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RESEARCH ARTICLE

In vitro modulation of seizure-like activity with beta-cyclodextrin-complexed rufinamide

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Objective: The global health concern of pharmacoresistant epilepsy necessitates innovative therapeutic strategies. Drug resistance often arises due to complex pharmacokinetic challenges. Beta-cyclodextrin, known for enhancing drug solubility and stability, offers a potential solution for improving the efficacy of antiseizure medications. This study aims to investigate the impact of beta-cyclodextrin-complexed rufinamide on seizure-like activity using an in vitro model of temporal lobe epilepsy.

Methods: Seizure-like neuronal activity was induced using a low-magnesium model. Local field potentials were recorded from transverse rat hippocampal slices. Rufinamide was solubilized using beta-cyclodextrin and administered at 100 micromolar concentration. The impact on various seizure-like parameters and time-resolved phase-amplitude coupling was assessed.

Results: Rufinamide increased the duration of the preictal phase while reducing the duration of ictal and postictal phases. The frequency of seizure-like events was higher in rufinamide. No significant change was observed in the firing rate of the first 10 ictal spikes, but the firing frequency of the second set of 10 ictal spikes was higher during rufinamide perfusion. Time-resolved phase-amplitude coupling maximum analysis did not reveal significant differences between the control and rufinamide treatment.

Conclusions: Beta-cyclodextrin-solubilized rufinamide significantly modulates seizure-like event dynamics, exhibiting both anticonvulsant and proconvulsant effects. While the compound shortened seizure-like activity, it increased the frequency of seizure-like events. Our observations suggest a need for further investigation into the solubilization method and its impact on rufinamide's bioavailability. Dose-dependent effects and underlying molecular mechanisms should also be explored to enhance the pharmacological properties of antiseizure medications.

Keywords: temporal lobe epilepsy, beta-cyclodextrin, rufinamide, *in vitro* epilepsy model, electrophysiology

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Introduction

Temporal lobe epilepsy is a prevalent neurological disorder characterized by recurrent, spontaneous seizures, frequently originating in the hippocampus, which results from excessive and synchronous neuronal activity [1]. Despite the availability of various antiseizure medications (ASMs), achieving optimal seizure control remains challenging for approximately one-third of the patients; this creates the need for continuous exploration of drug mechanisms and their effects on neural dynamics [2].

Rufinamide (RUF) is an ASM known for its efficacy in treating various types of seizures. It was approved in 2008 for the treatment of seizures associated with Lennox-Gastaut syndrome in patients aged 4 years and older, but it can also be used as adjunctive therapy for other types of epilepsy, including refractory focal seizures [3]. RUF is a triazole derivative, structurally different from other currently marketed ASMs, acting primarily by blocking the inactivated voltage-gated sodium channels, thereby preventing the generation of high-frequency action potentials and seizure propagation [4]. However, like many ASMs, RUF's clinical utility can be limited by its poor solubility in aqueous solutions, affecting its bioavailability and therapeutic effectiveness [5]. RUF's pharmacokinetic profile includes an inconsistent absorption rate, moderate protein binding, and a half-life that supports twice-a-day dosing with a variable therapeutic outcome. Peak plasma concentrations are achieved 4-6 hours after administration. It is metabolized in the liver and is primarily excreted in the urine [6].

Improving the solubility and bioavailability of many ASMs is crucial for enhancing their therapeutic potential. β-Cyclodextrin (β-CD), a cyclic oligosaccharide, has been widely used to improve the solubility of poorly soluble drugs through the formation of inclusion complexes [7]. By encapsulating hydrophobic molecules within its hydrophobic cavity, β-CD can significantly improve the aqueous solubility and stability of these drugs [8].

Previous studies have demonstrated the potential of cyclodextrin complexation to enhance the solubility of RUF. Szabó et al. (2017) studied the complexation of RUF with various cyclodextrins, including β-CD. They found that the complexation significantly improved the aqueous solubility of RUF, highlighting the potential for enhanced bioavailability and therapeutic efficacy of this method [9].

In this study, we investigated the effects of the RUF-β-CD complex on a low-magnesium *in vitro* model of

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temporal lobe epilepsy, a well-established method used for pharmacological testing. The removal of magnesium ions from the extracellular solution reduces the inhibition of NMDA receptors, thereby facilitating the occurrence of spontaneous and recurrent seizure-like events (SLEs) [10].

Our primary objective was to evaluate the impact of β-CD-solubilized RUF on the duration of the preictal, ictal, and postictal phases of SLEs, seizure frequency, and discharge frequency at the onset of seizures.

Additionally, we analyzed time-resolved phase-amplitude coupling (tPAC) to better understand the impact of RUF on the oscillatory behavior of hippocampal neurons. tPAC provides a dynamic measure of coupling between low and high frequency bands, capturing how this relationship evolves over time [11].

Methods

Animals

The Ethics and Research Committee of the University of Medicine, Pharmacy, Science, and Technology of Târgu Mureș approved all procedures involving laboratory animals (approval number: 1621/24.02.2022). Male Wistar rat pups (P7-13) were used in this experiment.

Chemicals and Solutions

All chemicals utilized for the preparation of solutions were procured from Sigma Aldrich (St. Louis, Missouri, USA). The artificial cerebrospinal fluid (ACSF) was used in three different formulations:

– Preparing ACSF (pACSF): NaCl 87 mM, saccharose 75 mM, glucose 25 mM, $NaHCO_3$ 25 mM, $MgSO_4$ 7 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 0.5 mM.
– Normal ACSF (nACSF): NaCl 129 mM, $NaHCO_3$ 21 mM, glucose 10 mM, KCl 3 mM, $MgSO_4$ 1.8 mM, $CaCl_2$ 1.6 mM, NaH_2PO_4 1.23 mM.
– Magnesium-Free ACSF (0MgACSF): similar to nACSF, but lacking $MgSO_4$ and with KCl increased to 5 mM to induce epileptiform activity.
– β-CD was dissolved in 0MgACSF at a 1% concentration to enhance the solubility of RUF. Then RUF was added to 0MgACSF at a concentration of 100 μM. The solution was continuously stirred at room temperature for 24 hours before use to ensure effective complexation, according to Szabó et al. (2017) [9].

Tissue Preparation

Rat pups were euthanised by quick decapitation, the brains were fastly removed and immersed in ice-cold nACSF. The frontal lobe and cerebellum were sliced off, and the remaining brain block was secured to a vibratome stage (Leica VT1000S, Leica Biosystems, Deer Park, IL, USA) using ethyl 2-cyanoacrylate. The block was submerged and sectioned into 400 μm thick transverse slices in an ice-cold pACSF. The hippocampal formations (including the dentate gyrus, Cornu Ammonis 1, 2 and 3 regions, and subiculum) were isolated using micro-scissors.

Incubation and Recording Setup

The hippocampal slices were incubated for 1 hour at 36°C and were then kept at room temperature in an interface-type holding chamber, previously filled with nACSF and bubbled with a carbogen gas mixture (5% CO_2–95% O_2). Each slice was transferred to a perfusion chamber inside a Faraday cage to prevent ambient electromagnetic interference. The slices were continuously perfused with carbonated and prewarmed (36°C) nACSF using a dual-channel in-line solution heater (TMP 5b, SuperTech, Switzerland) and a peristaltic pump (Minipuls 3, Gilson Medical Electronics, Villiers-le-Bel, France) at a 3 ml/min rate.

Recordings started with an accommodation period of 5 minutes in nACSF in order to record the baseline activity of the neuronal networks. It was followed by 0MgACSF perfusion, and the occurrence of at least five SLEs was monitored. Then, the sections were perfused with β-CD-complexed RUF dissolved in 0MgACSF. After recording at least five SLEs or, in their absence, after 20 minutes of exposure to the drug, we initiated a washout using 0MgACSF in order to assess the reversibility of RUF's effects and to confirm tissue viability in the event of complete suppression of epileptiform activity. The protocol concluded with a final washout using nACSF.

Electrophysiological Recordings

Local field potentials were recorded using microelectrodes (resistance ranging from 3 to 8 MΩ). These microelectrodes were fabricated from glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL, USA) using a two-stage pipette puller (PUL-2, WPI, Sarasota, FL, USA) and filled with nACSF. The tip of the electrode was inserted into the CA3 region of the hippocampus under microscopic guidance utilizing a hydraulic micromanipulator (WR88, Narishige, Tokyo, Japan). Recordings were conducted at a 5 kHz sampling rate with an amplifier (BioAmp SBA1-v6, SuperTech, Switzerland) equipped with a nominal preamplifier (bandwidth 0.16 Hz–2 kHz, 2k gain), and an integrated 50 Hz notch filter. The data were digitized using an A/D card (PCI 6036E, National Instruments, Austin, TX, USA).

Data Analysis

The recorded data were initially stored in .txt format on a computer hard disk for offline analysis and then converted to .smrx format, which is compatible with the data processing program (Spike 2 v8.01c, Cambridge Electronic Design). The transitions between perfused solutions were marked on the recordings (Figure 1). The preictal, ictal, postictal, and interictal periods, which are characteristic of SLEs, were identified, delineated, and measured (Figure 1). Phase identification was carried out based on the frequency variations of discharges (spikes). The preictal phase was

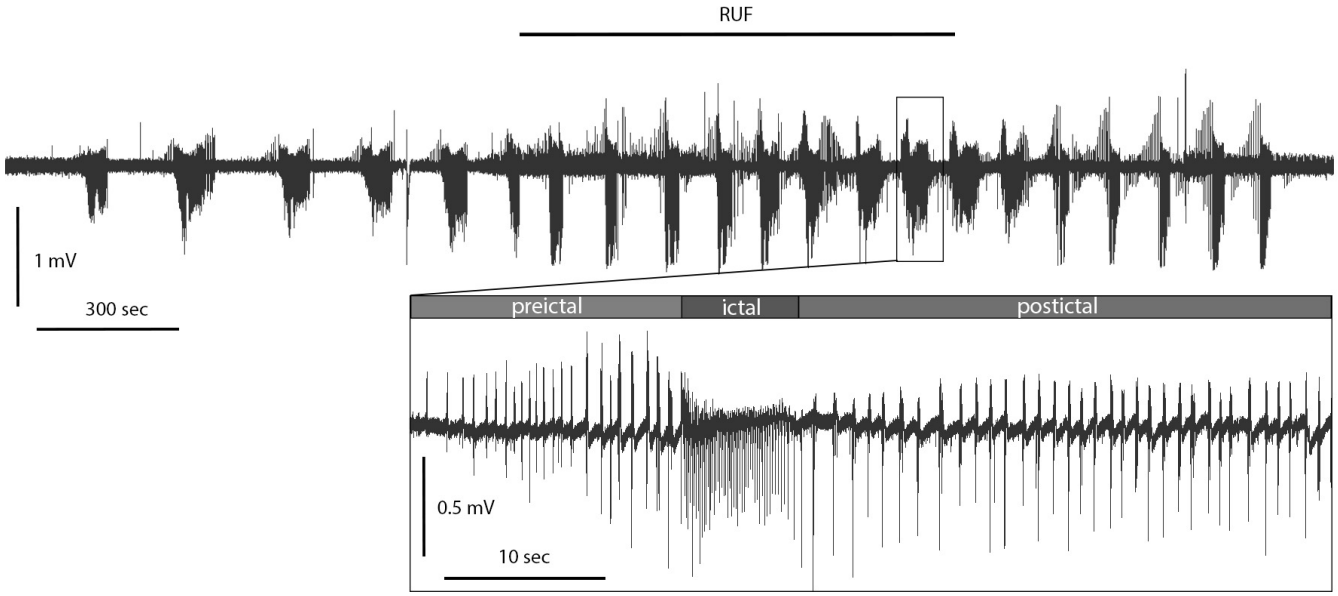

Fig. 1. Full recording of seizure-like events in 0MgACSF and β-CD – RUF complex.

initiated when the frequency increased above 0.2 Hz, continuing until the frequency abruptly increased above 1 Hz, marking the onset of the ictal phase. The beginning of the postictal phase was marked when the frequency decreased below 1 Hz, and interictal periods had spiking activity below 0.2 Hz [12].

Frequency Analysis

tPAC Max analysis was performed using the Brainstorm software (Brainstorm, Version: 3.231017, 2023, neuroimage.usc.edu/brainstorm) [13]. The .smrx files were imported into Brainstorm for detailed spectral analysis. Recordings were digitally resampled from a 5 kHz sampling rate to 2 kHz. A notch filter was applied at 50, 100, 150, 200, 250, 300, 350, and 400 Hz to eliminate electromagnetic artifacts. tPAC was analyzed to evaluate the dynamic interaction between the phase of low-frequency oscillations (1-12 Hz) and the amplitude of high-frequency oscillations (30-100 gamma, 100-200 ripple, and 200-400 fast ripple) on 5-second-long windows at the end of the preictal, beginning of ictal and the end of the postictal phase (Figure 2). Specifically, we focused on tPAC Max, the maximum value of phase-amplitude coupling observed within a specified time window, representing the peak strength of coupling between the phase of low-frequency oscillations and the amplitude of high-frequency oscillations.

Statistical Analysis

For comparative analysis between control (0MgACSF) and treated (β-CD+RUF) SLE groups, the parameters were normalized for each SLE, and induced changes were calculated as percentage changes; the mean value of each of the parameters measured in 0MgACSF for the given slice served as reference (100%). Data were statistically processed using t-tests and Mann Whitney tests, when appropriate. All statistical analyses were performed using GraphPad Prism v. 8.0.2. (Graph Pad Software, San Diego). A p-value of less than 0.05 was considered statistically significant.

Results

Recordings from six hippocampal slices were evaluated to determine the effects of β-CD-solubilized RUF on SLEs. The analysis focused on the durations of preictal, ictal, and postictal phases, as well as the frequency of SLEs and the firing rates of ictal spikes.

Regarding the phase durations, we found that the length of the preictal phase increased significantly after applying RUF (140.7±21.01 %, mean±SEM) compared to the control SLEs (100±9.03 %) (Figure 3A). Furthermore, a notable decrease in the length of the ictal phase was observed after RUF treatment (58.44±4.71 %) compared to control SLEs (95.88±4.29 %) (Figure 3B). The postictal phase length was also significantly reduced due to RUF (31.43±5.77 %) compared to control SLEs (88.35±8.60 %) (Figure 3C).

The absolute frequency of SLEs was found to be increased in the presence of RUF (25.89±1.91 vs. 17.00±1.14 SLE/hour) (Figure 3D).

We analyzed the firing rates of ictal spikes from the first 10 and the next 10 spikes (1-10 and 11-20 spikes). There was no significant change in the firing rate of the first 10 ictal spikes when comparing the control (107.80±5.63 %) and RUF (93.96±8.43 %) SLEs (Figure 4A). However, the firing frequency of the second set of 10 ictal spikes was higher during RUF perfusion (117.70±6.73 %) compared to 0MgACSF (99.52±3.20 %) (Figure 4B). In the case of control SLEs a significant deceleration of the firing activity was observed, the second set of 10 ictal spikes had significantly lower firing rate compared to the first set of 10 ictal spikes (first: 12.10±0.68 vs. second: 8.76±0.53 Hz, Mann-

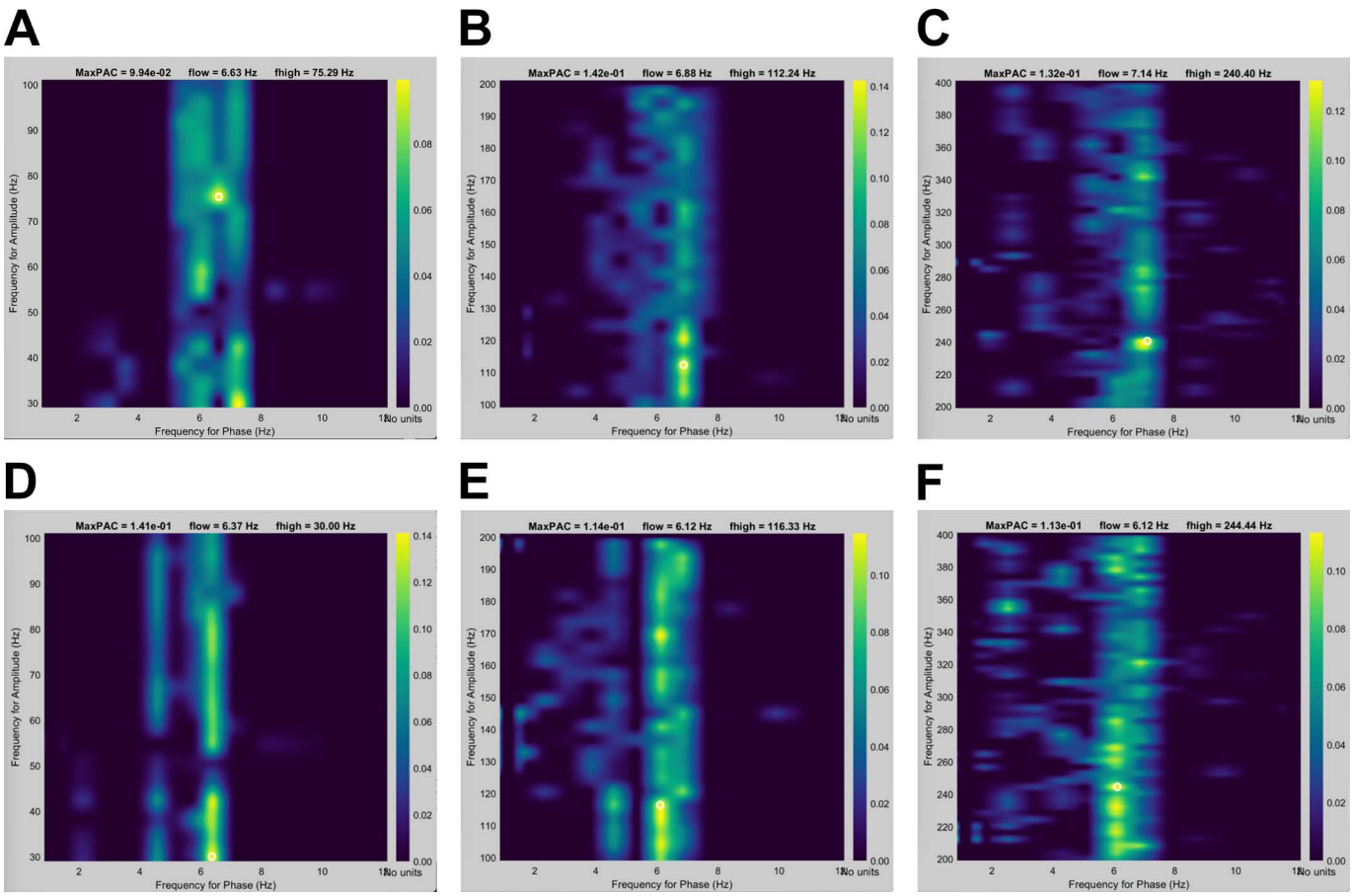

Fig. 2. tPAC comodulograms from the beginning of the ictal phase in 0MgACSF (A-C) and RUF (D-F), calculated for gamma (A, D), ripple (B, E) and fast ripple (C, F) frequencies.

Whitney test) (Figure 4C). There was no change of the firing frequency between the first and second set of ictal spikes in the case of RUF (10.25±0.93 vs. 10.02±0.70 Hz) (Figure 4D).

In the case of tPAC Max, no significant differences were observed between the control and RUF-treated conditions for any of the chosen phases and frequency ranges analyzed (Table I).

Discussions

Our findings indicate that RUF, when solubilized with β-CD, decreases the ictal and postictal durations of SLE. The firing rate of the second set of 10 ictal spikes is maintained compared to the first set of 10 discharges. RUF does not affect tPAC Max. Its anticonvulsant effect at 100 μM concentration is sustained by the decreased durations of the ictal and postictal phases. These findings are consistent with the observations of Gáll et al. (2017), who used dimethyl sulfoxide (DMSO) to solubilize RUF [14]. Shorter ictal durations indicate that RUF reduces the active seizure period, which is crucial for minimizing neuronal damage. The reduction in postictal duration suggests faster post-seizure recovery, implying that RUF contributes to quicker membrane stabilization. The prolongation of the preictal

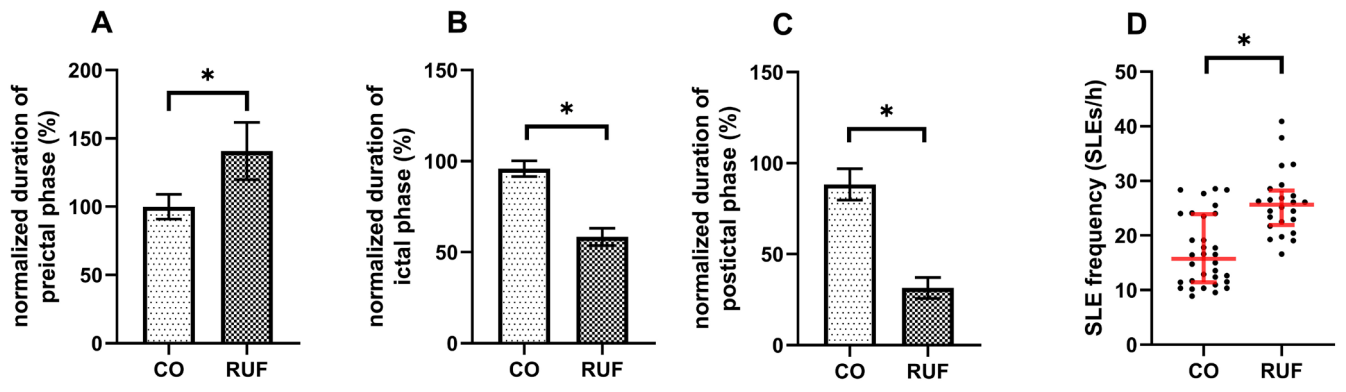

Fig. 3. 0MgACSF induced SLE durations and frequency are significantly influenced by application of β-CD (1%) complexed RUF (100 μM) compared to control (CO) SLEs. Each bar represents % change of parameters compared to CO: A. duration of the preictal phase, plot: Mean±SEM; B. duration of the ictal phase, plot: Mean±SEM; C. duration of the postictal phase, plot: Mean±SEM. D. raw SLE frequency/hour (Mann-Whitney nonparametric test), line at: median with interquartile range. n= 6

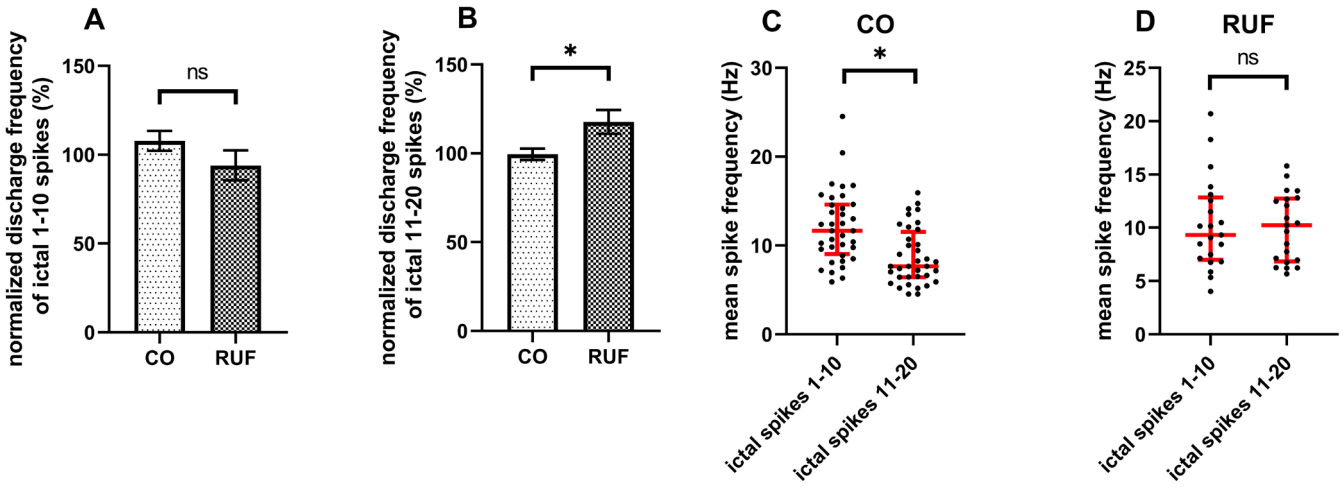

Fig. 4. Discharge frequency of the first 10 and second 10 ictal spikes. A. difference of the normalized discharge frequency of the first 10 ictal spikes between CO and RUF SLEs, plot: Mean±SEM; B. difference of the normalized discharge frequency of the second 10 ictal spikes between CO and RUF SLEs, plot: Mean±SEM; C. difference of the raw discharge frequency of CO SLEs between the first 10 and second 10 ictal spikes (Mann-Whitney nonparametric test), line at: median with interquartile range; D. difference of the raw discharge frequency of RUF SLEs between the first 10 and second 10 ictal spikes, line at: median with interquartile range. n= 6

Table I. Statistical analysis results of normalized tPAC Max values showing no significant difference between CO and RUF

		Frequency		
Phase		**Gamma**	**Ripple**	**fast Ripple**
Preictal	CO	100.40±4.68%	99.22±4.74%	99.20±3.55%
	RUF	107.60±4.70%	98.53±6.27%	108.90±6.69%
Ictal	CO	100.80±5.07%	99.14±3.55%	102.00±3.79%
	RUF	102.30±5.62%	98.92±6.37%	96.47±4.81%
Postictal	CO	99.57±4.35%	97.45±4.75%	104.10±5.06%
	RUF	84.94±8.36%	86.50±9.34%	95.72±10.67%

Description: CO- control group, RUF- rufinamide group; presented values include mean±SEM

phase duration in RUF suggests that this ASM delays the onset of ictal activity.

While the antiseizure effects of RUF are well-documented in the literature and clinical practice, there are no studies examining the effects of its solubilization with β-CD on SLEs. Additionally, tPAC has not been thoroughly studied in 0Mg *in vitro* epilepsy models, nor has the effect of RUF on tPAC.

Despite these antiseizure effects, the frequency of SLEs increased with RUF perfusion, a finding that contrasts with the results described by Gáll et al. (2017) using DMSO solubilization [14]. This counterintuitive result suggests that while RUF shortens individual seizures, it may increase the overall susceptibility to SLE occurrence. However, shorter synchronous neuronal activity may limit the number of symptomatic seizures a patient experiences (i.e., not all electrographic seizures may translate to symptoms). Increased neuronal excitability after RUF treatment has also been described in cellular cultures [15]. These results warrant further investigation to understand whether this effect is attributable to the different solubilization methods, which could influence the bioavailability of RUF even at the same concentrations. Understanding these underlying mechanisms is crucial for optimizing the therapeutic efficacy and safety of RUF in epilepsy treatment.

Regarding firing rates, the uniform discharge frequency during the first and second set 10 ictal spikes during RUF perfusion indicates a modulatory effect of RUF on neuronal excitability during the ictal phase as a stabilizing effect on neuronal activity during seizures.

Analysis of tPAC Max did not reveal differences between control and RUF-treated SLEs. This suggests that RUF's anticonvulsant effects may not be mediated through changes in oscillatory coupling. However, longer time sequences should be analyzed to more accurately validate these conclusions and ensure the robustness of the findings. Although we did not find any previous studies specifically investigating tPAC in an *in vitro* epilepsy model, PAC has been studied in the rat primary motor cortex under *in vitro* conditions, inducing simultaneous theta and gamma oscillations, and demonstrating strong cross-frequency coupling between those in layer V [16]. The oscillatory power was measurably influenced by $GABA_A$ and $GABA_B$ antagonists. These detectable changes of PAC in the context of network activity highlight the reliability of the method in identifying coupled oscillatory activity in neural tissues. By detecting coupled oscillations and demonstrating pharmacological modulation, PAC analysis proves to be a method worth further exploration. Furthermore, research using *in vitro* models like the pilocarpine and 4-aminopyrimidine models has highlighted the critical role of gamma oscillations and high-frequency oscillations (90-400 Hz) in the hippocampal formation during different stages of epileptogenesis [17]. These studies provide substantial evidence

supporting the use of PAC analysis in revealing changes in network dynamics that correlate with epileptogenesis and seizure activity.

Conclusion

β-CD-solubilized RUF at a 100 μM concentration significantly alters SLE dynamics *in vitro*, increasing the duration of the preictal phase while decreasing both ictal and postictal durations. Although, these modifications suggest improvements in certain SLE characteristics, the observed increase in SLE frequency highlights the necessity for further investigation. This study contributes to the evidence supporting the use of cyclodextrins to enhance the solubility and efficacy of ASMs, offering insights for developing improved therapeutic strategies in epilepsy management. Future research should explore dose-dependent effects and elucidate underlying molecular mechanisms. Additionally, in-depth frequency analysis using refined methods could provide valuable information regarding the effects of RUF and β-CD, enhancing our understanding of their therapeutic potential.

Authors' contribution

RJK (Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Resources, Visualization, Writing – original draft)
ZSAN (Data curation, Formal analysis, Investigation)
ASZ (Data curation, Formal analysis, Investigation)
ACS (Data curation, Formal analysis, Investigation)
AM (Data curation, Formal analysis)
HM (Data curation, Formal analysis)
EB (Data curation, Formal analysis)
AJB (Conceptualization, Methodology, Validation, Writing – review & editing)
ZSG (Conceptualization, Methodology, Resources, Validation, Writing – review & editing)
TSZ (Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing)
KOK (Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing)

Conflict of interest

None to declare.

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