RESEARCH ARTICLE

Flupirtine and diazepam combination terminates established status epilepticus: results in three rodent models

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Abstract

Objective: Status epilepticus (SE) is a neurological emergency requiring rapid termination of seizures. New treatment choices are needed for benzodiazepine-refractory SE or established SE (ESE). Previous studies have demonstrated that the potassium-channel opener flupirtine terminates seizures in neonatal animals. However, its effectiveness in adult ESE has not been tested. We tested whether flupirtine alone or in combination with the benzodiazepine diazepam would terminate ESE in three animal models. **Methods**: SE was induced by administration of lithium followed by pilocarpine, by electrical stimulation of the hippocampus or by diisopropylfluorophosphate (DFP) administration. Seizures were assessed by EEG recorded from the hippocampus and cortex. **Results**: Flupirtine alone did not terminate ESE within 60 min of administration in any of the three models of ESE. A combination of flupirtine and diazepam terminated ESE within 60 min in all the three models. The drug combination shortened the duration of ESE in all three models. Drug responsiveness was distinct between each model. **Conclusion**: A combination of the potassium channel opener flupirtine and diazepam is a potential therapy for ESE.

Introduction

The International League Against Epilepsy taskforce on classification of status epilepticus recently redefined status epilepticus (SE) as a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms that lead to abnormal, prolonged seizures (after time point t1). SE is a condition that can have long-term consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures.[1] SE afflicts 120,000-160,000 Americans each year and has a high rate of all-cause mortality (17%). This is especially so in the elderly, in whom the rate of all-cause mortality in the 30 days following an episode of SE is double that of the general population (38%).[2,3] In children, prolonged febrile SE can cause increased hippocampal T2 signal on MRI,[4] focal slowing on EEG[5] and even the development of epilepsy and mesial temporal sclerosis.[6] The rate of adverse outcomes of SE correlates with the duration of seizures; thus, early termination of SE is important.[7]

As reflected in the definition, SE is a dynamic condition in which a delay in effective control of seizures could lead to refractoriness and development of long term effects.[8] Benzodiazepines are an effective initial treatment for status epilepticus (SE).[9,10] However, in as many as 1/3rd of patients, seizures may continue despite treatment with adequate doses of benzodiazepines, a condition now referred to as established SE (ESE).[11] More effective therapies are needed for the termination of ESE.

An earlier study suggested that seizures in neonatal rats could be terminated with the anticonvulsant and analgesic drug flupirtine.[12] Flupirtine opens M-type potassium channels and facilitates GABA-A receptor function.[13] The activation of potassium channels leads to neuronal membrane hyperpolarization, and drugs that open the M-type (K_V 7.2-3, KCNQ2/3 potassium channels) are effective anticonvulsants.[14] Mutations of the KCNQ2/3 genes lead

to benign familial neonatal convulsions and epileptic encephalopathy.[15,16]

Previous studies did not address whether flupirtine can terminate established SE. The stage of SE (established SE vs. SE) and the method used to induce SE may impact the response to flupirtine. In addition, the previous studies were performed in neonatal animals, and KCNQ2/3 channel and GABA-A receptor expression change during brain development.[17,18] We tested whether flupirtine administered alone or in combination with the benzodiazepine diazepam can terminate or shorten established SE in adult animals.

Methods

All procedures on animals were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC). Adult male Sprague-Dawley rats weighing 250–325 g were housed with food and water ad libitum. Animals were stereotaxically implanted with bipolar insulated stainless steel electrodes in the left posterior ventral hippocampus, bilateral cortical electrodes, and a cerebellar reference electrode under ketamine/xylazine anesthesia and secured to the skull with dental acrylic, as described previously.[19,20] Electrode implantation was performed for each model and SE was induced 1 week later. Three methods for inducing SE were used in this study: lithium/pilocarpine, continuous hippocampal stimulation (CHS), and diisopropyl fluorophosphate (DFP). These methods have been described previously.[21–23] All chemicals were obtained from Sigma, St. Louis, MO, unless specified otherwise.

Lithium/pilocarpine method

The rats were administered 3 mmol/kg lithium chloride intraperitoneally (i.p.), and 20 h later, SE was induced by i.p. injection of 50 mg/kg pilocarpine. Thirty minutes prior to pilocarpine administration, 2 mg/kg scopolamine was given to each rat to reduce the peripheral effects of the pilocarpine. EEG activity was monitored continuously for 6 h, and behavior was observed visually. Only those animals that developed a stage 5 seizure on the Racine scale[24] within 60 min of pilocarpine administration were included in the study. Hippocampal and cortical electrodes were used to record seizures; both the electrodes showed similar EEG responses and hippocampal electrodes were used to determine the onset and end of SE.

Continuous hippocampal stimulation

Status epilepticus was induced with 1 msec duration biphasic square wave electrical pulses (50 Hz) in 10 sec trains applied every 11 sec for 90 min. Seizure activity following stimulation was monitored continuously for 6 h by electroencephalogram (EEG) recordings. Only those animals that continued to have continuous seizures (electrographic score III) for 30 min after stimulation were included in the study. This method has been described in detail in the past.[20] Seizures during the stimulation were recorded via cortical electrodes and both the hippocampal and cortical electrodes were used to record seizures after stimulation had ended.

DFP

The animals were pretreated with an oxime, pyridine-2-aldoxime methochloride (2-PAM, 50 mg/kg, interaperitoneal, i.p.) iodide and muscarinic receptor antagonist atropine (2 mg/kg, i.p.), 30 min before subcutaneous (s.c.) administration of DFP according to the protocol set up by the Department of Defense for the use of nerve agents in laboratory animals.[25] 2-PAM sequesters acetylcholine esterase, protecting it from organophosphate binding and atropine blocks peripheral muscarinic receptors, respectively.[26,27] EEG recording was initiated prior to administration of atropine and 2-PAM according to the guidelines. Both 2-PAM and atropine were dissolved in solution within one hour of injection. DFP was mixed into cold saline immediately prior to injection.

The evolution to ESE occurred in each model as described below in the results section. EEG activity was recorded continuously for at least 5 h following drug injection and inspected visually. ESE was considered terminated when the following two conditions were met: (1) the EEG returned to normal baseline or showed arrhythmic spikes (<2 Hz), and (2) there was no recurrence of electrographic or behavioral seizures in 1 h. All drugs were administered intraperitoneally unless noted otherwise.

We tested whether flupirtine alone or a combination of flupirtine and diazepam terminated ESE within 60 min of drug administration. The difference in proportions of seizure free animals was determined by Fisher's exact test. We also determined the mean duration of ESE following administration of a single dose of a drug or drug combination. This was compared with the duration of ESE following vehicle treatment (control) using a one-way ANOVA with a post hoc Tukey's test for multiple comparisons. Only drug versus control comparisons are reported.

The data were normally distributed except those for the combination therapy in DFP and lithium/pilocarpine models. Since mean and ANOVA assume normal distribution of observed population, which was not the case for animals treated with combination therapy in two of the three models tested, we also performed nonparametric Kaplan–Meier analysis with a pair-wise log-rank test to compare flupirtine (50 mg/kg) and diazepam
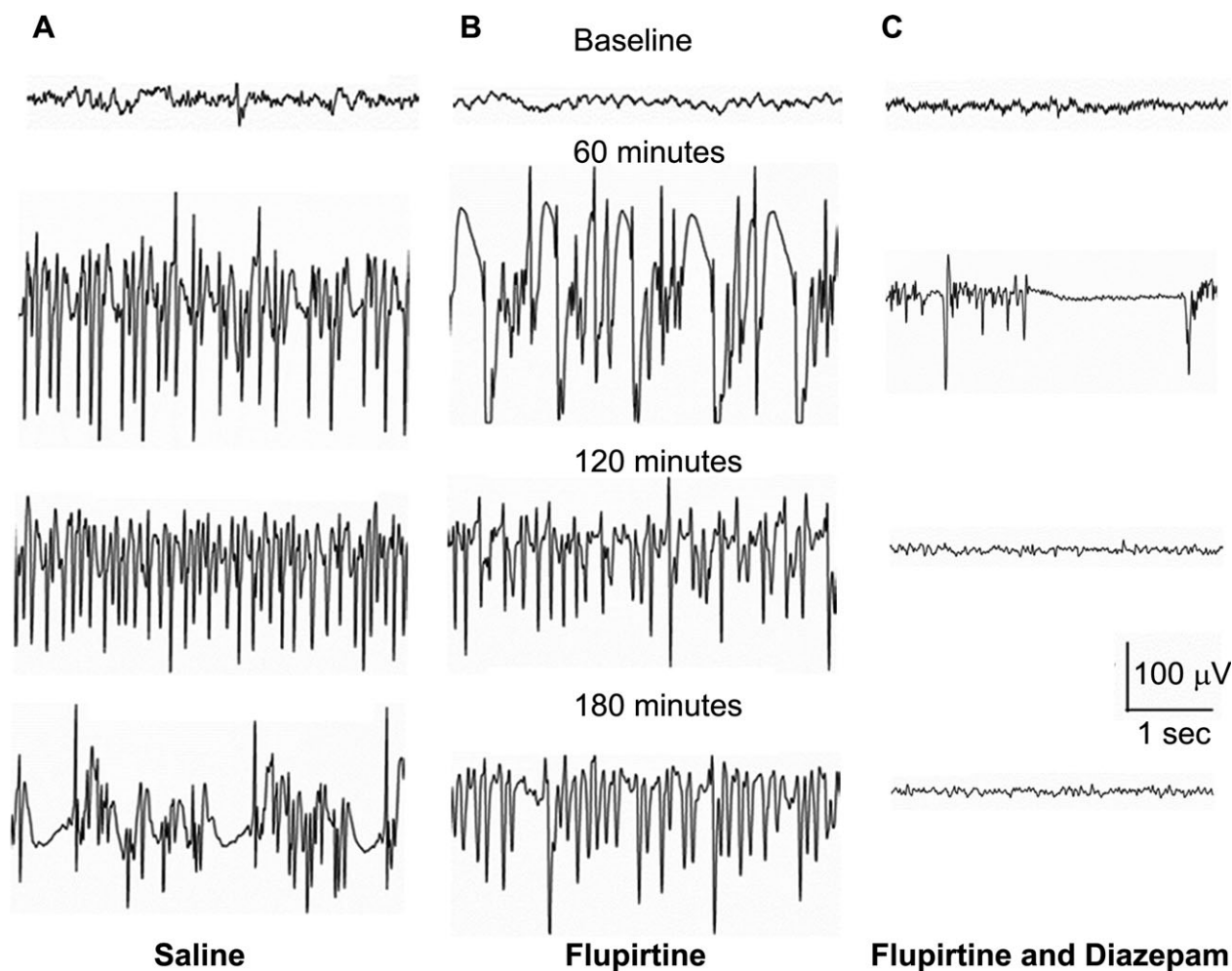
Figure 1. Li/pilocarpine-induced ESE. (A) EEG recording of seizures induced by pilocarpine in a lithium-pretreated animal, obtained from a bipolar metal electrode implanted in the left hippocampus. Vehicle was administered 10 min after the onset of continuous electrographic seizure activity and seizures continued unabated for 3 h. (B) EEG traces recorded from an animal treated with flupirtine (50 mg/kg). Seizures continued unabated for 3 h following flupirtine injection. (C) Treatment with a combination of flupirtine and diazepam. Seizures were terminated within 60 min, replaced by irregular spiking and suppression, followed by normal baseline EEG for the following 2 h.

(10 mg/kg) combination therapy with saline-treated controls in the three models.

Results

The initial evaluation of the effect of flupirtine treatment on ESE was performed in the lithium/pilocarpine model. Diazepam (10 mg/kg) or vehicle was administered 10 min after onset of continuous electrographic seizures, which also corresponded to the first stage 5 behavioral seizure. The onset of continuous electrographic seizures is associated with benzodiazepine resistance,[28] and this was confirmed in our sample. Onset of continuous electrographic seizure activity was associated with high-frequency spike-wave discharges and convulsive seizures (Racine behavioral seizure score 4 or 5).[24] Diazepam or

vehicle (control) was administered 10 min after the onset of continuous electrographic seizure activity. Electrographic and behavioral seizures continued unabated after treatment with diazepam (Fig. 2A). SE was not terminated in any animal within 60 min, as previously reported (0/5 animals in each group, $P > 0.05$, Fig. 2A).[22,28] Diazepam did not shorten the duration of seizures ($P > 0.05$). Thus, animals were in ESE 10 min after the onset of continuous electrographic seizures.

In this and subsequent experiments, flupirtine was administered 10 min after the onset of continuous electrographic seizure activity, since this marked the onset of benzodiazepine-refractory SE or ESE. Based on a previous study,[12] an initial dose of 50 mg/kg flupirtine (i.p.) was used to treat ESE. This dose did not terminate lithium/pilocarpine-induced ESE in any animal (0/5 animals,

Fig. 1 and 2A and C). Flupirtine (50 mg/kg) also did not reduce the mean SE duration (7.86 ± 1.5 h, $P > 0.05$, Fig. 2C). We then tested whether a 75 mg/kg dose would be effective, but it neither terminated nor shortened ESE (0/6 animals, seizure duration 8.07 ± 1.8 h, $P > 0.05$ for each, Fig. 2C). Thus, flupirtine treatment alone did not effectively treat lithium/pilocarpine-induced ESE.

A combination of diazepam with flupirtine could control ESE. Animals ($n = 7$) were treated with a combination of diazepam (10 mg/kg) and flupirtine (50 mg/kg). This combination reduced the frequency and amplitude of discharges, with periods of suppression. The discharges disappeared and normal EEG activity was restored (Fig. 1).

This flupirtine and diazepam combination terminated ESE within 1 h (from treatment) in five out of seven animals ($P < 0.05$, Fisher's exact test, Fig. 2A–C). The combination therapy reduced the mean duration of SE in this group (1.03 ± 0.51 h, $P < 0.05$, Fig. 2C). The data for the duration of SE in flupirtine (50 mg/kg) and diazepam (10 mg/kg) were not normally distributed. Hence, a

Kaplan–Meyer survival plot of animals treated with saline and combination therapy diazepam (10 mg/kg) and flupirtine (50 mg/kg) was plotted, and demonstrated a significant effect of therapy (Fig. 2B, $P = 0.0011$ Mantel–Cox log-rank test).

Diazepam (10 mg/kg) in combination with a lower dose of flupirtine (25 mg/kg) did not terminate ESE or shorten its duration (0/5 animals, duration 3.13 ± 0.5 h, $P > 0.05$, Fig. 2A and C). When diazepam (10 mg/kg) was administered in combination with a higher dose of flupirtine (75 mg/kg), all four animals died (15.6 ± 2.8 min). There was burst suppression present on EEG prior to death. Thus, combination treatment with flupirtine (50 mg/kg) and diazepam (10 mg/kg) could terminate lithium/pilocarpine-induced ESE within a narrow therapeutic window.

Electrical stimulation model

We then tested flupirtine in an electrical stimulation model of ESE, the continuous hippocampal stimulation
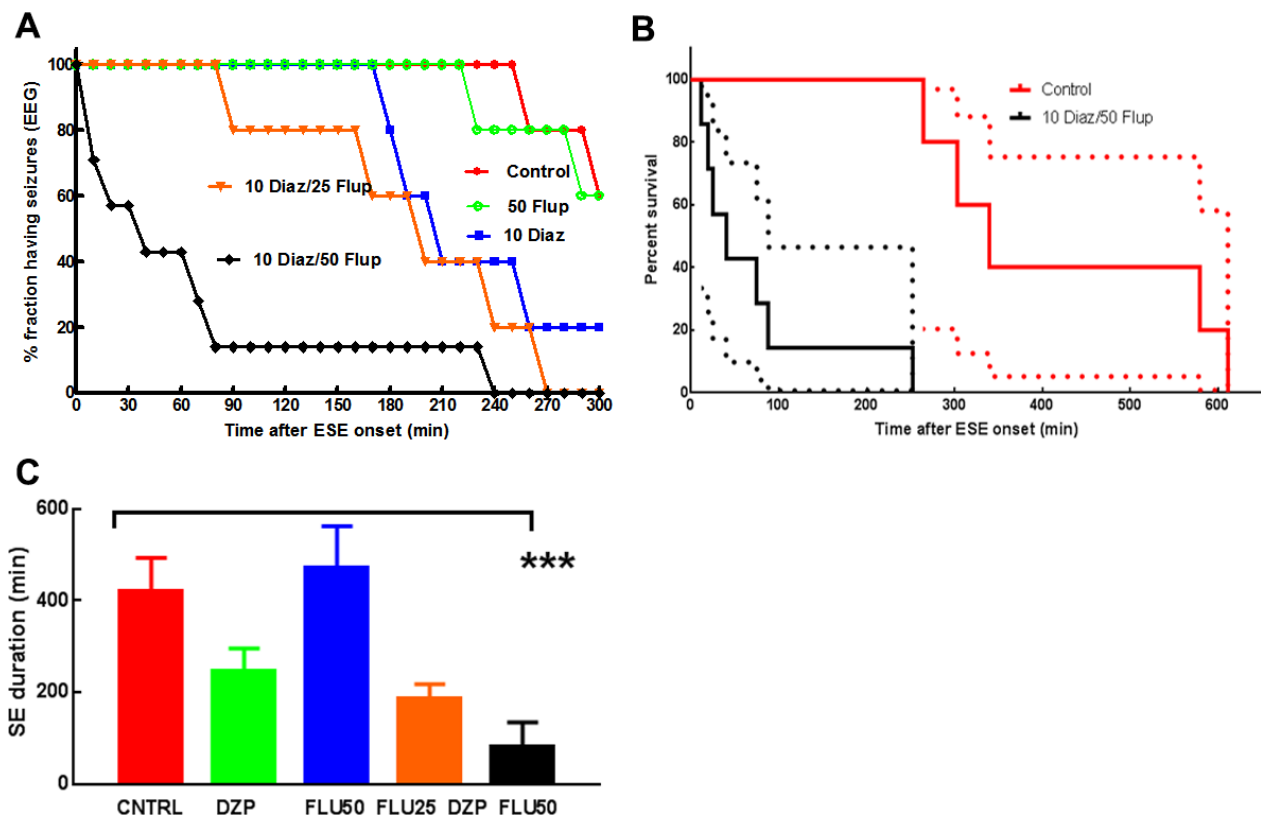

Figure 2. Flupirtine treatment of ESE induced by Li/pilocarpine. (A) Plot of the percentage of animals continuing to experience electrographic seizures over time: control (red line), (flupirtine 50 mg/kg, green), diazepam (10 mg/kg, blue), a combination of flupirtine (25 mg/kg) and diazepam (10 mg/kg, orange) and a combination of flupirtine (50 mg/kg) and diazepam (10 mg/kg). (B) The data for the duration of SE in flupirtine (50 mg/kg) and diazepam (10 mg/kg) combination therapy were not normally distributed. Hence, a Kaplan–Meyer survival plot of animals treated with saline and combination therapy was plotted. (C) The mean and SEM seizure duration for each treatment was compared with that for the saline control treatment using ANOVA with a post hoc Tukey's multiple comparison test, ***$P < 0.001$.
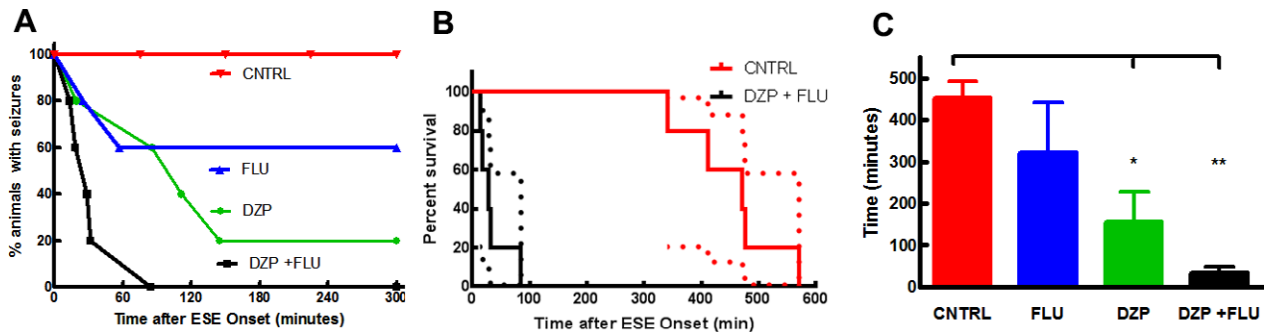
Figure 3. Flupirtine treatment of ESE induced with the CHS method. (A) Plot of the percentage of animals continuing to have seizures over time. (B) A Kaplan–Meyer survival plot comparing the duration of SE in saline-treated and diazepam and flupirtine combination-treated animals. (C) The mean and SEM seizure duration in animals treated with saline (control), flupirtine, diazepam or a combination of flupirtine and diazepam was compared using ANOVA with a post hoc Tukey's multiple comparison test, *$P < 0.05$, **$P < 0.01$.

(CHS) model.[20] After 90 min of electrical stimulation of the hippocampus, seizures continued for 6–9 h ($n = 5$, seizure duration 7.73 ± 0.6 h, Fig. 3C). Treatment with diazepam (10 mg/kg) 10 min after the onset of continuous electrographic seizure activity terminated seizures in only 1/5 animals ($P > 0.05$, Fig. 3A and C), but it shortened the duration of ESE (2.62 ± 1.64 h, $P < 0.05$, Fig. 3C).

We then tested the effect of flupirtine (50 mg/kg, $n = 5$) on CHS-induced ESE. In 2/5 animals, flupirtine terminated ESE within 60 min ($P > 0.05$, Fig. 3A and C). The mean duration of ESE in this group was not reduced (5.39 ± 2 h, $P > 0.05$, Fig. 3C). Treatment with diazepam and flupirtine in combination terminated ESE within 1 hour in 4/5 animals ($P < 0.05$, Fig. 3A and B). Kaplan–Meyer analysis revealed that combination therapy significantly reduced the duration of SE ($P = 0.0018$, Mantel–Cox log-rank test). The mean duration of ESE in the combination treatment group was 35.4 ± 12.9 min ($P < 0.05$, Fig. 3C). Thus, CHS-induced ESE was

effectively terminated with a combination of diazepam and flupirtine.

Organophosphate-induced SE

Next, we tested the efficacy of flupirtine (50 mg/kg) alone or in combination with diazepam (10 mg/kg) in terminating DFP-induced ESE. DFP is an organophosphate inhibitor of cholinesterase, and its analogs have been used as nerve agents.[23,29] Seizures in response to DFP were variable, and only those animals that developed continuous electrographic seizures with behavioral signs were selected for further study. We have previously reported that when diazepam (10 mg/kg) was administered 30 min after the start of continuous EEG seizure activity in this model, SE was not terminated within 60 or 120 min of seizure onset in any of the five animals tested, demonstrating the development of ESE.[23] The mean time to onset of continuous electrographic seizures was 18.5 ± 6.5 min ($n = 5$), and the duration of ESE,
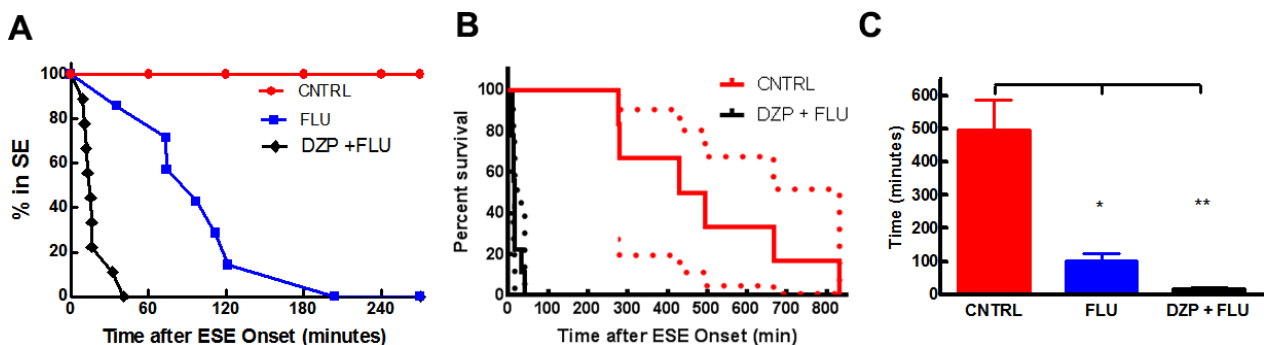

Figure 4. Flupirtine treatment of ESE induced with the DFP method. (A) Plot of the percentage of animals continuing to have seizures over time. (B) Because the duration of SE in combination therapy-treated animals were not normally distributed, a Kaplan–Meyer survival plot of SE duration was plotted for saline- and combination therapy, diazepam (10 mg/kg) and flupirtine (50 mg/kg)-treated animals. (C) The mean and SEM of seizure duration in animals treated with saline (control), flupirtine, or a combination of flupirtine and diazepam was compared using ANOVA with a post hoc Tukey's multiple comparison test, *$P < 0.05$, **$P < 0.01$.

beginning from onset of continuous electrographic seizures, was 8.56 ± 1.5 h (Fig. 4C). The tested drugs were administered 30 min after onset of continuous electrographic seizure activity.

Flupirtine (50 mg/kg) terminated DFP-induced ESE in only 1/6 animals within 60 min of treatment ($P > 0.05$, Fig. 4A), but it shortened DFP–induced ESE to 1.71 ± 0.3 h ($P < 0.05$, Fig. 4C). Combined treatment with diazepam (10 mg/kg) and flupirtine (50 mg/kg) was highly effective in terminating DFP-induced SE. All animals ($n = 9$) were seizure free within 60 min ($P < 0.05$, Fig. 4A and C), and the mean duration of SE was shortened (18.6 ± 3.6 min, $P < 0.05$, Fig. 4C). Kaplan–Meyer analysis also revealed that combination therapy significantly reduced the duration of SE ($P = 0.0002$, Mantel-Cox log-rank test). Thus, flupirtine in combination with diazepam was highly effective in terminating DFP-induced ESE.

Discussion

The primary goal of ESE treatment is prompt termination of seizures to limit adverse consequences that include hippocampal neuronal injury, cognitive and systemic adverse effects and increased mortality.[30] We found that a combination of diazepam and flupirtine rapidly terminated ESE (diazepam-refractory SE) in three different animal models. The combination of a potassium channel opener and a benzodiazepine could be a candidate therapy for patients who develop ESE.

In this study, flupirtine alone was ineffective in rapidly terminating ESE in all three models of ESE in adult rats, but in contrast, flupirtine was effective in terminating both kainate-induced and hypoxia-ischemia-induced seizures in P10 pups. Flupirtine was also more effective than diazepam or phenobarbital in the P10 kainate-induced seizure model.[12] Similarly, when flupirtine was administered to P10 animals prior to hypoxic and ischemic insult, seizures were prevented.[31] Finally, flupirtine could also terminate seizures when administered after the insult.[32] These differences in the efficacy of flupirtine likely result from differences in the developmental stage of the animal. KCNQ2/3 channel and GABA-A receptor expression in the brain is developmentally regulated.[17,33] Furthermore, this study tested treatment efficacy in ESE, a specific stage of SE in which benzodiazepines are ineffective.

It is likely that flupirtine is effective in treating SE through its actions on potassium channels and GABA-A receptors, as it has long been reported to open potassium channels. Initial studies suggested that it opened an inwardly rectifying potassium channel. More recent studies have indicated that it preferentially opens Kv7.2/3 channels. Flupirtine also potentiates GABA-A receptors.[13] It is important to consider that the therapeutic range of this drug varies depending on its nociceptive action. Multiple actions of flupirtine have been reported in the last 30 years, and many of these occur at concentrations ten to one hundred times higher than the therapeutic range observed in vivo (from 1 to 10 μmol/L).

Kv7.2/3 channels are critically involved in neuronal excitability and some forms of cholinergic excitation.[34] These channels are present at the axon initial segment and nodes of Ranvier, where they can modulate action potential generation and propagation.[35,36] These channels are inhibited by cholinergic muscarinic stimulation through the depletion of inositol diphosphate (IP2).[37,38] The cloning and identification of the molecular constituents of M channels has led to the development of a series of drugs that activate these channels. Three are well investigated, flupirtine, retigabine, and BMS 204352.[39] Retigabine was originally synthesized as a GABA-A receptor agonist, was later shown to open M channels at lower, therapeutic concentrations. Retigabine is an effective anticonvulsant in animal models of seizures, including maximal electrical shock, kindling model, audiogenic seizure model, and in in vitro seizures.[40,41] Retigabine was approved for clinical use as add on therapy for partial seizures. Retigabine has not been tested for the treatment of SE. Two other drugs are available to open potassium channels, flupirtine and BMS-204352. Flupirtine is structurally similar to retigabine, whereas BMS-204352 (Maxipost) is chemically distinct.[39]

Flupirtine provided additional benefit in terminating ESE when used in combination with diazepam, which served to hasten the termination of ESE. Thus, none of the animals treated with diazepam or flupirtine alone were seizure free within 3 h of onset of Li/pilocarpine-induced SE, but 80% were seizure free when treated with the two drugs in combination. Similarly, in the CHS and DFP models of ESE, the combination of flupirtine and diazepam acted faster than either drug alone. We determined SE termination within an hour of drug administration because intraperitoneal route for drug administration was used, which has slows drug absorption. In contrast, seizure termination within minutes from the onset of treatment is desirable under clinical settings, this could be obtained in the experimental setting with an intravenous drug administration.

A high dose of flupirtine used alone (75 mg/kg), for the treatment of SE caused respiratory arrest leading to death in the animals. This respiratory arrest could be due to actions of the drug on GABA-A receptor, KCNQ channels. It is possible that either one of these actions caused respiratory depression alone or in combination. In addition, SE can cause cardiorespiratory depression.

There is growing evidence that SE can be terminated more effectively by a combination of drugs that show synergistic action. It was previously reported that combinations of a benzodiazepine with ketamine or MK801 and valproate were more effective and less toxic than benzodiazepine monotherapy in this model of SE.[22,42] Similarly, treatment with the anticholinergic agent scopolamine combined with phenobarbital terminated refractory SE.[43] Recent studies have found that AMPA receptor antagonists such as perampanel or GYKI-52466 are also able to terminate seizures of SE in experimental animals.[44–46] Similar to the flupirtine-diazepam treatment used in this study, ketamine-diazepam treatment is also effective in terminating benzodiazepine-refractory SE.[22] The efficacy of ketamine-diazepam appears to be better than flupirtine-diazepam as 100% animals were seizure-free within one hour of drug administration (ketamine 50 mg/kg and diazepam 20 mg/kg).

Flupirtine alone was more effective in terminating CHS- and DFP-induced ESE than it was in terminating Li/pilocarpine-mediated ESE, suggesting a differential ESE model-dependent response to flupirtine. A similar ESE model-dependent response was reported for NMDA antagonists. CHS-induced ESE was terminated by ketamine and other NMDA antagonists, but these drugs were ineffective against Li/pilocarpine-induced SE.[21,22,47] When translating this evidence into clinical practice, it is possible that the etiology of ESE has an important role in determining the response to therapy. Emerging literature on refractory and super-refractory SE supports the notion that the ability of a particular treatment to terminate ESE is dependent on the underlying etiology of ESE.[48]

Author Contributions

Terry Zhang, Marko Todorovic and John Williamson performed experiments, analyzed EEG. Jaideep Kapur planned the study analyzed data, and wrote the manuscript.

Acknowledgments

Conflict of Interest

Jaideep Kapur has served as a consultant for EISAI pharmaceuticals. None of their products are discussed here.

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