Isobolographic characterization of interaction of levetiracetam with clobazam in the mouse 6 Hz psychomotor seizure model

Jarogniew J. Łuszczki1,2, Aleksandra Właź2, Ewa Marzęda1, Dominika Podgórska2, Dariusz Durmowicz1, Magdalena Florek-Łuszczki3

1 Isobolographic Analysis Laboratory, Institute of Rural Health, Lublin, Poland
2 Department of Pathophysiology, Medical University, Lublin, Poland
3 Department of Public Health, Institute of Rural Health, Lublin, Poland

Abstract

Introduction and objective: The aim of this study was to characterize the anticonvulsant effects of levetiracetam (LEV) in combination with clobazam (CLB – a second-generation antiepileptic drug), in the mouse 6 Hz psychomotor seizure model.

Materials and methods: 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. Isobolographic analysis for parallel dose–response relationship curves (DRRCs) was used to characterize the consequent anticonvulsant interactions between the drug combinations. Potential concurrent adverse-effect profiles of interactions between LEV and CLB were evaluated in the chimney (motor performance), passive-avoidance (long-term memory), and grip-strength (muscular strength) tests.

Results: LEV administered singly was associated with a DRRC that was parallel to that for CLB. With isobolography for parallel DRRCs, the combination of LEV with CLB at three fixed-ratios of 1:3, 1:1 and 3:1 exerted additive interaction. None of the combinations were associated with any concurrent adverse effects with regards to motor coordination, long-term memory or muscular strength.

Conclusions: LEV combined with CLB exerted additive interaction in the mouse 6 Hz psychomotor seizure model.

Key words

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

INTRODUCTION

Levetiracetam (LEV) is a unique second-generation antiepileptic drug (AED) which in preclinical studies is virtually ineffective in acute models of epilepsy i.e., the maximal electroshock (MES)- and pentylentetrazole (PTZ)-induced seizures [1, 2, 3], routinely used to screen for potential new AEDs [4, 5]. In contrast, LEV increased the threshold for electroconvulsions and suppressed seizures in kindled and genetically epileptic animals [1, 2, 3, 6, 7]. LEV has also shown protective activity against acute seizures induced by low frequency (6 Hz) long-duration (3 s) corneal electrical stimulation (a model of psychomotor or limbic seizures) [8, 9, 10, 11, 12]. Moreover, the drug attenuates spike-and-wave discharges in DBA/2 mice (an animal model of absence of epilepsy) [13], and it demonstrates potent anticonvulsant effects against audiogenic seizures in Krushinsky-Molodkina rats (a strain of rats selected for susceptibility to audiogenic seizures) [14].

Experimental evidence indicates that LEV is associated with favorable anticonvulsant pharmacodynamic interactions with numerous AEDs in various animal models, including: topiramate, oxcarbazepine, carbamazepine, diazepam, felbamate, clonazepam, valproate, phenobarbital and gabapentin [15, 16, 17, 18, 19, 20, 21, 22]. Previously, we found that LEV interacted synergistically with phenobarbital and produced additive interaction when combined with clonazepam, oxcarbazepine, tiagabine and valproate in the mouse 6 Hz-induced psychomotor seizure model [12].

Considering the fact that LEV is effective in the low frequency, long-duration corneal stimulation model (6 Hz psychomotor seizures), it was of pivotal importance to determine the interaction profile for LEV in combination with clobazam (CLB – a second-generation AED) that was also effective against 6Hz-induced psychomotor seizures in mice. The 6 Hz psychomotor seizures were reported to involve a minimal, clonic phase, followed by stereotyped and automatistic behaviours reminiscent of aura of patients with partial or limbic epilepsy [8, 9, 10, 11]. At present, the 6 Hz psychomotor seizure model is used for the early identification of anticonvulsant activity of new compounds effective against therapy-resistant epilepsy [11]. Therefore, the objective of this study was to evaluate potential interaction of LEV in combination with CLB in this model, and to use type I
isobolographic analysis for parallel dose-response relationship curves (DRRCs). Additionally, in order to determine the acute adverse-effect profiles for the combinations, the chimney test (a measure of motor performance impairment), the step-through passive avoidance task (a measure of long-term memory deficits), and the grip-strength test (a measure of skeletal muscular strength impairment) were used.

**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 21 ± 1°C, relative humidity 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 09.00-15.00. 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 in 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 CLB (Sanofi-Aventis Deutschland GmbH, Frankfurt-am-Main, Germany). 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 drug solutions were prepared on each day of experimentation and administered as follows: LEV – 60 min and CLB – 30 min before initiation of psychomotor seizures evoked by 6 Hz corneal electrical stimulation, evaluation of motor coordination, skeletal muscular strength and long-term memory tests. 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 [17, 23]. The times to the peak of maximum anticonvulsant effects for the AEDs 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 an ECT Unit 5780 (Ugo Basile, Comerio, Italy). 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 60-120 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 [9, 10]. 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 [9]. In the presented study, to determine the ED50 value, the AEDs were administered i.p. at the following dose ranges: CLB, 1-3 mg/kg and LEV, 5-30 mg/kg. Using the log-probit method, the median effective doses (ED50 values) were determined using a minimum of 8 mice per dose [24], after which mice were euthanized by CO2 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 [24]. Subsequently, from the respective linear equations the ED50 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 CLB based on the log-probit analysis according to Litchfield and Wilcoxon, was used, as described earlier [25, 26]. In this test, LEV had its DRRC parallel to that of CLB and interactions between LEV and CLB against 6 Hz-induced seizures were analyzed according to the methodology described by Tallarida [27], and Luszczki et al. [28]. Based on the ED50 values denoted previously for the AEDs administered alone, the median additive doses of mixtures of LEV with CLB (ED50 adds – 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 [29], as follows:

\[
x/ED_{50,LEV} + y/ED_{50,CLB} = 1 \]

where x and y are the doses of LEV and CLB, respectively, co-administered as a mixture that exerts the desired effect (50% effect for ED50). Subsequently, the proportions of the AEDs in the mixture were calculated and the respective mixtures of LEV with CLB at three fixed-ratios were administered to the animals. The anticonvulsant effects offered by LEV and CLB in combination, at three fixed-ratios of 1:3, 1:1 and 3:1, were evaluated and expressed as the experimentally-derived ED50 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 CLB in the mixture, the ED50 values were multiplied by the respective proportions of AEDs (denoted for purely additive mixture). Further details regarding these concepts have been published elsewhere [30].
Grip-strength test. The effects of LEV in combination with CLB at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1) on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. [31]. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 x 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tail so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The animals received the combinations of LEV with CLB at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1). The grip-strength test was used to determine the effects of AEDs on skeletal muscular strength, which was expressed in newtons (N) as means ± S.E.M. of 8 determinations (8 animals per group).

Chimney test. The effects of LEV in combination with CLB, at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1) on motor performance impairment were quantified with the chimney test of Boissier et al. [32]. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length), and motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. The animals received LEV in combination with CLB at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1). The acute adverse effects of AEDs in combination were expressed as the percentage of animals failing to perform the chimney test within 60 s.

Step-through passive avoidance task. On the first day before training, each animal was administered the combination of LEV with CLB at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1). The time before the commencement of the training session (after drug administration) was identical to that for the 6 Hz-induced seizure test. Subsequently, the animals were placed in an illuminated box (10 x 13 x 15 cm) connected to a larger dark box (25 x 20 x 15 cm) equipped with an electric grid floor. Entry of animals to the dark box was punished by an adequate electric foot shock (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation. On the following day (24 h later), the pre-trained animals did not receive any treatment and were placed again into the illuminated box and observed for up to 180 s. Mice that avoided the dark compartment for 180 s were considered to remember the treatment and were placed again into the illuminated box (10 x 13 x 15 cm). The step-through passive avoidance task was analyzed with one-way ANOVA followed by the post-hoc Bonferroni’s test for multiple comparisons. Qualitative variables from the chimney test were compared by use of the Fisher’s exact probability test. The data from the step-through passive avoidance task were statistically analyzed using the nonparametric Kruskal-Wallis test, followed by the post-hoc Dunn’s test.

RESULTS

Anticonvulsant effects of LEV and CLB 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 ED50 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 CLB revealed that LEV had its log-probit DRRC parallel to that of CLB (Tab.1; Fig. 1). With regards to LEV and CLB in combination, three fixed-ratio combinations of 1:3, 1:1 and 3:1 were examined in order to determine their ED50 values ( Tabs.2; Fig.1). All fixed-ratio combinations of LEV and CLB exerted a clear-cut anticonvulsant effect; the experimentally-derived ED50 values from the DRRCs for the mixture of both AEDs are presented in Table 2.

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 (mg/kg)</th>
<th>n</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEV</td>
<td>14.84 (9.15 – 24.08)</td>
<td>32</td>
<td>3.66</td>
</tr>
<tr>
<td>CLB</td>
<td>1.53 (1.11 – 2.12)</td>
<td>32</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Results are presented as median effective doses (ED50 values in mg/kg, with 95% confidence limits in parentheses) of LEV and CLB administered singly against 6 Hz-induced limbic seizures in mice. The AEDs were administered systemically (i.p.), as follows: LEV – 60 min and CLB – 30 min before the 6 Hz test.

Statistical analysis. The ED50 and ED50min 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 [24]. The obtained 95% confidence limits were transformed to standard errors of the mean (S.E.M.), as described previously [28, 30]. The experimentally-derived ED50 values for the mixture of LEV with CLB were statistically compared with their respective theoretical additive ED50 values by the use of unpaired Student’s t-test, according to Tallarida [27]. The results from the grip-strength test were analyzed with one-way ANOVA followed by the post-hoc Bonferroni’s test for multiple comparisons. Qualitative variables from the chimney test were compared by use of the Fisher’s exact probability test. The data from the step-through passive avoidance task were statistically analyzed using the nonparametric Kruskal-Wallis test, followed by the post-hoc Dunn’s test.

Software. A Microsoft 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 to determine the DRRCs of the AEDs administered alone and in combination from the log-probit linear regression analysis according to Litchfield and Wilcoxon [24]. The theoretically additive ED50 add values and their S.E.M. 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).
Doses of LEV and CLB and the mixture of LEV with CLB 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 [24]. 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, r² – coefficient of determination. Test for parallelism revealed that the experimentally determined DRRCs for LEV and CLB (administered alone) are parallel to one another (for more details see Table 1).

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

<table>
<thead>
<tr>
<th>FR</th>
<th>Dose of LEV (mg/kg)</th>
<th>Dose of CLB (mg/kg)</th>
<th>DRRC ED₅₀/µ</th>
<th>nᵣ</th>
<th>LEV</th>
<th>CLB</th>
<th>ED₅₀/µ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3</td>
<td>4.28 ± 0.25</td>
<td>7.51 ± 0.42</td>
<td>7.51 ± 0.42</td>
<td>24</td>
<td>1.01</td>
<td>1.01</td>
<td>7.51 ± 0.42</td>
</tr>
<tr>
<td>1:1</td>
<td>5.00 ± 0.50</td>
<td>7.51 ± 0.42</td>
<td>7.51 ± 0.42</td>
<td>24</td>
<td>1.01</td>
<td>1.01</td>
<td>7.51 ± 0.42</td>
</tr>
<tr>
<td>3:1</td>
<td>15.04 ± 1.57</td>
<td>7.51 ± 0.42</td>
<td>7.51 ± 0.42</td>
<td>24</td>
<td>1.01</td>
<td>1.01</td>
<td>7.51 ± 0.42</td>
</tr>
</tbody>
</table>

Data are presented as median effective doses (ED₅₀ or ED₅₀ mix) in mg/kg ± S.E.M. protecting 50% of animals tested against 6Hz-induced seizures. The ED₅₀ values were either experimentally derived from the mixture of two AEDs (ED₅₀ mix) or theoretically calculated (ED₅₀ add) from the equation of additivity [29]. The actual doses of LEV and CLB that comprised the mixtures at the three fixed-ratio combinations for both ED₅₀ and ED₅₀ mix 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; nᵣ - total number of animals used at those doses whose expected anticonvulsant effects ranged between 6 and 6 probits, denoted for the experimental mixture of drugs (nᵣ mix) and theoretically calculated (nᵣ add) from the equation of additivity.

Isobolographic analysis of interaction between LEV and CLB in the mouse 6 Hz psychomotor seizure model

Isobolographic analysis of interaction for parallel DRRCs revealed that all three fixed-ratio combinations of LEV with CLB at 1:3, 1:1, and 3:1 exerted additive interaction in the 6 Hz test in mice (Tab.2; Fig.2); their ED₅₀/µ values are shown in Table 2. Statistical analysis of data with Student’s t-test revealed that the ED₅₀/µ mix values did not differ significantly from their corresponding ED₅₀/µ add values (Tab.2; Fig.2).

Effects of LEV in combination with CLB on skeletal muscular strength, motor performance, and long-term memory in the grip-strength, chimney and step-through passive avoidance tests in mice

None of the studied combinations of LEV and CLB at the respective fixed drug dose ratios from the 6 Hz-induced psychomotor seizure test (i.e., 1:3, 1:1 and 3:1) impaired long-term memory, as determined in the passive avoidance task (Tab.3). Similarly, these combinations did not affect skeletal muscular strength as assessed by the grip-strength test (Tab.3), and did not alter motor performance in animals challenged with the chimney and step-through test (Tab.3). Moreover, LEV and CLB (at doses corresponding to their ED₅₀ values from the 6 Hz-induced psychomotor seizure test) produced no acute adverse effects in the grip-strength, chimney and step-through passive avoidance tests in mice (results not shown).

DISCUSSION

The presented results show that LEV and CLB produced a clear-cut anticonvulsant effect against 6 Hz psychomotor seizures in mice. The characterization of interactions of LEV
with CLB by using the type 1 isobolographic analysis for parallel DRRCs revealed that the combinations of LEV with CLB for all three fixed-ratios of 1:3, 1:1 and 3:1 were additive.

In the presented study, free plasma or total brain AED concentrations were not measured because, as documented earlier, LEV is not expected to interact pharmacokinetically with CLB [34]. Therefore, the observed additive interactions between LEV and CLB can be considered the consequence of pharmacodynamic interactions.

To explain the observed additive interactions between LEV and CLB one should consider molecular mechanisms of action of both AEDs. With regards to CLB (a 1,5-benzodiazepine), it enhances GABA(ergic) activity by binding to the α subunit of the GABA(α) receptor, and increasing the frequency of chloride channel conductance by allosteric activation of the GABA(α) receptor [35]. Moreover, CLB increases expression of glutamate transporter protein 1 (GLT1) and GABA transporter protein 3 (GAT3) in the brain [36].

In the case of LEV, molecular studies have revealed that the drug reduces voltage-operated K+ current and inhibits the delayed rectifier K+ current in neurons [37], reduces N-type and partially P/Q-type high voltage activated Ca2+ currents [38, 39], but not low-voltage-activated Ca2+ currents [40]. Moreover, LEV suppresses the inhibitory action of zinc and β-carbolines on GABA- and glycine-gated currents [41], blocks GABA(α) receptor run-down in neocortex, and thus increases GABA-ergic inhibitory neurotransmission in the brain [42]. Additionally, LEV inhibits ryanodine receptor (RyR) and inositol 1,4,5-triphosphate receptor (IP3R) mediated calcium-induced calcium release (CICR) in hippocampal neurons in culture [43], and thus, LEV by inhibiting Ca2+ release through both RyR and IP3R, affects a major second messenger system in neurons [43]. LEV activates renal outer medullary potassium (ROMK1) channels through a protein kinase A (PKA)-mediated phosphorylation [44]. The major physiological function of ROMK1 channels is to maintain the resting membrane potential during cellular excitation; therefore, LEV is capable of reducing neuronal excitability [44]. Molecular studies involving transgenic mice suggest that LEV binds to a synaptic vesicle protein 2A (SV2A), which is involved in vesicle neurotransmitter exocytosis [45]. At present, it is difficult to unequivocally ascertain which of the above-mentioned mechanisms of action are responsible for the additive interactions observed in the presented study. It is highly likely that all the above-discussed mechanisms are involved, at least in part, in these additive interactions between LEV and CLB in the mouse 6 Hz-induced psychomotor seizure model.

With regards to adverse effects of the AED combinations, results from the step-through passive avoidance task indicate that none of the combinations were associated with an effect on long-term memory. Furthermore, there were no effects on motor coordination and skeletal muscular strength as assessed by the chimney and grip-strength tests, respectively. These data might suggest that these tests of adverse effects may therefore not be sensitive; however, this is not the case. In a study of tiagabine co-administered with valproate, a significant impairment in motor coordination, as determined in the chimney test, was observed [46]. In studies of WIN 55,212-2 mesylate (a non-specific cannabinoid CB1 and CB2 receptor agonist) in combination with clonazepam, ethosuximide, phenobarbital and valproate, a significant impairment in motor coordination was observed [47, 48]. Similarly, adverse effects have been documented in the step-through passive avoidance task (tiagabine with gabapentin and vigabatrin with clonazepam and valproate) [49, 50]. Finally, in the grip-strength test, WIN 55,212-2 mesylate in combination with classical AEDs (clonazepam, carbamazepine, ethosuximide, phenytoin, phenobarbital and valproate) significantly reduced muscular strength [47, 48]. The above-described data clearly indicate that the experimental tests used in the presented study were sensitive for measurement of animal behaviour and adverse effect changes that might have occurred. Consequently, it can be concluded that the lack of adverse effects when LEV was combined with CLB testifies to a low toxic potential of these drug combinations.

Based on this preclinical study, one can conclude that the combination of LEV with CLB can potentially offer patients with limbic seizures a favourable combination and worthy of clinical evaluation. Nevertheless, because a substantial dose reduction of both drugs in the mixture can be anticipated, it can be expected that concurrent adverse effects would be significantly reduced, which would be a clinically desirable outcome [49, 50, 51, 52, 53].

Disclosure of conflicts of interest

The authors have no disclosures to declare.

Acknowledgements

This study was supported by research grants from the Medical University and the Institute of Rural Health in Lublin, Poland. Professor J. J. Luszczki is a Member of the Academy of Young Scientists in the Polish Academy of Sciences (Warsaw, Poland).

REFERENCES


