Proof-of-concept that network pharmacology is effective to modify development of acquired temporal lobe epilepsy

Alina Schidlitzki, Pablo Bascuñana, Prashant K. Srivastava, Lisa Welzel, Friederike Tewele, Kathrin Töllner, Christopher Käufer, Birthe Gericke, Rahel Felek, Martin Meier, Andreas Polyak, Tobias L. Ross, Ingo Gerhauer, Jens P. Bankstahl, Michael R. Johnson, Marion Bankstahl, Wolfgang Löscher,⁎

a Department of Pharmacology, Toxicology, and Pharmacy, University of Veterinary Medicine Hannover, 30559 Hannover, Germany
b Center for Systems Neuroscience, 30559 Hannover, Germany
c Department of Nuclear Medicine, Hannover Medical School, Germany
d Division of Brain Sciences, Imperial College London, London, UK
e Central Animal Facility & Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany
f Department of Pathology, University of Veterinary Medicine Hannover, Germany

ARTICLE INFO

Keywords:
Epileptogenesis
Levetiracetam
Topiramate
Seizures
RNA-seq
Kainate
Multimodal brain imaging
Neuroinflammation
Neurodegeneration
Blood-brain barrier leakage

ABSTRACT

Epilepsy is a complex network phenomenon that, as yet, cannot be prevented or cured. We recently proposed network-based approaches to prevent epileptogenesis. For proof of concept we combined two drugs (levetiracetam and topiramate) for which in silico analysis of drug-protein interaction networks indicated a synergistic effect on a large functional network of epilepsy-relevant proteins. Using the intrahippocampal kainate mouse model of temporal lobe epilepsy, the drug combination was administered during the latent period before onset of spontaneous recurrent seizures (SRS). When SRS were periodically recorded by video-EEG monitoring after termination of treatment, a significant decrease in incidence and frequency of SRS was determined, indicating antiepileptogenic efficacy. Such efficacy was not observed following single drug treatment. Furthermore, a combination of levetiracetam and phenobarbital, for which in silico analysis of drug-protein interaction networks did not indicate any significant drug-drug interaction, was not effective to modify development of epilepsy. Surprisingly, the promising antiepileptogenic effect of the levetiracetam/topiramate combination was obtained in the absence of any significant neuroprotective or anti-inflammatory effects as indicated by multimodal brain imaging and histopathology. High throughput RNA-sequencing (RNA-seq) of the ipsilateral hippocampus of mice treated with the levetiracetam/topiramate combination showed that several genes that have been linked previously to epileptogenesis, were significantly differentially expressed, providing interesting entry points for future mechanistic studies. Overall, we have discovered a novel combination treatment with promise for prevention of epilepsy.

1. Introduction

Epilepsy is one of the most common serious brain disorders, being characterized by spontaneous recurrent seizures (SRS) and high rates of comorbid health conditions (Devinsky et al., 2018). Approximately 20% of all epilepsies are a consequence of acute brain insults such as traumatic brain injury (TBI), stroke, CNS infections, and prolonged febrile or afebrile status epilepticus (SE) (Klein et al., 2018). Prevention or modification of acquired epilepsy in patients at risk is a major unmet medical need (Löscher et al., 2013; Klein et al., 2018). A latent period of
days to years often exists between the acute insult and the onset of clinically obvious epilepsy (Pitkänen et al., 2015). This post-injury interval offers a window of opportunity to interrupt or modify epileptogenesis with the aim to prevent epilepsy or reduce its severity. In view of the complexity of the processes underlying epileptogenesis, we have previously proposed that rationally chosen combinations of drugs that target multiple epileptogenic processes may be more effective to prevent or modify epilepsy than treatment with single, highly specific drugs (Löscher et al., 2013; White and Löscher, 2014). Clinical translation of such a network strategy would benefit from repurposing of approved drugs that are currently used for other indications.

Among the various clinically approved drugs that have been previously explored for antiepileptogenic effects following single drug treatment, two anti-seizure drugs, levetiracetam (LEV) and topiramate (TPM), have shown promising disease-modifying efficacy when administered during amygdala kindling or during the latent period after an epileptic brain insult in rodent models of acquired epilepsy (Löscher and Brandt, 2010; Löscher et al., 2016). Small clinical trials with LEV indicated that this drug may possess disease-modifying potential also in humans (Jehi et al., 2012; Klein et al., 2012a,b; Kaminski et al., 2014), whereas such trials are lacking for TPM. However, neither drug alone was capable of preventing epilepsy in preclinical studies (Löscher and Brandt, 2010).

LEV and TPM modulate, in a complementary fashion, a number of targets that are thought to be involved in epileptogenesis (Lösch et al., 2016; Rogawski et al., 2016). Several studies that investigated combinations of LEV and TPM for pharmacodynamic interactions on acute seizures in rodents reported synergistic (over-additive) anti-seizure efficacy without impairing motor coordination or learning, or affecting muscular strength (Kaminski et al., 2009; Sarhan et al., 2015). This is in line with the good tolerability of combinations of LEV and TPM in patients with epilepsy (Sarhan et al., 2015). We recently reported that treatment of mice with a combination of LEV (200 mg/kg b.i.d.) and TPM (30 mg/kg b.i.d.) over 3 days following a brain insult, induced by either intrahippocampal kainate or systemic administration of pilocarpine, was well tolerated (Klee et al., 2015).

In the present study, we tested the hypothesis that post-insult treatment with a combination of LEV and TPM is more efficacious to prevent or modify the development of epilepsy than either drug alone. For comparison, we also included a combination of LEV and phenobarbital (PB), for which a synergistic interaction was reported for acute seizure models (Kaminski et al., 2009). For the purpose of the present study, we used the intrahippocampal kainate mouse model of mesial temporal lobe epilepsy (TLE), the most common and often devastating type of epilepsy in humans. The mouse model exhibits several characteristics of mesial TLE in patients, including an epileptogenic focus in the ipsilateral hippocampus, development of SRS, and hippocampal pathology resembling hippocampal sclerosis (Guillemain et al., 2012; Lévesque and Avoli, 2013; Jeffersy et al., 2016). In view of the critique that most preclinical studies on antiepileptogenesis are underpowered and thus difficult to replicate (Pitkänen et al., 2013; Lapinlampi et al., 2017), we repeated all experiments at least once. Multimodal brain imaging, protein network and gene expression analyses were used to study the pathways and molecular processes that may underlie the promising antiepileptogenic effects of the LEV/TPM combination.

2. Materials and methods

2.1. Animals

Outbred male NMRI (Naval Medical Research Institute) mice, which originated from a colony of Swiss mice and are used as a general-purpose stock in many fields of research including pharmacology (Chia et al., 2005), were obtained from Charles River (Sulzfeld, Germany) at an age of 6–7 weeks (body weight 30–40 g). Mice were adapted to the laboratory conditions for 1–2 weeks before used in experiments, so that all mice were mid-adolescent at time of kainate injection. Animals were housed under controlled conditions (ambient temperature 22–24 °C, humidity 30–50%, lights on from 6:00 am to 6:00 pm). For imaging experiments, animals were housed in groups of up to 9 mice in individually ventilated cages under a 14/10-h light/dark cycle. Food (Altromin 1324 standard diet; Altromin, Lage, Germany) and water were freely available.

Experiments were performed according to the EU council directive 2010/63/EU and the German Law on Animal Protection (“Tierschutzgesetz”). Ethical approval for the study was granted by an ethical committee (according to §15 of the Tierschutzgesetz) and the governmental agency (Lower Saxony State Office for Consumer Protection and Food Safety; LAVES) responsible for approval of animal experiments in Lower Saxony (reference number for this project: 14/1659). All efforts were made to minimize both the suffering and the number of animals. A total of 254 mice were used for the present experiments. All animal experiments of this study are reported in accordance with ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Intrahippocampal kainate model in mice

In this model, a limbic status epilepticus (SE) is induced by unilateral injection of kainate into the CA1 sector of the dorsal hippocampus (Suzuki et al., 1995; Bouilleret et al., 1999). For this purpose, mice were anesthetized with chloral hydrate (500 mg/kg i.p.) and kainate monohydrate (0.21 μg in 50 nl saline), which was obtained from Sigma-Aldrich (Steinheim, Germany), was stereotaxically injected into the right CA1 area of the dorsal hippocampus as described previously (Twele et al., 2016a) and illustrated in Fig. S12. Stereotaxic coordinates were AP, −2.1; L, −1.6; and DV, −2.3 mm from bregma, using the mouse brain atlas of Paxinos and Franklin (2012). The correct location of the injection was repeatedly approved in the different batches of NMRI mice used for the present experiments, and coordinates were adapted if needed. Kainate was slowly injected over 60 s with a 0.5 μl microsyringe. After injection of kainate, the needle of the syringe was maintained in situ for additional 2 min to limit reflux along the injection track. For EEG recordings, the animals were immediately implanted with bipolar electrodes aimed at the site of kainate injection in the ipsilateral CA1, using the same coordinates as for kainate injection (see Twele et al., 2016a). A screw, placed above the left parietal cortex, served as the indifferent reference electrode. Additional skull screws, superglue (Pattex® Ultra Gel; Henkel, Düsseldorf, Germany), and dental acrylic cement (Paladur®; B. Braun Melsungen AG; Melsungen, Germany) anchored the entire headset. During all surgical procedures and for about 1 h thereafter, mice were kept on a warming pad to avoid hypothermia. Furthermore, mice received electrolyte/glucose solution (Stefrofundin® VG-5; B. Braun Melsungen AG; Melsungen, Germany) subcutaneously to compensate for loss of fluid and food during the day of surgery.

2.3. Drug treatment

We have recently shown that following intrahippocampal kainate injection in male NMRI mice, there is a latent period of 5–7 days before progressive development of spontaneous electrographic and clinical seizures (Twele et al., 2016a). Therefore, in a first experiment, mice were treated with TPM (30 mg/kg i.p.) and LEV (200 mg/kg i.p.) over 5 days, starting 6 h after kainate injection (Fig. 1). Six h was chosen to avoid that the drug treatment interfered with the kainate-induced SE development (Twele et al., 2016a; Schidlitzki et al., 2017).

The tolerability of the LEV + TPM combination after kainate-induced SE was proven by us in previous experiments (Klee et al., 2015). The doses used for each drug were chosen from previous studies in which respective drugs exerted disease-modifying effects in post-SE models of TLE when administered alone (Lösch and Brandt, 2010). The three times daily drug injection protocol chosen for the present experiments was based on the rapid elimination of TPM and LEV in...
rodents (Klee et al., 2015). During drug treatment, mice were weighed daily and observed for any obvious adverse effects.

Based on the promising data of the first experiment with the TPM and LEV combination (see Results), the experiment was repeated. Furthermore, we performed an experiment in which the doses of TPM and LEV were decreased by 50% to examine whether the anti-epileptogenic effect observed with the higher doses was also seen with the lower doses. In addition, experiments in which either LEV or TPM were administered alone were performed. These experiments were also repeated, so that overall 6 experiments were performed with the high doses of TPM and LEV and one experiment with the lower doses. In each drug experiment, a concurrent vehicle control group was used.

Finally, the experiment with the LEV+TPM combination was repeated again for multimodal imaging and gene-regulatory network analysis as shown in Fig. 1.

In an additional experiment, we examined whether the promising effect of the LEV/TPM combination extends to a combination of LEV and phenobarbital (PB). The experiment was performed exactly as described for TPM and LEV except that TPM was replaced by PB. Based on the pharmacokinetics of PB in mice, this drug was administered at a bolus of 25 mg/kg i.p., followed by 15 mg/kg i.p. t.i.d. We have shown previously that this LEV plus PB combination is well tolerated after kainate-induced SE (Klee et al., 2015). The experiment on the LEV plus PB combination was performed twice.

For each experiment, the aim was to have 16 mice for video/EEG recording (8 drug-treated and 8 vehicle-treated animals), which corresponded to the maximum capacity of our video/EEG setup (see below); in order to reach this aim, more (up to 24) mice were enrolled in each experiment to compensate for any losses during or after surgery. After SE, animals were randomly assigned to the treatment and vehicle groups. For subsequent video/EEG monitoring and analyses, all experiments were performed in a blinded fashion, so that it was not clear which group received drugs or vehicles.

### 2.4. Drug analysis in plasma

Mice eliminate most drugs much more rapidly than humans (Sakei et al., 2014). Thus, when comparing drug effects in rodents and humans, comparisons should be based on plasma drug levels rather than drug doses (Lösch, 2007). To evaluate whether the promising anti-epileptogenic efficacy of the LEV/TPM combination is obtained at clinically relevant plasma concentrations, additional groups of mice were treated once with the combination and blood was sampled by retroorbital puncture during short inhalation anesthesia with isoflurane at 0.5 h and 8 h following drug administration. Drug levels in plasma were determined by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method by an accredited laboratory (Labor Krone, Bad Salzuflen, Germany). Elimination half-life (t0.5) and area under the plasma concentration vs. time curves (AUC) were calculated using PK-Solutions (Summit Research Services, Montrose, CO, U.S.A.).

### 2.5. Video/EEG monitoring

As shown in Fig. 1, continuous (24 h/day) video/EEG monitoring for one week was performed after 4 and 12 weeks following kainate injection to record the different types of spontaneous electrographic and electroclinical seizures developing after a latent period following intrahippocampal kainate in mice (Twele et al., 2016a). For EEG-recording, mice were connected via a flexible cable to a system consisting of 4 one-channel bioamplifiers (ADInstruments Ltd., Sydney, Australia) and an analog-digital converter (PowerLab 8/30 ML870 or PowerLab 4/35 PL3504/P, ADInstruments). The data were recorded (sampling rate 200 Hz, time constant 0.1 s, low pass filter of > 60 Hz, 50 Hz notch filter) and analyzed with LabChart 8 for Windows software (ADInstruments). The EEG-recording was directly linked to simultaneous digital video-recording of four mice per system using four infrared board cameras (Sony) merged by one video quad processor (Monarcor TVSP-44COL). For video/EEG monitoring, mice were housed singly in clear plexiglass cages. For monitoring during the dark phase, infrared LEDs

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**Fig. 1.** Schematic illustration of the protocol used for the mouse experiments performed in this study. In all experiments, mice were randomly assigned to the drug and vehicle groups and experiments were performed in a blinded fashion. Each drug experiment was performed together with a vehicle experiment.
were mounted above the cages.

As described previously (Riban et al., 2002; Maroso et al., 2011; Twele et al., 2016b), the highly frequent electrophysiological seizures occurring in this model were differentiated into high-voltage sharp waves (HVSWs) and hippocampal paroxysmal discharges (HPDs). As recently arbitrarily defined by us for the mouse strain and model characteristics used in our studies (Twele et al., 2016b), HVSWs are characterized by sharp waves with high amplitudes of at least 3-times the EEG baseline, have a duration of at least 5 s, a frequency of at least 2 Hz, and an inter-event interval of at least 3 s. During the inter-event interval, there is either no epileptic EEG activity or isolated spikes or spike trains with an amplitude of < 3 times baseline occur, which was considered as interictal activity. HVSWs can either show no clear evolution or some evolution in frequency or pattern. HPDs always exhibit evolution in morphology and frequency and are often longer (> 20 s) than typical HVSWs. They typically start with large amplitude HVSWs, followed by a train of lower-amplitude spikes (≥ 2 times baseline) of at least 5 s of increased frequency (≥ 5 Hz). Similar to HVSWs, during inter-event intervals (at least 3 s) no epileptic EEG activity or isolated spikes or spike trains with an amplitude of < 2 times baseline are observed, which was considered as interictal activity. In addition to these typical HPDs, a second type was observed, which looked like a mixed event starting with HPD-like activity but then evolving into HVSW-like activity (see Twele et al., 2016b) and was assigned to HPDs when counting HVSWs and HPDs. During direct observation of epileptic mice or in the videos recorded during hippocampal HVSWs and HPDs, no clear behavioral alterations were seen, but subtle alterations may have been overlooked. For comparing the frequency of HVSWs and HPDs in vehicle and drug treated mice, they were counted visually in the EEG, using four 30-min periods (at 6 a.m. and 12, 6 and 11 p.m.) on day 1, 4 and 7 of the two one-week video-EEG recording periods shown in Fig. 1.

In addition to the highly frequent electrophysiological seizures, less frequent and generalized electroclinical seizures were observed in the EEG and videos. These clinical seizures, which were all associated with paroxysmal EEG activity in the hippocampal recordings, were rated by a modified Racine scale (Racine, 1972) as follows: stage 1, behavioral arrest and stereotyped sniffing; stage 2, head nodding and mouth or facial movements; stage 3, unilateral forelimb clonus; stage 4, rearing; stage 5, generalized tonic-clonic seizures with loss of righting reflexes. Stage 1–3 seizures were considered focal and stage 4 or 5 generalized convulsive seizures. For comparing the frequency of these electroclinical seizures in vehicle and drug-treated mice, they were counted visually in the video/EEG recordings over the periods of continuous recordings shown in Fig. 1. Furthermore, the duration of these seizures was determined.

During the 12-week duration of the experiments, several mice lost their electrode head assembly and had to be omitted from final EEG analyses. Furthermore, some mice died during severe generalized convulsive SRS.

2.6. Multimodal brain imaging

As shown in Fig. 1, one group (n = 8) of LEV + TPM treated mice (the 7th experiment) and respective vehicle controls (n = 7) were used for multimodal brain imaging by magnetic resonance imaging (MRI) and positron emission tomography (PET). The respective groups of mice were not implanted with EEG electrodes. The purpose of this experiment was to longitudinally determine the effect of drug treatment on blood-brain barrier (BBB) dysfunction developing after intrahippocampal kainate injection (Chen et al., 1999; Parathath et al., 2006; Zattoni et al., 2011; Zhang and Zhu, 2011) as well as on neuroinflammation as indicated by microglial activation (Zhang and Zhu, 2011; Brackhan et al., 2018) and neuronal degeneration (Janz et al., 2017) by MRI (day 2) and PET (day 7). Mice were scanned on a 7 T (300 MHz) small animal MR system (Bruker Pharmascan) 48 h after SE induction as previously reported (Breuer et al., 2017). A mouse brain receive-only coil array 11,765 V3 was used in combination with a quadrature MRI transmit-only coil with active decoupling T11070. The receive coil was placed on the animal bed in a defined position and mice were fixed with an incisor bar to allow comparable positioning between scan time points and animals. For imaging, animals were anesthetized with 1–2% isoflurane in humidified oxygen. T2-weighted 2D multi slice multi echo (MSME) and T1-weighted images were acquired using a 3D modified driven equilibrium Fourier transform method (MDEFT). T1 MDEFT sequences were acquired 10 min after end of contrast agent infusion with gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA [Magnevist®] 0.5 mmol/ml; Bayer HealthCare, Leverkusen, Germany) to identify BBB leakage, using a modified version of our previously rat-adjusted 20-min step-down infusion schedule (Breuer et al., 2017). T2-weighted MR imaging was repeated 37 days post-SE to determine neurodegeneration. PET imaging was performed 7 days after SE using a dedicated small animal PET/CT camera (Inveon PET/CT, Siemens Healthcare) following a previously described protocol (Brackhan et al., 2018) using the transplantor protein (TSPO) ligand [18F]GE180 (flutriacilamide) as novel 3rd generation in vivo marker for microglial activation (Boutin et al., 2015). In recent experiments, 7 days after SE proved to be a suitable time point for assessing neuroinflammation by PET in this model in mice (Brackhan et al., 2018). Briefly, [18F]GE180 was injected i.v. simultaneously with the start of a 60-min dynamic PET acquisition. List-mode data were histogrammed to 32 frames (5×2s, 4×5s, 3×10s, 8×30s, 5×60s, 4×300s, and 3×600s). Images were reconstructed using OSEM3D/fastMAP algorithm with standard corrections. In order to obtain anatomical information for image analysis, a routine fast computed tomography (CT) scan (Inveon CT, Siemens) was performed. MR and PET images were co-registered to an MRI template using PMOD software (PMOD Technologies, Zurich, Switzerland). A region of interest (ROI) template based on an atlas by Mirmione et al., 2006, was then applied to the co-registered images. T2 and T1 MRI regional values were normalized to cerebellum. [18F]GE180 uptake was calculated as percent injected dose per cubic centimeter of tissue (%ID/cc) in dorsal and ventral hippocampus, thalamus, amygdala, and cortex.

2.7. Histology and immunohistochemistry

Following the 2nd video/EEG recording period at 3 months post-SE, the mice were anesthetized and transcardially perfused with paraformaldehyde. Series of coronal brain sections (40 μm) were prepared for histology as described previously (Müller et al., 2009a,b). Naïve age-matched groups of mice were used as controls. Neurodegeneration in the hippocampus was determined in 6 sections (ranging from − 1.58 to − 2.30 mm from bregma) that were stained with thionin (for details see Polascheck et al., 2010). The left and right hippocampi were scanned in a meander form and neurodegeneration was rated by a score system as described previously (Gröticke et al., 2008). Scores were noted for each of the subregions of the hippocampal formation (CA1, CA2, CA3a, CA3c, and dentate hilus): score 0, no obvious damage; score 1, abnormal appearance of the structure without clear evidence of visible neuronal loss; score 2, lesions involving 20–50% of neurons; score 3, lesions involving > 50% of neurons. The extent of the granule cell dispersion (GCD) was visually assessed in the thionin-stained sections. Visual analysis was graded with a score system: score 0 = no GCD, score 1 = mild GCD, score 2 = moderate GCD, score 3 = severe GCD.

In the experiment, which was performed for multimodal brain imaging, immunohistochemical analyses, and gene expression analysis, mice were killed after the 2nd MR imaging at day 42 (i.e., 6 weeks) following kainate injection (cf., Fig. 1). In order to avoid interference with MR imaging, these animals were not implanted with EEG electrodes. Thus, we could not correlate the level of inflammatory markers (imaging, immunohistology, RNA seq) with seizure incidence/burden for individual mice. To allow performing immunohistochemical
analyses and gene-regulatory network analysis in the same brains, the brains were prepared as illustrated in Fig. S12. One part of the brain was used for immunohistochemical analyses of NeuN, glial fibrillary acidic protein (GFAP), and Mac-3 as described recently, using 2 μm brain sections (Walti et al., 2018). The other part of the brain was divided into ipsilateral and contralateral and used for gene-regulatory network analysis as described below. In addition to the kainate-injected groups that were either treated by vehicle or drugs, age-matched naïve mice were included for comparison. All histological and immunohistochemical analyses were performed in a blinded fashion.

In an additional experiment, we evaluated the extent of hippocampal neurodegeneration in the intrahippocampal kainate model before onset of drug treatment 6 h after kainate injection. For this purpose, groups of mice were anesthetized and perfused with paraformaldehyde 4 or 6 h after kainate injection. As described above, series of coronal brain sections (40 μm) were prepared for histology and 5–6 thionin-stained sections (at −1.56 to −2.18 from bregma) were semi-quantitatively scored for neurodegeneration. Naïve age-matched mice were used as controls. Additional sections (at −1.80 to −2.00 from bregma) were stained by Fluoro-Jade C (FJC), a sensitive and specific fluorescent marker of neuronal degeneration (Schmued et al., 2005), as described in detail previously (Grötticke et al., 2007). FJC-positive neurons were counted in 8 square fields of a defined size (90,000 μm²) that were posed subsequently in the respective region of each section at each section level to cover a large part of the region (resulting in different numbers of square fields per region): CA1, four fields; CA3a, two fields; CA3c, one field; dentate hilus, one field (for details see Polascheck et al., 2010).

2.8. Network analysis of protein targets of LEV, TPM and their combination

To explore known and predicted interactions between protein targets of LEV, TPM or their combination, the STITCH DataBase (http://stitch.embl.de/; version 5.0) for interaction networks of chemicals and proteins was used (Kuhn et al., 2008; Szklarczyk et al., 2016). STITCH (‘Search Tool for Interacting Chemicals’), in its fifth release, encompasses ~10 million proteins from 2031 eukaryotic and prokaryotic genomes. Its chemical space includes 430,000 compounds (not including different stereoisomers)(Szklarczyk et al., 2016). For the present analysis, the species was limited to mice (Mus musculus). The same analysis was performed for LEV and PB or their combination.

2.9. Gene expression analysis

Mice were killed 6 weeks after kainate injection and total RNA was extracted from the ipsilateral and contralateral hippocampus of mice (n = 6 per group) that were either treated with vehicle or LEV/TPM after SE as illustrated in Fig. 1. Sample preparation for RNA sequencing (RNA-seq) was performed according to the protocols recommended by the manufacturers (mRNA stranded, Illumina). Sequencing was done using Illumina HiSeq 4000 sequencer, with paired-end 75 bp nucleotide reads according to the protocol recommended by the vendor (30 million reads per sample). Raw reads were mapped to the reference mouse genome (mm10) using STARv2.1. To account for hidden covariate and batch effects we included the calculated 4 SVA factors (Leek et al., 2019) in the generalized linear model implemented in EdgeR. P-values were corrected for multiple testing using Benjamini-Hochberg False Discovery Rate (FDR). A cut-off of FDR ≤ 0.10 was applied to select differentially expressed genes. Functional enrichment analysis was performed using web server webgestalt (http://webgestalt.org) with significance set at FDR 5%.

2.10. Drugs

LEV (kindly provided by UCB Pharma, Brussels, Belgium) was dissolved in aqua ad injectabilia. TPM (kindly provided by Hexal, Holzkirchen, Germany) was dissolved in saline. PB (used as sodium salt) was purchased from Serva (Heidelberg, Germany) and dissolved in aqua ad injectabilia. All solutions were prepared freshly once a day. For injection of combinations of two substances, the injection volume was 5 ml/kg for each substance, for injection of one substance 10 ml/kg, respectively. Vehicle controls obtained drug vehicles with the same injection volume and number of injections as the drug treated groups.

2.11. Statistics

In all experiments, mice were randomly assigned to the drug and vehicle groups and experiments were performed in a blinded fashion. For the antiepileptogenesis studies, the sample size was restricted to 8 vehicle controls and 8 drug-treated mice, because we could perform video/EEG monitoring for only 16 mice in parallel. In this respect, it is important to note that we preferred individual vehicle control experiments together with each drug experiment vs. using historical controls to minimize the bias of batch-to-batch differences in animal responsiveness to the convulsant and seasonal effects on data (Lösch et al., 2017). Based on a sample size of 8 per group, the statistical power to determine a significant effect on seizure frequency was calculated as 0.81. As outlined above, all antiepileptogenesis experiments (except the experiment with the lower doses of LEV and TPM) were repeated once to assess the in-house reproducibility of the results. If results from the individual experiments did not differ significantly, they were combined for final analysis (see Results).

Depending on whether data were normally distributed or not, either parametric or nonparametric tests were used for statistical evaluation. For comparison of two groups and for intragroup comparison, either Student’s t-test or the Mann-Whitney U test was used. In case of more than two groups we used one- or two-way analysis of variance (ANOVA) with post hoc testing and correction for multiple comparisons. Depending on data distribution, either the ANOVA F-test, followed posthoc by Dunnett’s multiple comparison test, or the Kruskal-Wallis test followed posthoc by Dunn’s multiple comparisons test were used. Few outliers were detected and removed by Grubbs’s outlier test or the ROUT method (Motulsky and Brown, 2006). For comparison of frequencies in a 2 × 2 table, Barnard’s unconditional test (Barnard, 1947) was used, because this test preserves the significance level and generally is more powerful than Fisher’s exact test for moderate to small samples (Lydersen et al., 2009). Except Barnard’s unconditional test, all statistical analyses were performed with the Prism 8 software from GraphPad (La Jolla, CA, USA). All tests were used two-sided; a P ≤ .05 was considered significant.

3. Results

3.1. A combination of levetiracetam and topiramate causes significant disease modification in the intrahippocampal kainate mouse model of TLE

When LEV (200 mg/kg t.i.d.) and TPM (30 mg/kg t.i.d.) were administered during the latent period following a kainate-induced SE, as illustrated in Fig. 1, a significant effect on development of epilepsy was observed in two separate experiments. The two experiments did not differ from each other, so data were combined and illustrated in Fig. 2. As shown in Fig. 2A, based on periodic video-EEG monitoring at 1 and 3 months after kainate, combined treatment with LEV and TPM significantly reduced the frequency of electroclinical SRS by 80% (P = .0082; compared to vehicle controls), thus indicating that the rationally chosen drug combination exerted a disease-modifying efficacy. With respect to seizure incidence, all 14 vehicle controls developed SRS compared to 7/10 drug treated mice, which was significantly different (P ≤ .05), indicating that the drug combination exerted an anti-epileptogenic (epilepsy-preventing) effect in part of the animals. Including mice that could only be video-EEG recorded at 4–5 weeks post-SE (due to loss of EEG electrode assembly before starting the second
monitoring period at 12 weeks post-SE) in analysis of seizure incidence (cf., Fig. S1) resulted in 15/15 vehicle controls developing epilepsy compared to 9/12 drug-treated mice (P = .0466).

The temporal distribution and daily frequency of spontaneous electroclinical seizures recorded over the two weeks of continuous video-EEG monitoring at 4 and 12 weeks following SE is illustrated by heat maps in Fig. S1A. Considering ≥3 seizures per day as a high seizure frequency, 7/15 vehicle controls displayed such seizure frequencies compared to 0/12 drug treated mice (P = .0066).

In addition to incidence and frequency of electroclinical seizures, seizure load was significantly decreased by treatment, independently of whether seizure load was based on seizure duration (Fig. 2B) or seizure severity (Fig. 2C). However, in mice that developed SRS, the individual duration or severity of the seizures was not significantly reduced (Fig. S2).

When electroclinical seizures were classified according to severity by the Racine scale (Racine, 1972), mice treated with the drug combination exhibited significantly less generalized convulsive (stage IV and V) seizures than vehicle controls at 4 and 12 weeks after kainate (Fig. 3A, B), indicating that the treatment reduced progression from focal to generalized convulsive seizures. The frequency of focal (stage I-III) seizures was not significantly decreased (Fig. 3A, B).

In contrast to the significant group differences in incidence and frequency of electroclinical seizures, no such differences were observed for electrographic seizures (Fig. 2D). This suggests that the treatment inhibited the progression from electrographic to electroclinical seizures as observed during seizure evolution in this model (Heinrich et al., 2011; Twele et al., 2016a). As observed for electroclinical seizures, ~1/4 of the drug treated mice did not show any electrographic seizures (Fig. 2D), which was significantly different from controls (P ≤ .05). When electrographic seizures were further subdivided into high-voltage sharp waves (HVSWs) and hippocampal paroxysmal discharges (HPDs) as described previously (Riban et al., 2002; Twele et al., 2016b), no significant effects of treatment were observed (Fig. S3A–C).

Confirming our previous tolerability experiments with the LEV plus TPM combination (Klee et al., 2015), this drug combination was not associated with any obvious adverse effects when administered during the latent period after kainate. The good tolerability of the drug combination is also illustrated by body weight, which moderately (~15%) decreased in both drug and vehicle treated groups after SE, without any significant inter-group difference, and returned towards pre-SE values within about 10–11 days (Fig. S3D).
Fig. 3. Effects of treatment with a tolerable combination of levetiracetam (LEV) and topiramate (TPM) after status epilepticus (SE) vs. treatment with either drug alone on frequency of different types of electroclinical seizures. Each set of data (LEV + TPM; LEV alone; TPM alone) is from two independent experiments and data are shown as boxplots with whiskers from minimal to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Significant differences between groups are indicated by asterisk (*P < .05). Drug doses were 200 mg/kg LEV t.i.d. and 30 mg/kg TPM t.i.d. Seizure severity was rated according to Racine (1972) and, based on this rating, seizures were divided into focal (stage I-III) and secondarily generalized convulsive (stage IV and V) seizures. Seizure frequency is separately shown for 4 and 12 weeks following SE; frequency is shown as seizures per one week EEG-video recording. (A) Treatment with a combination of LEV and TPM reduces frequency of generalized convulsive seizures at 4 weeks post-SE. (B) Treatment with a combination of LEV and TPM reduces frequency of generalized convulsive seizures at 12 weeks post-SE. (C) Treatment with LEV alone does not decrease seizure frequency at 4 weeks post-SE. (D) Treatment with LEV alone does not decrease seizure frequency at 12 weeks post-SE. (E) Treatment with TPM alone does not decrease seizure frequency at 4 weeks post-SE. (F) Treatment with TPM alone does not decrease seizure frequency at 12 weeks post-SE.
3.2. The disease-modifying effect of levetiracetam and topiramate occurs at clinically relevant plasma concentrations and overall drug exposure

Although the doses of LEV (200 mg/kg t.i.d.) and TPM (30 mg/kg t.i.d.) used for the antiepileptogenesis experiments were quite high compared to clinically used doses, mice eliminate both drugs much more rapidly than humans, so high daily doses are needed to maintain effective drug levels (Klee et al., 2015). This is demonstrated by the pharmacokinetic data illustrated in Fig. 4, resulting from combined treatment with LEV (200 mg/kg) and TPM (30 mg/kg) in mice. When peak and trough levels of both drugs were determined in plasma, both drugs were eliminated with a half-life of 1.6 h, confirming previous pharmacokinetic experiments in mice (Klee et al., 2015). Therapeutically effective plasma concentrations (known from patients with epilepsy) were maintained for most of the 8 h dosing interval (Fig. 4A, B). Although peak plasma levels were above the therapeutic plasma concentration range determined in patients with epilepsy for both LEV and TPM (Patsalos et al., 2008), the area under the plasma concentration time curve (AUC) determined for TPM in the mouse experiments (Fig. 4C) was similar to the AUC determined in patients following treatment with a clinically used dose of TPM (200 mg orally; Doose et al., 1996). In contrast, the AUC for LEV was about 50% higher at the dose (200 mg/kg) used in mice compared to patients treated with a clinically used dose of LEV (1500 mg; Ramael et al., 2006), but maximum recommended daily dose of this drug is up to 3000 mg (Kaminski et al., 2009), so overall drug exposure (based on AUC) would be similar in mice and patients.

3.3. Treatment with either levetiracetam or topiramate alone does not cause significant disease modification

As with combined treatment, the experiments on treatment with either drug alone were repeated once (Fig. 1) and combined data from these experiments are illustrated in Figs. S4 and S5. In contrast to the significant effects of the drug combination, neither LEV nor TPM significantly altered the incidence or frequency of electroclinical seizures when they were administered alone. Similarly, incidence or frequency of electrographic seizures was not significantly altered by either treatment. Furthermore, in contrast to combined drug administration, neither drug alone reduced the progression from focal to generalized convulsive seizures (Fig. 3C–F).

3.4. Treatment with lower doses of levetiracetam and topiramate does not cause significant disease modification

Next, we examined whether lowering the doses of the TPM plus LEV

![Fig. 4. Pharmacokinetics of levetiracetam (LEV) and topiramate (TPM) after combined treatment of mice with 200 mg/kg LEV and 30 mg/kg TPM i.p. Plasma drug levels were determined 0.5 h and 8 h following drug administration in the morning in groups of 5 mice. Data in (A) and (B) are shown as boxplots with whiskers from minimal to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. (A) Plasma levels of LEV. The yellow area indicates the therapeutic plasma concentration range (10–40 μg/ml) known from patients with epilepsy (Patsalos et al., 2008). (B) Plasma levels of TPM. The yellow area indicates the therapeutic plasma concentration range (2–10 μg/ml) known from patients with epilepsy (Patsalos et al., 2008). The elimination half-life was estimated from the data in (A) and (B) and is indicated in the figs. (C) Area under the plasma concentration time curves (AUC) calculated from the data shown in (A) and (B). For comparison, data from clinical single-dose experiments with 1500 mg LEV p.o. (Ramael et al., 2006) and 200 mg TPM p.o. (Doose et al., 1996) are shown. Data are shown as means ± SEM. Significant differences to the mouse data are indicated by asterisks (** P < .01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
combination alters the antiepileptogenic or disease-modifying efficacy. For this purpose, doses were reduced by 50%, i.e., to 15 mg/kg TPM t.i.d. and 100 mg/kg LEV t.i.d. As shown in Fig. S6A, no significant effect of treatment was observed, so this experiment was not repeated. The lack of efficacy was not surprising, because reducing the doses by 50% will result in subeffective drug concentrations for several hours following each drug administration, particularly in case of TPM (see Fig. 4).

3.5. The disease-modifying effect of the levetiracetam plus topiramate high-dose combination does not extend to a combination of levetiracetam plus phenobarbital

In a mouse audiogenic seizure model, LEV was reported to enhance the anti-seizure effect of PB 16-fold, which was related to the known synergistic interaction between LEV and GABAergic compounds (Kaminski et al., 2009). This prompted us to investigate whether the beneficial effects of the LEV plus TPM combination extend to a combination of LEV plus PB. The experiment was repeated once and combined data from these experiments are illustrated in Fig. S6C,D. No significant effect on incidence or frequency of electocorticographic seizures was observed when mice were treated during the latent period with LEV (200 mg/kg t.i.d.) and PB (15 mg/kg t.i.d.). The temporal distribution and daily frequency of spontaneous electroclinical seizures recorded over the two weeks of continuous video-EEG monitoring at 4 and 12 weeks following SE is illustrated by heat maps in Fig. 5B. Considering ≥3 seizures per day as a high seizure frequency, 9/15 vehicle controls displayed such seizure frequency compared to 6/14 drug treated mice (P = .5246).

3.6. The levetiracetam plus topiramate high-dose combination does not prevent neurodegeneration

Treatment with TPM and LEV did not prevent the severe neuronal damage and characteristic GCD in the ipsilateral hippocampus following intrahippocampal injection of kainate (Fig. S7). Almost complete loss of neurons was observed in ipsilateral CA1, CA3c and dentate hilus of both vehicle and LEV/TPM treated mice (Fig. S7B-G). Furthermore, in both groups marked GCD was observed, which is characterized by dispersed granule cells that form a wider than normal granule cell layer (Suzuki et al., 2005). As shown in Fig. S7H, the severity of GCD in the ipsilateral hippocampus was not significantly affected by drug treatment. However, while all vehicle controls exhibited GCD, 3/11 drug-treated mice did not show GCD (Fig. S7D and H), which was significantly different from vehicle controls (P < .05). One of the three drug-treated mice without GCD did not develop SRS, whereas the other two developed SRS (and hippocampal neurodegeneration), arguing against any significant correlation between GCD and seizure development. No obvious neuronal loss or GCD were observed in the contralateral hippocampus in vehicle or drug-treated mice.

In addition to the brains analyzed 3 months after SE, we also performed immunohistological analyses of brains from the group that was perfused 42 days after kainate (see Fig. 1) to determine whether drug treatment induced transient neuroprotective effects as recently reported for a combination of AMPA and NMDA receptor antagonists in the same mouse model (Schidlitzki et al., 2017). Moreover, astroglial, as indicated by immunolabeling with glial fibrillary acidic protein (GFAP), and neuroinflammation as indicated by immunolabeling with Mac-3, which labels both activated microglia and blood-borne monocytes that infiltrated into the brain (Greter et al., 2015), were assessed. As in the mice perfused 3 months after kainate, marked neurodegeneration and GCD were observed in the ipsilateral hippocampus, which was not significantly reduced by treatment with LEV and TPM (Fig. S8). In addition to neuronal damage, marked astrogliosis (as indicated by GFAP labeling) was observed in the ipsilateral hippocampus, which was not reduced by drug treatment (Fig. S9). Only moderate Mac-3 labeling was observed 42 days after kainate, without significant difference between ipsilateral and contralateral brain regions, indicating that neuroinflammation had disappeared by 6 weeks following SE.

3.7. Intrahippocampal kainate causes neurodegeneration before onset of antiepileptogenic treatment

As shown in Fig. 1, treatment with the drug combinations started 6 h after intrahippocampal kainate injection. Based on the lack of any neuroprotective effect of drug treatments, we therefore thought that the excitotoxic kainate itself or the kainate-induced SE or both may have damaged hippocampal neurons over the 6 h before onset of drug treatment. When brain sections were stained by thionin at 4 or 6 h after intrahippocampal injection of kainate, neurons in the ipsilateral CA1, CA3, and dentate hilus showed pyknotic features, which was not observed in the contralateral hippocampus. Scoring of neuronal damage indicated significant neurodegeneration in CA1, CA3a, CA3c and dentate hilus, particularly 6 h after kainate (Fig. S10). GCD was not observed at 4 or 6 h after kainate. When dying neurons were labeled by Fluoro-Jade C (FJC), a significant number of FJC-positive neurons were observed in the ipsilateral CA1/CA2 sector (Fig. S10).

3.8. Multimodal brain imaging does not indicate neuroprotective or antiinflammatory effects of combined treatment with topiramate and levetiracetam

As shown in Fig. 1, in vivo multimodal brain imaging was performed during and after treatment with the LEV plus TPM combination to assess kainate-induced (i) BBB leakage, (ii) neuroinflammation, and (iii) neurodegeneration by magnetic resonance (μMR) and positron emission tomography (μPET) as recently described (Breuer et al., 2017; Brackhan et al., 2018). The contralateral brain hemisphere was used as control. The mice were not implanted with EEG electrodes to avoid any interference with MR imaging.

The timing of the different imaging modalities (see Fig. 1) was based on previous studies that showed that BBB leakage and neuronal damage occur early after SE (Chen et al., 1999; Parathat et al., 2006; Zatconi et al., 2011; Zhang and Zhu, 2011), while neuroinflammation as indicated by microglia activation and monocyte invasion occurs more delayed with maximal alterations being determined 5–7 days after SE (Brackhan et al., 2018). The late μMR imaging at 37 days after kainate was included to assess whether neurodegeneration further increased during progression of epilepsy.

In vehicle-treated kainate controls, infusion with the contrast agent gadolinium-diethyleneetriamine pentaacetic acid (Gd-DTPA) at 48 h after SE demonstrated ipsilateral BBB leakage (i.e. elevated T1 values) representing contrast agent extravasation, in the ipsilateral dorsal hippocampus (117%, P = .0014; Fig. 5A) and to a lesser extent in the ipsilateral thalamus (28%, P = .0200) and cortex (20%, P ≤ .05).

Increased MRI T2 signal intensity, representing edema, was ipsilaterally found at 48 h after SE in dorsal (13%, P = .0003; Fig. 5B) and ventral hippocampus (9%, P = .0021), as well as thalamus (2%, P = .0321) of vehicle-treated animals, while a decreased T2 signal, indicative of decreased water content, was observed in the amygdala (−10%, P = .0028).

On day 7 post-SE, PET scans with [18F]GE80 for visualization of translocator protein (TSPO) ligand expression as in vivo marker for microglial activation, SE revealed distinctly increased radiotracer uptake in the ipsilateral dorsal (52%, vehicle, P < .0001; Fig. 5C) and ventral (27%, P = .0006) hippocampus, thalamus (14%, P = .0133), and cortex (15%, P < .0001).

Atlas-based analysis of T2 MRI performed at 37 days post SE resulted in slightly reduced T2 signal, being indicative for neurodegeneration, in ipsilateral ventral hippocampus in vehicle-treated mice (−8%, P = .0027; Fig. 5D). To account for potential morphological alterations leading to discrepancy between atlas-defined ROI and actual
In this study, we investigated the effects of LEV and TPM on various aspects of brain function and structure in a mouse model of status epilepticus. We used multimodal brain imaging to assess blood-brain barrier impairment, neuroinflammation, and neurodegeneration. We observed no statistically significant differences between vehicle- and drug-treated animals for any imaging modality/parameter, suggesting a lack of LEV/TPM effects on kainate-SE-mediated BBB leakage, microglial activation, and neurodegeneration (Fig. 5A-D).

### 3.9. Protein-protein interaction network analysis

To explore potential mechanisms related to the antiepileptogenic effect of LEV/TPM, we performed a protein-protein interaction network analysis. Using the STITCH Database, we identified known and predicted drug-protein interactions for LEV and TPM alone and in combination. This analysis provided insights into the potential molecular targets and pathways affected by LEV/TPM, which could be further explored in future studies.
interactions for LEV and TPM alone are reported in Fig. 6A, B, respectively, and show that each drug individually interacts with different proteins including sodium channels, potassium channels, the kainate and AMPA subtypes of glutamate receptors, GABAA receptors, carbonic anhydrase subtypes, Trak1 (traffi cking kinesin-binding protein 1), and SV2A, all of which have been related to functional alterations during epileptogenesis (see Discussion). In combination, however, LEV and TPM interact in a larger drug-protein network (Fig. 6C) and therefore a consequence of combined treatment with LEV and TPM could be to impact a larger functional network of epilepsy-relevant proteins, providing a potential network pharmacology explanation for the observed antiepileptogenic and disease-modifying effect observed with this combination.

3.10. Gene expression analysis

To further explore potential mechanisms related to the anti-epileptogenic effect of LEV/TPM, changes in gene expression associated with LEV/TPM drug therapy after termination of treatment were determined. For this purpose, groups of mice (n = 6) treated with vehicle or LEV/TPM after SE were perfused 6 weeks after kainate injection (i.e., ~5 weeks after termination of drug treatment; see Fig. 1) and genome-wide gene expression data were generated from individual ipsilateral and contralateral whole hippocampus samples using high throughput RNA-sequencing (RNA-seq) (Methods). Differential gene expression was calculated using EdgeR (Robinson et al., 2010) following adjustment for hidden batch effects using Surrogate Variable Analysis (SVA; Leek, 2014). For the comparison of vehicle-treated versus LEV/TPM

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**Fig. 6.** Known and predicted protein interactions of levetiracetam (A) or topiramate (B) and their combination (C) in the mouse (*Mus musculus*), calculated by the STITCH Database. Drug-protein and protein-protein networks are shown by the confidence view of the database, in which stronger associations are represented by thicker lines. Drug-protein interactions are shown in green, protein-protein interactions in grey, and interactions between drugs in red. Predicted functional partners: Adipoq, adiponectin; Casp3, caspase 3; Cth, cystatin B; Car1, 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, subtypes of carbonic anhydrase; Fas, TNF receptor superfamily member 6; Gabra 1, 2, 3, 5, GABA<sub>A</sub> receptor, subunit alpha 1, 2, 3 and 5; Glul, glutamate-ammonia ligase (glutamine synthetase); Gria1, 2, 3, 4, glutamate AMPA receptor subtype 1, 2, 3, and 4; Grik1, 2, 3, 5, glutamate kainate receptor subtype 1, 2, 3, and 5; Fas, TNF receptor superfamily member 6; Kcnaj, 2, 3, 4, 5, voltage-gated potassium channels, shaker-related subfamily, members 1, 2, 3, 4, and 5; Kcnj10, inwardly rectifying potassium channel; Kcnq2, voltage-gated potassium channel, subfamily Q, member 2; Lep, leptin; Ptpg, protein tyrosine phosphatase, receptor type G; Scn1a, 1b, 2a1, 2b, 3a, 3b, 4a, 4b, 5a, 7a, 8a, 9a, 10a, 11a, subtypes of voltage-gated sodium channels; Scnn1a, 1b, 1g, nonvoltage-gated sodium channels; Sv2a, synaptic vesicle glycoprotein 2a; Trak1, trafficking kinesin-binding protein 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
treated mice, 21 genes were significantly (FDR < 10%) differentially expressed in hippocampus samples ipsilateral to the kainate injection (Fig. 7; Table S1). Although several (12 out of 19 known genes) have been individually linked to epilepsy and/or epileptogenesis in previous publications (cf. Fig. 7 and Table S1), functional enrichment analysis revealed no significant (FDR < 10%) pathway enrichments among the set of genes differentially expressed, suggesting classical GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways fail to capture the relevant functional processes related to the disease-modifying effect of LEV/TPM.

In addition to investigating differential expression in the hippocampus ipsilateral to the kainate injection, we also evaluated differential gene expression in the contralateral hippocampus for vehicle-treated and drug-treated (LEV/TPM) mice (Fig. 7, Table S1). In contrast to the ipsilateral hippocampus results described above, only a single gene (Gh; growth hormone) was significantly differentially expressed (downregulated), consistent with the intrahippocampal kainate model of epilepsy representing a unilateral focal epilepsy (Guillemin et al., 2012).

4. Discussion

In recent years, multitargeted or combinatorial therapies (“network pharmacology approaches”) have attained substantial therapeutic impact because such therapies modulate the activities of targets in complex diseases such as cancer, diabetes mellitus, hypertension, congestive heart failure, asthma, chronic obstructive pulmonary disease, and HIV-1 infection; similarly, such therapies are interesting for rare neurological diseases with complex etiologies unlikely to respond to single, target-specific treatments (Ainsworth, 2011; Boezio et al., 2017; Muhammad et al., 2018). Epilepsy is a complex distributed brain network phenomenon that currently cannot be prevented or cured (Klein et al., 2018). Epileptic networks are often conceptualized in terms of interacting brain regions, but there are multiple levels of interacting components during epileptogenesis, such as genes, transcription factors, proteins, neurons, different pathologic processes and others, which all function within networks, although at different levels of resolution (Scott et al., 2018). Targeting only one process within this network is less likely to halt the epileptogenic process than multitargeted approaches (Lösch et al., 2013).

There are at least two principal strategies that may be used to identify an efficient network approach for prevention or modification of epilepsy. One is the top-down approach used in the present study, in which rationally chosen combinations of drugs that are likely to affect different targets within an epileptogenic network are tested in animal models. For this strategy, repurposing (or repositioning) of generic drugs can be used to facilitate translation of promising drug combinations into clinical trials, because such drugs have been clinically used for years and have well known safety profiles (Ainsworth, 2011). The second strategy is a bottom-up approach that starts by identifying crucial genomic, transcriptomic or proteomic alterations in the network and then searches for drug targets that selectively affect these network changes (Swiney and Anthony, 2011; Eder and Herrling, 2016). The latter approach allows to develop novel, highly effective treatments, but the process of target validation is complex, long-lasting and associated with high attrition rates (Sams-Dodd, 2005; Swiney and Anthony, 2011).

Since we proposed network pharmacology as a novel approach for epilepsy prevention or modification in 2013 (Lösch et al., 2013), we have evaluated various rationally chosen combinations of repurposed drugs for tolerability and efficacy in the intrahippocampal kainate mouse model (Klee et al., 2015; Schidlitzki et al., 2017; Welzel et al., 2019), and the most effective combination is presented here. As described in the Introduction, the idea of evaluating a combination of LEV and TPM was primarily based on (i) promising disease-modifying efficacy of both compounds administered alone in rat models of acquired
epilepsy (Lösch and Brandt, 2010; Lösch et al., 2016), (ii) synergistic interaction of LEV and TPM in seizure models (Kaminski et al., 2009), and (iii) mechanisms of action that are relevant for targeting epileptogenesis. TPM acts at postsynaptic sites to inhibit glutamate-mediated excitatory neurotransmission, modulates a subset of GABA_A receptors, and blocks voltage-gated sodium currents (Rogawski et al., 2016), while LEV is thought to act mainly presynaptically by modulating synaptic vesicle protein SV2A and thus transmitter release (Lösch et al., 2016). However, several other cellular and molecular effects of TPM and LEV, which may be relevant for modulating epileptogenesis, have been reported in therapeutically relevant concentrations (Rogawski et al., 2016). Thus, as shown by in silico protein network analysis using the STITCH database (Szklarczyk et al., 2016), both LEV and TPM are not highly selective for a single target but interact, in a complementary fashion, with various receptors and ion channels that are thought to be relevant for epileptogenesis (Pitkänen et al., 2015; Mazarati and Sankar, 2016; Klein et al., 2018), including the kainate and AMPA subtypes of glutamate receptors, the GABA_A receptor, different types of sodium and potassium channels, subtypes of carbonic anhydrase, Trak1, and SV2A. As shown by the STITCH analysis, treatment with LEV and TPM combines these various targets, thus resulting in a network pharmacology approach that is likely to explain the promising disease-modifying effect of the combination.

Previous studies, in which LEV or TPM were administered alone during the latent phase following SE, indicated that both drugs may exert disease-modifying efficacy, although data were equivocal (cf., Lösch and Brandt, 2010; Klein and Tyrlíkova, 2017). In the present study, treatment with either drug alone, starting 6 h after SE, did not significantly modify epileptogenesis in the intrahippocampal kainate mouse model. Indeed, several previous studies indicated that epilepsy is difficult to prevent or modify in this model, including studies with glutamate receptor antagonists (Twele et al., 2015; Schidlitzki et al., 2017), the mTOR antagonist rapamycin (Shima et al., 2015), genetically engineered cells (All et al., 2017), and genetic manipulation of urokinase-type plasminogen activator receptor (Ndode-Ekane and Pitkänen, 2013) or BDNF-mediated TrkB signaling (Heinrich et al., 2011). This may be related to the double-hit insult produced in this model by the traumatic insult caused by surgical implantation of the EEG electrode into the hippocampus and the intrahippocampal injection of the excitotoxic kainate (Brackhan et al., 2018). This double-hit insult produces marked BBB disruption, neuroinflammation, and neuregeneration in the ipsilateral hippocampus and associated areas as reported previously (Riban et al., 2002; Pernot et al., 2011; Zattoni et al., 2011; Bitsika et al., 2016; Brackhan et al., 2018) and substantiated by the present data from multimodal brain imaging. Combined treatment with LEV and TPM during the latent period of the intrahippocampal kainate model significantly reduced the incidence and frequency of electroclinical SRS developing in the weeks after termination of treatment, indicating that this drug combination exerts an antiepileptogenic and disease-modifying effect, most likely by affecting a large network of protein targets as suggested by the STITCH analysis. Interestingly, the effects of the LEV plus TPM combination on development of SRS occurred in the absence of significant reduction of BBB disruption, neuroinflammation, or neuregeneration.

It has previously been shown that plasma and brain concentrations of either drug are not affected by combined treatment, excluding pharmacokinetic drug-drug interactions (Kaminski et al., 2009). In the present study, analysis of plasma levels of LEV and TPM indicated that the disease-modifying effect of the drug combination occurred at clinically relevant plasma concentrations and overall drug exposure derived from AUC calculation, which is often used for rodent vs. human comparisons and prediction of clinically effective drug levels or exposure (Klein et al., 2012b; Blanchard and Smoliga, 2015). The disease-modifying effect of the drug combination disappeared when doses of LEV and TPM were reduced by 50%.

Previous pharmacodynamic drug-drug interaction experiments in seizure models have shown that LEV does not only potentiate the anti-seizure effect of anti-glutamatergic drugs such as TPM but also the effect of GABA-potentiating drugs such as PB (Kaminski et al., 2009). This prompted us to evaluate whether the promising disease-modifying effect of the LEV/TPM combination is also observed with a combination of LEV and PB. No significant effects were observed with the latter drug combination, substantiating that the LEV/TPM combination stands out in this respect. This is also supported by a comparison of the protein networks affected by the LEV plus TPM combination vs. the LEV plus PB combination, using the STITCH database, which demonstrates that the network effect of the LEV plus PB combination is much more restricted in terms of targets thought to be critically involved in anti-epileptogenesis. Furthermore, in contrast to LEV plus TPM, no direct protein network interactions were observed for the LEV plus PB combination. However, the in silico STITCH analyses do not allow to determine in what way protein targets are affected by drug combinations. For this purpose, proteome analyses for determining protein expression and evaluation of biochemical pathways highlighted in the STITCH analysis will be needed. The lack of a significant disease-modifying effect of the LEV plus PB combination seems to indicate that in contrast to the synergistic potentiation of the anti-seizure effect of LEV by PB and other GABAergic drugs demonstrated in seizure models (Kaminski et al., 2009), combining LEV with a GABAergic drug does not potentiate its disease-modifying effect. The LEV/PB control was also important to exclude that the antiepileptogenic effect of the LEV/TPM treatment was, at least in part, due to attenuating or shortening of the kainate-induced SE by an anticonvulsant effect.

To further investigate potential mechanisms of action for the effect of LEV/TPM combination on epileptogenesis, we undertook gene expression analysis of LEV/TPM-treated vs. vehicle-treated post-SE mice at 5 weeks following termination of treatment. Genome-wide gene expression analysis of the ipsilateral hippocampus in treated and control mice revealed 19 known genes significantly differentially expressed. Twelve of these 19 genes have previously been implicated in epileptogenesis or epilepsy (Table S1) and could have potential disease-modifying effects in epilepsy including Nuclear receptor subfamily 4 group A member 1 (Nr4a1), a downstream target of CREB, thought to be a key regulator of epileptogenesis (Zhang et al., 2016), and somatostatin receptor 4 (Sstr4), a previously identified candidate for inhibition of epileptogenesis (Vezzani and Hoyer, 1999). These data thus suggest that drug-induced cellular differential expression plays a role in mediating the antiepileptogenic effects of this drug combination. Interestingly, in the contralateral hippocampus, only one gene (Gh) was significantly differentially expressed in LEV/TPM-treated mice.

In previous studies in which gene expression analysis was used to investigate the potential mechanisms underlying the disease-modifying effect of LEV in the amygdala kindling model, which we initially described in 1998 (Lösch et al., 1998), it was found that LEV prevents kindling-induced changes in several epilepsy-related genes, including brain-derived neurotrophic factor (BDNF), neuropeptide Y (NPY), thyrotropin-releasing hormone (TRH), and GFAP in the hippocampus of rats (Gu et al., 2004; Husum et al., 2004). To our knowledge, no such studies on gene or protein expression in TLE models are available for TPM or the combination of LEV and TPM.

An unexpected finding of the present study was the lack of any significant neuroprotective effect of the LEV plus TPM combination. Both drugs have been reported to exert neuroprotective effects in different brain injury models (Willmore, 2005; Shank and Maryanoff, 2008; Lösch and Brandt, 2010; Shetty, 2013). However, for any neuroprotective effect the timing of the treatment after brain injury is important. As shown here, the excitotoxic kainate exposure or the SE or both induced significant neuregeneration in the hippocampus before drug treatment started at 6 h following kainate. A similar observation of rapidly occurring neuronal damage in the hippocampus has recently been reported for a model in which kainate was injected into the cortex just above the hippocampus to avoid lesioning the hippocampus by the
injection cannula (Bedner et al., 2015). Furthermore, TUNEL positivity, indicating neuronal apoptosis, was observed in the ipsilateral hippocampus as early as 2 hours following intrahippocampal injection of kainate in mice (Pernot et al., 2011). The rapid onset of the kainate and/or SE indicating neuronal apoptosis, was observed in the ipsilateral hippocampus (Bedner et al., 2015). Furthermore, TUNEL positivity, could result in initial insult modification rather than an anti-epileptogenic or disease-modifying effect (Lösch and Brandt, 2010; Galanopoulou et al., 2012).

The present findings support previous studies (e.g., Brandt et al., 2003; Brandt et al., 2004) that neuroprotection may not be necessary for prevention or reduction of SRS in models of acquired epilepsy, but presumably the damage caused by the insult would still have lasting effects. Given the persistence of hippocampal damage in the present experiments, it will be important to study whether the promising LEV plus TPM drug combination can impact cognitive dysfunction that occurs in the intrahippocampal kainate mouse model (Grötteke et al., 2008).

The duration of treatment with the drug combinations evaluated here was restricted to 5 days, because the latent period in this model is 5–7 days (Twele et al., 2016a). Thus, treatment for 5 days should be sufficient to interfere with epileptogenesis. It has been demonstrated recently that a 5-day treatment with a combination of NMDA and AMPA glutamate receptor antagonists significantly retarded epilepto-genesis and GCD in the intrahippocampal kainate model (Schedlitzki et al., 2017). A short window of opportunity following brain injury was also demonstrated by Lippman-Bell et al. (2013), who reported that twice daily treatment of neonatal rats with the AMPA receptor antagonist NBQX over only two days following hypoxia-induced neonatal seizures in rats prevented subsequent development of epilepsy. Indeed, more recent experimental and clinical evidence suggests that the latent period and, thus, the window of therapeutic opportunity to interfere with epileptogenesis, is much shorter than previously thought; this may necessitate to start antiepileptogenic treatment shortly (within the first hours) after brain insult in a similar way as treatment of acute stroke (Lösch et al., 2015).

In conclusion, by combining two of the most effective anti-seizure drugs, LEV and TPM (Darhan et al., 2015), we have discovered a significant antiepileptogenic and disease-modifying effect in a mouse model of acquired TLE, whereas either drug alone was not effective. As demonstrated by the in silico analysis of protein network effects of the two drugs, LEV and TPM affect various epilepsy-related targets, thus providing a network pharmacology approach for epilepsy prevention or modification following brain injury. Genome-wide gene expression analysis of the ipsilateral hippocampus performed after termination of LEV/TPM treatment showed differential expression of several genes with potential disease-modifying activity, providing interesting entry points for future mechanistic studies. As a next step, it will be important to study this promising drug combination in other mouse and rat models of acquired epilepsy, including models of TBI-induced epilepsy. If the efficacy observed here translates to other models, which we expect, the LEV plus TPM combination may be a promising strategy for epilepsy prevention in patients at risk, which is a clinical problem of enormous need (Lösch et al., 2013).

Declaration of Competing Interest

The authors declare that they have no competing financial interests.

Acknowledgements

We appreciate the valuable comments of Drs. Pavel Klein and Detlev Boi inson during final revision of the manuscript. The authors thank E. Kaczmarek, K. Römermann, C. Brandt, M. Weilking, M. Johne, C. Hecker, K. Weber-Wilk, I. Leiter, A. Kanwischer, S. Eilert, P. Felsch, C. Bergen, and J. Pfeiffer for skillful technical assistance. We thank GE Healthcare for providing the general method and access to the precursor for $[^{18}F]GE180$ synthesis. This study was funded by the European Union’s Seventh’s Framework Programme (FP7/2007-2013) under grant agreement n°602102 (EPITARGET).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.104664.

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