ACUTE TOXICITY SCREENING OF THE LOPAC^{1280} LIBRARY IN ZEBRAFISH EMBRYOS

By

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A capstone project submitted for
Graduation with University Honors

May 11, 2017

University Honors
University of California, Riverside

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Abstract

There are thousands of compounds in commerce with limited toxicity data available. The use of zebrafish embryos as an alternative non-mammalian model organism allows for relatively inexpensive and efficient screening of a wide variety of compounds, while providing a complex physiological context with the potential for different organ systems to interact. Using a high-content assay to screen the LOPAC^{1280} library (a well-defined library of 1,280 pharmacologically active compounds), approximately 4% of the library was identified as acutely toxic, resulting in <85% survival in zebrafish embryos. The focus of our study was to find potential predictors of acute toxicity by exploring relationships between zebrafish embryo survival at 24 hours post-fertilization and the physicochemical properties of each acutely toxic compound, including mode of action, molecular weight, and partition coefficient (LogP). Our results suggest that there is no association between embryo survival and these compound properties.

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Introduction

There are thousands of commercially available compounds with limited information and literature available on how they impact survival of living organisms, particularly during early development (Zanella et al., 2010; Zhu et al., 2014). In order to study the impact of these compounds, an assay for alternative non-mammalian models is needed for both economical and ethical reasons. Because zebrafish embryos are relatively easy to breed, inexpensive, and are not considered "animals" prior to 72 hours post fertilization (hpf) (Coecke et al., 2007; Crofton et al., 2012), we are able to leverage zebrafish embryos as an alternative model system to test for developmental effects. In addition, zebrafish embryos provide a complex physiological context with the potential for different organ systems to interact.

There has been a lack of study on well-defined libraries, such as the LOPAC^{1280} (Library of Pharmacologically Active Compounds) library, which is a library of 1,280 marketed, failed, and well-characterized drugs (Figure 1). By screening this library, we are able to learn more about the impact specific drugs have on embryo development and examine potential relationships between these drugs. Therefore, for this study, we focused on specific compounds from the LOPAC^{1280} library that demonstrated acute toxicity in zebrafish embryos. Acute toxicity was quantified at <85% survival, which is a threshold chosen based on regulatory toxicity testing on zebrafish embryos in the past, driven by historical control of embryo survival in a 384-well plate. As the purpose of our study is to find what factors can predict toxicity, we identified acutely toxic compounds from the LOPAC^{1280} library to determine the potential relationship between the endpoint of survival and each acutely toxic compound's chemical structure and physical chemistry.

We focused on three properties of each acutely toxic compound: LOPAC-defined mode of action, molecular weight, and LogP.

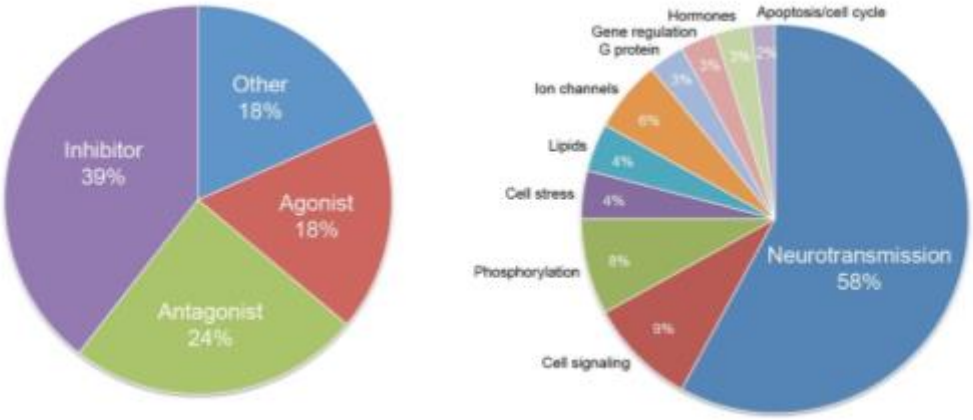

Figure 1. The LOPAC^{1280} library is a well-characterized library of 1,280 pharmacologically active compounds that offers a wide range of modes of action and targets different cellular components.

Methods

Animals. For this study, we used a transgenic (*fli1:egfp)* strain of zebrafish that stably express enhanced green fluorescent (eGFP) protein within vascular endothelial cells (Lawson and Weinstein, 2002), as this strain begins expressing eGFP at ~14 hours post-fertilization (hpf). Adult *fli1:egfp* zebrafish were maintained on a recirculating system with UV sterilization and mechanical/biological filtration units (Aquaneering, San Diego, CA, USA), and were kept under a 14-h:10-h light:dark cycle at a water temperature of ~27-28°C, pH of ~7.2, and conductivity of ~900-950 µS. Water quality was constantly monitored for pH, temperature, and conductivity using a real-time water quality monitoring and control system. Ammonia, nitrate, nitrite, alkalinity, and hardness levels were manually monitored weekly by test strip (Lifeguard Aquatics, Cerritos, CA). Zebrafish were fed once per day with dry diet (Gemma Micro 300, Skretting, Fontaine-

lès-Vervins, France). Adult males and females were bred directly on-system using in-tank breeding traps suspended within 3-L tanks. For all experiments described, newly fertilized eggs were collected within 30 min of spawning, rinsed, and reared in a temperature-controlled incubator (28°C) under a 14-h:10-h light:dark cycle. All embryos were sorted and staged according to previously described methods (Kimmel et al., 1995). All adult breeders were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (#20150035) at the University of California, Riverside.

Chemicals. Abamectin was purchased from ChemService, Inc. (West Chester, PA, USA), and a low-volume library of 1,280 pharmacologically active compounds (LOPAC^{1280}) was purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of abamectin (50 mM) were prepared by dissolving abamectin in high performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO) and stored at room temperature within 2-ml amber glass vials containing polytetrafluoroethylene (PTFE)-lined caps. Stock solutions (25 µl of 10 mM stock per compound) of the LOPAC^{1280} library (16 96-well racks containing 80 compounds per rack) were prepared and provided in DMSO by Sigma-Aldrich and stored at -30°C upon arrival; with the exception of partition coefficient (LogP) values, all compound-specific information was provided by Sigma-Aldrich within a Microsoft Excel spreadsheet following acquisition of the LOPAC^{1280} library. For each individual plate, working solutions of all treatments were freshly prepared by diluting stock solutions 1:1000 into embryo media (EM) (10 mM NaCl, 0.17 mM KCL, 0.66 mM $CaCl_2$, 0.66 mM $MgSO_4$), resulting in 0.1% DMSO within all vehicle control and treatment groups.

Assay Setup. Newly fertilized embryos were collected immediately following spawning and incubated in groups of approximately 50 per plastic petri dish until 5 hpf. Embryo media, vehicle control (0.1% DMSO), positive control (abamectin), or test solution (50 µl/well) was loaded into a black 384-well microplate containing 0.17-mm glass-bottom wells (Matrical Bioscience, Spokane, WA, USA). For the LOPAC^{1280} screens, vehicle (0.1% DMSO) and positive (6.25 µM abamectin) control groups each occupied two columns (32 wells per group) flanking the left and right sides of the plate (0.1% DMSO: columns 1 and 24; 6.25 µM abamectin: columns 2 and 23), whereas each LOPAC^{1280} compound occupied one column (16 wells) on each plate. At 5 hpf, 384 viable *fli1:egfp* embryos were manually arrayed into each well of a 384-well plate over a 45-min time period, resulting in one embryo per well. The plate was then covered with a plate lid, wrapped with parafilm to minimize evaporation, and incubated at 28°C under a 14-h:10-h light:dark cycle until 24 hpf. At 24 hpf, the plate was placed in a second incubator at 25°C for 1 h to acclimate the embryos to room temperature prior to imaging. At 25 hpf, the plate was then centrifuged for 2 min at 200 rpm to ensure all embryos were positioned at the well bottom.

Image Acquisition and Analysis. Using an image acquisition protocol optimized for our ImageXpress Micro (IXM) XLS Widefield High-Content Screening System equipped with MetaXpress 6.0.3.1658 (Molecular Devices, Sunnyvale, CA), a 4X objective and FITC filter cube were used to acquire one frame per well for assessment of survival. During the image acquisition period, internal temperature within the IXM system was maintained using previously described procedures (Raftery et al., 2014). Embryos were then euthanized by placing the plate at -30°C. Using images captured with

a 4X objective, survival was quantified using previously described procedures (Raftery et al., 2014). Treatments resulting in <85% survival were considered significant, as negative and vehicle control survival within this assay was always >90%.

Results

LOPAC^{1280} Library Screen. Embryos were exposed from 5-25 hpf to each compound at a single limit concentration of 10 µM. Compounds were identified as positive hits if exposure resulted in <85% survival. Based on a 5- to 25-hpf (20-h) exposure, approximately 4% (51 compounds) of the LOPAC^{1280} library was biologically active (relative to vehicle controls) due to significant effects on survival (Figure 2).

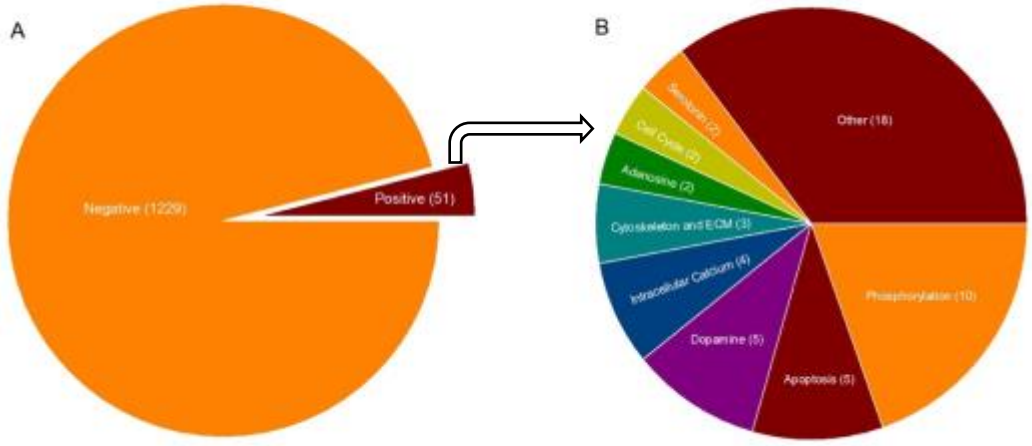

Figure 2. Approximately 4% (51 compounds) of the LOPAC^{1280} library was considered acutely toxic, resulting in <85% survival (A). LOPAC^{1280} hits were not associated with unique LOPAC^{1280}-defined classes of biological targets (B).

Data Mining. Partition coefficient (LogP) values were retrieved for all 1,280 compounds from the NCBI's PubChem database (https://pubchem.ncbi.nlm.nih.gov), and molecular weights for all compounds were correlated by endpoint to determine whether

hydrophobicity and/or chemical size predicted the potential for a positive hit within our assay. However, there was no association between physicochemical attributes (molecular weight and LogP) and the potential for acute toxicity, as compound LogP values and molecular weights ranged from -5.3 to 10 and 98 to 875 g/mol, respectively (Figure 3). We also correlated LogP and molecular weights separately with percent survival to determine a potential range for each physicochemical attribute where there was <85% survival. There was no clear association between these attributes and percent survival (Figure 4).

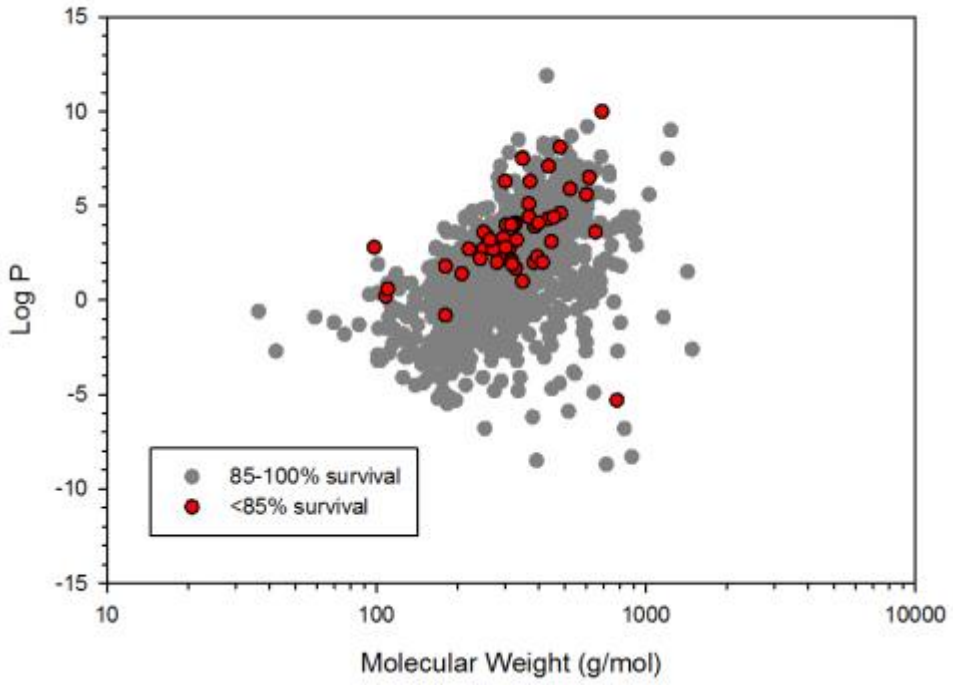

Figure 3. LOPAC^{1280}-positive hits are not associated with LogP and molecular weight. LogP values and molecular weights for all compounds were correlated to determine whether hydrophobicity and chemical size predicted the potential for a hit within our assay.

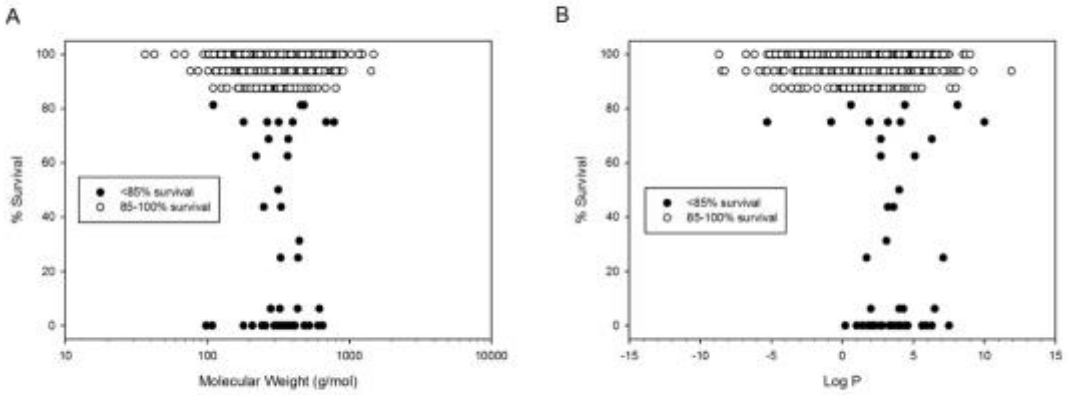

Figure 4. Survival (%) for LOPAC^{1280}-positive hits is not associated with molecular weight (g/mol) (A) nor LogP (B).

The entire LOPAC^{1280} library was clustered based on two-dimensional (2D) structural similarity using NCBI's Single Linkage algorithm (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?p=clustering). Within this model, a Tanimoto Similarity score of 0.68 or higher denotes a statistically significant 2D structural similarity at the 95% confidence interval. Based on this analysis, the potential for acute toxicity was not associated with 2D compound structural similarity (data not shown).

Discussion

Approximately 4% of the LOPAC^{1280} library was identified as acutely toxic compounds, or positive hits, resulting in <85% survival in zebrafish embryos. There was no association between the acutely toxic compounds and the three attributes that we examined for each compound: mode of action, molecular weight, and LogP. Therefore,

the predictive power and domain of applicability of zebrafish embryos for high throughput screening of the LOPAC^{1280} library are unclear.

The variability of predictors of acute toxicity may be influenced by non-biological issues including compound concentration and water solubility, exposure duration, and timing of exposure relative to different stages of embryonic development. Because the dose of each LOPAC^{1280} library stock solution was limited to 10 μM, we are uncertain about the effects of lower or higher concentrations on embryo survival. High water solubility of each compound, as indicated by a LogP value that is less than 0, may also limit uptake into the embryo, affecting the internal dose. These limitations may increase the possibility of false-negatives for positive hits in the library. The exposure duration and timing of exposure are other important factors that could be better controlled to target the exact window of susceptibility during zebrafish embryo development.

Future studies may produce more conclusive results if compounds were more effectively delivered to maximize the internal dose for some representative compounds from the LOPAC^{1280} library with minimal variability in the zebrafish embryo system. Because our exposures were limited to the first 25 hpf of the zebrafish embryos, other studies may begin exposures later on within the 72 hpf during which major development occurs, which would allow for a more maximized physiological context.

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