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Measuring drug absorption improves interpretation of behavioral responses in a larval zebrafish locomotor assay for predicting seizure liability

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Methotrexate (PubChem CID: 126,941)
Pentetrazol (PubChem CID: 5917)
Picrotoxin (PubChem CID: 31,304)
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ABSTRACT

Introduction: Unanticipated effects on the central nervous system are a concern during new drug development. A larval zebrafish locomotor assay can reveal seizure liability of experimental molecules before testing in mammals. Relative absorption of compounds by larvae is lacking in prior reports of such assays; having those data may be valuable for interpreting seizure liability assay performance.

Methods: Twenty-eight reference drugs were tested at multiple dose levels in fish water and analyzed by a blinded investigator. Responses of larval zebrafish were quantified during a 30 min dosing period. Predictive metrics were calculated by comparing fish activity to mammalian seizure liability for each drug. Drug level analysis was performed to calculate concentrations in dose solutions and larvae. Fifteen drug candidates with neuronal targets, some having preclinical convulsion findings in mammals, were tested similarly.

Results: The assay has good predictive value of established mammalian responses for reference drugs. Analysis of drug absorption by larval fish revealed a positive correlation between hyperactive behavior and pro-convulsive drug absorption. False negative results were associated with significantly lower compound absorption compared to true negative, or true positive results. The predictive value for preclinical toxicology findings was inferior to that suggested by reference drugs.

Discussion: Disproportionately low exposures in larvae giving false negative results demonstrate that drug exposure analysis can help interpret results. Due to the rigorous testing commonly performed in preclinical toxicology, predicting convulsions in those studies may be more difficult than predicting effects from marketed drugs.

1. Introduction

Mitigating undesirable effects of new therapeutics is a key goal of preclinical toxicological investigation. Generating toxicological data early-on in drug development improves detection of toxicities and thus the chance of identifying the best molecules to move forward into later stage development. Effects on the central nervous system (CNS) are important to consider when developing drugs for neurological or psychiatric indications (Easter et al., 2009). In vitro assays with primary dissociated neurons or brain slice cultures (Humpel, 2015) offer high-throughput platforms for detecting convulsive effects, but these cannot be used to interrogate nervous system response in the context of a whole organism, the role of metabolism, or that of the blood brain barrier. The zebrafish (*Danio rerio*) offers a model that bridges a gap between preclinical studies performed in vitro or with invertebrates (e.g. *Caenorhabditis elegans*) and those performed in mammals. Assays using larval zebrafish can afford high-throughput data from a vertebrate possessing a neuroanatomy and neurochemistry significantly homologous to the human nervous system (Panula et al., 2010; Rico et al., 2011). For example, multiple reports have been published on the use of zebrafish for discovering mechanisms and/or personalized treatments of epileptiform diseases (Griffin et al., 2017; Pode-Shakked et al., 2016; Sicca et al., 2016; Wager et al., 2016) or for discovering novel anti-convulsants (Barbalho, Carvalho, Lopes-Cendes, & Maurer-Morelli, 2016; Pisera-Fuster, Otero, Talevi, Bruno-Blanch, & Bernabeu, 2017; Sheng et al., 2016); for reviews see Griffin, Krasniak, & Baraban, 2016; Cunliffe, 2016; and Grone & Baraban, 2015. A larval zebrafish locomotor assay has been proposed as an alternative method to de-risk seizure liability of drug candidates (Koseki, Deguchi, Yamashita, Miyawaki, & Funabashi, 2014; Winter et al., 2008). Such an assay, if

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used early in drug development, could allay mammalian testing with convulsive compounds, streamline the testing of safer candidates by providing rapid results, and allow more efficient internal prioritization. This can be accomplished using relatively small amounts of test article before chemistry efforts are dedicated to generate larger amounts. In addition, given an established zebrafish laboratory with sufficient capabilities to track and monitor behavior, the larval zebrafish assay is relatively easy to perform, allowing for quantitative analysis of drug effects.

Here we describe the characterization of a larval zebrafish locomotor assay designed to predict convulsive activity of drug candidates. Based on established CNS activities of 28 reference drugs in mammals, the assay has predictive value (sensitivity 70%, specificity 100%, positive predictive value 100%, and negative predictive value 57%). Analysis of drug absorption from the dose solution provides further insight into dose responses, and into those treatments which failed to elicit a positive predictive response. We also tested 15 drug candidates with neurological targets, representing 7 mechanisms of action. Mammalian data on these candidates was derived from preclinical toxicology studies where doses exceeded predicted therapeutic levels by large margins. Comparing the performance of the zebrafish assay on this subset to that from the 28 reference drugs gives a real-world perspective on how such an assay may perform if used to mitigate toxicity in the drug development pipeline.

2. Methods

2.1. Animal husbandry

Adult wild-type zebrafish (*Danio rerio*) were housed in a continual-flow housing system (Tecniplast ZebTEC stand-alone rack with Active Blue technology). Water conditions were monitored independently of the rack monitors and maintained as follows: temperature (28 ± 1 °C), pH (7.5 ± 0.5), and conductivity (950 μS). General husbandry and breeding were conducted using standard conditions (Westerfield, 2000). Embryos were collected from breeding tanks 2–3 h after removing the male/female separation barrier. Larval fish were housed in 10 cm petri dishes (≤ 50 fish/50 mL) and kept in an incubator on a 14:10 light:dark cycle at 28 ± 1 °C. Larval fish water (pH = 7 ± 0.5) was 60 μg/mL Instant Ocean sea salts (Blacksburg, VA). All experiments were conducted in compliance with AbbVie's Institutional Animal Care and Use Committee (IACUC). AbbVie operates under the National Institutes of Health Guide for Care and Use of Laboratory Animals in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). No animal health concerns were observed in these studies.

2.2. Test compound selection and classification

Reference drugs (listed in Table 1) were selected to ensure inclusion of positive (n = 20) and negative (n = 8) controls for mammalian preclinical and/or clinical seizure liability based on research using the following resources: PubMed®, Google Scholar®, PharmaPendium®, Go3R® and ProQuestDialog®. All reference drugs were either synthesized in-house, or purchased from Sigma Aldrich®. A grading scheme was applied to the reference drugs to sort them into 3 liability categories (Fig. 1A): no liability was assigned to drugs having no reports for seizure found in the literature; moderate liability was assigned to drugs reportedly associated with seizure upon overdosing or under predisposing conditions e.g. fever, head trauma, or co-dosing with another pro-convulsant; high liability was assigned to drugs used as convulsive agents, that are associated with seizure at therapeutic doses and that have seizure on the label.

Drug candidates with neuronal targets (listed in Table 2) were selected from discovery programs with CNS or pain indications. Information on convulsions associated with these compounds was derived from 155 toxicology studies conducted on mice, rats, dogs, and non-human primates, most of them dosed orally. These studies included the following: GLP with and without recovery, dose-range finding, dose-escalation, single dose or repeat dose studies lasting up to 52-weeks. Convulsions in these mammalian studies, when observed, were at doses above predicted therapeutic doses. No convulsions were reported in human phase I studies. A grading scheme was applied to the drug candidates to sort them into 3 categories (Fig. 1B): no liability was assigned to compounds having no reports of convulsion or only reported in conjunction with a lethal dose; moderate liability was assigned to compounds associated with convulsions only after repeat dosing; high liability was assigned to compounds associated with convulsions after a single dose.

2.3. Conducting the locomotor assay

Test compounds were initially dissolved in 100% DMSO and then in fish water to create a 2× dosing solution for the highest dose group; this 2× dose contained 0.6% DMSO. The upper limits of solubility, as perceived by eye, for each compound determined its highest target dose (0.3, 1, or 3 mM). The pH was adjusted, if needed, to be near 7 (6.5–7.5) using sodium bicarbonate or hydrochloric acid. Dilutions were made in fish water containing 0.6% DMSO to create 4 additional treatments, being half-log serial dilutions. Seven day post fertilization larvae (7 dpf) were placed individually in separate wells of a 48-well microplate in 0.25 mL fish water. For any given experiment, larvae were derived from 3 or more clutches which were randomly mixed together before plating, minimizing clutch-specific effects on locomotion. After allowing 10–15 min of acclimation time, larvae were dosed by adding 0.25 mL of either test compound or vehicle (fish water with 0.6% DMSO); the final DMSO concentration was 0.3% in all treatments. Reference drugs were tested, and data analyzed, by a blinded investigator; drug candidates were tested and analyzed by an investigator blinded to their mammalian preclinical convulsive category. Each dose group, including the vehicle group, consisted of 8 larvae; there was a different vehicle-treated group on each microplate in every instance of the locomotor assay. For 30 min, activity of each larva was tracked in the dark using Viewpoint™ Zebrabox® Behavioral Tracking System equipped with Zebralab Quantization® software version 3, 22, 3, 11. The detection sensitivity was set to 11 with the transparency option selected. Activity results were integrated every 10 s for each larva for the entire monitoring period. Activity thresholds, although not used in the final behavioral assessment were: burst = 50, freezing = 5. At the end of the monitoring period, viability of each fish was evaluated using a dissecting stereoscope or compound microscope; dead fish, confirmed by absence of heartbeat, were not included in behavioral data. Larvae and dose solutions were then collected for drug-level analysis.

2.4. Locomotor assay data analysis

The maximum activity of each larva for any of the 10 s periods during the 30 min assay was determined, and group averages of those values calculated. The maximum activity used here was reported as 'actinteg' from Zebralab Quantization®; this is an integral of activity over time. Based on a Student's *t*-test, if the average maximum activity of the group was significantly higher than that of the vehicle group from the same microplate the respective treatment was labeled as moderately convulsive to zebrafish larvae. If that average value was also greater than, or equal to the 90th percentile of maximum activity of vehicle-treated larvae from historical data, the treatment was labeled as highly convulsive to zebrafish larvae. The historical data on vehicle-treated larvae was collected from 4 different days of experimentation conducted over 1 month using 15 separate microplates and 120 larvae. In addition to the Student's *t*-test, Dunnett's multiple comparison procedure was conducted on each compound. This procedure compared the 5 dose groups to the co-plated vehicle-treated group.

Table 1
Effects of reference drugs in the zebrafish locomotor assay. LogP = log of octanol/water partition coefficient. Reported mechanisms are listed for each drug (▲ = increases activity; ▼ = decreases activity). The targeted in-water dose level is indicated at the top of each column, under which the larval fish exposures are indicated in the corresponding row (pmoles per larva, blank cell = dose level not tested, n.m. = drug levels not measured, ≤ drug levels were below detectable limits, given here in μM as follows methotrexate 0.022, picrotoxin 0.73, baclofen 0.023, vitamin B6 0.006, dexamethasone 0.012). Asterisks and bold font indicate significant zebrafish hyperactivity compared to the vehicle group, based on Student's *t*-test (* = moderate convulsive result, ** = high convulsive result). A summary decision for the convulsive activity of each drug is provided for zebrafish next to that based on literature reports for mammalian convulsive liabilities.

Compound	LogP	Mechanism of action	Zebrafish exposure and convulsive result						Convulsive activity	
			Target in water dose (mM)						Zebrafish	Mammals
			0.01	0.03	0.1	0.3	1	3		
4-Aminopyridine	0.32	K + Channel▼, glutamate▲		n.m.	n.m.	**8.1****	**29.3****	**160****	High	High
Bicuculline	− 2.14	GABA- A▼	**n.m.****	**32.8****	**66.9****	**134****			High	High
Gabazine	0.85	GABA- A▼		n.m.	n.m.	2.2	2.6	**42.1***	Moderate	High
Kainic acid	− 1.18	Glutamate▲	n.m.	n.m.	0.5	0.8	3		None	High
Methotrexate	− 3.62	Folic acid▼		n.m.	n.m.	<	<	**29.9****	High	High
Pentetrazol	1.19	GABA- A▼		n.m.	n.m.	52	139	**448****	High	High
Picrotoxin	− 4.6	GABA- A▼		n.m.	n.m.	< **	< **	< **	High	High
Quinolinic acid	1.08	NMDA▲	n.m.	**n.m.****	**n.m.****	**n.m.****	**n.m.****		High	High
Strychnine	0.7	Glycine, acetylcholine▼	n.m.	n.m.	4.7	17	42		None	High
Baclofen	− 1.14	GABA -B▲	n.m.	n.m.	<	<	<		None	Moderate
Clozapine	3.56	Serotonergic▼, dopaminergic▼, adrenergic▲		n.m.	n.m.	**244***	Lethal	Lethal	Moderate	Moderate
Donepezil	4.95	Reversible cholinesterase inhibitor	n.m.	n.m.	41	75	**241****		High	Moderate
Maprotiline	4.52	Alpha2 adrenergic▼, amine transporter▼, histamine ▼	5.6	13	49	Lethal	Lethal		None	Moderate
Naloxone	− 0.62	Opioid ▼	n.m.	n.m.	5.4	**14.5***	30		Moderate	Moderate
Naproxen	2.82	NSAID		n.m.	n.m.	16	27	**70.1****	High	Moderate
Pilocarpine	− 0.2	Acetylcholine analogue		n.m.	n.m.	4.8	13	35	None	Moderate
Scopolamine	0.29	Antimuscarinic		n.m.	n.m.	11	**31.7****	91	High	Moderate
Verapamil	5.17	L-type Ca channel ▼	n.m.	3.4	33	91	Lethal		None	Moderate
Vitamin B6	− 0.2	Coenzyme		n.m.	n.m.	<	0.7	**18.6****	High	Moderate
Buspirone	2.19	5-HT-1a ▲, D2 ▼	n.m.	n.m.	**28.2****	**86.8****	169	n.t.	High	Moderate
Bromocriptine	− 1.97	Dopamine D2 ▲	n.m.	5.2	6.1	11			None	None
Clonidine	2.85	Alpha2-adrenergic ▲		n.m.	n.m.	12	21	88	None	None
Dexamethasone	− 3.9	Glucocorticoid	n.m.	<	1.2	3			None	None
Penicillin	− 6.42	Antibiotic		n.m.	n.m.	1.1	37	41	None	None
Pindolol	1.67	Adrenergic beta ▼	n.m.	n.m.	n.m.	n.m.	n.m.		None	None
Tetracycline	− 5.01	Antibiotic	n.m.	n.m.	2.3	2.8	3.9		None	None
Trazodone	3.4	Serotonin 5-HT-2 ▼	n.m.	n.m.	95	166	322		None	None
Valproate	− 1.24	GABA ▲		n.m.	n.m.	n.m.	n.m.	n.m.	None	None

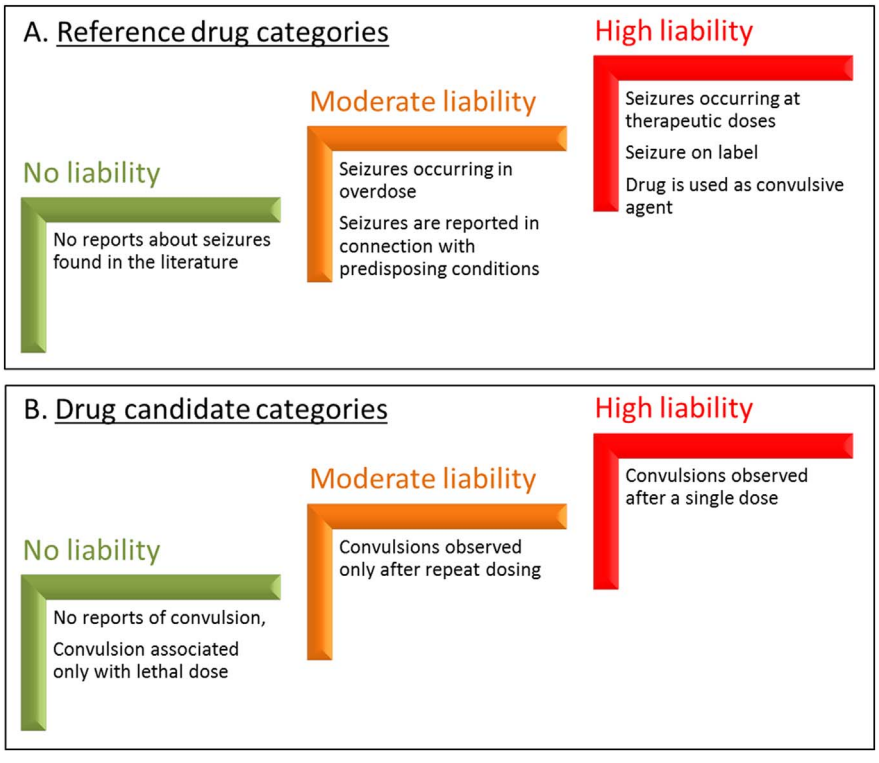

Fig. 1. Seizure or convulsive liability categories for (A) reference drugs based on drug literature, and for (B) drug candidates based on preclinical findings.

Table 2
Effects of drug candidates in the zebrafish locomotor assay. See legend for Table 1.

Compound	LogP	Mechanism of action	Zebrafish exposure and convulsive result						Convulsive activity	
			Target in water dose (mM)						Zebrafish	Mammals
			0.003	0.01	0.03	0.1	0.3	1		
1	2.16	Histamine H3 Receptor ▼		n.m.	n.m.	1.8	19	45	None	Moderate
2	0.37	Histamine H3 Receptor ▼	n.m.	n.m.	19	59	124		None	None
3	2.15	Histamine H3 Receptor ▼	n.m.	n.m.	1.8	4.5	22		None	Moderate
4	0.07	Neuronal Nicotinic Receptor ▲		n.m.	n.m.	8.3	42	217	None	High
5	2.11	Neuronal Nicotinic Receptor ▲		n.m.	n.m.	4.9	28	**61.5****	High	Moderate
6	3.08	Neuronal Nicotinic Receptor ▲		0.2	0.3	2.8	Lethal	Lethal	None	None
7	2.56	Neuronal Nicotinic Receptor ▲	n.m.	n.m.	0.5	3.8	11		None	High
8	1.78	Neuronal Nicotinic Receptor ▲		n.m.	n.m.	6.5	30	88	None	None
9	− 1.6	Dopamine D3 Receptor ▼		n.m.	n.m.	5.3	18	**84.1****	High	Moderate
10	3.31	Dopamine D3 Receptor ▼		n.m.	n.m.	3.8	10	20	None	Moderate
11	4.24	Dopamine D3 Receptor ▼		n.m.	n.m.	0.6	2.7	24	None	None
12	1.15	Serotonin 5-HT6 Receptor ▼		n.m.	n.m.	0.7	3.3	29	None	High
13	2.14	CaV3 Calcium Channel ▼	n.m.	n.m.	1.4	6.5	17		None	Moderate
14	4.86	Sphingosine 1-phosphate Receptor ▲		n.m.	n.m.	86	160	435	None	None
15	2.67	NaV1.7 Sodium Channel ▼		n.m.	n.m.	10	33	162	None	None

2.5. Drug level analysis

After the locomotor assay, three fish from each of three treatment groups (maximum tolerated dose plus 2 lower serial dilutions) were rinsed in 50 mL of larval fish water 2 times and placed together into a pre-weighed vial. The majority of water was then carefully removed and the vial placed on dry ice. After determining the mass of the 3 fish, deionized water (300 μL) was added and they were homogenized using an Omni Bead Ruptor 24® (Omni International™). An aliquot of fish homogenate and set of standards were pipetted into a 96-well plate and subjected to protein precipitation extraction on a Microlab Star robot® (Hamilton Robotics™). A dilution series was made to generate a range of standards from 1000 to 0.1 ng/mL. The dosing solutions were pipetted into a 96-well plate and subjected to protein precipitation extraction as well. Acetonitrile, containing 6 reference compounds (12.5 nM verapamil, 1000 nM dexamethasone, 200 nM diclofenac, 250 nM carbutamide, 125 nM lidocaine, and 100 nM tolbutamide) as internal standards, was used as an organic solvent to precipitate out all biological proteins from fish and dose solutions. Where one of these 6 reference compounds was the actual compound to be analyzed, it was excluded from the reference mix. Supernatant from protein precipitation was transferred into mass spectrometry plates and diluted with the mobile phase of the liquid chromatography–mass spectrometry (LC-MS) column. The liquid chromatography analysis was performed using reverse phase or HILIC chromatography in either positive or negative ion mode using gradient elution. The tandem mass spectrometry analysis was carried out on SCIEX™ triple quadrupole mass spectrometer with an electrospray or atmospheric pressure ionization interface. Data acquisition and evaluation were performed using Analyst® software.

Results from LC-MS were used to calculate dose solution and larval homogenate concentrations. Dose solution concentrations were compared to desired (targeted) levels. Larval homogenate concentrations were used to estimate drug exposure per larva (μmoles/larva). Absorption was reported as a function of larval exposure per dose solution concentration. All absorption percentiles are based on the entire dataset (reference drugs and drug candidate values combined). For interpreting the impact of compound solubility, exposure, and absorption on assay performance, each dose-level treatment of a given compound was considered independently of other doses. As such, no effect on behavior from a low dose was considered a false negative result, even though higher doses of the same compound may have yielded a true positive result.

3. Results

3.1. Locomotor assay results

Assay data from 120 vehicle-treated fish from 4 days of experimentation on 15 separate microplates gave an average maximum activity of 650 (± 371.6; range = 6 to 1515). The 90th percentile of those values was 1129; this was used as the minimum average activity for labeling treatments as highly convulsive. Data, analyzed blindly to drug identity (reference drugs) or to mammalian convulsive category (drug candidates) exhibited a low frequency of convulsive calls from the zebrafish assay. Out of 215 treatments (5 dose levels for 43 compounds) only 29 treatments from 16 compounds were labeled convulsive (asterisks in Tables 1 and 2) using Student's *t*-test. These tended to be at the higher end of the dose range for those compounds. Representative results are shown in Fig. 2. The significance of most treatment effects was conserved between Student's *t*-test and Dunnett's test. The exceptions were losses of significant effects on activity from clozapine, gabazine, and drug candidate 9, when analyzed by Dunnett's.

3.2. Predictive value based on reference drugs

The 28 reference drugs used to estimate the predictive value of the locomotor assay represent multiple mechanisms (Table 1). Twenty of the 28 were categorized as having convulsive potential in mammals based on our criteria (9 with high liability, 11 with moderate liability); the remaining 8 were categorized as having no seizure liability in mammals. Variable solubility among the 28 drugs, as perceived by eye during preparation, resulted in different high target doses, and therefore slightly different dose ranges, as indicated in Table 1. Considering moderate and high zebrafish results collectively, this locomotor assay predicted convulsions for 7/9 of the high seizure liability drugs, 7/11 of the moderate seizure liability drugs, and 0/8 of the no seizure liability drugs. The overall assay predictive metrics based on these drugs are: 70% sensitivity, 100% specificity, 100% positive predictive value, and 57% negative predictive value (Table 3A). These values are within the range of those of prior reports using zebrafish to predict mammalian seizure liability (Koseki et al., 2014; Winter et al., 2008).

3.3. Predictive value for preclinical convulsions

The 15 drug candidates used to evaluate the assay's ability to forewarn on convulsions in preclinical toxicology represent 7 neuronal mechanisms (Table 2). Nine of the 15 were categorized as convulsive

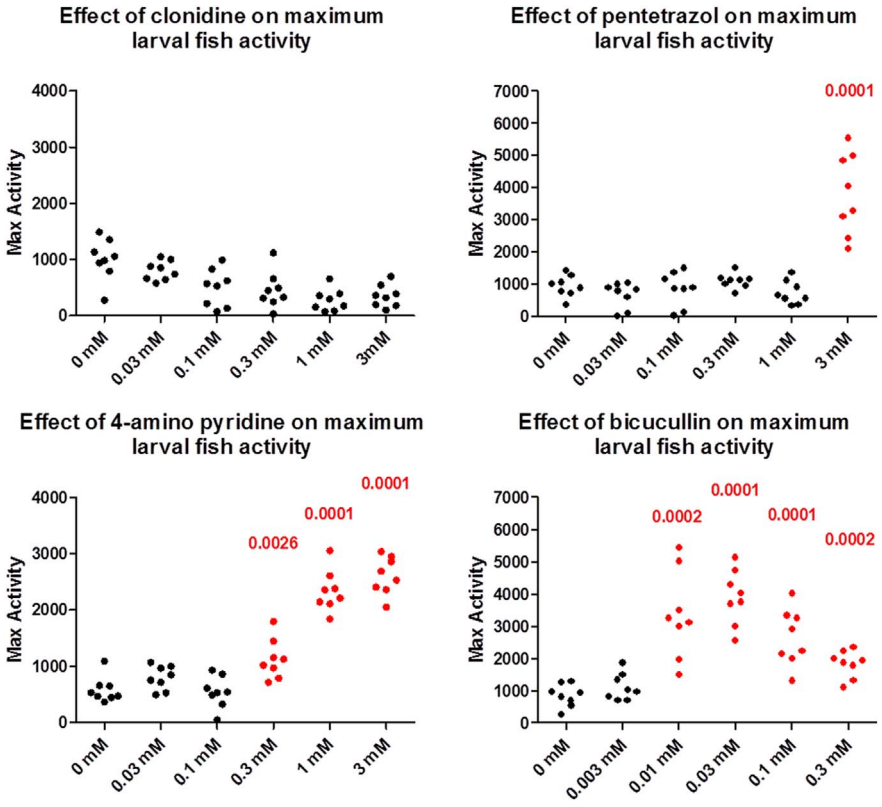

Fig. 2. Example results of the zebrafish locomotor assay from 4 reference drugs. Each dot represents the maximum activity for a single larva over 10 s during 30 min of dosing. N = 8 larvae per dose level, red dots indicate that the group average was significantly higher than that of the co-plated vehicle-treated larvae (0 mM); p-values, in red, are from Student's *t*-test. Max activity is maximum integral of activity as reported by Viewpoint™ Zebralab Quantization® software.

Table 3
Predictive metrics of the zebrafish locomotor assay. (A) Predictive metrics of the assay based on reports of seizure liability for 28 reference drugs. (B) Predictive metrics of the assay based on findings from preclinical toxicology studies. TP = true positive, FP = false positive, TN = true negative, FN = false negative.

A. Convulsive activity of 28 drugs in mammals and fish		Expected based on mammalian data	
		Active	Not active
		20	8
Zebrafish assay	Active	14	0
	Not active	6	8
Positive predictive value = 100.0			
Negative predictive value = 57.1			
Sensitivity = 70.0			
Specificity = 100.0			

B. Convulsive activity of 15 drug candidates in mammals and fish		Expected based on mammalian data	
		Active	Not active
		9	6
Zebrafish assay	Active	2	0
	Not active	7	6
Positive predictive value = 100.0			
Negative predictive value = 46.2			
Sensitivity = 22.2			
Specificity = 100.0			
Positive predictive value = TP/(TP + FP)			
Negative predictive value = TN/(FN + TN)			
Sensitivity = TP/(TP + FN)			
Specificity = TN/(FP + TN)			

based on findings from mammalian toxicology studies (3 of high liability, 6 of moderate liability); the remaining 6 were categorized as having no convulsive liability. The zebrafish locomotor assay predicted convulsions for 0/3 of the high liability compounds, 2/6 of the moderate liability compounds, and 0/6 of the no liability compounds. The overall predictive metrics for this subset of drug candidates are: 22% sensitivity, 100% specificity, 100% positive predictive value, and 46% negative predictive value (Table 3B). This subset reflected a lower sensitivity, and a lower negative predictive value than expected based on the reference drugs.

3.4. Compound solubility, exposure, and absorption relative to assay performance

The average solubility for all treatments analyzed was 81.8% (± 31.8); solubility was calculated by dividing the dose solution concentration, as measured by LC-MS, by the target concentration for each treatment. There was no significant difference in solubility among treatments giving false negative, true negative or true positive results (Fig. 3A). No difference in solubility among the different prediction categories is an indication that, in general, false negative results are not driven by poor compound solubility. There was also no significant difference in solubility between reference drugs and drug candidates (Fig. 3B). This indicates that poor solubility, in general, is not a reason for the higher rate of false negative predictions in the drug candidate subset (78% vs 30% for reference drugs).

Examination of exposures (amount of compound per larva) revealed a significant positive correlation with dose solution concentration (Fig. 4). This indicates that, in general, more compound is absorbed given a higher dose level in the water. For compounds that elicited a convulsive result, exposure and activity level were positively correlated; $R^2 = 0.44$ (Fig. 5A). For compounds that did not elicit a convulsive result, even though their exposure range was equivalent to that of active compounds, there was not a relationship between exposure and activity level; $R^2 = 0.0015$ (Fig. 5B). This indicates that, in general, the behavioral metric of this assay is responsive to dose-dependent exposures of pro-convulsive treatments.

Comparing exposures among treatments with different translational value, there was significantly less exposure in those larvae dosed with treatments yielding false negative results compared with those associated with true negative or true positive results (Fig. 6A). This

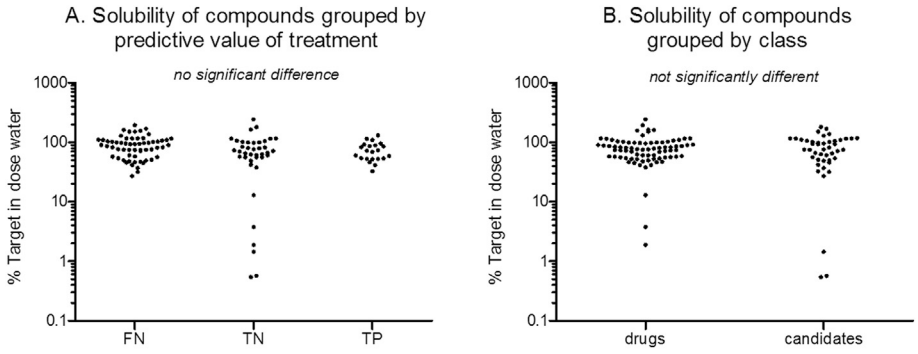

Fig. 3. Solubility comparisons. Each dot represents a treatment (typically, 3 dose-level treatments per compound were measured). (A) Solubility of compounds grouped by predictive value of treatment (FN = false negative; TN = true negative; TP = true positive). (B) Solubility of compounds grouped by compound class (drugs = reference drugs; candidates = drug candidates).

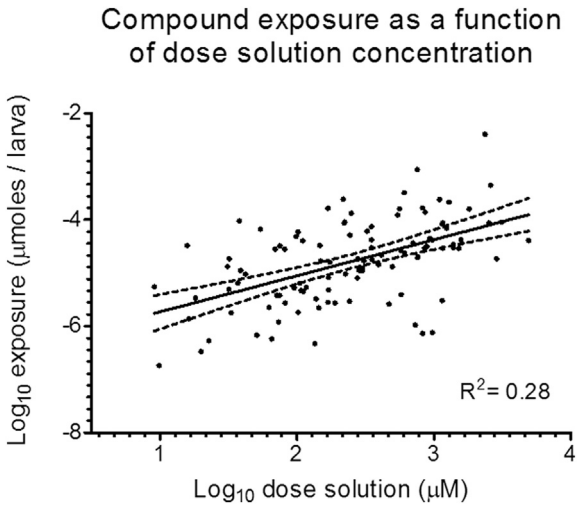

Fig. 4. Exposure as a function of dose solution concentration. Each dot represents a treatment. Relationship between dose solution concentration (µM) and exposure of larvae (µmoles/larva) reveals a significant positive correlation. Dotted lines mark the 95% confidence range for the regression (solid line). Five outlier treatments were removed from this dataset using a studentized residual > 2 after the linear fit on the $\log_{10}$ scale; these outliers were from 2 true negative compounds (bromocriptine and drug candidate 14) in which solubility was very low but exposure was relatively high, resulting in aberrant absorption calculations.

indicates that lower exposure may play a role in failure to predict mammalian response to a convulsive drug. There was not a significant difference between exposures of true negative and true positive groups.

Since exposure, as measured here, is a function of both compound solubility and ability to be absorbed by the larvae, we calculated absorption for each treatment by dividing the exposure by the dose solution concentration. Like exposure, absorption was significantly lower in larvae dosed with treatments yielding false negative results (Fig. 6B). This indicates that regardless of solubility, the capacity of a compound to penetrate and remain in the larval fish plays a key role in the predictive value of this assay.

There was not a significant difference between exposure levels for reference drugs and drug candidates (Fig. 7A). This indicates that, in general, disparate absorption of compounds between those classes did not play a role in the inferior performance of the assay when tested on drug candidates. Since a higher false negative rate drove that inferior performance, we compared only the exposures in larvae dosed with treatments that yielded a false negative result. Like the broader datasets, these more specified groups were not significantly different (Fig. 7B).

4. Discussion

4.1. Historical perspective

This report follows two other reports describing the predictive value of larval zebrafish on mammalian seizure liability from reference drugs (Koseki et al., 2014; Winter et al., 2008). Both of those reports quantified high-speed movement over 1 h after dosing; the latter (Koseki et al., 2014) added prolonged exposure (4 h) and a flashing light stimulus to improve predictive value. Although our assay differs slightly, measuring maximum activity over time versus velocity, and only for 30 min after dosing, we have very similar predictive values to those prior reports. The present report adds to this body of knowledge by contributing drug analysis data, as well as a demonstration of real-world preclinical toxicology predictions.

4.2. Physiochemical considerations

Considering that different types of seizures (clonic, tonic, absence, etc.) are associated with disparate behaviors in mammals, it is likely that drugs acting through myriad mechanisms, as tested here, could induce a spectrum of larval fish behavioral responses, some of which would be undetectable by this assay. Since maximum activity is used to indicate seizure liability, this assay is insensitive to seizures that do not induce hyperactivity. Such seizures, if present in larval fish, would give a false negative result from the assay. This disadvantage is one of the

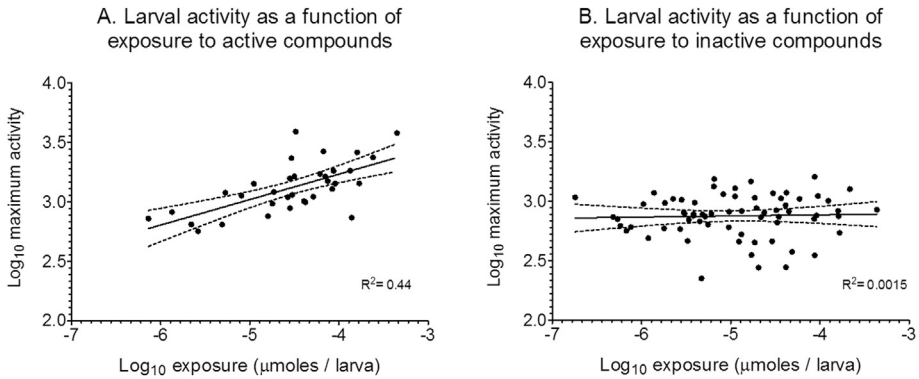

Fig. 5. Larval activity as a function of exposure to compounds. Each dot represents a treatment; maximum activity is the average of 8 larvae in each treatment group. Dotted lines mark the 95% confidence range for the regression (solid line). (A) Illustrates a significant positive correlation between exposure to compounds causing hyperactivity and average maximum activity. (B) No correlation existed between exposure to compounds that did not cause hyperactivity and maximum activity in those treatments.

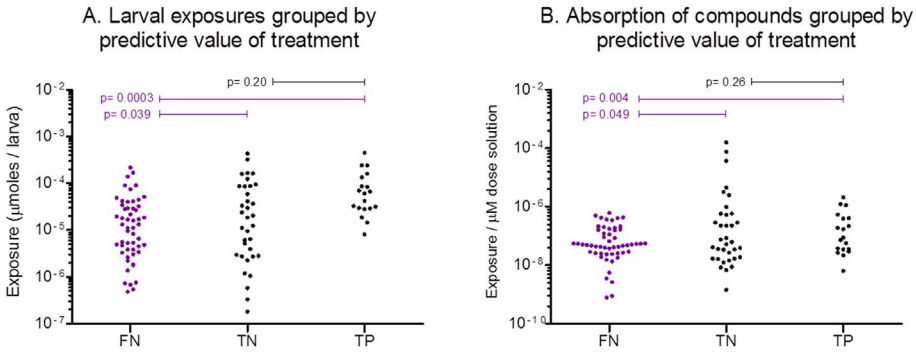

Fig. 6. Exposure and absorption are lower in treatments yielding false negative results. Each dot represents a treatment. P-values are from Student's *t*-test. (A) Exposure of larvae (µmoles/larva) grouped by predictive value of treatment. (B) Absorption of compounds, calculated as exposure per dose solution concentration, grouped by predictive value of treatment.

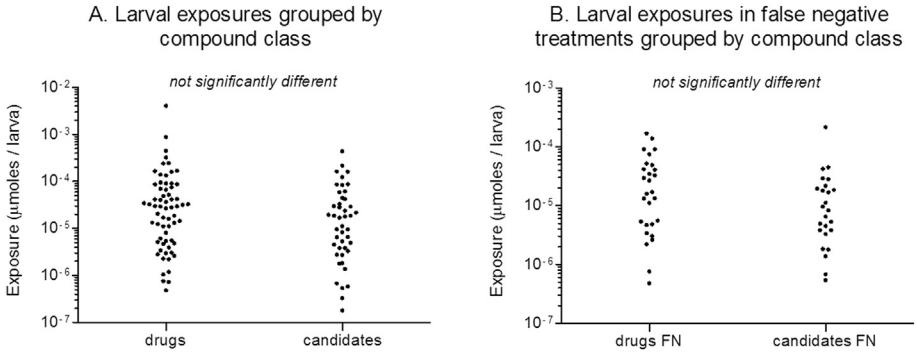

Fig. 7. Exposures are not different between reference drugs and drug candidates. Each dot represents a treatment. (A) Exposure of larvae grouped by compound class. (B) Exposure of larvae from treatments yielding false negative results, grouped by compound class.

costs of a high-throughput assay and should be considered as a potential reason for the false negative results discussed below. Another point of consideration concerning false negative results is that drugs detected at high levels in total larval homogenate may not have reached the CNS due to the blood brain barrier. In such cases no seizure is possible and would result in a false negative associated with high drug absorption. Lastly, the lipophilicity of a drug may be a factor in its absorption by larval fish. Zebrafish embryos have been shown to absorb higher levels of lipophilic dyes, compared with hydrophilic dyes (de Koning et al., 2015). Correspondingly, in the compounds we tested, high LogP values tended to be associated with high exposure levels (see Tables 1 and 2). However, this was only a trend in our small dataset; other factors likely play a role in absorption and retention of chemical matter, such as metabolism and active transport.

4.3. False negative results from reference drugs

Kainic acid, a glutamatergic agonist, is commonly used to induce seizure in rodent models (Eppsa & Weinshenkera, 2013), and it has been demonstrated that similar effects are manifested when dosed i.p. in adult zebrafish (Alfaro, Ripoll-Gómez, & Burgos, 2011). Demonstration of kainic acid activity on the CNS of larval zebrafish is limited to positive effects on acetylcholinesterase production (Cortés-Castell et al., 2014), and a reported protection against i.p.-administered kainic acid later in life (Peres-Menezes, Pacheco-Rico, & Souza-Da Silva, 2014). In our experiment, kainic acid treatment afforded a false negative result; having no effect when one was expected. This result is likely explained by the very low exposure levels of this drug detected in the larvae (Table 1). Indeed, the absorption levels for the three kainic acid doses were all below the 5th percentile of those calculated (not shown). These low values are a result of low exposure and high solubility; kainic acid had > 77% solubility in all three doses analyzed. The reason for the apparent low absorption is unknown, possible explanations include transporter activity and metabolism (only drug levels for the parent compound were analyzed by LC-MS).

Strychnine, a glycine receptor antagonist, commonly used to induce convulsions in mammalian models (Philippe, Angenot, Tits, & Frédérich, 2004), had moderate exposures (Table 1) and absorption (~50th percentile) however it was a false negative in this assay. Strychnine causes reduced motility in larval zebrafish, presumably through a weakening of tail power (Mirat, Sternberg, Severi, & Wyart, 2013); in that study, tail beat frequency did not slow after strychnine treatment, but the heading, speed, and distance traveled with each tail beat was decreased. The proposed mechanism behind this is that the rhythmic tail movement relies on glycine receptor-coordinated bursts of nerve signaling that move rostrocaudally, alternating from side to side along the spinal cord, and that these are impaired by strychnine (Wiggin, Peck, & Masino, 2014). Such a decrease in motility may have contributed to the false negative result for strychnine in our assay.

The other four false negative results came from reference drugs labeled as moderately convulsive in mammalian models. As such higher exposures, or prolonged periods of dosing may be needed to increase zebrafish activity, compared to drugs categorized as highly convulsive in mammals. Pilocarpine, although detected with moderate exposure at the high dose (Table 1) had poor solubility (< 50%) and absorption (< 25th percentile) for all doses; therefore may have been under-dosed. Baclofen was detectable in water, returning very high solubility values (100%) but was not detectable in larval homogenates, indicating that, like kainic acid, there is poor/no uptake, high metabolism, or that an active transport mechanism prevents retention. Verapamil and maprotiline were both very soluble (75–100%), and well-absorbed (> 80th percentile) but both were lethal at the highest doses tested (Table 1). If there was a positive effect on activity during those high dose treatments, it was not recorded since data from larvae that were dosed a lethal treatment were not analyzed.

4.4. Low exposures from poorly absorbed compounds impact predictive value

False negative results, as a group, were generated from treatments with significantly lower exposure and absorption compared to true negatives and true positives. Of the six false negative reference drugs discussed above, five had limited exposures due to solubility, absorption, or general toxicity. In many cases, such conditions are also applicable to the drug candidates that gave false negative results.

Absorptions calculated from all dose levels for four out of the seven drug candidates that gave false negative results were below the 50th percentile (these compounds are labeled numbers 3, 7, 10, and 12 in Table 2). Perhaps if higher levels could have been reached, those would have driven a positive response and improved the predictive value with regard to preclinical toxicology findings. For example, there were 9 drug candidates that generated absorption values higher than the 50th percentile. If only those 9 are considered, the 100% positive predictive value and specificity remain, while the negative predictive value increases from 46% to 57%, and the sensitivity increases from 22% to 40%. So, by including drug level analysis in future studies investigators may be able to proactively identify possible false negatives by monitoring relative absorptions among their compounds. As a point of reference, the 50th percentile for absorption of the compounds interrogated in this report was 5.4×10^{-8} (= μmoles per larva / μM in dose solution). Furthermore, exposure can be increased by direct compound injection or by improving the formulation of compounds which demonstrate poor absorption after standard dosing.

4.5. Value for predicting convulsion in preclinical toxicology

The drug candidates had equivalent solubility and absorptive capacity to reference drugs yet the assay returned a much higher false negative rate (78% vs 30% for reference drugs). The rigorous nature of toxicology testing, including dose levels and durations that push tolerability to the limit, may have placed the assay at a disadvantage for predicting those findings. It seems that the effects of such dosing conditions, resulting in high exposures and declining general health of the animals (potentially predisposing them to convulsions) cannot be easily predicted by the zebrafish locomotor assay, as employed here. Preclinical mammalian data on these drug candidates were used to define safe doses for humans, applying a 10 × safety margin from the NOAEL observed in preclinical studies. None of the candidates in this report were halted in development based solely on convulsive preclinical findings and none of them that reached Phase I were associated with seizure or convulsion in the clinic. Thus, this subset of compounds differs markedly from reference drugs with established seizure liabilities.

5. Conclusion

Since the zebrafish locomotor assay is relatively high-throughput and uses small amounts of test article, it is feasible to analyze many compounds in early-stage drug development and, with rapid turnaround, provide data that aids their prioritization. Taking into consideration compound solubility and larval fish exposures can augment the quality of those data, revealing potential false negative results. Early indication of convulsive liability exposes potential risks and can inform the design of subsequent in vivo testing, improving the probability of success as molecules progress through development.

Disclosure

Financial support for these experiments was provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the manuscript. All authors are employees of AbbVie and have no additional conflicts of interest to disclose.

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