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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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Strychnine (PubChem CID: 441,071)
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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.