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Authors
Niquet, J
Baldwin, R
Suchomelova, L
et al.

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Treatment of experimental status epilepticus with synergistic drug combinations

Jerome Niquet, Roger Baldwin, Lucie Suchomelova, Lucille Lumley, Roland Eavey, and Claude G. Wasterlain

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SUMMARY

During status epilepticus (SE), synaptic γ-aminobutyric acid A receptors (GABA_\text{A}Rs) become internalized and inactive, whereas spare N-methyl-D-aspartate receptors (NMDARs) assemble, move to the membrane, and become synaptically active. When treatment of SE is delayed, the number of synaptic GABA_\text{A}Rs is drastically reduced, and a GABA_\text{A} agonist cannot fully restore inhibition. We used a combination of low-dose diazepam (to stimulate the remaining GABA_\text{A}Rs), ketamine (to mitigate the effect of the NMDAR increase), and valproate (to enhance inhibition at a nonbenzodiazepine site) to treat seizures in a model of severe cholinergic SE. High doses of diazepam failed to stop electrographic SE, showing that benzodiazepine pharmacoresistance had developed. The diazepam-ketamine-valproate combination was far more effective in stopping SE than triple-dose monotherapy using the same individual drugs. Isobolograms showed that this drug combination’s therapeutic actions were synergistic, with positive cooperativity between drugs, whereas drug toxicity was simply additive, without positive or negative cooperativity. As a result, the therapeutic index was improved by this drug combination compared to monotherapy. These results suggest that synergistic drug combinations that target receptor changes can control benzodiazepine-refractory SE.

KEY WORDS: Refractory status epilepticus, Cholinergic seizures, Polytherapy, Diazepam, Ketamine, Valproate.
ketamine (which has multiple actions including NMDAR antagonism) has been described.6,7 We explored the hypothesis that combinations of a GABA_A agonist with an NMDAR antagonist and an antiepileptic drug (AED) acting at a nonbenzodiazepine site would be able to restore the balance between inhibition and excitation and stop benzodiazepine-refractory SE. Our results show that seizures that are refractory to deeply anesthetic doses of diazepam can be stopped when the same benzodiazepine (at a 20-fold lower dose) is combined with the NMDA antagonist ketamine and with the AED valproate. They also suggest that this three-drug combination shows positive cooperativity (synergism) in its therapeutic effects but not in its toxic side-effects.

**Materials and Methods**

**Animals**

Male Wistar rat (200–300 g; Simonsen, Gilroy, CA, U.S.A.) were housed in a temperature- and humidity-controlled room with 12 h light–dark cycles and had free access to food and water. All experiments were conducted with the approval and in accordance with the regulations of the West Los Angeles VA Medical Center Institutional Animal Care and Use Committee.

**Induction of SE and treatment**

As described previously,7 rats received lithium chloride (5 mEq/kg, s.c.; #L-0505; Sigma, St. Louis, MO, U.S.A.) and, 16 h later, pilocarpine hydrochloride (320 mg/kg, i.p.; #P6503; Sigma) and scopolamine methyl bromide (1 mg/kg, i.p.; #S8502; Sigma). Seizures started 7.6 ± (standard deviation) 2.7 min after pilocarpine injection, and the second Racine scale stage 3 or higher seizure occurred 8–20 min later. At the end of the second stage 3 or higher seizure, all animals received scopolamine (10 mg/kg, i.p.; #S1013; Sigma) to remove the original seizure trigger without stopping SE, and either sham injection (SE control group), one drug, or a combination of three drugs intraperitoneally. Drugs included diazepam (#321312 Hospira), ketamine (#RL3760 Hospira), and sodium valproate (#P4543 Sigma).

**Implantation of electrodes**

Under isoflurane anesthesia, the animals were implanted with skull screws, which served as recording electrodes.7 The BioPac Systems MP150 was used to record electrocardiography studies. Sampling rate was 200 Hz.

**Acute video-EEG monitoring**

EEG recordings were analyzed with AcqKnowledge.7 Seizures were classified according to a modified Racine scale.8

**Outcome measures** were described previously7 and included posttreatment EEG power integral; Hjorth function over 1–6 h post-treatment7; number of posttreatment seizures; cumulative seizure time (subtracting interictal time); number of posttreatment spikes; duration of SE; time to the first minute of seizure-free EEG; composite seizure score (number of seizures + EEG power integral over the first hour posttreatment); and time needed for EEG amplitude to fall below two times the pre-pilocarpine EEG baseline.

**Toxicity studies**

The following scale was used: 0, normal gait; 1, ataxic; 2, unable to walk but able to crawl, righting reflex preserved; 3, righting reflex partially impaired; 4, complete loss of righting reflex; 5, no response to tail pinch; and 6, loss of corneal reflex. The toxicity score was the sum of measures taken every 15 min for the first hour after injection.

**Construction of three-dimensional isobolograms** for toxicity and efficacy: We defined the ED_{50} as the dose that reduced a particular measure of seizure severity by 50%, and the TD_{50} as the dose that induced half-maximal toxicity. Dose–response curves were fit by the method of Chou.10 The variances of the TD_{50}’s were calculated by the method of Tallarida (p 29, Equation 2.9).11 The theoretical dosage sum was calculated from Tallarida (p 59, Equation 4.1, and its variance from p. 60, Equation 4.2). For each isobologram, a plane was fit through the ED_{50}’s or TD_{50}’s using SciDavis (http://scidavis.sourceforge.net). The observed toxicity or efficacy of the triple drug combination was then placed in the same three-dimensional plot with the plane connecting TD_{50}’s or ED_{50}’s for individual drugs. Drug synergism (positive cooperativity) is indicated if the observed toxicity or efficacy of the combination lies below the additivity plane in the isobologram. When the effects are simply additive, the values fall in the additivity plane. Negative cooperativity would result in values above that plane. Significance was determined by a modified t-test (Tallarida, Section 4.3, p 60–62).11

Toxicity curves were straightforward, but efficacy curves required some extrapolations because, even at toxic doses, monotherapy often reduced EEG seizures by <50%. The ED_{50} doses for monotherapy could be underestimated as a result. However, because this would work against our hypothesis, it should not detract from our conclusion that positive cooperativity was observed.

**Statistical analyses**

EEG were analyzed with AcqKnowledge, statistical comparisons used a Kruskal-Wallis test followed by Dunn’s multiple comparison test, or two-way analysis of variance (ANOVA) with Dunnett’s test (GraphPad version 6).

**Results**

**Treatment with combinations of a GABA_A agonist, an NMDA antagonist, and an antiepileptic drug (AED)**

The time needed for EEG power to return to twice the preseizure baseline is a good measure of SE termination. Monotherapy with diazepam, ketamine, or valproate had no
effect, but a combination of the same drugs reduced that time (Fig. 1A) from $4.7 \pm 0.3$ (diazepam 1 mg/kg), $5.2 \pm 1.8$ (ketamine) and $4.8 \pm 3.8$ (valproate) h to 27 ± 14 min (mean ± standard deviation [SD], p < 0.01 for all). The untreated SE control group value was 4.8 ± 3.9 h and high-dose diazepam (20 mg/kg) was 3.1 ± 1 h (data not shown). The fact that drugs that failed to shorten SE when given alone were so effective in combination raised the possibility of synergism between drugs. The combined seizure score (Fig. 1B), and Hjorth function,
another measure of seizure severity (Fig. 1C), also showed greater therapeutic efficacy for the drug combination compared to monotherapy. When EEG power integral during SE before treatment was plotted against EEG power integral during the 4 h following treatment, the combination diazepam-ketamine-valproate was better at stopping SE than any monotherapy at a 3–5 times higher dose (p < 0.01), supporting our hypothesis (Fig. 1D). However, within all groups, individual rats with a lower EEG power integral before treatment had a better response than did rats with a higher pretreatment EEG power integral, suggesting that some seizure-dependent pharmacoresistance was still present.

**Isobolograms**

To determine whether positive or negative cooperativity was present, we looked at both drug toxicity and drug efficacy. The TD50 was 17.5 mg/kg for diazepam, 80.4 mg/kg for ketamine, 780 mg/kg for valproate, and for the three-drug combination diazepam 4.8 mg/kg + ketamine 47.6 mg/kg + valproate 151 mg/kg. The toxicity of the combination of diazepam + ketamine + valproate was located precisely in the additivity plane connecting the TD50’s of the individual drugs (Fig. 2A), suggesting that toxicity was simply additive, without evidence of positive or negative cooperativity. Figure 2B shows the efficacy of the diazepam-ketamine-valproate combination as measured by the time it took to return EEG power to twice the preseizure baseline. ED50’s were 83.0 mg/kg for diazepam, 27.5 mg/kg for ketamine, 71.9 mg/kg for valproate, and for the combination diazepam 0.3 mg/kg + ketamine 3 mg/kg + valproate 9.1 mg/kg. The point reflecting the effect of the three-drug combination was far below the additivity plane (p < 0.001). In other words, that combination reduced EEG power during SE at much lower concentrations than would be expected from adding individual values for the three component drugs. This suggests strong positive cooperativity between the three drugs in reducing EEG power and stopping seizure activity.

**Discussion**

Our results show that the diazepam + ketamine + valproate combination stopped seizures at a time when pharmacoresistance to high doses of diazepam (5–20 mg/kg) was clearly present (Fig. 1D). Overcoming pharmacoresistance is one of our most difficult problems in treating SE. Rats that had a greater EEG power integral (e.g., a greater seizure burden) at the time of treatment responded less well than those with a lesser seizure burden (Fig. 1D), suggesting that it is the accumulation of seizures, rather than the accumulation of time, which is associated with an increase in pharmacoresistance. Combination therapy overcame pharmacoresistance but may not have abolished it, since the decrease in response with increased pretreatment EEG power integral was also seen in the combination therapy group.

The diazepam-ketamine-valproate combination potentiated the therapeutic response without potentiating drug toxicity, so that the therapeutic index was improved by switching from monotherapy to polytherapy. Figure 2A shows that toxicity was simply additive between the three drugs of the combination, and Figure 2B shows that the therapeutic response was synergistic. As a result of that synergy, this three-drug combination delivered a greater therapeutic response than monotherapy for the same level of drug-induced toxicity. This increased therapeutic index might be particularly useful when cardiovascular depression limits the amount of drugs delivered in the intensive care unit, or when respiratory depression is the limiting factor because respiratory support is not readily available. Similar
synergism between scopolamine and two types of GABA<sub>AR</sub> agonists has recently been reported. The comparison of triple therapy with dual therapy is discussed in a separate paper.

We did not measure drug brain concentrations, and cannot rule out a pharmacokinetic explanation for our results. Such an explanation is unlikely for the following reasons. No pharmacokinetic interaction has been found between valproate and ketamine, or ketamine and diazepam when given acutely. Valproate mildly inhibits the metabolism of diazepam, but since the elimination half-life of diazepam is close to 48 h, the likelihood of a significant change in brain concentration within an hour after treatment (when we observe highly significant differences in EEG power) is small. It displaces diazepam from its albumin binding sites, which could increase free serum levels, but increasing the diazepam dose up to 20 mg/kg (which should increase free levels far more) has no effect on seizures.

These results have therapeutic implications: our traditional reliance on monotherapy in the treatment of epilepsy may need to be reevaluated. Current therapeutic guidelines for SE recommend benzodiazepine monotherapy as initial treatment, but the valuable stimulation of synaptic GABA<sub>AR</sub>Rs that benzodiazepines provide may treat only half of the problem. The synergistic effect that we observed is compatible with the hypothesis that pharmacoresistance is affected by both GABA and glutamate receptor trafficking, and that treatment is most effective when it targets both changes. These results imply that future clinical trials of the treatment of SE must include a polytherapy arm.

These results are highly relevant to organophosphate- and nerve agent–induced SE, which represents a significant terrorist and military threat. The model we used mimics the effect of 1.2 LD<sub>50</sub> of soman. In battlefield or terrorist situations involving nerve agent–induced seizures, treatment is often delayed, and in animal models, pharmacoresistance to cholinergic seizures develops quickly. Current therapeutic kits for nerve agent–induced seizures contain a benzodiazepine (diazepam or midazolam), but in experimental models, delayed administration of benzodiazepines is poorly effective. Consideration should be given to the use of synergistic drug combinations, which show higher efficacy at time-points when benzodiazepine monotherapy cannot overcome pharmacoresistance.

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Disclosure of Conflict of Interest

Jerome Niquet and Claude Wasterlain have a patent pending on polymedication for nerve agent seizures (UC Case No. 2012-172-2). Other authors have no conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References