AUTHENTICATION OF KEY RESOURCES PLAN - PROJECT 2 – PRECLINICAL MODEL FOR ANTIEPILEPTOGENIC THERAPY SCREENING IN POST-TRAUMATIC EPILEPSY

1. Z944 AUTHENTICATION

Since the original description of Z944 structure, in vitro preclinical properties and animal efficacy (Trignham et al., 2012) it has been synthesized multiple times and in multiple batches. The starting materials are all commercially available and are provided with certificates of analysis from the supplier. Each synthetic batch of Z944 is characterized to confirm the chemical structure and batch purity by multiple analytical assessments, including mass spectroscopy, MS-HPLC, infrared spectroscopy, UV spectrum, thermal analysis (melting point) and that are compared to existing values for an internal Z944 standard.

Since 2009 synthetic batches of Z944 all exhibit the expected structural/chemical integrity and to have purities of >95% (generally >98%). Testing of different batches of Z944 in vitro and in animal models (via i.p., oral and mini-pump routes of administration) have not shown any deviation from expected properties as these relate to known off-target effects or acute/chronic toxicities (LeBlanc et al., 2016; Matschke et al., 2015; Casillas-Espinosa et al 2015; Cain et al., 2015; Ranzato et al., 2015). The synthetic scheme for Z944 in the current proposal is the same as that for its GMP manufacture utilized in three human clinical trials to date.

Publications utilizing different batches of Z944

Matschke, LA, M Bertoune, J Roeper, TP Snutch, WH Oertel, S Rinné, N Decher (2015) A concerted action of L-type and T-type Ca^{2+} channels regulates pacemaking in neurons of the locus coeruleus. Molecular and Cellular Neurosciences, 68: 293-302.

Casillas-Espinosa, PM, A Hicks, A Jeffreys, TP Snutch, TJ O'Brien, KL Powell (2015) Z944, a novel selective T-type calcium channel antagonist delays the progression of epileptogenesis in the amygdala kindling model of temporal lobe epilepsy. PLOS One, Aug 14;10(8):e0130012. doi: 10.1371/journal.pone.0130012. PMID:26274319

Cain, SM, JR Tyson, KL Jones, TP Snutch (2015) Thalamocortical neurons display suppressed burst-firing due to an enhanced I_h current in a genetic model of absence epilepsy. Pflugers Archives – European Journal of Physiology 467: 1367-1382. PMID: 24953239.

Ranzato, E, VMagnelli, S Martinotti, Z Waheed, SM Cain, TP Snutch, C Marchetti, B Burlando (2014) Epigallocatechin-3-gallate elicits Ca^{2+} spike in mcf-7 breast cancer cells: essential role of Cav3.2 T-type calcium channels. Cell Calcium 56:285-295.

Tringham, E, KL Powell, SM Cain, K Kuplast, J Mezeyova, M Weerapura, C Eduljee, X Jiang, P Smith, J-L Morrison, NC Jones, E Braine, G Rind, M Fee-Maki, D Parker, H Pajouhesh, M Parmar, TJ O'Brien, TP Snutch (2012) T-Type calcium channel blockers that attenuate thalamic burst firing and suppress absence seizures. Science Translational Medicine 4: 121ra19.

LeBlanc BW, Lii TR, Huang JJ, Chao YC, Bowary PM, Cross BS, Lee MS, Vera-Portocarrero LP, Saab CY (2016) T-type calcium channel blocker Z944 restores cortical synchrony and thalamocortical connectivity in a rat model of neuropathic pain. Pain. 2016 157:255-63. PMID: 26683108

2. AUTHENTICATION OF OTHER COMPOUNDS WE ARE USING IN THIS GRANT

In this grant we are using also the following compounds for testing for antiepileptogenic effects:
1) Sodium Selenate will be purchased by SIGMA (Cat No S0882) with documentation of 95% purity.
2) Deferiprone (3-hydroxy-1,2-dimethyl-4(1H)-pyridone) will be purchased from SIGMA (379409) with documentation of 98% purity.
3) Kineret (rrIL-1ra) (SOBI) will be purchased from the pharmacy since it is clinically approved for use in humans.
4) VX-765 will be purchased from InvivoGen (Cat No: inh-vx765-1) with documentation of 97% purity.
5) Levetiracetam will be purchased from Medchem Express (HY-B0106), with >98% purity documentation.
All the compounds are being purchased from established sources for chemicals with documented high purity (95 – 98%). The compounds will be dissolved in vehicles appropriate for delivery to rats via subcutaneous osmotic minipumps and will be handled under sterile conditions. These compounds have been used by us or our collaborators in previous experimental studies in animals which produced the anticipated effects, in regards to their known mechanisms, without any adverse or paradoxical effects. Within this consortium, we plan to share common protocols to avoid having high variability in outcomes. We also plan to obtain pharmacokinetics and blood levels of the drugs, through our collaboration with Dr. Jim Cloyd and Lisa Coles (University of Minnesota). This is a manner will be used for quality control, to ensure proper and rationale delivery of the drug

but will also inform on the therapeutic or active levels of the drugs. Such levels can therefore be used by other investigators as a reference point in their experiments to interpret them and compare their conditions with ours and facilitate comparisons and interpretation. To ensure that the results of our experiments can be translated and compared with the results that could be obtained in different labs, we plan to:

(a) adopt rigorous and unbiased practices in study design, data collection and analysis, through the use of blinded, randomized, dose-response and vehicle response experiments and adherence to the ARRIVE guidelines[6], the guidelines reported by Landis et al [7] and the recommendations of the AES/ILAE Translational research Task Force of the ILAE [2, 3].
(b) We will perform pharmacokinetics (PK) and PK modeling and determination of therapeutic levels to provide information on the MCFA levels that are needed to reproduce the effect observed in our studies.
(c) We plan to investigate whether factors that could interfere with the levels of some of these compounds could affect the results we obtain. For instance we will study the levels after separation of the animals for monitoring and compare them with nonfasting or with supplementation of glucose to determine if metabolic state may affect their levels or efficacy. We will report these findings in our publications and will adapt the therapeutic levels of the drugs according to these data.
(d) Publications that will stem from our studies will report the source, quality control and purity, vehicle preparation and method and dose of administration of the compounds.
(e) Although our experiments will be done by subcutaneous osmotic minipumps that allow continuous delivery of the drug, all of the compounds we have selected have been tested in humans in phase I or phase II trials, which suggests that they can be dissolved in FDA-approved formulations . usual
(f) We will perform this study through a collaboration of 6 independent research groups, Einstein (Drs. Moshé and Galanopoulou: experts in in vivo models of epilepsy, neurotherapeutics), University of Minnesota (Drs. Cloyd and Coles: experts in pharmacokinetics and formulation optimization), University of Melbourne (Dr. Terence O'Brien), UCLA (Dr. Rick Staba), University of Eastern Finland (Dr. Asla Pitkänen), and University of British Columbia (Dr. Terrance Snutch)

All the materials and chemicals used here otherwise are from established suppliers with documentation of their purity. The source of the chemicals and the composition of vehicles will be written in our publications.will be written in the publications from our group. United States Pharmacopeia grade reference standards will be purchased for use as internal standards in the measurement of drug concentrations from Sigma-Aldrich.

3. METHODS TO HARMONIZE THE EXPERIMENTAL PROCEDURES, PROTOCOLS, REPORTS AND METHODS OF ANALYSIS OF THE DATA DERIVED FROM THIS CONSORTIUM

In this consortium, we have a collaboration among the following investigators to perform the animal studies.
Drs. Galanopoulou and Moshé (Einstein)
Dr. Staba (UCLA)
Dr. O'Brien (Melbourne)
Dr. Pitkänen (University of Eastern Finland)
Drs. Cloyd and Coles (Minnesota)
Dr. Snutch (U British Columbia)
Dr. Agoston (Uniformed Services University)

Due to the nature of this multicentre study, the main experimental work (animal experiments, MRI, video EEG, blood collection for analysis) will need to be shared in 4 centers (Einstein, UCLA, Melbourne, UEF). For this reason, we plan to prepare and adopt common protocols and reporting forms so that results do not present with variability that confounds results. We therefore plan to dedicate the first 3 months from the start of the funding period, if awarded, teleconferences, emails, and webconferences, face to face meetings, if possible, discussing how to develop this. Harmonization will be done on the following.

1) Experimental protocols. We plan to share protocols amongst us so that people are trained to follow the same procedures in techniques included in the grant. These will include methods for blood collection, methods for LFP injury
2) EEGs: Wide band EEGs will be acquired and utilized by all 3 centers to utilize the same technology as UCLA in detecting pHFOs and rHFOSs. Amplifiers from Intan will be purchased and UCLA will train post-docs from the other 3 centers to perform the surgery with the microelectrodes and recordings.
3) EEG interpretation of seizures or other patterns is an issue that we will dedicate time to agree on the same definitions and classifications of patterns. This will be done in year 1 of the grant through webconferences, face-to-face meeting and teleconferences with exchange of data. Dr. Galanopoulou is also the co-leader of the TASK1 working groups of the AES/ILAE Translational Research Task Force, which has as a main goal

the development of common definitions and classifications for EEG patterns from rodents. Therefore, once this classification is available it will be introduced in our work here.

4) LFP induction: All 4 centers will use the AmScien instruments Fluid Percussion Device, the same anesthesia and method of induction and monitoring.
5) MRIs : There are differences across centers on the source and type of magnet, coils and methods they utilize (see Table 1 on MRI Instrumentation. Einstein will purchase surface coils for the experiments planned in Years 4-5 and the Investigators who are overseeing the MRI Centers in these Instititutions have agreed an protocols that could provide a solution to these technical differences, that will include the same type of anesthesia (isoflurane), montoring= of vitals, image acquisition for anatomical images using 3D-FISP pulse sequence, T2*, unwrapped phase and SWI using 3D-multiecho GRE, diffusion tensor MRI and fiber tracking. Furthermore, beyond the local, on site analysis of images, MRI data will be transferred to the IAC (Director Dr. Toga) for centralized analyses.
6) Testing for Neuroscore, blood collection, and animal husbandries. Standardized protocols will be written with relevant training if needed so that investigators handling the animals can adopt the same procedures.
7) Blood and biospecimen collection for drug levels, plasma biomarkers and miRNAs. Standardized written protocols will be written with sufficient detail to describe how the procedure should be done and how the specimens should be handled. The centers responsible for the analysis of these studies (Dr. Cloyd for pharmacokinetics, Dr. Pitkänen for miRNAs, Dr. Agoston for plasma biomarkers) will be responsible to write these protocols.
8) Animals. W agreed to include only Sprague-Dawley male rats from local providers. There are differences expected in genotype, breeding and nutritional status, which may affect the outcomes, since the research centers are international. However, we will accept this level of heterogeneity, since even humans have significant genetic, epigenetic, habitual or life style areas of differences that could affect TBI outcomes. We have randomized the study so that each center can contribute the same number of animals.
9) Procedural issues for the antiepileptogenesis study. To maintain the rigor of the study across all centers, it will be important to apply the same protocols across all centers. A system for blinding, unmasking, and workflow will be agreed upon so as to create standardized written protocols that will facilitate the work. The investigators responsible for blinding the experimenters at their sites will be holding the log with the identifiable data and provide it at pre-determined study points (e.g. when study with data collection is over) to the statistician who will analyze the data (IAC) and who will not be involved in the blinding, handling of the animals or other data collection. At the onset of the grant the investigators will agree on the composition and format of a central database that will be de-identfied (located at IAC but accessible through internet to all involved centers). This de-identified database will be populated through the course of the study and unmasked only at the conclusion of the study, i.e, when all study subjects have enrolled and completed testing and data have been analyzed. Dr. Galanopoulou will be responsible to oversee these procedural aspects.
10) We realize that even if we address the areas of differences within our group, efforts to replicate the goals of our study in different centers may be challenged by unanticipated differences. We will make every effort to publish with sufficient detail the methodological details of our study so that it is clearly communicated what will be done during the study.

Table 1	***Project 2.*** *MRI Instrumentation*			
	UEF	***U Melbourne***	***UCLA***	***Einstein***
Magnet	*7T/16cm*	*4.7T/33cm*	*7T/30cm*	*9.4 T 31 cm*
Console	Bruker Pharmascan	*Bruker Biospec*	*Bruker Biospec*	*Agilent Direct Drive*
Gradients	*600 mT/m risetime 150 us*	*440mT/m risetime 150 us*	*400 mT/m rise time~110us*	*600 mT/m, 180 us risetime*
Head Rf-coils	*Actively decoupled volume transmitter and quad. surface coil receivers (Bruker)*	*Actively decoupled volume transmit and 4-channel surface coil receiver (Bruker)*	*Actively decoupled volume transmitter and single channel surface coil receivers*	*Actively decoupled volume transmitters (M2M), 4 channel array coils (M2M)*

4. miRNA ANALYSIS

Plasma quality

The hemolysis can be detected by existence of miR-23a and miR-451a in the sample.
Ref: http://www.sciencedirect.com/science/article/pii/S0003269715003681

RNA quality

RNA extraction: RIN >7.0 (Bioanalyzer 2100, Agilent Technologies) as well as OD260/230 and OD260/280 ratio ~2.0 (NanoDrop, Thermo Scientific).

For miRNAs there is no standardized method for quality control. miRNAs are generally well-preserved are not as susceptible to RNases due to their size. The RIN number and 260/280 ratio will be checked before and after transportation as well as prior to use, if samples have been stored for a longer period of time (>1 year).

miRNA-Seq

Exiqon's guidelines (miR-seq): RIN 7-10 indicates high quality RNA. It is possible to do miRNA-Seq in samples that have RIN 5-10. With RIN<5 sequencing is possible but sequencing of degraded material may contribute to results.

RT-qPCR: Housekeeping genes GAPDH for mRNA, U6 for miRNA in brain tissue, miR-425 for miRNA analysis in plasma.

Internal controls: GAPDH and U6 are commonly used. Studies in Pitkänen lab indicate that miR-425 has a stable expression over a period of 2 d to 2 months post-TBI.

Exiqon will perform the RNA isolation for microRNA-Seq. Exiqon has developed its own qPCR based quality control panel for serum and plasma RNA samples. The method is based on miRCURY LNA Universal RT microRNA PCR system and it uses four synthetic spike-ins. The degree of hemolysis will be evaluated. After library generation, Reverse Transcription (RT) and PCR pre-amplification the insert rate of the desired RNA type is evaluated using Bioanalyzer DNA high sensitivity chip. After sequencing, reads are compared to a number of reference sources (miRBase, Rfam). Before differential expression analysis results are normalized to compensate sample specific effects.

5. REVERSE PHASE PROTEIN MICROARRAY (RPPA) (Agoston Lab)

RPPA and Antibody Quality control

All the details of the sample preparation and RPPA methods and quality control experiments done in the RPPA used in Dr. Agoston's lab are described in[4]. Antibodies used in RPPA are tested in Western blots using the appropriate biomaterial (e.g., serum) and blocking peptides or recombinant proteins to distinguish between specific and non-specific bands. Only antibodies that did not show residual nonspecific bands were chosen [4]. Antibodies used in this RPPA also had to bind to their targets in a 1:1 ratio.

Slides used in the RPPA were tested for false positive signal and the best out of 6 tested nitrocellulose coated slides was used. Use of replicates in the same array, placed apart from each other, prevented loss of data due to position effect. In the signal analysis, the amount of protein was estimated by employing a regression approach using all available spots within the dynamic range; saturated data and data close to the noise level were removed. Exclusion of bad data was done using the following criteria: signal to noise ≤2, replicate spot variability present.

Several criteria are used in determining the acceptability of each dilution series; (1): visual inspection of the scanning image to identify if smears and blotches affect the quality of the intensity measurement; (2): if the parameters of the regression identify high variability, or too few points within the dynamic range, or non-linearity of the data (low value of R2), and (3): the slope of the log–log plot should theoretically be equal to −1.

6. ANTIBODIES

We will use the antibodies listed in Table 2, which have been referenced in key references in the relevant fields, including those of our collaborators or our PI groups. These antibodies have been characterized by Western blot using brain extracts and show binding to the appropriate bands. When not available, quality control experiments or specificity assays will be done using antigen adsorption assays or by demonstrating lack of binding on tissues from gene-specific knockout mice.

Table 2. Targets studied in Aim 1A and 1B.

Drug	Targets (antibody, source)
Sodium selenate	Total tau (Tau-5, Millipore), p-Ser198 tau, p-Ser202-tau (AT8, ThermoFisher Scientific), p-Thr231-tau (AT180, Pierce), and p-Ser262-tau (ab64193, Abcam), PR55 (539521, Millipore)[5, 10, 12].
Deferiprone	H&E stain[14], Modified Perls stain, total iron assay[1], ceruloplasmin (ABN39, SIGMA) [1].
Kineret (rrIL-1ra) / VX-765	IL-1ra (PA5-21776, ThermoFisher Scientific), IL-1β (NBP1-42767, Novus), NFkB (8242S, Cell Signaling Technology), glial fibrillary astrocytic protein (GFAP) (MS1376P1, ThermoFisher Scientific), MBP (SMI-94, Covance Research products), Iba1 (016-20001, Wako Chemicals) [9, 11, 13].
Levetiracetam	anti-SV2 antibody (Developmental Studies Hybridoma Bank) [8].

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