Methylated flavonoids as anti-seizure agents : Naringenin 4′,7-dimethyl ether attenuates epileptic seizures in zebrafish and mouse models

Methylated flavonoids as anti-seizure agents: naringenin 4′,7-dimethyl ether attenuates epileptic seizures in zebrafish and mouse models

Daniëlle Copmans[a*], Adriana M. Orellana-Paucar[a,b,1*], Gert Steurs[c], Yifan Zhang[a], Annelii Ny[a], Kenn Foubert[d], Vasiliki Exarchou[d], Aleksandra Siekierska[a], Youngju Kim[c], Wim De Borggraeve[c], Wim Dehaen[c], Luc Pieters[d], Peter A. M. de Witte[a#]

[a]Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven - University of Leuven, Leuven, Belgium

[b]Carrera de Bioquímica y Farmacia, Facultad de Ciencias Químicas, Universidad de Cuenca, Cuenca, Ecuador

[c]Molecular Design and Synthesis, Department of Chemistry, KU Leuven - University of Leuven, Leuven, Belgium

[d]Natural Products & Food Research and Analysis (NatuRA), Department of Pharmaceutical Sciences, University of Antwerp, Antwerp, Belgium

[1]Current affiliation: Facultad de Ciencias Médicas, Universidad de Cuenca, Cuenca, Ecuador

[*]Authors contributed equally to this work.

[#]Corresponding author: Prof. Dr. Peter A. M. de Witte, Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Herestraat 49 bus 824, 3000 Leuven, Belgium. E-mail: peter.dewitte@kuleuven.be

ABSTRACT

Epilepsy is a neurological disease that affects more than 70 million people worldwide and is characterized by the presence of spontaneous unprovoked recurrent seizures. Existing anti-seizure drugs (ASDs) have side effects and fail to control seizures in 30 % of patients due to drug resistance. Hence, safer and more efficacious drugs are sorely needed. Flavonoids are polyphenolic structures naturally present in most plants and consumed daily with no adverse effects reported. These structures have shown activity in several seizure and epilepsy animal models through allosteric modulation of $GABA_A$ receptors, but also via potent anti-inflammatory action in the brain. As such, dietary flavonoids offer an interesting source for ASD and anti-epileptogenic drug (AED) discovery, but their pharmaceutical potential is often hampered by metabolic instability and low oral bioavailability. It has been argued that their drug-likeness can be improved via methylation of the free hydroxyl groups, thereby dramatically enhancing metabolic stability and membrane transport, facilitating absorption and highly increasing bioavailability. Since no scientific data is available regarding the use of methylated flavonoids in the fight against epilepsy, we studied naringenin (NRG), kaempferol (KFL), and three methylated derivatives, i.e., naringenin 7-*O*-methyl ether (NRG-M), naringenin 4′,7-dimethyl ether (NRG-DM), and kaempferide (4′-*O*-methyl kaempferol) (KFD) in the zebrafish pentylenetetrazole (PTZ) seizure model. We demonstrate that the methylated flavanones NRG-DM and NRG-M are highly effective against PTZ-induced seizures in larval zebrafish, whereas NRG and the flavonols KFL and KFD possess only a limited activity. Moreover, we show that NRG-DM is active in two standard acute mouse seizure models, i.e., the timed i.v. PTZ seizure model and the 6-Hz psychomotor seizure model. Based on these results, NRG-DM is proposed as a lead compound that is worth further investigation for the treatment of generalized seizures and drug-resistant focal seizures. Our data therefore highlights the potential of methylated flavonoids in the search for new and improved ASDs.

KEYWORDS

epilepsy, drug discovery, methylated flavonoids, naringenin, zebrafish, mouse

Chemical compounds studied in this article

Naringenin (PubChem CID: 932); Naringenin 7-*O*-methyl ether (PubChem CID: 73571); Naringenin 4′,7-dimethyl ether (PubChem CID: 14057196); kaempferol (PubChem CID: 5280863); kaempferide (PubChem CID: 5281666)

1. INTRODUCTION

Epilepsy is one of the most common neurological diseases affecting more than 70 million people worldwide (Ngugi et al., 2010; Singh and Trevick, 2016). It is characterized by the presence of spontaneous unprovoked recurrent seizures and involves risks of comorbidities, anxiety, depression and increased mortality. Moreover, patients suffer from social stigma and the stress of living with an unpredictable disease that can lead to loss of autonomy in daily life (Moshé et al., 2015). Even though over 25 anti-seizure drugs (ASDs) are on the market, seizures cannot be controlled in 30 % of patients due to drug resistance (Dalic and Cook, 2016; Franco et al., 2016; Moshé et al., 2015). In fact, the probability of achieving seizure freedom did not lower since the 70s, despite the availability of new-generation ASDs (Franco et al., 2016). In addition, existing ASDs have many adverse effects, ranging from gastrointestinal problems to hepatotoxicity and even cognitive impairment (Löscher et al., 2013; Moshé et al., 2015). Hence, there is an unmet need for the development of new and safe ASDs that can control seizures effectively and prevent seizure-induced neurological damage (Löscher, 2017; Łukawski et al., 2016).

Significantly, inflammatory mediators such as cytokines and prostaglandins, released from brain cells and peripheral immune cells are involved in the origin of seizures, as well as in epileptogenesis (de Vries et al., 2016; Dey et al., 2016; Löscher et al., 2013; Vezzani et al., 2013). Thus, in the search for more effective ASDs with novel mechanisms of action, anti-inflammatory agents have been proposed not only as potential ASDs but also as candidate anti-epileptogenic drugs (AEDs) that can prevent or favorably modify the development of epilepsy (Dey et al., 2016; Vezzani, 2015).

Of interest, flavonoids and their glycosides have been shown to exert mild to potent activity in several seizure and epilepsy animal models (Sucher and Carles, 2015; Zhu et al., 2014). Investigating the underlying mechanism of action, it was found that these structures modulate allosterically $GABA_A$ receptors by binding to the benzodiazepine receptor site (Hanrahan et al., 2011; Johnston, 2015). However, flavonoids are also known to exert potent anti-inflammatory effects in the brain by means of free-radical-scavenging activity (Diniz et al., 2015), or by directly modulating key components of the neuroinflammatory cascade (Spencer et al., 2012). Accordingly, this neuroprotective activity has been invoked to explain their anticonvulsant effects as well (Diniz et al., 2015; Golechha et al., 2014, 2011; Lin et al., 2015).

Flavonoids are polyphenolic structures that are daily consumed due to their widespread distribution in almost all terrestrial plants with no adverse effects reported (Jäger and Saaby,

2011; Tapas et al., 2008). As such, dietary flavonoids and chemical derivatives thereof offer a possibly interesting source for ASD and AED chemical discovery work (Jäger and Saaby, 2011; Marder and Paladini, 2002; Singh et al., 2014). Although their pharmaceutical potential is often hampered by metabolic instability and low oral bioavailability, it has been argued that their drug-likeness can be improved via methylation of the free hydroxyl groups, dramatically enhancing the metabolic stability and membrane transport, facilitating absorption and highly increasing the bioavailability (Koirala, 2016; Koirala et al., 2016). Of importance, this methylation also abrogates their chemical anti-oxidant capacity, but leaves the general protective activity of the compounds against oxidative stress intact (Deng et al., 2006).

Since to the best of our knowledge no scientific data is available regarding the use of methylated flavonoids in the fight against epilepsy, we studied the anti-seizure activity of naringenin (NRG) and kaempferol (KFL), and of some of their common methylated derivatives, i.e., naringenin 7-*O*-methyl ether (NRG-M), naringenin 4′,7-dimethyl ether (NRG-DM), and kaempferide (4′-*O*-methyl kaempferol) (KFD) (Fig. 1A).

KFL belongs to the chemical subgroup of the flavonols that feature a double bond in the heterocyclic benzopyran moiety (C ring) resulting in planar A (fused aromatic ring) and C rings (Hanrahan et al., 2011). Interestingly, KFL is a key active constituent of the extract of *Crinum jagus* L. that is used in traditional Cameroonian medicine as an antiepileptic remedy. After isolation, the compound was found to improve convulsions-induced by pentylenetetrazole (PTZ) and to protect mice against PTZ-induced kindling development (Taiwe et al., 2016).

NRG on the other hand belongs to the flavanones (Ferreira et al., 2014), a subgroup with a puckered conformation of the C ring due to the lack of the double bond (Hanrahan et al., 2011). NRG is found in citrus fruits and has strong anti-inflammatory and anti-oxidant activities (Alam et al., 2014; Miler et al., 2016; Patel et al., 2014; Renugadevi and Prabu, 2009). It can pass the blood brain barrier, which enables the compound to exert neuroactive properties (Patel et al., 2014; Youdim et al., 2003). Recently, NRG was shown to delay seizure onset, to ameliorate induced morphological brain alterations, and to reduce microglia-derived neuro-inflammation in a kainic acid-induced mouse seizure model (Park et al., 2016). Of interest, NRG and KFL feature a highly similar hydroxylation pattern (Fig. 1A), allowing for a better comparison between the biological activity of the two different flavonoid subgroups.

To evaluate the anti-seizure activity of the compounds, we used a zebrafish PTZ seizure model (Afrikanova et al., 2013; Baraban et al., 2005), a standard assay in preliminary ASD discovery due to the high level of translation to rodents (Afrikanova et al., 2013; Buenafe et al., 2013;

Orellana-Paucar et al., 2012). Zebrafish are recognized as an important organism for modeling human diseases with respect to the 3R principle (Doke and Dhawale, 2015; Grone and Baraban, 2015) because it is a lower vertebrate model that is highly conserved with regard to the human reference genome (Howe et al., 2013), physiology, and pharmacology (MacRae and Peterson, 2015).

In this study, we show that the methylated NRG-M and NRG-DM are highly effective against PTZ-induced seizures in larval zebrafish, whereas NRG, KFL, and KFD possess only a limited activity. NRG-DM was further investigated in standard acute rodent seizure models, i.e., the mouse timed i.v. PTZ seizure model and the mouse 6-Hz psychomotor seizure model, and showed dose-dependent seizure reduction in both. Thus, highlighting the potential of methylated flavonoids as anti-seizure agents.

2. MATERIAL AND METHODS

2.1. Experimental animals

All animal experiments carried out were approved by the Ethics Committee of the University of Leuven (approval numbers 101/2010, 061/2013, and 150/2015) and by the Belgian Federal Department of Public Health, Food Safety & Environment (approval number LA1210199) in accordance with the EU Directive 2010/63/EU.

2.1.1. Zebrafish

Adult zebrafish (*Danio rerio*) stocks of AB strain (Zebrafish International Resource Center, Oregon, USA) were maintained at 28.0 °C, on a 14/10 hour light/dark cycle under standard aquaculture conditions. Fertilized eggs were collected via natural spawning and raised in embryo medium (1.5 mM HEPES, pH 7.2, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, and 0.6 μM methylene blue) at 28.0 °C, under constant light.

2.1.2. Mice

Male C57Bl/6 mice (9 weeks old, Charles River Laboratories) and male NMRI mice (weight 18-20 g, Charles River Laboratories) were housed in polyacrylic cages under a 14/10-hour

light/dark cycle at 22 °C. The animals were fed a pellet diet and water *ad libitum* and were allowed to acclimate for one week before experimental procedures were conducted.

2.2. Compound preparation and calculated LogP values

(±)-Naringenin, naringenin 7-*O*-methyl ether, kaempferol, and kaempferide (4′-*O*-methyl kaempferol) were purchased (analytical standard, Sigma-Aldrich). Naringenin 4′,7-dimethyl ether was synthesized as previously described (Kim et al., 2007). For experiments with zebrafish larvae, dry samples were dissolved in 100 % dimethyl sulfoxide (DMSO, spectroscopy grade) as 100-fold concentrated stocks and diluted in embryo medium to a final concentration of 1 % DMSO content. Control groups were treated with 1 % DMSO (vehicle, VHC) in accordance with the final solvent concentration of tested compounds. For mice experiments, a mixture of polyethylene glycol M.W. 200 (PEG200) and 100 % DMSO (spectroscopy grade, Acros Organics) (PEG200:DMSO 1:1) was used as solvent and VHC.

Calculated LogP values (cLogP) of the compounds were estimated with the interactive LogP calculator XLogP (version 3) (Cheng et al., 2007) based on the compound structure and were provided by PubChem as 2.4, 2.7, 3, 1.9, 2.2 for NRG, NRG-M, NRG-DM, KFL, and KFD, respectively.

2.3. Zebrafish pentylenetetrazole seizure model

2.3.1. Toxicity evaluation

Maximum tolerated concentration (MTC) was determined prior to further experiments and used as the highest test concentration. MTC was investigated by exposing 12 larvae of 7 days post-fertilization (dpf) to a range of concentrations in a 100 µL volume during 18 hours (28 °C). After 1 hour and 18 hours of exposure touch response, morphology, posture, edema, signs of necrosis, swim bladder, and heartbeat were investigated. MTC was defined as the highest concentration at which no larvae showed signs of toxicity or locomotor impairment. In case no MTC was reached, the maximum soluble concentration was used.

2.3.2. Behavioral analysis

Experiments were performed as described before (Afrikanova et al., 2013; Orellana-Paucar et al., 2012). In brief, a single 7-dpf larva was placed in each well of a 96-well plate and treated with VHC (1 % DMSO) or compound in a 100 µL volume. Larvae were incubated in dark for 1 hour at 28 °C, whereafter 100 µL of either VHC (embryo medium) or 40 mM PTZ was added to each well. Next, within 5 minutes the larval behavior was video recorded in an automated tracking device (ZebraBox Viewpoint, France) during 30 minutes. Total locomotor activity per time period was measured in actinteg units (ZebraLab software Viewpoint, France).

2.3.3. Electrophysiology

Non-invasive local field potential (LFP) recordings (Zdebik et al., 2013) were measured from the optic tectum of 7-dpf zebrafish larvae pre-incubated with VHC only, PTZ only, compound and VHC, or compound and PTZ. Larvae were incubated for 1 hour with VHC (1 % DMSO) or the compound (MTC) in a 100 µL volume (28 °C). After incubation, an equal volume of VHC (embryo medium) or PTZ was added to the well for 15 minutes (28 °C) prior to the recording. A larva was embedded in 2 % low melting point agarose (Invitrogen) and the signal electrode [an electrode inside a blunt soda-glass pipet (Hilgenberg, Germany)] was positioned on the skin covering the optic tectum. Electrophysiological recordings (room temperature) were performed as described before (Sourbron et al., 2017; Zhang et al., 2017). Manual analysis was done with Clampfit 10.2 software (Molecular Devices Corporation, USA). An electrical discharge was considered as epileptiform if it corresponded to a poly-spiking event comprising at least 3 spikes with a minimum amplitude of three times the baseline amplitude and a duration of at least 100 milliseconds.

2.4. Determination of whole body compound concentrations in zebrafish

2.4.1. Equipment

LC-MS analysis was performed using an Agilent 1100 HPLC system, consisting of a G1311A quaternary pump and solvent module, a G1322A vacuum degasser, a G1313A autosampler (ALS), a G1315A diode-array (DAD) detector (operating at 280 nm) and a G1316A thermostatted column compartment (TCC, kept at a constant temperature of 25 °C). The HPLC system was equipped with a Grace Prevail reversed-phase C18 3µm column (length: 150 mm,

ID: 2.1 mm) and coupled to an Agilent 6110 single-quadrupole mass spectrometer with an electrospray ionization (ESI) source (capillary voltage: 3500 V), operating in the positive mode. Samples (injection volume: 10 µL) were run in a mixture of methanol and water + 0.1 % formic acid (HPLC grade, Acros Organics) using a specific gradient program for each compound. For NRG (t_R: 21.8 minutes), a linear gradient program was used, going from 40 % to 100 % methanol over a period of 40 minutes. For NRG-DM (t_R: 31.2 minutes), a similar gradient was employed, starting initially at 50 % methanol and reaching 100 % over the same time period. All samples were run at a flow rate of 0.12 mL/minute. Data were acquired and processed using Agilent LC/MSD ChemStation software rev. B.04.03-SP2[105]. Further analysis was performed using Microsoft Excel 2016 and GraphPad Prism 5.

2.4.2. Sample preparation

Whole body compound concentrations were determined from extracts of 25 larvae of 7 dpf that were incubated for 1 hour (dark incubation at 28 °C) with 12.5 µM NRG-DM or 50 µM NRG (100 µL volume per larva in a 24-well plate), in accordance with the experimental conditions. Extraction protocol was based on literature (Kislyuk et al., 2017). After incubation, larvae were washed to remove excess compound by flushing them with 15 mL of ultrapure water on a 74 µm polyester mesh (Costar). Next, larvae were anesthetized on an ice-cold petridish and transferred to 1.5 mL Eppendorf tubes with acid-washed glass beads (diameter: 710–1180 µm, Sigma Aldrich) and 270 µL extraction medium (methanol:water 2:1, ultrapure water and HPLC grade methanol, Sigma Aldrich) within 3 minutes. The samples were homogenized by 30 minutes of ultrasonication (30 cycles of sonication and intermission of 30 seconds each, high energy input setting, Diagenode Bioruptor Plus, Seraing, Belgium) at 4 °C. After centrifugation (14000 g, 15 minutes), the supernatans was collected and stored on 4 °C (short-term) or on -20 °C (long-term) until further processing. Supernatants were filtered over a Millipore 0.45 µm syringe filter and the filter was rinsed three times with 2 mL methanol. The filtrate was lyophilized and the obtained solid residue was dissolved in 30 µL of the LC eluent. Two samples were pooled together and injected onto the LC-MS system to avoid the injection of air bubbles.

2.4.3. Quantification of whole body compound concentrations

Quantification of the integrated signals was achieved by comparison with a calibration curve. For NRG, a linear calibration curve (A_{NRG} = 215334c, R^2 = 0.9998) was obtained in a concentration range from 0.17 to 170 µg/mL (two-fold dilution series). For NRG-DM, two different calibration curves were generated, as two columns were used for the quantification. For both columns, a linear calibration curve ($A_{NRG\text{-}DM}$ = 127529c, R^2 = 0.9987 and $A'_{NRG\text{-}DM}$ = 171078c, R^2 = 0.9998) was obtained in a concentration range from 1.47 to 93.85 µg/mL (two-fold dilution series). NRG had a limit of detection (LOD) of 170 ng/mL (0.20 ng/larva, 0.82 ng/mg body weight) and NRG-DM had an LOD of 1470 ng/mL (1.76 ng/larva, 7.07 ng/mg body weight). The mean body weight (± SD) of a single 7-dpf zebrafish larva was 249 µg (± 18 µg) and was measured from 3 batches of larvae by weighing 25 larvae (n = 3 per batch) after removal of excess water on filter paper.

2.5. Mouse timed i.v. pentylenetetrazole seizure model

100 µL (weight-adjusted) of VHC (PEG200:DMSO 1:1) or treatment was i.p. injected in C57Bl/6 mice (10 weeks old, 23-30 g), 30 minutes prior seizure induction by continuous i.v. PTZ infusion through a catheter (7.5 mg/mL, 150 µL/minute) connected to a syringe in an automatic pump. During PTZ infusion mice were observed in a transparent polyacrylic cage for the onset of the following behavioral events: ear-, tail- and myoclonic twitch, forelimb clonus, falling, tonic hind limb extension and death. PTZ doses needed to trigger the behavioral parameters were calculated based on the time latencies.

2.6. Mouse 6-Hz psychomotor seizure model

Experiments were performed as described before (Buenafe et al., 2013; Orellana-Paucar et al., 2013). In brief, 100 µL (weight-adjusted) of VHC (PEG200:DMSO 1:1) or treatment was i.p. injected in NMRI mice (25-32 g), 30 or 120 (in case of phenytoin, as reported by Barton and colleagues (Barton et al., 2001)) minutes prior seizure induction by corneal electrical stimulation (6 Hz, 0.2 ms pulse width, 3 s duration, 44 mA). VHC-treated mice typically displayed stun, twitching of the vibrissae, forelimb clonus, and Straub tail for at least 45 seconds. Mice displaying normal exploratory and locomotion behavior within a time period of 40 seconds were considered as protected against psychomotor seizures.

3. RESULTS

3.1. Anti-seizure analysis of compounds using the zebrafish PTZ seizure model

We first explored the anti-seizure activity of the flavanone NRG, the flavanol KFL and their methylated derivatives, NRG-M, NRG-DM, and KFD, by analyzing their effects on the locomotor and brain activity of zebrafish larvae exposed to the GABA-antagonist PTZ. The flavonoids were tested at their maximum tolerated concentration (MTC) (i.e. 50 µM, 25 µM and 12.5 µM in case of NRG, NRG-M and NRG-DM, respectively) or their maximum soluble concentration (25 µM in case of KFL and KFD) and two two-fold dilutions thereof.

Upon exposure to PTZ the larvae exhibited a dramatically increased seizure-like behavior and locomotor activity (Fig. 1B-K) as documented before (Afrikanova et al., 2013; Baraban et al., 2005). Furthermore, all compounds except for NRG induced a significant reduction in the PTZ-induced seizure behavior as analyzed during a 30-minute tracking period, at least at the highest concentration used (Fig. 1B-F). Overall, the most efficient compounds were NRG-M and NRG-DM that reduced PTZ-induced activity to ca 25-35 % of the PTZ control activity, whereas KFL and KFD only had limited inhibitory activity. A more detailed analysis of the 30-minute tracking period split into 5-minute intervals confirmed the overall activity as observed for the active compounds (Fig. 1G-H, Fig. 1J-K), but also revealed some effect by NRG, at least at the highest concentration tested (50 µM) (Fig. 1I).

In absence of PTZ, a significant increase in larval locomotion in comparison to VHC-treated controls was observed for 25 µM NRG-M ($p \leq 0.01$).

To confirm these results, we then incubated the zebrafish larvae with the compounds at their MTC and measured the epileptiform activity in the zebrafish larval optic tectum by non-invasive local field potential recordings. A significant reduction of larvae with PTZ-induced epileptiform activity was observed, in comparison to PTZ-treated controls, when the larvae were pre-exposed to NRG-DM ($p \leq 0.001$), NRG-M ($p \leq 0.01$) and KFL ($p \leq 0.05$) (Fig. 2A). Moreover, NRG-DM and NRG-M also significantly lowered the number ($p \leq 0.001$) and cumulative duration ($p \leq 0.001$) of PTZ-induced epileptiform events (Fig. 2B-C). Consistent with the behavioral observations described, NRG-M showed convulsive activity, significantly increasing the percentage of larvae that displayed epileptiform activity in comparison to VHC-treated controls ($p \leq 0.001$). Likewise, NRG exposure increased the number of larvae with epileptiform activity proportionally ($p \leq 0.05$) (Fig. 2A).

3.2. Determination of whole body concentrations of naringenin 4′,7-dimethyl ether and naringenin in zebrafish

To investigate whether the methylation of naringenin affects the uptake in zebrafish, we determined the whole body concentrations of NRG-DM and NRG after exposure under the experimental conditions. Compound concentrations were measured from extracts of 25 larvae (n = 3) that were incubated 1 hour with either 12.5 µM NRG-DM or 50 µM NRG. Larvae exposed to NRG-DM had a mean whole body concentration (± SD) of 279.4 ng/mg (± 81.3 ng/mg), equal to an uptake of 69.7 ng/larva (± 20.3 ng/larva), while no quantifiable amounts of compound were detected for larvae exposed to NRG. Hence, the absorption of NRG-DM was observed to be more than 340-fold higher than that of NRG in the experimental conditions used.

3.3. Anti-seizure analysis of naringenin 4′,7-dimethyl ether in the mouse timed i.v. pentylenetetrazole seizure model

We selected NRG-DM for further investigations in acute rodent seizure models. First, NRG-DM was tested in the mouse timed i.v. PTZ seizure model, a standard model for ASD discovery that is capable of identifying molecules with varied mechanisms of action (Mandhane et al., 2007). Mice treated with 12.5 mg/kg NRG-DM (i.p. administered), 30 minutes before continuous i.v. PTZ injection, required a higher PTZ dose than VHC-treated controls to elicit tail twitch ($p \leq 0.05$), falling ($p \leq 0.01$), tonic hind limb extension ($p \leq 0.05$), and death ($p \leq 0.001$). Also at 25, 6.25, and 2 mg/kg NRG-DM, a significant higher PTZ dose was needed to induce death in comparison to controls ($p \leq 0.05$, $p \leq 0.001$, and $p \leq 0.01$, respectively). As expected, mice treated with positive control valproate (100 mg/kg, i.p. administered 30 minutes before i.v. PTZ injection), required a higher PTZ dose than VHC-injected controls for several behavioral traits, namely, tail twitch ($p \leq 0.05$), forelimb clonus ($p \leq 0.05$), falling ($p \leq 0.05$), and death ($p \leq 0.001$) (Fig. 3, Table 1).

3.4. Anti-seizure analysis of naringenin 4′,7-dimethyl ether in the mouse 6-Hz psychomotor seizure model

To investigate whether NRG-DM has potential to control drug-resistant seizures, of particular interest for refractory epilepsies, it was additionally tested in the mouse 6-Hz psychomotor seizure model (Barton et al., 2001). This model involves electrical induction of drug-resistant seizures by a low frequency, long duration corneal stimulation (6 Hz, 0.2 ms rectangular pulse

width, 3 s duration) at a current intensity of 44 mA (Barton et al., 2001; Walrave et al., 2015). Treatment with 6.25 mg/kg NRG-DM (i.p. administered), 30 minutes before electrical stimulation, showed protection against psychomotor seizures in all mice ($p \leq 0.01$). Protection was defined as the mouse displaying normal exploratory and locomotion behavior within a time period of 40 seconds. At 25, 12.5, and 2 mg/kg NRG-DM, protection was observed for 5 out of 6 mice ($p \leq 0.05$). As expected, treatment with positive control, valproate (300 mg/kg, i.p. administered 30 minutes before electrical stimulation), led to complete protection ($p \leq 0.01$), whereas treatment with negative control, phenytoin (10 mg/kg, i.p. administered 120 minutes before electrical stimulation), showed no significant protection (3 out of 6 mice protected) (Fig. 4A). When comparing seizure duration in all conditions, a significant reduction is observed between the control group (mean duration 70 seconds) and mice treated with 6.25 mg/kg NRG-DM ($p \leq 0.05$, mean duration 15 seconds), and with 300 mg/kg valproate ($p \leq 0.001$, mean duration 0 seconds). A non-significant reduction in seizure duration was observed for 25, 12.5, and 2 mg/kg NRG-DM (mean durations 26, 31, and 38 seconds, respectively), as well as for 10 mg/kg phenytoin (mean duration 43 seconds).

4. DISCUSSION

Using a PTZ-induced zebrafish seizure model we demonstrated in the present study that non-methylated flavonoids like NRG and KFL possess only limited anti-seizure activity, as shown by both behavioral testing and LFP measurements. Of interest, methylation of KFL (forming KFD or 4′-*O*-methyl kaempferol) increased only marginally the activity (as shown in the behavioral test), whereas methylation of NRGs (forming naringenin 7-*O*-methyl ether (NRG-M), and naringenin 4′,7-dimethyl ether (NRG-DM)), had a clear impact on the outcome.

Significantly, it has been observed that methylation of flavonoids dramatically favors their metabolic stability and membrane transport, thereby facilitating absorption and positively affecting their bioavailability (Koirala, 2016; Koirala et al., 2016). In the present study however we were not able to prove any metabolic advantage of the methylated *vs* the non-methylated compound as any clear metabolic turnover of NRG-DM was lacking (results not shown) and no quantifiable amounts but also no metabolites could be detected in case of NRG. Although zebrafish larvae are capable of extensive biotransformation after long incubations with compounds (96 hours) (Brox et al., 2016), it should be underscored that we used short incubation periods (1 hour) throughout this study, thereby decreasing the probability of any

metabolic turnover. This limited exposure could also account for the discrepancy between the relative lack of activity, as observed for NRG and KFL in this study, and the clear anti-seizure properties in acute and chronic epilepsy rodent models of the compounds, as described in earlier reports after oral and i.p. administration of relative large doses (Park et al., 2016; Taiwe et al., 2016).

Conversely, we observed that the whole-body uptake of NRG-DM by zebrafish larvae was at least 340-fold higher than that of NRG, and although the actual brain concentrations of the individual compounds are not known, it is anticipated that the difference in activity between the compounds relates to their differential uptake. The dramatically enhanced absorption of dimethylated NRG in comparison to its nonmethylated counterpart is therefore in agreement with earlier observations that methylation of flavonoids facilitates absorption (Koirala, 2016; Koirala et al., 2016). However, the discrepancy between the recovery of the two compounds is nonetheless surprising, especially in view of the fact that NRG-DM and NRG have similar physicochemical properties (M_r: 300 and 270, cLogP : 3.0 and 2.4, respectively), and that small molecules with LogP > 1 are expected to exhibit good bioavailability in zebrafish larvae (Milan et al., 2003). Although any mechanistic background is lacking to explain these results, it is known that some compounds including poly-phenolics can aspecifically interfere with many cellular proteins (Baell and Walters, 2014), in case of larval skin possibly resulting in poor absorption characteristics.

In spite of the fact that methylation of flavonoids could raise the biological activity by increasing compound bioavailability (Koirala et al., 2016), as seen for NRG-DM in this study, it could also confer new pharmacological activity to the original structure. Therefore, the low activity as observed for the non-methylated NRG and KFL, but also in case of the monomethylated KFD, might also point to an inappropriate pharmacological profile of the compound that is unable to phenotypically rescue the PTZ-induced pathological state. The clear convulsive properties of NRG-M are also in accordance with this assumption as likely this adverse effect is due to an unknown off-target activity not activated by the other compounds, or to differences in affinities for a common target. Often, when commonly used ASDs are overdosed or inadequately chosen, seizure aggravation and induction have been reported, thus revealing a sensitive balance between anti- and pro-seizure activities (Gayatri and Livingston, 2006; Sazgar and Bourgeois, 2005).

Probably, also the site of methylation (4′ versus 7) plays an important role in the pharmacological spectrum, as KFD (4′-*O*-methyl kaempferol) did not have a positive impact

on the PTZ-induced abnormal brain activity (as measured during LFP recordings), whereas NRG-M (naringenin 7-*O*-methyl ether) had a clear effect on this read-out. But obviously, also the difference in chemistry between the flavanone NRG and the flavanol KFL might have accounted for this discrepancy.

From all compounds studied in the zebrafish model, NRG-DM was most effective against PTZ-induced seizures and epileptiform discharges and did not induce convulsant effects. Hence, NRG-DM is considered a promising anti-seizure hit and was further investigated in standard acute mouse seizure models that are commonly used for ASD discovery. The mouse timed i.v. PTZ seizure model (Mandhane et al., 2007; Nutt et al., 1986) is a rodent correlate of the zebrafish PTZ model that can identify molecules with varied mechanism of action (Mandhane et al., 2007). In this model generalized myoclonic, clonic, and tonic seizures are induced by continuous PTZ infusion through the tail vein (Bialer and White, 2010; Löscher, 2009; Mandhane et al., 2007). NRG-DM treatment enhanced the mean PTZ dose needed to trigger multiple seizure parameters, including death, for which a concentration-dependent effect was seen. These results confirm NRG-DM's anti-seizure activity in a mammalian model, thus providing an additional example of the translation of results from zebrafish to rodents, which has been previously reported (Afrikanova et al., 2013; Buenafe et al., 2013; Leclercq et al., 2015; Orellana-Paucar et al., 2013). Moreover, these results show that NRG-DM is active against generalized seizures. Finally, besides anticonvulsant assessment this mouse model also allows the determination of (pro)convulsant drug activity, if any, which is seen as a significant reduction in the seizure threshold of one of the behavioral parameters. In line with the results obtained in the zebrafish PTZ seizure model, NRG-DM did not show convulsant properties in the mouse timed i.v. PTZ seizure model.

The activity of NRG-DM was also investigated in the mouse 6-Hz psychomotor seizure model in which drug-resistant focal seizures are electrically induced through the cornea with a current intensity of 44 mA (Barton et al., 2001). The 6-Hz (44 mA) model is considered a gold standard in current ASD discovery efforts, such as the U.S. Epilepsy Therapy Screening Program, as it can detect compounds with novel anti-seizure mechanisms and potential against drug-resistant focal seizures as exemplified by levetiracetam (Barton et al., 2001; Wilcox et al., 2013). In this study, NRG-DM treatment ameliorated the induced psychomotor seizures resulting in protection of mice at all doses tested. Thus, NRG-DM is shown to have a broad utility against both generalized seizures and drug-resistant focal seizures. Based on its prominent anti-seizure activity in zebrafish and mouse seizure models, we propose NRG-DM as a lead compound that

is worth further investigations in the treatment of generalized seizures and, of particular interest, for the treatment of drug-resistant focal seizures.

The molecular mechanisms by which NRG-DM but also NRG-M exert their pronounced anti-seizure activity and to what extent they are substantially different from the parent compound NRG is presently unknown. NRG has only a mild affinity for the $GABA_A$-receptor benzodiazepine site (Jäger et al., 2007), and thus its anti-seizure activity is unlikely due to this target modulation solely. However, the compound also possesses strong anti-oxidant and anti-inflammatory activities (Alam et al., 2014; Park et al., 2016; Raza et al., 2013), which are known to be relevant for its anti-seizure and neuroprotective activity (de Vries et al., 2016; Dey et al., 2016; Shin et al., 2011). For instance, in a mouse kainic acid-induced seizure model, NRG attenuated interleukin 1β (IL-1β) production (Park et al., 2016), an important pro-inflammatory mediator in epileptogenic foci (Dey et al., 2016), and also a targeted inhibition of the Toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling pathway was suggested to be an important mechanism of action (Dou et al., 2013). Although methylation abolishes the chemical anti-oxidant properties of flavonoids (i.e., scavenging reactive oxygen species) (Deng et al., 2006), the activity that triggers cellular anti-oxidant mechanisms remains intact. For instance, it has been reported that also NRG-M downregulates TNF-α, IL-6, and IL-1β in lipopolysaccharide-stimulated macrophages *in vitro* by suppressing the activation of the ERK1/2, JNK, and NF-κB signaling pathways (Soromou et al., 2012). So, methylation (and possibly also double methylation as in the case of NRG-DM) does not destroy the anti-inflammatory activity of NRG, although the potency and spectrum of inhibitory actions exhibited by the compounds might vary. Moreover, methylation can also confer a distinctive pharmacological characteristic to flavonoids. This is exemplified by hispidulin, a methylated apigenin, that exhibits a different profile of activity at $GABA_A$ receptors as compared to the parent compound (Johnston, 2015), and by NRG-M and NRG-DM that inhibit voltage-gated potassium channels $K_v1.3$ in T-lymphocytes, whereas NRG did not (Teisseyre et al., 2009). Although further research is needed to elucidate the molecular anti-seizure targets of NRG-DM and NRG-M, these data as well as our results indicate that methylation of flavonoids yields a distinct pharmacological spectrum that can be particularly relevant for anti-seizure drug discovery.

5. CONCLUSIONS

In this study, the potential of methylated flavonoids as a source for anti-seizure drug discovery was investigated using NRG, KFL, and three common methylated derivatives as a case study. We demonstrate that the methylated flavanones NRG-DM and NRG-M are highly effective against PTZ-induced seizures in larval zebrafish, whereas the flavanone NRG and the flavonols KFL and KFD possess only a limited activity. Even though a differential compound uptake might explain the results, it does not exclude the possibility that methylation of flavonoids can alter their pharmacological target(s). In addition, the promising anti-seizure hit NRG-DM is shown to be active in two standard acute mouse seizure models, i.e., the timed i.v. PTZ seizure model and the 6-Hz psychomotor seizure model. Based on these results, we propose NRG-DM as a lead compound worth investigating further for the treatment of generalized myoclonic, clonic, and tonic seizures, and, of particular interest, for the treatment of drug-resistant focal seizures. Our data therefore highlights the potential of methylated flavonoids in the search for new and improved ASDs.

ABBREVIATIONS

AED, anti-epileptogenic drug; ASD, anti-seizure drug; cLogP, calculated LogP; dpf, days post-fertilization; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; IL-1β, interleukin 1β; IL-6, interleukin 6; KFD, kaempferide; KFL, kaempferol; LFP, local field potential; LOD, limit of detection; MTC, maximum tolerated concentration; NRG, naringenin; NRG-DM, naringenin 4′,7-dimethyl ether; NRG-M, naringenin 7-*O*-methyl ether; NF-κB, nuclear factor κB; PEG200, polyethylene glycol M.W. 200; PTZ, pentylenetetrazole; TNF-α, tumor necrosis factor-α; TLR4, Toll-like receptor 4; VHC, vehicle

AUTHOR INFORMATION

[#]Corresponding author

Prof. Dr. Peter A. M. de Witte, Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Herestraat 49 bus 824, 3000 Leuven, Belgium. E-mail: peter.dewitte@kuleuven.be

Author contributions

PAMW, LP, WD, WDB, DC, and AOP were responsible for study design. YK synthesized naringenin 4′,7-dimethyl ether. AS optimized the electrophysiology method. Experiments were performed by DC, AOP, GS, YZ, AN, KF, and VE. DC, AOP, and GS were responsible for data acquisition and analysis. DC, AOP, GS, and PAMW wrote the manuscript. DC prepared the figures and table. All authors edited and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported in part by the Research Foundation–Flanders (FWO Vlaanderen) postdoctoral fellowship [Aleksandra Siekierska, project number 12G3616N]. We thank Dr. Laura Walrave, Dr. Jessica Coppens, and Prof. Dr. Ilse Smolders (Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Center for Neurosciences, Vrije Universiteit Brussel (VUB), Brussels, Belgium) for sharing their expertise on acute mouse seizure models. We thank Dr. Stanislav Kislyuk and Prof. Dr. Deirdre Cabooter for their advice on the determination of internal compound concentrations in zebrafish larvae. Concerning the graphical table of contents, we thank Nina Dirkx for the design and somersault18:24 BVBA (www.somersault1824.com) for providing scientific illustrations. Finally, we thank Bart Van Huffel for general maintenance of the LC-MS apparatus.

REFERENCES

Afrikanova, T., Serruys, A.S.K., Buenafe, O.E.M., Clinckers, R., Smolders, I., de Witte, P.A.M., Crawford, A.D., Esguerra, C. V., 2013. Validation of the Zebrafish Pentylenetetrazol Seizure Model: Locomotor versus Electrographic Responses to Antiepileptic Drugs. PLoS One 8, 1–9. doi:10.1371/journal.pone.0054166

Alam, M.A., Subhan, N., Rahman, M.M., Uddin, S.J., Reza, H.M., Sarker, S.D., 2014. Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their

mechanisms of action. Adv. Nutr. 5, 404–17. doi:10.3945/an.113.005603

Baell, J., Walters, M.A., 2014. Chemistry: Chemical con artists foil drug discovery. Nature 513, 481–483. doi:10.1038/513481a

Baraban, S.C., Taylor, M.R., Castro, P.A., Baier, H., 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 131, 759–768. doi:10.1016/j.neuroscience.2004.11.031

Barton, M.E., Klein, B.D., Wolf, H.H., White, H.S., 2001. Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. Epilepsy Res. 47, 217–227. doi:10.1016/S0920-1211(01)00302-3

Bialer, M., White, H.S., 2010. Key factors in the discovery and development of new antiepileptic drugs. Nat. Rev. Drug Discov. 9, 68–82. doi:10.1038/nrd2997

Brox, S., Seiwert, B., Küster, E., Reemtsma, T., 2016. Toxicokinetics of Polar Chemicals in Zebrafish Embryo (Danio rerio): Influence of Physicochemical Properties and of Biological Processes. Environ. Sci. Technol. 50, 10264–10272. doi:10.1021/acs.est.6b04325

Buenafe, O.E., Orellana-Paucar, A., Maes, J., Huang, H., Ying, X., De Borggraeve, W., Crawford, A.D., Luyten, W., Esguerra, C. V., De Witte, P.A.M., 2013. Tanshinone IIA exhibits anticonvulsant activity in zebrafish and mouse seizure models. ACS Chem. Neurosci. 4, 1479–1487. doi:10.1021/cn400140e

Cheng, T., Zhao, Y., Li, X., Lin, F., Xu, Y., Zhang, X., Li, Y., Wang, R., 2007. Computation of Octanol - Water Partition Coefficients by Guiding an Additive Model with Knowledge. J. Chem. Inf. Model. 47, 2140–2148. doi:10.1021/ci700257y

Dalic, L., Cook, M.J., 2016. Managing drug-resistant epilepsy: challenges and solutions. Neuropsychiatr. Dis. Treat. 12, 2605–2616. doi:10.2147/NDT.S84852

de Vries, E.E., van den Munckhof, B., Braun, K.P.J., van Royen-Kerkhof, A., de Jager, W., Jansen, F.E., 2016. Inflammatory mediators in human epilepsy: A systematic review and meta-analysis. Neurosci. Biobehav. Rev. 63, 177–190. doi:10.1016/j.neubiorev.2016.02.007

Deng, D., Zhang, J., Cooney, J.M., Skinner, M.A., Adaim, A., Jensen, D.J., Stevenson, D.E., 2006. Methylated polyphenols are poor “chemical” antioxidants but can still effectively protect cells from hydrogen peroxide-induced cytotoxicity. FEBS Lett. 580, 5247–5250.

doi:10.1016/j.febslet.2006.08.051

Dey, A., Kang, X., Qiu, J., Du, Y., Jiang, J., 2016. Anti-Inflammatory Small Molecules to Treat Seizures and Epilepsy: From Bench to Bedside. Trends Pharmacol. Sci. 37, 463–484. doi:10.1016/j.tips.2016.03.001

Diniz, T.C., Silva, J.C., de Lima-Saraiva, S.R.G., Ribeiro, F.P.R. de A., Pacheco, A.G.M., de Freitas, R.M., Quintans-Júnior, L.J., Quintans, J. de S.S., Mendes, R.L., Almeida, J.R.G. da S., 2015. The role of flavonoids on oxidative stress in epilepsy. Oxid. Med. Cell. Longev. 2015, 171756. doi:10.1155/2015/171756

Doke, S.K., Dhawale, S.C., 2015. Alternatives to animal testing: A review. Saudi Pharm. J. 23, 223–229. doi:10.1016/j.jsps.2013.11.002

Dou, W., Zhang, J., Sun, A., Zhang, E., Ding, L., Mukherjee, S., Wei, X., Chou, G., Wang, Z.-T., Mani, S., 2013. Protective effect of naringenin against experimental colitis via suppression of Toll-like receptor 4/NF-κB signalling. Br. J. Nutr. 110, 599–608. doi:10.1017/S0007114512005594

Ferreira, A., Pousinho, S., Fortuna, A., Falcão, A., Alves, G., 2014. Flavonoid compounds as reversal agents of the P-glycoprotein-mediated multidrug resistance: biology, chemistry and pharmacology. Phytochem. Rev. 14, 233–272. doi:10.1007/s11101-014-9358-0

Franco, V., French, J.A., Perucca, E., 2016. Challenges in the clinical development of new antiepileptic drugs. Pharmacol. Res. 103, 95–104. doi:10.1016/j.phrs.2015.11.007

Gayatri, N., Livingston, J., 2006. Aggravation of epilepsy by anti-epileptic drugs. Dev. Med. Child Neurol. 48, 394–398. doi:10.1017/S0012162206000843

Golechha, M., Chaudhry, U., Bhatia, J., Saluja, D., Arya, D.S., 2011. Naringin Protects against Kainic Acid-Induced Status Epilepticus in Rats: Evidence for an Antioxidant, Anti-inflammatory and Neuroprotective Intervention. Biol. Pharm. Bull. 34, 360–365. doi:10.1248/bpb.34.360

Golechha, M., Sarangal, V., Bhatia, J., Chaudhry, U., Saluja, D., Arya, D.S., 2014. Naringin ameliorates pentylenetetrazol-induced seizures and associated oxidative stress, inflammation, and cognitive impairment in rats: possible mechanisms of neuroprotection. Epilepsy Behav. 41, 98–102. doi:10.1016/j.yebeh.2014.09.058

Grone, B.P., Baraban, S.C., 2015. Animal models in epilepsy research: legacies and new directions. Nat. Neurosci. 18, 339–343. doi:10.1038/nn.3934

Hanrahan, J.R., Chebib, M., Johnston, G.A.R., 2011. Flavonoid modulation of GABA(A) receptors. Br. J. Pharmacol. 163, 234–45. doi:10.1111/j.1476-5381.2011.01228.x

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Eliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Mortimer, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Roest Crollius, H., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. doi:10.1038/nature12111

Jäger, A.K., Almqvist, J.P., Vangsøe, S.A.K., Stafford, G.I., Adsersen, A., Van Staden, J., 2007. Compounds from Mentha aquatica with affinity to the GABA-benzodiazepine receptor. South African J. Bot. 73, 518–521. doi:10.1016/j.sajb.2007.04.061

Jäger, A.K., Saaby, L., 2011. Flavonoids and the CNS. Molecules 16, 1471–1485. doi:10.3390/molecules16021471

Johnston, G.A.R., 2015. Flavonoid nutraceuticals and ionotropic receptors for the inhibitory neurotransmitter GABA. Neurochem. Int. doi:10.1016/j.neuint.2015.07.013

Kim, J., Park, K.-S., Lee, C., Chong, Y., 2007. Synthesis of a Complete Series of O-Methyl Analogues of Naringenin and Apigenin. Bull. Korean Chem. Soc. 28, 2527–2530. doi:10.5012/bkcs.2007.28.12.2527

Kislyuk, S., Kroonen, J., Adams, E., Augustijns, P., de Witte, P., Cabooter, D., 2017. Development of a sensitive and quantitative UHPLC-MS/MS method to study the whole-body uptake of pharmaceuticals in zebrafish. Talanta 174, 780–788. doi:10.1016/j.talanta.2017.06.075

Koirala, N., 2016. Enhancing the Pharmaceutical Properties of Flavonoids via Methylation and Glycosylation. SOJ Biochem 2, 1–2.

Koirala, N., Thuan, N.H., Ghimire, G.P., Thang, D. V., Sohng, J.K., 2016. Methylation of flavonoids: Chemical structures, bioactivities, progress and perspectives for biotechnological production. Enzyme Microb. Technol. 86, 103–116. doi:10.1016/j.enzmictec.2016.02.003

Leclercq, K., Afrikanova, T., Langlois, M., De Prins, A., Buenafe, O.E., Rospo, C.C., Van Eeckhaut, A., de Witte, P.A.M., Crawford, A.D., Smolders, I., Esguerra, C. V., Kaminski, R.M., 2015. Cross-species pharmacological characterization of the allylglycine seizure model in mice and larval zebrafish. Epilepsy Behav. 45, 53–63. doi:10.1016/j.yebeh.2015.03.019

Lin, T.Y., Lu, C.W., Wang, S.J., Huang, S.K., 2015. Protective effect of hispidulin on kainic acid-induced seizures and neurotoxicity in rats. Eur. J. Pharmacol. 755, 6–15. doi:10.1016/j.ejphar.2015.02.041

Löscher, W., 2017. The Search for New Screening Models of Pharmacoresistant Epilepsy: Is Induction of Acute Seizures in Epileptic Rodents a Suitable Approach? Neurochem. Res. 42, 1926–1938. doi:10.1007/s11064-016-2025-7

Löscher, W., 2009. Preclinical assessment of proconvulsant drug activity and its relevance for predicting adverse events in humans. Eur. J. Pharmacol. 610, 1–11. doi:10.1016/j.ejphar.2009.03.025

Löscher, W., Klitgaard, H., Twyman, R.E., Schmidt, D., 2013. New avenues for anti-epileptic drug discovery and development. Nat. Rev. Drug Discov. 12, 757–776. doi:10.1038/nrd4126

Łukawski, K., Gryta, P., Łuszczki, J., Czuczwar, S.J., 2016. Exploring the latest avenues for antiepileptic drug discovery and development. Expert Opin. Drug Discov. 11, 369–382. doi:10.1517/17460441.2016.1154840

MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14, 721–731. doi:10.1038/nrd4627

Mandhane, S.N., Aavula, K., Rajamannar, T., 2007. Timed pentylenetetrazol infusion test: A comparative analysis with s.c.PTZ and MES models of anticonvulsant screening in mice. Seizure 16, 636–644. doi:10.1016/j.seizure.2007.05.005

Marder, M., Paladini, A.C., 2002. GABA-A-Receptor Ligands of Flavonoid Structure. Curr. Top. Med. Chem. 2, 853–867. doi:10.2174/1568026023393462

Milan, D.J., Peterson, T.A., Ruskin, J.N., Peterson, R.T., MacRae, C.A., 2003. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. Circulation 107, 1355–1358. doi:10.1161/01.CIR.0000061912.88753.87

Miler, M., Živanović, J., Ajdžanović, V., Oreščanin-Dušić, Z., Milenković, D., Konić-Ristić, A., Blagojević, D., Milošević, V., Šošić-Jurjević, B., 2016. Citrus flavanones naringenin and hesperetin improve antioxidant status and membrane lipid compositions in the liver of old-aged Wistar rats. Exp. Gerontol. 84, 49–60. doi:10.1016/j.exger.2016.08.014

Moshé, S.L., Perucca, E., Ryvlin, P., Tomson, T., 2015. Epilepsy: new advances. Lancet 385, 884–898. doi:10.1016/S0140-6736(14)60456-6

Ngugi, A.K., Bottomley, C., Kleinschmidt, I., Sander, J.W., Newton, C.R., 2010. Estimation of the burden of active and life-time epilepsy: A meta-analytic approach. Epilepsia 51, 883–890. doi:10.1111/j.1528-1167.2009.02481.x

Nutt, D.J., Taylor, S.C., Little, H.J., 1986. Optimizing the pentetrazol infusion test for seizure threshold measurement. J. Pharm. Pharmacol. 38, 697–698. doi:10.1111/j.2042-7158.1986.tb03114.x

Orellana-Paucar, A.M., Afrikanova, T., Thomas, J., Aibuldinov, Y.K., Dehaen, W., De Witte, P.A.M., Esguerra, C. V., 2013. Insights from zebrafish and mouse models on the activity and safety of ar-turmerone as a potential drug candidate for the treatment of epilepsy. PLoS

One 8, 1–14. doi:10.1371/journal.pone.0081634

Orellana-Paucar, A.M., Serruys, A.-S.K., Afrikanova, T., Maes, J., De Borggraeve, W., Alen, J., León-Tamariz, F., Wilches-Arizábala, I.M., Crawford, A.D., De Witte, P.A.M., Esguerra, C. V, 2012. Anticonvulsant activity of bisabolene sesquiterpenoids of Curcuma longa in zebrafish and mouse seizure models. Epilepsy Behav. 24, 14–22. doi:10.1016/j.yebeh.2012.02.020

Park, J., Jeong, K.H., Shin, W.-H., Bae, Y.-S., Jung, U.J., Kim, S.R., 2016. Naringenin ameliorates kainic acid-induced morphological alterations in the dentate gyrus in a mouse model of temporal lobe epilepsy. Neuroreport 27, 1182–1189. doi:10.1097/WNR.0000000000000678

Patel, K., Singh, G.K., Patel, D.K., 2014. A review on pharmacological and analytical aspects of naringenin. Chin. J. Integr. Med. doi:10.1007/s11655-014-1960-x

Raza, S.S., Khan, M.M., Ahmad, A., Ashafaq, M., Islam, F., Wagner, A.P., Safhi, M.M., Islam, F., 2013. Neuroprotective effect of naringenin is mediated through suppression of NF-κB signaling pathway in experimental stroke. Neuroscience 230, 157–171. doi:10.1016/j.neuroscience.2012.10.041

Renugadevi, J., Prabu, S.M., 2009. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. Toxicology 256, 128–134. doi:10.1016/j.tox.2008.11.012

Sazgar, M., Bourgeois, B.F.D., 2005. Aggravation of epilepsy by antiepileptic drugs. Pediatr. Neurol. 33, 227–234. doi:10.1016/j.pediatrneurol.2005.03.001

Shin, E.-J., Jeong, J.H., Chung, Y.H., Kim, W.-K., Ko, K.-H., Bach, J.-H., Hong, J.-S., Yoneda, Y., Kim, H.-C., 2011. Role of oxidative stress in epileptic seizures. Neurochem. Int. 59, 122–137. doi:10.1016/j.neuint.2011.03.025

Singh, A., Trevick, S., 2016. The Epidemiology of Global Epilepsy. Neurol. Clin. 34, 837–847. doi:10.1016/j.ncl.2016.06.015

Singh, P., Singh, D., Goel, R.K., 2014. Phytoflavonoids: antiepileptics for the future. Int. J. Pharm. Pharm. Sci. 6, 51–66.

Soromou, L.W., Zhang, Z., Li, R., Chen, N., Guo, W., Huo, M., Guan, S., Lu, J., Deng, X., 2012. Regulation of inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 murine macrophage by 7-O-methyl-naringenin. Molecules 17, 3574–3585. doi:10.3390/molecules17033574

Sourbron, J., Smolders, I.J., De Witte, P.A., Lagae, L., 2017. Pharmacological Analysis of the Anti-epileptic Mechanisms of Fenfluramine in scn1a Mutant Zebrafish. Front. Pharmacol. 8, 1–13. doi:10.3389/fphar.2017.00191

Spencer, J.P.E., Vafeiadou, K., Williams, R.J., Vauzour, D., 2012. Neuroinflammation: modulation by flavonoids and mechanisms of action. Mol. Aspects Med. 33, 83–97. doi:10.1016/j.mam.2011.10.016

Sucher, N.J., Carles, M.C., 2015. A pharmacological basis of herbal medicines for epilepsy. Epilepsy Behav. 52, 308–318. doi:10.1016/j.yebeh.2015.05.012

Taiwe, G.S., Tchoya, T.B., Menanga, J.R., Dabole, B., De Waard, M., 2016. Anticonvulsant activity of an active fraction extracted from Crinum jagus L. (Amaryllidaceae), and its possible effects on fully kindled seizures, depression-like behaviour and oxidative stress in experimental rodent models. J. Ethnopharmacol. 194, 421–433. doi:10.1016/j.jep.2016.10.023

Tapas, A.R., Sakarkar, D.M., Kakde, R.B., 2008. Flavonoids as Nutraceuticals: A Review. Trop. J. Pharm. Res. 7, 1089–1099. doi:10.4314/tjpr.v7i3.14693

Teisseyre, A., Duarte, N., Ferreira, M.J.U., Michalak, K., 2009. Influence of the multidrug transporter inhibitors on the activity of KV1.3 voltage-gated potassium channels. J. Physiol. Pharmacol. 60, 69–76.

Vezzani, A., 2015. Anti-inflammatory drugs in epilepsy: does it impact epileptogenesis? Expert Opin. Drug Saf. 14, 583–592. doi:10.1517/14740338.2015.1010508

Vezzani, A., Friedman, A., Dingledine, R.J., 2013. The role of inflammation in epileptogenesis. Neuropharmacology 69, 16–24. doi:10.1016/j.neuropharm.2012.04.004

Walrave, L., Maes, K., Coppens, J., Bentea, E., Van Eeckhaut, A., Massie, A., Van Liefferinge, J., Smolders, I., 2015. Validation of the 6Hz refractory seizure mouse model for intracerebroventricularly administered compounds. Epilepsy Res. 115, 67–72. doi:10.1016/j.eplepsyres.2015.06.003

Wilcox, K.S., Dixon-Salazar, T., Sills, G.J., Ben-Menachem, E., White, H.S., Porter, R.J., Dichter, M. a., Moshé, S.L., Noebels, J.L., Privitera, M.D., Rogawski, M. a., 2013. Issues related to development of new antiseizure treatments. Epilepsia 54, 24–34. doi:10.1111/epi.12296

Youdim, K.A., Dobbie, M.S., Kuhnle, G., Proteggente, A.R., Abbott, N.J., Rice-Evans, C.,

2003. Interaction between flavonoids and the blood-brain barrier: in vitro studies. J. Neurochem. 85, 180–192. doi:10.1046/j.1471-4159.2003.01652.x

Zdebik, A. a., Mahmood, F., Stanescu, H.C., Kleta, R., Bockenhauer, D., Russell, C., 2013. Epilepsy in kcnj10 morphant zebrafish assessed with a novel method for long-term EEG recordings. PLoS One 8, 6–10. doi:10.1371/journal.pone.0079765

Zhang, Y., Vanmeert, M., Siekierska, A., Ny, A., John, J., Callewaert, G., Lescrinier, E., Dehaen, W., de Witte, P.A.M., Kaminski, R.M., 2017. Inhibition of glutamate decarboxylase (GAD) by ethyl ketopentenoate (EKP) induces treatment-resistant epileptic seizures in zebrafish. Sci. Rep. 7, 7195. doi:10.1038/s41598-017-06294-w

Zhu, H.L., Wan, J.B., Wang, Y.T., Li, B.C., Xiang, C., He, J., Li, P., 2014. Medicinal compounds with antiepileptic/anticonvulsant activities. Epilepsia. doi:10.1111/epi.12463

FIGURE LEGENDS

Figure 1. Behavior-based anti-seizure analysis of naringenin 4′,7-dimethyl ether (NRG-DM), naringenin 7-*O*-methyl ether (NRG-M), naringenin (NRG), kaempferide (KFD), and kaempferol (KFL) in the zebrafish pentylenetetrazole (PTZ) seizure model. Larvae were incubated with compound or vehicle (VHC) for one hour. (A) Overview of compounds. (B-F) PTZ-induced seizure-like behavior expressed as mean actinteg values during 30 minutes. (G-K) Percentage of PTZ-induced activity, normalized to PTZ-control (VHC + PTZ), as a function of time. Data is shown as the mean ±SEM from three or four independent experiments, each with n = 10-12 per condition. Statistical analysis: (B-F) one-way ANOVA with Dunnett's multiple comparison test, (G-K) two-way ANOVA with Bonferroni posttests (GraphPad Prism 5). Significance levels: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 2. Electrophysiology-based anti-seizure analysis of naringenin 4′,7-dimethyl ether (NRG-DM), naringenin 7-*O*-methyl ether (NRG-M), naringenin (NRG), kaempferide (KFD), and kaempferol (KFL) in the zebrafish pentylenetetrazole (PTZ) seizure model. Noninvasive local field potential recordings from the optic tectum of larvae pre-exposed to vehicle (VHC) and PTZ (VHC + PTZ, n = 19), VHC only (VHC + VHC, n = 17), compound and PTZ (n = 12), or compound and VHC (n = 11-12). Larvae were pre-incubated with compound or VHC for 1 hour, approximately. (A) Larvae are considered to show epileptiform brain activity when three or more epileptiform events occurred during a 10-minute recording. Epileptiform discharges are quantified by the number (B) and cumulative duration (C) of events per 10-minute recording. Data is shown as the mean ± SD. Statistical analysis: (A) Fisher's exact test with Bonferroni correction, (B-C) Kruskal-Wallis test with Dunn's multiple comparison test (GraphPad Prism 5). Significance levels: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 3. Evaluation of the anti-seizure activity of naringenin 4′,7-dimethyl ether (NRG-DM) in the mouse timed i.v. pentylenetetrazole (PTZ) seizure model. Radar graphic depicts mean PTZ doses required to induce seven characteristic behavioral parameters (ear twitch (ET), myoclonic twitch (MT), tail twitch (TT), forelimb clonus (FC), falling (F), tonic hind limb extension (THE), and death (D)), normalized to 100 % for vehicle (VHC)-treated controls. VHC (n = 8 (A) and 7 (B)), NRG-DM (n = 5), and positive control valproate (n = 5) were i.p. injected 30 minutes prior to continuous i.v. PTZ infusion.

Figure 4. Evaluation of the anti-seizure activity of naringenin 4′,7-dimethyl ether (NRG-DM) in the mouse 6-Hz psychomotor seizure model. Drug-resistant focal seizures were induced by electrical stimulation (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA) through the cornea, 30 minutes after i.p. injection of vehicle (VHC, n = 6), positive control valproate (n = 6), or NRG-DM (n = 6), and 120 minutes after i.p. injection of negative control phenytoin (n = 6). Number of mice protected against seizures are depicted (A) and defined by a seizure duration shorter than 40 seconds (s) (B). Data is shown as the mean ± SD. Statistical analysis: (A) Fisher's exact test, (B) Kruskal-Wallis test with Dunn's multiple comparison test (GraphPad Prism 5). Significance levels: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

TABLE LEGENDS

Table 1. Anti-seizure activity of naringenin 4′,7-dimethyl ether (NRG-DM) in the mouse timed i.v. pentylenetetrazole (PTZ) seizure model. PTZ doses that evoked ear twitch (ET), myoclonic twitch (MT), tail twitch (TT), forelimb clonus (FC), falling (F), tonic hind limb extension (THE), and death (D), are expressed as mean ± SD. Mice were i.p. injected with vehicle (VHC, n = 8 (group 1) and 7 (group 2)), NRG-DM (n = 5), or valproate (n = 5), 30 minutes before continuous i.v. PTZ infusion. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism 5). Significance levels: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

FIGURES

Figure 1.

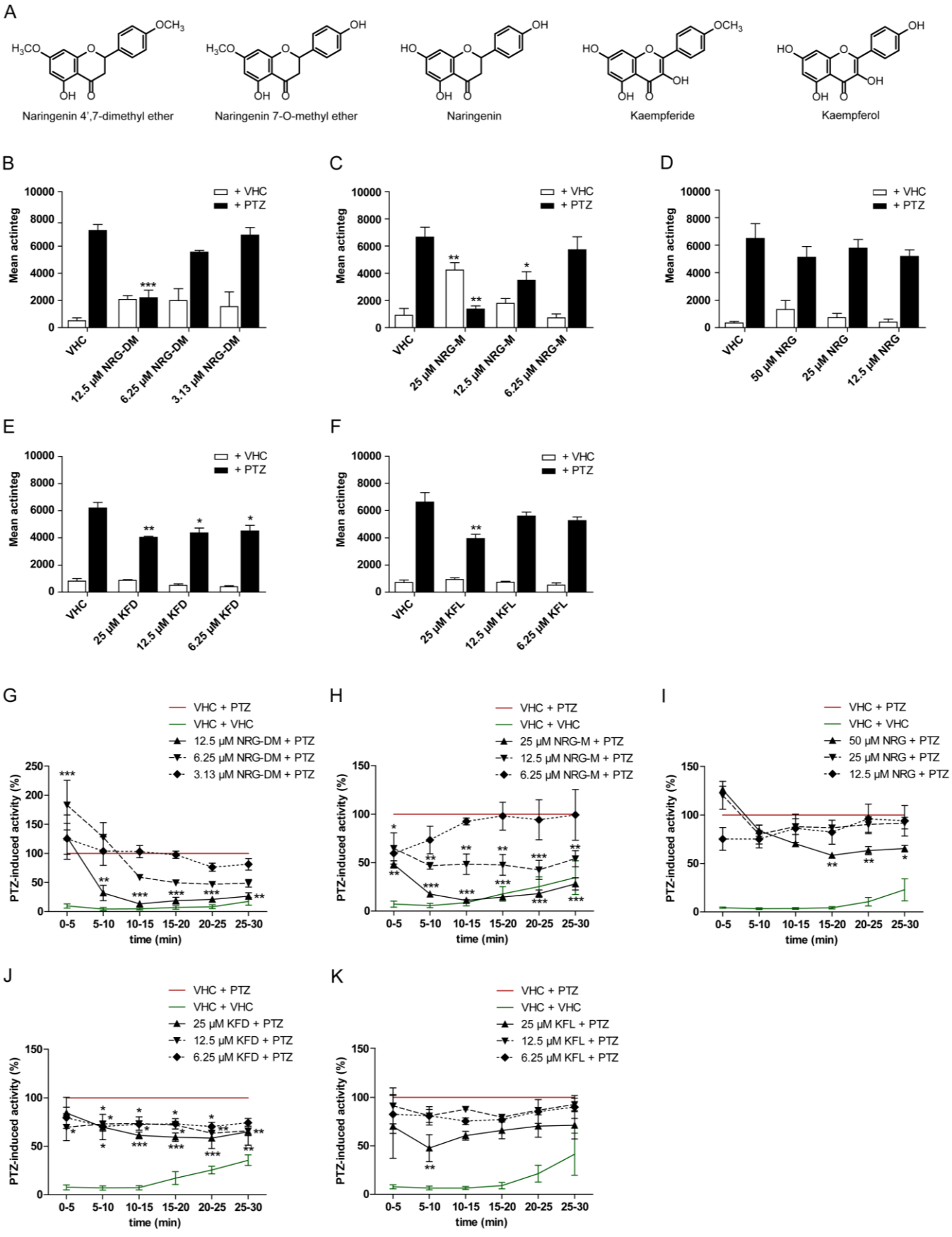


Figure 2.

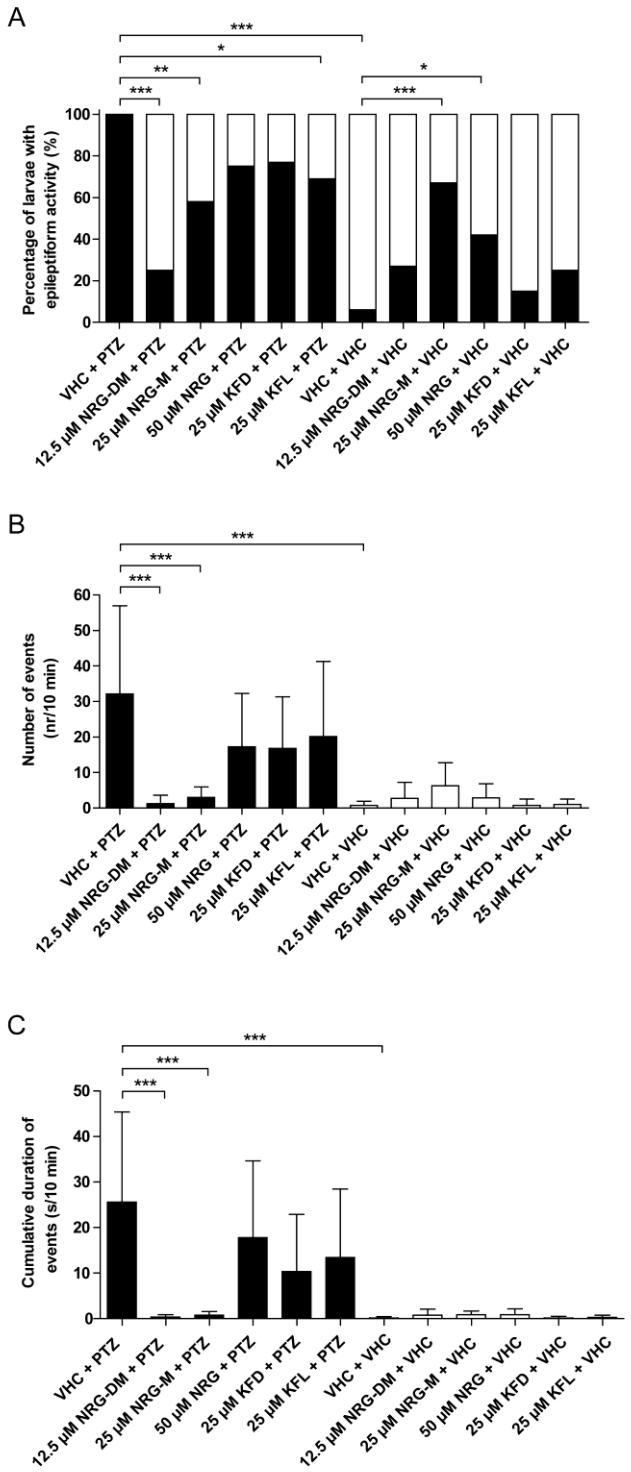

A
Percentage of larvae with epileptiform activity (%)
B
Number of events (nr/10 min)
C
Cumulative duration of events (s/10 min)
VHC + PTZ
12.5 µM NRG-DM + PTZ
25 µM NRG-M + PTZ
50 µM NRG + PTZ
25 µM KFD + PTZ
25 µM KFL + PTZ
VHC + VHC
12.5 µM NRG-DM + VHC
25 µM NRG-M + VHC
50 µM NRG + VHC
25 µM KFD + VHC
25 µM KFL + VHC

Figure 3.

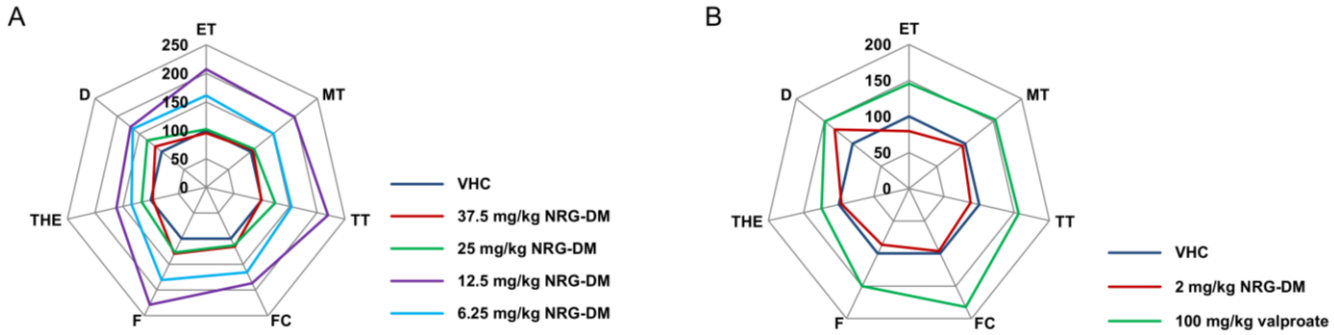
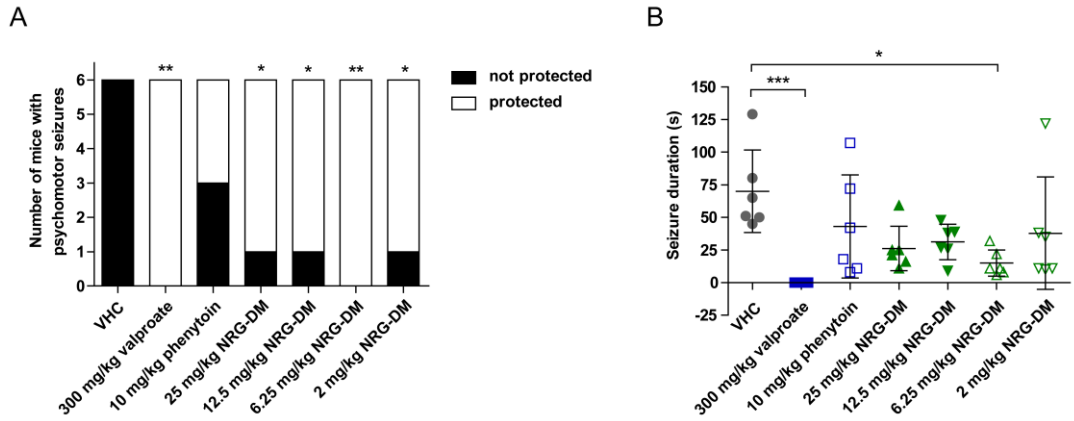


Figure 4.



TABLES

Table 1.

	mean PTZ dose (mg/kg) required (± SD)							
	VHC group 1	**37.5 mg/kg NRG-DM**	**25 mg/kg NRG-DM**	**12.5 mg/kg NRG-DM**	**6.25 mg/kg NRG-DM**	**VHC group 2**	**2 mg/kg NRG-DM**	**100 mg/kg valproate**
ET	47 ± 14	44 ± 7	48 ± 13	97 ± 57	75 ± 67	43 ± 8	34 ± 5	63 ± 33
MT	55 ± 17	58 ± 10	60 ± 16	109 ± 58	84 ± 71	48 ± 10	46 ± 6	74 ± 34
TT	48 ± 13	48 ± 8	60 ± 17	106 ± 58*	74 ± 59	46 ± 8	40 ± 7	72 ± 32*
FC	64 ± 16	74 ± 4	72 ± 15	119 ± 55	106 ± 69	67 ± 18	62 ± 3	118 ± 62*
F	68 ± 17	88 ± 6	87 ± 18	156 ± 63**	123 ± 71	69 ± 26	63 ± 8	110 ± 31*
THE	110 ± 22	106 ± 16	127 ± 31	178 ± 64*	148 ± 57	109 ± 24	118 ± 32	152 ± 48
D	133 ± 18	153 ± 43	176 ± 25*	227 ± 15***	219 ± 3***	135 ± 24	192 ± 42**	218 ± 3***