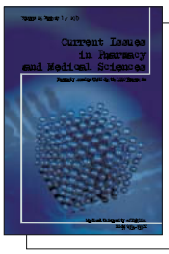

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Additive interaction of levetiracetam with lamotrigine in the mouse 6 Hz psychomotor seizure model – an isobolographic analysis

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ABSTRACT

The aim of this study was to characterize the anticonvulsant effects of levetiracetam (LEV) in combination with lamotrigine (LTG – a second-generation antiepileptic drug), 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 and isobolographic analysis for parallel dose-response relationship curves (DRRCs) was used to characterize the consequent anticonvulsant interactions between the drug combinations. Results indicated that LEV administered singly was associated with a DRRC that was parallel to that for LTG. With isobolography for parallel DRRCs, the combination of LEV with LTG at three fixed-ratios of 1:3, 1:1 and 3:1 exerted additive interaction. LEV combined with LTG exerted additive interaction in the mouse 6 Hz psychomotor seizure model.

Keywords: 6 Hz psychomotor seizure model; Antiepileptic drugs; Drug interactions; Lamotrigine; Levetiracetam; Isobolographic analysis

INTRODUCTION

Levetiracetam (LEV) is a unique second-generation antiepileptic drug (AED), which is ineffective in the acute seizure models, including maximal electroshock (MES)- and pentylenetetrazole (PTZ)-induced seizures [8, 9, 11, 22]. In contrast, LEV increased the threshold for electroconvulsions and suppressed seizures in kindled and genetically epileptic animals [8, 9, 22]. LEV exerted antiseizure activity against low frequency (6 Hz) long-duration (3 s) corneal electrical stimulation (a model of psychomotor or limbic seizures) [1, 15, 40]. Additionally, LEV suppressed **a**bsence seizures in DBA/2J mice [29], and protected rats against audiogenic seizures [39].

In preclinical studies, LEV exerted favorable anticonvulsant pharmacodynamic interactions with numerous AEDs, including: topiramate, oxcarbazepine, carbamazepine, diazepam, felbamate, clonazepam, valproate, phenobarbital and gabapentin [4-6, 10, 18-20, 30, 36]. Isobolographic analysis revealed that LEV interacted synergistically with lacosamide and phenobarbital and exerted additive interaction when combined with clona- zepam, clobazam, oxcarbazepine, tiagabine and valproate in the mouse 6 Hz-induced psychomotor seizure model [1, 7, 27, 40].

Considering the fact that LEV is effective in the acute 6 Hz seizure model, it was of pivotal importance to determine the interaction profile for LEV in combination with lamotrigine (LTG – a second-generation AED) in this psychomotor (6 Hz) seizure model in mice. At present, the 6 Hz seizure test is used for the early identification of anticonvulsant activity of new compounds and combinations of available AEDs effective against therapy-resistant epilepsy [1, 15]. Therefore, the objective of this study was to evaluate potential interaction of LEV in combination with LTG in this seizure model and to use type I isobolographic analysis for parallel dose-response relationship curves (DRRCs).

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MATERIALS AND METHODS

Animals. All experiments were performed on adult male Swiss mice weighing 22–26 g. 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 9.00 a.m. and 3.00 p.m. 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 First Local Ethics Committee at the Medical University of Lublin and conformed to the Guide for the Care and Use of Laboratory Animals (License No.: 46/2008).

Drug administration. The following AEDs were used in this study: LEV (UCB Pharma, Braine-l'Alleud, Belgium) and LTG (Glaxo Wellcome, Greenford, Middlesex, UK). The drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and administered intraperitoneally (i.p.) as a single injection in a volume of 5 ml/kg body weight. Fresh AED solutions were prepared on each day of experimentation and administered at 60 min before initiation of psychomotor seizures evoked by 6 Hz corneal electrical stimulation. The pretreatment times before testing of these AEDs were based upon information about their pharmacokinetic and pharmacological data in the literature and our previous experiments [10, 19].

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 an ECT Unit 5780 (Ugo Basile, Comerio, Italy). Ocular anesthetic (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 behavioral 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 [15]. Animals resumed their normal exploratory behavior after the seizure. The experimental endpoint was protection against the seizure: an animal was considered to be protected if it resumed its normal exploratory behavior 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 behavior when placed in the cages [15]. In the present study, to determine the ED_{50} value, the AEDs were administered i.p. at the following dose ranges: LTG, 5–12 mg/kg and LEV, 5–30 mg/kg. Using the log-probit method the median effective doses (ED_{50} values) were determined using a minimum of 8 mice per dose [13] after which mice were euthanized by CO_2 narcosis.

Isobolographic analysis of interactions. Isobolographic analysis is considered the method of choice for evaluating and characterizing drug interactions for various fixed drug dose ratio combinations (usually, at three fixed-ratios of 1:3, 1:1 and 3:1). The original isobolographic analysis has a fundamental presumption requiring the parallelism of two DRRCs of the investigated drugs administered separately. The percent protection of animals against psychomotor seizures per dose of an AED administered alone and the DRRC for each investigated AED were fitted using log-probit linear regression analysis according to Litchfield and Wilcoxon [13]. Subsequently, from the respective linear equations the ED_{50} values of AEDs administered alone were calculated. To precisely and correctly analyze the experimental data with isobolography, the test for parallelism of DRRCs for LEV and LTG based on the log-probit analysis according to Litchfield and Wilcoxon, was used as described earlier [23, 25]. In this test LEV had its DRRC parallel to that of LTG and interactions between LEV and LTG against 6 Hz-induced seizures were analyzed according to the methodology described by Tallarida [38], and Luszczki et al. [26]. Based on the ED_{50} values denoted previously for the AEDs administered alone, the median additive doses of mixtures of LEV with LTG ($ED_{50\ add}$s – i.e., doses of the two-drug mixtures, which theoretically should protect 50% of the animals tested against 6 Hz-induced seizures) for three fixed-ratio combinations of 1:3, 1:1 and 3:1 were calculated from the equation of additivity presented by Loewe [14], as follows: $x/ED_{50\_LEV} + y/ED_{50\_LTG} = 1$; where x and y are the doses of LEV and LTG, respectively, co-administered as a mixture that exerts the desired effect (50% effect for ED_{50}). Subsequently, proportions of the AEDs in the mixture were calculated and the respective mixtures of LEV with LTG at three fixed-ratios were administered to animals. The anticonvulsant effects offered by LEV and LTG in combination, at three fixed-ratios of 1:3, 1:1 and 3:1, were evaluated and expressed as the experimentally-derived $ED_{50\ mix}$ values,

corresponding to the doses of two-drug mixture, sufficient for the 50% protective effect against 6 Hz-induced seizures in mice. Finally, to determine the separate doses of LEV and LTG in the mixture, the $ED_{50\ mix}$ values were multiplied by the respective proportions of AEDs (denoted for purely additive mixture). Further details regarding these concepts have been published elsewhere [21].

Statistical analysis. The ED_{50} and $ED_{50\ mix}$ values (with their respective 95% confidence limits) for AEDs administered alone or in combination at the fixed-ratios of 1:3, 1:1 and 3:1 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 (S.E.) as described previously [21, 26]. The experimentally-derived $ED_{50\ mix}$ values for the mixture of LEV with LTG were statistically compared with their respective theoretical additive $ED_{50\ add}$ values by the use of unpaired Student's *t*-test, according to Tallarida [38].

Software used. 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 DRRCs of the AEDs administered alone and in combination from the log-probit linear regression analysis according to Litchfield and Wilcoxon [13]. The theoretically additive $ED_{50\ add}$ values and their S.E. at the fixed-ratio combinations of 1:3, 1:1 and 3:1 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).

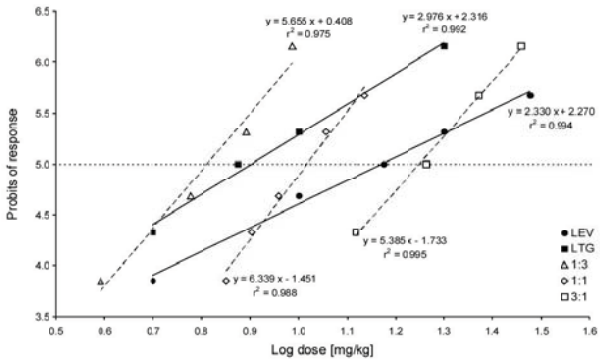
RESULTS

ANTICONVULSANT EFFECTS OF LEVETIRACETAM AND LAMOTRIGINE ADMINISTERED SEPARATELY AND IN COMBINATION IN THE MOUSE 6 HZ PSYCHOMOTOR SEIZURE MODEL. The AEDs administered alone produced a clear-cut anticonvulsant effect against 6 Hz psychomotor seizures and their experimentally-derived ED_{50} values are presented in Table 1. The equations of log-probit DRRC for the studied AEDs, when administered separately and in combination, are presented in Figure 1. The test for parallelism of DRRCs between LEV and LTG revealed that LEV had its log-probit DRRC parallel to that of LTG (Table 1; Figure 1). With regards to LEV and LTG in combination, three fixed-ratio combinations of 1:3, 1:1 and 3:1 were examined so as to determine their $ED_{50\ mix}$ values (Table 2; Figure 1). All fixed-ratio combinations of LEV and LTG exerted a clear-cut anticonvulsant effect and the experimentally-derived $ED_{50\ mix}$ values from the DRRCs for the mixture of both AEDs are presented in Table 2.

Table 1. Anticonvulsant effects of levetiracetam (LEV) and lamotrigine (LTG) administered singly against psychomotor (6 Hz-induced) seizures in mice

Drug	ED_{50} (mg/kg)	*n*	S.E.
LEV	14.84 (9.15 – 24.08)	32	3.66
LTG	7.98 (5.15 – 12.36)	24	1.78
*Test for parallelism: LEV vs. LTG		S.R. = 1.239	f ratio S.R. = 1.489

Results are presented as median effective doses (ED_{50} values in mg/kg; with 95% confidence limits in parentheses) of LEV and LTG administered singly against 6 Hz-induced limbic seizures in mice. The AEDs were administered systemically (i.p.), as follows: LEV and LTG – 60 min before the 6 Hz test. *n* – total number of animals used at doses whose expected anticonvulsant effects ranged between 4 and 6 probits (16% and 84%); S.E. – standard error of ED_{50}; S.R. – slope function ratio (S_{LEV}/S_{LTG}); f ratio S.R. – factor for slope function ratio. Test for parallelism of two DRRCs was performed according to Litchfield and Wilcoxon [13]. If S.R. value is lower than f ratio S.R. value, the examined two DRRCs are parallel one another.
* All detailed calculations required to perform the test for parallelism of two DRRCs were presented in the Appendix to the paper by Luszczki et al. [21].



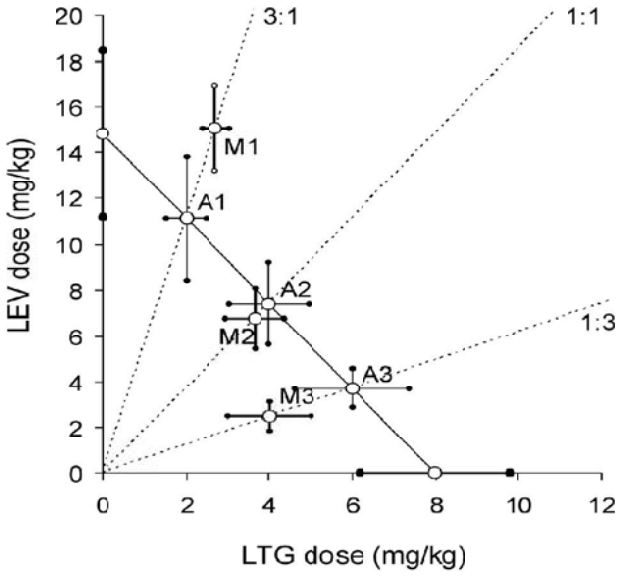
Doses of LEV, LTG and the mixture of both drugs at three fixed-ratio combinations of 1:3, 1:1 and 3:1 were transformed into logarithms, whereas the protective effects offered by the AEDs against 6 Hz-induced seizures were transformed into probits [13]. Linear regression equations of DRRCs are presented on the graph; where y – is the probit of response, and x – is the logarithm (to the base 10) of a drug dose, r^2 – coefficient of determination. Test for parallelism revealed that the experimentally determined DRRCs for LEV and LTG (administered alone) are parallel to one another (for more details see Table 1).

Fig. 1. Log-probit dose-response relationship curves (DRRCs) for levetiracetam (LEV) and lamotrigine (LTG) administered alone, and the combinations of both drugs at the fixed-ratios of 1:3, 1:1 and 3:1 in the mouse psychomotor (6 Hz)-induced seizure model

Table 2. Isobolographic analysis of interactions (for parallel DRRCs) between levetiracetam (LEV) and lamotrigine (LTG) in the mouse 6 Hz-induced limbic seizure model

FR	$ED_{50\ mix}$	n_{mix}	LEV	LTG	$ED_{50\ add}$	n_{add}	LEV	LTG
1:3	6.49 ± 0.76	24	2.48	4.00	9.70 ± 2.25	52	3.71	5.99
1:1	10.42 ± 0.95	32	6.78	3.64	11.41 ± 2.72	52	7.42	3.99
3:1	17.79 ± 2.19	24	15.09	2.70	13.13 ± 3.19	52	11.13	2.00

Data are presented as median effective doses (ED_{50} values in mg/kg ± S.E.) protecting 50% of animals tested against 6Hz-induced seizures. The ED_{50} values were either experimentally determined from the mixture of two AEDs ($ED_{50\ mix}$) or theoretically calculated ($ED_{50\ add}$) from the equation of additivity [14]. The actual doses of LEV and LTG that comprised the mixtures at all three fixed-ratio combinations for both $ED_{50\ mix}$ and $ED_{50\ add}$ values are presented in separate columns. Statistical evaluation of data was performed by using unpaired Student's *t*-test. FR – fixed-ratio of drug dose combinations; *n* – total number of animals used at those doses whose expected anticonvulsant effects were ranged between 4 and 6 probits, denoted for the experimental mixture of drugs (n_{mix}) and theoretically calculated ($n_{add} = n_{\_LEV} + n_{\_LTG} - 4$) from the equation of additivity.



The median effective dose (ED_{50}) for levetiracetam (LEV) is plotted graphically on the Y-axis, whereas the ED_{50} value of lamotrigine (LTG) is plotted on the X-axis. The solid lines on the X and Y axes represent the S.E. for the ED_{50}s of AEDs administered alone. The straight line connecting these ED_{50} values on a graph represents the theoretical line of additivity for a continuum of different fixed dose ratios. The dotted lines starting from the point (0,0) correspond to the fixed-ratios of 1:3, 1:1, and 3:1 for the combination of LEV with LTG. The open circles (o) depict the experimentally derived $ED_{50\ mix}$ values (± S.E. as the error bars) for the total dose expressed as the proportion of LEV and LTG that produced a 50% anticonvulsant effect. The closed circles (?) depict the additively calculated $ED_{50\ add}$ values (± S.E. as the error bars) for the total dose expressed as the proportion of LEV and LTG that produced a 50% anticonvulsant effect. The experimentally-derived $ED_{50\ mix}$ values of the mixture of LEV with LTG for all the fixed-ratios of 1:3, 1:1, and 3:1 are placed close to the theoretical line of additivity, indicating the additive interaction. For more details see Table 2. The X- and Y-coordinates for all points presented on the isobologram are as follows: A1 (2.0; 11.13), A2 (3.99; 7.42), A3 (5.99; 3.71), M1 (2.70; 15.09), M2 (3.64; 6.78), and M3 (2.48; 4.00).

Fig. 2. Isobologram illustrating additive interactions between levetiracetam (LEV) and lamotrigine (LTG) in the psychomotor (6Hz)-induced seizures in mice

ISOBOLOGRAPHIC ANALYSIS OF INTERACTION BETWEEN LEVETIRACETAM AND LAMOTRIGINE IN THE MOUSE 6 HZ PSYCHOMOTOR SEIZURE MODEL. The isobolographic analysis of interaction for parallel DRRCs revealed that all three fixed-ratio combinations of LEV with LTG at 1:3, 1:1, and 3:1 exerted additive interaction in the 6 Hz test in mice (Table 2; Figure 2) and their $ED_{50\ mix}$ values are shown in Table 2. Statistical analysis of data with Student's *t*-test revealed that the $ED_{50\ mix}$ values did not differ significantly from their corresponding $ED_{50\ add}$ values (Table 2; Figure 2).

DISCUSSION

The present results show that LEV and LTG produced a clear-cut anticonvulsant effect against 6 Hz psychomotor seizures in mice. The characterization of interactions of LEV with LTG by using the type I isobolographic analysis for parallel DRRCs revealed that the combinations of LEV with LTG for all three fixed-ratios of 1:3, 1:1 and 3:1 were additive. In the present study we did not measure free plasma or total brain AED concentrations because, as documented earlier, LEV is not expected to interact pharmacokinetically with LTG [33]. Therefore, the observed additive interactions between LEV and LTG can be considered the consequence of pharmacodynamic interactions.

To explain the observed additive interactions between LEV and LTG one should consider molecular mechanisms of action of both AEDs. With regards to LTG, the drug acts at voltage-dependent sodium channels to decrease the presynaptic release of the excitatory neurotransmitter glutamate [2]. LTG blocks veratridine-evoked, but not potassium-elicited release of endogenous glutamate [12]. Moreover, LTG decreases voltage gated calcium currents [37], and probably, this effect contributes also to a decrease in glutamate release. In case of LEV, molecular studies have revealed that the drug reduces voltage-operated K^+ current and inhibits the delayed rectifier K^+ current in neurons [28], reduces N-type and partially P/Q-type high-voltage activated Ca^{2+} currents [16, 31], but not low-voltage-activated Ca^{2+} currents [41]. Moreover, LEV suppresses the inhibitory action of zinc and β-carbolines on $GABA_A$- and glycine-gated currents [35], blocks $GABA_A$ receptor run-down in neocortex and thus, increases GABA-ergic inhibitory neurotransmission in the brain [32]. Molecular studies involving transgenic mice suggest that LEV binds to a synaptic vesicle protein 2A (SV2A), which is involved in vesicle neurotransmitter exocytosis [17].

Considering the above-mentioned mechanisms of action of both AEDs, it is difficult to unequivocally ascertain which of these mechanisms are responsible for the additive interactions observed in the present study. It is highly likely that all mechanisms are involved, at least in part, in this additive interaction between LEV and LTG in the mouse 6 Hz-induced psychomotor seizure model.

Experimental evidence indicates also that LEV combined with lacosamide (LCM – a third-generation AED) at the fixed-ratios of 1:3, 1:1 and 3:1 interacted synergistically in the mouse 6 Hz model [7]. More specifically, a pharmaceutical composition comprising LEV and LCM (two licensed AEDs) has been patented based on their synergistic anticonvulsant interaction in the mouse 6 Hz model [7]. The synergistic interaction between LEV and LCM was assessed with isobolographic analysis, suggesting that this method is a perfect tool for researchers, who apply for a patent. On the other hand, a new trend of patenting synergistic AED interaction has appeared in experimental epileptology. At present, a question arises whether all synergistic combinations between AEDs that were elaborated in preclinical studies should be patented. If so, clinicians should conduct clinical trials providing evidence that the combinations of two or three AEDs are clinically favorable.

Additionally, the type I isobolographic analysis revealed that LEV interacted synergistically with phenobarbital and produced additive interaction when combined with clonazepam, clobazam, oxcarbazepine, tiagabine and valproate in the mouse 6 Hz-induced psychomotor seizure model [1, 40].

Based on this preclinical study, one can conclude that the combination of LEV with LTG can potentially offer the patients with limbic seizures a favorable combination and worthy of clinical evaluation. Nevertheless, because a substantial dose reduction of both drugs (LEV and LTG) in the mixture can be anticipated, it can be expected that concurrent adverse effects would be significantly reduced and this is a clinically desirable outcome [3, 24, 34].

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DISCLOSURE OF CONFLICTS OF INTEREST

The authors have no disclosures to declare.

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