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Network pharmacology for antiepileptogenesis: Tolerability and neuroprotective effects of novel multitargeted combination treatments in nonepileptic vs. post-status epilepticus mice

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ABSTRACT

Network-based approaches in drug discovery comprise both development of novel drugs interacting with multiple targets and repositioning of drugs with known targets to form novel drug combinations that interact with cellular or molecular networks whose function is disturbed in a disease. Epilepsy is a complex network phenomenon that, as yet, cannot be prevented or cured. We recently proposed multitargeted, network-based approaches to prevent epileptogenesis by combinations of clinically available drugs chosen to impact diverse epileptogenic processes. In order to test this strategy preclinically, we developed a multiphase sequential study design for evaluating such drug combinations in rodents, derived from human clinical drug development phases. Because pharmacokinetics of such drugs are known, only the tolerability of novel drug combinations needs to be evaluated in Phase I in öhealthy" controls. In Phase IIa, tolerability is assessed following an epileptogenic brain insult, followed by antiepileptogenic efficacy testing in Phase IIb. Here, we report Phase I and Phase IIa evaluation of 7 new drug combinations in mice, using 10 drugs (levetiracetam, topiramate, gabapentin, deferoxamine, fingolimod, ceftriaxone, α-tocopherol, melatonin, celecoxib, atorvastatin) with diverse mechanisms thought to be important in epileptogenesis. Six of the 7 drug combinations were well tolerated in mice during prolonged treatment at the selected doses in both controls and during the latent phase following status epilepticus induced by intrahippocampal kainate. However, none of the combinations prevented hippocampal damage in response to kainate, most likely because treatment started only 16–18 h after kainate. This suggests that antiepileptogenic or disease-modifying treatment may need to start earlier after the brain insult. The present data provide a rich collection of tolerable, network-based combinatorial therapies as a basis for antiepileptogenic or disease-modifying efficacy testing.

1. Introduction

Recently, the old simplistic notion that epileptic seizures must be either "focal/partial" or "generalized" in nature has been supplanted by concepts of epileptic networks, suggesting that the development and clinical manifestations of epilepsy are the consequence of pathologies of brain network dynamics and functional connectivity that may involve abnormal network pathways (Kramer and Cash, 2012; Holmes and Tucker, 2013; Khambhati et al., 2015; Smith and Schevon, 2016; Klein et al., 2018; Scott et al., 2018). The epileptic network is characterized by pathologic, seizure-generating 'foci' embedded in a web of structural and functional connections, resulting in a complex relationship between foci and the surrounding network that drives seizure dynamics (Holmes and Tucker, 2013). Based on this concept, which also applies to other brain diseases, developing new therapies that act on individual drug targets may be less effective than multitargeted drugs or drug combinations, i.e., "network pharmacology" (Hopkins, 2008; Ainsworth, 2011; Löscher et al., 2013; Boezio et al., 2017).

We recently proposed network pharmacology as a novel strategy for antiepileptogenesis, i.e., drug administration during the latent period

Abbreviations: DMSO, dimethyl sulfoxide; GCD, granule cell dispersion; HPβCD, hydroxypropyl-β-cyclodextrin; PEG, polyethylene glycol; SE, status epilepticus; TBI, traumatic brain injury; TLE, temporal lobe epilepsy

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following an epileptogenic brain insult with the aim to prevent or modify development of epilepsy (Löscher et al., 2013; White and Löscher, 2014). Rather than creating new multitargeted drugs, we suggested to repurpose clinically established drugs to form rationally chosen drug cocktails for antiepileptogenesis. In a first series of experiments, we chose 8 clinically approved drugs that target major mechanisms of epileptogenesis, i.e., neuroinflammation, neurodegeneration and development of abnormal neuronal hyperexcitability (Klee et al., 2015). Because preclinical trials on disease-modifying or antiepileptogenic drug effects are extremely complex and cost- and time-expensive (Löscher and Brandt, 2010), we decided to organize our study of the antiepileptogenic potential of rationally chosen drug combinations along the lines of human clinical drug development, starting with Phase I (pharmacokinetics, tolerability, safety) and the safety or tolerability part of Phase II (Klee et al., 2015). We used data from the literature for preclinical pharmacokinetics of individual drugs. To de-risk the experiments, only small groups of mice were used for the tolerability experiments, starting in nonepileptic mice (Phase I). Drug combinations that were tolerated were then tested in epileptic mice (Phase IIa) and in mice during the latent period preceding epilpsy (Phase IIb), which was induced by status epilepticus (SE)(Klee et al., 2015). Tolerability experiments in mice after brain injury (SE) were done because kindled or epileptic animals often exhibit increased adverse effects in response to drug administration, which may result in treatment discontinuation or even mortalities (Löscher, 2016). Only drug combinations that were tolerated in all three phases of the tolerability experiments were then chosen for preclinical antiepileptogenesis trials in large groups of mice. A first preclinical antiepileptogenesis trial with such a drug combination was recently completed, indicating that this preclinical study design (or "algorithm") may result in the discovery of new, effective drug cocktails (Schidlitzki et al., 2018).

In the present study, 10 clinically established drugs with diverse mechanisms (Fig. 1), recently proposed for antiepileptogenic drug combinations from a clinical perspective (Klein and Tyrlikova, 2017), were chosen for combination and evaluated for tolerability, using a slightly modified study design for drug testing (Fig. 2). Based on our previous results, which showed no difference in tolerability in epileptic mice vs. mice tested during the latent period after SE (Klee et al., 2015), we omitted tolerability testing in mice with chronic epilepsy and used only mice during the latent period for Phase IIa. Furthermore, we included testing for early signals of antiepileptogenic or disease-modifying efficacy by evaluating whether the drug cocktails reduced or prevented hippocampal neurodegeneration after SE (Fig. 2). In clinical trials, Phase I studies include determination of pharmacokinetics. This was not necessary for the drugs tested here, because all the drugs are clinically approved, with pharmacokinetic data for both rodents and humans available in the literature (see Results). As in our previous study (Klee et al., 2015), the duration of drug administration was restricted to 3 days (see Discussion). We chose the intrahippocampal kainate SE model in mice, because this model exhibits several features of temporal lobe epilepsy (TLE), the most common type of acquired epilepsy in humans, and has the added advantage of low mortality and high frequency of spontaneous seizures (Guillemain et al., 2012; Löscher, 2017). Overall, 7 drug combinations with 2–4 drugs from different mechanistic categories were evaluated.

2. Materials and methods

2.1. Animals

Male NMRI mice were obtained from Charles River (Sulzfeld, Germany) at the age of 6–8 weeks. In total, 245 mice were used (86 animals for preliminary experiments and pharmacokinetic analyses, 115 nonepileptic animals and 44 kainate-treated animals). All male mice were housed singly to avoid fighting. All animals were housed under controlled conditions (ambient temperature 22–24 °C, humidity

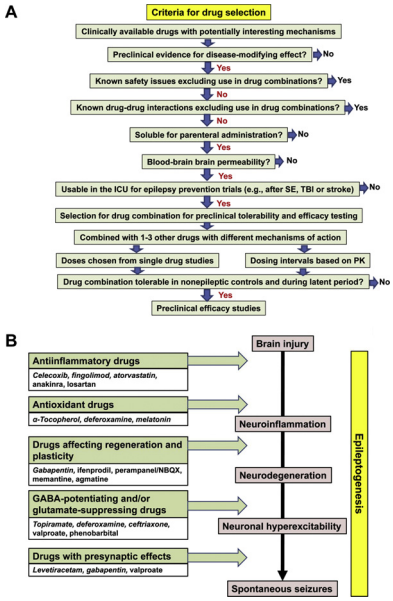


Fig. 1. Criteria for selection of drugs used in the present study. A. Flow chart illustrating the selection process that led to the drugs included in the present study. B. Drug mechanisms that we chose for the present approach and how they may interfere with epileptogenesis after brain injury. Nineteen drugs that were selected as shown in A and that act by mechanisms known to interfere with epileptogenesis are indicated. Ten of these drugs (shown in italics) were used for the 7 drug combinations that were evaluated in the present experiments, while combinations of the other drugs (except agmatine) were examined in previous experiments (Klee et al., 2015; Schidlitzki et al., 2017). Except NBQX, which was used as a surrogate for perampanel (Schidlitzki et al., 2017), all drugs shown are clinically approved for diverse indications and were repurposed for antiepileptogenesis. Mechanisms of epileptogenesis are certainly much more complex than illustrated here, but neuroinflammation, neurodegeration, and neuronal hyperexcitability are considered hallmarks of the epileptogenic process (Vezzani et al., 2013; Löscher et al., 2015). For drug mechanisms, see reviews by Vezzani et al. (2013), Friedman et al. (2014), Rogawski et al. (2016), and Klein and Tyrlikova (2017).

30–50%, lights on from 6:00 am to 6:00 pm) and adapted to the laboratories for at least one week before being used in the experiments. Food (Altromin 1324 standard diet) 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 government agency (Lower Saxony State Office for Consumer Protection and Food Safety; LAVES) responsible for approval of animal experiments in Lower Saxony. The Animal License allowed the use of chloral hydrate as anesthetic (see below). All efforts were made to minimize both the suffering and the number of animals.

2.2. Choice of drugs, drug doses, drug vehicles, and parenteral route of administration

As illustrated in Fig. 1A, drug combinations were chosen with the rationale to combine compounds that, based on their mechanism(s) of action, may result in "network pharmacology" when combined together as recently described (Löscher et al., 2013). There are hundreds of clinically approved drugs with mechanisms that may interfere with epileptogenesis; however, as shown in Fig. 1A, drugs chosen for our studies had to fulfill several characteristics, which limited the number of suitable drugs. The doses used for each drug were chosen based on previous studies in which the drug exerted disease-modifying effects in post-SE models of TLE or other models of brain injury when administered alone (see Table 1). Since we wanted to administer all drugs

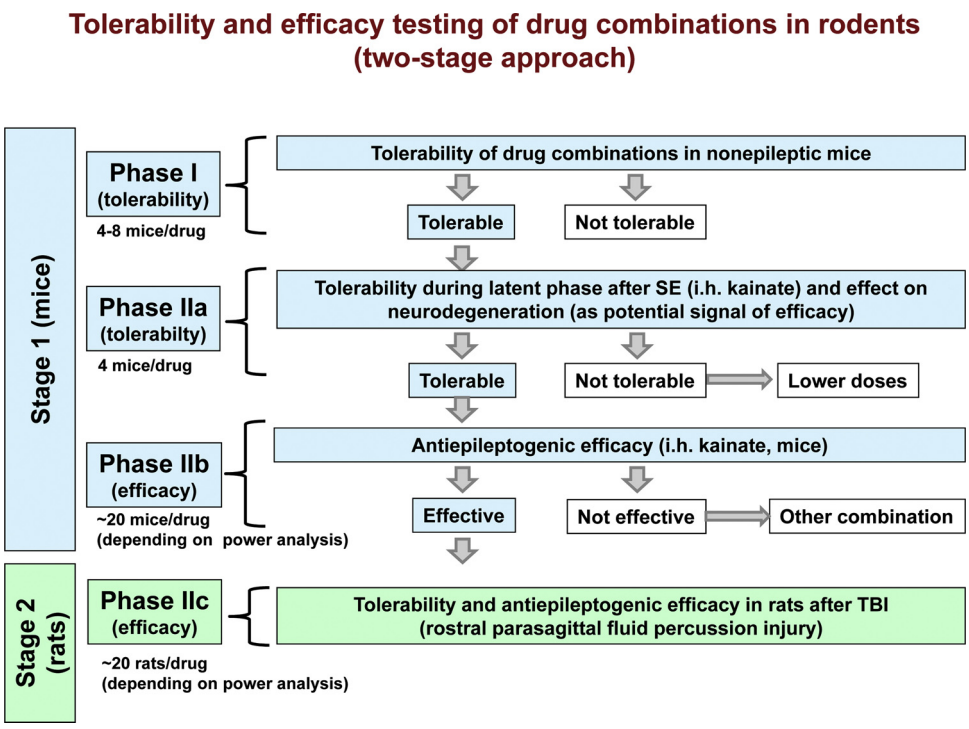


Fig. 2. Study design ("algorithm") for testing drug combinations in a 2-stage approach in mice and rats. Abbreviations: i.h., intrahippocampal; SE, status epilepticus; TBI, traumatic brain injury. Modified from Klee et al. (2015).

parenterally (i.p. or s.c.), we first had to find suitable vehicles allowing to dissolve (or emulsify) drugs in an adequate injection volume (Table 2). For hydrophilic drugs, sterilized water or saline were used for solutions (Tables 1 and 2). However, several hydrophobic drugs were difficult to dissolve in tolerable vehicles, so various solvents had to be evaluated (Table 2), including Solutol® HS 15 (a non-ionic surfactant that is 70% lipophilic, consisting of polyglycol mono- and diesters of 12-hydroxystearic acid, and 30% hydrophilic, consisting of polyethylene glycol), Miglyol® 812 (medium-chain caprylic and capric triglycerides), sulfobutylether-β-cyclodextrin (Captisol®), hydroxypropyl-β-cyclodextrin (HPβCD) and polyethylene glycol (PEG) 400 (cf., Strickley, 2004). Because of its numerous pharmacological effects (e.g., Jacob and Herschler, 1986; Castro et al., 1995; Colucci et al., 2008; Jacob and de la Torre, 2009; Turner et al., 2011; Hall et al., 2014), we avoided the use of dimethylsulfoxide (DMSO) as a solvent for hydrophobic drugs if possible. When we had to use it, its maximum concentration was limited to 4–5% as recommended by most Institutional Animal Care and Use Committees (IACUCs). Similarly,

Table 1
Drug combinations, vehicles, routes of administration, and doses finally used for drug testing. Based on pharmacokinetics (see Table 3), all drugs were administered twice daily at the indicated doses, except for fingolimod, which was administered once daily. Selection of doses was based on the literature shown.

	Drug cocktail	Respective vehicles	References for selection of dosages
A	(1) Levetiracetam (200 mg/kg i.p.) + (2) Gabapentin (200 mg/kg i.p.), (3) Topiramate (30 mg/kg i.p.)	(1) Aqua ad injectabilia i.p.* (2) Aqua ad injectabilia i.p.* (3) 0.9% NaCl i.p.	(1) Klee et al. (2015) (2) Cilio et al. (2001) (3) Klee et al. (2015)
B	(1) Levetiracetam (200 mg/kg i.p.), (2) α-Tocopherol (250 mg/kg s.c.)	(1) Aqua ad injectabilia i.p., (2) 10% Ethanol absolute + 90% Miglyol® 812 s.c.	(1) Klee et al. (2015) (2) Ambrogini et al. (2014); Betti et al. (2011)
C	(1) Levetiracetam (200 mg/kg i.p.) + (2) Deferoxamine (40 mg/kg i.p.), (3) Melatonin (10 mg/kg s.c.)	(1) Aqua ad injectabilia i.p.* (2) Aqua ad injectabilia i.p.* (3) 10% Ethanol absolute + 10% Solutol® HS 15 + 80% Aqua ad injectabilia s.c.	(1) Klee et al. (2015) (2) Panter et al. (1992); Gusakov et al. (1993); Liu et al. (2011) (3) Lima et al. (2011); Petkova et al. (2014)
D	(1) Levetiracetam (200 mg/kg i.p.) + (2) Deferoxamine (40 mg/kg i.p.), (3) Celecoxib (10 mg/kg s.c.)	(1) Aqua ad injectabilia i.p.* (2) Aqua ad injectabilia i.p.* (3) 10% Ethanol absolute + 10% Solutol® HS 15 + 80% Aqua ad injectabilia s.c.	(1) Klee et al. (2015) (2) Panter et al. (1992); Gusakov et al. (1993); Liu et al. (2011) (3) Jung et al. (2006)
E	(1) Levetiracetam (200 mg/kg i.p.) + (2) Deferoxamine (40 mg/kg i.p.), (3) Gabapentin (200 mg/kg i.p.) + (4) Fingolimod (1 mg/kg i.p.)	(1) Aqua ad injectabilia i.p.* (2) Aqua ad injectabilia i.p.* (3) Aqua ad injectabilia i.p.* (4) Aqua ad injectabilia i.p.*	(1) Klee et al. (2015) (2) Panter et al. (1992); Gusakov et al. (1993); Liu et al. (2011) (3) Cilio et al. (2001) (4) Gao et al. (2012)
F	(1) Levetiracetam (200 mg/kg i.p.), (2) Atorvastatin (10 mg/kg i.p.), (3) Ceftriaxone (200 mg/kg s.c.)	(1) Aqua ad injectabilia i.p., (2) 4% DMSO + 10% Solutol® HS 15 + 86% PBS i.p., (3) Aqua ad injectabilia s.c.	(1) Klee et al. (2015) (2) Lee et al. (2008); Piermartiri et al. (2009)and, and (2010); (3) Goodrich et al. (2013)

* Two drugs (#1 and 2 in A, C, D and E or #3 and 4 in E) dissolved together in the same vehicle (water).

Table 2
Solubility of drugs. Abbreviations: DMSO, dimethyl sulfoxide; HPβCD, hydroxypropyl-β-cyclodextrin; PBS, phosphate-buffered saline.

Drug	Dosage	Intended injection volume	Vehicle	Solubility	Comments
Water-soluble drugs					
Levetiracetam	200 mg/kg	3 ml/kg	Aqua ad injectabilia	+	
Topiramate	30 mg/kg	5 ml/kg	0.9% NaCl	+	
Gabapentin	200 mg/kg	3 ml/kg	Aqua ad injectabilia	+	
Deferoxamine	40 mg/kg	3 ml/kg	Aqua ad injectabilia	+	Used as mesylate salt
Fingolimod	1 mg/kg	5 ml/kg	Aqua ad injectabilia	+	Used as hydrochloride
Ceftriaxone	200 mg/kg	3 ml/kg	Aqua ad injectabilia	+	Used as disodium salt hemi(heptahydrate)
Drugs not soluble in water					
α-Tocopherol	250 mg/kg	5 ml/kg	2-10% Ethanol absolute + 90-98% 0.9% NaCl	–	No emulsion, phase separation
			10% Ethanol absolute + 90% Miglyol® 812	+	↓injection volume or ↓ % ethanol not feasible for injections due to viscosity
Melatonin	10 mg/kg	3 ml/kg	5-10% Ethanol abs. + 90-95% Aqua ad injectabilia	–	
			5-10% Ethanol abs. + 90-95% NaCl 0.9%	–	
			10% Ethanol absolute + 90% Miglyol® 812	+	Emulsion
			10% Ethanol absolute + 10% Solutol® HS 15 + 80% Aqua ad injectabilia	+	Emulsion
Celecoxib	10 mg/kg	3 ml/kg	0.1 mM PBS	–	
			10-35% Ethanol absolute + 65-90% 0.1 mM PBS (heated up to 35 °C)	–	
			10% Ethanol absolute + 90% Miglyol® 812	+	Emulsion
			10% Ethanol absolute + 10% Solutol® HS 15 + 80% Aqua ad injectabilia	+	Emulsion
Atorvastatin	10 mg/kg	5 ml/kg	Captisol® (40% in Aqua ad injectabilia)	–	Used as hemicalcium salt sesquihydrate
			Captisol® (40% in 25 mM PBS, pH 7.4 / 8.0)	–	
			15% Ethanol absolute + 85% Captisol® (40% in 25 mM PBS, pH 7.4)	–	
			10% Ethanol absolute + 10% Solutol® HS 15 + 80% 25 mM PBS	–	
			40% PEG 400 + 60% Aqua ad injectabilia	–	
			HPβCD (10% HPβCD, glucose, bidest. water)	–	
			2% DMSO + 98% Aqua ad injectabilia	–	
			5% DMSO + 95% 0.9% NaCl	–	
			2% DMSO + 98% 25 mM PBS (pH 8.0)	–	
			2% DMSO + 10% Solutol® HS 15 + 88% 25 mM PBS (pH 8.0)	(-)	
			4% DMSO + 10% Solutol® HS 15 + 86% Aqua ad injectabilia	–	
			4-5% DMSO + 10% Solutol® HS 15 + 85-86% 25 mM PBS (pH 8.0)	+	
			4% DMSO + 10% Solutol® HS 15 + 86% 25 mM PBS (pH 7.4)	+	

concentrations of other solvents were limited to tolerable levels (cf., Strickley, 2004; Turner et al., 2011).

In general, based on previous experiments, we wanted to avoid administering any drug as suspension, because drug absorption following parenteral administration of drug suspensions in mice is highly variable and lower compared to administration of drug solutions (Löscher et al., 1990). The vehicles chosen for each drug are shown in Table 1. All drugs were administered as solutions except α-tocopherol, which was emulsified in Miglyol® 812 (90%) and ethanol (10%). Water-soluble drugs were mixed in one aqueous solution shortly before injection, to reduce the number of injections over the period of treatment. This was possible for levetiracetam and deferoxamine, levetiracetam and gabapentin, and gabapentin and fingolimod (Table 1). Some experiments were repeated by using different vehicles (see Table 2) and the best tolerated vehicle solutions were used for final analysis (Table 1). Furthermore, based on preliminary experiments using the oral, i.p. and s.c. routes of drug and drug vehicle administration in mice, we chose for each drug and drug vehicle the route that was best tolerated during repeated drug administration. During repeated s.c. administration of Miglyol® 812, local bumps developed, indicating inadequate absorption of Miglyol® 812, even though the s.c. injection site was changed at each injection timepoint. These bumps did not stay at the local injection site, but migrated subcutaneously to the shoulder and front leg regions. Thus, if possible, we used other solvents to replace Miglyol® 812 and, if not possible (α-tocopherol; see Tables 1 and 2) did not use this vehicle in a higher injection volume than 3 ml/kg per drug combination.

Overall, 10 clinically approved drugs were administered in 6 combinations, ranging from 2 to 4 drugs per combination. A 7th combination, consisting of levetiracetam, α-tocopherol, deferoxamine and celecoxib was not tested during the latent period following kainate, because experiments in nonepileptic mice indicated poor tolerability, mainly due to the high volume of Miglyol® 812 (6 ml/kg) that had to be used to emulsify α-tocopherol and celecoxib for s.c. administration. Oral administration of this drug combination resulted in significant weight loss and was therefore also not possible. Levetiractam was included in all combinations, because clinical studies with administration after traumatic brain injury (TBI) indicated favorable tolerability, pharmacokinetics and preliminary evidence of efficacy (Klein et al., 2012a,b; Pearl et al., 2013).

2.3. Pharmacokinetics of drugs in mice

Rodents such as laboratory mice or rats eliminate most drugs much more rapidly than humans (Martignoni et al., 2006; Löscher, 2007; Sakei et al., 2014). Thus, both doses and dosing intervals have to be adapted to the rapid drug metabolism of rodents when testing clinically approved drugs in mice or rats. For most drugs, pharmacokinetic data for rodents were available from the literature (see Results). These data were then used to decide about the dosing intervals of daily injections. One drug (celecoxib) was chosen to determine the effect of different drug vehicles on pharmacokinetics following s.c. administration. For this purpose, the poorly water-soluble celecoxib, which proved difficult to dissolve in conventional vehicles, was dissolved either in 10% ethanol and 10% Solutol® HS 15 plus 80% water or in 10% ethanol and 90% Miglyol® 812 (cf., Strickley, 2004) and administered s.c. in groups of mice. Blood samples were taken at different time points after drug administration for drug analysis in plasma. Each mouse was used for two blood samplings, taken from the retrobulbar venous plexus under isoflurane anesthesia, and was sacrificed under anesthesia after the second blood withdrawal. Celecoxib level was determined in plasma by HPLC, using the method described by Emami et al. (2008) with slight modifications. Pharmacokinetic parameters were calculated by PK Solutions 2.0™(Summit Research Services, Montrose, CO, USA).

2.4. Drug testing in healthy (nonepileptic) control mice (Phase I)

All drug combinations were tested in nonepileptic control mice (Phase I in Fig. 2) and were compared with administration of vehicles without drugs. Based on rapid drug elimination in rodents (see Results), all drugs were administered twice daily, except for fingolimod, which was administered once daily. To minimize both the suffering and the number of animals, only relatively small group sizes were used (n = 4–8), so drug combination testing for tolerability represented a screening approach. Several of the animal groups (4/7) were used for two tolerability experiments; the interval between two experiments in the same animal was at least 2 weeks.

2.5. Drug testing during the latent period following status epilepticus (Phase IIa)

In an additional experiment (Phase IIa in Fig. 2), all drug combinations that were tolerated in Phase I were administered during the latent period following SE induced by intrahippocampal kainate injection. Drug treatment started 16–18 h after kainate injection, which corresponds to the average duration of SE in this model (Twele et al., 2016). In addition, this time interval was used to allow comparison of drug tolerability with the tolerability obtained in our previous study on other drug combinations, in which treatment was started at a similar interval after kainate injection (Klee et al., 2015). Each animal was only used for one experiment. For each drug-treated group, a drug vehicle control group was used for comparison, receiving the same vehicles as the drug group. All drugs were administered twice daily, except for fingolimod, which was administered once daily. As for Phase I, only relatively small group sizes (n = 4 per group) were used to minimize both the suffering and the number of animals. Vehicle and drug-treated groups were evaluated in parallel in one experiment.

2.6. Induction of status epilepticus by focal administration of kainate in mice

As described in the Introduction, we chose the intrahippocampal kainate SE model in mice for our experiments, because this model exhibits several features of TLE and has the advantage of low mortality and high frequency of spontaneous seizures, which is important for studies on antiepileptogenic drug effects (Guillemain et al., 2012; Löscher, 2017). Furthermore, similar to most types of clinical TLE, the epileptic focus is restricted to the ipsilateral hippocampus without the widespread bilateral brain damage observed in more traditional TLE models such as the pilocarpine model (Löscher, 2017). Our previous tolerability studies on drug combinations have not indicated any tolerability difference between the intrahippocampal kainate and systemic pilocarpine mouse models of TLE (Klee et al., 2015). An added advantage of the intrahippocampal kainate model is that the convulsant is directly injected into the brain, thus preventing the inter-individual variation caused by restricted penetration through the blood-brain barrier in models with systemic administration of kainate or pilocarpine.

For intrahippocampal injection of kainate, mice were anesthetized with chloral hydrate (500 mg/kg i.p.) and kainate (0.21 µg in 50 nl saline) was stereotaxically injected into the right CA1 area of the dorsal hippocampus as described previously (Gröticke et al., 2008; Twele et al., 2016). Kainate was slowly injected over 60 s with a 0.5 µl microsyringe at the following stereotaxic coordinates: AP, – 2.1 mm; L, − 1.6 mm; and DV, − 1.8 mm, respectively (Paxinos and Franklin, 2012). After injection, the needle of the syringe was maintained in situ for additional 2 min to limit reflux along the injection track. During all surgical procedures and for about 1 h thereafter mice were kept on a warming pad to avoid hypothermia. During the days following kainate, all animals were given 0.5 ml Sterofundin® ISO s.c. once daily and pellet pap b.i.d. to facilitate post-SE recovery. SE induction and subsequent

treatment was performed without concomitant EEG/video monitoring, because we have previously shown that all male NMRI mice exhibit limbic SE with continuous activity of spikes or spikes-and-waves and polyspikes in the ipsilateral hippocampal EEG, intermittent generalized convulsive seizures, and develop epilepsy with a latent period of 5–7 days (Twele et al., 2016).

2.7. Assessment of tolerability of drug cocktails (Phase I and IIa)

Tolerability of the drug combinations in mice was assessed using a modified Irwin screen test battery (Irwin, 1968). Before starting the drug and vehicle control experiments, mice were habituated to the procedures by performing the Irwin test two to three times, measuring rectal body temperature three to four times, and training the mice on the rotarod four to five times.

In a first set of experiments (Phase I in Fig. 2), the drug combinations were tested in healthy (nonepileptic) control animals. Together with each drug-treated group, a control group of mice received the respective vehicle solutions. In a second set of experiments (Phase IIa in Fig. 2), the drug combinations and respective vehicles were administered during the latent period following intrahippocampal injection of kainate, starting 16–18 h after kainate injection as in our previous tolerability studies with other drug combinations (Klee et al., 2015). Drugs (or drug vehicles) were administered b.i.d. (inter-administration interval 10–14 h) over three days, except for fingolimod, which was administered once daily (Table 1). Water-soluble drugs were injected as mixed solutions, to reduce the number of injections over the period of treatment (see above), whereas all other drugs were injected separately.

The tests were performed over four days (including the day after termination of treatment to determine whether any of the observed adverse drug effects outlasted the treatment); behavioral scoring was carried out by trained experimenters in a blind fashion, i.e., the experimenter was unaware at any time of the experiment whether a mouse belongs to the vehicle or treatment group. Mice were brought into the laboratory at least 30 min before the morning injection. Before injections, the body weight and temperature were measured. On the first day, tests were performed 30 min, 90 min, 4 h, and 8 h after the morning injection. On the second and third day, tests were performed at the two time points (in some cases three time points on day 2) which had yielded the most prominent effects on day 1. On the fourth day, animals did not receive an injection and only one test was performed in the morning (i.e. about 14 h after the last injection).

The tests were performed in a certain order escalating from non-invasive to invasive in about 2–3 minutes as described recently (Klee et al., 2015). Each subject was first observed in a transparent box (22.2 cm × 16.2 cm × 14 cm) for body position, tail elevation, piloerection, spatial locomotion, transfer arousal, stereotyped behavior, and ataxia. Invasive measures included fear responses such as finger approach, touch escape, and elevating at the tail. In a modified hanging wire test (Coughenor et al., 1977), mice were put upon a grid, which was rotated by 180°. Cut-off was after 5 s. Each measure was scored using a rating system from score 0 up to 5 with score 2 representing "normal" appearance, behavior or reaction (Suppl. Fig. 1).

Motor performance impairment was also assessed in the rotarod test (Dunham and Miya, 1957). Mice had to walk on a turning rod (6 rpm) for 60 s. Mice that were unable to stay on the rod during three sequential 1-min trials were considered to exhibit deficits. The body temperature of the mice was measured thirty min after the beginning of the test battery.

For subsequent group comparisons and statistical analyses, the test results were transferred into a "summation score system" (Klee et al., 2015). An Irwin screen score of 2 was considered as "normal" and therefore transferred into score 0. Deviations (i.e., increase [3–5 points] or decrease [0–1 points] of a parameter) from normal were rated with up to 2 points. Body temperature was scored as change in rectal temperature, with temperature measured before the morning injection as a reference. Deviations (i.e. increase or decrease) in body temperature were scored with 0.5 points per 0.5 °C with a maximum score of 2 points. Body weight was scored as change in body weight, with the weight measured before the first injection on day 1 as a reference. A decrease in weight (accessed in the evening of day 1, 2 and 3, or in the morning of day 4, respectively) was scored with 0.5 points per 5% reduction with a maximum score of 2 points. The result of the rotarod test was scored with a 0 for succeeding in at least one of the trials and a 2 for failing in all three attempts. The score points were added up for each test time point for presenting the development of the drug effects over time. A mean day score was calculated by dividing all score points of a day by the number of the respective test time points of the day. Mean day scores were added up to obtain a sum of the effects during the whole treatment.

To ensure principles of animal welfare, animals were closely observed during all experiments for pain, distress, and discomfort using welfare score sheets for humane endpoints (Stokes, 2002; Fentener et al., 2015; Lidster et al., 2016). Distress was rated from 0 (normal) to 3 (severe), using a distress scoring system (Morton and Griffiths, 1985; Lloyd and Wolfensohn, 1999). Treatments inducing a maximum of score 1 (reduced food and water intake, loss of body weight of less than 10%, reduced locomotor activity, normal response to environmental stimuli, normal grooming but dull coat) were considered tolerable. Similarly, treatments transiently inducing score 2 (markedly reduced food and water intake, loss of body weight of up to 20%, loss of spontaneous movement, reduced response to environmental stimuli, reduced grooming) were considered tolerable. However, if score 2 persisted for more than two days, the treatment was interrupted, except when drugs in a given cocktail were known to produce adverse effects (e.g., weight loss or sedation) that interfered with the distress scoring system (cf., Lidster et al., 2016). Reaching score 3 (no food and water intake, loss of body weight of > 20%, loss of spontaneous movement, loss of response to environmental stimuli, loss of grooming, red eye and nose exudates) led to immediate interruption of the experiment. None of the drug cocktails tested necessitated premature termination of the experiment (see Results).

2.8. Assessment of drug effects on hippocampal neurodegeneration following kainate (Phase IIa)

In Phase IIa, the mice were anesthetized and perfused with paraformaldehyde 7–8 days after kainate injection. Series of coronal brain sections (40 µm) were prepared for histology as described previously (Bröer et al., 2016). Naive age-matched groups of mice were used as controls. Neurodegeneration in the hippocampus was evaluated using 5–6 thionin-stained sections (at −1.56 to −2.18 from bregma), which were semi-quantitatively scored using a score system that was applied to detect potential differences to controls (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.

Additional sections (at -1.80 to -2.00 mm 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 (Gröticke et al., 2007). FJC-positive neurons were counted in 8 square fields of a defined size (90 µm^2) 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/CA2, four fields; CA3a, two fields; CA3c, one field; dentate hilus, one field (for details see Polascheck

Table 3
Overview of the drug and drug vehicles used in this study.

Compound/ vehicle	Vendor / Supplier	Compound / vehicle number (vendor)	CAS Registry Number
Drugs			
Levetiracetam	Provided by UCB Pharma	–	102767-28-2
Topiramate	Provided by Hexal	–	97240-79-4
Gabapentin	Provided by Pfizer	–	60142-96-3
Deferoxamine (mesylate salt)	Sigma-Aldrich	D9533	138-14-7
Fingolimod (FTY20)	Sigma-Aldrich	SML0700	162359-56-0
Ceftriaxone (disodium salt hemi(heptahydrate))	Sigma-Aldrich	PHR1382	104376-79-6
(+)-α-Tocopherol	Sigma-Aldrich	T3634	59-02-9
Melatonin	Sigma-Aldrich	M5250	73-31-4
Celecoxib	Provided by Pfizer	–	169590-42-5
Atorvastatin (hemicalcium salt sesquihydrate)	Sigma-Aldrich	PHR1422	344423-98-9
Kainate	Sigma-Aldrich	K0250	58002-62-3
Vehicles			
Aqua ad injectabilia	B. Braun Melsungen AG; Vendor: Tierärztebedarf J. Lehnecke GmbH	308452	–
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418	67-68-5
Phosphate-buffered saline (PBS) tablets	Sigma-Aldrich	P4417	–
Ethanol absolute (EMSURE®)	Merck KGaA; Vendor: Sigma-Aldrich	1009831000	64-17-5
Miglyol® 812	Caesar & Loretz GmbH	3274	52622-27-2
Solutol® HS 15 (former name, now Kolliphor® HS 15)	BASF SE	–	70142-34-6
Sulfobutyl-ether-β-cyclodextrin sodium salt (Captisol®)	Provided by PIQUR	–	182410-00-0
Polyethylene glycol (PEG) 400 (ROTIPURAN®)	Carl Roth GmbH + Co. KG	0144.1	25322-68-3
Hydroxypropyl- β-cyclodextrin (HPβCD)	Provided by Roquette-Pharma	–	128446-35-5

et al., 2010).

2.9. Drugs

Drugs, drug doses, and vehicles chosen for drug solutions are shown in Table 1. In case of drugs that were used as salts, all doses (in mg/kg body weight) refer to the free acid or base forms of the respective drugs. All drugs except melatonin and ceftriaxone were prepared freshly once a day. Melatonin and ceftriaxone were prepared freshly b.i.d., because of limited stability of the solutions. For injection of combinations, the injection volume was 3 ml/kg for all substances except topiramate and atorvastatin, which were not soluble below an injection volume of 5 ml/kg. All injection volumes were kept as low as possible to avoid total injection volumes of > 10-12 ml/kg in mice.

Sources for drugs with product numbers and CAS registry numbers are shown in Table 3. In short, kainate for induction of SE was purchased from Sigma-Aldrich (Steinheim, Germany). Levetiracetam was kindly provided by UCB Pharma (Brussels, Belgium), topiramate by Hexal (Holzkirchen, Germany) and gabapentin and celecoxib by Pfizer (Sandwich, Kent, UK). Fingolimod (FTY720, fingolimod hydrochloride), atorvastatin (used as hemicalcium salt sesquihydrate), α-tocopherol (D-α-tocopherol, vitamin E), melatonin, deferoxamine (used as mesylate salt), and ceftriaxone (used as disodium salt hemi(heptahydrate)) were purchased from Sigma-Aldrich.

Sources for drug vehicles with product numbers and, if available, CAS registry numbers are shown in Table 3. In short, aqua ad injectabilia was purchased from Lehnecke (Schortens, Germany), dimethyl sulfoxide (DMSO) from Sigma-Aldrich, ethanol absolute (EMSURE®) from Sigma-Aldrich, Miglyol® 812 from Caesar & Loretz GmbH (Hilden, Germany), Solutol® HS 15 from BASF SE (Ludwigshafen, Germany), and polyethylene glycol 400 (ROTIPURAN®) from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Captisol® (sulfobutyl-ether-β-cyclodextrin sodium salt) was kindly provided by PIQUR Therapeutics AG (Basel, Switzerland) and HPβCD by Roquette-Pharma (Frankfurt, Germany).

2.10. Statistics

Statistical differences in tolerability scores between treatment and vehicle groups were calculated using the Mann-Whitney *U* test. Statistical differences in body weight and body temperature within each group were calculated using one-way analysis of variance (ANOVA) for paired data with Sidak's multiple comparisons test as a posthoc test. Statistical differences in body weight and body temperature between vehicle- and drug-treated groups were calculated by two-way ANOVA with Dunnett's multiple comparison test as a posthoc test; some pairwise comparisons were performed with the Mann-Whitney *U* test. Significance of differences in neurodegeneration was calculated by Kruskal-Wallis ANOVA for nonparametric data with Dunn's multiple comparison test as a posthoc test. A $P \leq 0.05$ was considered significant.

3. Results

3.1. Pharmacokinetics of drugs

To allow a comparison of elimination half-lives in mice, rats, and humans, a literature research was performed for the 10 drugs. Data are shown in Table 4.

As expected, all drugs were eliminated much more rapidly by rodents than by humans. As a consequence, twice daily administration of drugs, as performed in this study, may result in only transient maintenance of effective drug levels, even if relatively high doses of drugs are used. The only exceptions were α-tocopherol and fingolimod, which, although more rapidly eliminated by rodents than humans, still have a relatively long half-life of 9 (α-tocopherol) or 23 (fingolimod) h in rodents; fingolimod was therefore only injected once daily.

Celecoxib proved to be difficult to dissolve in any conventional vehicle, so we evaluated whether the vehicles that we finally chose affected the pharmacokinetics of the drug in mice. As shown in Fig. 3, plasma concentrations of celecoxib following s.c. administration of 10 mg/kg dissolved in ethanol (10%), Solutol® HS 15 (10%) and water (80%) rapidly declined with an average half-life of 4.03 h in male mice, which is similar to the half-life of celecoxib in male rats reported by Paulson et al. (2000) (Table 4).

However, following s.c. administration of celecoxib in a vehicle consisting of 10% ethanol and 90% Miglyol® 812, which resulted in similar peak plasma concentrations (about1 μg/ml) compared to the

Table 4
Species differences in elimination half-life of test drugs.

Drug	Elimination half-life (h)			References
	Rat	Mouse	Human	
Antiepileptic drugs with diverse mechanisms				
Levetiracetam	2-3	1.5	6-11	Löscher, 2007; Markowitz et al., 2010
Topiramate	2-5	?	20-30	Löscher, 2007
Gabapentin	2-3	?	5-7	Löscher, 2007
Antioxidant drugs				
α-Tocopherol	9	?	30	Abuasal et al., 2012; Traber et al., 2015
Melatonin	0.33	?	˜0.75	Cavallo and Ritschel, 1996; Harpsoe et al., 2015; Yeleswaram et al., 1997
Anti-glutamatergic drugs				
Deferoxamine	?	0.09	6.1 (slow phase)	Allain et al., 1987; Panter et al., 1992
Ceftriaxone	0.6-2.3	0.7-1.2	6-9	Patel et al., 1981; Klesel et al., 1984; Matsui et al., 1984; DrugBank Version 5.1.1, 2018 ; Gill et al., 1998
Antiinflammatory drugs				
Celecoxib	3.7 (male), 14 (female)	4 (male)	11-16	Paulson et al., 2000; Shi and Klotz, 2008; present study
Fingolimod	23	˜19	144-216	David et al., 2012 Kovarik et al., 2004 David et al., 2015 Mao et al., 2014 Meno-Tetang et al., 2006
Atorvastatin	4.8	˜0.6	7-14	Lennernas, 2003; Zheng et al., 2010; Reddy et al., 2012; DrugBank Version 5.1.1, 2018

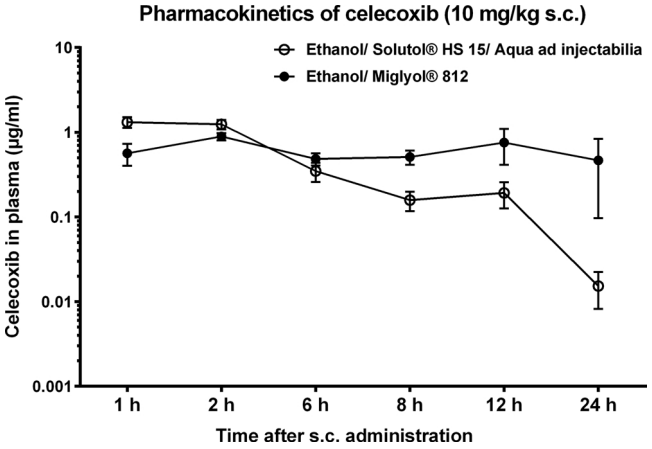


Fig. 3. Plasma concentrations following s.c. administration of celecoxib prepared either as a solution in ethanol, Solutol® HS 15 and water or an emulsion in ethanol and Miglyol® 812 in male mice. Data are shown as means ± SEM of 6–8 mice per time point. Note the lack of any obvious elimination of celecoxib following s.c. administration of the preparation using ethanol and Miglyol® 812, indicating that the use of Miglyol® 812-containing medium-chain triglycerides resulted in a depot preparation of celecoxib. Following s.c. administration of the celecoxib solution in ethanol, Solutol® HS 15 and water, an average half-life of 4 h was calculated for celecoxib in male mice.

solution in ethanol, Solutol® HS 15 and water, no obvious elimination was observed over the 24 h of the experiment (Fig. 3), indicating that the use of Miglyol® 812-containing medium-chain triglycerides resulted in a depot preparation. To avoid accumulation of drug levels during repeated administration, we therefore chose the celecoxib solution in ethanol, Solutol® HS 15 and water for the tolerability experiments.

3.2. Tolerability of drug combinations in healthy (nonepileptic) controls (Phase I) and mice following SE (Phase IIa)

For illustrating how data looked before transformation to summation scores (see Methods), individual data from the Irwin screen at one time point (30 min after 1st administration) of the first day of the experiment are illustrated for combination A (levetiracetam plus gabapentin plus topiramate) in control mice in Suppl. Fig. 2A. Furthermore, data from rotarod (Suppl. Fig. 2B) and rectal body temperature measurements (Suppl. Fig. 2C) recorded during the trial period are illustrated. After the first drug application, no obvious differences were observed between the treated and the vehicle group in most of the parameters scored. Drug-treated mice exhibited significant ataxia (Suppl. Fig. 2A), which, however, did not result in more failures in the rotarod test (Suppl. Fig. 2B). Furthermore, treated mice exhibited reduced balance, touch escape and elevating at tail response (Suppl. Fig. 2A). Body temperature was significantly decreased by drug treatment (Suppl. Fig. 2C). On the second and third day, altered touch escape and ataxia were the only adverse effects that were evident, resulting in significant rotarod failures on day 2. On the fourth day, when the animals were no longer injected, no significant differences to vehicle controls were observed. Over all test days, the body weight of vehicle and drug-treated mice was stable except for day 4, at which body weight of drug-treated mice (36.8 ± 0.68 g) was slightly, but statistically significant lower than body weight of vehicle-treated controls (39.4 ± 0.79 g; $P = 0.0257$). Overall, this drug combination was considered tolerable when administered twice daily over three days, because no serious or unexpected adverse effects occurred and all mice survived the treatment period without seriously impaired general behavior or wellbeing.

Based on the individual assessment of behavioral differences in individual mice in the various tests, summation scores were calculated for each group as described in Methods to facilitate the evaluation of the time course of adverse effects over the treatment period as well as group comparisons. The summation scores for the data shown in Suppl. Fig. 2 are illustrated under “healthy controls” in Fig. 4A–C. The time course of summation scores shown in Fig. 4A and B indicates that drug treatment differed significantly from vehicle control over the three days of the treatment period, mainly as a result of ataxia. The overall summation scores in Fig. 4C demonstrate the significant difference between drug- and vehicle-treated groups.

When mice were treated with combination A during the latent period following SE, adverse effects were similar to those observed in healthy controls. As shown in Fig. 4D–F, drug-treated mice differed significantly from vehicle controls, mainly as a result of ataxia. Thus,

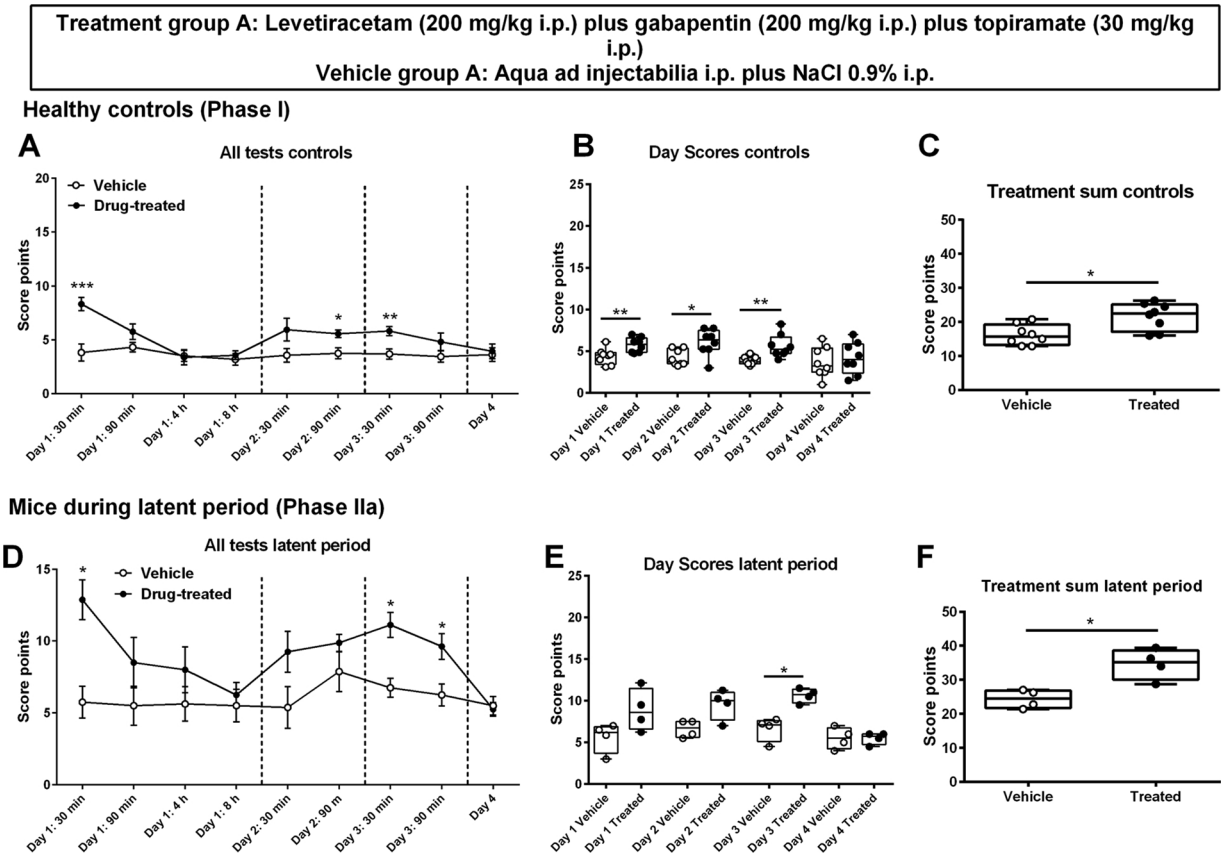


Fig. 4. Summation scores for treatment A (levetiracetam, gabapentin and topiramate) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. "A" and "D"show the 9 individual testing periods during and after the drug trial, "B" and "E" the daily scores, and "C" and "F" the summed scores for the whole trial period. In A and D, data are shown as means ± SEM of 8 vehicle controls and 8 drug-treated mice for the experiment in nonepileptic animals, and 4 vehicle controls and 4 drug-treated mice for the experiment in the latent period, respectively. Significant differences between vehicle and drug groups are indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001). In B, C, E and F, data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Significant differences between vehicle and drug groups are indicated by asterisks (*P < 0.05; **P < 0.01).

this drug combination was considered tolerable when administered twice daily over three days in mice during the latent period.

Data for treatment group B (levetiracetam plus α-tocopherol) are shown in Fig. 5. No significant adverse effects were observed in controls, except occasional hunched body position and stereotypic rearing. Over all test days, the body weight of vehicle and drug-treated mice was stable except for day 4, at which body weight of drug-treated mice (38.71 ± 0.85 g) was significantly lower than body weight of vehicle-treated controls (41.1 ± 0.56 g; P = 0.0335). The summation scores illustrated in Fig. 5A–C indicated that, except for day 1, no significant behavioral differences were observed between drug-treated and vehicle-treated mice. This was similar when drug treatment was performed during the latent period after SE (Fig. 5D–F). Thus, this drug combination was considered to be tolerated.

Data for treatment group C (levetiracetam plus deferoxamine plus melatonin) are shown in Fig. 6. No significant adverse effects were observed in any group, so this treatment was excellently tolerated.

Data for treatment group D (levetiracetam plus deferoxamine plus celecoxib) are shown in Fig. 7. No significant adverse effects were observed in any group, so this treatment was also excellently tolerated.

Data for treatment group E (levetiracetam plus deferoxamine plus gabapentin plus fingolimod) are shown in Fig. 8. Except for significant impairment of balance in the first 0.5 h following the first drug administration on day 1, no significant adverse effects were observed in any group, so this treatment was also well tolerated.

Data for treatment group F (levetiracetam plus atorvastatin plus ceftriaxone) are shown in Fig. 9. Except for transient alterations in balance, touch escape and transfer arousal during the first or second day of treatment, no significant adverse effects were observed in any group, so this treatment was well tolerated.

As recently shown (Schidlitzki et al., 2017), mice after a brain insult (SE in this study) may be more susceptible to drug adverse effects than healthy controls. When the tolerability of the 6 drug combinations evaluated in the present experiments was directly compared between healthy controls and mice during the latent period, only group A (levetiracetam, gabapentin, topiramate) resulted in significantly higher scores in the latent period group (Fig. 10). Thus, overall all 6 drug combinations were sufficiently well tolerated at the selected doses to allow drug efficacy studies.

As described in Methods, a 7th drug combination (levetiracetam, α-tocopherol, deferoxamine and celecoxib) was not tolerated in nonepileptic controls at the selected doses and vehicles used and was therefore not evaluated during the latent period.

3.3. Effects of drug combinations on hippocampal neurodegeneration in mice following SE (Phase IIa)

The hypothesis of these experiments was that first signals of antiepileptogenic or disease-modifying efficacies of drug combinations may be obtained when determining potential neuroprotective effects of the treatments, because several of the drugs used in this study have shown such effects in previous studies (see Discussion). Intrahippocampal kainate is known to produce severe neuronal damage in the ipsilateral hippocampus (Bouilleret et al., 1999). This was also observed in the

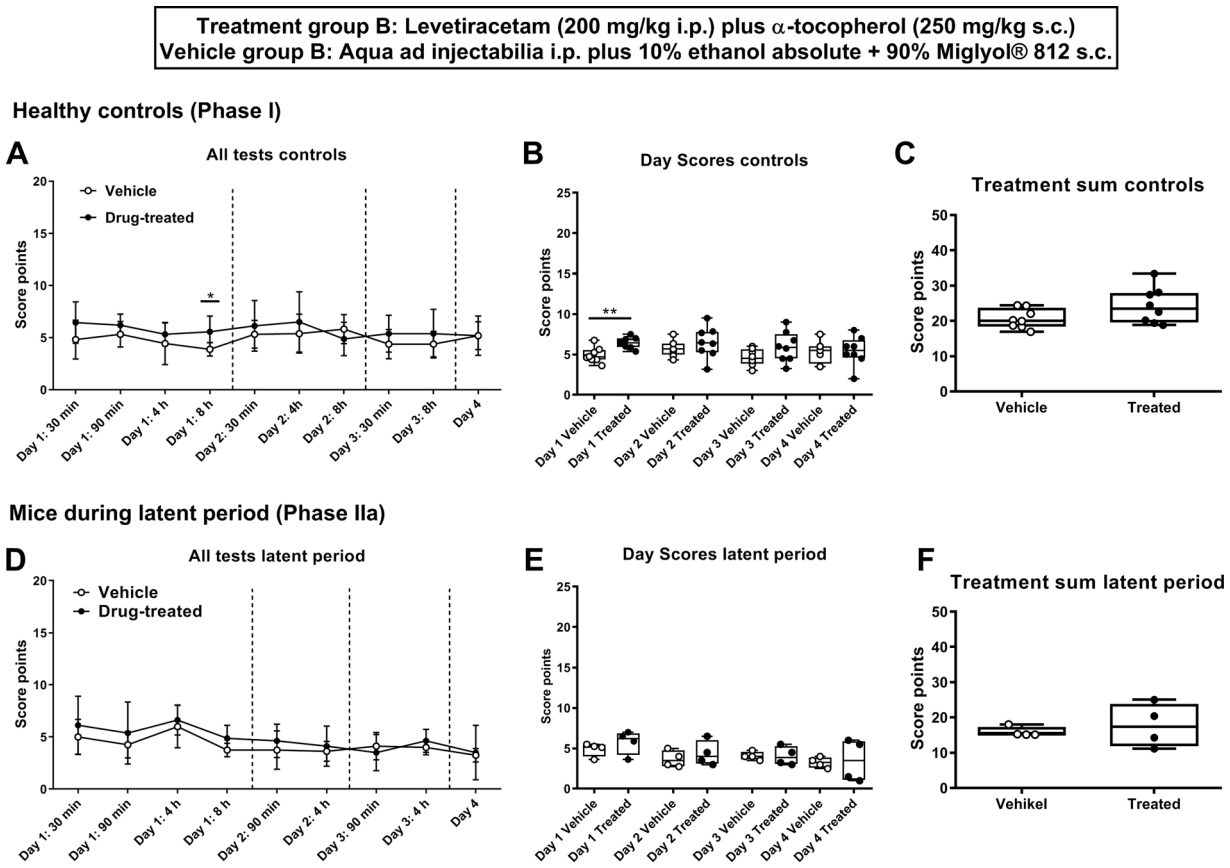


Fig. 5. Summation scores for treatment B (levetiracetam plus α-tocopherol) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. See Fig. 7 legend for further details.

present experiments when the neuronal cell layers of the hippocampus were evaluated 7–8 days after kainate injection in vehicle-treated controls (Fig. 11). Severe neuronal loss was observed in CA1, CA2, CA3a, CA3c and dentate hilus of the ipsilateral hippocampus, whereas no damage was seen in the contralateral hippocampus (see CA3c as an example in Fig. 11J). Examinations of serial sections of the mice used in these experiments indicated that the hippocampal neurodegeneration extended on average over an area ranging from −1.14 to −3.11 mm from bregma, which was both observed in vehicle and drug-treated animals. The most affected region was the ipsilateral dentate hilus, in which almost complete loss of neurons was observed in most mice. In addition to neuron loss, significant granule cell dispersion, a typical consequence of intrahippocampal kainate injection (Bouilleret et al., 1999), was observed in the ipsilateral hippocampus (Fig. 11C,I), but not in the contralateral hippocampus (not shown). However, the granule cell dispersion observed at 1 week following kainate (Fig. 11C,I) was much less marked than in brain sections taken at several weeks after kainate injection (Bouilleret et al., 1999).

Unexpectedly, none of the drug combinations administered during the latent period exerted any significant neuroprotective effect (Fig. 11). To make sure that we did not miss subtle neuroprotective effects by scoring the hippocampal damage in thionin-stained sections, we counted FJC-labeled neurons in additional sections of the hippocampus (Fig. 11). FJC is a sensitive and specific fluorescent marker of dying neurons (Schmued et al., 2005). As shown in Fig. 11D and K–N, significant numbers of FJC-positive dying neurons were observed in CA1/CA2, CA3a, and CA3c but not dentate hilus of vehicle-treated controls when analyzed 7–8 days after intrahippocampal kainate injection. The lack of FJC-positive neurons in the hilus was obviously due to the fact that most hilar cells had died and been phagocytosed before FJC labeling was performed, which is indicated by the almost complete loss of hilar neurons in the thionin-stained sections (Fig. 11H). None of the drug combinations significantly altered the number of FJC-positive neurons compared to vehicle-treated controls, substantiating the lack of any significant neuroprotective effect.

In an additional analysis, we examined whether the individual body temperature during treatment was associated with the extent of neurodegeneration in the mice of the various treatment groups. No association was found (data not shown).

4. Discussion

4.1. Combinatorial therapies for complex diseases

Multitargeted and combinatorial therapies (“network approaches”) have achieved considerable therapeutic efficacy by modulating the activities of targets in complex diseases such as HIV-1 infection, cancer, diabetes mellitus, hypertension, congestive heart failure, asthma and chronic obstructive pulmonary disease, and rare neurological diseases with complex etiologies unlikely to respond to single, target-specific therapeutics but requiring intervention at multiple points within a perturbed disease system (Ainsworth, 2011; Boezio et al., 2017; Muhammad et al., 2018). Epilepsy is a complex network phenomenon that, as yet, cannot be prevented or cured (Klein et al., 2018). Although networks in epilepsy are frequently conceptualized at the level of interacting brain regions forming an epileptic network, there are in fact multiple levels with interacting components (genes, proteins, neurons, different pathologic processes and so on), all functioning within networks, albeit at different levels of resolution (Scott et al., 2018). As an example, the pathologic processes induced by brain injury (as illustrated in a simplified manner in Fig. 1B) form a complex interacting network; so only targeting for instance neuroinflammation alone is

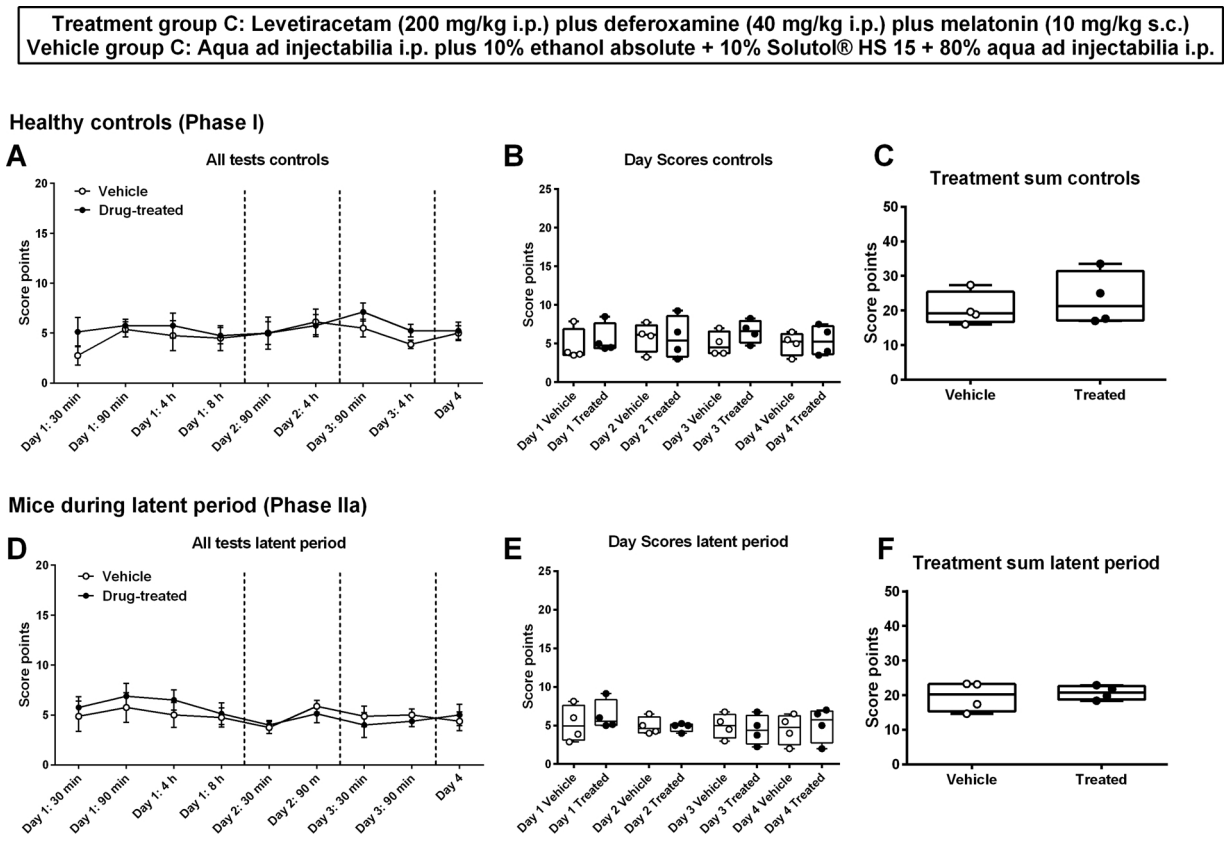


Fig. 6. Summation scores for treatment C (levetiracetam, deferoxamine and melatonin) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. See Fig. 7 legend for further details.

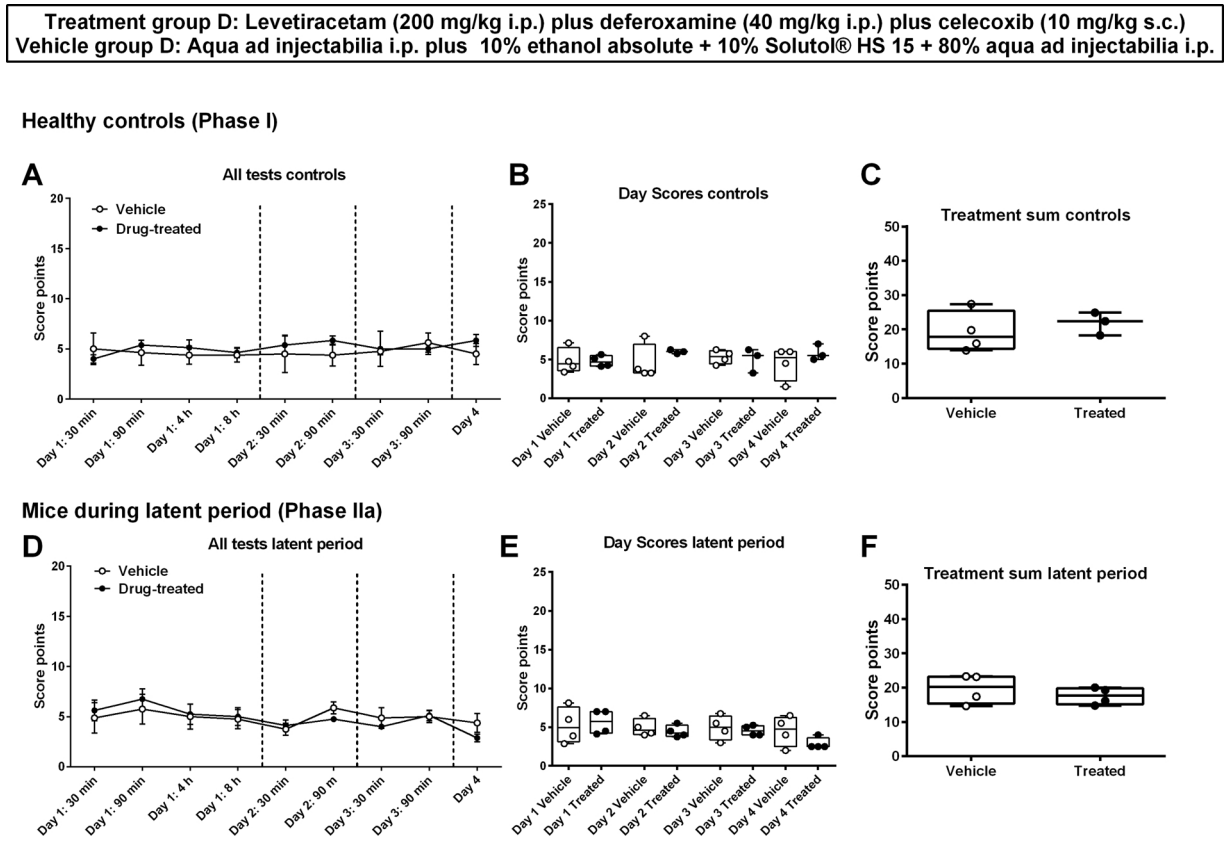


Fig. 7. Summation scores for treatment D (levetiracetam, deferoxamine and celecoxib) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. See Fig. 7 legend for further details.

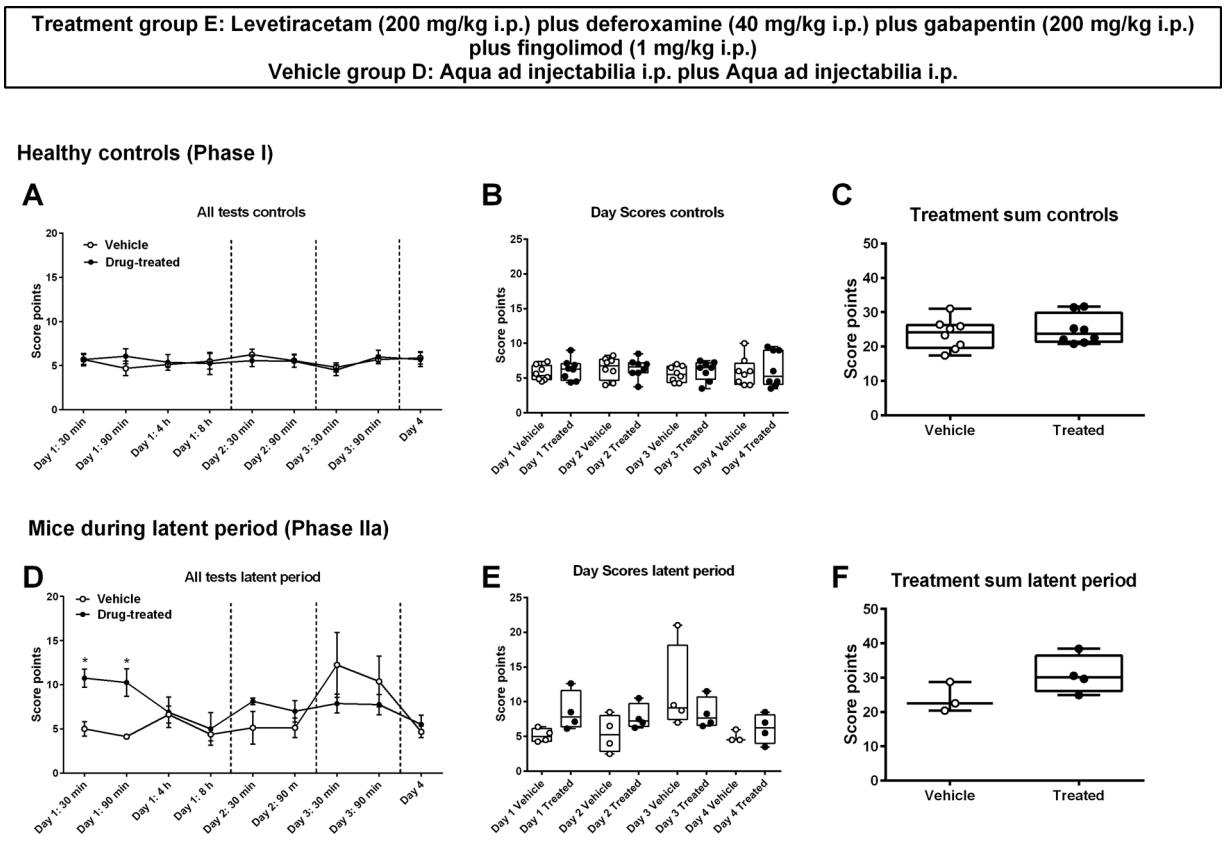


Fig. 8. Summation scores for treatment E (levetiracetam, deferoxamine, gabapentin and fingolimod) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. See Fig. 7 legend for further details.

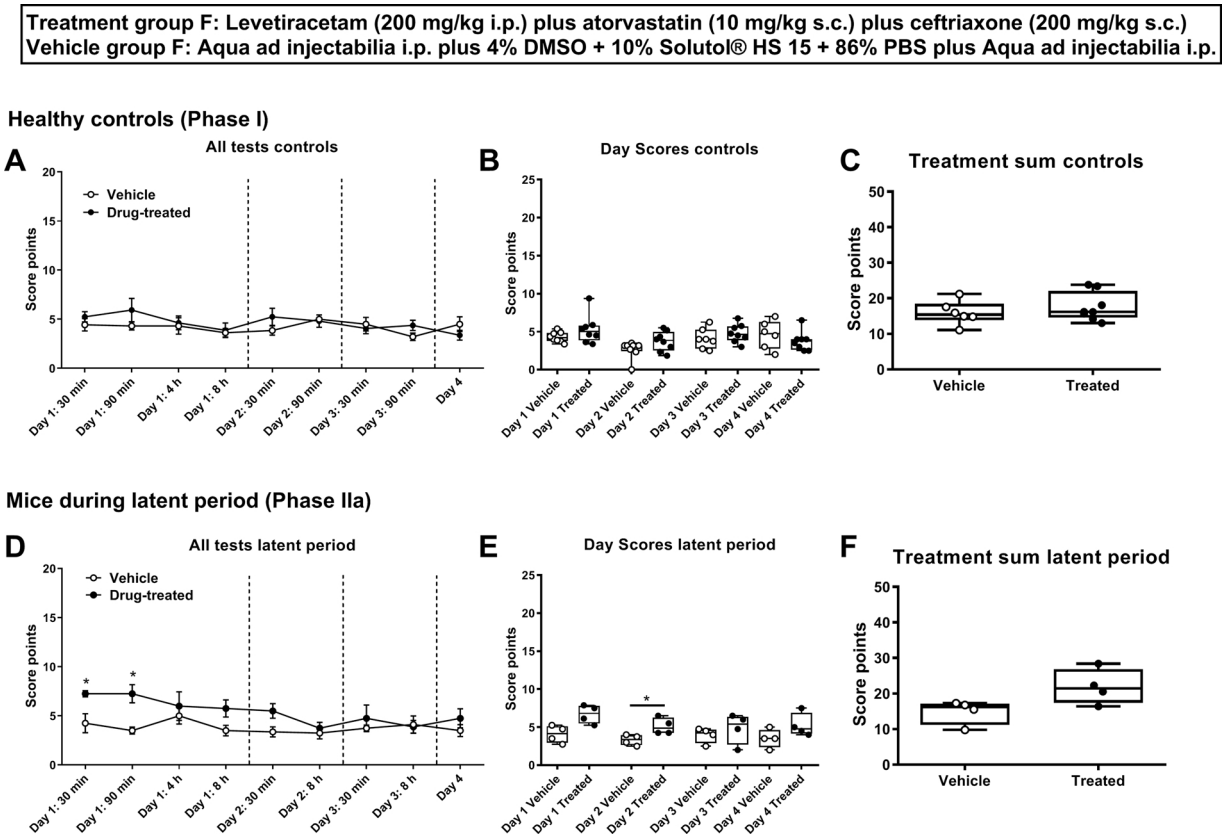


Fig. 9. Summation scores for treatment F (levetiracetam, atorvastatin and ceftriaxone) in naive (nonepileptic; A–C) mice and mice during the latent period (D–F). Groups receiving the same vehicles as the drug-treated groups were used for control. See Fig. 7 legend for further details.

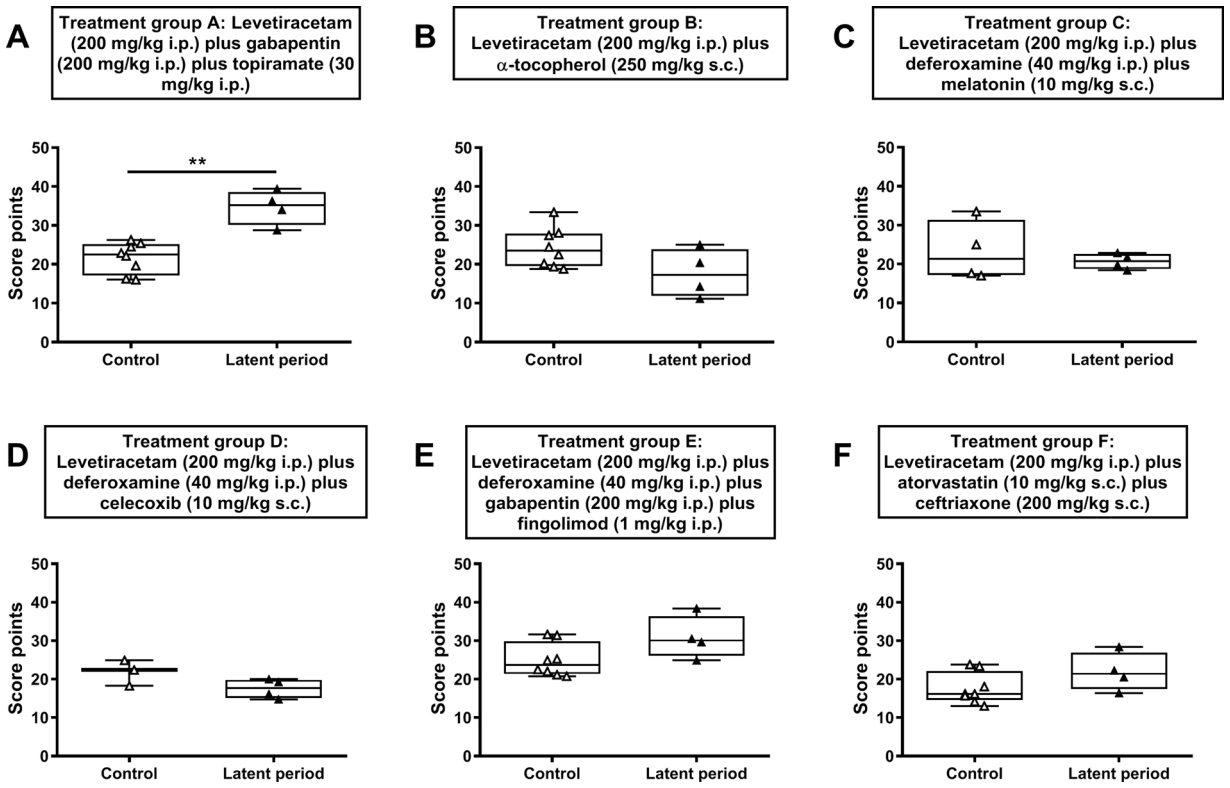


Fig. 10. Comparison of overall summation scores of the six drug combinations in nonepileptic mice vs. mice during the latent period. Significant differences between nonepileptic mice and mice during the latent period are indicated by asterisks (**P < 0.01). For further details, see Figs. 6–11.

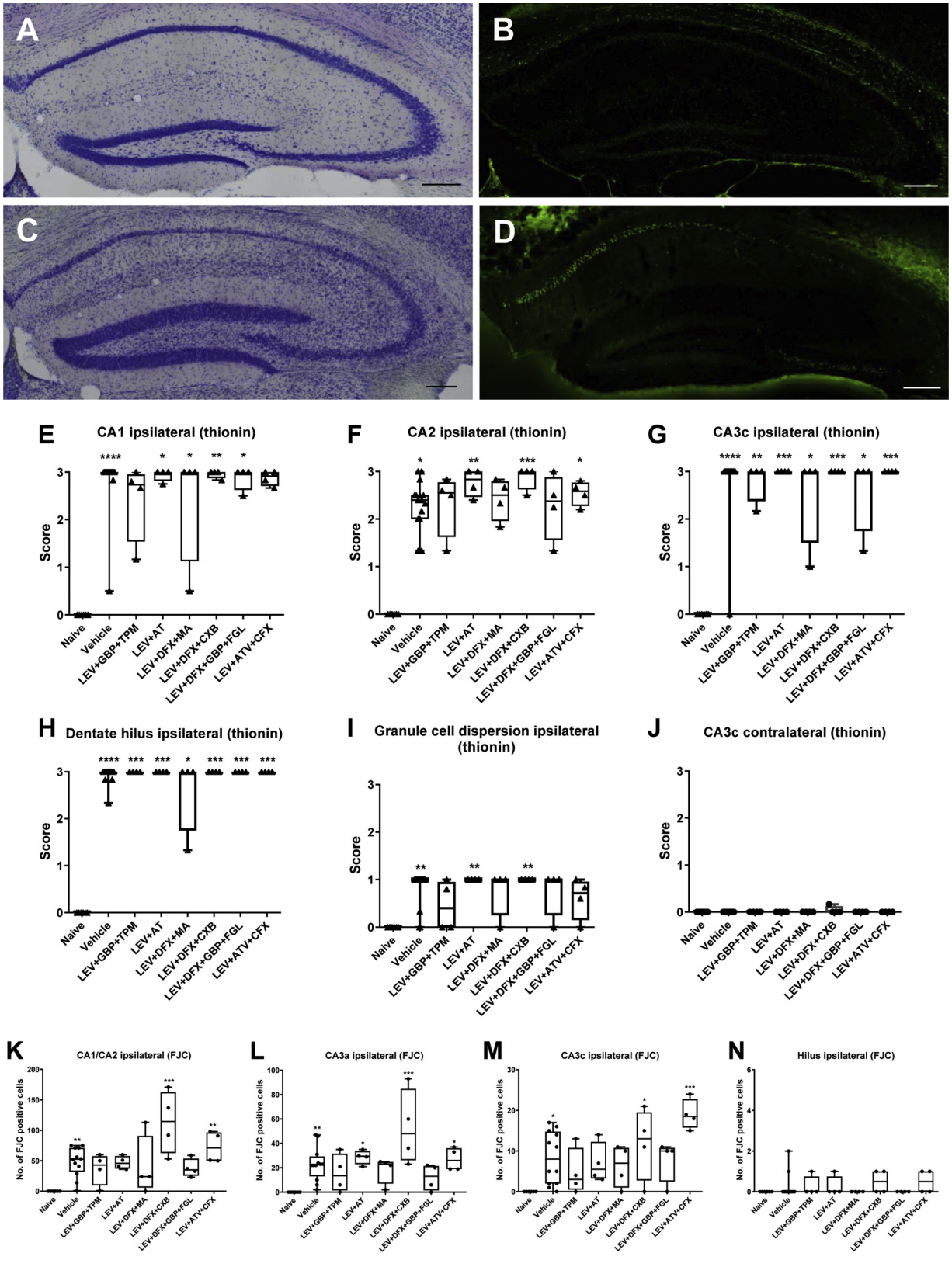
unlikely to halt the epileptogenic process.

There are two principal strategies for identifying an efficacious network approach for antiepileptogenesis. One is the strategy used for our previous (Klee et al., 2015) and present studies, in which rationally chosen drug combinations that affect different targets within an epileptogenic network are tested in animal models. This top-down approach is relatively unspecific, thus resembling phenotypic drug screening (Swinney and Anthony, 2011; Eder and Herrling, 2016; Moffat et al., 2017). The second approach starts by identifying specific targets or critical nodes in the network and then searches for drugs (or develops new compounds) that selectively affect these targets or nodes (Swinney and Anthony, 2011; Eder and Herrling, 2016). This bottom-up target-based approach can very effectively develop novel treatments for a validated target or node, but the process of target validation is complex and associated with a high degree of uncertainty (Sams-Dodd, 2005; Swinney and Anthony, 2011). Indeed, in different fields of drug development, the latter paradigm has not resulted in increased productivity over the traditional phenotypic approach, but the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeded that of target-based approaches (Sams-Dodd, 2005; Swinney and Anthony, 2011; Eder et al., 2014; Moffat et al., 2017). Driven by advances in biology, engineering, and informatics, new paradigms integrate phenotypic with target-based algorithms into comprehensive, systems-level approaches offering value-added strategies for optimized drug discovery (Waldman and Terzic, 2013). An alternative relatively rapid approach for developing new effective combinatorial treatments is repurposing (or repositioning) of generic drugs, which have been on the market for years, have well known safety profiles and are easy and cheap to obtain for clinical trials because their original patents have expired (Ainsworth, 2011). If they involve new formulations, new routes of administrations, applications to new disorders, or doses that are considerably lower than those used in monotherapy, they can still be covered by patents (Nosengo, 2016). Furthermore, the repositioning approach can be used for developing new indications for discontinued clinical compounds (Ciallella and Reaume, 2017).

4.2. Drug selection for the present study

The 10 drugs used for this study were chosen both on practical and theoretical grounds. First, all drugs should be clinically approved, thus allowing relatively rapid translation from preclinical to clinical trials, including repurposing of these drugs for antiepileptogenesis (Klein and Tyrlikova, 2017). For approved drugs, pharmacokinetic data in both animals and humans are available in the literature, so we could use such data for deciding about dosing intervals. Second, all drugs should be tolerable enough for using them in epilepsy prevention trials in patients after stroke or TBI. Third, for all drugs, some indication of a disease-modifying effect when administered after brain injury should be available from the preclinical or clinical arena (see Table 1). Fourth, the drugs should act on different processes thought to be involved in epileptogenesis (cf., Fig. 1B), thus allowing a network approach. In the 6 combinations that were tolerated in the present study (Table 1), at least two of the four drug categories shown in Fig. 1B were combined, because combining drugs within only one category was considered less promising, based on recent experience with antiinflammatory drugs (Noe et al., 2013). One limiting factor when choosing drugs for such a potentially antiepileptogenic network approach is the fact that as yet no drug exists for which antiepileptogenic or disease-modifying efficacy has been demonstrated in patients. As a consequence, animal models cannot be validated by a drug with a known antiepileptogenic effect, so the translational value of animal models is not known at present.

The only exception may be statins such as atorvastatin, which was therefore included in one of the present drug combinations. Several previous studies reported antiepileptogenic efficacy of statins in different experimental models of epileptogenesis (Scicchitano et al., 2015). The preclinical effects of statins were successfully translated to a clinical study in that Guo et al. (2015) reported that acute statin use



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reduces the risk of poststroke early-onset seizures, and may also prevent the progression of initial poststroke seizures to chronic epilepsy, findings that, if reproducible, would be the first proof-of-concept of antiepileptogenesis in humans (Siniscalchi and Mintzer, 2015). Antiepileptogenic effects of statins are also suggested by two other clinical studies (Pugh et al., 2009; Etminan et al., 2010). In addition to effects on serum cholesterol levels, statins exhibit immunomodulatory, anti-inflammatory, and anti-excitotoxic properties (Scicchitano et al., 2015), which could explain the antiepileptogenic effects that have been observed both experimentally and clinically.

Fig. 11. Hippocampal neurodegeneration in the intrahippocampal kainate model. A-D: Representative photomicrographs of thionin (left graphs) and Fluoro Jade C (FJC)-labeled (right graphs) ipsilateral sections of the hippocampus. A and B show sections of the hippocampus of a control mouse, illustrating the normal cytoarchitecture of the hippocampus in the thionin-stained section and lack of FJC-positive neurons in the FJC-labeled section. C and D show sections of the hippocampus of a mouse 7 days after intrahippocampal kainate injection. Note the thinning and loss of neurons within the CA1, CA2, CA3a and CA3c layers, the loss of dentate hilus cells, and the dispersion of the granule cell layer in the thionin-stained section. FJC-labeled dying neurons (green) are visible in the CA1 layer in the FJC-labeled section (D). Scale bars indicate 200 µm. Also, astrogliosis is evident. E-J: Semiquantitative analysis of the neuronal damage observed at 1 week following intrahippocampal kainate in ipsilateral thionin-stained hippocampal sections of the different treatments groups of this study. Data from naive mice are shown for comparison. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Group size was 4 for each drug-treated group, 16 for the vehicle group (because the different vehicle groups were combined), and 6 for the naive group. Significant differences between naive mice and kainate-treated mice are indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). None of the drug-treated groups differed significantly from the vehicle-treated group. No neurodegeneration was observed in the contralateral hippocampus (shown for the CA3c in J). K-N: Quantitative analysis of FJC-labeled neurons counted at 1 week following intrahippocampal kainate in ipsilateral FJC-labeled hippocampal sections of the different treatments groups of this study. Data from naive mice are shown for comparison. For further details see above.

The antiepileptic drug levetiracetam was included in all of the present drug combinations because (i) preclinical evidence indicating disease-modifying activity in post-SE models of TLE (Löscher and Brandt, 2010; Kaminski et al., 2014: Löscher et al., 2016) and (ii) clinical studies with administration after TBI indicated favorable tolerability, pharmacokinetics and preliminary evidence of efficacy (Klein et al., 2012a,b; Pearl et al., 2013). In a Phase 2 study, Klein et al. determined pharmacokinetics of this drug in patients with TBI and found that pharmacokinetics of levetiracetam did not differ significantly from previously published oral and i.v. pharmacokinetics in healthy humans (Klein et al., 2012a). Furthermore, levetiracetam was safe and well tolerated, in doses and with plasma levels similar to those in animal studies on antiepileptogenesis (Klein et al., 2012b). The study was not controlled or randomized and was not powered to show efficacy. However, there was a signal of reduced post-traumatic epilepsy incidence in levetiracetam-treated adults compared with an observational group (Klein et al., 2012a, 2012b; Pearl et al., 2013). This study set the stage for implementation of a prospective study to prevent posttraumatic epilepsy in an at-risk population (Pearl et al., 2013).

Our hypothesis was that combining levetiracetam with different mechanistic categories of drugs reported to exert disease-modifying effects in post-SE TLE models when administered alone may increase the chance to halt epileptogenesis (Löscher and Brandt, 2010; Löscher et al., 2013; White and Löscher, 2014). As described for network pharmacology (Hopkins, 2008; Ainsworth, 2011), we decided to limit the number of drugs in the combination to a maximum of 3-4.

4.3. Selection of suitable vehicles

Since we wanted to administer all drugs parenterally (i.p. or s.c.), we first had to find suitable vehicles allowing to dissolve (or emulsify) drugs in an adequate injection volume. Many CNS-active drugs are poorly water-soluble, because such drugs are typically highly lipophilic in order to enable penetration through the blood-brain barrier (Pardridge, 2005). I.p. administration of such drugs as suspension, as often done in preclinical studies, may lead to low and highly variable drug absorption compared to administration of drug solutions (Löscher et al., 1990), so we wanted to avoid drug suspensions. By using different solubilizing excipients and delivery strategies (Strickley, 2004; Turner et al., 2011; Kalepu and Nekkanti, 2015), all poorly water-soluble drugs used in this study could be injected parenterally as solution or emulsion. A further factor that had to be considered when preparing drug solutions for drug combinations was that the total injection volume should not exceed 10 ml/kg in mice to avoid volume effects (Löscher et al., 1990), which further complicated dissolving poorly water-soluble drugs in a sufficiently low vehicle volume.

4.4. Selection of doses and dosing intervals

As in our previous tolerability studies on other drug combinations (Klee et al., 2015), all drugs were administered at doses shown to exert disease-modifying effects when administered alone; in other words, we did not use lower dosages when combining these drugs. The reason for this was twofold. First, without almost any previous experience in the effects of combinatorial treatments in post-SE models of TLE, we wanted to start with drug combinations at doses that have a maximal chance to interfere with epileptogenesis in subsequent studies as illustrated in Fig. 2. Furthermore, based on our previous experience with the NBQX/ifenprodil combination (Schidlitzki et al., 2017), we expected that such combinations at the chosen dosages may cause serious adverse effects, particularly during the latent period, which then would lead to dose adjustments as shown in Fig. 2. Based on available pharmacokinetic data in rodents, all drugs except fingolimod were administered twice daily for the present experiments.

4.5. Duration of treatment

The duration of treatment with the drug combinations was restricted to 3 days, starting 16–18 h after kainate injection. As in our previous tolerability study of drug combinations for antiepileptogenesis (Klee et al., 2015), there were several reasons for restricting the duration of treatment to 3 days. First, for an antiepileptogenic treatment to be clinically manageable, it should be administered when the patient with a potentially epileptogenic brain injury is still hospitalized. Otherwise, compliance may be too low (Schmidt et al., 2014). Thus, the antiepileptogenic effect should be reached after a few days of treatment, or, even better, after one administration. We demonstrated previously that this is not completely unrealistic by showing that one low dose of the NMDA receptor antagonist MK-801 (injected shortly after diazepam) exerts marked disease-modifying effects in the kainate model of TLE when injected 90 min after onset of SE (Ebert et al., 2002; Brandt et al., 2003). Second, in a study by Lippman-Bell et al. (2013), twice daily treatment of neonatal rats with the AMPA receptor antagonist NBQX over only two days prevented development of epilepsy in a model in which epilepsy develops after hypoxia-induced neonatal seizures in rats. Third, when mice were treated with a combination of NBQX and the NMDA receptor antagonist ifenprodil over 5 days, starting 6 h after intrahippocampal kainate injection in adult mice, disease-modifying effects were observed (Schidlitzki et al., 2017). Fourth, accumulating experimental and clinical evidence indicates that the latent period and, thus, the window of opportunity to prevent epilepsy, may be much shorter than previously thought, necessitating starting antiepileptogenic treatment within the first hours after brain insult in a similar way as treatment of acute stroke (Löscher et al., 2015). For the antiepileptogenesis studies illustrated in Fig. 2 (Phase IIb), we plan to use a treatment period of 5 days.

4.6. Tolerability of the drug combinations

In the present study, 7 rationally chosen multitargeted combinations of 10 clinically approved drugs, using 2–4 drugs per combinations, were evaluated for tolerability and early signal of disease-modifying efficacy (i.e., neuroprotection) in naive mice and mice following SE, using the logistics of Phase I and Phase IIa of clinical trials. In previous

experiments we evaluated a drug combination of two glutamate receptor antagonists with different subreceptor selectivity for antiepileptogenic efficacy in large groups of mice, using the intrahippocampal kainate model of epilepsy. There was high mortality, which we had not foreseen because we had not first performed adequate tolerability assessment (Schidlitzki et al., 2017). Thus, we decided to start evaluation of novel drug combinations by evaluating the tolerability using a screening approach with relatively small groups of mice, before planning powered efficacy experiments. In our experience the group size was large enough to exclude any serious adverse effects or toxicity, such as marked loss of body weight or mortality. Six of the 7 drug combinations were sufficiently well tolerated at the selected doses both in naive mice (Phase I) and in mice during the latent epileptogenic period after kainate-induced SE (Phase IIa). This will allow us to evaluate the antiepileptogenic or disease-modifying potential of these drug combinations in future experiments.

4.7. Lack of neuroprotective effects of the drug combinations

Based on previous experiments in which several of the drugs used here were reported to exert neuroprotective activity in different rodent models of brain injury (Jain, 2011; Grupke et al., 2015; Loane et al., 2015; Walker, 2015), we thought that evaluating the drug combinations for neuroprotective activity would lead to an early signal of disease-modifying efficacy. However, unexpectedly none of the drug combinations reduced neuronal damage in the intrahippocampal kainate model, when drug treatment was started 16–18 h after kainate injection. One likely explanation for this finding is that the excitotoxic kainate exposure and/or the SE induced significant neurodegeneration in the hippocampus before onset of drug treatment, so the treatment started too late to reduce the neuronal damage. Indeed, in a model in which kainate was injected stereotactically into the cortex just above the right dorsal hippocampus and hippocampal slices were prepared 4 h and 6 h post kainate injection, FJC-positive dying neurons in the ipsilateral hippocampus were observed at these early time points (Bedner et al., 2015). Thus, in order to target this neuronal damage, treatment should start before 4 h after kainate. This, however, may lead to initial insult modification (Löscher and Brandt, 2010; Galanopoulou et al., 2012), i.e., by shortening the initial insult (SE) or decreasing its severity, thus reducing its long-term consequences. Instead the aim of our experiments is to identify novel combinations of drugs that prevent the development of epilepsy when administered during the latent period following an epileptogenic brain injury or at least modify epileptogenesis in such a way that the epilepsy that develops is milder, easier to treat, non-progressive and without cognitive decline and drug-resistance (Löscher and Brandt, 2010).

In addition to the possibility that treatment started too late after kainate, the lack of any significant neuroprotective effect may be due to the rapid elimination of most drugs in mice. This may lead to only transient maintenance of effective drug levels, even if relatively high doses of drugs are used. Thus, we plan to inject drugs three times daily (instead of two times daily) during the planned antiepileptogenesis experiments. Furthermore, the stress caused by the repeated tolerability assessment performed in the present study in Phases I and IIa may have had a negative effect on the neuroprotective activity of the treatments. This stress will be minimized in the planned Phase IIb efficacy studies.

It is important to note that the lack of any significant neuroprotective effect of the drug combinations tested here does not necessarily exclude that these combinatorial treatments exert antiepileptogenic or disease-modifying effects on the development of spontaneous seizures in the intrahippocampal kainate model. This will be evaluated in Phase IIb of our two-stage approach (Fig. 2). In this respect, it is interesting to note that the definition of “aniepileptogenesis” by Pitkänen and Engel (2014) does not include neuroprotection, but “antiepileptogenesis” is rather defined by a process that counteracts the effects of epileptogenesis, including prevention, seizure modification, and cure. Even though one may consider epilepsy-associated hippocampal damage part of the epilepsy syndrome, particularly in TLE, this damage may be more important for comorbidities such as cognitive decline than for development of spontaneous recurrent seizures (SRS). One example in this regard is our previous finding that the NMDA receptor antagonist MK-801, injected after kainate-induced SE, completely prevented hippocampal damage in part of the rats, but not the development of SRS (Brandt et al., 2003), which, to our knowledge, was the first published evidence that hippocampal damage is not a prerequisite of development of SRS.

4.8. Drug-drug interactions

In studies with multiple drugs, both pharmacodynamic and pharmacokinetic interactions can occur, often depending on the dose or concentration ratios of drugs to be combined (Zaccara and Perucca, 2014; Roberts and Gibbs, 2018). Given the fact that plasma concentrations were not measured in this study, pharmacokinetic drug-drug interactions can be a complex confounder. Thus, for promising drug combinations identified in subsequent efficacy studies (Fig. 2), pharmacokinetic drug-drug interactions should be assessed.

4.9. Two-stage approach for testing rationally chosen drug combinations for antiepileptogenic efficacy

In a recent commentary by the American Epilepsy Society Basic Science Committee and the International League Against Epilepsy Working Group on Recommendations for Preclinical Epilepsy Drug Discovery, it was highlighted that adverse effect reporting is often limited in preclinical studies on new treatments for epilepsy, including antiepileptogenic treatments (Galanopoulou et al., 2012), which may critically limit the successful translation of preclinical findings to the clinic. In our previous (Klee et al., 2015) and present studies, we addressed the complex issue of tolerability of drug combinations by a pragmatic approach as illustrated in Fig. 2. Multitargeted drug combinations that were found sufficiently tolerable by this approach will next be tested for antiepileptogenic potential in the intrahippocampal kainate mouse model of mesial TLE (Fig. 2). For this purpose, the combinations will be administered 3 times daily over 5 days, using a design that was recently described for a combination of NBQX and ifenprodil (Schidlitzki et al., 2017). If any of the present drug combinations exerts an antiepileptogenic effect, we will lower the doses to determine minimally needed doses for such an effect. Furthermore, drug plasma concentrations occurring at such doses will be determined to facilitate clinical translation (cf., Klein and Tyrlikova, 2017).

Rationally chosen drug combinations used for our approach are of course not limited to the drugs used in our previous (Klee et al., 2015) and present studies but may involve compounds with additional mechanisms to target the complex network involved in epileptogenesis (Löscher et al., 2013). The focal kainate mouse model of TLE was chosen for the first efficacy experiments (Phase IIb), because this model permits a “medium-throughput drug screening” due to a high frequency of spontaneous recurrent seizures allowing for short EEG recording periods (Klein et al., 2018). If a drug combination is effective in this model, it will be evaluated in a rat model of post-TBI TLE (Phase IIc), because it is presently not known whether antiepileptogenic drug effects identified in a post-SE model will translate to a TBI model or even to patients with TBI (Pitkänen et al., 2011). The goal to develop antiepileptogenic interventions would be greatly facilitated by the identification of reliable biomarkers of epileptogenesis that could be used to create cost-effective, high-throughput screening models for potential antiepileptogenic compounds, as well as enrich patient populations and serve as surrogate endpoints for clinical trials (Pitkänen and Engel, 2014). We have recently found that alterations in seizure threshold and behavior predict development of epilepsy in the rat pilocarpine model (Brandt et al., 2015; Bröer and Löscher, 2015), so we would start with

these phenotypic biomarkers.

4.10. Conclusions

In conclusion, using a study design developed by us for preclinical testing of multitargeted combinations of repurposed drugs, we report results of Phase I and Phase IIa evaluation of 7 combinations of 10 drugs in mice, demonstrating that 6 of these combinations are well tolerated at the selected doses, thus allowing proceeding to Phase IIb efficacy testing (Fig. 2). Compared to the tolerability studies reported here, evaluation of antiepileptogenic or disease-modifying potential of the drug combinations is much more laborious and time-consuming, because relatively large groups of animals have to be treated, and because the animals have to be followed with continuous video-EEG monitoring to identify any antiepileptogenic or disease-modifying effect, as recently described for the NBQX/ifenprodil combination (Schidlitzki et al., 2017). The latter study and the favorable outcome of a subsequent preclinical antiepileptogenesis trial with another drug combination (Schidlitzki et al., 2018) indicates that the study design developed by us for preclinical drug testing results in new, effective drug cocktails with the potential for relatively rapid translation to clinical trials. Together with our previous studies (Klee et al., 2015; Schidlitzki et al., 2017), we have now characterized the tolerability of 19 repurposed drugs (see Fig. 1) in 11 novel drug combinations, thus providing a rich collection of network-based combinatorial therapies as a basis for antiepileptogenic or disease-modifying efficacy testing.

Conflictof interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eplepsyres.2019.02.010.

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