Disorders of the Nervous System

Large-Scale Phenotype-Based Antiepileptic Drug Screening in a Zebrafish Model of Dravet Syndrome

Matthew T. Dinday,1 and Scott C. Baraban1,2

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1Department of Neurological Surgery, Epilepsy Research Laboratory, University of California San Francisco, San Francisco, California 94143, 2Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California San Francisco, San Francisco, California 94143

Abstract

Mutations in a voltage-gated sodium channel (SCN1A) result in Dravet Syndrome (DS), a catastrophic childhood epilepsy. Zebrafish with a mutation in scn1ab recapitulate salient phenotypes associated with DS, including seizures, early fatality, and resistance to antiepileptic drugs. To discover new drug candidates for the treatment of DS, we screened a chemical library of ~1000 compounds and identified 4 compounds that rescued the behavioral seizure component, including 1 compound (dimethadione) that suppressed associated electrographic seizure activity. Fenfluramine, but not huperzine A, also showed antiepileptic activity in our zebrafish assays. The effectiveness of compounds that block neuronal calcium current (dimethadione) or enhance serotonin signaling (fenfluramine) in our zebrafish model suggests that these may be important therapeutic targets in patients with DS. Over 150 compounds resulting in fatality were also identified. We conclude that the combination of behavioral and electrophysiological assays provide a convenient, sensitive, and rapid basis for phenotype-based drug screening in zebrafish mimicking a genetic form of epilepsy.

Key words: antiepileptic; drug discovery; epilepsy; high throughput; pharmacology; zebrafish

Significance Statement

Dravet syndrome is a catastrophic childhood epilepsy that is resistant to available medications. Current animal models for this disease are not amenable to high-throughput drug screening. We used a zebrafish model for Dravet syndrome and screened >1000 compounds. We report the identification of compounds with the ability to suppress seizure behavior and electrographic seizure activity. This approach provides an example of precision medicine directed to pediatric epilepsy.

Introduction

Dravet syndrome (DS) is a devastating genetic epileptic encephalopathy that has been linked to more than >300 de novo mutations in a neuronal voltage-gated sodium channel (SCN). Children with DS are at a higher risk for sudden unexplained death in epilepsy and episodes of
uncontrolled status epilepticus (Dravet et al., 2005; Ceulemans et al., 2012). Delayed language development, disruption of autonomic function, and motor and cognitive impairment are also associated with this disease. Seizure management includes treatment with benzodiazepines, valproate, and/or stiripentol (Caraballo et al., 2005; Chiron and Dulac, 2011). Some reduction in seizure activity has been reported with the use of bromides and topiramate, or a ketogenic diet (Lotte et al., 2012; Wilmshurst et al., 2014; Dressler et al., 2015). Despite these options, available antiepileptic drugs (AEDs) do not achieve adequate seizure control in most DS patients (Dravet et al., 2005; Chiron and Dulac, 2011; Dressler et al., 2015), making the identification of new drugs a critical unmet need. High-throughput screening offers a powerful tool to identify new drug candidates for these patients. However, commonly available screening approaches rely on in vitro cell-based assays (Masimirembwa et al., 2001; Snowden and Green, 2008; Ko and Gelb, 2014) and do not recapitulate the complicated neural networks that generate seizures in vivo. Given the need for new treatments for children with DS, and the growing number of genetic epileptic encephalopathies that are medically intractable (Leppert, 1990; Epi4K Consortium, 2012; Ottmann and Risch, 2012), we developed an alternative phenotype-based in vivo drug-screening strategy. While cell-based in vitro screening assays can efficiently identify compounds that bind specific targets, whole-organism-based screens are more likely to reliably predict therapeutic outcomes as they maintain the complex neural circuitry involved in the underlying disease process. Whole-organism screens do not require well validated targets to identify compounds that yield a desirable phenotypic outcome, but can be prohibitively costly and time consuming in mammals. As a simple vertebrate with significant genetic similarity to human, zebrafish are now recognized as an ideal cost-effective alternative to achieve rapid in vivo phenotype-based screening (All et al., 2011).

Using scn1a mutant zebrafish larvae with a gene homologous to human and spontaneously occurring seizures (Baraban et al., 2013), we screened, in a blinded manner, a repurposed library of ~1000 compounds for drugs that inhibit unprovoked seizure events. We also screened two compounds (hyperzine A and fenfluramine) that were discovered in rodent-based assays using acquired seizure protocols and that were recently suggested as potential treatments for DS (Boel and Casaer, 1996; Coleman et al., 2008; Ceulemans et al., 2012; Bialer et al., 2015). Only 20 compounds in the repurposed drug library reduced swim behavior to control levels. However, many of these compounds were toxic or were not confirmed on retesting, and only four compounds advanced to a second-stage in vivo electrophysiology assay. Of these compounds (cytarabine, dimethadione, theobromine, and norfloxacain) only dimethadione, a T-type calcium channel antagonist previously reported to have anticonvulsant activity (Lowson et al., 1990; Zhang et al., 1996), reduced ictal-like electrographic discharges seen in scn1Lab mutant larvae. This two-stage phenotype-based screening approach, using a genetic DS model with >75% genomic similarity to human, is a sensitive, rapid means to successfully identify compounds with antiepileptic activity.

Materials and Methods

Zebrafish

Zebrafish were maintained in a light- and temperature-controlled aquaculture facility under a standard 14:10 h light/dark photoperiod. Adult zebrafish were housed in 1.5 L tanks at a density of 5-12 fish per tank and fed twice per day (dry flake and/or flake supplemented with live brine shrimp). Water quality was continuously monitored: temperature, 28-30°C; pH 7.4-8.0; conductivity, 690-710 mS/cm. Zebrafish embryos were maintained in round Petri dishes (catalog #FB0875712, Fisher Scientific) in “embryo medium” consisting of 0.03% Instant Ocean (Aquarium Systems, Inc.) and 000002% methylene blue in reverse osmosis-distilled water. Larval zebrafish clutches were bred from wild-type (WT; TL strain) or scn1Lab (didys552) heterozygous animals that had been backcrossed to TL wild-type for at least 10 generations. Homozygous mutants (n = 6544), which have widely dispersed melanosomes and appear visibly darker as early as 3 dpf (Fig. 1b), or WT larvae (n = 71) were used in all experiments at 5 or 6 dpf. Embryos and larvae were raised in plastic petri dishes (90 mm diameter, 20 mm depth) and density was limited to ~60 per dish. Larvae between 3 and 7 dpf lack discernible sex chromosomes. The care and maintenance protocols comply with requirements outlined in the Guide for the Care and Use of Animals (ebrary Inc., 2011) and were approved by the Institutional Animal Care and Use Committee (protocol #AN108659-01D).

Seizure monitoring

Zebrafish larvae were placed individually into 1 well of a clear flat-bottomed 96-well microplate (catalog #260836, Fisher Scientific) containing embryo media. Microplates were placed inside an enclosed motion-tracking device and acclimated to the dark condition for 10-15 min at room temperature. Locomotion plots were obtained for one fish per well at a recording epoch of 10 min using a DanioVision system running EthoVision XT software (DanioVision, Noldus Information Technology); threshold detection settings to identify objects darker than the background were optimized for each experiment. Seizure scoring was performed using the following three-stage scale (Baraban et al., 2005): Stage 0, no or very little swim activity; Stage I, increased, brief bouts of swim activity; Stage II, rapid “whirlpool-like” circling swim behavior; and Stage III, paroxysmal whole-body clonus-like convulsions, and a brief loss of posture. WT fish are normally scored at Stage 0 or I. Plots were analyzed for distance traveled (in millimeters) and mean velocity (in millimeters per second). As reported previously (Winter et al., 2008; Baraban et al., 2013), velocity changes were a more sensitive assay of seizure behavior. For electrophysiology studies, zebrafish larvae were briefly paralyzed with
α-bungarotoxin (1 mg/ml) and immobilized in 1.2% agarose; field recordings were obtained from forebrain structures. Epileptiform events were identified post hoc in Clampfit (Molecular Devices) and were defined as multi-spike or polyspike upward or downward membrane deflections greater than three times the baseline noise level and >500 ms in duration. During electrophysiology experiments zebrafish larvae were continuously monitored for the presence (or absence) of blood flow and heart beat by direct visualization on an Olympus BX51WI upright microscope equipped with a CCD camera and monitor.

**Drugs**

Compounds for drug screening were purchased from MicroSource Discovery Systems, Inc. (PHARMAKON 1600) and were provided as 10 mM DMSO solutions (Table 1). Test compounds for locomotion or electrophysiology studies were dissolved in embryo media and were tested at an initial concentration of 100 μM, with a final DMSO concentration of <2%. In all drug library screen studies, compounds were coded and experiments were performed by investigators who were blind to the nature of the compound. Baseline recordings of seizure behavior were performed prior to treatment, and the percentage change in state was used to identify compounds with potential anticonvulsant activity.

**Figure 1.** Locomotion assay to identify drugs that rescue the scn1Lab mutant epilepsy phenotype. **a**, Schematic of the phenotype-based screening process. Chemical libraries can be coded and aliquoted in small volumes (75 μL) into individual wells containing one mutant fish. The 96-well microplate is arranged so that six fish are tested per drug; with one row of six fish maintained as an internal control (red circles) on each plate. **b**, Representative images for WT and scn1Lab mutant zebrafish larvae at 5 dpf. Note the morphological similarity but darker pigmentation in mutant larvae. **c**, Box plot of mean velocity (in millimeters per second) for two consecutive recordings of mutant larvae in embryo media. Experiments were performed by first placing the mutant larvae in embryo media and obtaining a baseline locomotion response; embryo media was then replaced with new embryo media (to mimic the procedure used for test compounds), and a second locomotion response was obtained. The percentage change in velocity from baseline (recording 1) versus experimental model (recording 2) is shown. In the box plot, the bottom and top of the box represent the 25th percentile and the 75th percentile, respectively. The line across the box represents the median value, and the vertical lines encompass the entire range of values. This plot represents normal changes in tracking activity in the absence of a drug challenge. **d**, Plot of locomotor seizure behavior for scn1Lab mutants at 5 dpf for the 1012 compounds tested. Threshold for inhibition of seizure activity (positive hits) was set as a reduction in mean swim velocity of ≥44%; the threshold for a proconvulsant or hyperexcitable effect was set at an increase in the mean swim velocity of ≥44% (green dashed lines).
Table 1. List of compounds from the PHARMAKON 1600 library used in this screen.

| Compound                        | Compound                        |
|---------------------------------|---------------------------------|
| ABACAVIR SULFATE                | AMPROLIUM                       |
| ABAMECTIN (avermectin B1a shown) | AMSACRINE                       |
| ACADESINE                       | ANASTROZOLE                     |
| ACARBOSE                        | ANCITABINE HYDROCHLORIDE        |
| ACETABUTOLOL HYDROCHLORIDE      | ANETHOLE                        |
| ACECLIDINE                      | ANIRACETAM                      |
| ACECLOFENAC                     | ANISINDIONE                     |
| ACENOCOUMAROL                   | ANTAZOLINE PHOSPHATE            |
| ACETAMINOPHEN                   | ANTHELINE                       |
| ACETOHYDROXYAMIC ACID           | ANTIHYDROXYAMIC ACID            |
| ACETOPHENAZINE MALEATE          | APOMORPHINE HYDROCHLORIDE       |
| ACETYLCHOLINE CHLORIDE          | APRAZOLAM                       |
| ACETYLGLYCINE                   | ARGinine HYDROCHLORIDE          |
| ACETYLGLYCINE                   | ARMODAFINIL                     |
| ACYCLOVIR                       | ARTENOLOL                       |
| ADAPALENE                      | ATORVASTATIN CALCIUM            |
| ADELMLIDROSE                    | ATROPAUSINE                     |
| ADENINE                         | ATROPINE SULFATE                |
| ADENOSINE                       | AUROTHIOGLUCOSE                 |
| ADENOSINE PHOSPHATE             | AVOBENZONE                      |
| ADIPHENINE HYDROCHLORIDE        | AZACITIDINE                     |
| ALKLOMIDE                       | AZASERINE                       |
| ALAPROCLATE                     | AZATADINE MALEATE               |
| ALBENDAZOLE                     | AZATHIOPRINE                    |
| ALBUTEROL (+/-)                 | AZELAIC ACID                    |
| ALENDRONATE SODIUM              | AZITHROMYCIN                    |
| ALEXIDINE HYDROCHLORIDE         | BECKAMycin SULFATE              |
| ALLANTOIN                       | BEMOTRIZINOL                    |
| ALLOPURINOL                     | BENAZEPIL HYDROCHLORIDE         |
| ALMOTRIPTAN                     | BENDROFLUMETHIAZIDE             |
| alpha-TOCHOPHEROL               | BENORILATE                      |
| alpha-TOCHOPHERYL ACETATE       | BENSERAZIDE HYDROCHLORIDE       |
| ALPRAZOLAM                      | BENZALKONIUM CHLORIDE           |
| ALRESTATIN                      | BENZETHIONIUM CHLORIDE          |
| ALTIAZIDE                       | BENZOCAINE                      |
| ALTRETAMINE                     | BENZOIC ACID                    |
| ALVERINE CITRATE                | BENZONATATE                     |
| AMANTADINE HYDROCHLORIDE        | BENZOYL PEROXIDE                |
| AMINONIOGLUTETHIMIDE            | BENZTHIAZIDE                    |
| AMINOPROPIA ACID                | BENZYL ALCOHOL                  |
| AMINOHIPPURIC ACID              | BENZYL BENZOATE                 |
| AMINOLEVULINIC ACID HYDROCHLORIDE | BEPREDIL HYDROCHLORIDE         |
| AMINOSALICYLATE SODIUM          | BERGAPTEIN                      |
| AMITRIPTYLINE HYDROCHLORIDE     | beta-CAROTENE                   |
| AMLEXANOX                       | BETAHISTINE HYDROCHLORIDE       |
| AMLODIPINE BESYLATE             | BETAINA HYDROCHLORIDE           |
| AMODIAQUINE DIHYDROCHLORIDE     | BETAMETHASONE                   |
| AMORFOLINE HYDROCHLORIDE        | BETAMETHASONE 17,21-DIPROPIONATE |
| AMOXICILLIN                     | BETAMETHASONE VALERATE          |

(Continued)
| Compound                        | Compound                        |
|--------------------------------|--------------------------------|
| BETAMIPRON                     | CEFOTAXIME SODIUM               |
| beta-NAPHTHOL                  | CEFOTETAN                       |
| BETAZOLE HYDROCHLORIDE         | CEFFOXITIN SODIUM               |
| BETANECHOL CHLORIDE            | CEFPIRAMIDE                     |
| BEZAFIBRATE                    | CEFSULODIN SODIUM               |
| BICALUTAMIDE                   | CEFTIBUTEN                      |
| BIOTIN                         | CEFTRIAXONE SODIUM TRIHYDRATE   |
| BISACODYL                      | CEFUROXIME AXETIL               |
| BISOCTRIZOLE                   | CEFUROXIME SODIUM               |
| BISORCIC                       | CELECOXIB                       |
| BITHIONATE SODIUM              | CEPHALEXIN                      |
| BLEOMYCIN (bleomycin B2 shown) | CEPHALOTHIN SODIUM              |
| BRETYLIUM TOSYLATE             | CEPHAPIRIN SODIUM               |
| BRINZOLAMIDE                   | CEPHRADINE                      |
| BROMHEXINE HYDROCHLORIDE       | CETYLPYRIDINIUM CHLORIDE        |
| BROMOCRIPTINE MESYLATE         | CHENODIOL                       |
| BROMPHENIRAMINE MALEATE        | CHLORAMBUCIL                    |
| BROXYQUINOLINE                 | CHLORAMPHENICOL                |
| BUDESONIDE                     | CHLORAMPHENICOL HEMISUCCINATE  |
| BUMETANIDE                     | CHLORAMPHENICOL PALMITATE      |
| BUPIVACAINE HYDROCHLORIDE      | CHLORCYCLIZINE HYDROCHLORIDE    |
| BUPROPION                      | CHLORHEXIDINE                   |
| BUSULFAN                       | CHLOROCRESEL                    |
| BUTACAINE                      | CHLOROQUANIDE HYDROCHLORIDE     |
| BUTAMBEN                       | CHLOROQUINE DIPHOSPHATE         |
| BUTOCONAZOLE                   | CHLOROTHIAZIDE                  |
| CAFFEINE                       | CHLOROXINE                      |
| CAMPHOR (1R)                   | CHLOROXYLENOL                   |
| Candesartan                    | CHLORPHENIRAMINE (S) MALEATE    |
| Candesartan Cilexil            | CHLORPROMAZINE                  |
| CANDICIDIN                     | CHLORPROPAMIDE                  |
| CANRENOIC ACID, POTASSIUM SALT| CHLORPROMAZINE SODIUM           |
| CANRENONE                      | CILASTOROL                      |
| CAPECITABINE                   | Cimetidine                      |
| CAPREOMYCIN SULFATE            | CINCHOPHEN                      |
| CAPSAICIN                      | CINNARAZINE                     |
| CAPTAMINE                      | CINOSACIN                       |
| CAPTOPRIL                      | CINTRIAMIDE                     |
| CARBACHOL                      | CIPROFIBRATE                    |
| CARBENICILLIN DISODIUM         | CIPROFLOXACIN                   |
| CARBENOXOLONE SODIUM           | CISPLATIN                       |
| CARBETAPENTANE CITRATE         | CITALOGRAM HYDROBROMIDE         |
| CARBIDOPA                      | CITICOLINE                      |
| CARBOXINAMINE MALEATE          | CLARITHROMYCIN                  |
| CARBOPLATIN                    | CLAVULANATE LITHIUM             |
| CARISOPRODOL                   | CLEMASTINE                      |
| CARMUSTINE                     | CLIDINIUM BROMIDE               |
| CARNITINE (di) HYDROCHLORIDE   | CLINDAMYCIN HYDROCHLORIDE       |
| CARPROFEN                      | CLOHEXACIN                      |
| CARVEDILOL                     | CLOIONOL                        |
| CEFACLOR                       | CLOBETASOL PROPIONATE           |
| CEFADROXIL                     | CLOFARABINE                     |
| CEFAMANDOLE NAFATE             | CLOFIBRATE                      |
| CEFAMANDOLE SODIUM             | (Continued)                     |
| CEFAZOLIN SODIUM               | (Continued)                     |
| CEFEPINE HYDROCHLORIDE         |                                |
| CEFMENOXIME HYDROCHLORIDE      |                                |
| CEFMETAZOLE SODIUM             |                                |
| CEFOPERAZONE                   |                                |
| CEFORANIDE                     |                                |

(Continued)
| Compounds from the PHARMAKON 1600 library used in this screen. (continued) |
|-----------------------------------------------|
| CLOMIPHENE CITRATE                            |
| CLONIDINE HYDROCHLORIDE                       |
| CLOPIDOGREL SULFATE                           |
| CLORSULON                                     |
| CLOSANTEL                                     |
| CLOTRIMAZOLE                                  |
| CLOXACILLIN SODIUM                            |
| CLOXYQUIN                                     |
| CLOZAPINE                                     |
| COENZYME B12                                  |
| CORTISONE ACETATE                             |
| COTININE                                      |
| CRESOL                                        |
| CROMOLYN SODIUM                               |
| CRYOFLURANE                                   |
| CYCLAMIC ACID                                 |
| CYCLIZINE                                     |
| CYCLOBENZAPRINE HYDROCHLORIDE                 |
| CYCLOHEXIMIDE                                 |
| CYCLOPENTOLATE HYDROCHLORIDE                  |
| CYCLOPHOSPHAMIDE HYDRATE                      |
| CYCLOSERINE (D)                               |
| CYCLOSPORINE                                  |
| CYCLOTHIAZIDE                                 |
| CYPERMETHRIN                                  |
| CYPROTERONE ACETATE                           |
| CYSTEAMINE HYDROCHLORIDE                      |
| CYTARABINE                                    |
| DACARBAZINE                                   |
| DACTINOMYCIN                                  |
| DANAZOL                                       |
| DANThRON                                      |
| DANTROLENE SODIUM                             |
| DAPSONE                                       |
| DAPTOMYCIN                                    |
| DASATINIB                                     |
| DAUNORUBICIN                                  |
| DECIMEMIDE                                    |
| DEFEROXAMINE MESYLATE                         |
| DEFLAZACORT                                   |
| DEHYDROACETIC ACID                            |
| DEHYDROCHOLIC ACID                            |
| DEMECLOCYCLINE HYDROCHLORIDE                  |
| DERAoxib                                      |
| DESIPRAMINE HYDROCHLORIDE                     |
| DESOXYCORTICOSTERONE ACETATE                  |
| DESVENLAFAXINE SUCCINATE                      |
| DEXAMETHASONE                                 |
| DEXAMETHASONE ACETATE                         |
| DEXAMETHASONE SODIUM PHOSPHATE                |
| DEXIUPROFEN                                   |
| DEXLANSOPRAZOLE                               |
| DEXPROPRANOLOL HYDROCHLORIDE                  |
| DEXTROMETHORPHAN HYDROBROMIDE                 |
| DIAPERIDINE                                   |
| DIBENZOTHIOPHENE                              |
| DIBUCAINA HYDROCHLORIDE                       |
| DICHLORPHENINE                                |

(Continued)

| Compounds from the PHARMAKON 1600 library used in this screen. (continued) |
|-----------------------------------------------|
| DICHLORVOS                                   |
| DICLAZURIL                                   |
| DICLOFENAC SODIUM                            |
| DICLOXACILLIN SODIUM                         |
| DICUMAROL                                    |
| DICYCLOMINE HYDROCHLORIDE                    |
| DIENESTROL                                   |
| DIETHYLCARBAMAZINE CITRATE                   |
| DIETHYSTILBESTROL                            |
| DIFLOXACIN HYDROCHLORIDE                     |
| DIFLUNISAL                                   |
| DIGITOXIN                                    |
| DIGOXIN                                      |
| DIHYDROERGOTAMINE MESYLATE                   |
| DIHYDROSTREPTOMYCIN SULFATE                  |
| DILAZEP DIHYDROCHLORIDE                      |
| DIMENHYDRINATE                               |
| DIMERCAPROL                                  |
| DIMETHADIONE                                 |
| DIOXYBENZONE                                 |
| DIPHENHYDRAMINE HYDROCHLORIDE                |
| DIPHENYLPRALINE HYDROCHLORIDE                |
| DIPYRIDAMOLE                                 |
| DIPYRONE                                     |
| DIRITHROMYCIN                                |
| DISOPYRAMIDE PHOSPHATE                       |
| DISULFIRAM                                   |
| DOBUTAMINE HYDROCHLORIDE                     |
| DOCETAXEL                                    |
| DONEPEZIL HYDROCHLORIDE                      |
| DOPAMINE HYDROCHLORIDE                       |
| DOXEPIN HYDROCHLORIDE                        |
| DOXOFYLLINE                                  |
| DOXYCYCLINE HYDROCHLORIDE                    |
| DOXYLAMINE SUCINNATE                         |
| DROFENINE HYDROCHLORIDE                      |
| DROPERIDOL                                   |
| DROSPIRENONE                                 |
| DYCLOLONINE HYDROCHLORIDE                    |
| DYPHYLLINE                                   |
| ECAMSULE TRIETHANOLAMINE                     |
| ECONAZOLE NITRATE                            |
| EDETATE DISODIUM                             |
| EDITOL                                       |
| EDOXUDINE                                    |
| EMETINE                                      |
| ENALAPRIL MALEATE                            |
| ENALAPRILAT                                  |
| ENOXACIN                                     |
| ENROFLAXACIN                                 |
| ENTACAPONE                                   |
| EPHEDRINE (1R,2S) HYDROCHLORIDE               |
| EPINEPHRINE BITARTRATE                       |
| EPRINOMECTIN                                 |
| ERGOCALCIFEROL                              |
| ERGONOVINE MALEATE                           |
| ERYTHROMYCIN                                 |
| ERYTHROMYCIN ESTOLATE                        |
| ERYTHROMYCIN ETHYLSCUCCINATE                 |
| ESCITALOPRAM OXALATE                         |
| ESOMEPRAZOLE POTASSIUM                       |

(Continued)
| Compound                  | Compound                        |
|--------------------------|---------------------------------|
| ESTRADIOL                | FLUVASTATIN                     |
| ESTRADIOL BENZOATE       | FOLIC ACID                      |
| ESTRADIOL CYPIONATE      | FOSCARNET SODIUM                |
| ESTRADIOL DIPROPIONATE   | FOSSOMYCIN CALCIUM              |
| ESTRADIOL VALERATE       | FTTAXILIDE                      |
| ESTRAMUSTINE             | FULVESTRANT                     |
| ESTRIOLE                 | FURAZOLIDONE                    |
| ESTROPIRATE              | FUROSEMIDE                      |
| ETHACRYNIC ACID          | FUSIDIC ACID                    |
| ETHAMBUTOL HYDROCHLORIDE | GABOXADOL HYDROCHLORIDE         |
| ETHAM VERINE HYDROCHLORIDE | GADOTERIDOL                  |
| ETHINYL ESTRADIOL        | GALANTHAMINE                    |
| ETHIONAMIDE              | GALLAMINE TRIETHIODIDE          |
| ETHISTERONE              | GANCICLOVIR                     |
| ETHOPROPAZINE HYDROCHLORIDE | GATIFLOXACIN               |
| ETHYL PARaben            | GEFTINIB                        |
| ETODOLAC                 | GEMFIBROZIL                     |
| ETOPOSIDE                | GENTAMICIN SULFATE              |
| EUCALYPTOL               | GENTIAN VIOLET                  |
| EUCATROPINE HYDROCHLORIDE | GLIMEPIRIDE                  |
| EUGENOL                  | GLUCONOLACTONE                  |
| EVANS BLUE               | GLUCOSAMINE HYDROCHLORIDE       |
| EXEMESTANE               | GLUTAMINE (D)                   |
| EZETIMIBE                | GRAMICIDIN                      |
| FAMCICLOVIR              | GRANISETRON HYDROCHLORIDE       |
| FAMOTIDINE               | GRISEOFULVIN                    |
| FAMPRIDINE               | GUAIFENESIN                     |
| FASUDIL HYDROCHLORIDE    | GUANABENZ ACETATE               |
| FEBUXOSTAT               | GUANETHIDINE SULFATE            |
| FENBENDAZOLE             | HALAZONE                        |
| FENBUFEN                 | HALCINONIDE                     |
| FENDILINE HYDROCHLORIDE  | HALOPERIDOL                     |
| FENOFIBRATE              | HEPTAMINOL HYDROCHLORIDE        |
| FENOPROFEN               | HETACILLIN POTASSIUM            |
| FENOTROL HYDROBROMIDE    | HEXACHLOROPHENE                 |
| FENOPIRIDE HYDROCHLORIDE | HEXYLMESORCINOL                 |
| FEXOFENADINE HYDROCHLORIDE | HISTAMINE DIHYDROCHLORIDE    |
| FIPEXIDE HYDROCHLORIDE   | HOMATROPINE BROMIDE             |
| FIROCOXIB                | HOMATROPINE METHYLBROMIDE       |
| FLOXURIDINE              | HOMOSALATE                      |
| FLUCONAZOLE              | HYCANTHONE                      |
| FLUCORTISONE ACETATE     | HYDRAZINE HYDROCHLORIDE         |
| FLUFENAMIC ACID          | HYDRASTINE (1R, 9S)            |
| FLUNARIZINE HYDROCHLORIDE | HYDROCLOROTHIAZIDE              |
| FLUNDAROL                | HYDROCORTISONE                  |
| FLUMEQUINE               | HYDROCORTISONE ACETATE          |
| FLUMETHASONE             | HYDROCORTISONE BUTYRATE         |
| FLUMETHAZONE PIVALATE    | HYDROCORTISONE HEMISUCCINATE    |
| FLUNISOLIDE              | HYDROCORTISONE PHOSPHATE TRIETHYLAMINE |
| FLUNIXIN MEGLUMINE       | HYDROFLUMETHAIZIDE              |
| FLUCINOLONE ACETONIDE    | HYDROQUINONE                    |
| FLUCINONIDE              | HYDROXYAMPHETAMINE HYDROBROMIDE|
| FLUOROMETHOLONE          | HYDROXYCHLOROQUINE SULFATE      |
| FLUOROURACIL             | HYDROXYPROGESTERONE CAPROATE    |
| FLUOXETINE               | HYDROXYTOLUIC ACID              |
| FLUPHENAZINE HYDROCHLORIDE | HYDROXYUREA                 |
| FLURANDRENOLIDE          | HYDROXYZINE PAMOATE             |
| FLURIPROFEN              | HYOSCYAMINE                     |
| FLUROFAMIDE              | IBANDRONATE SODIUM              |
| FLUTAMIDE                | IBUROFEN                        |
|                         | IDOXURDINE                      |
| IDOXURIDINE             | LOMUSTINE          |
|------------------------|-------------------|
| IMIPRAMINE HYDROCHLORIDE | LORATADINE          |
| IMIQUIMOD              | LORNOXICAM         |
| INAMRINONE             | LOSARTAN           |
| INDAPAMIDE             | LOVASTATIN         |
| INDOMETHACIN           | LUMIRACOXIB        |
| INDOPOFEN              | MANFENIDE HYDROCHLORIDE |
| INOSITOL               | MALATHION          |
| IODIPAMIDE             | MANGAFODIPIR TRISODIUM |
| IODIXANOL              | MANIDIPINE HYDROCHLORIDE |
| IODOQUINOL             | MANNITOL           |
| IOHEXOL                | MAPIRTILINE HYDROCHLORIDE |
| IOPANIC ACID           | MEBENDAZOLE        |
| IOPTHALIC ACID         | MEBEVERINE HYDROCHLORIDE |
| IOVERSOL               | MEHYDROLIN NAPHTHALENESULFONATE |
| IOXILAN                | MECAMYLAMINE HYDROCHLORIDE |
| IPRATROPIUM BROMIDE    | MECHLORETAMINE     |
| IRBESARTAN             | MECLIZINE HYDROCHLORIDE |
| ISONIAZID              | MECLOXYCLINE SULFOSALICYLATE |
| ISOPROPAMIDE IODIDE    | MECLOFENAMATE SODIUM |
| ISOPROTERENOL HYDROCHLORIDE | MECLOFENOXATE HYDROCHLORIDE |
| ISOSORBIDE DINIRATE    | MEDROXYPROGESTERONE ACETATE |
| ISOSORBIDE MONONITRATE | MEDRYSONE         |
| ISOTRETINON            | MEFENAMIC ACID     |
| ISOXICAM               | MEFEXAMIDE         |
| ISOXSUPRINE HYDROCHLORIDE | MEfloQUINE     |
| ITOPRIDE HYDROCHLORIDE | MEGESTROL ACETATE  |
| IVERMECTIN             | MEGLUMINE          |
| KANAMYCIN A SULFATE    | MELOXICAM SODIUM   |
| KETOCONAZOLE           | MELPERONE HYDROCHLORIDE |
| KETOPROFEN             | MELPHALAN          |
| KETOROLAC TROMETHAMINE | MEMANTINE HYDROCHLORIDE |
| KETOTIFEN FUMARATE     | MENADIONE          |
| Labetalol HYDROCHLORIDE | MEPARTRICIN     |
| LACTULOSE              | MEPENZOLATE BROMIDE|
| LAMIVUDINE             | MEPHENESIN         |
| LANATOSIDE C           | MEPHENTERMINE SULFATE |
| LANSOPRAZOLE           | MEPIVACAINE HYDROCHLORIDE |
| LEFLUNOMIDE            | MEQUINOL           |
| LETROZOLE              | MERBROMIN          |
| LEUCOVORIN CALCIUM     | MERCAPTOPURINE     |
| LEVAMISOLE HYDROCHLORIDE | MEROPENEM      |
| LEVOCETIRIZINE DIHYDROCHLORIDE | MESNA     |
| LEVOFLOXACIN           | METO-ERYTHRITOL    |
| LEVOMENTHOL            | MESTRALON          |
| LEVONORDEFRIN          | METAPROTERENOL     |
| LEVONORGESTREL         | METARAMINOL BITARTRATE |
| LEVOSIMENANDAN         | METAXALONE         |
| LEVOTHYROXINE          | METHACHOLINE CHLORIDE |
| LIDOCAINE HYDROCHLORIDE | METHACYCLINE HYDROCHLORIDE |
| LINCOMYCIN HYDROCHLORIDE | METHAPYRILENE HYDROCHLORIDE |
| LINDANE                | METHAZOLAMIDE      |
| LINEZOLID              | METHENAMINE        |
| LIOTHYRONINE           | METHICILLIN SODIUM |
| LIOTHYRONINE (L- isomer) SODIUM | METHIMAZOL          |
| LISINOPRIL             | METHOCARBAMOL      |
| LITHIUM CITRATE        | METHOTREXATE(+/-)  |
| LOBELINE HYDROCHLORIDE | METHOXAMINE HYDROCHLORIDE |
| LOFEXIDINE HYDROCHLORIDE | METHOXASALEN |
| LOMEFLOXACIN HYDROCHLORIDE | METHSCOPOLAMINE BROMIDE |
| LOMERIZINE HYDROCHLORIDE | METHYCLOTHIAZIDE  |

(Continued)
Table 1. List of compounds from the PHARMAKON 1600 library used in this screen. (continued)

| Compound                          | Compound                          |
|-----------------------------------|-----------------------------------|
| METHYLBENZETHIONIUM CHLORIDE      | NOMIFENSINE MALEATE               |
| METHYLDOPA                        | NOREPINEPHRINE                    |
| METHYLERGONOVINE MALEATE          | NORETHINDROME                     |
| METHYLPROPIOSULFATE               | NORETHINDRONE ACETATE             |
| METHYLPERDOSULFATE                | NORETHYNOBREL                     |
| METHYLPERDOSULFATE SODIUM SUCCINATE | NORFLOXACIN                    |
| METHYLTHIOURACIL                  | NORGESTREL                        |
| METOCLOPRAMIDE HYDROCHLORIDE      | NORTRIPTYLINE                     |
| METOPROLOL TARTRATE               | NOSCAPINE HYDROCHLORIDE           |
| METRONIDAZOLE                     | NOVOBIOCIN SODIUM                 |
| MEXILETINE HYDROCHLORIDE          | NYLIDRIN HYDROCHLORIDE            |
| MICONAZOLE NITRATE                | NYSTATIN                          |
| MИDODRINE HYDROCHLORIDE           | OCTOPAMINE HYDROCHLORIDE          |
| MИGLITOL                          | OFLOXACIN                         |
| MИLNICIPRAN HYDROCHLORIDE         | OLMESARTAN                        |
| MINAPRINE HYDROCHLORIDE           | OLMESARTAN MЕDOXОMІL              |
| MINOCYCLINE HYDROCHLORIDE         | OLSALАЗINE SODIUM                 |
| MИNOXИDIL                         | OLSЕLTAMІVIR PHOSPHATE            |
| MITОMYCIN C                      | OМЕGA-3-АСID ESTЕRS (ЕPA shown) |
| MITОTANE                          | OНDANSEТRON                       |
| МITOXАНTRONE HYDROCHLORIDE       | ORLISTAT                          |
| MОLSІDОMІNE                       | ORPHЕNADRІNE CITRATE              |
| MОNENSIN SODIUM (monensin A is shown) | QUАBAІN                        |
| MONOBENZОNE                      | OXАCILLІN SODIUM                  |
| MОРАNТЕL CITRATE                  | OXАLIPLATІN                       |
| MOXАLACTAM DISODIUM               | OХАRBАZЕРІНЕ                     |
| MOXIFLOXАСІN HYDROCHLORIDE        | ОХЕТХАЗАІНЕ                       |
| МYСОРОХЕНОLATE MОFЕTІL            | ОХІВЕNДАZОLЕ                   |
| МYСОРОХЕНОLIC АСІD               | ОХІDОPАMІNЕ HYDROCHLORIDE         |
| NАBUMЕTONE                        | ОХІLІNІС ACІD                   |
| NАDІDІE                           | OXYBENZONE                        |
| NАДОLОL                           | OXYМЕТАZОLІNE HYDROCHLORIDE       |
| NАFСІLLІN SODIUM                  | OXYPHЕНІNУТАZОNЕ               |
| NАFRОNYL ОXАLАTE                  | OXYPHЕНІСYLІMІNE HYDROCHLORIDE    |
| NАLBУPHІNЕ HYDРОСHLОРІDЕ          | OXYQUІNOLІНЕ HЕМІSУLFАTE         |
| NАLІDІСІC АСІD                   | OXYТЕТРАСУLІNЕ                   |
| NАLОXОNЕ HYDРОСHLОРІDЕ            | PAНІТАXЕL                        |
| NАLТRХОNЕ HYDРОСHLОРІDЕ           | PALІРЕDІОN                     |
| NАРАХОلزمE HYDРОСHLОРІDЕ          | PAРААВЕRІNЕ HYDРОСHLОРІDЕ         |
| NАРРОXЕN(+)                       | PARАСHLОРOPЕPHЕNOL              |
| NАРРОXOЛ                          | PARАRОСАНІLІNЕ PАМОАТЕ           |
| NАTEGІNІDІE                      | PARАGLYNE HYDРОСHLОРІDЕ           |
| NЕFАZОDОNЕ HYDРОСHLОРІDЕ          | PARОМОМОСYC SУLFAТЕ             |
| NЕFОPAМ                          | PARӨXЕТІNЕ HYDРОСHLОРІDЕ         |
| NЕLАRА BIN                       | PЕMЕТРЕХЕD                     |
| NЕОМІСИН SУLFAТЕ                 | PЕNСІСLOVІR                   |
| NЕОСТИГМІNЕ BРОMІDЕ              | PЕNІСІLLАMІNЕ               |
| NEВІRАPIN                         | PЕNІСІLІN G ПОТАSSІUM           |
| NIАСІN                           | PЕNІСІLІN V ПОТАSSІUM            |
| NIАРІDІРІNЕ HYDРОСHLОРІDЕ         | PЕNТОLІNІUM TАRTRАTE          |
| NIСЕРОLІNЕ                       | PЕNТОXІФІLІNЕ                |
| NIСLОСАMІDЕ                      | PЕRGОLІDЕ MЕSYLАTE                |
| NIСОTІNІVІL АLСОHОL TAРTRAТЕ     | PЕRHЕXІLІNЕ MАLEАTE             |
| NIФЕDІРІNЕ                       | PERІСІAZІNЕ                   |
| NIФUРСОL                         | PERІНDОРРІR ЕРBUМІNЕ             |
| NIЛUТАМІDЕ                       | PЕРФЕNАZІNЕ                   |
| NIМОDІРІNЕ                       | PHЕNАСЕМІDЕ                   |
| NIТАZОХАNІDЕ                     | PHЕNАZОРРІRІDІNE HYDРОСHLОРІDЕ   |
| NIТRЕНІDІNЕ                      | PHЕNЕLЗІNЕ SУLFAТЕ            |
| NIТRОРАNТОІN                     | PHЕNІNІDІONE                 |
| NIТRУΡАZОNЕ                     | PHЕNІRАMІNЕ MАLEАTE           |
| NIТРОMІDЕ                       | (Continued)                   |

(Continued)
Table 1. List of compounds from the PHARMAKON 1600 library used in this screen. (continued)

| Compound                                      |
|-----------------------------------------------|
| PHENOLPHTHALEIN                               |
| PHENTOLAMINE HYDROCHLORIDE                    |
| PHENYL AMINOSALICYLATE                        |
| PHENYL BUTAZONE                               |
| PHENYLEPHRINE HYDROCHLORIDE                   |
| PHENYL MERCURIC ACETATE                       |
| PHENYLPROPANOLAMINE HYDROCHLORIDE             |
| PHENYTOIN SODIUM                              |
| PHTHALYLSULFATHIAZOLE                         |
| PHYSOSTIGMINE SALICYLATE                      |
| PILOCARPINE NITRATE                           |
| PIMOZIDE                                      |
| PINDOLOL                                      |
| PIOGLITAZONE HYDROCHLORIDE                    |
| PIPERACETAZINE                                |
| PIPERACILIN SODIUM                            |
| PIPERAZINE                                    |
| PIPERIDOLATE HYDROCHLORIDE                    |
| PIPERINE                                      |
| PIPOBROMAN                                    |
| PIRACETAM                                     |
| PIRENERONE                                    |
| PIRENZEPINE HYDROCHLORIDE                     |
| PIROCTONE OLAMINE                             |
| PIROXICAM                                     |
| PITAVASTATIN CALCIUM                          |
| PIZOTYLINE MALATE                             |
| POLYMYSYN B SULFATE                           |
| POTASSIUM p-AMINOBENZOATE                     |
| PRAMIPEXOLE DIHYDROCHLORIDE                   |
| PRAMOXINE HYDROCHLORIDE                       |
| PRASUGREL                                     |
| PRAZIQUANTEL                                  |
| PRAZOSIN HYDROCHLORIDE                        |
| PREDNICARBATE                                 |
| PREDNISOLONE                                  |
| PREDNISOLONE ACETATE                          |
| PREDNISONE                                    |
| PRILOCAINE HYDROCHLORIDE                      |
| PRIMAQUINE DIPHOSPHATE                        |
| PRIMIDONE                                     |
| PROADIFEN HYDROCHLORIDE                       |
| PROBENEcid                                    |
| PROBUCOL                                      |
| PROCAINAMIDE HYDROCHLORIDE                    |
| PROCAINE HYDROCHLORIDE                        |
| PROCARBAZINE HYDROCHLORIDE                    |
| PROCHLORPERAZINE EDISYLATE                    |
| PROCYCLIDINE HYDROCHLORIDE                    |
| PROGESTERONE                                  |
| PROGLUMIDE                                    |
| PROMAZINE HYDROCHLORIDE                       |
| PROMETHAZINE HYDROCHLORIDE                    |
| PRONETALOL HYDROCHLORIDE                      |
| PROPafenONE HYDROCHLORIDE                     |
| PROPHETHINE BROMIDE                           |
| PROPIOLACTONE                                 |
| PROPOFOL                                      |
| PROPYLTHIOURACIL                              |
| PSEUDOEPHEDRINE HYDROCHLORIDE                 |

(Continued)
Table 1. List of compounds from the PHARMAKON 1600 library used in this screen. (continued)

SILDENAFIL CITRATE
SIMVASTATIN
SIROLIMUS
SISOMICIN SULFATE
SODIUM DEHYDROCHOLATE
SODIUM NITROPRUSSIDE
SODIUM OXYBATE
SODIUM PHENYLACETATE
SODIUM PHENYLACETATE
SODIUM SALICYLATE
SPARFLOXACIN
SPARTEINE SULFATE
SPECTINOMYCIN HYDROCHLORIDE
SPIPERONE
SPIRAMYCIN
SPIRAPRIL HYDROCHLORIDE
SPIRONOLACTONE
STAVUDINE
STREPTOMYCIN SULFATE
STREPTOZOSIN
SUCINYL SULFATHIAZOLE
SULBACTAM
SULCONAZOLE NITRATE
SULFABENZAMIDE
SULFACETAMIDE
SULFACHLORPYRIDAZINE
SULFADIAZINE
SULFAMETHOXINE
SULFAMERAZINE
SULFAMETER
SULFAMETHAZINE
SULFAMETHIZOLE
SULFAMETHOXAZOLE
SULFAMETHOXYPYRIDAZINE
SULFAMONOMETHOXINE
SULFANILATE ZINC
SULFANITRAN
SULFAPYRINDINE
SULFAQUINOXALINE SODIUM
SULFASALAZINE
SULFATHIAZOLE
SULFINPYRAZONE
SULFISOXAZOLE
SULINDAC
SULMAZOLE
SULCOTIDIL
SULPIRIDE
SUPROFEN
SURAMIN
TACROLIMUS
TAMOXIFEN CITRATE
TANDUTINIB
TANNIC ACID
TAZOBACTAM
TEGASEROD MALEATE
TEMLISARTAN
TEMEFOS
TEMOCYLAMIDE
TENIPOSIDE
TENOXICAM
TERBUTALINE HEMISULFATE
TERCONAZOLE
TERFENADINE
TESTOSTERONE
TESTOSTERONE PROPIONATE
TETRACAINE HYDROCHLORIDE
TETRACYCLINE HYDROCHLORIDE
TETRAHYDROZOLINE HYDROCHLORIDE
TETROQUINONE
THALIDOMIDE
THEOBROMINE
THEOPHYLLINE
THIABENDAZOLE
THIAMPHENICOL
THIMEROSAL
THIOGUANINE
THIEMIDAZINE HYDROCHLORIDE
THIOPENTONE
THIOPENTONE
THIOTHIXENE
THIRAM
THONZONIUM BROMIDE
THONZYLAMINE HYDROCHLORIDE
TIAPRIDE HYDROCHLORIDE
TIBOLONE
TIGECYCLINE
TILARGININE HYDROCHLORIDE
TILETAMINE HYDROCHLORIDE
TILMICOSIN
TIMOLOL MALEATE
TINIDAZOLE
TOBRAMYCIN
TODRALAZINE HYDROCHLORIDE
TOLAZAMIDE
TOLAZOLINE HYDROCHLORIDE
TOLBUTAMIDE
TOLMETIN SODIUM
TOLNAFTATE
TOLPERISONE HYDROCHLORIDE
TOSYLCLORAMIDE SODIUM
TRANEXAMIC ACID
TRANLYCYPROMINE SULFATE
TRAZODONE HYDROCHLORIDE
TRETINOIN
TRIACETIN
TRIAMCINOLONE
TRIAMCINOLONE ACETONIDE
TRIAMCINOLONE DIACETATE
TRIAMTERENE
TRICHLORMETHIAZIDE
TRIFLUOPERAZINE HYDROCHLORIDE
TRIFLUROMAZINE HYDROCHLORIDE
TRIFLURIDINE
TRIHEXYPHENYLHYDROCHLORIDE
TRILOSTANE
TRIMEPRAZINE TARTRATE
TRIMETHOBENZAMIDE HYDROCHLORIDE
TRIMETHOPRIM
TRIMETOCINE
TRIMIPRAMINE MALEATE
TRIOXASALEN
TRIPELENAMINE CITRATE
(Continued)
were obtained from mutants bathed in embryo media, as described above; a second locomotion plot was then obtained following a solution change to a test compound and an equilibration period of 15–30 min. Criteria for a positive hit designation were as follows: (1) a decrease in mean velocity of ≥44% (e.g., a value based on the trial-to-trial variability measured in control tracking studies; Fig. 1c); and (2) a reduction to Stage 0 or Stage I seizure behavior in the locomotion plot for at least 50% of the test fish. Each test compound classified as a “positive hit” in the locomotion assay was confirmed, under direct visualization on a stereomicroscope, as the fish being alive and/or moving in response to external stimulation or a visible heartbeat following a 60 min drug exposure. Toxicity (or mortality) was defined as no visible heartbeat or movement in response to external stimulation in at least 50% of the test fish. Hyperexcitability was defined as a compound causing a ≥44% increase in swim velocity and/or Stage III seizure activity in at least 50% of the test fish. Hits identified in the primary locomotion screen were selected from the PHARMAKON 1600 library and re-screened using the method described above. Select compound stocks that were successful in two primary locomotion assays, and were not classified as toxic in two independent clutches of zebrafish, were then purchased separately from Sigma-Aldrich for further testing. Drug concentrations between 0.5 and 1 mM were used for electrophysiology assays to account for more limited diffusion in agar-embedded larvae.

### Data analysis
Data are presented as the mean and SEM, unless stated otherwise. Pairwise statistical significance was determined with a Student’s two-tailed unpaired t test, ANOVA, or Mann–Whitney rank sum test, as appropriate, unless stated otherwise. Results were considered significant at $p < 0.05$, unless otherwise indicated.

### Results
A first-stage behavioral screen for antiepileptic activity
Locomotion tracking is a reliable and rapid strategy with which to monitor behavioral seizures in freely swimming larval zebrafish (Baraban et al., 2005, 2013; Winter et al., 2013). In these locomotion plots, high-velocity movements of ≥20 mm/s correspond to paroxysmal whole-body convulsions, referred to as Stage III, and are consistently observed in unprovoked scn1Lab mutant larvae but not in age-matched wild-type siblings. Using automated locomotion tracking, we performed a phenotype-based screen to identify compounds that significantly reduce mutant swim behavior to levels associated with Stage 0 or Stage I (e.g., activity equivalent to that seen in normal untreated WT zebrafish). In a 96-well format, we tracked mutant swim activity at baseline, and then again after addition of a test compound (100 μM); each compound was tested on six individual mutant larvae (Fig. 1a), and larvae were sorted based on pigmentation differences (Fig. 1b). Mutant swim activity between two consecutive recording epochs in embryo media is tracked on every plate as an internal control. A box plot showing the change in swim velocity in untreated mutants is shown in Figure 1c ($n = 112$) and defined as the control. Based on an SD of 21.8 for these data, we set the detection threshold as any compound that inhibits movement (measured as a change in mean velocity) by >2 SDs (or ≥44%). This approach was previously validated using standard antiepileptic drugs in this model (Baraban et al., 2013). Next, we screened a repurposed library in which all compounds have reached the clinical evaluation stage (PHARMAKON 1600 Collection; http://www.msdiscoveý .com/pharma.html). Among the 1012 compounds screened (Fig. 1d) only 20 (or 1.97%) were found to significantly inhibit spontaneous seizure behavior in scn1Lab mutants. All 20 compounds were subsequently retested in a separate clutch of scn1Lab mutants at a concentration of 100 μM (Fig. 2a, trial 2; $N = 6$ fish/compound). A total of 154 compounds were classified as “toxic” (Table 2); 55 compounds were classified as “hyperexcitable” (Table 3). Representative locomotion tracking raw data plots for gemfibrozil, a toxic nonsteroid

### Table 1. List of compounds from the PHARMAKON 1600 library used in this screen. (continued)

| Compound                        |
|---------------------------------|
| TRIPROLIDINE HYDROCHLORIDE      |
| TRISODIUM ETHYLENEDIAMINE TETRACETATE |
| TROLEANOMYCIN                   |
| TROPICAMIDE                     |
| TROPISETRON HYDROCHLORIDE       |
| TRYPTOPHAN                      |
| TUA MineHEPTANE SULFATE         |
| TUBOCURARINE CHLORIDE           |
| TYROTHRICIN                     |
| URACIL                          |
| URAPIDIL HYDROCHLORIDE          |
| UREA                            |
| URETHANE                        |
| URSODIOL                        |
| VALDECOXIB                      |
| VALGANCICLOVIR HYDROCHLORIDE    |
| VALPROATE SODIUM                |
| VALSARTAN                       |
| VANCOMYCIN HYDROCHLORIDE        |
| VENLAFAXINE                     |
| VIDARABINE                      |
| VIBLASTINE SULFATE              |
| VINORELBINE                     |
| VINPOCETINE                     |
| VIOMYCIN SULFATE                |
| VORICONAZOLE                    |
| VORINOSTAT                      |
| WARFARIN                        |
| XYL AZINE                      |
| XYLOMETAZOLINE HYDROCHLORIDE    |
| YOHIMBINE HYDROCHLORIDE         |
| ZALCITABINE                     |
| ZAPRINAST                       |
| ZIDOVUDINE [AZT]                |
| ZIPRASIDONE MESYLATE            |
| ZOMEPIRAC SODIUM                |
| ZOPICLOLINE                     |

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nuclear receptor ligand, and mepivacaine, a hyperexcitable proconvulsant anesthetic, are shown in Figure 2.

A second-stage electrophysiology assay for antiepileptic activity

Extracellular recording electrodes are a reliable, reproducible, and sensitive approach to monitor electroencephalographic activity in agar-immobilized larval zebrafish (Baraban et al., 2005; Baraban, 2013). Field electrodes offer high a signal-to-noise ratio and can be placed, using direct visualization in transparent larvae, into specific CNS structures (i.e., telencephalon or optic tectum). Using a local field electrode, we can efficiently monitor the occurrence of electrographic seizure events in the same zebrafish that were previously tested in the locomotion assay. Based on a positive nontoxic result in two independent locomotion assays, four drugs moved on to electrophysiology testing at concentrations between 500 μM and 1 mM (Fig. 3). Consistent with a “false-positive” classification, spontaneous epileptiform discharge activity was observed for three of these drugs: norfloxacin, theobromine, and cytarabine. Dimethadione, previously shown to inhibit spontaneous epileptiform discharges in thalamocortical slices at concentrations between 1 and 10 mM (Zhang et al., 1996), suppressed burst discharge activity in scn1Lab mutant larvae (Fig. 3a, b). To identify whether any of these four compounds exert nonspecific effects on behavior, they were also tested on freely swimming WT zebrafish larvae (5 dpf) at a concentration of 500 μM. Comparing the total distance moved during a 10 min recording epoch before, and after, the application of a test compound failed to reveal any significant changes in locomotor activity (Fig. 3c).

Figure 2. Positive hits identified in the locomotion assay. a, Heat map showing the results of individual zebrafish trials (1-6) for compounds tested at a concentration of 100 μM in the locomotion-tracking assay. Raw data values for individual fish are shown within the color-coded boxes for one sample trial. Mean velocity data are shown at right for “trial 1” and “trial 2”; six fish per trial. Note: only drugs highlighted in bold type were classified as positive nontoxic hits in two independent trials and moved on to further testing. b, Representative raw locomotion data plots for six individual scn1Lab mutant larvae at baseline (top) and following the addition of a compound resulting in fatality (bottom, gemfibrozil) or hyperactivity (bottom, mepivacaine). Movement is color coded, with low-velocity movements shown in yellow, and high velocity movements shown in red.
| Table 2: List of compounds exhibiting toxicity. |
|-----------------------------------------------|
| ABACAVIR SULFATE                              |
| ACIPIMOX                                      |
| ADENOSINE PHOSPHATE                           |
| ALAPROCLATE                                   |
| AMLEXANOX                                    |
| AMOROLFINE HYDROCHLORIDE                      |
| ANTAZOLINE PHOSPHATE                          |
| ARTEMETHER                                    |
| ASCORBIC ACID                                 |
| ATORVASTATIN CALCIUM                          |
| AUROTHIOGLUCOSE                               |
| AZELAIC ACID                                  |
| BENORILATE                                    |
| BENZONATE                                     |
| BETAIN HYDROCHLORIDE                          |
| BETAMIPRON                                    |
| BROMHEXINE HYDROCHLORIDE                      |
| BUDESONIDE                                    |
| BUPIVACAINE HYDROCHLORIDE                     |
| BUSULFAN                                      |
| BUTOCONAZOLE                                  |
| CAPSAICIN                                     |
| CARPROFEN                                     |
| CEFORANIDE                                    |
| CEFOTAXIME SODIUM                             |
| CEFOXITIN SODIUM                              |
| CEPHALEXIN                                    |
| CHLORAMBUCIL                                  |
| CHLORAMPHENICOL HEMISUCCINATE                 |
| CHLOORGUANIDE HYDROCHLORIDE                   |
| CHLORPHENIRAMINE (S) MALEATE                  |
| CINCHOPHEN                                    |
| CINNARAZINE                                   |
| CINTRIAMIDE                                   |
| CIPROFLOXACIN                                 |
| CLIDINIUM BROMIDE                             |
| CLOZAPINE                                     |
| COLISTIMETHATE SODIUM                         |
| CRYOFLURANE                                   |
| CYCLOPHOSPHAMIDE HYDRATE                      |
| CYCLOTHIAZIDE                                 |
| CYPERMETHRIN                                  |
| DAUNORUBICIN                                  |
| DECIMEMIDE                                    |
| DEXTROMETHORPHAN HYDROBROMIDE                 |
| DICHLOROPHEN                                  |
| DIETHYLCARBAMAZINE CITRATE                    |
| DIOXYBENZONE                                  |
| DIRITHROMYCIN                                 |
| DISOPYRAMIDE PHOSPHATE                        |
| DISULFIRAM                                    |
| ECONAZOLE NITRATE                             |
| EDETA TE DISODIUM                             |
| EMETINE                                       |
| ENALAPRILAT                                   |
| ERYTHROMYCIN                                  |
| ETHINYL ESTRADIOL                             |
| ETHIONAMIDE                                   |
| ETHOPROPAZINE HYDROCHLORIDE                   |
| ETHYL PARABEN                                 |
| EUGENOL                                       |
| FIPEXIDE HYDROCHLORIDE                        |

(Continued)
Assessment of huperzine A and fenfluramine for antiepileptic activity

Next, we tested two additional compounds that were not in our drug library, but have recently been described as potential antiepileptic treatments for DS. Huperzine A, a small-molecule alkaloid isolated from Chinese club moss with NMDA-type receptor blocking and anticholinesterase activity, has purported antiepileptic actions against NMDA- or soman-induced seizures (Tonduli et al., 2001; Coleman et al., 2008). In the locomotion assay, huperzine A failed to significantly alter scn1Lab seizure behavior at any concentration tested (Fig. 4a,b). In contrast, huperzine A was effective at 1 mM in the acute pentylenetetrazole (PTZ) assay (Fig. 4b). Fenfluramine is an amphetamine-like compound that has been reported to successfully reduce seizure occurrence in children with DS as a low-dose add-on therapy (Ceulemans et al., 2012). In the locomotion assay, fenfluramine significantly reduced mutant mean swim velocity at concentrations between 100 and 500 μM (Fig. 4c,d). A 1 mM fenfluramine was toxic in the scn1Lab and PTZ assays (Fig. 4d). The fenfluramine-treated scn1Lab mutant exhibited a suppression of spontaneous electrographic seizure discharge to levels similar to controls at 500 μM, but only a partial reduction in electrographic activity at 250 μM (Fig. 4e).

Discussion

Zebrafish and humans share extensive genomic similarity. With regard to disease, 84% of genes known to be associated with disease states in humans have a zebrafish homolog (Howe et al., 2013). This genetic similarity and the characteristic of zebrafish larvae to exhibit quantifiable seizure behaviors or electrographic seizure discharge that is fundamentally similar to that recorded in humans (Jirsa et al., 2014) make this an ideal system for drug discovery. Behavioral assays customized for auto-

Table 2: List of compounds exhibiting toxicity.

| QUININE SULFATE | RETINYL PALMITATE | RIFAMPIN | RITONAVIR | ROFECOXIB | RUFOXACIN HYDROCHLORIDE | SACCHARIN | SALICIN | SENNOSIDE A | STAVUDINE | STREPTOMYCIN SULFATE | SULFADIAZINE | SULINDAC | SULOCTIDIL | TANNIC ACID | TELMISARTAN | TENOXICAM | THEOPHYLLINE | TILETAMINE HYDROCHLORIDE | TILMICOSIN | TIMOLOL MALEATE | TOLBUTAMIDE | TOLNAFTATE | TRAZODONE HYDROCHLORIDE | TRETOININ | TRIFLUPROMAZINE HYDROCHLORIDE | TROPISERON HYDROCHLORIDE | VALDECOXIB | VORINOSTAT | ZALCITABINE |

Table 3: List of compounds exhibiting hyperexcitable or pro-convulsant activity.

| ADENOSINE PHOSPHATE | ALBUTEROL (+/-) | ALEXIDINE HYDROCHLORIDE | AMANTADINE HYDROCHLORIDE | AMINOHIPPURIC ACID | AMINOLEVULINIC ACID HYDROCHLORIDE | AUROTHIOLIGLOSE | AZACITIDINE | BENZOYL PEROXIDE | BETAZOLE HYDROCHLORIDE | BROMHEXINE HYDROCHLORIDE | BUSULFAN | CEFSULODIN SODIUM | CEFUROXIME AXETIL | CHLOROQUININE HYDROCHLORIDE | CYSTHEAMINE HYDROCHLORIDE | ECAMSULE TRIETHANOLAMINE | ECONAZOLE NITRATE | EDOXUDINE | ENROFLOXACIN | ESTRADIOL CYPIONATE | ETHINYL ESTRADIOL | ETHOPROPAZINE HYDROCHLORIDE | ETOPOSIDE | FASUDIL HYDROCHLORIDE | FEBUXOSTAT | FLUMETHASONE | FLUOROMETHOLONE | FURAZOLIDONE | GANCICLOVIR | GLUCONOLACTONE | GRANISETRON HYDROCHLORIDE | HALAZONE | HEXACHLOROPHENE | IODIPAMIDE | LABETALOL HYDROCHLORIDE | MEPIVACAINE HYDROCHLORIDE | MITOXANTRONE HYDROCHLORIDE | MORANTEL CITRATE | NOCODAZOLE | OFLOXACIN | PENTOLINIUM TARTRATE | PERINDOPRIL ERBUMINE | PIOGLITAZONE HYDROCHLORIDE | PRAMIPEXOLE DIHYDROCHLORIDE | PROGLUMIDE | RIFAMPIN | SERATRODAST | SERTRALINE HYDROCHLORIDE | SIBUTRAMINE HYDROCHLORIDE | SUCINYLFLUORFIAZOLE | TACROLIMUS | TETROQUINONE | TIMOLOL MALEATE | UPACIL |
mated evaluation of locomotion (Winter et al., 2008; Creton, 2009; Baxendale et al., 2012; Baraban et al., 2013; Raftery et al., 2014) make moderate-to-high-throughput phenotype-based drug screening in zebrafish possible. Using this approach and a zebrafish \textit{scn1} mutant (Baraban et al., 2013), we successfully identified antiepileptic compounds. Here we report results from screening 1000 compounds from a repurposed drug library and present data that will be periodically updated on-line using this open-access publishing mechanism.

As a model system, the \textit{scn1}Lab mutant zebrafish has many advantages. First, in contrast to transient and variable knockdown of gene expression using antisense morpholino oligonucleotides (Teng et al., 2010; Finckbeiner et al., 2011; Mahmood et al., 2013), \textit{scn1}Lab mutants carry a stable and heritable amino acid substitution at position 1208 in the third domain of \textit{SCN1A} that shares 76% homology with humans (Schoonheim et al., 2010; Baraban et al., 2013). Mutations in this channel are one of the primary genetic causes underlying DS (Claes et al., 2003; Escayg and Goldin, 2010; De Jonghe, 2011; Saitoh et al., 2012). As zebrafish possess two \textit{scn1} genes (Novak et al., 2006), homozygous mutants for \textit{scn1}Lab are comparable to the haploinsufficient clinical condition, and there is no variability from larvae to larvae, or clutch to clutch, with respect to gene inactivation, as is commonly observed with morpholino injections (Kok et al., 2015). Although crosses of heterozygotes produce only one-quarter homozygous \textit{scn1}Lab mutants per mating, there are virtually no limitations on maintaining a large colony of healthy, adult breeders for these types of large-scale screens. Second, it is possible to observe and monitor seizure-like behavior consisting of rapid movements and whole-body convulsions in freely swimming \textit{scn1}Lab mutants as early as 4 dpf that persist for the life of the larvae (~12 dpf). These behaviors are comparable to those observed with exposure to a common convulsant agent (PTZ) and classified as Stage III (Baraban et al., 2005).

Figure 3. Electrophysiology assay to identify drugs that rescue the \textit{scn1}Lab mutant epilepsy phenotype. \textit{a}, Representative field electrode recording epochs (5 min in duration) are shown for the “positive” compounds identified in the locomotion assay. All recordings were obtained with an electrode placed in the forebrain of agar-immobilized \textit{scn1}Lab larvae that was previously tested in the locomotion assay. A suppression of epileptiform electrographic discharge activity was noted in mutants exposed to dimethadione. \textit{b}, Bar plot showing the mean number of epileptiform events in a 10 min recording epoch for \textit{scn1}Lab larvae exposed to cytarabine (N = 6), dimethadione (N = 6), theobromine (N = 6), and norfloxacin (N = 6). The mean ± SEM is shown. The fish shown were tested in the locomotion assay first. \textit{c}, Bar plot showing the total distance traveled before (black bars) and after (white bars) exposure to a test compound; 10 min recording epoch and six fish per drug. The mean ± SEM is shown.
addition, clear evidence for epileptiform discharge generated in the CNS of immobilized *scn1Lab* mutant larvae has been obtained at ages between 4 and 8 dpf (Baraban et al., 2013). Both zebrafish measures of seizure activity are sensitive to inhibition by AEDs commonly prescribed to children with DS (e.g., valproate, benzodiazepines, and stiripentol), but are resistant to many antiepileptic compounds (e.g., phenytoin, carbamazepine, ethosuximide, decimemide, tiletamine, primidone, phenacemide, and vigabatrin). Pharmacoresistance is defined as the inability to control seizure activity with at least two different AEDs (Berg, 2009), and, with demonstrated resistance to eight

Figure 4. Evaluation of putative antiepileptic drugs in *scn1Lab* mutants. a, Locomotion tracking plots for *scn1Lab* zebrafish at baseline and following huperzine A administration. Total movement is shown for a 10 min recording epoch. b, Plot showing the change in mean velocity for three different huperzine A concentrations (blue bars). Each bar is the mean change for six fish. The threshold for a positive hit is shown as a dashed line. WT fish exposed to PTZ and huperzine A are shown in red (*N* = 7). c, d, Same for fenfluramine. Note that 1 mM fenfluramine was toxic, as indicated. e, Representative field recordings from *scn1Lab* mutant larvae at 5 dpf. Electrophographic activity is shown for a 5 min recording epoch (top traces); high-resolution traces are shown below, as indicated. Note that abnormal burst discharge activity persists in *scn1Lab* mutants exposed to 250 μM fenfluramine. The fish shown were tested in the locomotion assay first.
AEDs, our model clearly fits this definition. This level of model validation has not been possible with morpholinos probably owing to the high degree of variability, or off-target effects, associated with this technique (Kok et al., 2015).

Our screening results highlight the stringency of our approach with a positive hit rate of only 1.97% on the first-stage locomotion assay, and successful identification of 1 compound (of 1012 compounds) with known antiepileptic activity (i.e., dimethadione, a T-type channel antagonist). In additional testing, we confirmed an antiepileptic action for fenfluramine (serotonin uptake inhibitor). Similar to ethosuximide, a reduction in regenerative burst discharges associated with neuronal T-type calcium currents could be the underlying mechanism for dimethadione in DS mutants; however, it is worth noting that T-type channel blockers ethosuximide and flunarizine were not similarly effective (Baraban et al. 2013; this article), and that dimethadione can cause arrhythmia owing to blockade of cardiac human ether-a-go-go-related gene potassium channels (Azarbayjani and Danielsson, 2002; Danielsson et al., 2007). Modulation of serotonin [5-hydroxytryptamine (5-HT)] signaling by blocking uptake or increasing release from neurons by acting as substrates for 5-HT transporter (sertraline) proteins (Fuller et al., 1988; Gobbi and Mennini, 1999; Baumann et al., 2000; Rothman et al., 2010) may be the mechanism of action for fenfluramine in patients with DS, though a detailed analysis of precisely how fenfluramine modulates excitability via this signaling pathway has not been performed. Nonetheless, both drugs probably exert a direct effect on network excitability (at neuronal or synaptic levels, respectively) to suppress electrographic discharge and the associated high-velocity seizure behavior seen in *scn1Lab* mutants, and may be potential targets for clinical use. In contrast, three other drugs identified in the primary locomotion assay were not effective in suppressing electrical events and were designated as false positives. This is not altogether surprising given that the xanthine alkaloid (theobromine), chemotherapeutic (cytarabine), and antibiotic (norfloxacin) mechanisms for these compounds would not be consistent with seizure inhibition. Moreover, the variability inherent in behavioral experiments performed on different zebrafish larvae, in different microplates, and on different days may contribute to these false-positive designations in locomotion assays, and is evident in the range of mean velocity values seen during tracking episodes from control studies (Fig. 1c) or in the failure of many of the initial 20 lead compounds to be confirmed on subsequent retesting (see Fig. 2a). This is a limitation of locomotion-based screening assays and is another reason why a secondary electrophysiology assay on the same zebrafish is a critical advantage of our approach.

An additional advantage of *in vivo* screening with zebrafish larvae is the simultaneous identification of compounds resulting in toxicity. Zebrafish-based anticonvulsant drug-screening assays based primarily on *in situ* hybridization detection of early gene expression at 2 dpf (Baxendale et al., 2012) do not routinely monitor spontan-eneous swim behavior, heart rate, or response to external stimuli. Lacking these real-time measures of toxicity, compounds observed to induce fatality in a freely swimming *scn1Lab*-based behavioral assay (e.g., gemfibrozil, sulocartil, pimozide, or doxycybenzone) were mistakenly classified as seizure-suppressing compounds in the PTZ-based c-Fos *in situ* hybridization assay. Indeed, 41% of the “anticonvulsant” compounds positively identified at 2 dpf in Baxendale et al. (2012) were toxic, proconvulsant, or simply not effective in *scn1Lab* mutant assays at 5–6 dpf. Similarly, it is critical to monitor blood flow and heart activity even in the agar-immobilized electrophysiology assay as compounds effective in suppressing electrical activity can also be toxic. These discrepancies highlight the potential problems associated with zebrafish drug-screening strategies that do not encompass multiple readouts and suggest the need for a note of caution when comparing screening results from different laboratory groups. While any lead compound identified in a zebrafish-based screening assay will, ultimately, need to be independently replicated and/or validated in additional mammalian model systems, the ability to rapidly identify such compounds, while simultaneously identifying potential negative side effects, makes genetically modified zebrafish a unique resource for drug discovery in an age of personalized medicine.

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