Development of a *scn1a* Null Zebrafish Model for Screening Potential Anti-Epileptic Natural Products

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ABSTRACT

Epilepsy affects around 50 million people in the world. Thus, it is imperative that new more effective and safe treatments be found. This study was designed to use zebrafish to screen potential anti-epileptic natural products. We utilized two \textit{scn1Lab null} zebrafish models (morpholino-injected fish where \textit{scn1Lab} was transiently knocked down and a transgenic \textit{scn1Lab} $^{-/-}$ fish line). Alternatively, we used AB wildtype fish to test compounds in a chemically-induced seizure model. Natural compounds including cannabidiol, CBD, and \textit{Tapinanthus globiferous}, TG, extracts were screened for their anti-seizure activity. When \textit{scn1Lab} $^{-/-}$ fish were treated with CBD (0.075 and 0.3 mg/L) from 5 to 7 days post fertilization (dpf), a non-significant reduction of seizure-like activity, compared to control, was found. Additional exposures are needed to verify this reduction. In wildtype fish where seizures were induced by treatment of pentylenetetrazole, TG extracts were tested for anti-epileptic potential from 5 to 6 dpf. Our results showed that one extract of TG, AF.1.11.TG.4 (0.2, 1, and 5 mg/L) showed some promise in reducing seizure-like activity. We conclude that certain extracts of TG show promise as anticonvulsants, and CBD requires further research to verify its reduction. The transgenic \textit{scn1Lab} $^{-/-}$ fish once acquired and raised proved to a better model for screening natural products for Dravet-specific seizures than morpholino-based approaches.
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<tr>
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<td>THC</td>
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<td>Voltage-gated sodium channel</td>
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1. INTRODUCTION

1.1 Epilepsy

Epilepsy, which affects 50 million people worldwide, is a central nervous system disorder in which the activity of the brain is abnormal (World Health Organization, 2018). Abnormal brain activity results in seizures or periods of unusual behavior, sensations, and sometimes loss of awareness. In half of the people with epilepsy, there are no discernable causes for the condition. In others, epilepsy may be caused by genetic factors, head trauma, infectious diseases, and prenatal injuries. Seizure symptoms include temporary confusion, staring spells, uncontrollable jerking movements of the arms and legs, loss of consciousness, and psychic symptoms such as fear and anxiety (Sourbron, 2015). Epilepsy is most commonly treated with anti-epileptic drugs (AEDs). Common side-effects of these pharmaceuticals include fatigue, dizziness, weight gain, and skin rashes. However, many of these medications also have more serious side-effects such as speech problems, memory or cognitive problems, depression, loss of coordination, and inflammation of certain organs. Over the last decade, advances have been made towards the development of new AEDs. This is based on the fact that current, available AEDs do not provide optimal therapy for patients with epilepsy (French et al., 2004).

1.2 Dravet Syndrome

Dravet Syndrome (DS), also known as severe myoclonic epilepsy of infancy (SMEI), is a severe form of epilepsy. It appears during the first year of life with frequent febrile seizures. Febrile seizures are fever-related seizures and are rare beyond the age of
However, as the condition progresses, other forms of seizures usually occur such as myoclonus and status epilepticus. In 15 to 25 percent of DS cases, a family history of either epilepsy or febrile seizures exists. Around 80% of cases are due to a mutation in the SCN1A gene, which belongs to a family of genes that provide instructions for making sodium channels. This gene is required for the proper function of brain cells. Around 10% of the cases are idiopathic. People with Dravet Syndrome may suffer from deterioration of intellectual development around age 2, lack of coordination, poor development of language, hyperactivity, and difficulty relating to others (Escayg et al., 2010). The seizures may be triggered by slight increases in temperature, various illnesses, and light. People with DS require constant care; the condition greatly impacts the lives of the patients as well as the family’s quality of life. It is estimated that 10-20 percent of people with DS pass away before adulthood with most deaths occurring before the age of 10 years (National Institutes of Health, 2018).

Treatment of DS includes combinations of medications due to its resistant nature. However, some of the common anticonvulsant medications worsen the seizures in DS patients. Therefore, it is important to explore newer more viable drug options in order to reduce adverse side effects and occurrence of seizures.

### 1.3 Scn1Lab gene

It is estimated 80% of DS cases are due to de novo mutations in the SCN1A gene. One of the primary monogenic causes of DS includes mutations in Na,1.1 (SCN1A), a voltage gated sodium channel. Specifically, it is the Type I channel (over 9 exist), and the mutations are found in the α subunit which is responsible for passing the Na⁺. Studies
reported that the altered sodium channels are mainly expressed in the GABAergic
(inhibitory) interneurons, leading to impaired inhibitory neurotransmission that would
explain the seizures (Mayo Clinic, 2018). Voltage gated sodium channels (VGSC) are
essential for neuronal excitability by initiating and propagating the rising phase of the
action potential (Frank et al., 2003). Therefore, it seems plausible that mutations in
VGSCs have a role in epilepsy because those channels play a part in controlling electrical
excitability. Membrane depolarization activates the VGSC, and this causes the voltage-
dependent conformational change that increases the permeability to sodium ions. This
step further depolarizes the cell. The channel then closes and the permeability to the
sodium ions decreases and this causes inactivation. The membrane potential is then
allowed to return to resting level. The exact mechanisms for how mutations in the SCN1A
gene cause epilepsy are still being researched. However, by using a morpholino and
chemical mutagenesis, we can develop model organisms with the unique features of DS;
this allows for the study of the molecular and behavior basis of this specific disease.

1.4 Zebrafish as a model

Zebrafish (Danio rerio) are tropical fresh-water fish in the minnow family. Originally, these fish were found in rivers, ponds, and puddles in India. Over the past two
decades, zebrafish have become an increasingly popular option for modeling human
diseases. Zebrafish provide advantages over other animals including high fecundity,
quick maturation, and the availability of many transgenic mutant strains. Furthermore, the
embryos develop outside of the parent organism facilitating microscopic visualization
throughout early development. It is also estimated that around 84% of genes known to
human disease are also expressed in zebrafish (Kundap et al., 2017). These attributes make zebrafish a useful model for studying human diseases.

Specific to studies of epilepsy drug discovery, zebrafish have been shown to be an effective model because they exhibit seizure phenotypes (French et al., 2004). Zebrafish have homologs for 85% of human epilepsy genes (Hortopan et al., 2010). Larval zebrafish respond to convulsant drugs (e.g. pentylenetetrazole and allylglycine) (Afrikanova et al., 2013; Leclercq et al., 2015), recapitulate seizures of similar duration, frequency and electrical discharges as mammals (Hortopan et al., 2010) and have corresponding induced expression of c-fos (Rahn et al., 2014; Buenafe et al., 2013). Furthermore, zebrafish genetic mutations in VCSCs (Baraban et al., 2013; Zhang et al., 2015) and chromodomain helicase DNA binding protein 2 (chd2) (Suls et al., 2013) spontaneously develop seizures like their mammalian counterparts while also providing a much easier model to screen for idiopathic epilepsy therapies. Zebrafish movement can be tracked via the ViewPoint Zebrabox, which enables researchers to analyze behavioral aspects of the seizures in zebrafish. In recent studies, zebrafish have been used as a model for DS using a scn1a antisense morpholino and scn1Lab−/− approaches. Currently, fenfluramine and clemizole have emerged from zebrafish screens as promising forms of treatment for this particular form of epilepsy (Baraban et al., 2013).

Pentylenetetrazole (PTZ) is used in zebrafish to induce generalized epileptic seizure-like conditions and to study the mechanisms of seizures. PTZ, a GABA receptor agonist, can induce convulsive effects by inhibiting the activity of GABA and GABA\textsubscript{A} receptors (Kundap et al., 2017). Exposure to PTZ induces a concentration-dependent response of stereotype behavioral changes such as increase in movement and certain
swimming patterns. The use of PTZ alongside natural products with hypothesized antiepileptic activity, allows for comparison of drug response and efficacy.

Many zebrafish mutant strains have been propagated following random mutagenesis. The transgenic *scn1Lab* fish line was generated following a chemical mutagenesis assay using N-ethyl-N-nitrosourea (ENU). ENU is an alkylating agent (to usually thymine) that produces small, random, single nucleotide mutations. The protocol entails exposing an adult male zebrafish to water containing ENU. After the mutagenesis, a three-generation mutant screen is performed; researchers look for phenotypes that are characteristic and can be linked to specific mutations (Figure 1). For example, in *scn1Lab* mutants, the fish show darker pigmentation, lack of a swim bladder, slight curvature of the body, and seizures (Mayo Clinic, 2018). It is important to understand the genetic basis for a specific phenotype. Therefore, researchers typically carry out genetic mapping and positional cloning experiments, which allow researchers to pinpoint the location of the associated gene and identify the responsible mutation.
1.5 Morpholino

The use of morpholino oligos allows for the transient knockdown of the SCN1A gene by blocking translation at the ribosome. Morpholino oligos are designed to specifically bind to selected target sites in order to block access of cell components to that specific target site. This property is exploited to block translation. However, the same property can be used to block splicing, block miRNAs or their targets, and block ribozyme activity (GeneTools, 2018). Morpholinos do not typically cause degradation of their RNA targets. Instead, they work through hindering the biological activity of the target RNA until that RNA is degraded naturally. Morpholinos must also be administered via microinjection; the morpholino is then incorporated via cell division during development. A morpholino differs greatly from a regular nucleic acid. For instance, a

Figure 1: Overview of ENU mutagenesis screen (Deo, 2008)
morpholino oligo has methylenemorpholine rings which replace the ribose or deoxyribose sugar moieties and non-ionic phosphorodiamidate linkages replace the anionic phosphates of DNA and RNA (Figure 2). A 25-base morpholino oligo strongly and specifically binds to its complementary 25-base pair target site in a strand of RNA because each morpholine ring positions one of the standard DNA bases (GeneTools, 2018).

There is a great benefit to utilizing morpholinos. Morpholinos combine the properties of stability, nuclease-resistance, efficacy, long-term activity, water-solubility, low toxicity and specificity (GeneTools, 2018). For instance, morpholino oligo is not recognized by enzymes or signaling proteins, which makes it completely stable to nucleases, and it does not trigger an immune response (GeneTools, 2018). Through this, oligo degradation as well as inflammation and interferon induction is avoided. These problems are quite common in other gene knockdown reagents. There are negative qualities to morpholinos as well. Although morpholino oligos are more soluble than other non-ionic structural types, some morpholinos with high G content (greater than 30%) do have limited solubility. Solubility also varies with the oligo sequence and is difficult to predict. An important factor to consider when using morpholinos in zebrafish is that it produces a transient knockdown; this is due to the fact that the morpholino is diluted as the fish develops.
Various preparations of natural products in the form of injectable extracts, infusions, fluid extracts or tea bags are widely used in different cultures around the world to treat various diseases including epilepsy. *Tapinanthus globiferus* (TG) is a species of mistletoe in the Loranthaceae family. This plant is hemiparasitic and is found in parts of western Africa. Mistletoe grows on various types of trees such as shea butter tree, the cocoa tree, the sweet orange tree, hog-plum tree, and rubber trees. These various trees are valuable to the farming community, so TG is seen as a nuisance. That said, TG also has been used to treat ailments. Traditionally, the leaves of TG are crushed up, soaked in either cold water or beer and then the liquid is orally consumed (Adesina et al, 2013). There is not a substantial number of controlled studies which substantiate the effectiveness of TG in the treatment of epilepsy. However, TG has traditionally been used to treat some forms of epilepsy, and other mistletoes have shown success in treating epilepsy (Adesina et al., 2013). For this reason, we are interested in exploring the anti-seizure properties of TG.

Following the exploration of TG, we were interested in studying the properties of Cannabidiol (CBD). The potential for cannabis as a treatment for seizures is growing.

**Figure 2:** Phosphodiester DNA and a morpholino structures (Corey et al., 2001).
However, this progress is hindered by its schedule I classification by the United States federal government. Cannabis is not recognized as an accepted form of medical treatment; therefore, research on its safety and effectiveness as a medical drug is restricted (Drug Enforcement Administration, n.d.). More research is necessary for the progression of treatment of epilepsy; effective and safe treatments should be more accessible to all people. This entails conducting sufficient research on cannabis in order to determine its effectiveness, safety, and mechanism of action.

CBD is a constituent of cannabis. CBD, which is similar in structure to Δ9-tetrahydrocannabinol, (THC) (Figure 3), is the major nonpsychoactive component of *Cannabis sativa*. Over the past few years, increasing public and political pressure has supported the legalization of medical marijuana. One of the main reasons for this effort is related to the treatment of refractory epilepsy such as DS. CBD possesses pharmacological effects which are mediated through G protein coupled receptors, cannabinoid type I (CB₁) and cannabinoid type II (CB₂), which are highly expressed in the hippocampus and other parts of the central nervous system (Welty et al., 2014). However, CBD has very low affinity at CB₁ and CB₂ unlike THC. When CB₁ receptors are activated, they inhibit synaptic transmission through action on voltage-gated calcium and potassium channels, which are known to modulate epileptiform and seizure activity (Falenski et al., 2009). CBD induces a bidirectional change in intracellular calcium levels that depends on cellular excitability. Under normal physiological Ca^{2+} conditions, CBD slightly increases intracellular Ca^{2+}, whereas CBD reduces intracellular Ca^{2+} under high-excitability conditions (Rosenberg et al., 2015). CBD shows great promise in the treatment of epilepsy. Recently, the first cannabis-based pharmaceutical, Epidiolex, was
approved by the FDA. It is the first in a new class of treatments with a new mechanism of action against epilepsy. Epidiolex is an oral solution of marijuana plant-derived CBD.

Figure 3: Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) structures

This study was designed to use zebrafish to screen potential anti-epileptic natural products such as TG and CBD. We used AB wildtype fish to test compounds in a chemically-induced seizure model. Also, we utilized two \( \text{scn1Lab} \) null zebrafish models; these models were created through morpholino injections or chemical mutagenesis.

1.7 Study Goals

- Test mistletoe extracts in chemically-induced seizure model to assess reduction in seizure-like activity.
- Perform microinjection of an antisense morpholino directed against the \( \text{scn1Lab} \) gene to produce DS phenotypes.
- Characterize the phenotypic features of \( \text{scn1Lab}^{-/-} \) and \( \text{scn1Lab}^{+/+} \) transgenic fish.
- Determine if CBD and clemizole (expected positive control) will reduce seizure-like activity in \( \text{scn1Lab}^{-/-} \) fish.
2. METHODS AND MATERIALS

2.1 Zebrafish culture and egg collection

AB wild-type zebrafish were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR) and raised and tested under the approved IACUC protocol. The DeWitte Lab at The University of Leuven kindly provided the transgenic (scn1Lab +/-) fish, and they were raised under the approved IACUC protocol as well. The zebrafish were kept in the Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with each unit containing ~0.3 L of water per fish. The habitats contained zebrafish appropriate water (pH 7.0-7.6, 340 parts per million (ppm) Instant Ocean, Cincinnati, OH) in a climate (25-28°C) and light (14 hours of light and 10 hours of dark) controlled room. They were fed Gemma Micro 300 (Skretting Nutreco Company, Westbrook, ME) two times every day. The fish used for breeding did not have any form of disease and were also of a healthy breeding age (4-19 months post fertilization). To prepare for egg collection, the fish were transferred to breeding tanks at a 1:1 ratio of males to females and were then left overnight to produce fertilized eggs.

The following morning, after the lights turned on, the fish were returned to their normal holding tanks. The eggs which had fallen through the protective metal grate to the bottom of the breeding tank were collected by pouring the water from the breeding tanks through a sieve. The breeding tanks were rinsed with water and the water was then poured through the sieve; this was repeated three times to ensure that every egg had been collected.
Unwanted debris and unfertilized/dead eggs were removed after the eggs were transferred to a petri dish; this was done by using a transfer pipette. The water in the petri dish was then replaced with zebrafish water (60 ppm Instant Ocean, pH 7.5) and placed in an incubator at 28°C for five days. Nonviable eggs were removed daily.

2.2 Chemically-induced seizures

At 5 days post fertilization (dpf), healthy larvae were transferred to 96-well plates (1 larva per well). The larvae were chosen based on lack of deformities. The water in each well was removed and replaced with 150 µL of a dosing solution. TG extracts were provided from Dr. Jordan Zjawiony. The zebrafish (n=12/treatment/plate; 2 plates) were exposed to: control (0.05% DMSO), 25 µM diazepam (DZP; 7 mg/L), and an extract from TG (0.2, 1, and 5 mg/L). The TG extract that was tested was AF.1.11.TG.4. Once the dosing was complete, the plates were covered with aluminum foil and placed back in the incubator.

Following a 24-hour exposure to the dosing solutions (6 dpf), morphological changes were noted to determine if TG extracts had any toxic side effects including a response to touch, pericardial edema, yolk sac edema, a curved body axis and/or a non-inflated swim bladder (Dietrich, 2017). If any of these morphologies were observed, then that larva was excluded from further data analysis. PTZ (Sigma Aldrich; 50 µL of 20 mM pentylenetetrazol) was added to each well, except for the first row which was the control, to yield a final concentration of 5 mM PTZ. Following addition of PTZ, behavioral analysis was conducted. Viewpoint ZebraBox tracked larval movement for 15 minutes
with the lights on at 100% with a threshold of 27 and activity parameters were set:
inactivity (< 5 mm/sec), small (5-9 mm/sec), and large (>9 mm/sec).

2.3 Morpholino injections

Numerous trials with amounts ranging from approximately 10-60 ng of *scn1a*-MO were conducted. A translation blocking MO (ATG MO: 5′-CTGAGCAGCCATATTGACATCCTGC-3′) was used to achieve partial knockdown of zebrafish *scn1Lab*. Standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as a negative control. Phenol red (0.5 µL) was added to the morpholino solution to ensure visibility of the injected solution. All MOs were designed and synthesized by GeneTools LLC (Philomath, Oregon, USA) and injected into one-to-two cell stage embryos. MOs were tagged with green fluorescent protein (GFP) to ensure that MOs were incorporated into the injected fish. In each trial, there were (n≈24) for each treatment group: non-injected, control MO, and *scn1Lab* MO. Following injection, embryos were placed in petri dishes with zebrafish water. Subsequently, the petri dishes were placed in the incubator.

2.3a Survival and fluorescent checks

Embryos were checked daily for mortality and fluorescence until 4 dpf. Embryos expressing green fluorescent protein (GFP) showed that the morpholino had been successfully incorporated into the genome of the zebrafish. Non-fluorescing and nonviable eggs were removed from the petri dishes. At 4 dpf, the remaining larvae were
then transferred to 96 well plates with approximately 300 µL of zebrafish water so that the water level filled each well completely to the top.

2.3b Higher temperature exposure

Following the transfer of larvae to a 96 well plate on 4 dpf, a subset of larvae were place in an incubator at 34.0°C for 10 minutes and then returned to the incubator at 28.0°C. This was done to visualize hyperthermia-induced abnormalities/seizures.

2.4 Viewpoint data collection and analysis

In the morpholino assay, larvae were screened on 4, 5, and 6 dpf for developmental deformities (lack of touch response, yolk sac edema, pericardial edema, lack of swim bladder inflation, and body axis) and then the plate was placed in the ViewPoint Zebrabox. The larvae were acclimated for 5 minutes before the recording of behavior. The ViewPoint Zebrabox tracked larval movement for 45 minutes with the lights on at 100%. The ZebraBox software creates an excel sheet that includes the duration each larva spends in the inactive (<5 mm/sec), small (5-9 mm/sec), and large (>9 mm/sec) movements over the 45-minute period with intervals of 15 minutes. Movement that meets the large activity requirements is indicative of seizure activity and was the data which was used to test the morpholino knockdown’s effectiveness. After the final recording on 6 dpf, the larvae were euthanized with buffered MS-222 and placed in RNAlater and stored at -80.0°C.
The data collected from the ViewPoint ZebraBox was analyzed using GraphPad Prism 5.0 (La Jolla, CA). The data sets were checked for normality using a Kolmogorov-Smirnov test. If the data passed the normality test, then statistically significant differences between control and treatment groups were determined using a t-test. All tests used $p < 0.05$ for determination of statistical significance.

In the chemically-induced seizure assay, the parameters for the ViewPoint Zebrabox remained the same as in the morpholino assay. However, behavior was tracked on 6 dpf for 15 min.

In the CBD/clemizole experiment, transgenic $scn1Lab$ mutants were utilized. Homozygous $scn1Lab^{-/-}$ larvae showed a darker appearance and sometimes a slight curvature of the body. No phenotypic difference between WT $scn1Lab^{+/+}$ and heterozygous $scn1Lab^{+/−}$ mutants was observed (Figure 8). At 3-12 dpf, the zebrafish were observed for 15 min for behavioral changes using ViewPoint ZebraBox. Pictures were taken for the characterization of physical differences between heterozygous and homozygous $scn1Lab$ mutants. Behavioral data at 4, 5, 6, and 7 dpf was utilized for analysis purposes.

### 2.5 Transgenic $scn1Lab$ mutants

Zebrafish $scn1Lab$ mutant eggs were provided from the University of Leuven. Upon arrival, the bleached eggs (20 egg/bottle; 6 bottles = 120 larvae) were dechorinated with forceps. The fish were then transferred to petri dishes with 50% Danieau’s solution and 50% system water. The petri dishes were then placed in the incubator at 28.0°C.
When the larvae reached 5 dpf, they were transferred to the tank system. Around three months of age, the zebrafish were spawned.

**2.5a Exposure assays**

Zebrafish larvae from a pool of homozygotes, heterozygotes, and wild type (\( scn1Lab^{-/-} \), \( scn1Lab^{+/-} \), \( scn1Lab^{+/+} \)) were separated based on morphological characteristics. On 3dpf, the zebrafish (\( n=6/\text{treatment} \)) were plated in a 96 well plate. After plating, the plate was covered in aluminum foil and placed inside the incubator at 28°C. On 5 dpf, the larvae were exposed to: control (0.05% DMSO), 1.8 mg/L clemizole (5 \( \mu \)M), 0.075 mg/L CBD low (0.25 \( \mu \)M) and 0.3 mg/L CBD high (1 \( \mu \)M). Throughout the experiment, the water was static and not changed. Behavior was tracked using the ViewPoint ZebraBox before and after treatment; large duration data from 6 dpf and 7 dpf was utilized for data analysis. The Viewpoint ZebraBox tracked larval movement for 15 minutes with the lights on at 100% as described in section 2.4.
3. RESULTS

3.1 Chemically-induced seizure

Results for the mistletoe assay are shown in Figure 4. Diazepam (7 mg/L; 25 µM) showed a significant decrease in large activity when compared to the control group treated with PTZ (5 mM). Only 0.2 mg/L of AF.1.11.TG.4 showed a significant decrease in large movement compared to 5 mM PTZ. 1 and 5 mg/L of the extract failed to significantly decrease large movement when compared to 5 mM PTZ.

![AF.1.11.TG.4 seizure activity](image)

**Figure 4. AF.1.11.TG.4 seizure activity.** Zebrafish larval behavior was analyzed using the Viewpoint Zebbrabox (15 min recording with 100% light) to record duration of large activity (>9mm/sec), following a 24-hr exposure to AF.1.11.TG.4, induced by 5 mM PTZ to determine if this compound has anticonvulsant properties (n=10-12 per treatment group, Student t-test p≤0.05). Asterisks indicate a significant difference compared to 5 mM PTZ group.
3.2 Dravet Syndrome induced by morpholino

Pictures depicting both control-MO and *scn1Lab*-MO injections are shown (Figure 5). In Figure 5A, two embryos were successfully injected with the control-MO.

In Figure 5B, two different embryos showed a successful *scn1Lab*-MO injection.

Figure 5A-B: Images of morpholino injections. Panel A shows the injection of a control-MO into two embryos, and panel B shows the injection of a *scn1Lab*-MO. Red arrows indicate morpholino injection site.
3.2a Survival and fluorescent checks

Fluorescent checks were done starting at 1 dpf through 4 dpf. These checks were to ensure that the morpholino was still incorporated into the embryo. Non-injected fish were not included in the table because they did not receive a morpholino injection and thus did not fluoresce. Table 1 displays percent of successfully injected embryos at 1 dpf.

**Table 1: Percent fluorescing embryos following morpholino injections for normal conditions (10 ng MO at 28°C), higher temperature (10 ng MO at 34°C), and increased MO amounts at 28°C. Trials under normal conditions (n=60-90/treatment group; 3 trials included in data set), higher temperatures and increased MO amounts (n=60/treatment group; 1 trial included in data set) were performed.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MO</th>
<th># of fish injected</th>
<th># of fish fluorescing at 1 dpf</th>
<th>% fluorescent at 1 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ng MO at 28°C</td>
<td>Control</td>
<td>197</td>
<td>61</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>Scn1a</td>
<td>205</td>
<td>99</td>
<td>48%</td>
</tr>
<tr>
<td>10ng MO at 34°C</td>
<td>Control</td>
<td>59</td>
<td>6</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>Scn1a</td>
<td>60</td>
<td>8</td>
<td>13%</td>
</tr>
<tr>
<td>30-60ng MO at 28°C</td>
<td>Control</td>
<td>59</td>
<td>20</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Scn1a</td>
<td>89</td>
<td>26</td>
<td>29%</td>
</tr>
</tbody>
</table>
Survival checks were done by calculating percent survival at 4 dpf. Calculations were determined by taking the number of living fish on 4 dpf and dividing by the total number of fish in that group at 0 dpf. Data under normal conditions (28°C), increased temperature conditions (34°C for 10 min), and increased amounts of morpholino is depicted in Figure 6.

Figure 6: Percent survival following morpholino injection for normal conditions (A), higher temperature (B), and increased morpholino amounts (C). Percent survival taken on 4 dpf. Trials under normal conditions (n=60-90/treatment group; 3 trials included in data set), higher temperature and increased MO amounts (n=60/treatment group; 1 trial included in data set) were performed.
3.2b ViewPoint data and analysis

The results for three trials of morpholino injections under normal conditions are shown in Figure 7A. *scn1Lab*-MO (10 ng) did not increase large movement compared to the non-injected treatment group. The results for higher temperature are shown in Figure 7B. Exposing the fish to a heat of 34°C failed to increase large movement in the *scn1Lab*-MO injected group. Figure 7C shows results for zebrafish that were injected with high amounts of both control and *scn1Lab* morpholino. Increased amounts of morpholino did not significantly increase large movement compared to the non-injected group.
Figure 7: Seizure activity following morpholino injection for normal conditions (a), higher temperature (b), and increased morpholino amounts (c). Zebrafish larval behavior was analyzed using the ViewPoint Zebrabox (45 min recording with 100% light) to record duration of large activity (>9mm/sec) on 4 dpf, 5 dpf, and 6 dpf.
### 3.3 *scn1Lab* mutant zebrafish

Transgenic fish were spawned a total of three times and the number of homozygous *scn1Lab* \(^{-/-}\) fish were noted in **Table 2**. Eggs were collected at 0 dpf and determination between WT/heterozygous and homozygous took place at 4 dpf. The percent of *scn1Lab* \(^{-/-}\) fish was calculated by taking the number of *scn1Lab* \(^{-/-}\) fish and dividing that number by the total number of eggs.

**Table 2: Percentage of homozygotes produced from *scn1Lab* \(^{ +/-}\) mating.** Adult *scn1Lab* \(^{ +/-}\) were spawned three separate times. *scn1Lab* \(^{-/-}\) were identified by hyperpigmentation and curved body axes.

<table>
<thead>
<tr>
<th># of adult <em>scn1Lab</em>(^{ +/-})-spawned</th>
<th>Total # of eggs</th>
<th># of <em>scn1a</em>-(^{-/-})</th>
<th>% <em>scn1a</em>-(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>110</td>
<td>24</td>
<td>22%</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>11</td>
<td>22%</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>8</td>
<td>13%</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td><strong>220</strong></td>
<td><strong>43</strong></td>
<td><strong>20%</strong></td>
</tr>
</tbody>
</table>
Figure 8 shows the phenotypic differences between $scn1Lab$ heterozygous and homozygous fish. The $scn1Lab$ $-/-$ shows darker pigmentation towards the head region and along the edges of the body as well as a lack of a swim bladder.

**Figure 8:** Heterozygous and homozygous $scn1Lab$ mutant morphology. A) Example of a heterozygous mutant. B) Example of a homozygous mutant.
Figure 9 demonstrates the difference in large activity duration between homozygous (-/-) and heterozygous (+/-) *scn1Lab* mutants. The percentage of large activity duration of *scn1Lab* <sup>+/+</sup> to *scn1Lab* <sup>−/−</sup> (heterozygous/homozygous) was calculated at 4, 5, 6, and 7 dpf. 4 and 6 showed the largest difference between the heterozygotes and the homozygotes at 63% and 66%, respectively. At 5 and 7 dpf the percent difference was 45% and 40%, respectively.

Figure 9: Comparison of large activity duration between homozygous (*scn1Lab* <sup>−/−</sup>) and heterozygous (*scn1Lab* <sup>+/−</sup>) mutant zebrafish. Large activity duration was measured in a ViewPoint Zebrabox (15 min recording with 100% light) at 4, 5, 6 and 7 dpf. (n=24 on 4 and 5 dpf; n=6 on 6 and 7 dpf).
The results of two different concentrations of CBD is shown in **Figure 10**. At 24 hours post treatment (hpf), there was a slight decrease in large activity duration between control -/- and clemizole -/- . However, neither of the concentrations of CBD (0.075 and 0.3 mg/L) showed a significant decrease in the duration of large activity when compared to control -/- . While not significant, CBD did show a reduction close to that of clemizole. Also, CBD and clemizole treatment reduced -/- activity to that of control +/- . On 7dpf, the clemizole -/- treatment group showed an increase in activity. Neither of the concentrations of CBD showed a significant decrease in the duration of large activity on 7dpf.

**Figure 10**: Seizure activity following clemizole and CBD exposure in *scn1Lab* mutant zebrafish. *Scn1Lab* mutants that were homozygous (-/-) and heterozygous (+/-) were in a ViewPoint ZebraBox (15 min recording with 100% light). The duration of large activity following a 24 and 48 exposure to CBD and clemizole was analyzed to determine if CBD shows promise in reducing seizure-like activity (n=6)
4. DISCUSSION

The goal of this study was to utilize a *scn1Lab* deficient zebrafish model to screen for potential anti-epileptic natural products. Dravet Syndrome is a highly AED resistant form of epilepsy, thus, a screen for compounds or extracts that may show effectiveness against seizure activity is necessary. Two natural products were of great interest in this research project – CBD and mistletoe. Previous research in our laboratory demonstrated that the mistletoe compound, AF.1.11.TG.4, showed promising anti-convulsant results. Previous research in our laboratory also showed that CBD is not useful in generalized epilepsy; generalized epilepsy trials were conducted using PTZ to induce seizures that represent a general form of epilepsy. However, it is important to note that while one drug may not work for one form of epilepsy it may work for another. In this case, we hypothesized that CBD may show anticonvulsant properties in a *scn1Lab* deficient zebrafish model.

*Tapinathus globiferus*, TG, extracts were used in generalized epilepsy assays. The most promising extract was AF.1.11.TG.4. Four trials were conducted using the same concentrations of extract – 0.2, 1, and 5 mg/L. Success of the assay was based on the positive control, diazepam, significantly decreasing PTZ-induced seizure activity. The TG extract was run in multiple trials and in only one of the four trials was the duration of large activity decreased. Because AF.1.11.TG.4 was not toxic at the highest concentration tested and chemically-induced seizure activity was not reduced completely to control levels, higher concentrations of this extract can be tested for determination of more effective concentrations. Additionally, longer term exposures should be conducted to
determine if acute or chronic exposures cause toxicities (Dietrich, 2017). It is important to note that CBD was also unsuccessful in treating generalized epilepsy. However, previous research conducted by Devinsky et al. (2017) indicated that CBD may show promise in reducing seizure-like activity in human DS patients. Thus, TG could potentially be useful in treating drug-resistant forms of epilepsy, so further experimentation utilizing DS models should be done.

A knockdown \textit{scn1Lab} model in zebrafish was created through the use of an antisense oligonucleotide morpholino. Various concentrations ranging from approximately 10-60ng of the \textit{scn1Lab}-MO and exposure to higher temperatures did not increase movement in the MO-injected zebrafish either. Fish were exposed to higher temperatures to visualize hyperthermia-induced abnormalities because fevers are known causes of seizures. Some of the MO-injected zebrafish did possess the desired phenotypes—hyperpigmentation, curved body axis, and non-inflated swim bladders. These phenotypes are characteristic features of \textit{scn1Lab} mutant zebrafish as noted in previous research (Sourbron et al., 2016; Zhang et al., 2015). Digital imaging of the morpholino injection told us that the MO was successfully injected into the embryo. After injection, fluorescent screens also proved that the morpholino was still in the embryo. However, the knockdown was deemed unsuccessful because behavioral experiments using ViewPoint data demonstrated that the MO-injected zebrafish did not show an increase in the duration of large activity when compared to the non-injected fish. Typically, inhibition of translation is measured through the use of a western blot. However, there is no available antibody to this protein in zebrafish.
In research conducted by Zhang et al. (2015), automated video-based behavioral tracking system was used to simultaneously monitor and quantify the locomotor activity of freely swimming *scn1Lab* morphants. The *scn1Lab* morphants displayed spontaneously increased total movement as compared to control larvae from 3 to 7 dpf. The increase in total movement was initially observed at 3 dpf and became more pronounced at 4 and 5 dpf. Movement was extremely minimal on 4 dpf, high in 5 dpf, and slightly lower than 5 dpf at 6 dpf. However, in our experiments, the *scn1Lab* group did not show an increase in movement. The control-MO and non-injected groups should have demonstrated similar duration in large activity, but they did not. This indicates possible adverse effects from the morpholino or injection procedure itself. Also, in our results, we did not see larval sensitivity to hyperthermia as seen in Zhang et al. (2015) results.

Zhang et al. (2015) also performed higher-resolution video recording to capture more subtle larval seizure behaviors not detected by the automated tracker. They found that *scn1Lab* morphant larvae displayed not only increased total movement in *scn1Lab* morphants but also abnormal behavior. Larvae displayed jerking behavior and sudden stiffening and relaxation of the entire body. Higher-resolution video recording was not done in our experiments. If further experimentation were to be done, this could be a viable option for tracking behavior.

Because the *scn1Lab* knockdown model was unsuccessful, chemically mutated transgenic fish were utilized. Fish with the *scn1Lab* gene knocked out were used in the CBD assay. We hypothesized that CBD would be an effective anti-convulsant compound against DS due to previous research (Cunha et al., 1980; Devinsky et al., 2017).
Experimental results obtained from ViewPoint data demonstrated that both 0.075 mg/L CBD (0.25 µM) and 0.3 mg/L CBD (1 µM) did not significantly decrease the duration of large activity. In the homozygous group, clemizole decreased large activity duration on 6 dpf, but lost its efficacy on 7 dpf. This experiment was only conducted once with 6 homozygous and 6 heterozygous/wildtype per treatment group. Also, the activity of the CBD groups and clemizole groups are extremely close in 6 dpf. Therefore, further experimentation is necessary to determine whether or not CBD is an effective anti-epileptic drug for DS. In addition, various concentrations of CBD need to be tested. Increasing the amount of time for the behavioral experiments from 15 min to 45 min will increase the potential to observe seizure-like activity in each treatment group. In our experiment, we used 6 fish per treatment. Thus, increasing the number of fish in each treatment group could increase significance as well. Because both concentrations of CBD seemed to lose efficacy at 7 dpf, daily dosing may be necessary in later experiments.

Clemizole 1.8 mg/L (5µM) was used as a positive control in the CBD/clemizole assay with *scn1Lab* fish. Clemizole was used based on previous research conducted by Baraban et al. (2013). Their group found that clemizole was effective in suppressing spontaneous seizure activity in their mutant DS lines. Although clemizole did not significantly decrease large activity movement in our experiment, there was some decrease in large movement as compared to the control group. Clemizole seemed to lose its efficacy on 7 dpf in the homozygous group; this could be due to natural metabolism of the drug. Clemizole also showed toxic effects in the heterozygous group on 7 dpf. Another promising anti-epileptic drug, fenfluramine, has been found to significantly
reduce epileptiform discharges in \textit{scn1Lab} morphants (Zhang et al., 2015). Thus, fenfluramine could be used in future experimentation as a comparison to CBD.

Although CBD failed to significantly decrease large activity movement in our experiment, Cunha et al. (1980) found that CBD administration inhibited the effect of PTZ in rats, decreased the astrocytic hyperplasia, decreased neuronal damage in the hippocampus caused by the seizures and selectively reduced the expression of the NR1 subunit of NMDA (Cunha et al., 2015). Because CBD shows potential in larger animal models, this assay may show successful results in human studies. Multiple small studies of CBD safety in humans in both placebo-controlled and open trials have demonstrated that it is well tolerated across a wide dosage range. No significant central nervous system side effects, or effects on vital signs or mood have been seen at doses up to 1,500 mg/day (p.o.) or 30 mg (i.v.) in both acute and chronic administration (Devinsky et al., 2014). Thus, CBD is still a viable option in treating drug-resistant forms of epilepsy, so further experimentation utilizing DS models should be done.

In conclusion, three different anti-epilepsy in vivo drugs screens were used in this research project. PTZ-induced screens represent a generalized seizure model and are relatively easy to conduct using diazepam as a positive control. In this screen, TG failed to decrease PTZ-induced activity. However, this result does not necessarily suggest that extracts will not be effective in DS epilepsy. Therefore, we targeted \textit{scn1Lab} knockdown/out for a syndrome specific screening. Despite multiple manipulations, the morpholino-based approach did not generate the expected seizure phenotypes. Thus, the transgenic \textit{scn1Lab} fish were a far better model for routine screening. Initial results for both the positive control clemizole and CBD suggest the assay is working as expected.
and that CBD inhibits *scn1Lab*–mediated seizures. Further work with higher sample sizes and more concentrations will be needed to confirm these initial promising results.

5. REFERENCES


