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ORIGINAL ARTICLE

Interactions of levetiracetam with ethosuximide in the mouse 6 Hz psychomotor seizure model – a type II isobolographic analysis

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Abstract

The aim of the present study was to characterize the anticonvulsant effects of levetiracetam in combination with ethosuximide in the mouse 6 Hz psychomotor seizure model.

Limbic (psychomotor) seizure activity was evoked in albino Swiss mice by a current (32 mA, 6 Hz, 3 s stimulus duration) delivered via ocular electrodes; type II isobolographic analysis was used to characterize the consequent anticonvulsant interactions between the drug combinations for fixed-ratios of 1:1, 1:2, 1:5 and 1:10.

With type II isobolographic analysis, the combinations of levetiracetam with ethosuximide for the fixed-ratios of 1:5 and 1:10 were supra-additive (synergistic; $P<0.05$ and $P<0.01$, respectively) in terms of seizure suppression, while the combinations for the fixed-ratios of 1:1 and 1:2 were additive in the mouse 6 Hz psychomotor seizure model.

The combinations of levetiracetam with ethosuximide for the fixed-ratios of 1:5 and 1:10 appear to be particularly favorable combinations exerting supra-additive interaction in the mouse 6 Hz psychomotor seizure model. Finally, it may be concluded that because of the synergistic interactions between levetiracetam and ethosuximide, the combination might be useful in clinical practice.

Key words

6 Hz psychomotor seizure model, antiepileptic drugs, drug interactions, ethosuximide; levetiracetam; isobolographic analysis

INTRODUCTION

There is no doubt that monotherapy with one of the currently available antiepileptic drugs is the treatment of choice for approx. 70% of epileptic patients [1]. However, there is still 30% of epileptic patients, who require the application of two or more antiepileptic drugs to suppress their seizure attacks [1, 2]. In such a situation, polytherapy based on rational selection of antiepileptic drugs for specific seizure types and diverse molecular mechanisms of action, seems to be a favorable treatment regimen for these epileptic patients [2, 3]. In the case of polytherapy, certain antiepileptic drug combinations seem to be more favourable than others, especially if antiepileptic drugs with diverse molecular mechanisms of action are combined [2, 4]. From the theoretical point of view, the most advantageous antiepileptic drug combination is that between two antiepileptic drugs that are synergistic in relation to their therapeutic (anticonvulsant) activity, and thus supra-additive in seizure suppression and with concomitant infra-additivity (antagonism) in relation to their adverse effects [2, 5].

Experimental evidence indicates that levetiracetam combined with lacosamide and phenobarbital exerted supra-additive (synergistic) interactions in mice challenged with the 6 Hz psychomotor seizure model [6, 7]. Moreover, isobolographic analysis revealed both, supra-additive and additive interactions between levetiracetam and carbamazepine, phenytoin, topiramate and vigabatrin in the mouse 6 Hz model [8]. Additionally, the combinations of levetiracetam with clonazepam, clobazam, lamotrigine, oxcarbazepine, tiagabine and valproate exerted additive interaction in the mouse 6 Hz model [7, 9, 10]. It is widely accepted that the mouse 6Hz model (low frequency (6 Hz) long-duration (3 s) corneal electrical stimulation) is an experimental animal model of psychomotor or limbic seizures in humans [11].

Considering the above-mentioned combinations, an attempt was made to determine types of interaction between levetiracetam and ethosuximide in the mouse 6 Hz psychomotor seizure model. Generally, antiepileptic drugs in combination should possess diverse and complementary molecular mechanisms of action with respect to their anticonvulsant effects. Additionally, their adverse-effect profiles should also be different [2, 3]. Clinicians should avoid combining antiepileptic drugs that act on the same molecular target or receptors in the brain. In light of these facts, levetiracetam seems to be an optimal antiepileptic drug which, due to its unique anticonvulsant molecular mechanisms of action, can be combined with ethosuximide. This is the reason for levetiracetam in combination with ethosuximide being tested in the presented study.

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MATERIALS AND METHODS

Animals and experimental conditions. All experiments were performed on adult male Swiss mice weighing 22–26 g, purchased from a licensed breeder (J. Kolacz, Warsaw, Poland). The mice were kept in colony cages with free access to food and tap water under standardized housing conditions (natural light-dark cycle, temperature of 21 ± 1°C, relative humidity of 55 ± 5%). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of 8 mice per group. Each mouse was used only once. All tests were performed between 09.00 and 15.00. Procedures involving animals and their care were conducted in conformity with current European Communities Council Directive of 24 November 1986 (86/609/EEC) and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures listed were approved by the local Ethics Committee at the Medical University of Lublin (License No.: 46/2008) and conformed to the Guide for the Care and Use of Laboratory Animals.

Drug administration. The following antiepileptic drugs were used in this study: levetiracetam (UCB Pharma, Braine-l'Alleud, Belgium) and ethosuximide (Sigma, St. Louis, MO, USA). The drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and were administered intraperitoneally (i.p.) as a single injection in a volume of 5 ml/kg body weight. Fresh drug solutions were prepared on each day of experimentation, and administered as follows: ethosuximide – 45 min and levetiracetam – 60 min before initiation of psychomotor seizures evoked by 6 Hz corneal electrical stimulation, evaluation of motor coordination, grip-strength and long-term memory tests. The control animals received an equivalent volume of vehicle (1% Tween 80). The pretreatment times before testing of these antiepileptic drugs were based on information from the literature and our previous experiments [12]. The times to the peak of maximum anticonvulsant effects for all antiepileptic drugs were used as the reference times in all behavioural tests.

Six-Hertz (6 Hz) seizure model. Psychomotor (limbic) seizures were induced via corneal stimulation (6 Hz, 0.2 ms rectangular pulse width, 32 mA, 3 s duration) delivered by a S48 Square Pulse Stimulator and CCU1 Constant Current Unit (Grass Technologies, West Warwick, RI, USA). Ocular anaesthetic (0.5% tetracaine) was applied to the mouse corneas 15 min before stimulation. Animals were manually restrained and released immediately following the stimulation and observed for the presence or absence of seizure activity. Before stimulation, the corneal electrodes were wetted with saline to provide good electrical contact. Immediately following stimulation, mice were placed separately in Plexiglas cages (25 × 15 × 10 cm) for behavioural observation. Following the stimulation, the animals exhibited a "stunned" posture associated with rearing and automatic movements that lasted from 60 to 120 s in untreated animals. The low frequency (6 Hz) long-duration (3 s) seizures were characterized by immobility or stun, jaw and forelimb clonus, twitching of the vibrissae, and an elevated tail or Straub-tail [11]. Animals resumed their normal exploratory behaviour after the seizure. The experimental endpoint was protection against the seizure: an animal was considered to be protected if it resumed its normal exploratory behaviour within 10 s after stimulation. Protection in the 6 Hz model was defined as the absence of a seizure. Mice not experiencing seizures exhibited normal exploratory behaviour when placed in the cages [11]. In the present study, to determine the $ED_{50\,mix}$ values, the antiepileptic drugs were administered i.p. at the following dose ranges: ethosuximide, 8–30 mg/kg and levetiracetam, 2–25 mg/kg. Using the log-probit method, the median effective doses ($ED_{50\,mix}$ values) were determined using a minimum of 8 mice per dose [13] after which the mice were euthanized by CO_2 narcosis.

Isobolographic analysis of interactions. Isobolographic analysis of interaction is a mathematical method allowing for the precise characterization of interactions between drugs in both preclinical and clinical studies [14]. This method allows determination of the interactions between the drugs applied at various doses. Of note, the proportions of drugs used in two-drug mixture should be constant and determined before experimental verification of interactions [14]. This is the reason that several fixed-ratio combinations were tested isobolographically for each antiepileptic drug combination. In the present study, there were selected 4 standard fixed-ratio combinations of 1:1, 1:2, 1:5 and 1:10, which are routinely used when testing interactions with type II isobolographic analysis. To perform the isobolographic analysis of the interactions between levetiracetam and ethosuximide (as regards their anticonvulsant activities against psychomotor seizures), the antiepileptic drugs in numerous fixed-ratio combinations were administered to animals. Subsequently, the experimentally derived $ED_{50\ mix}$ values (± S.E.M.) for the mixture were determined using log-probit analysis [13]. Moreover, theoretically additive $ED_{50\ add}$ values (± S.E.M.) were calculated from the equation presented by Porreca et al. [15], as follows: $ED_{50\ add} = ED_{50\ levetiracetam} / P_{levetiracetam}$; where, $P_{levetiracetam}$ is the proportion of the drug, fully effective against psychomotor (6 Hz) seizures (levetiracetam) in the total amount of two-drug mixture. It should be noted that for two-drug mixtures the equation presented above is true when: $P_{levetiracetam} + P_{ethosuximide} = 1$; where, $P_{ethosuximide}$ is the proportion of the drug that is virtually ineffective in the 6 Hz-induced seizure test (ethosuximide). The proportions of antiepileptic drugs in the mixture are based on a mass quantity of antiepileptic drugs (for instance, a fixed-ratio combination of 1:1 comprised equal amounts of levetiracetam and ethosuximide). This particular kind of type II isobolographic analysis allows for the acceptance of mass quantity of drugs in the mixture as a basis for constructing the notation of fixed-ratio combinations. For instance, for the fixed-ratio of 1:2 for levetiracetam + ethosuximide combination, the proportion of levetiracetam was ⅓ = 0.3333, while the proportion of ethosuximide was ⅔ = 0.6666, in the total amount of the mixture. Subsequently, the theoretical amount of pure additive ($ED_{50\,add}$) mixture for the fixed-ratio of 1:2 was calculated as follows: ED_{50} of levetiracetam divided by $P_{levetiracetam}$. Hence, $ED_{50\ add}$ = 14.84 / 0.3333 = 44.52 mg/kg (Tab. 1).

On the other hand, the fixed-ratio of 1:2 provides information that the second drug used in the mixture (a virtually ineffective antiepileptic drug) is administered at doses 2-times higher than that for the first fully effective

antiepileptic drug in the mixture. A more detailed description and the theoretical background relating to isobolographic analysis, including equations showing how to calculate $ED_{50\ add}$ values and their S.E.M., has been presented in our previous studies [16]. Finally, to determine the separate doses of levetiracetam and ethosuximide in the mixture, the $ED_{50\ mix}$ values were multiplied by the respective proportions of antiepileptic drugs (denoted for purely additive mixture).

Statistical analysis. The ED_{50} and $ED_{50\ mix}$ values (with their respective 95% confidence limits) for antiepileptic drugs administered alone or in combination for the fixed-ratios of 1:1, 1:2, 1:5 and 1:10 in the mouse 6 Hz-induced seizure test were calculated by computer-assisted log-probit analysis, according to Litchfield and Wilcoxon [13]. The obtained 95% confidence limits were transformed to standard errors of the mean (S.E.M.), as described previously [16]. The experimentally-derived $ED_{50\ mix}$ values for the mixture of levetiracetam with ethosuximide were statistically compared with their respective theoretical additive $ED_{50\ add}$ values by the use of unpaired Student's *t*-test, according to Tallarida [14]. Results were considered statistically significant if $P<0.05$.

Software utilized. Microsoft's Excel spreadsheet was used to perform calculations and to graphically illustrate the results as isobolograms. This spreadsheet was programmed to compute all calculations automatically, and determine the dose-response relationship curves of levetiracetam administered alone and in combination with ethosuximide from the log-probit linear regression analysis, according to Litchfield and Wilcoxon [13]. The theoretically additive $ED_{50\ add}$ values and their S.E.M. for the fixed-ratio combinations of 1:1, 1:2, 1:5 and 1:10 were also calculated with this programmed spreadsheet. All statistical tests were performed using commercially available GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Anticonvulsant effects of levetiracetam and ethosuximide administered separately and in combination in the mouse 6 Hz psychomotor seizure model. Levetiracetam administered alone produced a clear-cut anticonvulsant effect against 6 Hz psychomotor seizures and its experimentally derived ED_{50} value was 14.84 (9.15–24.08) mg/kg. In contrast, ethosuximide administered singly at doses up to 250 mg/kg produced only 25% protection (2 out of 8 mice) against 6 Hz-induced seizures in mice (results not shown).

Type II isobolographic analysis of interactions between levetiracetam and ethosuximide in the mouse 6 Hz psychomotor seizure model. Isobolographic analysis revealed that the combination of levetiracetam with ethosuximide for the fixed-ratios of 1:5 and 1:10 was supra-additive (synergistic) in the mouse 6Hz-induced psychomotor seizure model ($P<0.05$ and $P<0.01$; Tab. 1; Fig. 1). In contrast, the antiepileptic drug combination for fixed-ratios of 1:1 and 1:2 displayed additive interaction (Tab. 1; Fig. 1).

Table 1. Isobolographic characterization of interactions between levetiracetam and ethosuximide in the mouse 6 Hz psychomotor seizure model

Antiepileptic drug combination	Fixed-ratio	$ED_{50\ mix}$	n_{mix}	$ED_{50\ add}$	n_{add}
Levetiracetam + ethosuximide	1:1	23.9±2.6	24	29.7±7.3	30
Levetiracetam + ethosuximide	1:2	25.5±2.6	16	44.5±11.0	30
Levetiracetam + ethosuximide	1:5	30.1±3.4*	24	89.0±22.0	30
Levetiracetam+ethosuximide	1:10	48.4±9.1**	32	163.2±40.3	30

Data are presented as median effective doses (ED_{50} in mg/kg ± S.E.M.) protecting 50% of animals tested against 6 Hz psychomotor seizures. ED_{50} values were either experimentally determined from the mixture of two antiepileptic drugs ($ED_{50\ mix}$), or theoretically calculated from the equation of additivity ($ED_{50\ add}$). Statistical evaluation of the data was performed using the unpaired Student's *t*-test with Welch's correction; n – total number of animals at those doses whose expected anticonvulsant effects ranged between 4–6 probits, denoted for the experimental mixture of drugs (n_{mix}) and theoretically calculated (n_{add}) from the equation of additivity.
*$P<0.05$ and **$P<0.01$ vs. the respective $ED_{50\ add}$, indicating supra-additive (synergistic) interaction

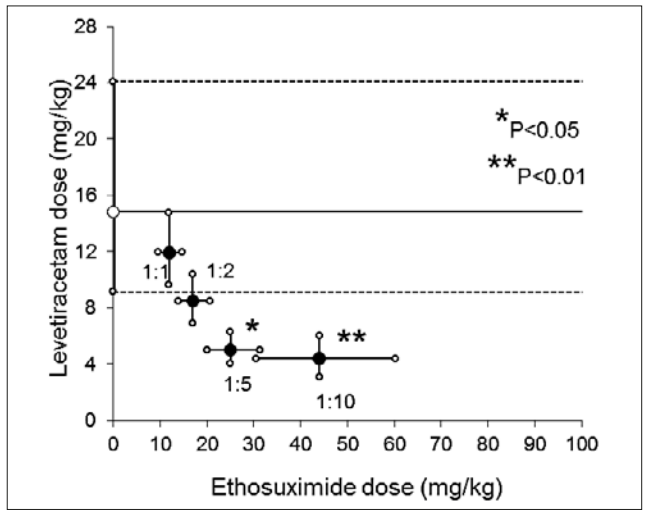

Figure 1. Isobologram showing interactions between levetiracetam and ethosuximide in the mouse 6 Hz psychomotor seizure model
Levetiracetam doses plotted graphically on the Y-axis; doses of ethosuximide plotted on the X-axis. The thick line is parallel to the X-axis, representing the ED_{50} value for levetiracetam administered alone, and defines the theoretical dose-additive line for a continuum of different fixed dose ratios. Dotted lines represent S.E.M. values for levetiracetam administered alone. Closed circles (•) depict the experimentally-derived $ED_{50\ mix}$ values for total doses of mixtures expressed as proportions of levetiracetam and ethosuximide that produced median anticonvulsant effects. The S.E.M. represents an actual calculation of the vertical and horizontal component of the error. Experimental $ED_{50\ mix}$ values for the mixture of levetiracetam + ethosuximide for the fixed-ratios of 1:5 and 1:10 are significantly below the theoretical line of additivity, indicating supra-additivity (*$P<0.05$ and **$P<0.01$). Fixed-ratio combinations of 1:1 and 1:2 indicate additive interactions.

DISCUSSION

The characterization of interactions of levetiracetam with ethosuximide by using the type II isobolographic analysis revealed that, while the combinations of levetiracetam with ethosuximide (for the fixed-ratios of 1:1 and 1:2) were additive, the combinations of levetiracetam with ethosuximide (for the fixed-ratios of 1:5 and 1:10) were supra-additive (synergistic) and potentially useful combinations.

In the presented study, ethosuximide was considered as a virtually ineffective drug in the mouse 6 Hz-induced psychomotor seizure model. The results for the antiepileptic drug administered alone in the 6 Hz-induced seizure model were in contrast to those reported earlier by Barton et al. [11], who found that ethosuximide exerted a clear-cut anticonvulsant effect in the mouse 6 Hz model and the experimentally-derived median effective dose (ED_{50} value)

for ethosuximide was 167 (114–223) mg/kg [11]. In our study, it was reported that ethosuximide was ineffective in terms of suppression of psychomotor seizures because the drug administered i.p. at doses up to 250 mg/kg produced only moderate anticonvulsant effects (i.e., 2 out of 8 mice (25%) were protected against 6 Hz psychomotor seizures). Considering the above-discussed facts a considerable discrepancy was reported between the results presented herein and those documented earlier by Barton et al. [11] in the mouse 6 Hz model. The observed discrepancy may be explained through different pretreatment times used in both studies. More specifically, in the present study, ethosuximide was administered systemically at 45 min. prior to the test, whereas in the study by Barton et al. [11], ethosuximide was administered at 15 min. before seizure initiation. As regards the pretreatment time for ethosuximide, it was documented in other experiments that the time to peak of maximum anticonvulsant effects for the drug was established at 45 min. after its i.p. administration in mice [17]. This is the reason that ethosuximide was administered in the present study at 45 min. before 6 Hz-induced seizures.

Although the hypothesis of different pretreatment times is highly speculative to explain inactivity of ethosuximide in the presented study, it could be, however, partially confirmed by the results presented for levetiracetam. In this study, levetiracetam was administered i.p. at 60 min. before the 6 Hz-induced psychomotor seizures and its ED_{50} value was 14.84 (9.15–24.08) mg/kg. Quite similar results were reported by Barton et al. [11], who found that the ED_{50} value for levetiracetam administered i.p. at 60 min prior to the test was 19.4 (9.90–36.0) mg/kg. The lack of any significant difference between the ED_{50} values for levetiracetam in both experimental studies confirms the hypothesis that the pretreatment times play a particular role in the anticonvulsant activity of ethosuximide in the mouse 6 Hz model.

It is worth mentioning that synergistic interactions between levetiracetam and ethosuximide (for the fixed-ratios of 1:5 and 1:10) were observed for doses of the antiepileptic drugs considerably lower than their ED_{50} values, as determined by Barton et al. [11] in the mouse 6 Hz psychomotor seizure model. More specifically, synergistic interaction between levetiracetam and ethosuximide for the fixed-ratio of 1:10 was observed when levetiracetam and ethosuximide were administered at doses of 4 and 44 mg/kg, respectively. This is the reason that in the presented study it was accepted that ethosuximide was virtually ineffective and type II isobolographic analysis was used to characterize the interactions of levetiracetam with ethosuximide in the mouse 6 Hz seizure model.

This study documents that adding a drug that is virtually ineffective when given alone to a drug fully effective, the combination of two antiepileptic drugs exerted synergistic interaction and added efficacy when given as add-on. This phenomenon one can try to explain by taking into account the molecular mechanisms of action of the tested drugs. The mixture of two antiepileptic drugs exerted the anticonvulsant effect through diverse molecular mechanisms of action of particular drugs used in mixture.

In the case of levetiracetam, several molecular mechanisms of action seem to be involved in its anticonvulsant action. For instance, levetiracetam reduces voltage-operated potassium current and inhibits the delayed rectifier potassium current in neurons [18]. The drug reduces N-type and partially P/Q-type high-voltage activated calcium currents [19, 20]. Levetiracetam suppresses the inhibitory action of zinc and β-carbolines on $GABA_A$- and glycine-gated currents [21]. Levetiracetam blocks $GABA_A$ receptor run-down in neocortex, and thus increases GABA-ergic inhibitory neurotransmission in the brain [22]. The drug binds to a synaptic vesicle protein 2A (SV2A), which is involved in vesicle neurotransmitter exocytosis [23].

With respect to ethosuximide, the drug binds to the inactivated state of low-threshold T-type calcium channels and selectively inhibits pathological firing without any effect on normal neuronal activity [24, 25]. Moreover, ethosuximide decreases the calcium-activated potassium current in neurons and partially reduces the non-inactivating sodium current [24, 25].

It is important to note that in the case of the observed synergistic interactions between levetiracetam and ethosuximide in the mouse 6 Hz model, it can be concluded that the T-type calcium channel blocking properties of ethosuximide and levetiracetam-evoked inhibition of SV2A proteins and reduction of glutamate release, contribute to the suppression of psychomotor seizures.

In the presented study, two antiepileptic drugs with diverse mechanisms of action were combined in an attempt to produce synergistic interaction between antiepileptic drugs. It should be stated that the doses of both antiepileptic drugs used in mixture were generally lower than doses of antiepileptic drugs administered alone, which produced the same anticonvulsant effect. However, along with the reduction of drug doses, a risk of the appearance of adverse effects of particular antiepileptic drugs in mixture decreases, which is important for epileptic patients [24].

In the present study, neither blood nor total brain antiepileptic drug concentrations were measured because of ethical reason. Previously, it has been documented that ethosuximide significantly diminished total brain concentrations of levetiracetam in experimental mice [12]. More specifically, it has been found that ethosuximide (97.4 mg/kg, i.p.) significantly reduced total brain concentrations of levetiracetam (195 mg/kg, i.p.) [12]. In contrast, levetiracetam had no impact on total brain concentrations of ethosuximide in mice [12]. However, it should be stressed that despite the reduction in total brain concentrations of levetiracetam, the observed interaction between levetiracetam and ethosuximide for the fixed-ratio of 1:2 was supra-additive (synergistic) in the mouse pentylenetetrazole-induced clonic seizure model [12]. Since pharmacokinetic interactions were observed earlier in experimental animals, we did not repeat experiments because of ethical reasons. Nevertheless, in the presented study, doses of levetiracetam and ethosuximide were substantially low i.e., the combination of levetiracetam with ethosuximide for the fixed-ratio of 1:10 comprised levetiracetam at a dose of 4 mg/kg and ethosuximide at a dose of 44 mg/kg. In the case, it was less probable that ethosuximide would pharmacokinetically reduce total brain concentrations of levetiracetam in mice. However, to confirm this hypothesis more advanced studies are required.

It is noteworthy that none of the tested combinations for the fixed-ratio of 1:10 produced impairment of motor coordination, disturbed learning and changed skeletal muscular strength in the animals (results not shown), which was in agreement with the authors' earlier studies reporting no acute adverse effects in animals [12].

CONCLUSION

The combination of levetiracetam with ethosuximide can potentially offer some epileptic patients with limbic seizures the favorable combination, worthy of clinical evaluation. Because of a substantial dose reduction of both drugs in the mixture, it can be expected that concurrent adverse effects would be significantly reduced and this is a clinically desirable outcome.

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