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Discovery of the first Vitamin K analog as a potential treatment of pharmaco-resistant seizures

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Abstract

Despite the availability of more than 25 anti-seizure drugs on the market, approximately 30% of patients with epilepsy still suffer from seizures. Thus, the epilepsy therapy market has a great need for a breakthrough drug that will aid pharmaco-resistant patients. In our previous study, we discovered a vitamin K analog,

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4 **2h**, which displayed modest anti-seizure activity in zebrafish and mouse seizure models.

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6 However, there were limitations for this compound due to its pharmacokinetic profile.

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9 In this study, we developed a new series of vitamin K analogs by modifying the
10 structure of **2h**. Among these, compound **3d** shows full protection in a rodent
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12 pharmacoresistant seizure model with limited rotarod motor toxicity and favorable PK
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14 properties. Furthermore, the brain/plasma concentration ratio of **3d** indicates its
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16 excellent permeability to the brain. The resulting data shows **3d** can be further
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18 developed as a potential anti-seizure drug in the clinic.
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25 **Introduction**

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28 Epilepsy is one of the most common neurological disorders and affects an
29 estimated 70 million people worldwide.¹ Many people with epilepsy have more than
30 one type of seizure and may have additional neurological issues. Anti-seizure drugs
31 (ASDs) are the mainstay treatment for epilepsy, effectively controlling seizures for
32 around 70% patients with epilepsy. Driven by the limited efficacy and toxicity of the
33 established anti-seizure agents, development of ASD has exploded in the past quarter-
34 century, with more than 25 ASDs on the market.² Nevertheless, around 30% of patients
35 have inadequate control of their seizures, a percentage unchanged since 1881.³⁻⁴
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37 Currently, there are two main challenges for the treatment of epilepsy; the first and the
38 most important one is drug resistance, or pharmacoresistance, which refers to patients
39 who do not achieve adequate seizure control after trials of at least two appropriate ASDs.
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41 Clinically, there are limited therapeutic intervention for pharmacoresistant epilepsy, but
42 only for patients with disabling focal epilepsy.⁴ The second major concern for ASDs is
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4 tolerability, as people with epilepsy often require lifelong drug treatment.⁵ However,
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6 none of the ASDs currently on the market are free from side effects. In fact, these side-
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8 effects have been found to harm the quality of life of epilepsy patients more than the
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10 seizures themselves, and can lead to low adherence and discontinuation of treatment.⁶
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12 New third-generation ASDs are marketable due to lowered toxicity and adverse drug
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14 interactions; however, they are only marginally (if at all) more efficacious than older
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16 drugs.⁷ Thus, the epilepsy therapy market is in great need of a breakthrough drug that
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18 would aid medication-resistant patients and be better tolerated.⁸⁻⁹ This leaves room for
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20 new drugs and drug combinations to gain market share quickly.
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28 Pentylentetrazol (PTZ), a chemical convulsant, induces seizures in various animal
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30 models of epilepsy, including worms, flies, frogs, zebrafish and mice,¹⁰⁻¹¹ among which,
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32 the zebrafish PTZ seizure model is widely used for the initial screen of ASDs, since it
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34 matches well with rodent models and is amenable to higher-throughput screening.¹² As
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36 epilepsy comes in many forms, it is also prudent that efficacy of the leading candidate
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38 is tested in a wide range of rodent models, particularly in animal models of medication-
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40 resistant epilepsy.^{7, 13} For over 40 years, the National Institute of Neurological
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42 Disorders and Stroke (NINDS) has supported the Epilepsy Therapy Screening Program
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44 (ETSP), which plays a key role in the identification of novel anti-seizure agents to
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46 address the unmet needs in epilepsy. The current workflow focuses on
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48 pharmacoresistant and etiologically relevant models
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50 (<https://panache.ninds.nih.gov/CurrentModels.aspx>).¹⁴⁻¹⁵ Here in this article, our
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52 compounds were screened for acute seizure models including the mouse 6 Hz seizure,
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4 the maximal electroshock (MES) seizure, and subcutaneous PTZ seizure threshold (s.c.
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6 PTZ) models. The 6 Hz models are believed to mimic partial seizures observed in
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8 humans which are used to screen compound's ability to block psychomotor seizures.
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10 This model exhibits pharmacoresistance when the stimulation intensity is increased
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12 from 22 to 44 mA.¹⁶⁻¹⁷ The 6 Hz 44 mA model is resistant to ASDs that target voltage-
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14 gated ion channels. Levetiracetam and valproic acid, while still effective in this model,
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16 they are significantly less potent at this stimulation intensity.¹⁸⁻¹⁹ Therefore, the 6 Hz
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18 44 mA model provides a high fidelity threshold assay early in the drug identification
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20 process. MES is a model for generalized tonic-clonic seizures and used for screening a
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22 compound's ability to prevent seizure spread when all neuronal circuits in the brain are
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24 maximally active. These seizures are highly reproducible and are electrophysiologically
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26 consistent with human seizures (<https://panache.ninds.nih.gov/CurrentModels.aspx>).
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28 The mouse 6 Hz and MES seizure models are included in the current ETSP workflow
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30 for pharmacoresistant epilepsy, and the s.c. PTZ-induced seizure model detects the test
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32 compound's ability to protect animals from exhibiting clonic, forebrain seizures.
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44 In our previous study, we discovered compound **2h**, which was effective in the 6
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46 Hz 32 mA mouse seizure model, but had limitations in the pharmacokinetic (PK) profile
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48 due to short $t_{1/2}$ and limited central nervous system exposure (**Figure 1**). Moreover, **2h**
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50 did not show any protection in mice when given the higher 44 mA stimulation intensity
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52 in the 6 Hz model.²⁰ Therefore, in this study, we modify the structure of **2h** to develop
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54 a new series of Vitamin K (VK) analogs. To accelerate the discovery process, a two-
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56 tier whole organism screening process was first set up using the zebrafish PTZ model,
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which is amenable to higher-throughput examination to establish structure activity relationship that correlates well with higher animal models.¹¹ After establishing the structure-activity relationship (SAR) in the zebrafish model, top candidates were examined for *in vivo* anti-seizure activity and PK profiles in mice. Compound **3d** shows titratable full protection in the 6 Hz seizure model at 32 and 44 mA, with limited motor toxicity and favorable PK properties, making **3d** a leading ASD candidate.



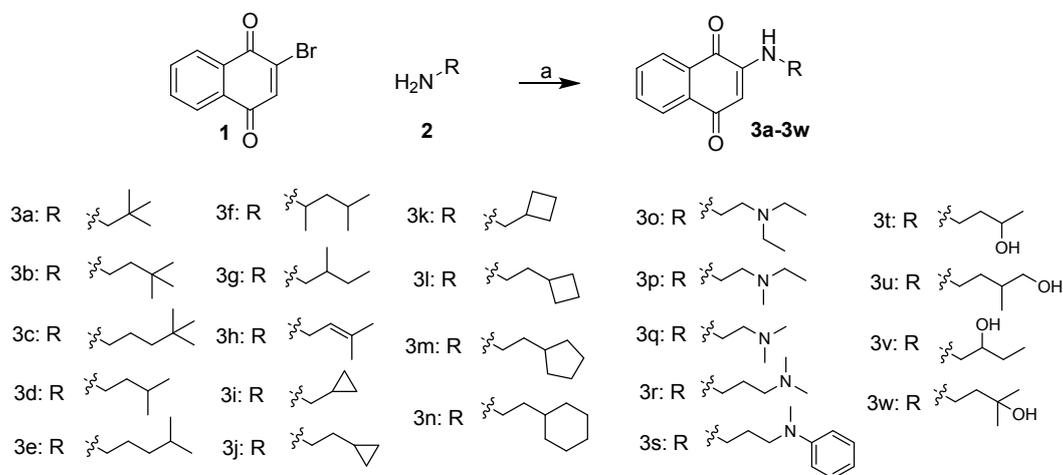
	Vitamin K3 (VK3)	Analog 2h	new VK analog
In Vivo Stability	Mouse i.v. $t_{1/2} = 27$ mins	Mouse i.v. $t_{1/2} = 1.10$ hrs	Mouse i.v. $t_{1/2} = 4.47$ hrs
Tolerability	Toxic (6 μ M)	Good (>20 μ M)	Excellent (> 40 μ M)
Effective in Zebrafish	Yes $EC_{50} \sim 3$ μ M ²⁰	Yes $EC_{50} \sim 20$ μ M ²⁰	Yes $EC_{50} \sim 5$ μ M
Effective at 6Hz 32 mA	N/A (Toxic)	Yes	Yes
Effective at 6Hz 44 mA	N/A (Toxic)	No	Yes

Figure 1. Evolution of a new VK analogs with excellent seizure protection and PK profile. VK3 and Analog **2h** data are previously published.²⁰

Chemistry

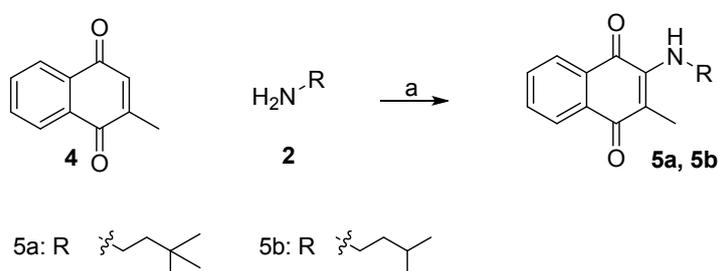
Scheme 1 shows the synthesis of **3a-3w**. 2-bromonaphthalene-1,4-dione (**1**) was treated with various fatty amines (**3a-3n**), diamines (**3o-3s**) and alcohol amine (**3t-3w**) to yield desired compounds. 2-methylnaphthalene-1,4-dione (**4**) was reacted with 3,3-dimethylbutan-1-amine or 3-methylbutan-1-amine to obtain **5a** or **5b**, respectively (**Scheme 2**).

Scheme 1



Reagent and condition: (a), different saturated and unsaturated fatty amines, ethanol, room temperature, overnight, 50-65% yield.

Scheme 2



Reagent and condition: (a), 3-methylbutan-1-amine or 3,3-dimethylbutan-1-amine, methanol and dichloromethane, room temperature, overnight, 58-65% yield.

Results and Discussion

Discovery of A New VK Analog with Excellent Seizure Protection and PK Profile

In our previous study, we tested VK1, VK2, and VK3, and found that the smallest VK, VK3, was protective and could reduce PTZ-induced locomotor activity in zebrafish. However, the lethal dose of VK3 in zebrafish is 7 μ M, which is similar to its

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4 effective does (3-6 μM), therefore, VK3 shows significant toxicity.²⁰ Further
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6 modification achieved VK analog **2h**, which exhibited clear anti-seizure activity in
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8 zebrafish at the concentration of 20 μM with no observable toxicity. In 6 Hz 32 mA
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10 mouse models, **2h** displayed full protection at 15 min; however, further testing in the
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12 higher intensity 6 Hz 44 mA model showed that **2h** was no longer protective.²⁰ The
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14 short half-life (1.06 h) of **2h** correlates to its acute but short protective duration in the
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16 mouse seizure model (effective within 15 min but the protection drops off sharply in
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18 less than a half-hour). While the compound is stable, displaying no catabolic conversion
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20 even after two hours in mouse serum, its plasma concentration rapidly diminishes *in*
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22 *vivo*, suggesting either rapid hepatic metabolism or diminished renal reuptake. This
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24 finding is not surprising, as alkynes are known to be oxidized and excreted rapidly by
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26 the liver and kidney; therefore, we modified alkynes of **2h** to produce a new series of
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28 VK analogs, and at the same time, also modified 2-position (R_1 group) (**Figure 1**). In
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30 detail, substitution of alkynes with aliphatic chains yields compounds **3a-3h**, while with
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32 cyclic aliphatic group yields **3i-3j**. The compounds **3o-3s** and **3t-3u** were the structures
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34 containing tertiary amine and oxhydryl, respectively. Substitution of hydrogen of **3b**
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36 and **3d** with methyl yields **5a** and **5b**, respectively (**Scheme 1**).

47 48 49 **Anti-seizure Activity of VK Analogs in Zebrafish**

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52 Previous studies showed that zebrafish displays analogous electrophysiological
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54 and molecular features associated with mammalian seizure models when exposed to the
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56 convulsant agent PTZ.¹¹ The level of seizure severity in the brains of zebrafish, as
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58 determined by field potential measurements, corresponds with increased swimming
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4 activity, which is measured as distance traveled. This zebrafish assay has been used to
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6 screen greater than 500,000 mutagenized fish for seizure-resistant genes,²¹ and was
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8 further validated against rodent models of epilepsy with 13 current ASDs.¹² Therefore,
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10 we initially used the zebrafish PTZ-induced seizure model to screen our compounds.
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12 Zebrafish larvae (7 days post-fertilization, dpf) were treated with synthesized
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14 candidates at concentrations of 10 μ M and 5 μ M for 1 h prior to seizure induction with
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16 15 mM PTZ, 5 mM and 10 mM VPA were used as positive controls. Total swimming
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18 distance in 15 min was recorded after PTZ-induced seizures.²⁰ PTZ treatment induced
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20 a 7-fold increase in the total distance traveled compared to the average control zebrafish
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22 (**Figure 2a** and **2b**). The level of seizure protection by compounds at 10 μ M are shown
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24 in **Figure 2a**. Previous lead compound **2h** did not significantly reduce distance traveled
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26 at the lower 10 μ M concentration; which we had previously observed seizure protection
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28 at 20 μ M in our earlier study.²⁰ Among compounds with aliphatic chains (**3a-3h**), the
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30 compounds **3b**, **3d**, **3e**, **3f**, **3g** and **3h** protect against PTZ-induced seizures by reducing
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32 the total distance traveled by more than 50%. It should be noted that 10 μ M **3d**, **3f** and
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34 **3l** display better activity than 10 mM VPA, which was used as positive control.
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36 Compound **3b** with substitution of 2,2-dimethylbutyl shows better suppressive activity
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38 than **3a** with one carbon shorter and **3c** with one carbon longer, which indicates 2
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40 carbons is the optimal length. This is also consistent with the activity of **3d** and **3e**.
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42 Within compounds bearing cyclic aliphatic group, **3l** and **3m**, with cyclobutyl and
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44 cyclopentyl respectively, reduce distance traveled greater than 50% as compared to
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46 zebrafish treated with PTZ only. The activity difference between **3k** and **3l** is also
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4 consistent with the conclusion drawn from **3a-3c**. Unfortunately, compounds with
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6 tertiary amine (**3o-3s**), oxhydryl (**3t-3w**) as well as compounds **5a** and **5b** with methyl
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8 group in 2-position show no protection against PTZ-induced seizures. Among all of the
9
10 tested compounds, **3d** and **3l** display the best activity in zebrafish, by reducing the
11
12 distance traveled by 77.4% and 89.0%, respectively. **3d** and **3l** also show the best
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14 activity at 5 μ M (**Figure 2b**, **Table 1**). The mean distance traveled per 1 min of the
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16 most active compounds, **3d** and **3l**, is also shown in **Figure 2c**, from which we can
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18 observe that these two compounds significantly reduced distance traveled in every
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20 minute. The SAR is summarized in **Figure 3**.
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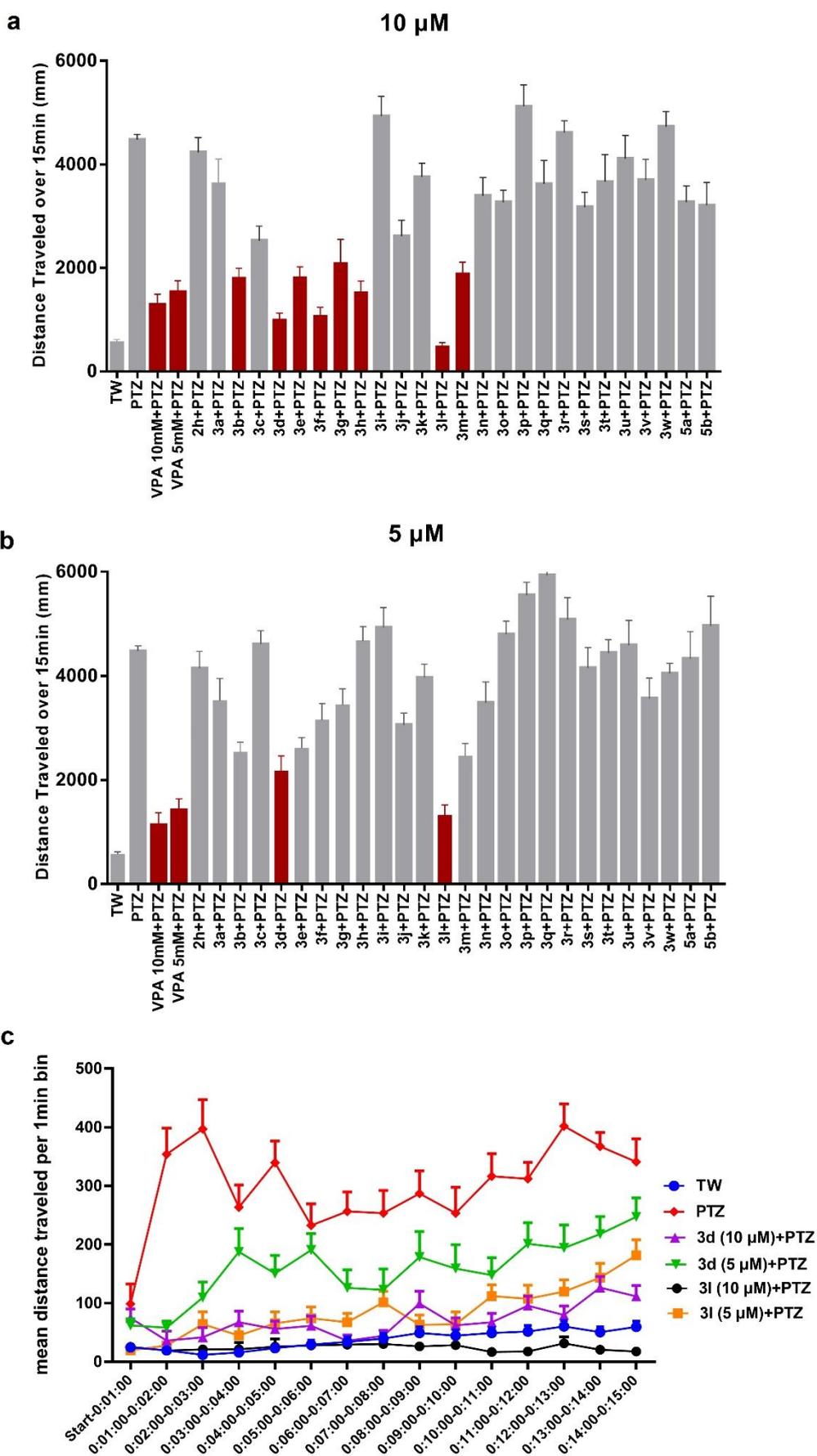
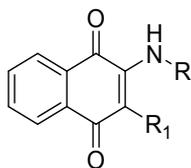


Figure 2. (a) and (b) Total mean distance traveled over 15 min. Synthesized compounds were tested at 10 μM and 5 μM , respectively. 5 mM and 10 mM VPA were used as positive controls. Data represents the mean distance traveled, error bar represents SEM, $n = 8$ for each group. The red color shows $> 50\%$ percentage of reduced travel distance (c) Mean distance traveled per 1 min after treatment of **3d** and **3l**, data are shown as mean distance traveled, error bar represents SEM, $n = 8$ for each group. With the exception of **3d** at 10 μM + PTZ, all time points are significantly different from the positive PTZ run $p < 0.01$. TW = tank water only control.

Table 1. Reduction of PTZ-induced distance traveled after treatment of designed compounds at the concentration of 10 μM and 5 μM .



Compounds No.	R	R ₁	Percentage of reduced travel distance (%) ^a	
			10 μM	5 μM
2h		H	5.4 \pm 0.37	7.4 \pm 0.58
3a		H	18.4 \pm 2.30	21.2 \pm 2.53
3b		H	59.2 \pm 5.52	43.4 \pm 3.30
3c		H	43.5 \pm 4.81	-3.0 \pm 0.17
3d		H	77.4 \pm 8.97	51.5 \pm 6.79
3e		H	59.1 \pm 6.22	41.6 \pm 3.20
3f		H	75.8 \pm 8.66	29.6 \pm 2.97
3g		H	52.9 \pm 11.10	23.0 \pm 2.01

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4	3h		H	65.6 ± 8.83	-4.4 ± 0.26	
5						
6	3i		H	-10.1 ± 0.79	-10.1 ± 0.79	
7						
8						
9	3j		H	41.6 ± 4.84	31.7 ± 2.38	
10						
11	3k		H	16.1 ± 1.14	11.4 ± 0.73	
12						
13						
14	3l		H	89.0 ± 11.34	70.4 ± 10.50	
15						
16	3m		H	57.4 ± 6.29	45.0 ± 4.75	
17						
18						
19	3n		H	24.1 ± 2.47	22.2 ± 2.58	
20						
21						
22	3o		H	27.0 ± 1.93	-7.2 ± 0.38	
23						
24	3p		H	-14.3 ± 1.18	-23.9 ± 1.08	
25						
26	3q		H	19.1 ± 2.40	-32.4 ± 2.01	
27						
28	3r		H	-2.90 ± 0.15	-13.4 ± 1.13	
29						
30	3s		H	29.1 ± 2.65	7.3 ± 0.69	
31						
32						
33	3t		H	18.2 ± 2.64	0.78 ± 0.04	
34						
35						
36	3u		H	8.2 ± 0.90	-2.4 ± 0.26	
37						
38						
39	3v		H	17.4 ± 1.90	20.4 ± 2.28	
40						
41						
42	3w		H	-5.6 ± 0.34	9.5 ± 0.46	
43						
44						
45	5a			26.9 ± 2.55	3.3 ± 0.40	
46						
47	5b			28.3 ± 3.88	-10.8 ± 1.25	
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^a Assays were performed using eight zebrafish for each group. Values are the mean of ± SEM. n = 8 for each group. The negative number indicates increased distance traveled after treatment of designed compounds.

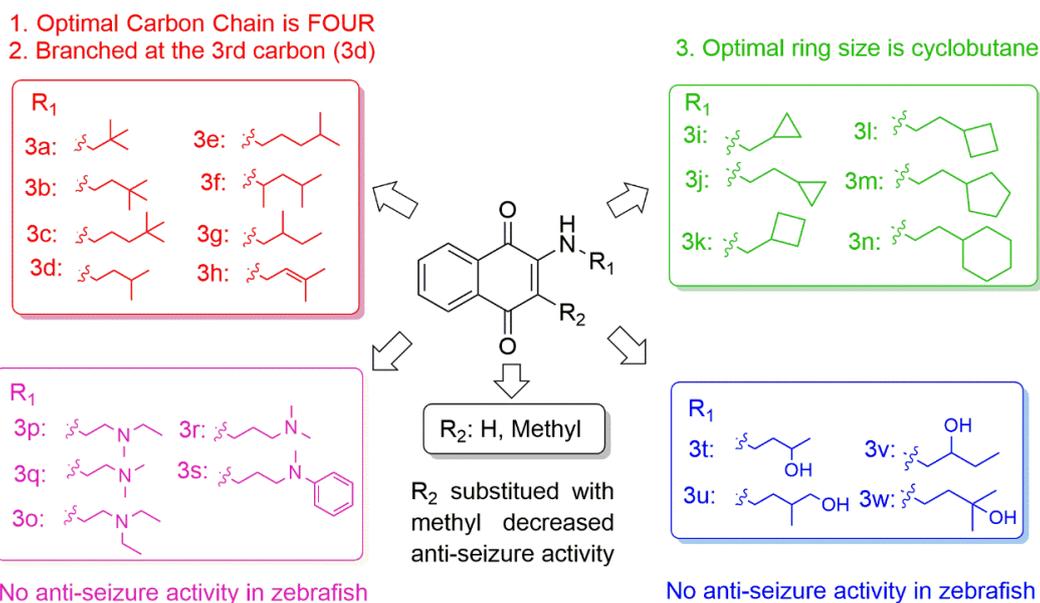


Figure 3. Structure and activity relationship study of VK analogs.

Toxicity of VK Analogs in HT-22 Neurons

As most epilepsy patients need to use ASDs throughout their lives, safety is one of the major concerns of ASDs. Therefore, we tested the toxicity of these compounds in HT-22 neuronal cells (**Table 2**). At the concentration of 50 μ M, compounds **3o-3r** with tertiary amine exhibit the highest toxicity with about 100% growth inhibition. Even when the concentration was decreased to 10 μ M, **3o-3r** still inhibit growth by 74.5%-86.2%. It is interesting that when methyl group of *N*-substituent of **3r** was changed to phenyl (**3s**), the percentage of inhibition is reduced to 10.25%. Compounds **3o-3s** with hydroxyl groups also show high toxicity at a concentration of 50 μ M with growth inhibition of 59.7%-76.7%, while inhibition drops to 19.4%-38.6% when the concentration is reduced to 10 μ M. Compounds **3i**, **3j** and **3k** with cyclic aliphatic groups show modest toxicity at 50 μ M (54.1%-62.3%) which also decrease at the concentration of 10 μ M. Compounds **3d** and **3l**, with the highest seizure protection in

the PTZ-induced zebrafish seizure model, do not display obvious toxicity in HT-22 cells (**Table 2**).

Table 2. Toxicity of VK analogs in HT-22-neurons.

Compounds No.	Percentage of inhibition (%) ^a	
	50 μ M	10 μ M
3a	22.42 \pm 6.63	10.78 \pm 1.94
3b	16.97 \pm 10.55	11.91 \pm 2.21
3c	6.75 \pm 1.29	3.81 \pm 5.08
3d	17.78 \pm 1.41	11.86 \pm 9.27
3e	21.34 \pm 3.42	28.77 \pm 5.90
3f	15.01 \pm 2.87	2.09 \pm 1.41
3g	41.21 \pm 2.98	14.81 \pm 4.24
3h	38.26 \pm 0.84	14.96 \pm 2.48
3i	54.10 \pm 6.32	16.83 \pm 4.98
3j	56.37 \pm 0.79	34.69 \pm 5.28
3k	62.34 \pm 0.07	33.70 \pm 5.31
3l	36.29 \pm 4.49	27.21 \pm 0.82
3m	43.76 \pm 2.83	35.37 \pm 4.70
3n	2.09 \pm 2.06	0.07 \pm 0.03
3o	100.05 \pm 0.21	83.84 \pm 0.33
3p	100.96 \pm 0.52	86.21 \pm 0.26
3q	99.78 \pm 0.16	82.63 \pm 3.28
3r	96.69 \pm 0.69	74.53 \pm 0.62
3s	10.25 \pm 6.61	11.53 \pm 1.71
3t	64.21 \pm 6.55	19.39 \pm 9.48
3u	76.65 \pm 3.41	25.66 \pm 2.59
3v	69.02 \pm 2.45	38.61 \pm 0.01
3w	59.67 \pm 0.11	25.52 \pm 2.66

5a	17.54 ± 2.83	10.15 ± 1.34
5b	21.39 ± 3.72	11.24 ± 2.56

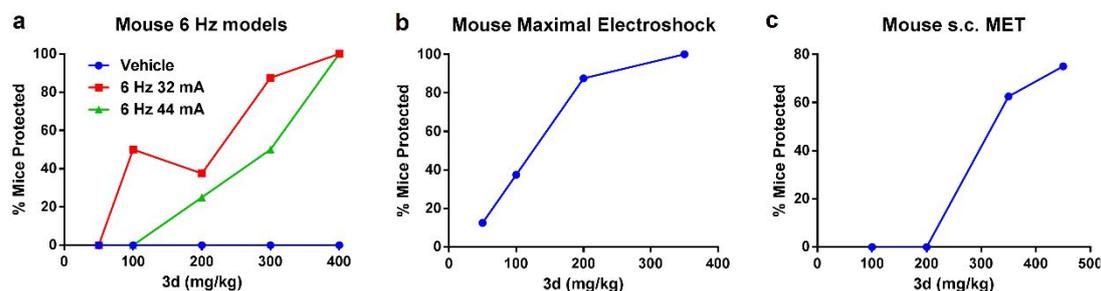
Note: ^a Values are the mean ± SEM, three experiments were performed for each data (n = 3).

***In Vivo* Anti-seizure Activity in Rodent Seizure Models**

The 6 Hz limbic seizure test using a stimulus intensity of 44 mA, is an acute model for pharmacoresistant seizures. Therefore, the most potent compounds **3d** and **3l** in zebrafish were tested for their anti-seizure activity in the 6 Hz rodent seizure models. Initial screening assays were performed using the lower stimulus intensities: 22 mA and 32 mA. Unfortunately, the most potent compound, **3l**, in the PTZ-induced zebrafish seizure model shows limited efficacy in the 6 Hz mouse seizure model at 22 mA, without full seizure protection up to 400 mg/kg (Supporting Information **Table S1**). Thus, we did not continue testing **3l** in the 6 Hz seizure model at 32 mA and 44 mA. First, a time course study was performed to establish a time to peak seizure protection of 1 h in 6 Hz models following ip administration (Supporting Information **Table S2**). Then, a dose-response quantitation study was performed at the 1 h peak response time to determine the ED₅₀. Compound **3d** is highly effective at both 32 mA and 44 mA, with full protection at the concentration of 400 mg/kg without obvious motor/sedative effects. EC₅₀ values of **3d** in the 6 Hz model at 32 mA and 44 mA are 152.7 and 263.7 mg/kg, respectively (**Figure 4a, Table 3**). Previously, **2h** did not show any protection in 6 Hz 44 mA model,²⁰ therefore, **3d** is more potent than both **3l** and **2h**. This is consistent with the established pharmacoresistant seizures; most drugs lose seizure

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4 protection effectiveness with increased mA, thus making this increased 44 mA voltage
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6 intensity a model of pharmacoresistance. Furthermore, only four FDA approved anti-
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8 seizure medications to date (valproic acid, levetiracetam, felbamate and cannabidiol)
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10 have shown activity in this particular model and are all used clinically to treat refractory
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12 or hard-to-control seizures seizures.²²⁻²³ We also compared the EC₅₀ and TD₅₀ (median
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14 toxic dose, a toxicology term that relates to the median toxic dose of a substance in
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16 which toxicity occurs in 50% of a species) of **3d** with these four drugs (**Table 4**). The
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18 reported EC₅₀ value of levetiracetam is over 1000 mg/kg in this model and it is one of
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20 the most commonly prescribed medicine for refractory seizures, compound **3d**'s EC₅₀
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22 value is comparable to all other anti-seizure medicines with EC₅₀ values ranging from
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24 100 mg/kg to 300 mg/kg. It should be noted that **3d** is well tolerated with a TD₅₀ value
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26 > 800 mg/kg, which is much higher than the other FDA approved drugs. Then **3d** was
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28 further screened in MES model and produced full protection at 350 mg/kg with EC₅₀
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30 value of 108.12 mg/kg (**Figure 4b**, **Table 3**). Result shows **3d** blocks seizures in the
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32 mouse 6 Hz and MES models in a dose-dependent manner, reaching full seizure
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34 protection at doses that do not produce motor impairment, which indicates its potential
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36 in the treatment of pharmacoresistant seizures. To provide more data of **3d**'s efficacy
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38 against clonic seizures, the ED₅₀ value was also determined in the s.c. PTZ mouse
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40 model at the 1h peak point. **3d** protects 6 out of 8 animals at the concentration of 450
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42 mg/kg with ED₅₀ value of 349.22 mg/kg (**Figure 4c**, **Table 3**). Of note, **3d** does not
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44 produce major motor impairment at the time of seizure testing, as the rotarod assay was
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46 performed immediately prior to 6 Hz stimulation (1 h after administration). However,
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4 other effects of treatment are noted following administration with **3d** that will be the
5
6 subject of ongoing investigation. Namely, **3d** administration is associated with writhing
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8 (abdominal constriction) both at the time of treatment and during the ~1 h observation
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10 period that followed. In addition, some animals also show discolored urine and lethargy
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12 following treatment (data not shown). Subsequent studies also reveal that higher doses
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14 of **3d** (e.g. greater than 500 mg/kg) are lethal in some animals after electric stimulation
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16 after 24h (data not shown). The mechanisms contributing to the lethality will be the
17
18 subject of future studies. Nonetheless, **3d** does not show signs of major motor
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20 impairment during the acute (~1h) observation period with a treatment dose up to 800
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22 mg/kg in mice (mouse rotarod assay) (Supporting Information **Table S2**).
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42 **Figure 4.** Compound **3d** in the acute seizure models, **3d** was injected via ip
43 administration 1 h prior to electrical stimulation or injection of Metrazol. The
44 percentage of mice protected is shown for each of the four dose levels for **3d** (n = 8).
45
46 (a) Dose-dependent effect of **3d** in mouse 6 Hz 32 mA and 44 mA seizure models. (b)
47 Dose-dependent effect of **3d** in the mouse MES seizure model. (c) Dose-dependent
48 effect of **3d** in the s.c. PTZ seizure model.
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58 **Table 3.** Effect of **3d** in 6 Hz mouse seizure models.
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Seizure model	Time of test	ED ₅₀ value (mg/kg)	95% confidence interval (mg/kg)
6 Hz 32mA mouse model	1 h	152.7	98.6-211.0
6 Hz 44mA mouse model	1 h	263.7	198.9-321.8
MES model	1 h	108.1	70.4-152.9
s.c. PTZ mouse model	1 h	349.2	256.0-432.1

Table 4. Comparison of **3d** and four FDA approved ASDs tested in the 6 Hz 44 mA seizure model.

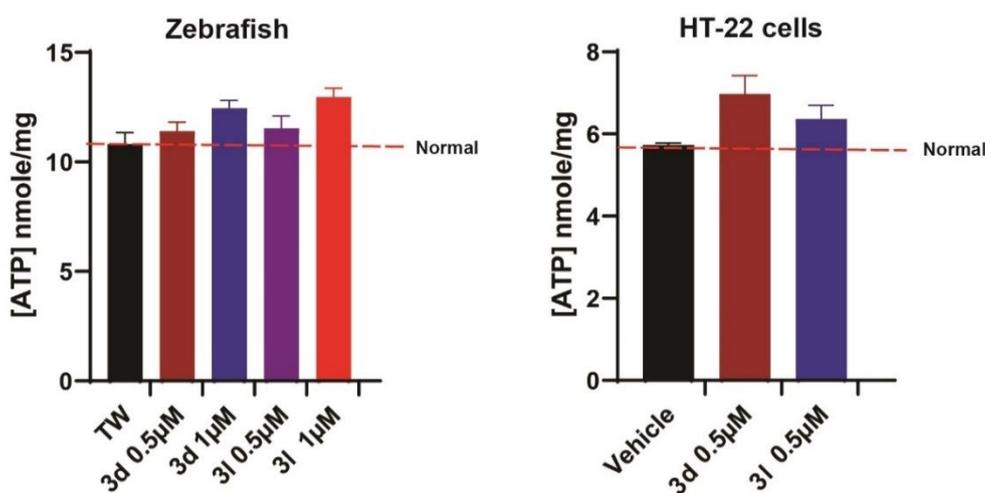
Compound	6 Hz 44 mA ED ₅₀ (mg/kg)	TD ₅₀ (mg/kg)
3d	263.7	>800
Valproic Acid ²³	289.1	390
Levetiracetam ²³	1089.3	>500
Felbamate ²²	97.5	452
Cannabidiol ²²	173.5	272

Vitamin K Analogs Increase ATP Levels

The exact molecular mechanisms of seizures are unknown and may involve multiple different factors. The 6 Hz 44 mA seizure model is particularly resistant to ASDs that target voltage gated sodium and potassium channels. To eliminate that our inhibitors are targeting these ion channels, recurrent epileptiform discharges (REDs) in brain slices derived from the kainite-induced status epilepticus model of temporal lobe epilepsy were examined *in vitro*.²⁴ Compound **3d** does not attenuate spontaneous REDs (Supporting Information **Figure S1**), which is consistent with the hypothesis that it has

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4 novel mechanism of action other than voltage gated sodium and potassium ion channel
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6 inhibitors. Importantly, neurons have high metabolic energy demands, as well as a low
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8 capacity to store ATP. A sudden change in ATP levels in neurons can impair Na/K
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10 ATPase activity and decrease neuronal membrane potential, which can give rise to an
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12 increase in neuronal excitability resulting in seizures.²⁵ In addition, heightened neuronal
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14 excitability impairs calcium sequestration resulting in an increased glutamate release
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16 into synaptic clefts, which can result in neuronal injury.²⁵ The ketogenic diet and
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18 valproic acid (VPA) are commonly used in combination for relapse seizures and the
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20 mechanism of action is hypothesized to be involved in modulating mitochondrial
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22 activity.²⁶⁻²⁹ In the 12 marked ASDs tested in the pharmaco-resistant seizure models,
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24 only VPA and levetiracetam displayed protection and both have mitochondrial
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26 effects.³⁰ VK is also known to play a role in the mitochondrial electron transfer chain
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28 and is capable of maintaining mitochondrial homeostasis. What's more, in our previous
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30 study, VK3 and its analog **2h** can increase ATP levels in HT-22 cells at the
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32 concentration of 5 μ M.²⁰ Hence, effects of **3d** and **3l** on ATP levels were determined
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34 for both zebrafish and neuronal cells (HT-22). In zebrafish, **3d** and **3l** increase ATP
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36 levels by 19.0% and 21.4% at the concentration of 1 μ M, respectively (**Figure 5**). In
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38 HT-22 cells, **3d** and **3l** increase ATP levels by 25.2% and 11.0% at the concentration
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40 of 0.5 μ M, respectively (**Figure 5**). Therefore, the most potent compounds at preventing
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42 seizures in zebrafish, **3d** and **3l**, significantly increase ATP levels in zebrafish as well
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44 as HT-22 cells, which indicates that maintaining mitochondrial homeostasis and ATP
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46 levels are likely to play a role in its mechanism of action. The VK analogs' molecular
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4 target likely involves proteins in the quinone oxidation/reduction process in the electron
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6 transport chain (ETC). VK in combination with vitamin C has been used in the clinic
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8 to enhance the oxidative phosphorylation process for the treatment of rare
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10 mitochondrial diseases with complex III mutations.³¹⁻³² VK has also been shown to play
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12 a key role as an alternative electron carrier that can rescue pink1 knock-out phenotype
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14 in *Drosophila*, and the VK synthase is a dominant enhancer of the phenotype.³³ VK is
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16 also involved in maintaining calcium ion homeostasis in cells through modulating γ -
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18 glutamyl carboxylation of VK-dependent proteins. Proteins such as growth arrest
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20 specific gene 6 (Gas-6) and protein S regulate calcium ion signaling in the central
21
22 nervous system. Gas-6 activation prevents amyloid beta protein and phospholipase
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24 A(2)-IIA induced calcium ion influx and attenuates neuronal death.³⁴⁻³⁵ Calcium ions,
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26 in turn, play a key role in the induction of seizures. Thus, the ability of VK to modulate
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28 the levels of calcium ion in neurons could be a major contributing factor in its ability
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30 to attenuate seizures.
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Figure 5. 3d and 3l significantly increase ATP concentrations (nmole/mg) in zebrafish

and HT-22 cells. Mean concentrations are plotted \pm SEM, $n = 7-13$. Treatment with compounds significantly increased ATP concentrations compared to controls ($p < 0.05$).

Tissue Distribution and PK Profile of **3d**

Compound **3d** displays promising activity in rodent models and was chosen for further evaluation. As epilepsy is a neurological disorder, effective drug candidates need to cross the blood-brain barrier. **3d** was expected to have excellent lipophilicity and blood-brain barrier permeation as shown by its predicted cLogP of 3.11 and a tPSA of 46.17 (molinspiration software <http://www.molinspiration.com/>). To verify the blood-brain barrier permeation of our compounds, **3d** was assessed for brain/plasma concentration ratio at the 0.5, 1, 2, 4, and 8 h time points, respectively. The brain/plasma ratio for **3d** is 2.64 at 0.5 h, and the ratio declines to 0.816 at 8 h, which indicates the excellent permeability of **3d** (Figure 6). The brain/plasma concentration ratio of **2h** is 0.325 at 0.5 h (Supporting information Table S3), which is much lower than **3d**.

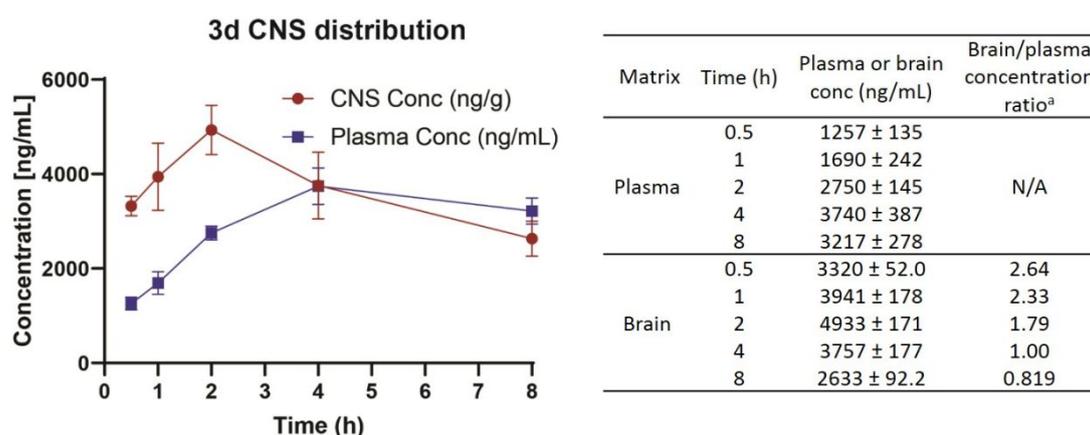


Figure 6. Brain/plasma concentration ratio of **3d** with i.p. administration at concentration of 400 mg/kg.

Improvement of the PK profile of **2h** is one of our goals to develop the new VK analogs as potential ASDs; therefore, we accessed the PK of **3d** in mice. As we used ip administration in rodent models, we initially studied the PK profile of **3d** with ip administration and compared with **2h**. Compound **2h** rapidly reaches its mean peak plasma concentration (C_{\max}) within 15 min. After that, its plasma concentration declines quickly with a short $t_{1/2}$ of 1.10 h (Table 5, Figure 7a). Unlike **2h**, **3d** reaches its C_{\max} within 60 min, and its plasma concentration declines more slowly with a much longer $t_{1/2}$ of 3.38 h (Table 5, Figure 7b). The area under the plasma concentration versus time curve (AUC) of **3d** is 1,726 hr*ng/mL, which is 1.8 times higher than **2h** (978 hr*ng/mL) (Table 5). Therefore, **3d** displays a more favorable PK profile with longer $t_{1/2}$ and higher AUC. A detailed PK study of **3d** was also conducted by i.v. and oral administration; parameters are shown in Figure 8 and Table 6. With i.v. and oral administration, $t_{1/2}$ of **3d** are 4.47 and 5.28 h, respectively, and oral bioavailability of **3d** is 35.9%. The PK profile indicates **3d** is also suitable for oral administration.

Table 5. PK parameters of **2h** and **3d**.^a

Cpd No.	Admini stration	Dose (mg/kg)	$t_{1/2}$ (h)	t_{\max} (h)	C_{\max} (ng/kg)	AUC _{last} (hr*ng/mL)
2h	ip	20	1.10 ± 0.08	0.25	1490 ± 79.3	978 ± 111.5
3d	ip	20	3.38 ± 0.16	1.00	320 ± 91.7	1716 ± 264.9

Note: ^a ip intraperitoneal administration. Three mice were used for each compound.

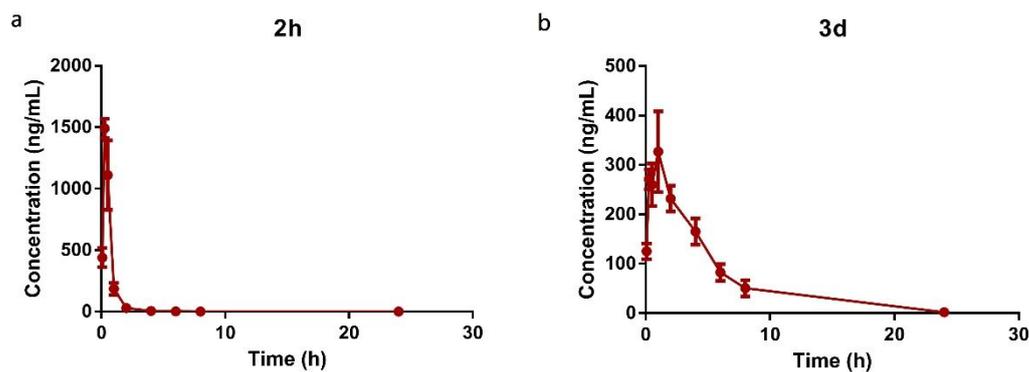


Figure 7. Comparison of PK profile of **2h** and **3d** by ip administration at 20mg/kg. 3 mice were used for each compound.

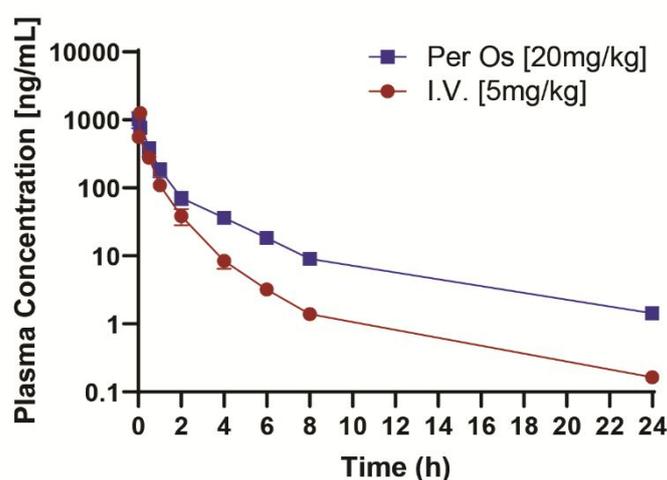


Figure 8. PK profile of **3d** with iv and oral administration three mice were used for each compound.

Table 6. PK parameters of **3d** with iv and oral administration.^a

Admini stration	Dose (mg/kg)	$t_{1/2}$ (h)	C_{max} (ng/kg)	AUC_{last} (hr*ng/mL)	F (%)
iv	5	4.47±0.54	1907±84.65	636±25.5	ND
po	20	5.28±0.55	1025±273.3	903±175.5	35.9±6.7

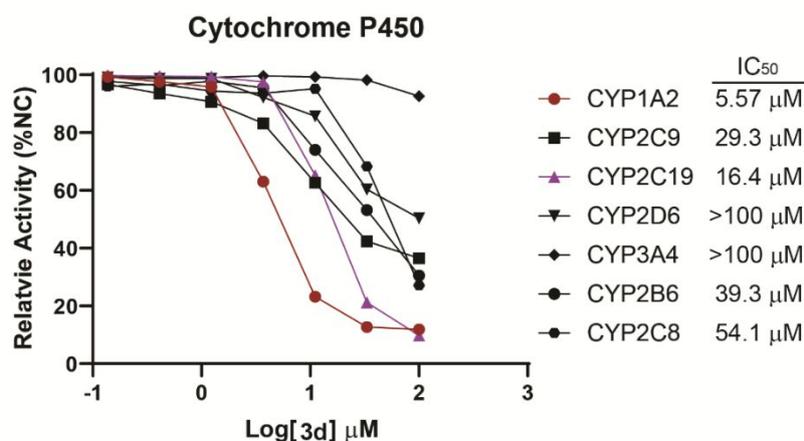
Note: ^a iv: intravenous injection administration; po: oral administration. Three mice

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4 were used for each experiment.
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7 **CYP450 Isoform and hERG Inhibition** 8 9

10 Cytochrome P450 (CYP450) isozymes play an important role in metabolism of
11 drugs. Assessment of the potential of a compound to inhibit a specific CYP450 enzyme
12 allows prediction of potential drug-to-drug interactions that could occur in humans as
13 the compound may alter the metabolism of a co-administered drug. Among all of the
14 CYP450 isoforms, there are six major CYP450s that metabolize 90 percent of drugs.
15 The two most significant are CYP3A4 and CYP2D6.³⁶ According to the FDA Drug-
16 Drug Interaction (DDI) Studies Guidance for Industry (www.fda.gov/media/108130/),
17 we evaluated compound **3d**'s potential to inhibit CYP isoforms including CYP1A2,
18 CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Among all the
19 tested isoforms, only CYP1A2 is moderately inhibited by **3d** with IC_{50} of 5.57 μ M, and
20 other CYP450 isoforms are all weakly inhibited by **3d** with $IC_{50} > 10 \mu$ M (**Figure 9**).
21 Therefore, **3d** does not significantly influence CYP450 enzymatic activity, which
22 indicates it can be safely co-administered with other drugs, with specific attention paid
23 to potential CYP1A2 interaction. We have also examined **3d**'s inhibitory activity
24 against hERG (human ether-à-go-go-related gene, a key potassium ion channel in the
25 heart) to determine potential cardiac-toxicity. hERG inhibitory IC_{50} of **3d** is higher than
26 30 μ M comparing to the positive control verapamil at 400 nM (Supporting Information
27 **Figure S2**). As plasma tissue binding ratio of **3d** in mouse is 98.5% (Supporting
28 Information **Table S3**), the free EC_{50} in mice is approximately 123 nM according to the
29 PK study (Supporting Information **Figure S3**) and give a free safety margin of 243
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24 **Figure 9.** IC₅₀ curves of **3d** in the inhibition of CYP450 isoforms.

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27 **PAINS Exclusion Assay**

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30 Promiscuous compounds appear as “frequent hitters” and display misleading assay
31 readouts. Thus, many of these compounds have been classified as Pan Assay
32 Interference Compounds (PAINS).³⁷ The quinone structure of VK3 contains the
33 potential reactive “Michael acceptor” and can be considered as a promiscuous PAINS
34 compound.³⁷⁻³⁸ Although, pharmacology studies above have shown that our analogs are
35 not promiscuous compounds (weak and differential CYP inhibition, little hERG
36 inhibition, and clear SAR in a whole organism), since **3d** is a VK3 analog, we tested
37 **3d** for its ability for Michael reaction. VK3 and **3d** were subjected to 2-mercaptoethanol,
38 respectively, VK3 rapidly reacts with 2-mercaptoethanol, while our lead compound **3d**
39 does not react with thiol, even over a 24 h period (Supporting Information **Figure S4**),
40 indicating **3d** is not a PAINS compound. The deactivation of the “Michael acceptor” in
41 **3d** may be due to amidation of the naphthoquinone. In addition, unlike VK3, amidated
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4 naphthoquinone does not increase oxygen consumption in cells unlike electron donors
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6 such as VK3 and methylene blue from our previous publication,³⁹ further indicating
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8 that an amidated naphthoquinone ring is also less likely to undergo redox cycle under
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10 physiological conditions.
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13 14 15 **Conclusions**

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18 In this study, we discover a new series of VK analogs with anti-seizure activity by
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20 the modification of VK3 analog **2h** in the group of alkynes. Among these, the best
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22 compounds, **3d** and **3l**, as assessed by the zebrafish PTZ-induced seizure model showed
23
24 significantly improved seizure protection compared to our previous lead compound **2h**
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26 at the 10 μ M and 5 μ M concentrations. Compound **3d** attenuates seizures in a dose-
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28 dependent manner in the mouse 6 Hz and MES models. Full seizure protection is also
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30 attained at doses that do not produce motor impairment in mice, which indicates its
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32 potential in the treatment of pharmaco-resistant epilepsy. Moreover, **3d** is also effective
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34 in the s.c. PTZ mouse model with an ED₅₀ of 349.22 mg/kg, which indicates its efficacy
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36 against clonic seizures. **3d** is well tolerated without obvious toxicity at the tested
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38 concentrations. Further PK studies show **3d** has a longer t_{1/2} and higher AUC than
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40 previous lead compound **2h** with ip administration. With iv and oral administration, t_{1/2}
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42 of **3d** are 4.47 and 5.28 h, respectively; oral bioavailability of **3d** is 35.9%, which
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44 indicates **3d** is suitable for oral administration. In addition to the *in vivo* anti-seizure
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46 efficacy and favorable PK profile, **3d** also shows excellent brain penetration.
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58 For the clinical safety and tolerability concern, **3d** does not show obvious toxicity
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4 at concentration of 50 μM in HT-22 neuronal cells. In mice seizure models, the LD_{50}
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6 for **3d** is greater than 800 mg/kg, which is extremely high for a small molecule and has
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8 sufficient safety index even for its EC_{50} at approximately 200 mg/kg. What is more, **3d**
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10 did not unduly influence CYP450 enzyme activity, it is expected to be safe with a low
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12 incidence of potential drug-to-drug interaction when co-administered with other
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14 therapeutic agents. Furthermore, **3d** has a predictive free hERG-safety margin of 243
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16 folds suggesting a high cardiac-safety index. All of our results indicate that **3d** can be
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18 further developed as a potential ASD with good tolerability for patients with
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20 pharmacoresistant epilepsy.
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28 **Experimental Section**

31 **Material and Methods**

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34 All chemical starting materials including reagents and solvents were purchased
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36 from Sigma-Aldrich, Fisher Scientific, or other chemical vendors and used as received
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38 without purification, unless otherwise noted. ^1H and ^{13}C NMR spectras were
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40 characterized using a Bruker Nanobay 400 MHz instrument in $\text{DMSO-}d_6$ with TMS as
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42 an internal standard. Chemical shifts (δ) in parts per million, and coupling constants (J)
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44 in hertz (Hz) are reported. Mass spectral were determined using Thermo LCQ Fleet
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46 mass spectrometer via electrospray ionization. Column purification was performed via
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48 Teledyne Isco Combiflash 200 on prepacked C18-Aq columns. All target compounds'
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50 purities were at least 95% detected at 254 nM via ESI-LCMS on an Agilent 1100 HPLC
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52 instrument using an ODS HYPERSIL column (5 μm , 4.6 mm \times 250 mm) with
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4 water/methanol gradient plus 0.1% formic acid (**3a-3w**: 0-13 mins from 0% to 100%
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6 methanol, 13-14 mins: from 100% to 0% methanol. **5a** and **5b**: 0-3minutes from 0% to
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8 90% methanol, 3-17 mins from 90% to 100% methanol, and 17-18 mins from 100% to
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10 0% methanol).
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15 **General Procedure A:**

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18 2-bromonaphthalene-1,4-dione (**1**, 0.24g, 1mmol) was suspended in 20 mL anhydrous
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20 ethanol, then different fatty amine was added (2 mmol). The mixture was stirred
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22 overnight at room temperature. The ethanol solvent was removed via rotovap in vacuum,
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24 and the crude product was purified by recrystallization or combi-flash column.
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29 **Synthesis of 3a-3x**

30 2-(neopentylamino)naphthalene-1,4-dione (**3a**)

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33 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2,2-
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35 dimethylpropan-1-amine gave **3c** as an orange-yellow solid, 56% yield. ¹H NMR (400
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37 MHz, DMSO-*d*₆) δ 7.99 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.93 (dd, *J* = 7.6, 1.3 Hz, 1H), 7.82
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39 (td, *J* = 7.5, 1.4 Hz, 1H), 7.72 (td, *J* = 7.5, 1.4 Hz, 1H), 7.18 (t, *J* = 6.8 Hz, 1H), 5.83 (s,
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41 1H), 3.03 (d, *J* = 6.9 Hz, 2H), 0.94 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 182.00,
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43 181.93, 149.68, 135.31, 133.60, 132.58, 130.77, 126.38, 125.73, 100.09, 53.10, 34.06,
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45 27.91 . ESI-MS *m/z*: 244.25 [M + H]⁺.
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53 2-((3,3-dimethylbutyl)amino)naphthalene-1,4-dione (**3b**)

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57 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 3,3-dimethylbutan-
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4 1-amine gave **3d** as an orange-yellow solid, 53% yield. ¹H NMR (400 MHz, DMSO-
5 *d*₆) δ 7.97 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.82 (td, *J* = 7.5, 1.3
6 Hz, 1H), 7.72 (td, *J* = 7.5, 1.4 Hz, 1H), 7.52 (t, *J* = 6.0 Hz, 1H), 5.62 (s, 1H), 3.24 –
7 3.09 (m, 2H), 1.55 – 1.44 (m, 2H), 0.94 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ
8 182.01, 181.54, 148.79, 135.29, 133.69, 132.56, 130.84, 126.33, 125.79, 99.45, 40.99,
9 38.97, 30.19, 29.58. ESI-MS *m/z*: 258.25 [M + H]⁺.

20 2-((4,4-dimethylpentyl)amino)naphthalene-1,4-dione (**3c**)

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22
23 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4,4-
24 dimethylpentan-1-amine gave **3e** as an orange-yellow solid, 63% yield. ¹H NMR (400
25 MHz, DMSO-*d*₆) δ 7.97 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.6, 1.3 Hz, 1H), 7.82
26 (td, *J* = 7.5, 1.4 Hz, 1H), 7.71 (td, *J* = 7.5, 1.4 Hz, 1H), 7.55 (t, *J* = 6.2 Hz, 1H), 5.66 (s,
27 1H), 3.15 (q, *J* = 6.9 Hz, 2H), 1.58-1.51 (m, 2H), 1.22-1.18 (m, 2H), 0.86 (s, 10H). ¹³C
28 NMR (101 MHz, DMSO-*d*₆) δ 182.03, 181.63, 148.94, 135.26, 133.69, 132.54, 130.85,
29 126.32, 125.77, 99.59, 43.09, 41.15, 30.42, 29.63, 23.20. ESI-MS *m/z*: 272.25 [M +
30 H]⁺.

45 2-(isopentylamino)naphthalene-1,4-dione (**3d**)

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47
48 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 3-methylbutan-1-
49 amine gave **3a** as an orange-yellow solid, 63% yield. ¹H NMR (400 MHz, DMSO-*d*₆)
50 δ 7.96 (ddd, *J* = 14.0, 7.7, 1.3 Hz, 2H), 7.82 (td, *J* = 7.6, 1.4 Hz, 1H), 7.72 (td, *J* = 7.5,
51 1.3 Hz, 1H), 7.56 (t, *J* = 6.1 Hz, 1H), 5.65 (s, 1H), 3.18 (q, *J* = 6.9 Hz, 2H), 1.68-1.51
52 (m, 1H), 1.50-1.45 (m, 2H), 0.91 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆)
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4 δ 182.03, 181.60, 148.90, 135.28, 133.67, 132.55, 130.85, 126.33, 125.77, 99.56, 40.66,
5
6
7 36.44, 25.97, 22.82. ESI-MS m/z : 244.25 $[M + H]^+$.

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10 2-((4-methylpentyl)amino)naphthalene-1,4-dione (**3e**)

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12
13 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4-methylpentan-1-
14
15 amine gave **3b** as an orange-yellow solid, 58% yield. 1H NMR (400 MHz, $DMSO-d_6$)
16
17 δ 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.82 (td, $J = 7.5, 1.4$ Hz,
18
19 1H), 7.71 (td, $J = 7.5, 1.4$ Hz, 1H), 7.54 (t, $J = 6.1$ Hz, 1H), 5.66 (s, 1H), 3.15 (q, $J =$
20
21 6.8 Hz, 2H), 1.61-1.49 (m, 3H), 1.23-1.17 (m, 2H), 0.86 (d, $J = 6.6$ Hz, 6H). ^{13}C NMR
22
23 (101 MHz, $DMSO-d_6$) δ 182.04, 181.62, 148.95, 135.25, 133.69, 132.53, 130.85,
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25 126.31, 125.76, 99.60, 42.56, 36.12, 27.69, 25.67, 22.89 . ESI-MS m/z : 258.25 $[M +$
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27 $H]^+$.

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34 2-((4-methylpentan-2-yl)amino)naphthalene-1,4-dione (**3f**)

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36
37 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4-methylpentan-2-
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39 amine gave **3f** as an orange-yellow solid, 58% yield. 1H NMR (400 MHz, $DMSO-d_6$) δ
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41 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.82 (td, $J = 7.6, 1.4$ Hz,
42
43 1H), 7.71 (td, $J = 7.5, 1.4$ Hz, 1H), 7.18 (d, $J = 8.9$ Hz, 1H), 5.71 (s, 1H), 3.64-3.57 (m,
44
45 1H), 1.68-1.55 (m, 2H), 1.34-1.25 (m, 1H), 1.15 (d, $J = 6.3$ Hz, 3H), 0.90-0.80 (m, 6H).
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51 ^{13}C NMR (101 MHz, $DMSO-d_6$) δ 182.15, 181.72, 148.18, 135.30, 133.61, 132.55,
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53 130.85, 126.35, 125.73, 99.43, 46.34, 44.65, 25.10, 23.02, 22.85, 20.02. ESI-MS m/z :
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55 258.25 $[M + H]^+$.

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59 2-((2-methylbutyl)amino)naphthalene-1,4-dione (**3g**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2-methylbutan-1-amine gave **3g** as an orange-yellow solid, 58% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.97 (dd, *J* = 7.6, 1.4 Hz, 1H), 7.93 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.82 (td, *J* = 7.5, 1.3 Hz, 1H), 7.71 (td, *J* = 7.5, 1.3 Hz, 1H), 7.58 (t, *J* = 6.3 Hz, 1H), 5.65 (d, *J* = 4.8 Hz, 1H), 3.15-2.93 (m, 2H), 1.81-1.73 (m, 1H), 1.47 – 1.34 (m, 1H), 1.17 – 1.07 (m, 1H), 0.94 – 0.78 (m, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 182.02, 181.62, 149.16, 135.27, 133.66, 132.54, 130.84, 126.33, 125.76, 99.76, 48.24, 33.43, 27.16, 17.55, 11.57. ESI-MS *m/z*: 244.25 [M + H]⁺.

2-((3-methylbut-2-en-1-yl)amino)naphthalene-1,4-dione(**3h**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and 3-methylbut-2-en-1-amine gave **3h** as an orange-yellow solid, 60% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.90 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.87 (dd, *J* = 7.6, 1.3 Hz, 1H), 7.75 (td, *J* = 7.5, 1.4 Hz, 1H), 7.65 (td, *J* = 7.5, 1.4 Hz, 1H), 7.54 (t, *J* = 6.0 Hz, 1H), 5.52 (s, 1H), 5.16-5.12 (m, 1H), 3.72 (t, *J* = 6.3 Hz, 2H), 1.64 (s, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 182.06, 181.62, 148.78, 135.43, 135.26, 133.64, 132.58, 130.84, 126.30, 125.79, 120.17, 100.10, 40.50, 25.80, 18.36. ESI-MS *m/z*: 242.17 [M + H]⁺.

2-((cyclopropylmethyl)amino)naphthalene-1,4-dione (**3i**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and cyclopropylmethanamine gave **3i** as an orange-yellow solid, 54% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.98 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.6, 1.3 Hz, 1H), 7.82 (td, *J* = 7.5, 1.4 Hz, 1H), 7.72 (td, *J* = 7.5, 1.4 Hz, 1H), 7.52 (t, *J* = 6.1 Hz, 1H), 5.72 (s,

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4 1H), 3.07 (t, $J = 6.4$ Hz, 2H), 1.18-1.09 (m, 1H), 0.50-1.46 (m, 2H), 0.29-0.25 (m, 2H).

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6 ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.08, 181.71, 148.95, 135.30, 133.65, 132.59,
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8 130.81, 126.34, 125.78, 99.88, 46.58, 10.04, 3.98. ESI-MS m/z : 228.17 $[\text{M} + \text{H}]^+$.

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12 2-((2-cyclopropylethyl)amino)naphthalene-1,4-dione (**3j**)

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15 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2-
16 cyclopropylethanamine gave **3j** as an orange-yellow solid, 55% yield. ^1H NMR (400
17 MHz, DMSO- d_6) δ 7.87 (ddd, $J = 13.8, 7.7, 1.3$ Hz, 2H), 7.73 (td, $J = 7.5, 1.4$ Hz, 1H),
18 7.63 (td, $J = 7.5, 1.4$ Hz, 1H), 7.42 (t, $J = 6.1$ Hz, 1H), 5.60 (s, 1H), 3.19-3.14 (m, 2H),
19 1.44-1.38 (m, 2H), 0.70-0.60 (m, 1H), 0.35-0.31 (m, 2H), 0.08-0.05 (m, 2H). ^{13}C NMR
20 (101 MHz, DMSO- d_6) δ 182.03, 181.63, 148.93, 135.27, 133.68, 132.54, 130.82,
21 126.32, 125.77, 99.65, 42.54, 32.79, 9.07, 4.65. ESI-MS m/z : 242.25 $[\text{M} + \text{H}]^+$.

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34 2-((cyclobutylmethyl)amino)naphthalene-1,4-dione (**3k**)

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37 Using the General procedure A, 2-bromonaphthalene-1,4-dione and
38 cyclobutylmethanamine gave **3k** as an orange-yellow solid, 57% yield. ^1H NMR (400
39 MHz, DMSO- d_6) δ 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.93 (dd, $J = 7.6, 1.3$ Hz, 1H), 7.82
40 (td, $J = 7.5, 1.4$ Hz, 1H), 7.71 (td, $J = 7.5, 1.4$ Hz, 1H), 7.50 (t, $J = 6.2$ Hz, 1H), 5.68 (s,
41 1H), 3.21 (t, $J = 6.7$ Hz, 2H), 2.71-2.60 (m, 1H), 2.05-1.97 (m, 2H), 1.887-1.80 (m,
42 2H), 1.77-1.68 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.03, 181.65, 149.16,
43 135.27, 133.65, 132.55, 130.83, 126.32, 125.75, 99.81, 47.52, 33.76, 26.01, 18.35. ESI-
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56 MS m/z : 242.25 $[\text{M} + \text{H}]^+$.

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59 2-((2-cyclobutylethyl)amino)naphthalene-1,4-dione (**3l**)

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4 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2-
5
6 cyclobutylethanamine gave **3l** as an orange-yellow solid, 49% yield. ¹H NMR (400
7
8 MHz, DMSO-*d*₆) δ 7.95 (ddd, *J* = 12.6, 7.7, 1.3 Hz, 2H), 7.82 (td, *J* = 7.5, 1.3 Hz, 1H),
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10 7.71 (td, *J* = 7.5, 1.4 Hz, 1H), 7.49 (t, *J* = 6.2 Hz, 1H), 5.63 (s, 1H), 3.09 (q, *J* = 6.8 Hz,
11
12 2H), 2.35-2.25 (m, 1H), 2.09-1.99 (m, 2H), 1.88-1.73 (m, 2H), 1.70-1.57 (m, 4H). ¹³C
13
14 NMR (101 MHz, DMSO-*d*₆) δ 182.01, 181.57, 148.90, 135.26, 133.68, 132.53, 130.83,
15
16 126.31, 125.77, 99.54, 40.49, 34.68, 33.69, 28.14, 18.64. ESI-MS *m/z*: 256.25 [M +
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18 H]⁺.
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2-((2-cyclopentylethyl)amino)naphthalene-1,4-dione (**3m**)

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28 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2-
29
30 cyclopentylethanamine gave **3m** as an orange-yellow solid, 51% yield. ¹H NMR (400
31
32 MHz, DMSO-*d*₆) δ 7.98 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.82
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34 (td, *J* = 7.5, 1.4 Hz, 1H), 7.72 (td, *J* = 7.5, 1.4 Hz, 1H), 7.55 (t, *J* = 6.1 Hz, 1H), 5.65 (s,
35
36 1H), 3.21-3.16 (m, 2H), 1.87-1.74 (m, 3H), 1.62-1.45 (m, 6H), 1.14-1.04 (m, 2H). ¹³C
37
38 NMR (101 MHz, DMSO-*d*₆) δ 182.05, 181.59, 148.89, 135.28, 133.69, 132.55, 130.86,
39
40 126.33, 125.78, 99.56, 41.77, 37.82, 33.86, 32.58, 25.13. ESI-MS *m/z*: 270.25 [M +
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42 H]⁺.
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2-((2-cyclohexylethyl)amino)naphthalene-1,4-dione (**3n**)

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53 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 2-
54
55 cyclohexylethanamine gave **3n** as an orange-yellow solid, 57% yield. ¹H NMR (400
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57 MHz, DMSO-*d*₆) δ 7.98 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.6, 1.3 Hz, 1H), 7.83
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(td, $J = 7.5, 1.4$ Hz, 1H), 7.72 (td, $J = 7.5, 1.4$ Hz, 1H), 7.53 (t, $J = 6.0$ Hz, 1H), 5.64 (s, 1H), 3.19 (q, $J = 6.7$ Hz, 2H), 1.74-1.60 (m, 5H), 1.51-1.45 (m, 2H), 1.36-1.04 (m, 4H), 0.96-0.87 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.05, 181.60, 148.90, 135.29, 133.70, 132.56, 130.87, 126.34, 125.78, 99.57, 35.36, 35.03, 33.06, 26.52, 26.19. ESI-MS m/z : 284.25 $[\text{M} + \text{H}]^+$.

2-((2-(diethylamino)ethyl)amino)naphthalene-1,4-dione (**3o**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and N1,N1-diethylethane-1,2-diamine gave **3t** as an orange-yellow solid, 61% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.98 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.83 (td, $J = 7.5, 1.4$ Hz, 1H), 7.72 (td, $J = 7.5, 1.4$ Hz, 1H), 7.22 (t, $J = 5.7$ Hz, 1H), 5.69 (s, 1H), 3.21 (q, $J = 6.3$ Hz, 2H), 2.63 (t, $J = 6.6$ Hz, 2H), 2.51 (q, $J = 7.1$ Hz, 5H), 0.96 (t, $J = 7.1$ Hz, 6H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 181.94, 181.70, 148.69, 135.36, 133.66, 132.63, 130.74, 126.36, 125.83, 99.95, 50.21, 46.79, 12.34. ESI-MS m/z : 273.25 $[\text{M} + \text{H}]^+$.

2-((2-(ethyl(methyl)amino)ethyl)amino)naphthalene-1,4-dione (**3p**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and N1-ethyl-N1-methylethane-1,2-diamine gave **3u** as an orange-yellow solid, 68% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.83 (td, $J = 7.5, 1.4$ Hz, 1H), 7.72 (td, $J = 7.5, 1.4$ Hz, 1H), 7.19 (t, $J = 5.6$ Hz, 1H), 5.69 (s, 1H), 3.24 (q, $J = 6.3$ Hz, 2H), 2.56 (t, $J = 6.5$ Hz, 2H), 2.42 (q, $J = 7.1$ Hz, 2H), 2.19 (s, 3H), 0.99 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 181.93, 181.72, 148.68,

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4 135.35, 133.64, 132.64, 130.75, 126.35, 125.83, 100.02, 54.17, 51.20, 41.54, 39.92,
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6 12.61. ESI-MS m/z: 259.17 [M + H]⁺.
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9
10 2-((2-(dimethylamino)ethyl)amino)naphthalene-1,4-dione (**3q**)
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12
13 Using the General procedure A, 2-bromonaphthalene-1,4-dione and N1,N1-
14 dimethylethane-1,2-diamine gave **3v** as an orange-yellow solid, 69% yield. ¹H NMR
15 (400 MHz, DMSO-*d*₆) δ 7.97 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.94 (dd, *J* = 7.6, 1.3 Hz, 1H),
16 7.82 (td, *J* = 7.5, 1.4 Hz, 1H), 7.72 (td, *J* = 7.5, 1.4 Hz, 1H), 7.17 (t, *J* = 5.7 Hz, 1H),
17 5.69 (s, 1H), 3.24 (q, *J* = 6.2 Hz, 2H), 2.49-2.48 (m, 2H), 2.19 (s, 6H). ¹³C NMR (101
18 MHz, DMSO-*d*₆) δ 181.93, 181.74, 148.69, 135.35, 133.62, 132.64, 130.75, 126.35
19 , 125.83, 100.04, 56.59, 45.48, 40.02. ESI-MS m/z: 245.17 [M + H]⁺
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32 2-((3-(dimethylamino)propyl)amino)naphthalene-1,4-dione (**3r**)
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35 Using the General procedure A, 2-bromonaphthalene-1,4-dione and N1,N1-
36 dimethylpropane-1,3-diamine gave **3w** as an orange-yellow solid, 61% yield. ¹H NMR
37 (400 MHz, DMSO-*d*₆) δ 7.97 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.93 (dd, *J* = 7.7, 1.3 Hz, 1H),
38 7.82 (td, *J* = 7.5, 1.4 Hz, 2H), 7.71 (td, *J* = 7.5, 1.4 Hz, 1H), 5.67 (s, 1H), 3.21 (q, *J* =
39 6.5 Hz, 2H), 2.28 (t, *J* = 6.6 Hz, 2H), 2.15 (s, 6H), 1.71 (p, *J* = 6.7 Hz, 2H). ¹³C NMR
40 (101 MHz, DMSO-*d*₆) δ 182.01, 181.62, 149.09, 135.26, 133.71, 132.54, 130.84,
41 126.31, 125.78, 99.56, 57.42, 45.63, 41.22, 25.56. ESI-MS m/z: 259.17 [M + H]⁺
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54 2-((3-(methyl(phenyl)amino)propyl)amino)naphthalene-1,4-dione (**3s**)
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57 Using the General procedure A, 2-bromonaphthalene-1,4-dione and N1-methyl-N1-
58 phenylpropane-1,3-diamine gave **3x** as an orange-yellow solid, 64% yield. ¹H NMR
59
60

(400 MHz, DMSO- d_6) δ 7.98 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.82 (td, $J = 7.5, 1.3$ Hz, 1H), 7.72 (td, $J = 7.5, 1.4$ Hz, 1H), 7.64 (t, $J = 6.1$ Hz, 1H), 7.16 – 7.07 (m, 2H), 6.75 – 6.65 (m, 2H), 6.58 (t, $J = 7.2$ Hz, 1H), 5.70 (s, 1H), 3.39 (s, 2H), 3.22 (q, $J = 6.8$ Hz, 2H), 2.87 (s, 3H), 1.82 (p, $J = 7.1$ Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.00, 181.70, 149.43, 149.03, 135.25, 133.66, 132.57, 130.89, 129.41, 126.33, 125.77, 116.11, 112.50, 99.85, 49.91, 38.34, 24.95. ESI-MS m/z : 321.17 $[\text{M} + \text{H}]^+$

2-((3-hydroxybutyl)amino)naphthalene-1,4-dione (**3t**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4-aminobutan-2-ol gave **3o** as an orange-yellow solid, 54% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.6, 1.3$ Hz, 1H), 7.82 (td, $J = 7.5, 1.4$ Hz, 1H), 7.71 (td, $J = 7.5, 1.4$ Hz, 1H), 7.59 (t, $J = 5.9$ Hz, 1H), 5.67 (s, 1H), 4.70 (d, $J = 4.7$ Hz, 1H), 3.75-3.68 (m, 1H), 3.24 (q, $J = 6.7$ Hz, 2H), 1.76-1.51 (m, 2H), 1.10 (d, $J = 6.2$ Hz, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 182.01, 181.62, 148.92, 135.27, 133.70, 132.54, 130.83, 126.31, 125.79, 99.58, 64.83, 39.91, 36.81, 24.20. ESI-MS m/z : 246.25 $[\text{M} + \text{H}]^+$.

2-((4-hydroxy-3-methylbutyl)amino)naphthalene-1,4-dione (**3u**)

Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4-amino-2-methylbutan-1-ol gave **3p** as an orange-yellow solid, 57% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.97 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.82 (td, $J = 7.5, 1.4$ Hz, 1H), 7.71 (td, $J = 7.5, 1.4$ Hz, 1H), 7.56 (t, $J = 6.0$ Hz, 1H), 5.66 (s, 1H),

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4 4.53 (t, $J = 5.2$ Hz, 1H), 3.28-3.14 (m, 4H), 1.70-1.55 (m, 2H), 1.40-1.31 (m, 1H), 0.88
5
6 (d, $J = 6.7$ Hz, 3H). ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ 182.04, 181.60, 148.89, 135.27,
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8 133.69, 132.54, 130.84, 126.31, 125.78, 99.60, 66.40, 40.56, 33.80, 31.43, 17.22. ESI-
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10 MS m/z: 260.25 $[\text{M} + \text{H}]^+$.

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15 2-((2-hydroxybutyl)amino)naphthalene-1,4-dione (**3v**)

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18 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 1-aminobutan-2-ol
19
20 gave **3q** as an orange-yellow solid, 53% yield. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 7.98
21
22 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.94 (dd, $J = 7.7, 1.3$ Hz, 1H), 7.82 (td, $J = 7.5, 1.4$ Hz, 1H),
23
24 7.72 (td, $J = 7.5, 1.4$ Hz, 1H), 7.24 (t, $J = 6.0$ Hz, 1H), 5.74 (s, 1H), 4.94 (d, $J = 5.2$ Hz,
25
26 1H), 3.67-3.60 (m, 1H), 3.2-3.02 (m, 2H), 1.53-1.30 (m, 2H), 0.90 (t, $J = 7.4$ Hz, 3H).
27
28 ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ 181.99, 181.79, 149.08, 135.32, 133.63, 132.61,
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30 130.78, 126.34, 125.79, 100.14, 69.51, 48.47, 28.10, 10.41. ESI-MS m/z: 246.25 $[\text{M} +$
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32 $\text{H}]^+$.

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39 2-((3-hydroxy-3-methylbutyl)amino)naphthalene-1,4-dione (**3w**)

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42 Using the General procedure A, 2-bromonaphthalene-1,4-dione and 4-amino-2-
43
44 methylbutan-2-ol gave **3r** as an orange-yellow solid, 58% yield. ^1H NMR (400 MHz,
45
46 $\text{DMSO-}d_6$) δ 7.95 (ddd, $J = 9.3, 7.7, 1.3$ Hz, 2H), 7.82 (td, $J = 7.5, 1.3$ Hz, 1H), 7.71
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48 (td, $J = 7.5, 1.4$ Hz, 1H), 7.63 (t, $J = 5.7$ Hz, 1H), 5.65 (s, 1H), 4.58 (s, 1H), 3.28-3.23
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50 (m, 2H), 1.71-1.68 (m, 2H), 1.16 (s, 6H). ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ 182.00,
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52 181.57, 148.82, 135.27, 133.74, 132.53, 130.83, 126.30, 125.80, 99.47, 69.12, 38.96,
53
54 29.78. ESI-MS m/z: 260.25 $[\text{M} + \text{H}]^+$.

General procedure B:

2-methylnaphthalene-1,4-dione (**3**, 0.24g, 1mmol) was dissolved in an mixed solution of 10 mL methanol and 10 mL dichloromethane, then 3-methylbutan-1-amine (0.19g, 2mmol) or 3,3-dimethylbutan-1-amine (0.20g, 2mmol) was added. The mixture was allowed to stir overnight at room temperature. After the reaction finished, ethanol was removed under vacuum, and the rude product was purified by combi-flash column.

2-((3,3-dimethylbutyl)amino)-3-methylnaphthalene-1,4-dione (5a**)**

Using the General procedure B, 2-methylnaphthalene-1,4-dione and 3,3-dimethylbutan-1-amine gave **5b** as an orange-yellow solid, 64% yield. ¹H NMR (400 MHz, Chloroform-*d*) δ 8.08 (dt, *J* = 7.8, 1.5 Hz, 1H), 7.98 (dq, *J* = 7.7, 1.5 Hz, 1H), 7.67 (tq, *J* = 7.7, 1.3 Hz, 1H), 7.56 (tq, *J* = 7.5, 1.3 Hz, 1H), 5.70 (d, *J* = 5.6 Hz, 1H), 3.59-3.54 (m, 2H), 2.25 (d, *J* = 1.8 Hz, 3H), 1.77-1.66 (m, 1H), 1.56-1.51 (m, 2H), 0.95 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 183.55, 182.54, 146.18, 134.31, 133.55, 131.77, 130.29, 126.19, 125.96, 111.98, 43.78, 39.82, 25.72, 22.50, 11.25. ESI-MS *m/z*: 272.17 [M + H]⁺

2-(isopentylamino)-3-methylnaphthalene-1,4-dione (5b**)**

Using the General procedure B, 2-methylnaphthalene-1,4-dione and 3-methylbutan-1-amine gave **5b** as an orange-yellow solid, 60% yield. ¹H NMR (400 MHz, Chloroform-*d*) δ 8.08 (dd, *J* = 7.8, 1.0 Hz, 1H), 7.98 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.67 (td, *J* = 7.6, 1.4 Hz, 1H), 7.56 (td, *J* = 7.5, 1.3 Hz, 1H), 5.65 (s, 1H), 3.59-3.54 (m, 2H), 2.25 (s, 3H), 1.57-1.53 (m, 2H), 0.97 (s, 9H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 183.52, 182.56,

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4 146.21, 134.29, 133.57, 131.75, 130.31, 126.19, 125.96, 111.93, 44.91, 42.08, 30.04,
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6 29.53, 11.23. ESI-MS m/z: 258.17 [M + H]⁺
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10 **Induction and Recording of Activity in Zebrafish**

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12
13 Zebrafish (AB strain) were from the Zebrafish International Resource Center
14
15 (supported by P40 RR012546 from NIH-NCRR). All zebrafish studies have been
16
17 approved by the Medical University of South Carolina Institutional Animal Care and
18
19 Use Committee (IACUC-2018-00278) and performed in accordance with the
20
21 guidelines. All experimental comparisons were from 7 dpf fish from the same clutch.
22
23 The method used here is a slightly modified version of the one characterized by Baraban
24
25 *et al.*,¹¹ which indicates that the total distance traveled by zebrafish after PTZ induction
26
27 of seizures quantitatively reflects seizure activity. 48-well Nunc plates (Cat # 12-565-
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29 322, FisherSci, Pittsburg, PA) were used to position a single zebrafish per well.
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31 Compounds were dissolved in DMSO and further diluted in E3 buffer (0.33 mM CaCl₂,
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33 5 mM NaCl, 0.33 mM MgSO₄, and 0.17 mM KCl). The larvae are treated at 37°C for
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35 1 h in sublethal concentrations of the compounds. After 1 h, PTZ (15 mM in 500 μL
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37 total volume per well) was added immediately to each well. The plate was immediately
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39 loaded into the Daniovision instrument (Noldus Information Technology) to track
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41 zebrafish movements over 15 min. At termination, zebrafish were monitored visually
42
43 for overt toxicity including failure to startle and increased mortality. The Ethovision
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45 XT software (Noldus) recorded live video images. The total movement in millimeters
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47 was measured in one-minute time bins and compared between groups using one-way
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49 ANOVA statistical analyses by mean ± SEM (GraphPad Prism Software).
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HT-22 Cell Toxicity Study

HT-22 neuronal cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM/high glucose) with 10% fetal bovine serum (FBS) and 1% of antibiotic-antimycotic (Amphotericin B, Penicillin, and Streptomycin; Invitrogen) at 37 °C in 5% CO₂. 2000 cells per well were plated in a clear 96-well plate, 12 h later, serially diluted compounds were added and incubated for 48 h. CellTiter-Blue (resazurin cell viability assay reagent) was added at a final concentration of 0.125 mg/mL to each well. The cell viability was determined after 2 h via resorufin fluorescence intensity at ex 560 nm/em 590 nm using a Tecan M200 Pro spectrophotometer. Data were processed and fitted via Prism (GraphPad) by subtracting the background and normalizing to the control wells.

6 Hz 32mA and 44mA Mouse Test

Investigational compounds were examined for their ability to attenuate psychomotor seizures resulting from a low-frequency (6 Hz), long-duration (3 sec) electric shock delivered through corneal electrodes.¹⁶ 8 male CF-1 mice were used per group per concentration. Experiments were conducted at stimulus intensities of 32 mA and 44 mA. Typically, the seizure was characterized by a brief stun followed immediately by jaw clonus, forelimb clonus, twitching of the vibrissae, and Straub tail lasting for at least 1 second. Animals not displaying this behavior were considered "protected". **3d** was dissolved in miglyol840/DMSO (95%/5%) and was dosed via ip administration. Quantification of the ED₅₀ value was conducted at the time of peak effect (TPE). To determine the TPE, mice were treated with the investigational

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4 compound and tested at 0.25 h, 0.5 h, 1.0 h, 2.0 h 4.0 h, or based on time-points from
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6 previous studies. Groups of $n = 8$ mice were tested with various doses of the
7
8 investigational compound. The data for each condition were presented as N/F, where N
9
10 = number of animals protected and F = number of animals tested. The ED_{50} , 95%
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12 confidence interval, the slope of the regression line, and the SEM of the slope are
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14 calculated by Probit analysis.⁴⁰
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20 **Maximal Electroshock (MES) Test**

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23 An alternating current at 60 Hz 50 mA was delivered via corneal electrodes for 2s
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25 to mice. An electrolyte solution containing 0.5% tetracaine HCl anesthetic agent was
26
27 applied to the eyes before the electric shock. 8 male CF-1 mice were used per group per
28
29 concentration. **3d** was suspended in 0.5% hydroxypropyl methylcellulose (HPMC) and
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31 was dosed via ip administration. If no hindlimb tonic extension was observed during
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33 the seizure, the animal was considered “protected” from the MES-induced seizures.¹⁹
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39 **Subcutaneous (s.c.) Pentylenetetrazole (PTZ) Test**

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42 The s.c. PTZ model determines the ability of potential anti-seizure compounds to
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44 attenuate a clonic forebrain seizure in mice.⁴¹ 1 h after ASD-treatment, 85 mg/kg PTZ
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46 was injected subcutaneously into the midline of the mouse neck. The mice were
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48 observed for the next 30 min to determine if clonic spasms of the fore and/or hindlimbs,
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50 jaws, or vibrissae had occurred lasting 3-5s in an isolation cage to minimize stress and
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52 other variables. 8 male CF-1 mice were used per group per concentration. **3d** was
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54 suspended in 0.5% HPMC and was dosed via ip administration. Mice without spasms
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4 or twitching were considered protected.¹⁹
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7 **Behavioral Toxicity in Mice**

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10 To assess the toxicity of compounds, all animals were visually inspected for signs
11 of movement impairment due to neurological or muscular dysfunction. The rotarod
12 procedure was used to evaluate motor impairment of each mouse tested.¹⁶ To examine
13 whether the mouse can maintain its normal equilibrium and movement for a defined
14 period of time, it is placed on a knurled rod that rotates at a constant speed of 6 rpm. If
15 the mouse fell three times within one minute, it was considered impaired. The rotarod
16 assessment was performed on the same animals prior to the 6 Hz assay.
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29 **Fluorometric ATP Assay**

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32 ATP content was measured by Biovision's ATP Fluorometric Assay Kit (K354-
33 100, Biovision, Milpitas, CA) as per our previous studies, with minor modifications
34 (PMID:26519465). Briefly, one 7 dpf zebrafish was placed in each well of a 48-well
35 plate. Fish were kept in a 28.5°C incubator and treated for 24 h with **3d** and **3l**. Stock
36 solutions were dissolved in DMSO. Solutions were further diluted in E3 buffer (5 mM
37 NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) to final concentrations of
38 0.5 μM and 1 μM in a total volume of 500 μL per well. DMSO was used in the control
39 wells. Twelve 7 dpf fish per condition were collected in one microfuge tube and flash
40 frozen in liquid nitrogen. Contents were stored at -80°C for later use. Fish were
41 resuspended in 200 μL ATP buffer, homogenized, and centrifuged at 12,000 x g for 5
42 min. The supernatant fractions were retained for ATP measurements following the
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4 manufacturer's protocol.
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7 HT-22 hippocampal neuronal cells were maintained in DMEM/high glucose
8 supplemented with 10% FBS (Hyclone, GE Healthcare, Chicago, IL), 1% antibiotic-
9 antimycotic (Amphotericin B, Penicillin, and Streptomycin; Invitrogen) and 1%
10 sodium pyruvate in a 37°C incubator with 5% CO₂. HT-22 were seeded in 2 mL volume
11 of media on 6 well plates (Grenier Bio-one, Monroe, NC) at a density of 1.5 x 10⁵ cells
12 per well for 24 h. In the same growth medium, the cells were treated for an additional
13 24 hours with 0.5 μM of **3d** and **3l**. The treated plates were maintained in a 37°C, 5%
14 CO₂ incubator. The cells were transferred to 15 mL falcon conical tubes after harvest,
15 and centrifuged at 1000 x g for 5 min. Cell pellets resuspended in 6 mL cold Hank's
16 Balanced Salt Solution (HBSS) (Hyclone, GE Healthcare, Chicago, IL) and centrifuged
17 for another 5 min. The HBSS solution was removed and lysates were transferred to an
18 -80°C freezer until further use. Cell lysates were dissolved in 100 μL ATP Buffer as
19 listed in the original protocol.
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41 Zebrafish supernatant fractions and cell lysates were measured in a Tecan M200
42 Pro spectrophotometer plate reader at ex 535 nm/em 587 nm. ATP content was
43 normalized by total protein using the Pierce BCA protein assay kit (#23255, Thermo
44 Fisher Scientific, Waltman, MA).
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52 **Brain/Plasma Concentration Ratio Test**

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56 Male CF-1 mice were housed in individual cages on a 12 h light and 12 h dark
57 cycle, with the room temperature at 22 ± 3°C. Before the PK studies, mice were fasted
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4 overnight. Water was provided ad libitum throughout the study and food was returned
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6 after the 6 h blood sample time. **3d** was dissolved in 95% Miglyol 840: 5% DMSO.
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9 Animals were dosed via ip injections and blood samples were collected at the indicated
10
11 time. Acetonitrile containing internal standard was added to plasma (3:1 ratio) to
12
13 precipitate out the proteins. The resulting supernatants were isolated via centrifugation
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15 at 3000 g for 10 min. Each sample supernatant was then analyzed via LCMS/MS. Serial
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17 dilution of the calibration standards and quality controls (1 mg/mL) were diluted in
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19 methanol : water (1:1, v/v) and spiked into blank plasma to give the standard curve
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21 from 1 ng/mL to 10 µg/mL for the compound and the quality control samples at three
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23 different concentration levels.
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30 For the tissue sample PK analysis, PBS buffer and tissue sample (3:1) homogenized
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32 to obtain each tissue homogenate sample. Subsequently, acetonitrile with the internal
33
34 standard was added to each tissue homogenate at 3:1 ratio, and the mixture was
35
36 vortexed, centrifuged (3000 g for 10 min) and supernatant isolated for analysis by LC-
37
38 MS/MS. Calibration standards curves were established similarly as described above.
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40 For each drug candidate, LC-MS/MS analysis was performed utilizing the multiple
41
42 reaction monitoring protocol to detect their characteristic ions. Plasma and tissue
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44 homogenate concentration were measured as described above. The brain/plasma
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46 concentration ratio was obtained by mean tissue concentration (ng/g) mean plasma
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48 concentration (ng/mL).
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57 **Pharmacokinetics**

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4 Male CD-1 Mice were maintained as described above. The dosing solution of each
5
6 test compound was prepared in a desired oral or intravenous formulation using 20%
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8 DMA:40% PEG300:40% H₂O; 5% DMSO:95% Neobee was used for ip administration.
9
10 Three animals are dosed via gavage needle for oral and ip administration at 20 mg/kg
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12 (20 mL/kg) or via tail vein injection for iv administration at 5 mg/kg (5 mL/kg). Blood
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14 samples (30-50 μ L per sample) were drawn via saphenous, jugular, or submandibular
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16 vein at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing. Blood samples were collected
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18 in Greiner MiniCollect K₂EDTA tubes and centrifuged at 15,000g for 5 min to obtain
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20 plasma samples. Three volumes of acetonitrile containing internal standard was added
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22 to one volume of plasma and samples were centrifuged at 3000 g for 10 min. The
23
24 resulting supernatants were removed for analysis by LC-MS/MS to determine the
25
26 targeted compound concentration. Calibration standard and quality control curves were
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28 determined as described in the above section and treated identically as the PK samples.
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30 The targeted compound and the internal standard were identified using LC-MS/MS
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32 under multiple reaction monitoring mode to detect their characteristic ions. Plasma
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34 concentrations at each time point were then plotted against the time for the analysis.
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36 Linear trapezoidal method was used to calculate AUC. PK parameters were fitted using
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38 the non-compartmental analysis. For intravenous administration PK parameters, $t_{1/2}$,
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40 C_0 , AUC, volume of distribution at steady-state (V_{ss}), total plasma clearance (CL_p),
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42 and mean residence time (MRT) were determined. For extravascular administration, $t_{1/2}$,
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44 C_{max} , t_{max} , AUC, mean residence time (MRT), and bioavailability (F%) were
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46 determined. All parameters were calculated for each animal as well as the combined
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4 mean, standard deviation, and coefficient of variation for the group.
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7 **CYP450 Inhibition Study**

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10 The CYP450 inhibition study was conducted by Touchstone Biosciences. 0.2
11 mg/mL mix-gender, pooled human liver microsomes (HLM), 100 mM potassium
12 phosphate buffer, and a 2 mM NADPH cofactor were used in the inhibition assay.
13 Individual CYP450 isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4,
14 CYP2B6, CYP2C8) were investigated where isoform-specific probe substrates (at
15 concentrations near the specific enzyme's K_m) were incubated individually with HLM
16 and compound **3d** or a corresponding positive control inhibitor. The organic solvent
17 was methanol (DMSO if compounds were not soluble in methanol) which was
18 maintained at a final concentration no greater than 1% in the assay. After incubation at
19 37°C for 15-30 min, the reaction was stopped by adding two volumes of acetonitrile
20 containing the internal standard. The resulting samples were centrifuged and the
21 supernatants were collected and analyzed for metabolites of the probe substrates by LC-
22 MS/MS at each of the tested concentrations. A decrease in the formation of the
23 metabolites compared to vehicle control was used to calculate percent inhibition at each
24 concentration or an IC_{50} value (test compound concentration which produces 50%
25 inhibition). The percent inhibition was calculated based on a decrease in the formation
26 of the metabolite of each specific substrate compared to vehicle control (negative
27 control), whereas an IC_{50} was calculated based on a sigmoidal dose-response model
28 with variable slope. Bottom and top parameter constraints were fixed to 0 and 100,
29 respectively. Compounds can be generally categorized as follows:
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Potent inhibitor: $IC_{50} < 1 \mu M$

Moderate inhibitor: $1 \mu M \leq IC_{50} \leq 10 \mu M$

Weak- or non-inhibitor: $IC_{50} > 10 \mu M$

Supporting Information:

The Supporting Information is available free of charge at <https://pubs.acs.org>. Time course experiment and toxicity **3l** in the acute, electrically evoked mouse seizure models; time course experiment and toxicity of **3d** in mouse; experiment method and result of plasma protein binding ratio of **3d** in mouse and human; Experiment method and result of spontaneous REDs assay and hERG inhibition assay; The reaction of VK3 and **3d** with 2-mercaptoethanol; Pharmacokinetic Data of **3d** at concentration of 400 mg/kg and 200 mg/kg with ip administration; 1H -NMR and ^{13}C -NMR spectrum of all target compounds (PDF); HPLC traces and purity of the target compounds.

Molecular Formula Strings (CSV).

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5
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7
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9
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22 23 **Competing interests**

24
25
26 C.J.C. and S.S.L.C. are the co-founders of Neuroene Therapeutics. The other
27
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29
30 of the authors and does not necessarily represent the official views of the National
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32 Institutes of Health.
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36 37 **Abbreviations used**

38
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40 ASDs, Anti-seizure drugs; PTZ, Pentylenetetrazol; NINDS, Neurological
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42 Disorders and Stroke; ETSP, Epilepsy Therapy Screening Program; MES, maximal
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44 electroshock seizure; s.c. PTZ, Subcutaneous PTZ seizure threshold; VK, Vitamin K;
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46 TD₅₀, median toxic dose; tPSA, Topological molecules polar surface area; C_{max}, mean
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48 peak plasma concentration; SEM, Mean and standard error of mean; TPE, Time of peak
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50 effect; HPMC, Hydroxypropyl methylcellulose; MRT, Mean residence time; F%,
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52 Bioavailability; RED, Rapid equilibrium dialysis.
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