**New experimental therapies for status epilepticus in pre-clinical development**

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**Abstract**

Starting with the established antiepileptic drug valproic acid, we have taken a novel approach to develop new antiseizure drugs that may be effective in status epilepticus. We first identified that valproic acid has a potent effect on a biochemical pathway, the phosphoinositide pathway, in *Dictyostelium discoideum* and we demonstrated that this may relate to its mechanism of action against seizures in mammalian systems. Through screening in this pathway, we have identified a large array of fatty acids and fatty acid derivatives with antiseizure potential. These were then evaulated in an *in vitro* mammalian system. One compound that we identified through this process is a major constituent of the ketogenic diet, strongly arguing that it may be the fatty acids that are mediating the antiseizure effect of this diet. We further tested two of the more potent compounds in an *in vivo* model of status epilepticus and demonstrated that they were more effective than valproate in treating the status epilepticus.

**Introduction**

Valproic acid is a branch chain fatty acid, 2-propylpentanoic acid. Its anti-seizure effects were discovered whilst using it as a solvent to dissolve a series of potential new antiepileptic drugs(Meunier, Carraz, Neunier, Eymard, & Aimard, 1963). Subsequently valproic acid and its salt, sodium valproate, have become widely used as broad-spectrum antiepileptic drugs. More recently, glycine, amide and carbamate derivatives of valproic acid have been shown also to have anti-seizure potential, and to be effective in status epilepticus(Mawasi, Shekh-Ahmad, Finnell, Wlodarczyk, & Bialer, 2015). This study also demonstrated that valproic acid and the related salts are effective in animal models of status epilepticus, both stopping the status epilepticus and providing varying degrees of neuroprotection.

Valproic acid is available as an intravenous formulation and has been used in the treatment of status epilepticus in humans. It is at least as effective as intravenous phenytoin in small studies, but it has a better cardiovascular safety profile (Trinka, 2007). In one, pseudo-randomised study, valproic acid was shown to be superior to levetiracetam in the treatment of status epilepticus(Alvarez, Januel, Burnand, & Rossetti, 2011).

There are, however, potential downsides to the treatment of status epilepticus with valproic acid. High dose valproate can precipitate hyperammonaemia and associated encepaholapthy(Lewis, Deshpande, Tesar, & Dale, 2012), and also fulminant liver failure in people with mitochondrial cytopathies(Engelsen et al., 2008).

Surprisingly, despite years of research, valproic acid’s mechanism of action has remained unclear. Here, we provide insight into valproic acid’s putative mechanism of action. Further, using a simple model system, *Dictyostelium*, we provide a method to screen a range of medium chain fatty acids and their derivatives for efficacy in seizures and status epilepticus. Lastly, we show that these therapies provide both neuroprotection and anti-seizure effects in *in vitro* and *in vivo* models of seizures and status epilepticus.

**Screening for antiepileptic drugs**

The modern era of antiepileptic drug discovery began in 1937 when a cat model of seizures (the maximal electroshock model) devised by Merritt and Putnam was used to screen over 700 compounds resulting in the discovery of the anti-seizure effect of phenytoin(Putnam & Merritt, 1937). Later, the National Institute of Neurological diseases (USA) started an antiepileptic drug screening programme(White, Wolf, Woodhead, & Kupferberg, 1998), which has screened over 25,000 compounds though animal seizure models; this programme has been remarkably effective and has identified many of the new antiepileptic drugs currently in use. The problem with this approach, however, is that efficacy in acute seizure models is usually demonstrated prior to any understanding of mechanism of action. Moreover, the screening takes place in acute seizure models rather than the spontaneous seizures of chronic epilepsy, and there is rarely testing of efficacy in models of status epilepticus.

The advantage of non-mammalian model systems is that it can be much more straightforward, through mutagenesis, to determine putative mechanisms of action(Cunliffe et al., 2015). The use of ex-vivo animal mammalian models (e.g. neuronal cultures, acute slice preparations) can also facilitate a mechanistic approach. We, therefore, set out to determine the mechanisms of action of valproate in these model systems, and through that to identify potential new antiepileptic drugs.

**Valproate and *Dictyostelium***

*Dictyostelium discoideum* is a social amoeba that shares many of its enzymes and biochemical pathways with mammalian systems and thus it has proven to be an excellent model for understanding specific aspects of cellular signalling in human disease(Eichinger et al., 2005; Williams et al., 2006). *Dictyostelium* permits rapid and easy genetic manipulation and screening of mutant libraries as a pharmacogenetic approach to understanding drug action.

During times of starvation *Dictyostelium* cells move towards one another, coalesce and form fruiting bodies. The mechanisms underlying this process are well understood. Cell surface receptors detect extracellular cyclic AMP and then activate an intracellular signalling cascade involving phosphoinositides, resulting in chemotaxis. We firstly found thatvalproic acid at clinically relevant concentrations blocks this chemotaxic cell movement(Xu et al., 2007) through rapidly reducing phosphoinositide production in moving *Dictyostelium* cells(Pawolleck & Williams, 2009; Xu et al., 2007). Importantly, the phosphoinositide pathway is a phylogenically conserved pathway that is present in mammalian cells. We next set out to determine where valproate acted in the phosphoinositide pathway. This is challenging in mammalian systems in which enzyme inhibitors are often non-specific and rarely completely effective. However, it is substantially easier in *Dictyostelium*, because of the ability to rapidly delete any gene of choice provided that the protein product is not vital. We have used this method to examine the effect of valproic acid on PI3Ks (phosphatidylinositol 3-kinases), a family of enzymes that are most commonly associated with the production of phosphoinositide(Chang et al., 2012). Here, we employed a single *Dictyostelium* cell line with all six genes encoding these proteins deleted . Valproic acid blocked phosphoinositide production in these cells in the absence of the PI3K enzymes, indicating that valproate did not work through changing the activity of these enzymes.

We have extended this work to mammalian neurons and *in vivo* animal seizure models(Chang, Walker, & Williams, 2014), and have shown that that acute seizures in a rat seizure model or in an *in vitro* model of seizure-like activity decrease hippocampal phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels and reduce protein kinase B (PKB/AKT) phosphorylation. These changes were reversed with VPA treatment. Moreover, valproic acid's effect on seizure-like activity was blocked by drugs that target phosphoinositide signaling.

***Dictyostelium* and drug discovery**

Another significant advantage of *Dictyostelium* over other model systems is that it can be used as a rapid throughput screen of drugs/chemicals, because the behaviour of cells can change rapidly following drug exposure. The observations that valproic acid reduces phosphoinositide production and that this likely plays a role in its antiseizure effect provides an approach to develop a rapid throughput assay, to identify compounds with a similar mode of action. Restricting this assay to compounds that are chemically similar to valproic acid (a branched chain fatty acid) enabled us to explore the correlation of the structure of branch and straight chain fatty acids in this assay to establish the structure-activity relationship of these compounds(Chang et al., 2012). The observation that only small changes in the structure (e.g. the addition of an extra carbon) has a profound effect on the compound’s activity indicated that these compounds were not working through some non-specific effect of fatty acids on this pathway but that a more precise interaction was taking place.

We thus identified a group of compounds that are effective in a non-mammalian assay, which relates to a mechanism of action of valproic acid, and had also established a structure-function relationship for efficacy in this assay(Chang et al., 2012). We next determined whether these compounds are effective in a mammalian system, using epileptiform activity induced in *ex vivo* hippocampal-entorhinal cortex slices by removing magnesium from the perfusion solution or by adding a convulsant(Armand, Louvel, Pumain, & Heinemann, 1998). We used these *in vitro* screens of epileptiform activity for four main reasons: 1) We avoided the confounders of drug metabolism and the blood-brain barrier; 2) Such a systems are simple and permits high throughput screening; 3) Such methods reduce animal use and 4) These models are resistant to many antiepileptic drugs; valproate only shows a partial effect. These *in vitro* mammalian systems refined our chemical space and identified a host of potential antiepileptic drugs(Chang et al., 2012, 2013, 2015).

**Mechanisms of the ketogenic diet and efficacy in status epilepticus**

One of the compounds that we identified in our *Dictyostelium* and *in vitro* seizure assays that had a particularly marked effect was decanoic acid (Chang et al., 2012; Chang et al., 2013). Decanoic acid and octanoic acid (which was not effective in our seizure models) are the main constituents of coconut oil, which is employed as the basis for medium chain fatty acids in the ketogenic diet used to treat epilepsy. The ketogenic diet, a low carbohydrate and high fat diet, came to prominence in the treatment of epilepsy in the 1920’s. It had been proposed that the production of ketones through the metabolism of fats was the underlying mechanism of this diet(Rho & Stafstrom, 2012). However, ketone production correlates poorly with antiepileptic effect of the diet and ketones have variable efficacy in seizure models. The mechanism of action of the diet has, therefore, remained unclear. The observation that decanoic acid is frequently used as a constituent of the diet and that there are high levels of decanoic acid in the blood of children on the diet(Haidukewych, Forsythe, & Sills, 1982) indicate that decanoic acid’s antiepileptic effect may be a major mechanism underlying this diet and may be a way to make a more palatable and better tolerated diet by avoiding the ketogenesis.

The ketogenic diet has been described to be effective in refractory status epilepticus(O’Connor et al., 2014; Thakur et al., 2014) and this, together with valproic acid’s well established use in status epilepticus, raises the question of whether the compounds that we have identified are also effective in status epilepticus. We tested two of the more efficacious compounds, nonanoic acid and 4 methyl-octanoic acid against status epilepticus induced in rats through constant stimulation of the perforant path (Chang et al., 2012). This leads to self-sustaining status epilepticus that is partially resistant to benzodiazepines and phenytoin but which responds to treatment with the anaesthetic propofol (Holtkamp, Tong, & Walker, 2001). Both nonanoic acid and 4 methyl-octanoic acid were significantly more effective than an equivalent dose of valproic acid in this model, and 4-methyloctanoic acid completely abolished behavioural and electrographic seizures, whilst valproic acid only had a partial effect(Chang et al., 2013). 4-methyloctanoic acid also resulted in significant neuroprotection.

**Conclusion**

We have shown here how *Dictyostelium* can be used to identify mechanisms of action of valproic acid, and to use these observations to screen an array of compounds, thus identifying an array of potential new antiepileptic drugs. Many of these were effective in *in vitro* and *in vivo* animal seizure models, opening up the path to producing novel antiepileptic drugs that may be more potent than valproate but lack some if its idiosyncratic side-effects.

Amongst the compound that we have identified is a medium chain fatty acid that is one of the constituents of the ketogenic diet. This fatty acid may, therefore, be a major contributor to the efficacy of the diet.

Since both valproic acid and the ketogenic diet are efficacious in status epilepticus, we tested two of the more potent compounds against an *in vivo* model of status epilepticus, and found that they were more potent than valproic acid.

These discoveries pave the way to the development of new treatments and treatment approaches not only to status epilepticus, but also to drug resistant epilepsy and possibly, through improved side-effect profiles, to the treatment of all epilepsies.

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**References**

Alvarez, V., Januel, J.-M., Burnand, B., & Rossetti, A. O. (2011). Second-line status epilepticus treatment: comparison of phenytoin, valproate, and levetiracetam. *Epilepsia*, *52*(7), 1292–6. doi:10.1111/j.1528-1167.2011.03056.x

Armand, V., Louvel, J., Pumain, R., & Heinemann, U. (1998). Effects of new valproate derivatives on epileptiform discharges induced by pentylenetetrazole or low Mg2+ in rat entorhinal cortex-hippocampus slices. *Epilepsy Research*, *32*(3), 345–355. doi:10.1016/S0920-1211(98)00030-8

Chang, P., Orabi, B., Deranieh, R. M., Dham, M., Hoeller, O., Shimshoni, J. A., … Williams, R. S. B. (2012). The antiepileptic drug valproic acid and other medium-chain fatty acids acutely reduce phosphoinositide levels independently of inositol in Dictyostelium. *Disease Models & Mechanisms*, *5*(1), 115–24. doi:10.1242/dmm.008029

Chang, P., Terbach, N., Plant, N., Chen, P. E., Walker, M. C., & Williams, R. S. B. (2013). Seizure control by ketogenic diet-associated medium chain fatty acids. *Neuropharmacology*, *69*, 105–14. doi:10.1016/j.neuropharm.2012.11.004

Chang, P., Walker, M. C., & Williams, R. S. B. (2014). Seizure-induced reduction in PIP3 levels contributes to seizure-activity and is rescued by valproic acid. *Neurobiology of Disease*, *62*, 296–306. doi:10.1016/j.nbd.2013.10.017

Chang, P., Zuckermann, A. M. E., Williams, S., Close, A. J., Cano-Jaimez, M., McEvoy, J. P., … Williams, R. S. B. (2015). Seizure control by derivatives of medium chain fatty acids associated with the ketogenic diet show novel branching-point structure for enhanced potency. *The Journal of Pharmacology and Experimental Therapeutics*, *352*(1), 43–52. doi:10.1124/jpet.114.218768

Cunliffe, V. T., Baines, R. A., Giachello, C. N. G., Lin, W.-H., Morgan, A., Reuber, M., … Williams, R. S. B. (2015). Epilepsy research methods update: Understanding the causes of epileptic seizures and identifying new treatments using non-mammalian model organisms. *Seizure*, *24C*, 44–51. doi:10.1016/j.seizure.2014.09.018

Eichinger, L., Pachebat, J. A., Glöckner, G., Rajandream, M.-A., Sucgang, R., Berriman, M., … Kuspa, A. (2005). The genome of the social amoeba Dictyostelium discoideum. *Nature*, *435*(7038), 43–57. doi:10.1038/nature03481

Engelsen, B. A., Tzoulis, C., Karlsen, B., Lillebø, A., Laegreid, L. M., Aasly, J., … Bindoff, L. A. (2008). POLG1 mutations cause a syndromic epilepsy with occipital lobe predilection. *Brain : A Journal of Neurology*, *131*(Pt 3), 818–28. doi:10.1093/brain/awn007

Haidukewych, D., Forsythe, W. I., & Sills, M. (1982). Monitoring octanoic and decanoic acids in plasma from children with intractable epilepsy treated with medium-chain triglyceride diet. *Clinical Chemistry*, *28*(4 Pt 1), 642–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7074833

Holtkamp, M., Tong, X., & Walker, M. C. (2001). Propofol in subanesthetic doses terminates status epilepticus in a rodent model. *Annals of Neurology*, *49*(2), 260–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11220748

Lewis, C., Deshpande, A., Tesar, G. E., & Dale, R. (2012). Valproate-induced hyperammonemic encephalopathy: a brief review. *Current Medical Research and Opinion*, *28*(6), 1039–42. doi:10.1185/03007995.2012.694362

Mawasi, H., Shekh-Ahmad, T., Finnell, R. H., Wlodarczyk, B. J., & Bialer, M. (2015). Pharmacodynamic and pharmacokinetic analysis of CNS-active constitutional isomers of valnoctamide and sec-butylpropylacetamide - Amide derivatives of valproic acid. *Epilepsy & Behavior : E&B*. doi:10.1016/j.yebeh.2015.02.040

Meunier, H., Carraz, G., Neunier, Y., Eymard, P., & Aimard, M. (1963). [Pharmacodynamic properties of N-dipropylacetic acid]. *Thérapie*, *18*, 435–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13935231

O’Connor, S. E., Richardson, C., Trescher, W. H., Byler, D. L., Sather, J. D., Michael, E. H., … Zupec-Kania, B. (2014). The ketogenic diet for the treatment of pediatric status epilepticus. *Pediatric Neurology*, *50*(1), 101–3. doi:10.1016/j.pediatrneurol.2013.07.020

Pawolleck, N., & Williams, R. S. B. (2009). Quantifying in vivo phosphoinositide turnover in chemotactically competent Dictyostelium cells. *Methods in Molecular Biology (Clifton, N.J.)*, *571*, 283–90. doi:10.1007/978-1-60761-198-1\_19

Putnam, T. J., & Merritt, H. H. (1937). Experimental determination of the anticonvulsant properties of some phenyl derivatives. *Science (New York, N.Y.)*, *85*(2213), 525–6. doi:10.1126/science.85.2213.525

Rho, J. M., & Stafstrom, C. E. (2012). The ketogenic diet: what has science taught us? *Epilepsy Research*, *100*(3), 210–7. doi:10.1016/j.eplepsyres.2011.05.021

Thakur, K. T., Probasco, J. C., Hocker, S. E., Roehl, K., Henry, B., Kossoff, E. H., … Cervenka, M. C. (2014). Ketogenic diet for adults in super-refractory status epilepticus. *Neurology*, *82*(8), 665–70. doi:10.1212/WNL.0000000000000151

Trinka, E. (2007). The use of valproate and new antiepileptic drugs in status epilepticus. *Epilepsia*, *48 Suppl 8*, 49–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18329999

White, H. S., Wolf, H. H., Woodhead, J. H., & Kupferberg, H. J. (1998). The National Institutes of Health Anticonvulsant Drug Development Program: screening for efficacy. *Advances in Neurology*, *76*, 29–39. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9408461

Williams, R. S. B., Boeckeler, K., Gräf, R., Müller-Taubenberger, A., Li, Z., Isberg, R. R., … Alexander, S. (2006). Towards a molecular understanding of human diseases using Dictyostelium discoideum. *Trends in Molecular Medicine*, *12*(9), 415–424. doi:10.1016/j.molmed.2006.07.003

Xu, X., Müller-Taubenberger, A., Adley, K. E., Pawolleck, N., Lee, V. W. Y., Wiedemann, C., … Williams, R. S. B. (2007). Attenuation of phospholipid signaling provides a novel mechanism for the action of valproic acid. *Eukaryotic Cell*, *6*(6), 899–906. doi:10.1128/EC.00104-06