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Seizure control by derivatives of medium chain fatty acids associated with the ketogenic diet show novel branching-point structure for enhanced potency

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Running title: Seizure control by medium chain fatty acid derivatives

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Nonstandard abbreviations: HDAC histone deacetylase; MCT medium chain triglyceride; MES maximum electric shock, PTZ pentelenetetrazol,

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Abstract

The medium chain triglyceride (MCT) ketogenic diet is a major treatment for drug resistant epilepsy but is problematic, particularly in adults, because of poor tolerability. Branched derivatives of octanoic acid, a medium chain fat provided in the diet have been suggested as potential new treatments for drug-resistant epilepsy, but the structural basis of this functionality not been determined. Here we investigate structural variants of branched medium chain fatty acids as new seizure-control treatments. We initially employ a series of methyl-branched octanoic acid derivatives, and using the GABA(A) receptor antagonist, pentelenetetrazol (PTZ), to induce seizure-like activity in rat hippocampal slices, we show a strong, branch-point specific activity that improves upon the related epilepsy treatment, valproic acid. Using low Mg^{2+} conditions to induce glutamate excitotoxicity in rat primary hippocampal neuronal cultures for the assessment of neuroprotection, we also show an identical structural dependence to that for seizure control suggesting a related mechanism of action for these compounds in both seizure control and neuroprotection. In contrast, the effect of these compounds on histone deacetylase (HDAC) inhibition, associated with teratogenicity, show no correlation with therapeutic efficacy. Furthermore, small structural modifications of the starting compounds provide active compounds without HDAC inhibitory effects. Finally, using multiple *in vivo* seizure models, we identify potent lead candidates for the treatment of epilepsy. This study therefore identifies a novel family of fatty acids, related to the MCT ketogenic diet, that show promise as new treatments for epilepsy control and, possibly, other MCT ketogenic diet-responding conditions such as Alzheimer's disease.

Introduction

Epilepsy is a common and severe neurological condition affecting up to 0.5-1% of the population worldwide (Bell and Sander, 2001). Despite the development of a range of new anti-epileptic drugs (AEDs) (Bialer et al., 2010) which, compared to older AEDs, have improved tolerability, reduced side effects, and fewer drug-drug interactions (Perucca, 2002), around 30% of people with epilepsy continue to experience seizures (Bialer and White, 2010). The development of new treatments for epilepsy therefore addresses a major unmet need.

As an alternative approach to drug treatment, a specialized diet has proven successful in controlling seizures in children with severe, drug resistant epilepsy (Kossoff and Rho, 2009;Neal et al., 2008;Neal et al., 2009;Sills et al., 1986;Vining et al., 1998). This medium chain triglyceride (MCT) ketogenic diet, first introduced in 1971 (Huttenlocher et al., 1971), was based around a reduction in dietary carbohydrate and an increase in medium chain fatty acid intake, in the form of triglycerides containing 81% octanoic acid (Caprylic acid) and 16% decanoic acid (Carpic acid) (Sills et al., 1986). This diet increases blood levels of octanoic acid and decanoic acid (Huttenlocher et al., 1971;Sills et al., 1986). Decanoic acid can directly inhibit seizure activity, and this may be an important mechanism for the diet's antiseizure effect (Chang et al. 2013). In contrast, octanoic acid has no direct effect on seizures. However, specific branched derivatives of octanoic acid provide more potent seizure control than a commonly used anti-epileptic drug, valproic acid (Chang et al., 2012; Chang et al., 2013). Although the diet remains a clear choice for the treatment of drug resistant epilepsy in children, its use in adults is severely limited due poor tolerability, hence development of these branched chain fatty acids may therefore provide an alternative to the diet by overcoming the side-effects and metabolic consequences of the diet, especially in adults.

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Here we investigate structural aspects of branched medium chain fatty acids in seizure control. We initially determined the seizure control efficacy and neuroprotective activity of a structured series of methyl-branched octanoic acid compounds. In these experiments we used rat entorhinal cortex-hippocampus slices exposed to the GABA(A) receptor antagonist pentylenetetrazol (PTZ), which is widely used to generate seizures and seizure-like (paroxysmal) activity (Armand et al., 1998). We also used dissociated hippocampal neurons in culture in the presence of low Mg^{2+} to generate high frequency epileptiform activity as an *in vitro* neuroprotection assay. This model results in glutamate-dependent excitotoxic cell death (Deshpande et al., 2008), , which mechanistically is similar to that observed *in vivo* during status epilepticus (DeLorenzo et al., 2005). We also measured HDAC inhibition; this can have teratogenic effects (Jentink et al., 2010) and has been proposed to explain the teratogenicity of the branched isomer of octanoic acid, valproic acid (2-propylpentanoic acid), a well-established epilepsy treatment (Eikel et al., 2006;Phiel et al., 2001). Based upon favorable potency and low HDAC inhibition, we also modified structures to generate more promising compounds. Finally, we analyzed *in vivo* efficacy of the most potent compounds, using distinct *in vivo* models to identify novel chemicals providing strongly enhanced seizure control activity compared to valproic acid, suggesting a potential new treatment for epilepsy and other MCT ketogenic diet-responsive conditions.

Materials and Methods

Animals

Hippocampal slice experiments: Male Sprague-Dawley rats were kept under controlled environmental conditions (24–25 °C; 50–60% humidity; 12 h light/dark cycle) with free access to food and tap water. All the experiments were performed in accordance with the guidelines of the animals (Scientific procedure) Act 1986.

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MES and 6Hz experiments: Adult male CF No 1 albino mice (18-25 g MES and 26-30 g 6Hz) and male Sprague-Dawley albino rats (100-150 g) were obtained from Charles River, Portage, Michigan, and were matched, where possible, for sex, age, and weight (Petty and Karler, 1965; Woolley et al., 1961). Animals were maintained on an adequate diet (Prolab RMH 3000), allowed free access to food and water (Davenport and Davenport, 1948) and allowed time to adjust after transit. All mice were housed in a dedicated facility, in plastic cages with controlled humidity, exchange of air and controlled lighting (12 h light/dark cycle). The animals were housed, fed, and handled in a manner consistent with the recommendations in the National Council Publication, "Guide for the Care and Use of Laboratory Animals". No insecticides capable of altering hepatic drug metabolism enzymes are used in the animal facility.

Compounds for analysis

Fatty acids used in this study were: VPA (Sigma), octanoic acid (Alfa Aesar), 2-methyloctanoic acid, 3-methyloctanoic acid, 6-methyloctanoic acid, and 7-methyloctanoic acid (all from Ukrorgsyntez Ltd), 4-methyloctanoic acid and 4-methylnonanoic acid (Alfa Aesar), 4-ethyloctanoic acid (Chemos GmbH), and *trans*-4-butylcyclohexane carboxylic acid (TCI). Compounds were prepared as 1000 times stocks (1 M) in dimethyl sulfoxide (DMSO), except for VPA which was dissolved in distilled water. Stocks were dissolved in artificial cerebrospinal fluid (aCSF) or media to achieve their final experimental concentrations of 1 mM. All compounds were over 95% purity.

Synthesis of 5-methyloctanoic Acid (adapted from Levene and Marker, 1931)

Magnesium turnings (0.209g, 8.55 mmol), iodine (0.002 g, ca) and anhydrous diethyl ether (2 ml) were stirred at room temperature for 10 min under an atmosphere of nitrogen. A solution of 1-bromo-4-methylheptane (1.5g, 7.78 mmol) in anhydrous diethyl ether (3 ml) was added dropwise over 5 min. The mixture was heated at reflux for 10 min then allowed to cool to room

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temperature. Dry ice (solid CO_2, 1.50 g, 34.08 mmol) was added and the mixture was allowed to warm to room temperature. Hydrochloric acid (1 M; 10 ml) was carefully added to the reaction mixture and the crude product was extracted with ethyl acetate (3 x 20 ml). The organic extracts were combined, washed with brine (20 ml), dried ($MgSO_4$) and evaporated under reduced pressure to give clear oil (0.884g). The crude product was dissolved in chloroform (20 ml) and extracted with NaOH (2M; 3 x 15 ml). The combined aqueous layers were acidified with conc. hydrochloric acid to pH 2, extracted from the aqueous layer with chloroform (3 x 20 ml). The organic extracts were combined, washed with brine (20 ml), dried ($MgSO_4$) and evaporated under reduced pressure to give 5-methyloctanoic acid (0.554g, 41%) as a colorless oil, (HRMS-ES (m/z) found 181.1203, calculated for $[C_9H_{18}NaO_2]^+$ 181.1199) ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.31 (s, 1H), 2.33 (t, *J* 7.6 Hz, 2H), 1.73 – 1.54 (m, 2H), 1.48 – 1.38 (m, 1H), 1.37 – 1.20 (m, 4H), 1.20 – 1.05 (m, 2H), 0.92 – 0.84 (m, 6H).^{13}C NMR (126 MHz, $CDCl_3$) δ 180.7 (CO), 39.3 (CH_2), 36.5 (CH_2), 34.6 (CH_2), 32.4 (CH), 22.4 (CH_2), 20.2 (CH_2), 19.6 (CH_3), 14.3 (CH_3).

In vitro electrophysiology

The preparation of entorhinal cortex-hippocampus slices and electrophysiological recording in CA1 were described previously (Armand et al., 1998; Chang and Walker, 2011). In brief, male rats (50-150 g) were decapitated after killing by intraperitoneal injection with an overdose of pentobarbitone (500 mg/kg). The brain was removed and preserved in oxygenated ice-cold sucrose solution in mM: NaCl 87, KCl 2.5, $MgCl_2$ 7, $CaCl_2$ 0.5, NaH_2PO_4 1.25, $NaHCO_3$ 26.2 sucrose 75, glucose 3. Transverse slices (350 µm) were prepared with a vibratome (VIBRATOME® 1500, Intracel Ltd) and were then stored in an interface chamber containing artificial cerebrospinal fluid solution (aCSF) in mM: NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1, $NaHCO_3$ 26.2 glucose 16.6. The slices were stored for over one hour. During the experiment, the slices were transferred from the interface chamber into a submerged recording chamber and continuously perfused with prewarmed (about 36°C) oxygenated (95% O_2, 5%

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CO_2) aCSF. A field potential recording was made by placing a glass microelectrode filled with aCSF solution in stratum radiatum of CA1. A bipolar stimulating electrode was positioned in the Schaffer collateral/commissural fiber pathway in stratum radiatum to confirm slice viability. Pentelenetetrazol (PTZ) (2 mM) was added to the perfusate and [K^+] was increased to 6 mM in order to induce epileptiform activity. PTZ-induced epileptiform discharges, population spikes consisted of positive field potentials, appeared 10-30 minutes after application of PTZ plus increase of [K^+]. Compounds were applied once the frequency and amplitude of the epileptiform discharges were stable over a period of 10 min. Anticonvulsant effects were evaluated by measuring the variation of frequency of the discharges every min.

In vitro neuroprotection assays

Primary hippocampal neuron cell culture

Primary hippocampal neuron cell culture was prepared from rat pups (postnatal day 0-1). After the brains were quickly removed and hippocampi isolated, the hippocampi were submerged in ice-cold HEPES buffered HBSS (Invitrogen). The tissue was then treated with 1% trypsin for 10 minutes at 37˚C to dissociate cells. After removal of residual trypsin, the tissue was triturated and plated at a density of $2x10^5$ cells/well onto a glial supported layer previously plated onto poly-L-lysine– coated cell culture coverslips (13 mm). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, and fed once weekly with NeuroBasal A medium supplemented with B-27 (Invitrogen) and Gibco® GlutaMAX™ Supplement (Invitrogen). Fourteen days after cell culture preparation, cultures were utilized for experimentation. Maintenance medium was replaced with HEPES buffered aCSF with or without $MgCl_2$ (2 mM), containing: 125 mM NaCl, 2.5 mM KCl, 25 mM HEPES, 2 mM $CaCl_2$, 30 mM glucose, pH 7.4, and osmolarity adjusted to 310 mOsm with sucrose. Low Mg^{2+} treatment with/without octanoic acid derivate was carried out with aCSF without added $MgCl_2$, whereas sham controls were treated with aCSF containing $MgCl_2$.

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Lactate dehydrogenase activity assay

Four hours after cells were exposed to a low Mg^{2+} environment in the absence or presence of compounds, neuronal cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released into the extracellular fluid from damaged cells using a commercial kit (TOX7; Sigma). Release of LDH into the extracellular fluid is an indication of compromised membrane integrity (Koh and Choi, 1987), thus providing a means to monitor cell toxicity without damaging cells in culture. The assay was conducted according to the manufacturer's protocol. In brief, duplicate samples of media (50 µl) were transferred to wells of a clear 96-well flat-bottom microtiter plate (Costar), and 20 µl of LDH mixture containing equal amounts of LDH cofactor, substrate, and dye solutions were added (all supplied in the TOX7 kit). The plates were incubated in the dark for 30 min at room temperature. LDH release was assayed by absorbance change (490 nm) using a microplate reader, with results normalized to sham control. Data were presented as mean percentage cell death ± SEM (compared to sham control).

Viability staining

Four hours after cells were exposed to the low Mg^{2+} environment in the absence or presence of compounds, cell death was visualized by staining with 10 µM propidium iodide (excitation at 488 nm; emission at 550 nm)(Sigma) and 10 µM Hoechst 33342 (excitation at 530 nm; emission at 620 nm) (Sigma). Propidium iodide is a cell-impermeant fluorescent biomarker that will fluoresce upon binding nuclear chromatin, thus fluorescence provides an indication of loss of cell membrane integrity as a marker for cell death. Hoechst 33342 is a cell-permeant nuclei stain that provides a total cell count. Following treatment, cells were stained for 30 min at room temperature, washed to remove unbound dye, and cell death and total cell count were visualized and quantified using Image J.

HDAC Activity Assay

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Histone deacetylase activity was analyzed by using an *in vitro* commercial assay kit, the HDAC fluorescent activity assay/drug discovery kit HDAC activity (Biomol, Plymouth Meeting, PA). The assay was conducted at room temperature according to the manufacturer's protocol, using Trichostatin A (a HDAC inhibitor; at 1 μM concentration) as a positive control. Data were derived from at least triplicate experiments with each experiment comprising at least duplicate measurements (n=6) normalized to controls (without valproic acid or fatty acids).

***In vivo* seizure model**

The 6 Hz "Psychomotor" Seizure Test

Adult male CF1 mice (18-25 g) were pretreated intraperitoneally (i.p.) with each compound at 100-150 mg/kg. After pretreatment, each mouse was given a drop of 0.5% tetracaine hydrochloride to each eye, following by low-frequency (6 Hz) stimulation (32 mA) for 3 seconds delivered through corneal electrodes. Animals were manually restrained during stimulation and released immediately following the stimulation and observed for the presence or absence of seizure activity at five time points (1/4, 1/2, 1, 2, and 4 hours). Typically, the 6 Hz stimulation results in a seizure characterized by a minimal clonic phase that is followed by stereotyped, automatistic behaviors, including twitching of the vibrissae, and Straub-tail. Animals not displaying such behaviors were considered protected.

Maximal Electroshock Test (MES)

The MES is a model for generalized tonic-clonic seizures (Loscher, 2011) which provides an indication of a compound's ability to prevent seizure spread when all neuronal circuits in the brain are maximally active. Animals are pretreated with compounds (100-125 mg/kg) by i.p. injection. Seizure activity was induced by delivery of 60 Hz of alternating current (50 mA in mice, 150 in rats) for 0.2s through corneal electrodes which had been primed with an electrolyte solution containing an anesthetic agent (0.5% tetracaine HCl). An animal is considered "protected" from MES-induced seizures upon abolition of the hind limb tonic extensor component of the seizure (Swinyard et al., 1995;White et al., 1995;White et al., 2007).

Subcutaneous Metrazol Seizure Threshold Test (scMET)

Subcutaneous injection of pentylenetetrazol produces clonic seizures in animals. The scMET test allowed detection of test compound efficacy in raising seizure threshold, thus demonstrating protection from clonic seizure induction. Animals were pretreated with each compound (100 mg/kg) in a similar manner to the MES test. Seizure activity was induced by administration of Metrazol (CD97: 85 mg/kg mice; 70 mg/kg rats) into skin in the midline of the neck. Animals were observed for the following 30 minutes for the presence or absence of a seizure. Animals were considered "protected" if they did not exhibit an episode of clonic spasms of the fore and/or hindlimbs, jaws, or vibrissae (3-5 seconds).

Corneal Kindled Mouse Model

This model involves the use of corneal kindled mouse model for prediction of efficacy in human partial seizures (Rowley and White, 2010). Adult male CF1 mice (18-25 g) mice were stimulated through corneal electrodes (3 mA, 60Hz, 3 seconds) after administration of 0.5% tetracaine hydrochloride to each eye. This procedure was carried out twice daily for an average of 12 days. Animals were considered kindled when they displayed five consecutive stage five seizures according to the Racine scale (Racine, 1972): Stage 1, facial automatisms; Stage 2: head nodding and more serve facial and mouth movements (jaw-opening); Stage 3: rats display forelimb clonus with a lordotic posture; Stage 4: bilateral forelimb clonus continues along with rearing; Stage 5: some rats will fall to one side first and then show evidence of forelimb clonus. After 5-7-days stimulation-free period once the mice were fully kindled, the test compounds were administrated (100 mg/kg, i.p.). Mice in each group were then tested at various time points (0.25, 0.5, 1, 2, 4 hours) after drug dosing. Mice displaying a seizure score < 3 are considered protected.

Statistical analyses

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In all data provided results are presented as mean ± SEM. Statistical comparisons were performed by using the one way ANOVA followed by Tukey for post hoc analysis using GraphPad.

Results

***In vitro* seizure control activity of methyl-branched octanoic acid derivatives in the PTZ/hippocampal model is structurally specific.** The discovery of a direct role for branched derivatives of octanoic acid, rather than the inactive unbranched compound, in multiple *in vitro* seizure models and an *in vivo* model of status epilepticus (Chang et al., 2012; Chang et al., 2013) suggests that improved activity may be found by examining the range of branching structures possible for these fatty acids. We thus initially examined the seizure control activity of a series of branched octanoic acid derivatives containing a methyl group side chain (Fig 1A) at a single concentration (1 mM) as an early indication of comparative activity. For these experiments, we induced seizure-like activity in rat hippocampal slices by the application of PTZ and monitored the frequency of burst activity in the CA1 region. Consistent with our previous study (Chang et al., 2013), octanoic acid has no effect on the frequency of burst discharges 20-40 minutes after application of PTZ (98.4±7.2% of baseline, n=4) (Fig 1B). In contrast, methyl-group substitution on the third to the seventh carbon of the octanoic backbone resulted in compounds that significantly reduced discharge frequency, and the position of the branching point related to activity (Fig 1B). Inhibition of seizure-like activity increased through substitution of the methyl group on the second carbon (2-MOA: 78.1±3.6% of baseline, n=5); to the third (3-MOA: 62.3±2.6% of baseline, n=5); and fourth carbon (4-MOA: 48.6±2.7% of baseline, n=5). Inhibition of seizure-like activity was greatest with the methyl branch chain located at the fifth position (5-MOA; 8.2±5.4% baseline, n=4). Inhibition of seizure-like activity then decreased compared to that of 5-methyloctanoic acid with the methyl branch chain was substituted on the

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6th (6-MOA: 25.4±7.9% of baseline, n=5); and then further reduced on the 7th position (7-MOA: 39.2±4.0% of baseline) (Fig. 1B). These potent compounds show enhanced seizure-activity control, compared to the established epilepsy treatment, valproic acid, application of which results in a small but significant reduction in discharge frequency in this model (77.1±2.0% of baseline) (Chang et al., 2013). These results therefore suggest a strong structure-relative effect of branched octanoic acid derivatives directly on controling seizures.

***In vitro* neuroprotective activity of methyl-branched octanoic acid derivatives in the low Mg^{2+} model is structurally specific.** The development of new anti-epileptic treatments has recently included the analysis of neuro-protective properties of these compounds, whereby novel treatments can reduce neuronal cell damage or death resulting from seizure activity. To investigate this effect in the series of branched-chain octanoic acid compounds, we used a model of glutamate receptor-dependent excitotoxic cell death, which is mechanistically similar to that observed *in vivo* during status epilepticus (DeLorenzo et al., 2005) this consisted of exposure of hippocampal neurons in culture to low Mg^{2+} (Deshpande et al., 2008). Cell death in this model occurs in a time-dependent manner, increasing from 30% to 100% with the increase of low Mg^{2+} exposure duration from 2 to 12h (Deshpande et al., 2008). We therefore induced epileptiform activity by exposure of neurons in culture to low Mg^{2+} conditions for 4 hours and monitored cell death by recording uptake of the propidium iodide (PI) dye as a marker for dead cells (Fig. 2A,B) and the release of lactose dehydrogenase (LDH) into the media as a biomarker for cellular cytotoxicity and cytolysis (Fig. 2C). In these experiments, exposure to low Mg^{2+} conditions significantly increased neuronal cell death, measured both by PI uptake (433 ± 30% of control) and LDH release (453 ± 38% of control). Application of OA provided no significant protection against neuronal injury in this model (PI uptake: 406±45% of control, LDH release: 326±62% of control). Similarly, addition of a methyl group to the second carbon, 2-MOA, did not provide significant protection measured either by PI uptake or by LDH release (407±45% of

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control; 286±56% of control respectively; Fig. 2A,B,C). In contrast, 3-MOA to 7-MOA all resulted in a significant attenuation of PI uptake and LDH release, with 4-MOA reducing PI uptake and LDH release to 197±11% and 186±57% of control respectively (Fig. 2A,B,C). In comparison, valproic acid, had an intermediate neuroprotective effect (PI uptake: 295±12% of control, and LDH release: 269±32% of control). Together these results indicate that the branching pattern of methyl-octanoic acid derivatives strongly regulates neuroprotective activity, similar to the structure-function relationship obtained from the *in vitro* PTZ-induced seizure model. These data also suggest that compounds containing branch points on the fourth to seventh carbon of the octanoic acid backbone have greater neuroprotective potential than valproic acid.

Physiochemical properties of methyl-branched octanoic acid derivatives do not correlate to efficacy. To examine a potential physiochemical basis of seizure control activity for these compounds, lipophilicity (LogP) and acidity (pKa) values were estimated for each compound. Data were derived using literature sources and predictive software. No gross trends were observed for either physical parameter over the octanoic acid series (Supplementary Fig 1). This suggests that seizure control activity and neuroprotective efficacy of these medium chain fatty acids were likely not due to purely physiochemical characteristics, but rather due to biological function.

Histone deacetylase inhibitory activity of methyl-branched octanoic acid derivatives is structurally specific. To investigate potential HDAC inhibitory potential of this series of branched octanoic acid derivatives, compounds were tested using an *in vitro* HDAC inhibition assay. In this assay, human HeLa cell nuclear extract was used as the source of HDAC activity. Valproic acid showed a relatively strong inhibition of HDAC activity (IC_{50}: 2.1±0.1 mM; Fig. 3A,B). Using this assay, OA showed strong inhibitory activity (IC_{50}: 6.4±0.3mM), but the addition of a methyl group to the second carbon to give 2-MOA caused a significant reduction in HDAC

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inhibitory activity (IC_{50}, 13.6±2.8 mM). Addition of a methyl group to the third carbon to give 3-MOA resulted in a similar small decrease in HDAC inhibition compared to octanoic acid (IC_{50}, 8.7±0.9 mM). Branched derivatives based upon the fourth - seventh carbon exhibited a highly significant reduction in HDAC inhibitory potency compared to octanoic acid (IC_{50}, 4-MOA: 30.5±1.4 mM; 5-MOA: 20.8±0.8 mM; 6-MOA; 20.2±2.8 mM; 7-MOA: 17.9±1.2 mM) (Fig. 3A,B). These data suggest that all branched chain octanoic acid compounds examined here are less likely than valproic acid to be teratogenic, and compounds branched on the fourth, fifth, sixth, and seventh carbon are unlikely to have any significant HDAC inhibitory effect at concentrations likely to be produced *in vivo*.

Related derivatives show no HDAC inhibition and strong activity in the PTZ/hippocampal model. Our previous studies indicated that compounds containing longer branch chains and extended backbones also have strong anti-seizure activity (Chang et al 2012; Chang et al 2013). To expand the chemical space examined in this study, we tested three compounds related to the octanoic acid series (Fig. 4A) using both the HDAC inhibitory assays (Fig. 4B) and the PTZ-induced hippocampal slice seizure model experiments (Fig. 4C) at the same concentration used for the previous compounds (1 mM). The formal addition of an 4-ethyl group to octanoic acid to produce 4-ethyloctanoic acid (4-EOA) rather than 4-methyloctanoic acid (4-MOA), showed no HDAC inhibition but enhanced epileptiform activity control in the PTZ-induced hippocampal slice model (Fig. 4A,B,C; 10.5±4.0% of baseline, n=8) over the related methyl-branched compound. Elongation of the backbone length from the same starting compound, to form 4-methylnonanoic acid (4-MNA), also showed no HDAC inhibition and enhanced epileptiform activity control (Fig. 4A,B,C; 2.0±2.9% of baseline, n=6) over the parent compound. Finally, analysis of a cyclic derivative, where a butyl group is attached to a cyclohexane carboxylic acid (4-butylcyclohexane carboxylic acid; 4-BCCA) again provided no HDAC inhibition with very strong epileptiform activity control (Fig. 4A,B,C; 1.7±1.5% of baseline, n=5).

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These compounds also did not show significant changes to physiochemical properties (Supplementary Fig 1). The enhanced activity of these compounds over valproic acid suggests further possible lead candidates for better epilepsy treatments.

***In vivo* seizure control activity of compounds derivatives suggest structurally specific lead candidates.** The advantage of *in vitro* models is that efficacy is not dependent on the pharmacokinetics and blood-brain-barrier penetration of the compounds. However, these *in vitro* models of epileptiform activity may not necessarily predict *in vivo* efficacy both because of pharmacokinetics and also because these are reduced models with only small portion of the brain circuitary intact. In the development of new anti-epileptics, *in vivo* models are therefore used both to better predict efficacy, and to provide evidence for physiochemical properties such as *in vivo* stability and blood brain barrier penetrance. We therefore examined efficacy of the most active compounds in multiple *in vivo* epilepsy models, in collaboration with the Anticonvulsant Screening Program (ASP), at the US National Institutes for Neurological Disorders and Stroke (NINDS). In these experiments, multiple doses were used to estimate ED_{50} levels and give comparative potencies compared to valproic acid. We first employed a low frequency (6 Hz), long duration (3 s) corneal stimulation model because numerous second generation anti-epileptic drugs show poor seizure control in this assay (Loscher, 2011). In this model, compounds are pre-administered to mice via i.p. injection and mice are challenged with sufficient current to elicit a psychomotor response (32 mA for 3s) (Toman et al., 1952). The model causes forelimb clonus and then automatistic behavior characteristic of limbic epilepsy in humans. Our initial analysis of methyl branched derivatives with the highest activity and lowest risk of HDAC inhibition, 4-MOA and 5-MOA, and the related 4-MNA showed limited seizure control (Table 1). However, analysis of 4-EOA and 4-BCCA showed an ED_{50} of 110 mg/kg and 81 mg/kg respectively, indicating that both are more potent than valproic acid which has an ED_{50} of 263 mg/kg in this model (Barton et al., 2001).

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Compounds showing potential efficacy in the 6Hz model were then tested in three further *in vivo* seizure models. The maximal electric shock (MES) seizure model is one of the primary preclinical models used in epilepsy drug development (Barton et al., 2001). Using this model, 4-EOA and 4-BCCA both had EC_{50} values of 100 mg/kg (Table 1). The subcutaneous MET (scMET) model, thought to determine the seizure threshold for clonic seizures, showed that 4-EOA and 4-BCCA showed EC_{50} values of 100 mg/kg and ~150 mg/kg respectively, whereas valproic acid had an equivalent effect at 191 mg/kg (Rowley and White, 2010). Finally, in the corneal kindled mouse model (CKM), that provides an *in vivo* model for temporal lobe epilepsy (White, 2003), 4-EOA and 4-BCCA had EC_{50} values of 71 mg/kg and 44 mg/kg respectively, compared with valproic acid that shows equivalent activity at 174 mg/kg. These data strongly suggest enhanced activity of 4-EOA and 4-BCCA over valproic acid in a range of distinct seizure models.

Discussion

The MCT ketogenic diet is a widely used and effective treatment for drug resistant epilepsy in children, but is rarely employed for adult treatment due to poor tolerability. Our recent discovery of the anti-seizure efficacy of branched derivatives of the major constituent of the diet, octanoic acid, that may provide lead candidates for pharmacological treatment of epilepsy based around this family of compounds, led us to investigate these structures in a more comprehensive manner.

In this study, we initially used a single-dose pentylenetetrazol (PTZ) model to identify differences in activity of a structured series of methyl-branched octanoic acid derivatives to provide a first indication of branching effects. PTZ is widely used as an epileptogenic agent. In the hippocampal slice model used here, PTZ triggers spontaneous interictal-like repetitive burst

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discharges (Hori and Katsuda, 1974;Rostampour et al., 2002), which are similar to seizure activity generated by increasing extracellular K^+ concentrations in the cerebral cortex *in vivo* (Futamachi et al., 1974;Heinemann et al., 1977;Moody et al., 1974). The induction of PTZ discharges in the *in vitro* preparation offers the advantage of a simplified model for studying the pharmacology of antiepileptic drugs (Piredda et al., 1985). We found a strong structure-related change in the control of PTZ-induced seizure-like activity in a rat hippocampal slice model, with highly significant inhibition of seizure activity by compounds branching at carbons 3-7. Our previous studies show that the currently used branched chain fatty acid, valproic acid, with a branch point on the second carbon, has a much smaller effect in this model (seizure activity is reduced to 77.1±2.0% of baseline at 1 mM) (Chang et al., 2013). These results suggest that alternative branch points on medium chain fatty acids may provide enhanced potency over valproic acid in seizure control. It is interesting to note that a recent study identified that orally dosed octanoic acid increased the threshold of seizure induction in a range of seizure models (Wlaz et al., 2012), however our data indicate no direct role of octanoic acid in seizure control, suggesting that metabolites and derivatives of octanoic acid may provide this therapeutic effect.

Neuroprotective activity may also prove useful in the reducing development of epilepsy, and cognitive and neurological decline following a prolonged seizure or brain insult (Dam, 1980; de Lanerolle et al., 1989; Barton et al., 2001). Similar to the seizure control data, we found a strong structural dependence of medium chain fatty acid-mediated neuroprotection in a low magnesium model. Compounds branched from the fourth to seventh carbon show greatest activity in protection against seizure-related cell death (Koh and Choi, 1987). This protective activity is greater than that shown by valproic acid, suggesting enhanced therapeutic efficacy for these compounds. A link between activity in the *in vitro* PTZ and neuroprotective models may relate to the suppression of seizure activity, or decreased ion fluxes, as opposed to a primary

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neuroprotective effect. Further studies will be necessary to determine if these active compounds function through a common mechanism for both seizure control and neuroprotection.

Direct inhibition of histone deacetylase (HDAC) by valproic acid (Gottlicher et al., 2001;Gurvich et al., 2004) is thought to give rise to teratogenicity (Jentink et al., 2010;Koren et al., 2006), causing a variety of major and minor malformations, including neural tube defects, cleft lip and palate, cardiovascular abnormalities, genitourinary defects, developmental delay, endocrinological disorders, limb defects, and autism (Alsdorf and Wyszynski, 2005). This biochemical activity provides a crucial side effect profile to consider in the development of carboxylic acid-based anti-epileptic drugs. Structural requirements for this activity for valproic acid congeners suggest a critical role for branching of the parent compound on the second carbon of the fatty acid backbone (Bialer, 2010; Bialer, 2007). Our data indicate that all compounds in our octanoic acid series show reduced HDAC inhibition, with branching at the fourth carbon providing the least activity, with around a fifteen-fold increase in IC_{50} values. This suggests that these compounds, especially 4-MOA (IC_{50} 30.5±1.4 mM), are unlikely to significantly inhibit HDAC activity *in vivo* at concentrations found in plasma for valproic acid (0.4-0.7 mM (DSMV IV, 2000) or octanoic acid (306 μM; Haidukewych et al., 1982). These data also support the enhanced safety of these novel branched fatty acids in seizure control, showing a reduced potential for HDAC activity associated with teratogenicity.

To provide lead candidates for seizure control treatment, we next focused on identifying new structures with low HDAC inhibitory activity (based on 4-MOA) to develop compounds showing activity equivalent or greater than that shown for 5-MOA. Addition of a longer branch chain (ethyl branch derivatives), lengthening the backbone (nonanoic acid), or incorporation of a ring structure - all provided equivalent (4-EOA) or enhanced (4-MNA, 4-BBCA) seizure control in the

PTZ induced hippocampal model compared to 5-MOA, and did not show HDAC inhibitory activity. These compounds therefore provided exciting new leads for further screening.

We next translated our *in vitro* results to multiple *in vivo* models (using a range of doses to determine potency), since these models are more relevant to human seizures and epilepsy. This is particularly important, since the efficacy of compounds *in vivo* is not necessarily predicted by *in vitro* models due to complex *in vivo* metabolic kinetics. Furthermore, compounds tested *in vitro* may not reach an effective brain concentration due to low blood-brain barrier (BBB) permeability. We first employed the 6Hz electric shock model (Barton et al., 2001), in which valproic acid, levetiracetam, phenytoin, lamotrigine, lacosamide, carisbamate, and retigabine have shown efficacy (Bialer et al., 2010). The methyl branched derivatives in this model show low potency in this model, that may mirror that shown for other established epilepsy treatments, such as topiramate (Rowley and White, 2010), or may be due to physiological restrictions such as half-life or blood brain barrier permeability and further studies will be necessary to address these questions. However, the enhanced potency of 4-EOA and 4-BCCA over valproic acid in this model provides an encouraging step forward in developing new treatments in this chemical space. Since these compounds also show significantly decreased IC_{50} values compared to valproic acid in the widely-used MES and scMET screening models and, importantly, in the corneal kindling model of temporal lobe epilepsy (Lothman and Williamson, 1994), these compounds have significant potential as anti-seizure drugs. This contrasts to orally-administered straight-chain octanoic acid (Wlaz et al., 2012), which increased the PTZ dose necessary to induce myoclonic twitch and clonic convulsions without an effect on tonic convulsions and increased the current intensity needed to induce psychomotor seizures in the 6Hz model, but had no effect in the MES model. Thus, our initial *in vitro* epilepsy model suggested novel branching structures that show efficacy in one (PTZ-induced hippocampal slice model), but only two compounds (4-EOA and 4-BCCA) show enhanced potency over valproic

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acid in multiple *in vivo* models, and therefore provide potential new structures for further analysis.

The discovery of this new chemical space for compounds with anti-seizure activity, comprising branched and cyclic derivatives of a medium chain fatty acid in the MCT ketogenic diet, has implications for other therapeutic areas. The MCT diet also provides a positive effect in decreasing brain excitability in young animals (de Almeida Rabello et al., 2008), as well as playing a neuro-protective role in traumatic brain injury and stroke (Gasior et al., 2006). Finally, the diet also has potential for other neurological disorders characterized by neuronal cell death (Stafstrom and Rho, 2012), such as Alzheimer's disease (Reger et al., 2004;Henderson et al., 2009) and Parkinson's disease (VanItallie et al., 2005). Future research into the use of these compounds for these conditions may provide important advances in therapy development.

In summary, a series of novel methyloctanoic acid derivatives were evaluated for seizure control initially using an *in vitro* model of seizure-like activity. Unlike the widely used short chain fatty acids, valproic acid, and related compounds branched on the second carbon, the potency of octanoic acid derivatives was enhanced with branching from the middle of the carboxylic acid backbone. A similar structure-activity relationship was also seen for neuro-protection against neuronal death induced by low magnesium levels. Small changes in structure to these active compounds to lengthen side chain and inclusion of a ring structure in the backbone further enhanced activity. A range of *in vivo* seizure control assays showed improved potency over valproic acid for these compounds. Given the remarkable value of the MCT diet in the treatment of epilepsies (Brandt et al., 2003;Huttenlocher et al., 1971;Neal et al., 2008;Neal et al., 2009;Sills et al., 1986), and its shortcomings in terms of adverse effects, there is interest in the search for derivatives of medium chain fatty acids found in the MCT diet with improved pharmacokinetic or safety profiles. We demonstrate that structurally specific medium chain fatty

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acids have potent anti-seizure and neuroprotective properties, with clear clinical implications in providing a new chemical space for the design of more potent and safer treatments for epilepsy.

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Author contributions

Participated in research design: P.Chang, A.M.E.Zuckermann, M.C.Walker and R.S.B.Williams

Conducted experiments: P.Chang, A.M.E. Zuckermann, S.Williams and M.Cano-Jaimez

Contributed new reagents or analytic tools: A. J. Close and J Spencer.

Performed data analysis: P.Chang, A.M.E. Zuckermann, J.P. McEvoy.

Wrote or contributed to the writing of the manuscript: P.Chang, A.M.E. Zuckermann, J.Spencer, J.P.McEvoy, M.C.Walker and R.S.B.Williams

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Footnotes

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Legends

Fig. 1. Branched methyloctanic acid derivatives show strong structure-specific potency in an *in vitro* model of epileptiform activity control. (A) The structure of the straight chain, eight carbon, octanoic acid, and derivatives branched at the second carbon to the seventh carbon. (B). Summary of the change in the frequency of PTZ-induced burst discharges in area CA1 recorded from the stratum pyramidal by application of octanoic acid and derivatives (1mM). The frequency of epileptiform activity is plotted against time. (C) Comparison of the mean frequency of PTZ-induced burst discharges, averaged from 20-40 min post compound addition (data shown as means ± SEM). *** indicates a significant difference at $P<0.001$ compared to OA. Data are provided for all compounds tested at 1mM from at least four to five independent repeats.

Fig. 2. Branched methyloctanic acid derivatives show strong structure-specific potency in an *in vitro* model of neuroprotection. (A) Primary rat hippocampal neurons, maintained under low Mg^{2+} conditions for 4 hours to trigger status epilepticus-induced cell damage, were treated with octanoic acid and derivatives, shown for control (without low Mg^{2+} treatment), following low Mg^{2+} treatment, and following treatment in the presence of octanoic acid (OA), 2-methyloctanoic acid (2-MOA), 4-methyloctanoic acid (4-MOA) and 5-methyloctanoic acid (5-MOA). Cells were co-stained with the nuclear dye Hoechst 33342 (Hoechst) to visualize total neuronal population, and the exclusion dye propidium iodide (PI) to visualize neuronal death. Scale bar = 20µm. (B) Quantification of change in ratio of PI staining to total cell number following treatment with the series of branched methyloctanic acid derivatives. (C) Comparison of the LDH release 4 hours after induction of status epilepticus in low Mg^{2+} model in hippocampal neurons in culture. All data shown as means ± SEM. ** and *** indicate a significant difference at $P<0.01$ and $P<0.001$

compared to low Mg^{2+} alone group. Data is provided for all compounds tested at 1mM from at least five independent experiments.

Fig. 3: Branched methyloctanic acid derivatives show structure-specific potency in an *in vitro* human HDAC enzyme activity assay. (A) Quantification of HDAC inhibition assay employing human nuclear enzyme extracts (from HeLa cells) as the source of HDAC activity shown as fitted dose−response curves (compared to valproic acid (VPA)). (B) Comparison of the IC_{50} of HDAC inhibition with different treatments, showing means ± SEM for three independent measurements at six concentrations (0.1 – 10 mM).** and *** indicate significant difference at P<0.01 and P<0.001 compared to VPA.

Fig. 4: Specific modified methyloctanic acid derivatives show no activity in an *in vitro* human HDAC enzyme activity assay and potency in a seizure control *in vitro* model of epileptiform activity. (A) The structure of various fatty acids with branch chain on the 4^{th} carbon, 4-ethyloctanoic acid (4-EOA), 4-methylnonanoic acid (4-MNA), and related 4-butylcyclohexane carboxylic acid (4-BCCA). (B) Quantification of HDAC inhibition assay employing human nuclear enzyme extracts (from HeLa cells) as the source of HDAC activity shown as fitted dose−response curves. (C) Summary of the change in the frequency of PTZ-induced burst discharges in area CA1 recorded from the stratum pyramidal by application of 4-EOA (n=8), 4-MNA (n=6), and 4-BCCA (n=5). ** and *** indicate significant difference at P<0.01 and P<0.001 compared to VPA.

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Compound	Species	Seizure model	dose (mg/kg)	animals (protected/tested)	animals (Toxic/tested)	ED_{50} (mg/kg)
4-MOA[a]	Mice	6Hz	100	0/4	0/4	----
5-MOA[a]	Mice	6Hz	300	1/4	0/4	----
	Rat	MES	300	2/4	0/4	----
4-MNA[a]	Mice	6Hz	100	0/4	0/4	----
4-EOA[a]	Mice	6Hz	150	7/8	----	110
	Rat	MES	125	12/16	4/15†	100
	Mice	scMET	200	8/8	4/8¥	142
	Mice	CKM	110	6/8	0/8	71
4-BCCA[a]	Mice	6Hz	100	3/4	0/4	81
	Rat	MES	100	4/8	1/8†	~100*
	Mice	scMET	150	4/8	0/8	~150*
	Mice	CKM	80	8/8	0/8	44
VPA	Mice	6Hz				263[b]
	Rat	MES				485[c]
	Mice	scMET				191[d]
	Mice	CKM				174[e]

Table 1: In vivo seizure control data for active compounds. Summary of *in vivo* seizure control in multiple models, with data provided by collaborative research with (a) NINDS; or previously determined by (b) (Barton et al., 2001); (c) (Loscher, 1999); (d) (Rowley and White, 2010); (e) by NINDS; *, based upon single dose data. --- not determined. † unable to grasp rotorod. ¥ ataxia/loss of righting reflex.

Figure 1

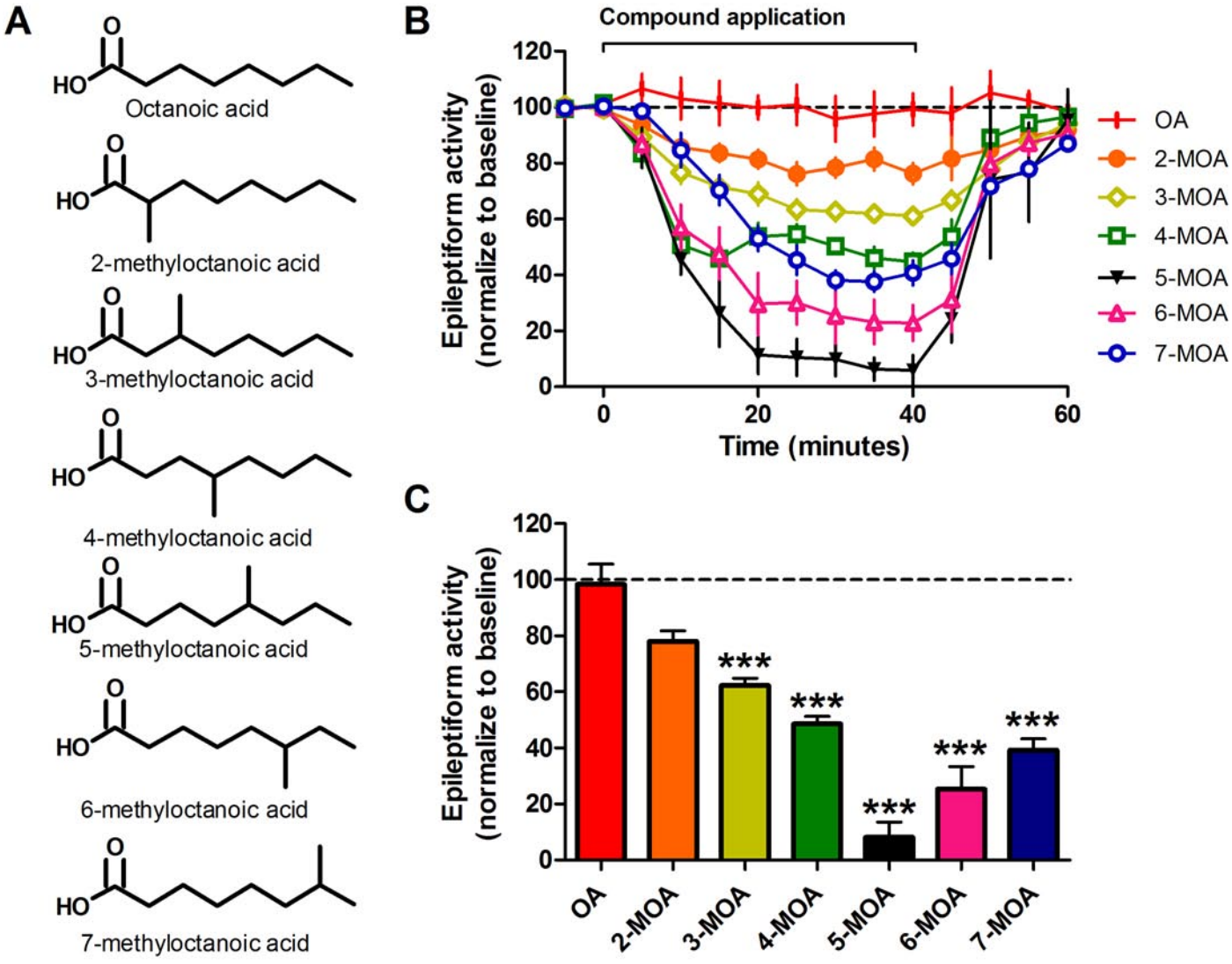

A
Octanoic acid
2-methyloctanoic acid
3-methyloctanoic acid
4-methyloctanoic acid
5-methyloctanoic acid
6-methyloctanoic acid
7-methyloctanoic acid
B
Compound application
Epileptiform activity (normalize to baseline)
Time (minutes)
OA
2-MOA
3-MOA
4-MOA
5-MOA
6-MOA
7-MOA
C
Epileptiform activity (normalize to baseline)
***

Figure 2

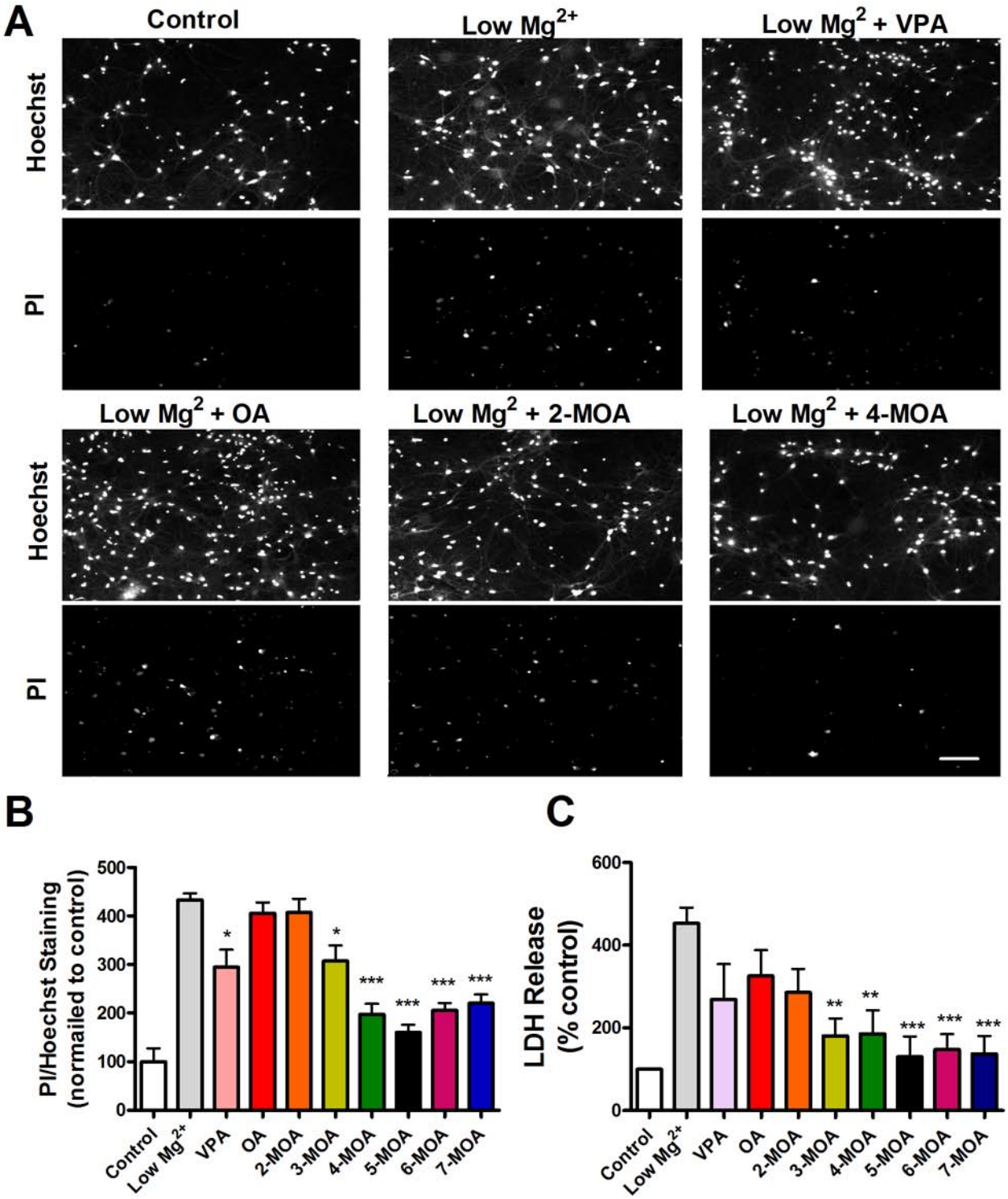

A
Control
Low Mg2+
Low Mg2 + VPA
Hoechst
PI
Low Mg2 + OA
Low Mg2 + 2-MOA
Low Mg2 + 4-MOA
Hoechst
PI
B
PI/Hoechst Staining
(normailed to control)
500
400
300
200
100
0
*
*
***
***
***
***
Control
Low Mg2+
VPA
OA
2-MOA
3-MOA
4-MOA
5-MOA
6-MOA
7-MOA
C
LDH Release
(% control)
600
400
200
0
**
**
***
***
***
Control
Low Mg2+
VPA
OA
2-MOA
3-MOA
4-MOA
5-MOA
6-MOA
7-MOA

Figure 3

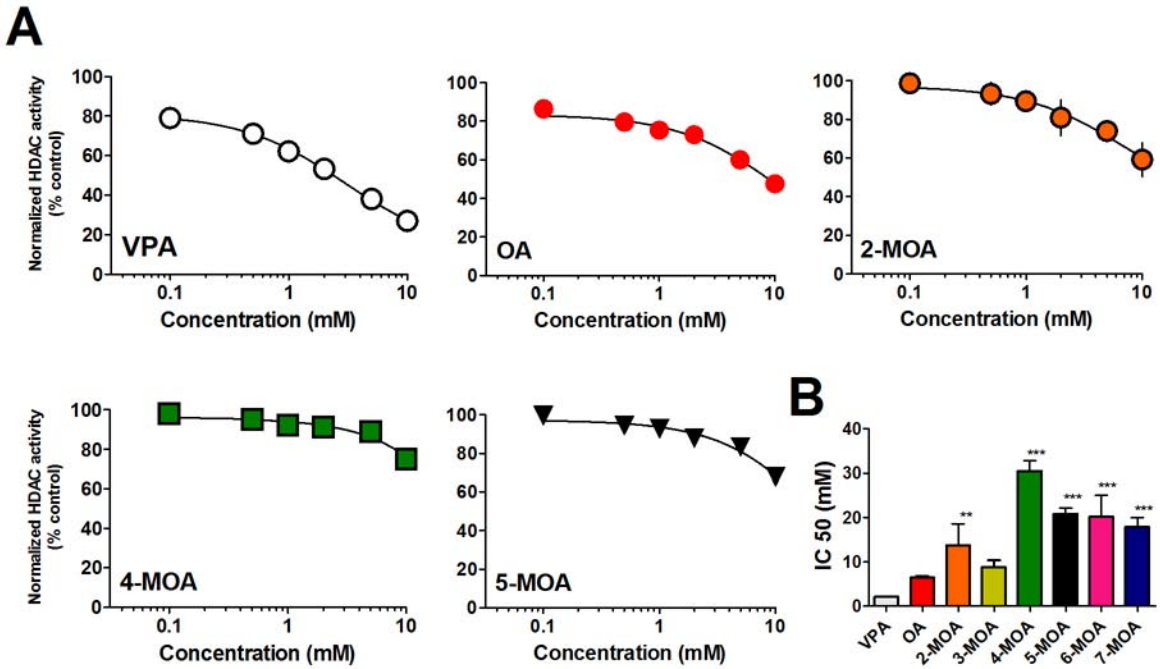
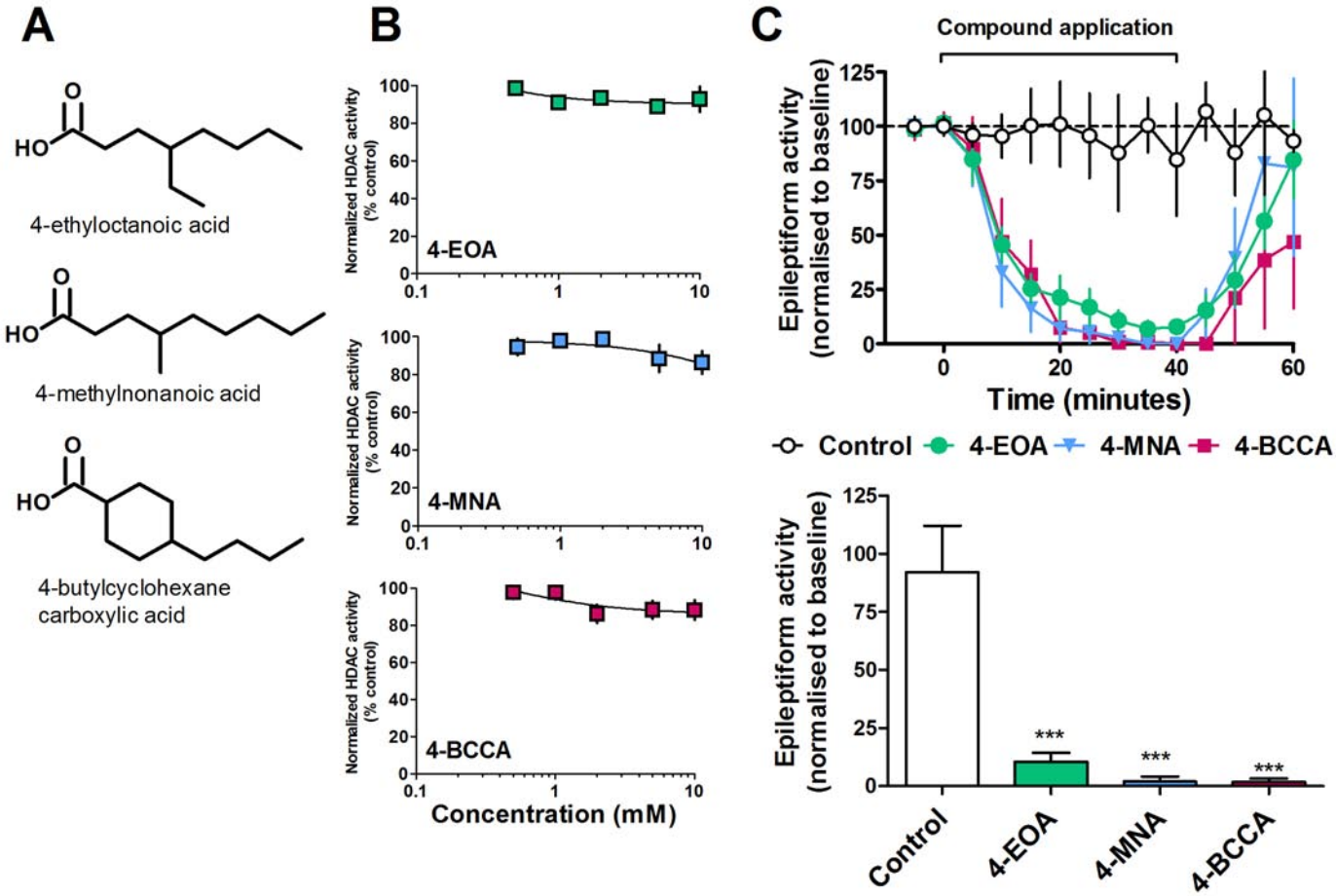

A
Normalized HDAC activity (% control)
VPA
OA
2-MOA
4-MOA
5-MOA
Concentration (mM)
B
IC 50 (mM)
VPA
OA
2-MOA
3-MOA
4-MOA
5-MOA
6-MOA
7-MOA

Figure 4


A
HO
O
4-ethyloctanoic acid
4-methylnonanoic acid
4-butylcyclohexane carboxylic acid
B
Normalized HDAC activity (% control)
4-EOA
4-MNA
4-BCCA
Concentration (mM)
C
Compound application
Epileptiform activity (normalised to baseline)
Time (minutes)
Control
4-EOA
4-MNA
4-BCCA
***