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Novel Vitamin K analogues suppress seizures in zebrafish and mouse models of epilepsy

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Abstract

Epilepsy is a debilitating disease affecting 1-2% of the world's population. Despite this high prevalence, 30% of patients suffering from epilepsy are not successfully managed by current medication suggesting a critical need for new anti-epileptic drugs (AEDs). In an effort to discover new therapeutics for the management of epilepsy, we began our study by screening drugs that, like some currently used AEDs, inhibit HDACs using a well-established larval zebrafish model. In this model, 7-day post fertilization (dpf) larvae are treated with the widely used seizure-inducing compound pentylenetetrazol (PTZ) which stimulates a rapid increase in swimming behavior previously determined to be a measurable manifestation of seizures. In our first screen, we tested a number of different HDAC inhibitors and found that one, NQN1, significantly decreased swim activity to levels equal to that of VPA. We continued to screen structurally related compounds including Vitamin K₃ (VK3) and a number of novel Vitamin K (VK) analogues. We found that VK3 was a robust inhibitor of the PTZ-induced swim activity, as were several of our novel compounds. Three of these compounds were subsequently tested on mouse seizure models at the National Institute of Neurological Disorders and Stroke (NINDS) Anticonvulsant Screening Program. Compound 2h reduced seizures particularly well in the minimal clonic seizure (6 Hz) and corneal kindled mouse models of epilepsy, with no observable toxicity. As VK3 affects mitochondrial function, we tested the effects of our compounds on mitochondrial respiration and ATP production in a mouse hippocampal cell line. We demonstrate that these compounds affect ATP metabolism and increase total cellular ATP. Our data indicate the potential utility of these and other VK analogues for prevention of seizures and suggest the potential mechanism for this protection may lie in the ability of these compounds to affect energy production.

INTRODUCTION

Epilepsy is a debilitating disease affecting approximately 1-2% of the world's population and is characterized by the periodic and unpredictable occurrence of seizures (Bialer and White, 2010). The initiation of seizure episodes are thought to result from increases in excitatory neurotransmitters (such as glutamate) and decreases in the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). However, the exact molecular

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mechanisms resulting in this imbalance are unknown. One important contributing factor to the occurrence of seizures may be the high energy demands of the nervous system. Because neurons have a low capacity to store ATP, any reduction in ATP levels can increase neuronal excitability. Decreased ATP can lead to impaired sodium-potassium ATPase activity and decreased neuronal membrane potential, both of which contribute to the increased neuronal excitability. Heightened excitability itself has the deleterious effect of exposing the neuron to damage by impairing calcium sequestration. Defective calcium transport can result in increased glutamate release into synaptic clefts, which may contribute to the occurrence of seizures (Bindoff and Engelsens, 2011, 2012). Thus, neurons are particularly vulnerable to defects in the mitochondrial respiratory chain, as this can lead to defects in ATP production by oxidative phosphorylation. Defects in the mitochondrial respiratory chain can also lead to increased reactive oxygen species (ROS) production. The brain is susceptible to ROS-induced damage because it has poor repair capacity by virtue of its lower antioxidant capacity but sustained high aerobic metabolic demand (Patel, 2002; Waldbaum and Patel, 2010). Increases in ROS have been hypothesized to lead to seizures as evidenced by studies using mice lacking mitochondrial superoxide dismutase (SOD2). Homozygous SOD2 knockout mice have been shown to display severe mitochondrial dysfunction and seizures and while heterozygous mice initially appear normal, they develop spontaneous and environmentally-induced seizures with age (Patel, 2002; Waldbaum and Patel, 2010). Furthermore, increases in ROS have the potential to directly damage neuronal tissue by attacking cellular proteins, lipids or DNA itself, and if sustained can lead to neuronal cell death (Patel, 2002).

Despite the high prevalence of epilepsy, 30% of patients do not have good control of their seizures (Duncan, 2002; Bialer and White, 2010; Loscher and Schmidt, 2011). Valproic acid (2-n-propylpentanoic acid, VPA, Depakene, Figure 1A) is one example of a broad-spectrum anti-epileptic drug (AED) used to treat all forms of seizures (Perucca, 2002). VPA is generally well tolerated, however, a high therapeutic dose is required and several side effects are associated with VPA, including acute hepatic failure, pancreatitis and teratogenesis (Lheureux and Hantson, 2009). Thus, VPA is contra-indicated for young children (Stewart et al. 2010) and pregnant women (Alsdorf and Wyszynski, 2005). Furthermore, VPA can induce a rapid decline in health in mitochondrial disease patients (Finsterer and Segall, 2010).

The mechanisms by which VPA (as an example AED) reduces seizure activity are not completely understood, but several pathways have been proposed. In the central nervous system, VPA enhances GABA-ergic transmission (Perucca, 2002), attenuates neuronal excitation and the high frequency repetitive firing associated with seizures (Rogawski and Loscher, 2004; Johannessen and Johannessen, 2003). VPA can also increase mitochondrial ATP production by serving as a substrate for beta-oxidation (Lheureux and Hantson, 2009); this is a possible mechanism for the anti-seizure activity of VPA, as maintaining or improving ATP levels would be beneficial in epilepsy. Interestingly, methylene blue (MB) is another AED that can improve mitochondrial ATP production, in this instance by acting as an alternative electron acceptor (Pelgrims et al., 2000; Furian et al., 2007). VPA also acts as an inhibitor of histone deacetylases (HDACs), which are proteins that regulate chromatin and the transcriptional state of DNA (Phiel et al., 2001), potentially linking epilepsy and VPA treatment with epigenetic changes (Hoffmann et al., 2008). Intrigued by VPA's HDAC inhibition activity, we hypothesized that other compounds that inhibit HDACs may possess similar anti-epileptic activity. To test this hypothesis we employed a high-throughput whole animal assay utilizing zebrafish larvae.

Zebrafish are an excellent animal model for use in drug screening assays as well as examination of developmental pathways (Zon and Peterson, 2005; Peterson and Fishman,

2011). They are highly fecund, producing hundreds of embryos that develop quickly and externally. Drugs can be easily taken up by developing zebrafish embryos by immersion in solutions and they are amenable to high-throughput analysis. Additionally, many behaviors can be monitored and quantified using commercially available recording devices. A number of recent studies highlight the utility of this animal model for the study of the genetic components of epilepsy as well as in screening for potential new AEDs (Baraban et al., 2005; Berghmans et al., 2007; Hortopan et al., 2010a; Hortopan et al., 2010b; Stewart et al., 2012; Baxendale et al., 2012). Many animal models of epilepsy, including worms, flies, frogs, zebrafish and mice (Hansen et al., 2004; Baraban et al., 2005), utilize the convulsant agent, pentylenetetrazol (PTZ), to induce seizures. Baraban et al. previously developed and extensively validated a zebrafish model of epilepsy, demonstrating that within minutes after exposure to PTZ, zebrafish larvae progress through a robust and stereotyped series of behaviors. This work also convincingly showed that PTZ-treated animals displayed the hallmark electrophysiological and molecular features associated with seizures in mammalian models (Baraban et al., 2005). Baraban et al. demonstrated that levels of seizure severity, as measured by field potential recordings from the brain, were tightly correlated with the high levels of swimming behavior of the animals indicating that the increased swim activity (as measured by distance traveled) represented a robust and quantitative measure of seizures in the larval zebrafish thereby establishing the parameter of distance traveled as a measure of seizure activity. This methodology has been used successfully in several studies on epilepsy (Baxendale et al., 2012; Orellana-Paucar et al., 2012; Mahmood et al., 2013), and was sensitive enough to screen >500,000 mutagenized fish for seizure-resistance (Baraban et al., 2007). Recently, the zebrafish PTZ seizure model was further validated with 13 AEDs, where the majority of AEDs caused the same response in zebrafish as assessed by behavioral (distance traveled) and electrographic assays. Afrikanova et al. (2013) showed that the zebrafish PTZ model correlates well with rodent models and that the zebrafish larval locomotor assay can be used to assess anticonvulsant activity of compounds.

Here we initially investigated the anticonvulsant activities of HDAC inhibitors using a zebrafish model system. Our results indicated that the HDAC inhibitor NQN1 was effective at reducing seizure-related behaviors in zebrafish. The Vitamin K (VK) family shares a naphthoquinone moiety with NQN1 and recent reports have suggested that VK has a role in nervous system function (Josey et al., 2013; Ferland, 2013). We went on to show that VK3 reduced seizure-activity in zebrafish, and directed by these results, we designed, synthesized and tested new VK3 analogues. Although we initially hypothesized that our positive compounds reduce seizures through HDAC inhibition, we did not observe any HDAC inhibitory activity. Thus, based on the reported actions for VK3 and other known AEDs, we hypothesized that our positive compounds may be reducing seizure activity by impacting energy metabolism (Vos et al., 2012; Wen et al., 2011; Pelgrims et al., 2000; Furian et al., 2007) and tested the effects of our compounds on energy metabolism of HT-22 neuronal cells. In addition, we tested our lead compounds for anticonvulsant activity and toxicity in mouse models of epilepsy. Our results suggest that these novel compounds may represent a promising new class of anti-seizure medication.

EXPERIMENTAL PROCEDURES

Chemicals

PTZ (Sigma P6500), 2-benzoylamino-1,4-naphthoquinone (NQN1), suberoylanilide hydroxamic acid (SAHA), diphenyl acetic hydroxamic acid (dPAHA), Tubastatin A, VPA (Sigma P4543), 2-propylpentane hydroxamic acid (VPHA), and Vitamin K₃ (VK3) were synthesized in the laboratory or obtained from commercially available sources (Inks et al., 2012). Vitamin K (VK) analogues were synthesized according to Josey et al. (Josey et al., 2013).

Zebrafish studies

Zebrafish (AB strain) were obtained from the Zebrafish International Resource Center (supported by P40 RR012546 from NIH-NCRR). Zebrafish were maintained and crossed according to standard methods (Westerfield, 2000). Fertilized eggs were collected and placed in E3 embryo medium and positioned in an incubator set at 28.5°C with a 14/10 h light/dark cycle (Kimmel et al., 1995). To determine the lethal dose of each compound, we used 96-well plates containing one zebrafish (7 days post-fertilization, dpf) per well in 100 µL of tank water. 100 µL of each compound (0.5 to 15 µM) was added to each well for 12 animals (one row) for a final volume of 200 µL. One row of larvae was used as DMSO only controls. The 96-well plate was placed on a warmer at 28.5°C and fish were observed for changes in phenotype, behavior and mortality initially after addition of compound, after 1 h treatment and after 5 h treatment. All zebrafish studies were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (AR #2850) and performed in accordance with the guidelines.

Induction and monitoring of seizures in zebrafish

We induced seizures in 7 dpf zebrafish larvae by addition of 15 mM PTZ as originally developed by Baraban et al. (Baraban et al., 2005). In a 48-well plate, one 7 dpf zebrafish was added per well. Larvae were dosed with each compound at a sub-lethal dose 1 h prior to PTZ treatment. Three control rows were included with each experiment – tank water only control, PTZ only and PTZ+VPA (4 mM final concentration of VPA). Seizures were induced by adding PTZ to wells to yield a final concentration of 15 mM. After 5 min, the plate was transferred to the Daniovision instrument (Noldus Information Technology) and the chamber light was turned on. After 2 min, MediaRecorder (Noldus) was used to record video for 15 min. A small number of videos were acquired at 25 frames per second, but the majority of data was acquired at 60 frames per second. After recording, fish were monitored visually for survival. Ethovision XT software (Noldus) was used to track the fish movement from the video images in order to calculate the total distance traveled over 15 min. Our methods were similar to those used in Baraban et al. (2005), which established that the distance traveled by fish after induction of seizures by PTZ reliably reflects seizure activity. All experimental comparisons were made between animals from the same clutch.

Toxicity studies

Using a 96-well plate, one zebrafish larva (7 dpf) was placed in each well in 100 µL of tank water. 100 µL of each compound was then added to each well for 12 animals (one row) for a final volume of 200 µL. One row of zebrafish larvae was used as DMSO only controls. The 96-well plate was then placed on a warmer plate at 28.5°C and the fish were observed for changes in phenotype, behavior and mortality initially after addition of compound, after 1 h of treatment and after 5 h of treatment. Toxicity was also measured in the mouse model by the NIH Anticonvulsant Screening Program at the National Institute of Neurological Disorders and Stroke (NINDS; Stables and Kupferberg, 1997), according to the established NIH experimental procedures. Compounds were delivered into mice by intraperitoneal (i.p.) injection at a dose of 100 mg/kg in sterile 5% DMSO, 95% Neobee (Josey et al., 2013). Acute toxicity was assessed by monitoring the animals for impaired neuromuscular function by placing treated mice on a rod rotating at 6 rpm. Compounds were considered toxic if the mouse fell off the rod three times in 1 min.

c-fos gene expression

Ten 7 dpf larvae were placed in 500 µL tank water in wells of a 48-well cell-culture plate and appropriate concentrations of drugs were added as in the behavior study (Table 1). After a 1 h pre-incubation period, PTZ was added to a final concentration of 15 mM in appropriate

wells and larvae were incubated for a further 45 min. Fish were quickly euthanized by incubating the plate in an ice-water bath for 15 min. Fish were removed from each well and all liquid removed before freezing at -80°C . RNA was extracted using Trizol (Invitrogen) followed by the RNeasy Mini kit (Qiagen). Frozen embryos were homogenized in 800 μL Trizol using in-tube pestles and a motorized homogenizer. Following a 5-min incubation at room temperature, 200 μL chloroform was added and the samples were centrifuged at 12,000 g for 10 min. The aqueous phase was transferred to a new tube and 250 μL 100% ethanol was added and the samples mixed. This mixture was then transferred to the Qiagen minicolumn assembly and the protocol followed as described with the kit. Samples were eluted in 30 μL RNase free water and concentration was determined using the Nanodrop instrument (Thermo Fisher). cDNA was prepared using the RETROscript kit (Ambion) with 500 ng total RNA. cDNA was then diluted 1:1 with sterile dH_2O and 2.5 μL of this cDNA was used in the qPCR reaction with SsoAdvanced SYBR Green Supermix (BioRad) and *c-fos* primers designed to span an intron-exon junction (*c-fos* F CACTGCAAGCTGAACTGACC; *c-fos* R GCGGCGAGGATGAACTCTAA) (300 mM each) in the BioRad CFX96 RealTime instrument. L13a and EF1a gene expression were used for normalization (Rahn et al., 2013). The following cycle conditions were used $95^{\circ}\text{C}/3$ min, 40 cycles of $95^{\circ}\text{C}/15$ s, $62^{\circ}\text{C}/30$ s, followed by 95°C 30 s and a dissociation curve. Samples were run in duplicate. The $2^{-\Delta\Delta\text{Ct}}$ method was used to quantify gene expression, whereby all gene C_t values were first normalized to C_t values of the geometric mean of the C_t of L13a and EF1a (Livak and Schmittgen, 2001). Treated samples were then normalized to the tank water control and converted to fold change.

Mouse studies

Mouse studies were performed by NIH Anticonvulsant Screening Program at the NINDS (Stables and Kupferberg, 1997) according to the established NIH experimental procedures outlined below. Compounds were delivered into mice by i.p. injection at a dose of 100 mg/kg in sterile 5% DMSO, 95% Neobee (Josey et al., 2013). One of four methods for seizure induction was subsequently administered to mice at 0.25, 0.5, 1, 2 and 4 h after treatment with compound. (1) Subcutaneous PTZ seizure threshold test. PTZ was administered at a concentration of 85 mg/kg, into the loose fold of skin in the midline of the neck. Mice were observed for 30 min for presence or absence of seizure (White et al., 1995). Mice were considered protected if they did not have clonic spasms (lasting approximately 3-5 s). (2) Maximal electroshock test. 60 Hz of 50 mA alternating current was delivered for 0.2 s by corneal electrodes. Mice were considered protected from seizures when the hindlimb tonic extensor was absent (White et al., 1995). (3) Minimal clonic seizure (6 Hz) test. 6 Hz of 32 mA or 44 mA alternating current was delivered for 3 s by corneal electrodes to elicit a psychomotor seizure. Mice were considered protected from seizures when the automatic behaviors were absent (Barton et al., 2001). (4) Corneal kindled mouse model. Mice were kindled electrically with a 3 s stimulation, 8 mA, 60 Hz, and corneal electrodes to a criterion of 10 consecutive Stage 5 seizures (facial clonus and head nodding progressing to forelimb clonus, and finally rearing and falling accompanied by a generalized clonic seizure as described by Racine, 1972). Animals generally reach Stage 5 after twice daily stimulation for 8 days. With continued stimulation once a day, animals usually progressed to a reproducible Stage 5 after 10-14 additional days. At least 72 hours after the mice were kindled, the test substance was administered i.p. and each animal was given the electrical stimulus indicated above. Following stimulation, the animals were observed for the presence or absence of the rearing and falling criteria of a Stage 5 seizure. Treated animals not displaying a Stage 4 or 5 seizure were considered protected.

Cell culture

HT-22 neuronal cells were grown in Dulbecco's Modified Eagle's Medium (DMEM/high glucose) supplemented with 10% fetal bovine serum and 1% of antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO₂. The HT-22 neuronal cell line is a subclone of the HT4 cell line, derived from mouse hippocampus (Morimoto and Koshland, 1990), kindly provided by Dr. David Schubert (The Salk Institute for Biological Studies).

Respirometry

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of HT-22 neuronal cells were performed on the XF-96 Extracellular Flux Analyzer (Seahorse Bioscience) using standard methods (Beeson et al., 2010). In brief, cells were cultured in 96 well Seahorse plates in DMEM high glucose media (Invitrogen) supplemented with 10% FBS, 10 mM HEPES, and 1% antibiotic-antimycotic (Invitrogen). The media was replaced with DMEM media supplemented with 25 mM glucose, 10 mM sodium pyruvate, 31 mM NaCl, and 2 mM glutamine at pH 7.4. Each of the four drug ports on the Seahorse sensor cartridge were filled with test compound (media only, 5 μ M NQN1, 10 μ M VK3, 5 μ M NQN1, 5 μ M analogue 2h, 5 μ M analogue 2j or 12.5 μ M MB), 10 μ M oligomycin, 1 μ M FCCP, and 5 μ M rotenone, which were injected into each well at 20, 80, 110, and 135 min, respectively. The pmol/min OCR rate and mpH/min ECAR for each well (12-89 wells/group) were measured. Wells were excluded from the analysis if their OCR or ECAR values surpassed the Tukey Outlier Rule (1.5 times greater than the interquartile range). In order to standardize the respiration rates across different plates, the data were normalized to the OCR or ECAR values at 17 min (last time point prior to injection of the test compounds) for each condition and then difference from control levels were calculated.

Fluorometric ATP assay

Cellular ATP levels were determined using a fluorometric ATP assay (BioVision). HT-22 cells were pretreated for 8 h with media only, 5 or 10 μ M NQN1, 5 or 10 μ M VK3, or 12.5 μ M MB. Cells were lysed with 100 μ L ATP assay buffer, sonicated for 20 s and centrifuged at 15,000 g for 2 min at 4°C. Protein concentrations of the supernatant were determined using the Bicinchoninic acid assay. 2-4 μ g of total protein lysate was used for ATP determination. A standard curve was generated with known ATP concentrations. ATP concentrations for each sample were determined and adjusted for total protein per mg lysate. Data were normalized to control samples from the respective plates.

Statistical Analyses

Statistical analyses were performed with JMP 10.0.2 software (SAS Institute). Multiple comparisons were made using a one-way ANOVA with a Kruskal-Wallis Test, followed by Dunn's Method to determine significant differences between all pairs or between control and experimental groups using the Dunn Method for Joint Ranking. Differences were considered statistically significant when $p < 0.05$. Data are represented as means $\pm$ standard error of the mean (SEM).

RESULTS

NQN1 reduced distance traveled in PTZ-treated zebrafish larvae

Because VPA had been shown to inhibit HDAC activity (Phiel et al., 2001), we decided to pursue other HDAC inhibitors as a potential new class of anti-epileptic drugs. We selected a panel of HDAC inhibitors (SAHA, dPAHA, NQN1, Tubastatin A and VPHA) with different HDAC isozyme inhibition profiles (Tessier et al., 2009, Bradner et al., 2010, Butler et al., 2010, Fass et al., 2010) for study in the zebrafish model. We first tested the toxicity of these

compounds on 7 dpf zebrafish larvae and determined the highest sub-lethal dose for each (Table 1). A pre-treatment experimental protocol was established in order to more accurately model the effectiveness of these drugs in preventing the initiation of seizures. Zebrafish larvae (7 dpf) were treated with the selected compounds at the determined sub-lethal concentrations for 1 h prior to inducing seizures with PTZ. Similar to previous larval zebrafish epilepsy studies, total distance traveled after seizures by each fish was measured and used as a proxy for seizure activity. Figure 1B shows representative traces of swimming behavior of individual zebrafish beginning 5 min after administration of PTZ to each well, and the total distance traveled (shown normalized to the control) for 15 min are given in Figure 1C. The swim behavior traces clearly show that compared to the control animal, PTZ induces a robust increase in total distance traveled by the fish (Figures 1B, 1C). Compared to the average control distance, treatment with PTZ induced a significant 4-fold increase in total distance traveled ($p < 0.0001$; Figures 1B, C). VPA was included as a positive control and as previously shown by Baraban et al. (2005), VPA significantly reduced distance traveled compared to PTZ alone ($p = 0.0007$) and to a level indistinguishable from control ($p = 1.0$). Of the five HDAC inhibitors tested in this initial screen, only NQN1 significantly suppressed PTZ-induced swim activity in zebrafish, reducing the seizure-associated swimming to a level not significantly different from the untreated control levels ($p = 0.2113$; Figure 1B, C). The concentration of NQN1 required to reduce swim levels in PTZ-treated fish was 1300 times less than the required concentration of VPA (Table 1). Neither VPA nor NQN1 alone significantly altered the swimming behavior (distance traveled) of the fish compared to controls ($p = 1.0$ and $p = 1.0$, respectively; Figures 1B, 1C). None of the other HDAC inhibitors tested reduced swim activity (data not shown).

We more closely examined the larval fish treated with our compounds to correlate our measure of seizures with a molecular marker for neuronal activity. *c-fos* (Gene ID:394198) expression has been shown to increase with seizure activity and was used previously to validate this zebrafish model of epilepsy (Baraban et al., 2005). We developed a new quantitative real-time PCR assay to measure *c-fos* gene expression in pools of zebrafish rather than in single fish as has been previously reported. We show that PTZ treatment increased *c-fos* expression 80-fold over control and that pre-treatment with VPA or NQN1 was able to blunt this increase in expression. Treatment with VPA or NQN1 alone did not increase *c-fos* expression to this extent (Figure 1D).

VK3 reduced PTZ-induced swim activity in 7 dpf zebrafish

Because NQN1 reduced swim activity to levels comparable to VPA treatment, we looked more closely at its chemical structure. NQN1 contains a central naphthoquinone moiety, which is the core motif of many natural products, but most notably it is the central structure for VK3 (Figure 2A). After first determining the highest non-lethal dose (Table 1), we tested VK3 in our zebrafish assay and demonstrated a robust inhibition of the PTZ-induced seizure swim behavior at the highest tolerated dose (6 μ M) as seen in the traces of the total distance traveled for representative fish (Figure 2B) and the normalized distance traveled (Figure 2C). 6 μ M VK3 reduced distance traveled after PTZ treatment more than 4-fold to a level not significantly different from the untreated control swim levels ($p = 1.0$). *c-fos* gene expression was also reduced by approximately 10-fold compared to PTZ alone after pre-treatment with VK3 (Figure 2D). We found declining activity of VK3 with reduction in dose (Figure 2B, C). At 3 μ M VK3, the PTZ-induced swim behavior was reduced 2-fold, which was not significantly different from control levels ($p = 0.5038$) or the PTZ levels ($p = 0.0996$). Treating the fish with 1.5 μ M VK3 only reduced distance traveled 0.5 fold from PTZ levels and was not significant from PTZ alone ($p = 1.0$). These data indicated that while both 3 μ M and 6 μ M VK3 were effective at blocking PTZ-induced seizure behavior in zebrafish, only the higher dose reduced the seizure behavior to control levels. We also tested

VK3 without PTZ and did not see any significant change in swim activity compared to control ($p=1.0$). Additionally, *c-fos* expression was not increased to the levels observed with PTZ treatment confirming that VK3 alone does not act as a sedative nor increase swim activity.

Novel Vitamin K analogues reduced PTZ-induced swim activity in zebrafish larvae

VK3 and NQN1 were effective in reducing PTZ-induced swimming in zebrafish, but testing revealed toxicity at higher concentrations (Table 1). In order to find new compounds that might be equally active but without the observed toxicity, we developed several new VK analogues (Figure 3A; Josey et al., 2013). Compound 2j was synthesized by modifying the core 1,4-naphthoquinone motif of VK by the addition of a benzyl amine group to the 2' position. 2q was generated by the further addition of chlorine at the meta position of the added benzene ring. 2h was generated by replacing the benzene group of 2j with a terminal alkyne, and 3n was created by replacing the benzene moiety of 2j with a trifluoromethyl group and the central methylene group with a carbonyl. A more detailed description of the synthesis and activity of these and other VK derivatives has been reported previously by our group (Josey et al 2013). We first determined the highest non-lethal dose in zebrafish for these compounds (Table 1). Compounds 2h and 2q did not display any toxicity at the concentrations we tested, however compounds 2j and 3n did display some toxicity although at higher doses than for VK3 and NQN1.

We then tested these analogues for activity in PTZ-treated zebrafish. Traces of the total distance traveled of an untreated control animal, animals exposed to VK analogues alone, to PTZ alone, and to the VK analogues in combination with PTZ are shown in Figure 3B. Quantification of PTZ-evoked swim behavior is shown in Figure 3C. We did not detect any differences in the total distance traveled when the fish were treated with any of the VK analogues alone (Figure 3C, $p=1.0$). Two compounds did however significantly reduce PTZ-induced swim activity 2.5-3 fold (2h $n=8$; $p=0.0018$ and 2j $n=16$; $p=0.0221$). Of these two analogues, only 2h reduced the induced swimming to a level indistinguishable from untreated controls ($p=1.0$); the distance traveled of fish treated with the 2j analog was significantly greater than control levels ($p=0.001$). Compounds 2q and 3n, although reducing the distance traveled after PTZ treatment more than two fold, did not reach statistical significance compared to PTZ levels (2q $n=8$; $p=0.3$ and 3n $n=16$; $p=0.06$). However, compound 2q did reach levels similar to control ($p=0.08$) where 3n remained different from control ($p=0.0004$). Together these data indicate that compound 2h was most effective at suppressing PTZ-induced seizure behavior, reducing distance traveled by more than half compared to PTZ alone, to a level comparable to control levels. We also examined *c-fos* gene expression and show that 2h, 2j, 2q, and 3n all reduced *c-fos* gene expression by about half, compared to PTZ alone. Use of these compounds in the absence of PTZ did not elicit a large change in *c-fos* expression (Figure 3D).

VK3 and NQN1 increased overall respiration and mitochondrial ATP turnover

Previous work has shown that VK increases electron transport and oxidative phosphorylation by acting as an alternative mitochondrial electron carrier in the electron transport chain (Vos et al., 2012). This suggests that VK could increase ATP production, and by extension potentially explain its anti-seizure activity. As many of our successful compounds were based on the core structure of VK, we were interested in testing the effects of these compounds on cellular respiration. Using Seahorse extracellular flux technology, we analyzed the effects of NQN1, VK3, and the 2j and 2h analogues on the mitochondrial function of mouse HT-22 cells. Additionally, we examined MB, another AED shown to act as an alternate electron acceptor (Wen et al., 2011; Pelgrims et al., 2000; Furian et al., 2007). The Seahorse instrument allows measurement of oxygen consumption rates (OCR) in real

time and with application of specific chemical inhibitors of enzyme complexes of the electron transport chain (ETC), detailed analysis of aspects of cellular respiration can be quantified. Basal cellular respiration was measured 1 h after treatment with 10 μ M VK3, 5 μ M NQN1, 5 μ M 2h, 5 μ M 2j or 12.5 μ M MB and the differences in OCR from control levels were calculated (Figure 4A). Addition of VK3 (21.57 ± 0.97 pmol/min; $p < 0.0001$, $n = 38$), NQN1 (24.35 ± 1.03 pmol/min; $p < 0.0001$, $n = 19$), analogue 2h (4.14 ± 0.64 pmol/min; $p < 0.0449$, $n = 12$) and MB (53.21 ± 5.92 pmol/min; $p < 0.0001$, $n = 18$) each significantly increased total cellular respiration compared to untreated control cells. The addition of 2j (-4.14 ± 2.9 pmol/min; $p = 1.0$, $n = 25$) did not significantly alter total cellular respiration of the cells compared to untreated control levels (Figure 4A).

Calculating the difference between basal respiration values and those after exposure to oligomycin, an inhibitor of the ATP synthase (complex V of the ETC), reveals OCR linked to ATP levels. Figure 4B shows significantly higher levels of ATP-linked respiration from the cells exposed to VK3 (12.2 ± 3.12 pmol/min; $p = 0.0056$), NQN1 (16.83 ± 1.83 pmol/min; $p < 0.0001$), and MB (19.76 ± 6.59 pmol/min; $p = 0.0006$), compared to untreated cells. ATP-linked respiration for cells treated with compound 2j was elevated but not significantly different to controls (6.58 ± 3.28 pmol/min; $p = 0.0924$), and treatment with analogue 2h significantly decreased levels (-18.4 ± 3.3 pmol/min; $p = 0.0254$; Figure 4B).

We used rotenone, a complex I inhibitor of the ETC, to show the level of OCR that is linked to non-mitochondrial respiration, and in doing so were able to calculate the OCR specifically resulting from mitochondrial respiration (basal respiration minus non-mitochondrial respiration). Treatment with 2j (9.09 ± 3.13 pmol/min; $p = 0.0430$), NQN1 (15.26 ± 2.29 pmol/min; $p = 0.0012$), or MB (19.22 ± 6.54 ; $p = 0.0038$) increased basal mitochondrial respiration compared to untreated control cells. VK3 mitochondrial respiration was elevated (8.11 ± 3.8 pmol/min) but failed to reach statistical significance ($p = 0.0684$) and treatment with analogue 2h significantly decreased OCR levels (-21.7 ± 5.3 pmol/min; $p = 0.0203$) compared to the basal mitochondrial respiration measured from control cells.

Using the extracellular flux analyzer it is also possible to measure extracellular acidification rates (ECAR, a measure of glycolysis). ECAR levels from cells treated with VK3 (1.89 ± 0.93 mpH/min; $p = 0.38$), NQN1 (1.75 ± 1.12 mpH/min; $p = 0.73$), analogue 2h (-5.4 ± 1.1 mpH/min; $p = 1.0$), or MB (-0.86 ± 1.26 ; $p = 1.00$) were not different from untreated control cells, however treating cells with 2j significantly increased glycolytic metabolism to 5.03 ± 1.11 mpH/min ($p = 0.0002$) compared to controls (Figure 4D).

VK3, NQN1, 2h and MB increase total cellular ATP

We hypothesized that the compounds found to be active at reducing swim activity may be acting on total ATP levels. To follow up on the respiration experiments, we measured total cellular ATP levels in the HT-22 cells using a fluorometric method. Addition of VK3, NQN1, 2h or MB significantly increased total cellular ATP levels 22-29% above control levels ($n = 4-12$; $p < 0.05$; Figure 5).

Novel Vitamin K analogues reduced seizures in mouse models of epilepsy

Compounds 2j, 2h and 2q were sent to the Anticonvulsant Screening Program at the NINDS (NIH), to test for anti-epileptic activities in four different mouse models of epilepsy. Pretreatment with 2j, 2h or 2q (100 mg/kg) had no effect on PTZ-induced seizures in mice (data not shown). However, all three compounds showed anti-epileptic activity with the 6 Hz model at 32 mA (Table 2) with the most promising compound being 2h, with 100% protection against 6 Hz seizures at 32 mA at 0.25 h. The 2j and 2q analogues also had some

anti-seizure activity. Additional testing of 2h with the 6 Hz model at 44 mA did not result in any protection (data not shown). There was some limited anti-epileptic activity for 2j and 2h with the maximal electroshock test (Table 3). Further testing was performed using 2h with the kindled mouse model and this compound showed activity protecting 2/4 mice from seizures (Table 4). No or low acute toxicity was observed in mice treated with 100 mg/kg of each compound, as assessed by rotorod assay (Table 5). Previously, we had treated mice with compounds 2j and 2q at 50 mg/kg i.p. daily for 3 weeks and did not observe any blood or major organ toxicity (Josey et al., 2013).

DISCUSSION

There is an unmet clinical need for new anti-epileptic drugs due to the intractance of seizures in many patients. In general, potential AEDs are tested on adult rodents, however since 70% of epilepsy occurs in childhood, there is a precedent for screening potential AEDs in younger animals, in addition to adults (Loscher and Schmidt, 2011). Use of the well-established zebrafish model of epilepsy allows us to address both of these needs by utilizing a higher-throughput assay on larval fish.

We initially began this study by following up on the observation that VPA can act as an HDAC inhibitor and initially hypothesized that activity against HDACs could be a mode of action for VPA and other AEDs. While we tested several known HDAC inhibitors with varying levels of specificity, only one HDAC inhibitor, NQN1, was effective at reducing swimming distance traveled (a measurement of seizure activity) to VPA-levels, at a concentration 1300 times lower than VPA. NQN1 (as well as VPA) reduced *c-fos* gene expression indicating that these compounds do indeed reduce seizures and that this reduction in seizures is quantifiable by measuring swim activity. None of the other HDAC inhibitors tested reduced PTZ-induced swim activity, including VPHA, which is the hydroxamic version of VPA that was previously shown to reduce seizures in rodents (Gravemann et al., 2008). NQN1 has been demonstrated to have specific inhibitory activity against HDAC 6 (Inks et al., 2013). However, none of the other successful compounds had any HDAC inhibitory activity (data not shown). This suggests that HDAC inhibition is not the molecular target for the anti-seizure activity of these compounds.

Because the core structure of NQN1 is a naphthoquinone similar to VK, we hypothesized that VK3 may exhibit similar activity to what was observed with NQN1. Testing VK3 using our zebrafish model showed that VK3 at 6 μ M reduced PTZ-induced swim activity to control levels and the level of swim activity inhibition was dose dependent (Figure 2). VK3 also reduced *c-fos* gene expression from PTZ treatment alone. VK3 has been noted to exhibit toxicity, and indeed we noticed toxicity in our larval zebrafish (Table 1). Because of this toxicity we developed and tested several VK analogues (analogues 2h, 2j, 2q and 3n; Figure 3). Several of these analogues reduced seizure activity in zebrafish to levels comparable to VPA and reduced *c-fos* gene expression; in addition they were effective without the toxicity observed with higher concentrations of VK3.

Although not clearly understood, one important contributing factor to the occurrence of seizures may be the high energy demands of the nervous system. Because neurons have a low capacity to store ATP, any alteration in mitochondrial function can increase neuronal excitability, which may contribute to seizures (Bindoff and Engelsens, 2011). Neurons are thus heavily reliant on mitochondria, the major source of ATP in the cell (Bindoff and Engelsens, 2011). Additionally, defects in Complex I of the mitochondrial ETC are often observed in patients with epilepsy (Waldbaum and Patel, 2010) further implicating the mitochondria and ATP production in the pathology of epilepsy. The widely used AED VPA can act as a substrate for beta-oxidation thereby increasing mitochondrial ATP production

(Lheureux and Hantson, 2009). MB, another AED, can improve mitochondrial ATP production by acting as an alternative electron acceptor (Pelgrims et al., 2000; Furian et al., 2007). We therefore hypothesized that the mechanism of action for VK3, NQN1, and our analogues, may be related to these drugs altering or enhancing mitochondrial energy production.

Measurements of cellular metabolism from HT-22 cells treated with VK3, NQN1 or MB demonstrated that these three compounds act in a similar manner to increase overall cellular respiration (Figure 4A), ATP-linked respiration (Figure 4B), and mitochondrial-dependent respiration (Figure 4C). Our data complements studies from other labs showing that MB and VK potentiate mitochondrial energy production by acting as alternative electron carriers (Wen et al., 2011, Vos et al., 2012, Shneyvays et al., 2005). VK3, MB and NQN1 did not increase ECAR (Figure 4D), suggesting that the elevated ATP levels we measured (Figure 5) are indeed due to increased mitochondrial oxidative phosphorylation, and not glycolysis.

We observed interesting differences in the actions of the two lead VK analogues (2h and 2j) on cellular metabolism. While the analogue 2h modestly increases basal cellular respiration, 2j did not (Figure 4A). Treatment with 2h decreased ATP-linked respiration and basal mitochondrial respiration, unlike 2j, which did not affect ATP-linked respiration but increased basal mitochondrial respiration (Figures 4B and C). These data also suggest that while not changing overall basal cellular respiration rates, compound 2j may drive cells toward glycolysis as shown by the increased ECAR (Figure 4d). VK3 was previously identified in a nutrient sensitized screen for its ability to shift cellular energy metabolism to glycolysis (Gohil et al., 2010). Although we did not observe this metabolic switch with VK3 in HT-22 cells, an increase in glycolysis with compound 2j suggests that switching metabolism to glycolysis could be a potential mechanism underlying its ability to increase total ATP levels (Figure 5). Treatment with analogue 2h increased total ATP levels (Figure 5), produced no change in glycolysis (Figure 4D) and decreased ATP-linked respiration (Figure 4B), suggesting that ATP utilization is decreased with 2h treatment. Decreased mitochondrial respiration could be accompanied by decreased ROS production, as ROS are a byproduct of oxidative phosphorylation. Accordingly, these VK analogues also protected neurons against glutamate toxicity by decreasing mitochondrial ROS generation (Josey et al., 2013). To further tease out the differences we describe for each compound, future studies would call for isolated mitochondrial experiments to examine the activity of different complexes of the ETC in the presence of these compounds.

Although we did not test MB in our anticonvulsant screen in the zebrafish, based on our observations of its actions in HT-22 cells, it is possible that MB may have some activity on cellular respiration in the zebrafish embryo. This may be of interest to those researching epilepsy in zebrafish as MB is often used in laboratories employing zebrafish as model organisms to reduce fungal growth in media used to rear embryos, albeit at much lower concentrations (0.002%; Westerfield, 2000).

Our lead VK analogues were tested by the Anticonvulsant Screening Program at the NINDS for anticonvulsant activity in mouse seizure models. All compounds (2j, 2h, 2q) showed good anticonvulsant activity in the minimal clonic (6 Hz) test at 32 mA (Table 2), whereas compounds 2j and 2h showed limited anticonvulsant activity in the maximal electroshock test (Table 3). Compound 2h did not protect against seizures in the minimal clonic (6 Hz) test at 44 mA (data not shown). Compound 2h was further tested in a corneal kindled mouse model and showed protection in 2 of 4 mice (Table 4). Each compound at 100 mg/kg showed no toxicity in mice (Table 5), and our previous studies showed that 50 mg/kg injected i.p. daily in mice for three weeks was not toxic (Josey et al., 2013).

The 2h compound was the most effective VK analog from the zebrafish screen, reducing PTZ-induced seizure behavior to control levels (Figure 3). Overall the data from the Anticonvulsant Screening Program suggests that it is also the most promising potential AED in the different mouse models (Tables 2-4). Analogue 2h's efficacy may in part be due to its superior bioavailability. 2h has the lowest CLogP values (a measure of hydrophobicity, 2.62) compared to analogues 2j and 2q (3.8 and 4.5, respectively; Josey, personal communication). Differences in CLogP hydrophobicity may explain in part why all three compounds showed some anti-seizure activity in the zebrafish, but limited results in the mouse model. Low solubility is likely more problematic for mammals where compounds must cross the blood brain barrier. Nonetheless, screening potential AEDs using zebrafish was useful for quickly finding potential lead compounds and directing the derivation of new VK analogues. Further studies are planned to test analogue 2h and related compounds in chronic seizure models.

In conclusion, our data suggest that energy production is a good target for developing new therapeutics for epilepsy. Our VK analogues may be valuable compounds to explore in the development of new AEDs, as these compounds are likely acting to alter energy production to reduce seizure activity. Our results reveal that compound 2h protects against seizures in the minimal clonic (6 Hz) and the corneal kindled mouse epilepsy models. Additionally, these compounds can now be used as tools to provide new insights into the basic mechanisms underlying epileptogenesis, and may also have the potential for treatment of other neurological disorders.

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Abbreviations

ATP	adenosine triphosphate
AED	anti-epileptic drug
DPF	days post fertilization
ECAR	extracellular acidification rate
ETC	electron transport chain
FCCP	trifluorocarboxylcyanide phenylhydrazone
DMSO	dimethyl sulfoxide
GABA	gamma-aminobutyric acid
HDAC	histone deacetylase
MB	methylene blue
NINDS	National Institute of Neurological Disorders and Stroke
NQN1	2-benzamido-1 4-naphthoquinone
OCR	oxygen consumption rate

PTZ	pentylenetetrazol
ROS	reactive oxygen species
SAHA	suberoylanilide hydroxamic acid
VPHA	2-propylpentane hydroxamic acid
VK	Vitamin K
VK3	Vitamin K ₃
VPA	valproic acid, 2-n-propylpentanoic acid
2j	VK analogue modified by the addition of a benzyl amine group to the 2' position of the 1,4-naphthoquinone motif of VK.
2q	VK analogue. It is a modification of analogue 2j with addition of a chlorine at the meta position of the added benzene ring.
2h	VK analogue. It is a modification of analogue 2j, with the addition of a terminal alkyne to the added benzene
3n	VK analogue. It was created by replacing the central methylene group of analogue 2j with a carbonyl group.

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HIGHLIGHTS

- NQN1, a compound with a naphthoquinone moiety, reduced seizures in zebrafish.
- VK3 shares similarity with NQN1 by also containing the naphthoquinone moiety.
- VK3 and VK analogues reduce seizures in zebrafish and mouse epilepsy models.
- VK analogues are non-toxic and reduce seizures at lower concentrations than VPA.
- Compounds that increase ATP production may be good therapeutics for epilepsy.

Figure 1.

A. Chemical structures of pentylene-tetrazol (PTZ), valproic acid (VPA) and NQN1. **B.** Recording traces of zebrafish larval movement over 15 min for each compound with and without PTZ. **C.** Total mean distance traveled over the 15 min recording period normalized to control. Treatment of zebrafish larvae with VPA (4 mM) or NQN1 (3 μ M) alone did not induce any increase in swim activity. PTZ significantly increases the distance traveled compared to control (* $p < 0.05$ compared to control). Pretreatment of zebrafish larvae with VPA or NQN1 significantly reduced PTZ-induced swim activity (# $p < 0.05$ compared to PTZ). Mean distance traveled $\pm$ SEM are shown, $n = 23-25$. **D.** Fold change of *c-fos* gene expression in treated zebrafish larvae. PTZ treatment increases *c-fos* gene expression 80-fold over control. Pretreatment with VPA or NQN1 blunt this increase and treatment of these compounds alone does not induce dramatic changes in *c-fos* expression. Fold change is plotted with SEM ($n = 2$).

Figure 2.

A. Structure of VK3. **B.** Recording traces of zebrafish larval movement over 15 min for VK3 with and without PTZ. **C.** Dose-dependent response of VK3 against PTZ-induced swim activity. Total mean distance traveled over the 15 min recording period. Zebrafish pretreated with VK3 prior to PTZ had a dose-dependent reduction in movement. 1.5 μ M and 3 μ M VK3 did not significantly reduce swim distance compared to PTZ and values remained significantly different from control (* $p < 0.05$ compared to control). 6 μ M VK3 significantly reduced distance traveled compared to PTZ (# $p < 0.05$ compared to PTZ). Treatment of VK3 alone had no effect on swim distance. Mean distance traveled $\pm$ SEM are shown, $n=8$ for each group. **D.** Fold change of *c-fos* gene expression in treated zebrafish larvae. PTZ treatment increases *c-fos* gene expression 80-fold over control. Pretreatment with 6 μ M VK3 blunts this increase in *c-fos* and treatment of VK3 alone did not induce dramatic changes in *c-fos* expression. Fold change is plotted with SEM ($n=2$).

Figure 3.

A. Structures of VK analogues that reduce PTZ-induced seizure activity in zebrafish. **B.** Recording traces of zebrafish larval movement over 15 min for all compounds with and without PTZ. **C.** Pre-treatment with VK analogues 2j (10 μ M) and 2h (20 μ M) significantly reduced swim activity from PTZ only levels (# $p < 0.05$ compared to PTZ). Compound 2q (20 μ M) reduced swim levels but was not significantly different from PTZ alone. Compound 3n (8 μ M) also reduced swim activity but to a level different from control but not from PTZ alone (* $p < 0.05$ compared to control). Treatment of compounds in the absence of PTZ did not increase swim activity. Mean distance traveled $\pm$ SEM are shown, $n = 8-40$. **D.** Fold change of *c-fos* gene expression in treated zebrafish larvae. PTZ treatment increases *c-fos* gene expression 80-fold over control. Pretreatment with 2j, 2h, 2q or 3n blunt this increase and treatment of compounds alone did not induce dramatic changes in *c-fos* expression. Fold change is plotted with SEM ($n = 2$).

Figure 4.

Cellular respiration is altered by VK3 and VK3 analogues. **A.** Treatment of HT-22 neurons with 5 μ M VK3, 5 μ M NQN1, 5 μ M 2h, or 12.5 μ M MB but not analogue 2j significantly increased basal cellular respiration compared to control (measured as oxygen consumption rates, OCR) (* p <0.05). **B.** Compared to control levels, ATP-linked respiration (revealed by exposure to oligomycin) was significantly increased in cells treated with VK3, NQN1, or MB (* p <0.05). ATP-linked respiration was significantly decreased after treatment with analogue 2h (* p <0.05) and levels after treatment with compound 2j were not significantly different from control values. **C.** Basal mitochondrial respiration was significantly increased after treatment with 2j, NQN1 or MB but not VK3 (* p <0.05). Treatment with analogue 2h significantly decreased basal mitochondrial respiration levels (* p <0.05). **D.** Glycolysis, as measured as media acidification (ECAR), did not change for VK3, NQN1, 2h or MB, but 2j showed significantly increased ECAR compared to control (* p <0.05). The OCR and ECAR levels are given as differences from the mean control values $\pm$ SEM, n =18-79.

Figure 5.

VK3 and its analogues significantly increase ATP levels. Total ATP levels (pmol/ μ g lysate) were measured from HT-22 neurons treated with 5 μ M VK3, 5 μ M NQN1, 5 μ M analogue 2h or 12.5 μ M MB. Each significantly increased ATP levels compared to control conditions (* $p < 0.05$). Mean values (relative to control values) are plotted $\pm$ SEM, $n = 7-13$.

Table 1

Compounds tested in the larval zebrafish swim assay. N.D. = not detectable.

Compounds tested	Lethal dose (mM)	Highest concentration tested for anti-seizure activity (mM)
Valproic Acid (VPA)	N.D.	4
Suberoylanilide hydroxamic acid (SAHA)	>0.015	0.015
Diphenyl acetic hydroxamic acid (dPAHA)	0.01	0.0075
2-Benzoylamino-1,4-naphthoquinone (NQN1)	0.005	0.003
Tubastatin A	>0.012	0.012
2-propylpentane hydroxamic acid (VPHA)	>0.05	0.05
VK3	0.007	0.006
2j	0.012	0.010
2h	>0.02	0.02
2q	>0.02	0.02
3n	0.012	0.008

Table 2

Pre-administration of test compounds (100 mg/kg) to mice via i.p. injection protects against minimal clonic seizures (6 Hz). The data for each treatment is represented as the number of animals protected / number of animals tested.

Time after treatment (h)	0.25	0.5	1.0	2.0	4.0
2j	0/4	0/4	1/4	1/4	1/4
2h	4/4	2/4	1/4	1/4	0/4
2q	1/4	0/4	1/4	0/4	0/4

Table 3

Pre-administration of test compounds (100 mg/kg) to mice via i.p. injection shows limited protection against maximal electroshock-induced seizures. The data for each treatment is represented as the number of animals protected / number of animals tested.

Time after treatment (h)	0.25	0.5	1.0	2.0	4.0
2j	0/4	0/4	0/4	2/4	0/4
2h	0/4	0/4	0/4	1/4	1/4
2q	0/4	0/4	0/4	0/4	0/4

Table 4

Pre-administration of test compound (100 mg/kg) to mice via i.p. injection shows protection against seizures in a corneal kindled mouse model. The data for each treatment is represented as the number of animals protected (N)/ number of animals tested (F).

Compound	Time after treatment (h)	N/F	Individual Seizure Scores	Avg. seizure score
2h	0.25	2/4	3,3,4,5	3.75

Table 5

Evaluation of mouse neurotoxicity after i.p. injection of 100 mg/kg compound. The compound is considered toxic if the animal falls of the rotarod three times during a 1 min period. The data for each treatment is represented as the number of animals displaying toxic effects / number of animals tested.

Time after treatment (h)	0.25	0.5	1.0	2.0	4.0
2j	0/12	0/12	0/12	0/12	0/12
2h	0/12	1/12	0/12	0/12	0/12
2q	0/12	0/12	0/12	0/12	0/12