8	0	0	
	250	61.6	12	1	8	0	0	
	500	123	12	2	17	0	0	

(*continued on next page*)

Table 3 (*continued*)

Drug name	Nominal concentration (μM)	Nominal concentration(mg/L)	Total number	Developmental toxicity		Lethality		Developmental toxicity findings
				Number	Incidence (%)	Number	Incidence (%)	
Trimethadione	5000	716	12	0	0	0	0	–
	10,000	1430	12	0	0	0	0	
Sodium valproate	31	4.47	12	0	0	0	0	DHB, HSR, LJA, NA,
	63	9.09	12	1	8	0	0	TA, FE, AE, DBC
	130	18.7	12	9	82	1	8	
	250	36.1	12	12	100	0	0	
Thalidomide	400	103	12	0	0	0	0	–
Cyclophosphamide monohydrate	630	164	12	0	0	1	8	DHB, HSR, LJA, NA, TA, FE, AE,
	1300	339	12	0	0	0	0	DBC
	2500	653	12	0	0	0	0	
	5000	1310	12	12	100	0	0	
Dasatinib	0.013	0.00634	12	0	0	0	0	HSR, NA, FE, AE, DBC
	0.025	0.0122	12	0	0	0	0	
	0.050	0.0244	12	0	0	1	8	
	0.10	0.0488	12	8	67	0	0	
13-*cis*-Retinoic acid	0.0063	0.00189	12	0	0	0	0	DHB, HSR, LJA, NA, TA, FE, AE,
	0.013	0.00391	12	11	100	1	8	DBC
Acitretin	0.00016	0.0000522	12	0	0	0	0	HR, DHB, HSR, LJA, NA, TA,
	0.00031	0.000101	12	7	58	0	0	FE, AE, DBC
	0.00063	0.000206	12	12	100	0	0	
	0.0013	0.000424	12	12	100	0	0	
	0.0025	0.000816	12	10	100	2	17	
Topiramate	31	10.5	12	0	0	0	0	HR, DHB, HSR, LJA, FE, DBC
	63	21.4	12	0	0	0	0	
	130	44.1	12	2	17	0	0	
	250	84.8	12	9	75	0	0	
	500	170	12	11	100	1	8	
Ibuprofen	31	6.39	12	0	0	0	0	HSR, LJA, NA, TA, FE, DBC
	63	13.0	12	0	0	0	0	
	130	26.8	12	9	100	3	25	
All-*trans*-retinoic acid	0.00031	0.0000931	12	0	0	0	0	DHB, HSR, LJA, NA, TA, FE, AE,
	0.00063	0.000189	12	4	36	1	8	DBC
	0.0013	0.000391	12	12	100	0	0	
	0.0025	0.000751	12	12	100	0	0	

(−): No developmental abnormality, DHB: decreased heartbeat, HSR: heart size reduction, HCA: heart chamber anomalies, EA: eye anomalies, OA: otic anomalies, LJA: lower jaw anomalies, NA: notochord anomalies, TA: tail anomalies, FE: facial edema, AE: abdominal edema, DBC: decreased blood circulation, HR: hatching retardation.

dechorionated embryos (24 hpf) or the larvae (from 48 hpf onwards) were individually transferred through four beakers (for about 10 s per beaker), each containing 500 mL of fresh dechlorinated tap water, to remove chemical residues on the body surface. After that, the embryos or larvae were homogenized with a silicone pestle in a 1.5-mL sampling tube containing a mixed solvent of the same composition as the eluent used to extract the test compound in liquid chromatography. The dechorionated embryos or the larvae were homogenized to extract the test compound. The mixture was centrifuged at 20,000×*g* for 10 min at 10 °C in a refrigerated centrifuge (CR21N, Hitachi Koki, Tokyo, Japan). The supernatant was collected in a volumetric flask. The extraction procedure was performed twice, and the supernatants from the two batches were mixed and brought up to a volume of 1 mL or 2 mL with a mixed solvent of the same composition as the eluent used to extract the test compound in liquid chromatography. They were then filtered through a membrane filter with a 0.2-μm pore size (Millex-LG, Merck KGaA, Darmstadt, Germany) to prepare analytical samples.

In these experiments, the Cw was measured. For testosterone, samples of the fresh aqueous solutions were taken at the start of exposure and at the time of renewal at 48 hpf, and old samples were taken before renewal at 48 hpf and at the end of exposure. For diethylstilbestrol, samples of the newly prepared aqueous solution and the old aqueous solution before renewal were collected, two aliquots of each. For drugs other than testosterone and diethylstilbestrol, aqueous solutions were collected at the start and end of exposure. The aqueous drug solutions were diluted with a solution of the same composition as the eluent for each drug to be within the concentration range of the calibration curve. They were then used as analytical samples.

2.5. Quantification of drug concentration

Preliminary studies have confirmed matrix effects (a loss in analytical response) derived from zebrafish embryos or larvae in the analysis of some drugs. For drugs for which the matrix effect has been confirmed in preliminary studies, a calibration curve was made by preparing standard solutions of the control or vehicle control that had been prepared in the same way, and had the same matrix content, as the analysis samples. For drugs for which it was confirmed that the matrix had no effect on the analytical results, a calibration curve was made by using a standard solution that did not contain the matrix.

A calibration curve was made for each target substance (regression equation by using the least-squares method: $Y = aX+b$, where Y is the analytical response and X is the concentration of the target substance) by using four or more concentrations of standard solution. When the calibration curve met the following criteria: (i) The correlation coefficient (r) was > 0.995; and (ii) the absolute value of the intercept (b) was

Table 4
NOECs of tested drugs.

Drug name	NOEC (mg/L)
Cytarabine	2430
Sumatriptan succinate	2070
Fluconazole	1530
Imatinib	24.7
Diclofenac sodium	3.20
Carbamazepine	47.3
Phenytoin	37.8
Testosterone	3.00
Diethylstilbestrol	0.268
Aspirin	36.0
Cisplatin	0.240
Caffeine	10.0
Methotrexate	59.1
Ribavirin	610
Hydroxyurea	380
5-Fluorouracil	52.0
Busulfan	15.5
Trimethadione	1430
Valproic acid	4.47
Thalidomide	103
Cyclophosphamide	653
Dasatinib	0.0244
13-*cis*-Retinoic acid	0.00189
Acitretin	0.0000522
Topiramate	21.4
Ibuprofen	13.0
All-*trans*-retinoic acid	0.0000931

within 5 % of the maximum analytical response and the linear regression line was treated as a straight line from the origin, the concentration of the target substance was quantified by using the absolute calibration curve method and one concentration of standard solution. When the calibration curve did not meet criterion (ii), the concentration of the target substance was quantified by substituting the analytical response into the regression equation (Y = aX+b).

Analytical samples and standard solutions were analyzed by high-performance liquid chromatography or liquid chromatography – tandem mass spectrometry analysis. The analytical methods and conditions of analysis for each drug are shown in Tables S2 and S3. For high-performance liquid chromatography, each drug was detected by an SPD-20AV UV–VIS (ultraviolet – visible light) detector (Shimadzu, Kyoto, Japan) using an LC-20AD solvent delivery system (Shimadzu) equipped with an L-column ODS (octadecyl–silica) column (length, 150 mm; inner diameter, 4.6 mm; particle size, 5 μm; Chemicals Evaluation and Research Institute, Japan, Tokyo, Japan) or L-column2 ODS column (length, 150 mm; inner diameter, 2.1 mm; particle size, 5 μm; Chemicals Evaluation and Research Institute, Japan). For liquid chromatography – tandem mass spectrometry analysis, an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu) and a Nexera X2 ultra-high-performance liquid chromatograph (Shimadzu) equipped with an ACQUITY UPLC BEH C18 column (length, 50 mm; inner diameter, 2.1 mm; particle size, 1.7 μm; Nihon Waters, Tokyo, Japan) were used.

2.6. Calculation of Ce

The wet weights of five embryos or larvae of the control or vehicle control every 24 hpf up to 120 hpf were individually measured by using a microbalance Cubis MSU 6.6S-DM (Sartorius Japan, Tokyo, Japan). Ce was then calculated by dividing the measured amount of drug in the whole embryo or larva by the average wet weight.

2.7. Values calculated by using obtained regression equation, and comparison with experimental Ce

The regression equation obtained from the plot of log [Ce/Cw] versus logD at each time point was used as the calculated value (Ce(cal)) for the nine drugs employed for the plots (Table S1, excluding caffeine). The values for logD shown in Table 1 were substituted into the regression equation for each drug, and the NOECs, which were determined from the results of the zebrafish developmental toxicity test shown in Table 3, were used as the Cw values. The NOECs are shown in Table 4.

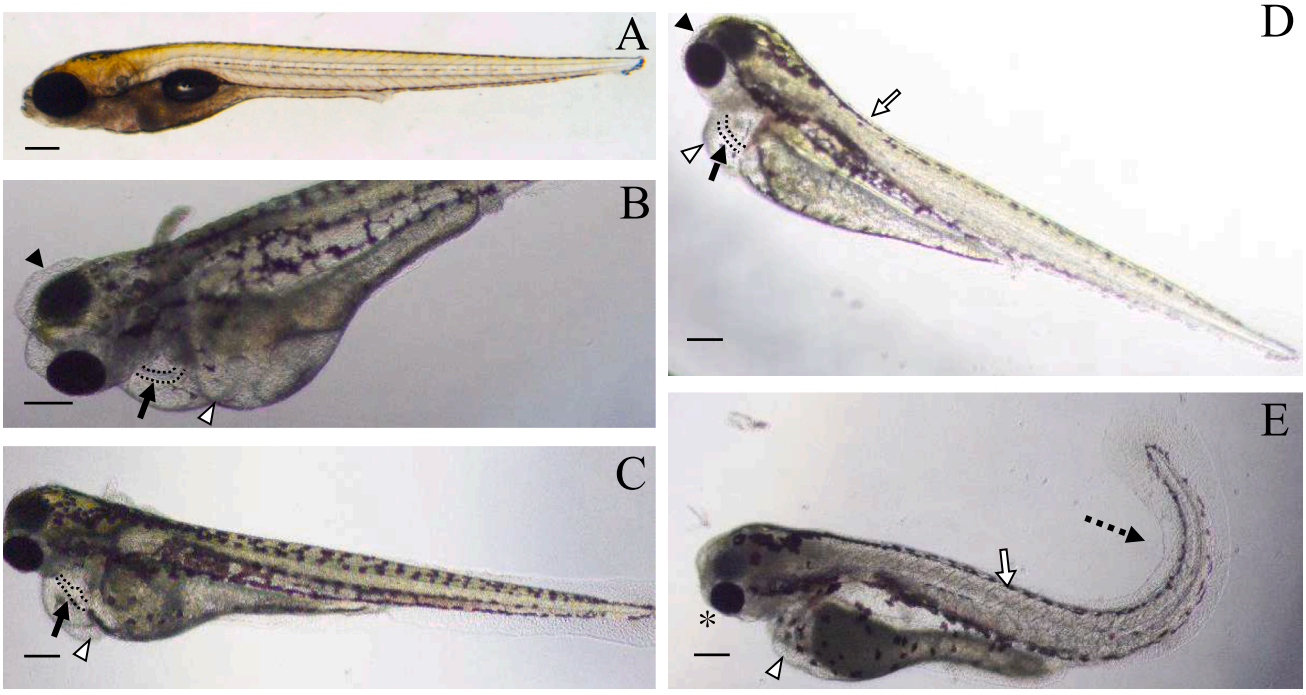

Fig. 1. Morphological abnormalities caused in zebrafish larvae by test drugs. Embryos/larvae were exposed to (A) vehicle (dimethyl sulfoxide), (B) 0.63 nM all-*trans*-retinoic acid, (C) 130 μM ibuprofen, (D) 400 μM carbamazepine, and (E) 400 μM methotrexate from 5 h post fertilization (hpf) until 120 hpf. Black arrows: reduced heart size; white arrows: notochord anomalies; black dotted arrow: tail anomalies; black arrowheads: facial edema; white arrowheads: abdominal edema; and asterisk: eye anomalies. The dotted line shows the outline of the heart. Scale bars indicate 200 μm.

Table 5
Positive/negative developmental toxicity results for ICH S5 positive-control reference drugs.

Drug name	Concentration used for judgment (μM)	MS_{50}	Positive/ negative	Previously published judgment
Cytarabine	10,000	0	N	N[a,c]
Fluconazole	10,000	14.2	P	P[c]
Imatinib	100	5.8	N	N[a], P[c]
Carbamazepine	400	25.5	P	P[a,c]
Phenytoin	300	0	N	I[c]
Aspirin	400	0.8	N	N[b]
Cisplatin	250	15.7	P	P[b,c]
Methotrexate	500	55.7	P	P[b,c]
Ribavirin	10,000	13.3	P	N[b],P[c]
Hydroxyurea	10,000	28.8	P	N[a], P[c]
5-Fluorouracil	2500	63.8	P	P[b,c]
Thalidomide	400	0	N	N[a], I[c]
Cyclophosphamide	5000	20.0	P	P[c]
Dasatinib	50	63.6	P	P[a,c]
13-*cis*-Retinoic acid	0.050	73.6	P	P[c]
Acitretin	0.0025	53.0	P	P[c]
Topiramate	500	57.3	P	P[a,c]
Ibuprofen	130	35.6	P	N[b], P[c]
All-*trans*-retinoic acid	0.20	68.3	P	P[b,c]
Trimethadione	10,000	0.8	N	N[a], P[c]
Valproic acid	310	53.3	P	P[b,c]
Busulfan	100	5.8	N	N[a], P[c]

P and N indicate that the zebrafish embryos were judged as positive (P) or negative (N) for developmental toxicity in zebrafish. Inconclusive (I) indicate that the drug caused some toxicity but not enough for estimation of developmental toxicity.
[a] Inoue et al., 2016.
[b] Yamashita et al., 2014.
[c] Weiner et al., 2024.

The estimated Ce(cal) for each drug was compared with the experimental Ce and the coefficient of determination (R^2) of the log [Ce(cal)] vs. log [Ce] plot was calculated to confirm the accuracy of the prediction. Ce(cal) was then calculated for 21 ICH S5 positive-control reference drugs (Table 1, excluding methotrexate, for which no human/mammalian AUC data are listed in the ICH S5 guideline).

2.8. Comparison of levels of exposure to ICH S5 positive-control drugs in zebrafish and humans/other mammals

The estimated Ce(cal) values at each time point (24, 48, 72, 96, and 120 hpf) for the 21 ICH S5 positive control reference drugs were used to obtain a calculated value for zAUC (zAUC(cal)) at 120 hpf by using Equation (1), based on the trapezoidal rule method (Chiou, 1978).

$$zAUC = \frac{(24-5)\left(Ce^{5}+Ce^{24}\right)+24\left(Ce^{24}+Ce^{48}\right))+24(Ce^{48}+Ce^{72})+24(Ce^{72}+Ce^{96})+24(Ce^{96}+Ce^{120})}{2} \quad (1)$$

where Ce^{t} : Ce at t hpf

The zAUC(cal) values for each of the drugs that we identified as positive (14 drugs) and negative (7 drugs) were then compared with the AUCs for rats, rabbits, and humans, as listed in the ICH S5 guidelines [ICH, 2020]. The AUCs used for comparison were those of rats and rabbits at the NOAEL and those of humans at the effective doses. In this comparison, the experimental zAUC calculated by Equation (1) using the measured values of Ce was also plotted to check the deviation from zAUC(cal).

3. Results

3.1. Concentration–response relationships of zebrafish embryos and larvae exposed to developmental toxicity agents

The results of the developmental toxicity studies for each drug are shown in Table 3, and the maximum NOEC for each drug are shown in Table 4. Additionally, examples of the morphological and functional abnormalities observed in the zebrafish embryos and larvae at the end of exposure to the developmental toxicity agents are shown in Fig. 1. Reduced heart size, abdominal or facial edema, kinked tail, and notochord anomalies were observed as typical abnormalities under all-*trans*-retinoic acid, ibuprofen, carbamazepine, and/or methotrexate. In contrast, cytarabine and trimethadione had no developmental toxicity at the upper limit concentration of 10,000 μM. No developmental toxicity was observed for phenytoin and thalidomide at 150 μM and 400 μM, respectively, likely because of their aqueous solubility. For the other drugs, concentration ranges inducing developmental toxicity or lethality were obtained, and a concentration dependence was observed for the incidence of effects and mortality. The results of our judgment of the ICH S5 positive-control reference drugs as “positive” or “negative” for human/mammalian developmental toxicity on the basis of the observed toxicity findings are shown in Table 5. Of the 22 positive-control reference drugs, 15 were correctly judged to be positive, whereas the remaining seven drugs were judged to be negative. Calculated from these results, the concordance of true positive (sensitivity) was 68 % (15/22) and the false negative rate was 32 % (7/22).

3.2. Drug concentration in zebrafish embryos or larvae and in aqueous solution

The experimental measured values for Ce every 24 hpf for drugs with relatively low liposolubility (logD<1) are shown in Fig. 2 and increased linearly over the time period. In contrast, for drugs with relatively high liposolubility (logD>1), Ce peaked between 48 and 96 hpf, after which it decreased over time (Fig. 3).

In addition, for 10 drugs (cytarabine, sumatriptan succinate, caffeine, fluconazole, imatinib, diclofenac sodium, carbamazepine, phenytoin, testosterone, diethylstilbestrol), a graph of the obtained Ce results at each time point plotted with log [Ce/Cw] on the vertical axis and logD of the drug on the horizontal axis is shown in Fig. 4. For all drugs except caffeine, plotting log [Ce/Cw] against logD gave a linear approximation. In a regression analysis of the plots, R^2 in the regression equation ranged from 0.87 to 0.96. Note that, at all time points, only the caffeine plot deviated clearly from the regression line and was above the regression line at all time points. Although the reason for this is unclear, it could have been caused by some property of caffeine, so it was excluded from the calculation of the regression equation.

The results of the time-weighted mean of Cw in each experiment and for each drug are shown in Table S1. The Cw of each drug ranged from 86.9 % to 110 % of each nominal concentration and mostly maintained the nominal concentration.

3.3. Results of comparison of Ce(cal) with the measured values of the nine drugs that displayed a linear approximation in the regression analysis

From the regression equation obtained from the plot of log [Ce/Cw] versus logD at each time point (Fig. 4), the calculated values of Ce(cal) at

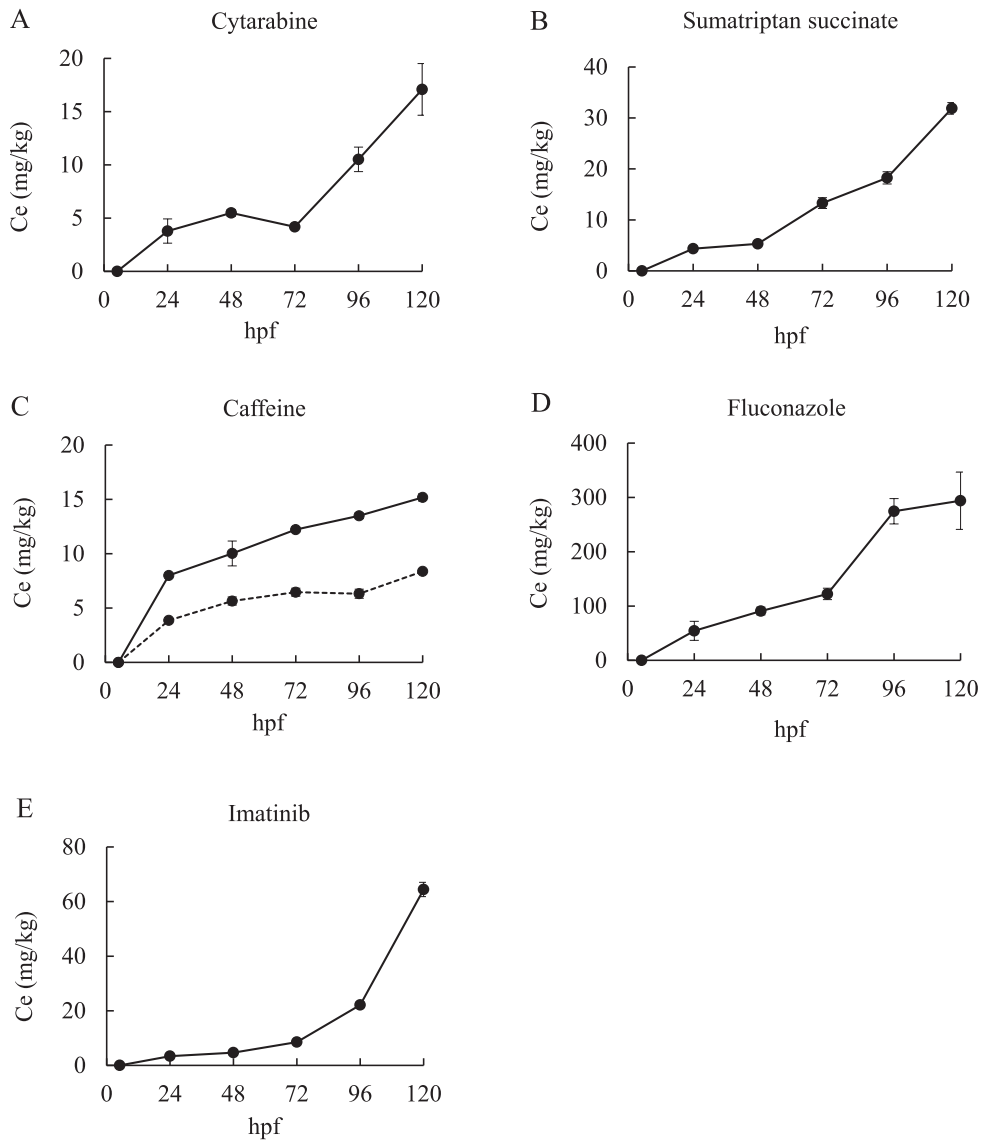

Fig. 2. Temporal changes in the concentrations of drugs with low liposolubility in whole embryos or larvae. Zebrafish embryos or larvae were exposed to each drug (cytarabine, sumatriptan succinate, caffeine, fluconazole, or imatinib) from 5 h post fertilization (hpf) until 120 hpf, and the drug concentration in whole embryos or larvae (Ce) was determined every 24 h from 24 hpf. (A) cytarabine (2430 mg/L), (B) sumatriptan succinate (2070 mg/L), (C) caffeine (solid line: 10.0 mg/L, dotted line: 5.00 mg/L), (D) fluconazole (1530 mg/L), (E) imatinib (24.7 mg/L). Each data point is the mean value of three replicates. Each error bar shows the standard deviation of three replicates.

each time point for the nine drugs included in the analysis are shown in Table S4, and the changes over time in the calculated and measured Ce for each drug are shown in Fig. 5. For most drugs, the pattern of change over time in Ce(cal) for each drug was generally similar to the measured Ce; however, there was poor agreement with the measured data for imatinib (Fig. 5C) and diclofenac sodium (Fig. 5G).

To confirm the prediction accuracy of Ce(cal), the results of plotting log [Ce(cal)] vs. log [Ce] at each time point are shown in Fig. S1. At all time points, the relationship between the two could be regressed to a linear equation. Overall, R^2 of the regression equation ranged from 0.72 to 0.89.

In addition, the results of Ce(cal) for 14 of the 21 ICH S5 positive-control reference drugs that were judged to be positive in the zebrafish developmental toxicity test are shown in Table S5 (as mentioned in section 2.7, methotrexate was removed from the calculations), and the calculated results of Ce(cal) for the seven drugs that were judged to be negative are shown in Table S6. The pattern of change in the calculated Ce(cal) over time was similar to the characteristics of the measured Ce (Fig. 3): The Ce for drugs with logD<1 increased over time up to 120 hpf, and that for drugs with relatively high fat solubility (logD>1) peaked between 48 and 96 hpf and decreased over time thereafter (Tables S5 and S6).

3.4. Calculation of zAUC and comparison with human/mammalian AUC

The zAUC(cal), measured zAUC, and human/mammalian AUC for the ICH S5 positive-control reference drugs are shown in Tables S7 and S8, and the relationship between zAUC (calculated and measured values) and the logarithm of human/mammalian AUC is shown in Fig. 6. The R^2 values for log [zAUC(cal)] of the 14 drugs judged to be positive in zebrafish compared with the log [AUC]s for rat, rabbit, and human were 0.73, 0.92, and 0.74, respectively (Fig. 6A, B, C), whereas those of the seven drugs judged negative in zebrafish were 0.49, 0.032, and 0.31, respectively (Fig. 6D, E, F). Measured Ce values for five ICH S5 positive control reference drugs (fluconazole, carbamazepine, cytarabine, imatinib, and phenytoin) were obtained, and the plots of the measured

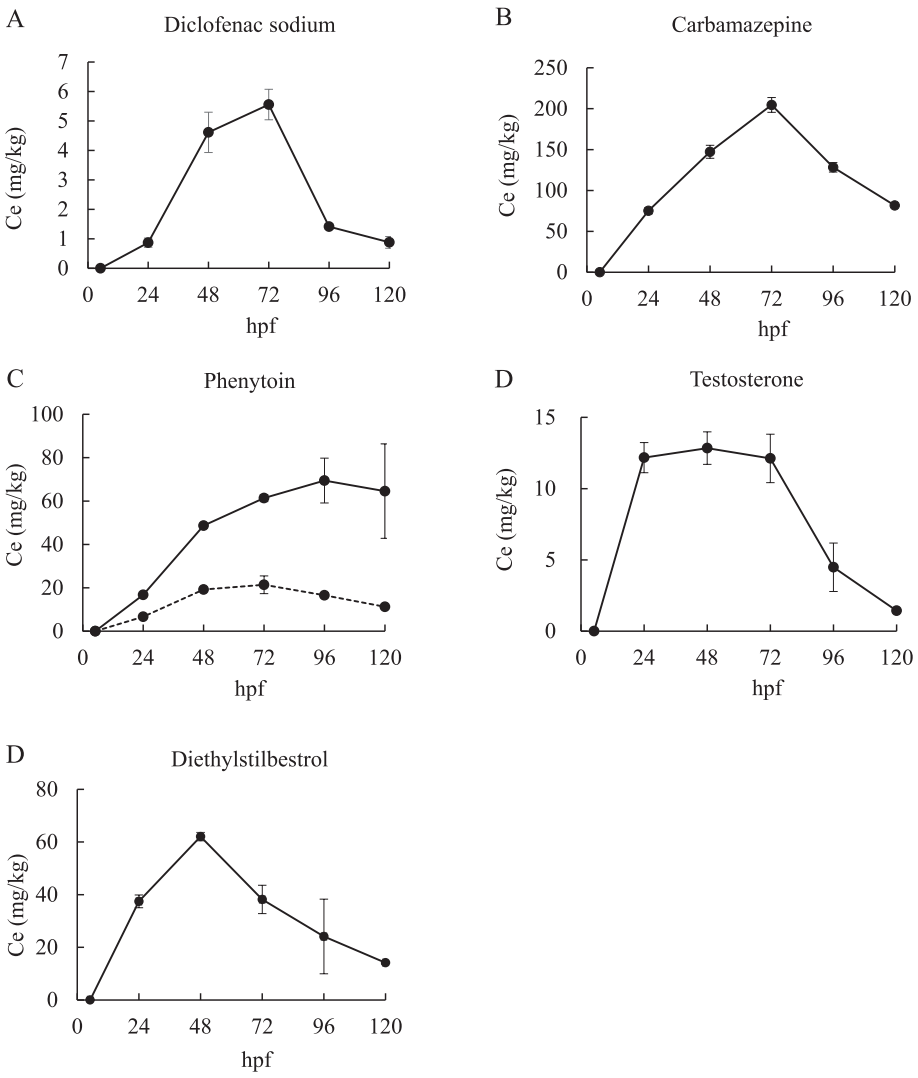

Fig. 3. Temporal changes in the concentrations of drugs with liposolubility in whole embryos or larvae. Zebrafish embryos or larvae were exposed to each drug from 5 h post fertilization (hpf) until 120 hpf, and the drug concentration in whole embryos or larvae (Ce) was determined every 24 h from 24 hpf. (A) diclofenac sodium (3.2 mg/L), (B) carbamazepine (47.3 mg/L), (C) phenytoin (solid line: 37.8 mg/L, dotted line: 18.9 mg/L), (D) testosterone (3.0 mg/L), (E) diethylstilbestrol (24.7 mg/L). Each data point is the mean value of three replicates. Each error bar shows the standard deviation of the three replicates.

values were close to those of the zAUC(cal) values (Fig. 6). Furthermore, regression analysis of the plots of log [zAUC(cal)] vs. log [zAUC] for these five drugs showed that R^2 for the regression equation was 0.92 (Fig. S2).

4. Discussion

As a first step in this study, developmental toxicity studies using zebrafish embryos and larvae were conducted on a total of 27 drugs—22 ICH S5 positive-control reference drugs and five other developmental toxicity agents—to confirm their concentration–response relationships. Concentration ranges inducing developmental toxicity or lethality were obtained for most of the drugs, and the incidences of effects and mortality were concentration dependent, resulting in experimental NOECs. As exceptions, cytarabine and sumatriptan succinate had no effect at the upper concentration limit used (10,000 μM), and thalidomide and phenytoin had no effect at concentrations near the solubility limit, so 10,000 μM, or concentrations near the solubility limit, were used as NOECs. It may be possible to detect developmental toxicity for these four drugs by dechorionation using protease or by raising the upper concentration limit or by forced exposure (e.g., by microinjection or electroporation), rather than exposure by the immersion used in this study (Mikami et al., 2019; Nishiyama et al., 2021). In addition, Liu et al. (2020) reported that craniofacial defects caused by imatinib, phenytoin and busulfan in zebrafish were detected by Alcian blue staining. Combining multiple methods such as these may be an effective means of reducing false negatives.

We investigated the developmental toxicity of ICH S5 positive-control reference drugs and determined it be positive or negative, resulting in a positive agreement rate of 68 % (15/22) and a false negative rate of 32 % (7/22). 15 drugs identified as positive had previously been reported as positive (Table 5) (Inoue et al., 2016; Yamashita et al., 2014; Weiner et al., 2024). Of the seven drugs identified as negative, four drugs other than Imatinib, trimethadione, busulfan were also identified as negative (or inconclusive) in previous papers (Table 5) (Inoue et al., 2016; Yamashita et al., 2014; Weiner et al., 2024). For phenytoin and thalidomide, which we identified as negative, no developmental toxicity was observed at concentrations considered to be at the water solubility limit (phenytoin: 150 μM, thalidomide: 400 μM), suggesting that low water solubility may have been the reason for the false negative results. It is well-known that thalidomide induced malformations in the pectoral fins and other organs of wild-type zebrafish (Ito et al., 2010; Siamwala et al., 2012; Gao et al., 2014); however, it was also reported that the effects of thalidomide by simple soaking in a

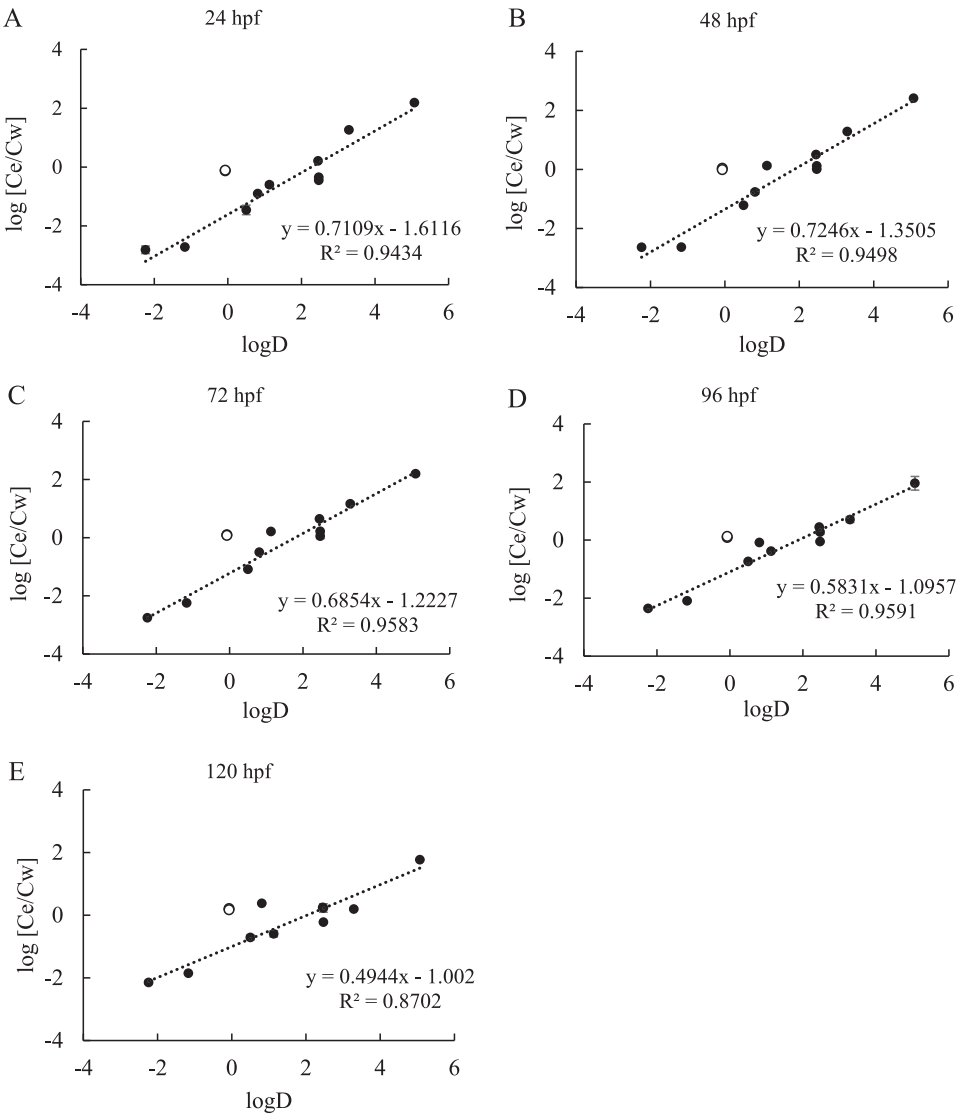

Fig. 4. Relationship between lipophilicity and drug concentration in zebrafish at each time point of exposure to various drugs. The influence of logD on the drug concentration in whole embryos and larvae (Ce) or in aqueous solution (Cw) for 10 drugs (cytarabine, sumatriptan succinate, caffeine, fluconazole, imatinib, diclofenac sodium, carbamazepine, phenytoin, testosterone, and diethylstilbestrol) is shown. Regression lines and equations were obtained from black plots. White data points (which were obtained with caffeine) were excluded from the calculation. Each data point is the mean of three replicates with the standard deviation. hpf: h post fertilization.

thalidomide solution were very weak (Mikami et al., 2019; Dong et al., 2023). Although cytarabine and aspirin did not have low solubility, few morphological abnormalities were observed at about the concentration range where lethality was observed, consistent with reports in previous papers (Inoue et al., 2016; Yamashita et al., 2014; Weiner et al., 2024). Although these drugs were identified as negative in zebrafish, there was no trend toward lower values of zAUC than those of the drugs identified as positive, suggesting that interspecies differences in toxicity sensitivity between zebrafish and human or mammalian species may be the cause of these findings. For imatinib, trimethadione, and busulfan, which we identified as negative, consistent with reports in previous papers by Inoue et al. (2016) and Yamashita et al. (2014), while Weiner et al. (2024) reported that these were correctly identified as positive. For imatinib and busulfan, Wener et al. (2024) increased the DMSO content of them to 1 % in their preparation, which may have allowed them to detect developmental toxicity due to the increased solubility. For trimethadione, it is unclear why our results differ from those of Weiner et al. (2024) but it is possible that differences in test conditions or observation items, or differences in the strain of zebrafish may be factors.

In the results for Ce measured every 24 h from 24 hpf onwards, Ce increased over time for highly water-soluble drugs with logD<1 (Fig. 2), whereas highly lipid-soluble drugs with logD>1 showed a peak in Ce between 48 and 96 hpf (Fig. 3). These temporal behaviors were almost the same as the previously reported Ce of drugs with logD<1 (caffeine and valproate sodium salt) and drugs with logD>1 (diethylstilbestrol, diclofenac sodium salt, and testosterone) in aqueous solution concentrations that caused developmental abnormalities or no abnormalities (Nawaji et al., 2018; Nawaji et al., 2020). In our previous report, we showed that the gradual decline in the Ce of fat-soluble compounds is caused by a decline in the total lipid concentration in whole embryos or larvae with aging, because of the energetic costs of growth and development (Nawaji et al., 2018). It appears that the declines observed here had the same cause. Furthermore, no peaks other than those of the tested drugs were observed on the chromatogram, so it is unlikely that the decline in the Ce levels of those drugs were caused by biotransformation.

We found a high correlation (R^2: 0.87–0.96) between log [Ce/Cw] and logD at all time points every 24 h up to 120 hpf (Fig. 4). The reason

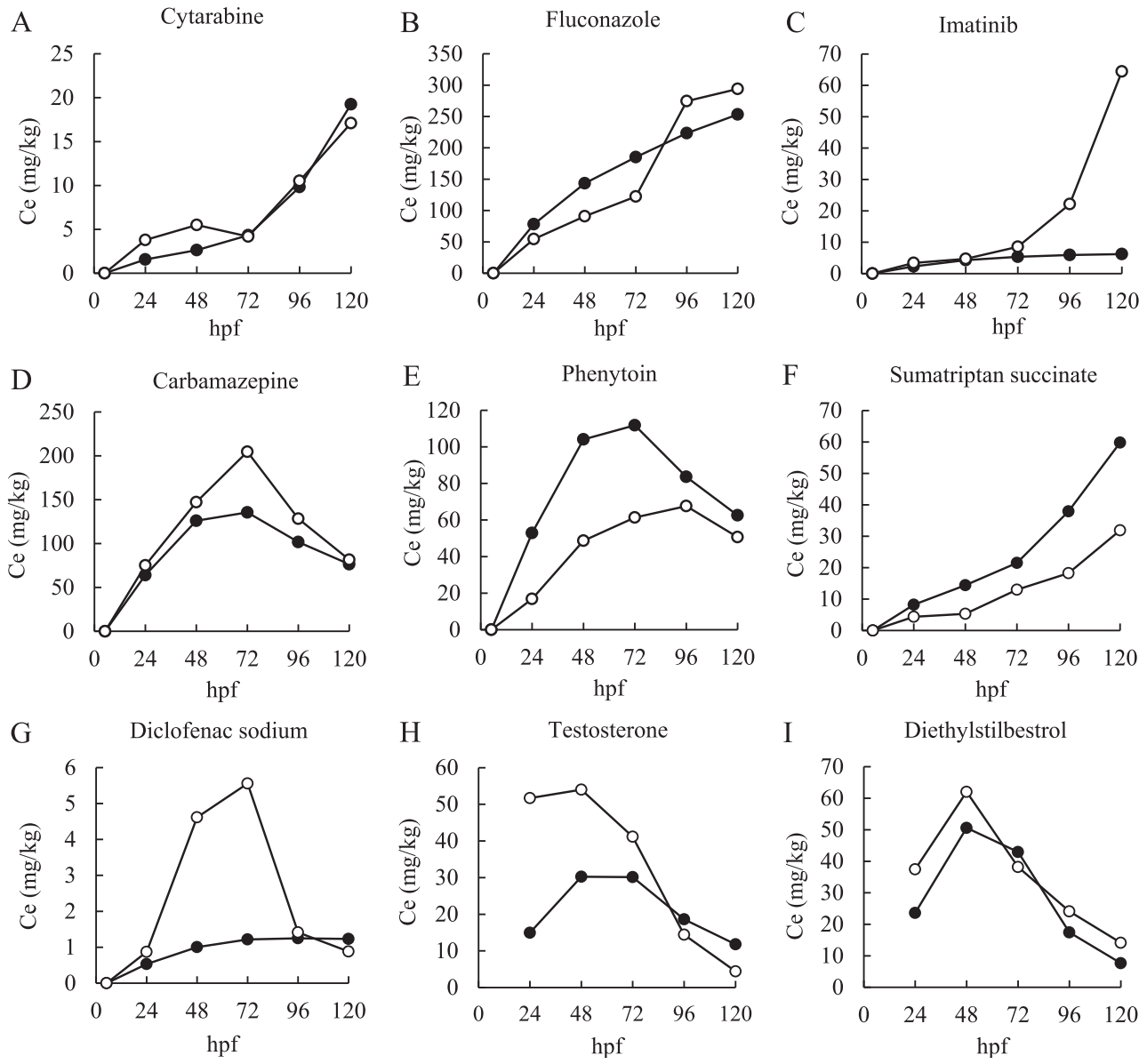

Fig. 5. Comparison of calculated and measured values of drug concentration in whole embryos or larvae. Drug concentration in whole embryos or larvae (Ce) after exposure to each of the nine drugs used in the regression analysis is shown. The average value of three replicates is shown by black data points for calculated Ce and white data points for measured Ce. hpf: h postf ertilization.

for this high correlation may be that the rate of drug concentration in embryos and larvae is strongly dependent on the total fat concentration (lipid content) in general (OECD, 2012); moreover, the total fat concentration in zebrafish embryos and larvae was nearly the same in each developmental stage. At all time points, the caffeine plot deviated well above the regression line, and caffeine was more highly concentrated than the other drugs in zebrafish embryos and larvae. In the caffeine exposure experiments, fish were exposed to two concentrations of caffeine in aqueous solution (5.00 mg/L and 10.0 mg/L); there was little variation in the Ce of the three samples taken at each time point (Fig. 2C), and the Ce/Cw ratio at both concentrations was almost the same (Fig. 4), so it is unlikely that the deviation from the regression line was caused by errors in experimental manipulation or other factors. The physicochemical properties of caffeine, or some intervening mechanism in the body, were likely the main causes of the deviation from the regression line, and caffeine was excluded from the calculations using the regression equation (Fig. 4). No findings have been reported that caffeine tends to accumulate more in animal tissues, or specifically in zebrafish, so it is unclear why caffeine was more highly concentrated than the other drugs in this study. Future analysis of several drugs with similar logD values to caffeine, or of drugs with similar bioactive effects to caffeine, may help to identify the cause. By using the regression equation obtained for the correlation between log [Ce/Cw] and logD at each time point in this study, it should be possible to estimate the drug concentration in zebrafish embryos and larvae if the logD of the developmental toxicity drug and the concentration of the drug in the aqueous solution used for exposure are known. In addition, because the drugs used in the analysis were drugs that showed pharmacological effects through various mechanisms of action, and the results were obtained in a concentration range at which no developmental toxicity was observed, it is highly likely that this correlation between log [Ce/Cw] and logD will hold true for a wide variety of drugs. Further data should be obtained using various categories of drugs in order to verify this.

To check the prediction accuracy of Ce(cal), we compared Ce(cal) with the measured temporal behaviors of Ce for the nine drugs that displayed a linear approximation in the regression analysis (Fig. 4). Five

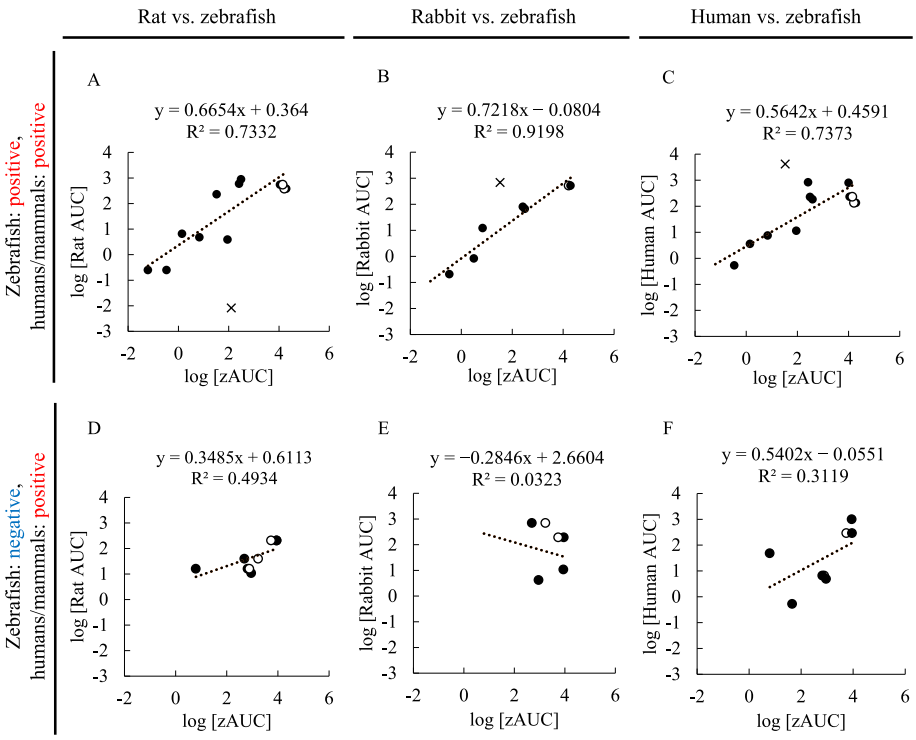

Fig. 6. Comparison of systemic exposure to drugs in zebrafish and in humans/mammals. Comparison of area under the time curve (AUC) in zebrafish (zAUC) with AUCs in (A) rat, (B) rabbit, and (C) human for positive-control reference drugs that were judged as positive in zebrafish in this study. Comparison of zAUC with AUCs in (D) rat, (E) rabbit, and (F) human for positive-control reference drugs that were judged as negative in zebrafish in this study. Black dots and crosses indicate zAUC based on calculated concentrations in whole embryos and larvae (Ce), and white dots indicate zAUC based on the mean values of measured Ce. Regression lines and equations were obtained from the black dots; crosses were excluded from the calculations. The cross in A represents data for ribavirin and the crosses in B and C represent data for valproic acid.

of the drugs were among the 21 ICH S5 positive-control reference drugs. The pattern of temporal behavior of Ce(cal) for each drug was similar to that of the measured Ce, and the coefficient obtained from the log [Ce (cal)] vs. log [Ce] regression analysis ranged from 0.72 to 0.89, indicating a relatively high precision of prediction. Some of the patterns of temporal behavior in Ce(cal) for imatinib and diclofenac sodium salt did not match the measured values (Fig. 5). This lack of agreement in the plots may have been caused by the presence of residuals from the log [Ce/Cw] vs. logD regression equation that were larger than the others.

The zAUC(cal) was determined on the basis of Ce(cal) at every 24 hpf for each of the 21 ICH S5 positive-control reference drugs and compared with the AUC at NOAEL in rats and rabbits and at the effective dose in humans. Log [zAUC(cal)] for 14 of the drugs that were identified as positive in this developmental toxicity study showed a relatively high positive correlation with that of rats, rabbits, and humans (R^2: 0.73–0.92, Fig. 6A, B, and C). For reference, the AUC for humans and the AUCs for rats or rabbits were analyzed in the same way as Fig. 6, and the results are shown in Fig. S3. The R^2 value of the regression equation between the logarithm of the human AUC and the logarithm of the rat and rabbit AUCs was 0.79 and 0.66, respectively. Although the R^2 values in each comparison cannot be simply compared, The R^2 values obtained in this study (Fig. 6A, B, and C) were equal to or higher than the R^2 values between rat or rabbit AUC and human AUC (Fig. S3). In the analysis of Fig. 6A, B, and C, two plots were excluded as outliers: ribavirin in comparison with the rat AUC and also valproic acid in comparison with the human AUC. Ribavirin, which is a nucleic acid analog, had a much lower rat AUC (0.00828 μg•h/mL) than the other drugs, indicating a very high sensitivity to toxicity in rat compared with the sensitivity in zebrafish. Yamashita et al. (2014) indicated that prolonged exposure to ribavirin due to mammalian-specific erythrocyte accumulation of ribavirin triphosphate (RTP) may have increased the susceptibility to ribavirin toxicity in humans/mammals, causing a difference in the susceptibility seen in zebrafish. In mammalian erythrocytes almost all ribavirin is phosphorylated and converted to RTP, but the absence of 5′-nucleotidase and alkaline phosphatase, which hydrolyze RTP to ribavirin in mammalian denucleated erythrocytes, has led to the long-term accumulation of RTP within erythrocytes (Page and Connor, 1990). On the other hand, because the erythrocytes of teleost fish, including zebrafish, have nuclei even in their mature form, zebrafish erythrocytes may retain dephosphorylation activity, and thus RTP accumulation in the erythrocytes is unlikely to occur. Therefore, it is understandable that ribavirin deviates markedly from the regression line in the comparison between zebrafish and rats. (For rabbits and humans, the AUC data for ribavirin are not known, because the ICH S5 guideline does not contain AUC data for ribavirin.) Valproic acid, an antiepileptic drug, is known to be a representative developmental toxicity drug that causes severe developmental abnormalities in humans, mammals, and zebrafish (Alsdorf and Wyszynski, 2005). The plot for valproic acid was uncharted in Fig. 6B and C because it was so high, but it is unclear why the blood levels of valproic acid are higher in humans. It is possible that the accumulation may be higher because of binding affinity to certain tissues, or other factors, but further studies are needed to determine the cause of the deviation of valproic acid from the plot. As mentioned above, except in the case of drugs that deviated from the plot, the zAUC could be used as an indicator of the exposure of zebrafish to drugs that were judged positive for developmental toxicity in both zebrafish and human/mammals. The dose–response relationship in zebrafish may be similar to that in rats, rabbits, and humans for developmentally toxic drugs, confirming the importance of the use of zebrafish as a mammalian alternative method. The R^2 value of the regression equation using the log [zAUC(cal)] vs. log [zAUC] regression analysis was high at 0.92 (Fig. S2), suggesting that, at least for the ICH S5 positive-control reference drugs (five drugs), the influence of the discrepancy between Ce(cal) and the measured Ce values on the comparison of zAUC and AUCs in

other species was small.

On the other hand, for the seven drugs that we identified as negative, the normal logarithm of zAUC(cal) was poorly correlated with the normal logarithm of AUC for rats, rabbits, and humans (Fig. 6D, E, F). From our experimental results and those in previously published reports, the reasons for the negative results are likely low water solubility, interspecies differences in toxicity sensitivity between zebrafish and humans/mammals, and low drug uptake in embryos and larvae. zAUC is the amount of exposure at the NOEC, and the NOEC of each drug deviated from the measured value because of the various factors (low water solubility, interspecies differences in toxicity sensitivity, and low drug uptake), leading to a low correlation with the human/mammal AUC. In addition, only seven drugs were used in this study and the range of the plots was narrow; therefore, further experiments are mandatory to fully understand the correlation in exposure levels between zebrafish and mammals/humans.

5. Conclusion

In conclusion, the normal logarithm of zAUC(cal) for the 14 ICH S5 positive-control reference drugs identified as positive in zebrafish showed a relatively high positive correlation with that in rats, rabbits, and humans. This suggests that zAUC may be useful as an indicator of exposure to developmental toxicity drugs in zebrafish embryos and larvae. Furthermore, our results suggest that zebrafish may have an exposure–response relationship similar to that of rats, rabbits, and humans. As far as we know, our study is the first report of a relationship in drug exposure between zebrafish and mammals, using an original exposure index in the developmental toxicity test with zebrafish embryos and larvae. The findings obtained here provide important information on the relationship between the concentration used in the predictive method and the exposure level at which an adverse outcome occurs in the species in which exposure levels are being predicted. This information is required for use of this method as an alternative EFD test in the ICH S5 guidelines. However, because zAUC(cal) is a value calculated on the basis of a regression equation, it is necessary to collect actual Ce data and zAUC data for many drugs in the future to verify whether a similar correlation can be established.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tasuku Nawaji, Naohiro Mizoguchi, and Ryuta Adachi are currently employed by CERI.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100187.

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