Interactions between pregabalin and phenobarbital in the mouse maximal electroshock-induced seizure model: an isobolographic analysis

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Abstract: The aim of this study was to characterize the anticonvulsant effects of pregabalin (PGB – a third-generation antiepileptic drug) in combination with phenobarbital (PB – a classical antiepileptic drug) in the mouse maximal electroshock (MES)-induced seizure model by using the type I isobolographic analysis for non-parallel dose-response relationship curves (DRRCs). Tonic hind limb extension (seizure activity) was evoked in adult male albino Swiss mice by a current (sine-wave, 25mA, 500V, 50Hz, 0.2s stimulus duration) delivered via auricular electrodes. Potential adverse-effect profiles of interaction of PGB with PB at the fixed-ratio of 1:1 in the MES test with respect to motor performance, long-term memory and skeletal muscular strength were measured together with total brain PB concentrations. In the mouse MES model, PGB administered singly had a DRRC non-parallel to that for PB. With type I isobolographic analysis for non-parallel DRRCs, the combination of PGB with PB at the fixed-ratio of 1:1 exerted additive interaction. In combination, neither motor coordination, long-term memory nor muscular strength were affected. Pharmacokinetic estimation of total brain PB concentrations revealed that PGB did not affect total brain concentrations of PB in experimental animals. In conclusion, the additive interaction between PGB and PB is worthy of consideration while extrapolating the results from this study to clinical settings.

Key words: pregabalin, phenobarbital, isobolographic analysis, maximal electroshock, pharmacodynamic/pharmacokinetic interaction

INTRODUCTION

Although many new (second-generation) antiepileptic drugs (AEDs) have been introduced in the last decade, there is still a clear need for AEDs with improved efficacy and tolerability that are also easy to use in clinical practice. At present, less than half of all patients become seizure-free with the first AED tried, and approx. 30% remain uncontrolled on either their first or second AED [14]. The remaining patients are difficult to control from the beginning, and will still experience seizures even when receiving a combination of currently available AEDs. Therefore, some novel (third-generation) AEDs with improved efficacy and novel mechanisms of action are urgently needed to provide effective combination treatment for patients with epilepsy [21].

Pregabalin (PGB; (S)-(+-)3-(aminomethyl)-5-methylhexanoic acid or (S)-(+-)3-isobutyl GABA) is a third-generation AED recently licensed as an adjunct therapy for partial (simple and complex) seizures, with or without secondary generalization, in patients over 18 years of age [6, 10, 12].

Experimental evidence indicates that PGB exhibits anticonvulsant activity in the maximal electroshock (MES)-induced tonic seizure and pentylentetrazole (PTZ)-induced clonic seizure models in rodents [38]. PGB reduced the incidence of seizures in DBA/2 audiogenic mice, but the drug did not reduce the incidence of spontaneous absence seizures in genetically susceptible rats (GAERS) [38]. PGB prevents seizures in hippocampal kindled rats [38], and protects the animals against seizures induced by picrotoxin or bicuculline [38].

The aim of this study was to determine the interaction profile of PGB (a third-generation AED) in combination with phenobarbital (PB – a classical AED used in patients with generalized tonic-clonic seizures and partial onset seizures) in the mouse MES model. Generally, the mouse MES model is considered as an animal model of tonic-clonic seizures and partial convulsions, with or without secondary generalization in humans [16, 17]. Thus, it was appropriate to determine the interaction profile of PGB with PB in the mouse MES model.

Additionally, 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 to determine the acute adverse-effect potential for the combination of PGB with PB. Finally, to ascertain whether the observed interaction was pharmacodynamic in nature or that pharmacokinetic interaction also contributed, total brain PB concentrations were measured with fluorescence polarization immunoassay.
MATERIALS AND METHODS

Animals and experimental conditions. All experiments were performed on adult male albino Swiss mice (weighing 22-26 g, 6-weeks-old) purchased from the licensed breeder (Dr. T. Gorzkowska, 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 21 ± 1°C, relative humidity 55 ± 3%). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups, each group consisting of 8 mice. Individual mice were used only once. All tests were performed between 09:00-15:00. Procedures involving animals and their care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. 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 described in this manuscript were approved by the Local Ethics Committee at the Medical University of Lublin (License No.: 21/2007).

Drugs. The following AEDs were used in this study: PGB (Lyrica®, Pfizer Ltd., Sandwich, Kent, UK) and PB (Polfa, Krakow, Poland). The AEDs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in saline and administered by intraperitoneal (i.p.) injection in a volume of 0.005 ml/g body weight. The AEDs were administered 60 min before seizures and behavioural tests, as well as before brain sampling for the measurement of AED concentrations. The time to the peak of maximum anticonvulsant effects for the AEDs was used as the reference time in all behavioural tests. The route of systemic (i.p.) administration and these pretreatment times were chosen based upon information about their biological activity from the literature [36] and pilot studies.

Maximal electroshock seizure test. The protective activities of PGB and PB administered separately were evaluated and expressed as their median effective doses (ED₅₀, in mg/kg), protecting 50% of mice against MES-induced seizures (sine-wave, fixed current intensity of 25 mA, maximum stimulation voltage of 500 V, frequency of 50 Hz). Electroconvulsions were produced by a current (0.2 s stimulus duration) delivered via standard auricular electrodes by a Hugo Sachs generator (Rodent Shocker, Type 221, Freiburg, Germany). The criterion for the occurrence of seizure activity was tonic hindlimb extension. The animals were administered with different drug doses in order to obtain a variable percentage of protection against MES-induced seizures, allowing the construction of a dose-response relationship curve (DRRC) for PGB and PB administered alone, according to Litchfield and Wilcoxon [15]. The anticonvulsant activity of the mixture of PGB with PB at the fixed-ratio of 1:1 was evaluated and expressed as median effective doses (ED₅₀ values) against MES-induced seizures. This experimental procedure has been described in detail elsewhere [22, 25, 31, 32, 41].

Isobolographic analysis of interactions. The percent protection of animals against MES-induced seizures per dose of an AED administered alone and the DRRC for each investigated AED in the mouse MES model were fitted using log-probit linear regression analysis according to Litchfield and Wilcoxon [15]. Subsequently, from the respective linear equations the median effective doses (ED₅₀) of AEDs administered alone were calculated. To precisely and correctly analyze the experimental data with isobolography, the test for parallelism of DRRCs for PGB and PB based on the log-probit analysis was used [18, 19, 23, 24]. The test for parallelism was performed according to Litchfield and Wilcoxon [15], as described previously in detail [23]. In this test, PGB had its DRRC non-parallel to that of PB (Table 1). Therefore, the interactions between PGB and PB against MES-induced seizures were analyzed according to the methodology described by Tallarida [35] and Luszczki [18-20]. Based upon the ED₅₀ values denoted previously for the AEDs administered alone, median additive doses of the mixture of PGB with PB – i.e., doses of the mixture, which theoretically should protect 50% of the animals tested against MES-induced seizures (ED₅₀mix) – were calculated from 2 equations of additivity presented by Tallarida [35]. For the lower line of additivity, the equation at a 50% effect for the combination of PGB with PB is as follows: \( y = \text{ED}_{50_{PB}} - \frac{\text{ED}_{50_{PB}}}{\text{ED}_{50_{PGB}} / \text{ED}_{50_{PGB}} - x} \). To calculate the curve-fitting parameters (Hill coefficients) for PB and PGB, respectively. Similarly, for the upper line of additivity, the equation at a 50% effect for the combination of PGB with PB is as follows: \( y = \text{ED}_{50_{PB}} \left( \frac{\text{ED}_{50_{PGB}}}{\text{ED}_{50_{PGB}} - x} \right) \). The homogenates were centrifuged at 10,000 g for 10 min. and the supernatant samples (75 μl) analyzed by fluorescence polarization immunoassay using a TDx analyzer and reagents (PB), exactly as described by the manufacturer (Abbott Laboratories, North Chicago, IL, USA). Total brain concentrations of PB were expressed in μg/ml of brain supernatants as means ± S.D. of at least 8 separate brain preparations.

Chimney test. The effects of the studied AEDs (PGB and PB) administered alone at their ED₅₀ values and in combination (administered at doses corresponding to their ED₅₀mix values)
at the fixed-ratio of 1:1 from the MES-induced seizure test) on motor coordination impairment were quantified with the chimney test of Boissier et al. [3]. In the chimney test, animals had to climb backwards up a plastic tube (3 cm inner diameter, 30 cm length). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Data were presented as a percentage of animals that failed to perform the chimney test. This experimental procedure has been described in detail earlier [22, 24, 25, 41].

**Step-through passive avoidance task.** On the first day before training, each animal received either the studied AEDs administered alone or the respective combination of PGB with PB, at doses corresponding to their ED_{50max} values at the fixed-ratio of 1:1 from the MES-induced seizure test. Subsequently, animals were placed in an illuminated box (10 × 13 × 15 cm) connected to a larger dark box (25 × 20 × 15 cm) equipped with an electric grid floor. Entry of animals into the dark box was punished by an adequate electric footshock (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 as having remembered the task. The time that the mice took to enter the dark box was noted and the median latencies (retention times) with 25th and 75th percentiles were calculated. The step-through passive avoidance task gave information about ability to acquire the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory [39]. This experimental procedure has been described in detail earlier [29, 30].

**Grip-strength test.** The effects of the studied AEDs administered alone (PGB and PB) and in combination (administered at doses corresponding to their ED_{50max} values at the fixed-ratio of 1:1 from the MES-induced seizure test) on muscular strength in mice were quantified by the grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tails until they relinquished grip on the grid. The maximal force exerted by the mice before losing grip was recorded. The mean of 3 measurements for each animal was calculated and the mean maximal force of 8 animals per group was determined. The skeletal muscular strength in mice was expressed in N (newtons) as means ± S.E.M. of at least 8 determinations. This experimental procedure has also been described in detail earlier [22, 25, 41].

**Statistics.** The ED_{50} and ED_{50max} values (with their respective 95% confidence limits) for PGB and PB administered alone or in combination at the fixed-ratio of 1:1 in the MES-induced seizure test were calculated by computer-assisted log-probit analysis according to Litchfield and Wilcoxon [15]. In the isobolographic analysis for non-parallel DRRCs, the experimentally derived ED_{50max} value for the mixture of PGB with PB at the fixed-ratio of 1:1 was statistically compared with their respective theoretically additive ED_{50add} values by using the unpaired Student’s t-test, according to the method described by Tallarida [35]. Total brain AED concentrations were statistically analyzed using the unpaired Student’s t-test. Qualitative variables from the chimney test were compared by using the Fisher’s exact probability test. Median retention times obtained in the passive avoidance task were statistically evaluated using Kruskal-Wallis non-parametric ANOVA. The mean values of skeletal muscular strength from the grip-strength test were analyzed statistically with one-way ANOVA followed by the Bonferroni’s post-hoc test for multiple comparisons. Differences among values were considered statistically significant if P<0.05. 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 PGB and PB administered separately and in combination in the mouse MES model.** PGB administered alone (i.p., 60 min. before test) at doses ranging between 50-250 mg/kg produced a clear-cut anticonvulsant effect that increased from 12.5% - 75% against MES-induced seizures (Fig. 1). The equation of DRRC for PGB allowed determination of the ED_{50} value for the AED, which was 142.14 ± 32.54 mg/kg (Fig. 1, Table 1). Similarly, PB administered singly (i.p., 60 min. before test) at doses ranging between 15 - 35 mg/kg produced a definite antiseizure activity that increased from 25%-75% in the mouse MES model (Fig. 1). The equation of DRRC for PB allowed determination of the ED_{50} value for PB that amounted to 26.17 ± 2.07 mg/kg (Fig. 1, Table 1). The test for parallelism of DRRCs between PGB and PB revealed that the AEDs had their DRRCs non-parallel to one another (Fig. 1, Table 1). The combination of PGB with PB at the fixed-ratio of 1:1 exerted antiseizure activity in the MES test, and the experimentally derived ED_{50max} value from the DRRC for the mixture of both AEDs was 28.32 ± 4.26 mg/kg (Fig. 1, Table 2).

**Isobolographic analysis of interaction between PGB and PB in the mouse MES model.** Type I isobolographic analysis of interaction for non-parallel DRRCs revealed that the mixture of PGB with PB at the fixed-ratio of 1:1 exerted additive interaction in the MES test in mice (Fig. 2). The experimentally derived ED_{50max} value for this fixed-ratio combination was 28.32 ± 4.26 mg/kg, whereas the additively calculated ED_{50add} values were 35.90 ± 27.78 mg/kg (for the lower ED_{50add}) and 132.42 ± 15.96 mg/kg (for the upper ED_{50add}) (Table 2). Thus, the ED_{50max} value did not significantly differ from the ED_{50add} Values (Table 2, Fig. 2).

**Total brain PB concentrations.** Total brain concentration of PB administered singly at a dose of 4.4 mg/kg was 1.40 ± 0.18 µg/ml of brain supernatant, and did not significantly differ from the total brain concentration of PB (4.40 mg/kg) co-administered with PGB (23.92 mg/kg), which amounted to 1.54 ± 0.19 µg/ml of brain supernatant.

**Effects of PGB, PB, and their combination on motor performance in the chimney test, long-term memory in the step-through passive avoidance task, and skeletal muscular strength in the grip-strength test**
Anticonvulsant effects of pregabalin (PGB) and phenobarbital (PB) administered singly against maximal electroshock (MES)-induced seizures in mice.

Results are presented as median effective doses (ED50 values in mg/kg ± S.E.M.) of PGB and PB administered singly against MES-induced seizures in mice. The drugs were administered systemically (i.p.), as follows: PGB and PB – 60 min before the MES-induced seizures; n – total number of animals used at doses whose expected anticonvulsant effects ranged between 4 - 6 probits (16% and 84%); CFP – q/ratio of q and p values; S.R. – slope function ratio for the respective 2-drug combination; S.PGB and S.PB are slopes for the antiepileptic drugs administered alone; f ratio S.R. – factor for slope function ratio for the respective 2 drug combinations. The slope function ratio S.R. = q/p; CFP – coefficient of determination.

Statistical evaluation of data was performed with unpaired Student’s t-test.

Isobolographic analysis of interactions (for non-parallel DRRCs) between pregabalin (PGB) and phenobarbital (PB) at fixed-ratio 1:1 against maximal electroshock (MES)-induced seizures.

Data are presented as median effective doses (ED50 values in mg/kg ± S.E.M.) for 2-drug mixtures, determined either experimentally (ED50 values), or theoretically calculated (ED50 values) from the equations of additivity (35), protecting 50% of the animals against MES-induced seizures. The actual doses of PGB and PB that comprised the mixtures at the fixed-ratio of 1:1 for the ED50 add and ED50 mix values are presented in separate columns. PB – dose of PB in the mixture; PGB – dose of PGB in the mixture; n – total number of animals used at doses whose expected anticonvulsant effects ranged between 16% - 84% (i.e., 4 and 6 probits). Statistical evaluation of data was performed with unpaired Student’s t-test.

Figure 1 Log-probit dose-response relationship curve (DRRC) analysis of pregabalin (PGB) and phenobarbital (PB) administered alone and in combination against maximal electroshock (MES)-induced seizures in mice.

Figure 2 Isobologram showing additive interaction between pregabalin (PGB) and phenobarbital (PB) against maximal electroshock (MES)-induced seizures in mice.
was additive, whereas the combination of GBP with PB at the fixed-ratios of 5:1, 7:1, and 10:1 exerted supra-additive (synergistic) interactions in the mouse MES model [4]. Pharmacokinetic verification of interaction between GBP and PB at the fixed-ratio of 10:1 revealed that GBP had no impact on free (non-protein bound) plasma concentrations of PB in experimental animals [4]. In contrast, PB significantly elevated the plasma GBP concentrations in mice [4]. Thus, the synergistic interaction between GBP and PB at the fixed-ratio of 10:1 was accompanied with a pharmacokinetic increase in plasma GBP concentration in experimental animals. It should be stressed that in this study the AED concentrations were measured in brain homogenate because only the estimation of AED concentrations in biophase (brain homogenate or cerebrospinal fluid) provide us with certainty about the exact nature of the interaction observed between AEDs at the site where the AEDs exert their activity, i.e., in the brain [7, 28]. Recently, it has been documented that 2-phosphonomethyl-pentanedioic acid (2-PMPA – a glutamate carboxypeptidase II inhibitor) elevated free plasma valproate concentration, but the compound did not alter total brain valproate concentration in mice [26]. Moreover, a pharmacokinetic study has revealed that loreclezole (a second-generation AED) significantly increased free plasma concentrations of valproate, whereas concentrations of valproate in the brain homogenates remained unchanged when combined with loreclezole [27]. In contrast, valproate significantly elevated total brain concentrations of loreclezole in experimental animals, whereas the plasma concentration of loreclezole after co-administration with valproate remained unchanged in mice [27]. In considering the above-discussed facts, one can ascertain that the evaluation of total brain concentrations of AEDs provide information on the exact nature of interaction between the AEDs in preclinical studies.

Pharmacokinetic verification of interaction in the present study revealed that GBP did not significantly alter total brain concentrations of PB in experimental animals. With regard PGB, the drug has an ideal pharmacokinetic profile because it neither binds to plasma proteins nor replaces other AEDs from plasma proteins [2, 40]. PGB undergoes a negligible (2%) metabolic transformation in the liver, and the drug is excreted virtually unchanged by the kidneys. PGB neither inhibits nor activates liver enzymes such as cytochrome P450 system [2, 36, 40]. Considering the favourable pharmacokinetic profile of PGB, it is unlikely that PB would be able to affect total brain GBP concentrations in experimental animals. Comparing the nature of interaction between GBP and PB with PB, one can ascertain that PGB exerted additive interaction with PB, whereas GBP exerted both additive and supra-additive interactions with PB in the mouse MES model. Thus, some fixed-ratio combinations of GBP with PB were superior to that for PGB with PB in the mouse MES model. The apparent discrepancy between the interaction profiles of PGB and GBP with PB resulted from different isobolographic methods used for the analysis of interactions. It should be stressed that the interaction of GBP with PB was analyzed with type I isobolographic analysis, whereas the interaction between PGB and PB was examined with type I isobolographic analysis. In experimental studies, GBP was considered to be virtually ineffective in the mouse MES model [1, 8]. In contrast, PGB exerted a clear-cut anticonvulsant activity in the MES test with an ED$_{50}$ value of 142.14 mg/kg. In experimental studies, type II isobolographic analysis is

### Table 3

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Motor performance (%)</th>
<th>Retention time (s)</th>
<th>Grip-strength (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td>100</td>
<td>180 (180; 180)</td>
</tr>
<tr>
<td>PGB (142.14) + vehicle</td>
<td>100</td>
<td>180 (180; 180)</td>
<td>96.3 ± 6.01</td>
</tr>
<tr>
<td>PB (26.17) + vehicle</td>
<td>100</td>
<td>180 (180; 180)</td>
<td>97.8 ± 6.44</td>
</tr>
<tr>
<td>PGB (23.92) + PB (4.40)</td>
<td>100</td>
<td>180 (175; 180)</td>
<td>94.8 ± 6.25</td>
</tr>
</tbody>
</table>

Results are presented as:
1) percentage of mice without impairment of motor coordination in the chimney test; 2) median retention times (in s; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice; 3) mean strengths (in newtons ± S.E.M.) from the grip-strength test, assessing skeletal muscular strength in mice. Each experimental group consisted of 8 mice. Statistical analysis of data from the passive avoidance task was performed with non-parametric Kruskal-Wallis ANOVA; results from the grip-strength test were analyzed with one-way ANOVA followed by the Bonferroni’s post-hoc test for multiple comparisons. The Fisher’s exact probability test was used to analyze the results from the chimney test. All drugs were administered ip. at times scheduled for the MES test, and at doses corresponding to their ED$_{50}$ values (when administered alone) and ED$_{max}$ values at fixed-ratio 1:1 (when administered in combination) against MES-induced seizures in mice (for more details see legends to Tables 1 and 2).

### DISCUSSION

The presented results indicate that PGB combined with PB at the fixed-ratio of 1:1 exerted an additive interaction in the mouse MES model. To explain the exact characteristics of the interaction between PGB and PB, one should consider their anticonvulsant mechanisms of action. As mentioned in the Introduction, PGB binds with high affinity and specificity to the δ-subunit of P/Q-type voltage-gated calcium channels and, by decreasing Ca\textsuperscript{2+} influx at nerve terminals, the drug reduces the release of excitatory neurotransmitters in the brain. Although PGB is a substituted analogue of γ-aminobutyric acid (GABA), the drug is inactive at GABA receptors, including GABA_A, benzodiazepine, and GABA_B$_{\text{radioligand binding sites}}$ [9]. GBP does not alter GABA concentration in brain tissue [11].

With respect to PB, the drug, by facilitating GABA-mediated inhibition through the allosteric modulation of neuronal postsynaptic GABA_A receptors [5, 33], hyperpolarizes the postsynaptic neuronal cell membrane, and thus, disrupts epileptiform transmission [37]. Moreover, PB at relatively low concentrations inhibits responses mediated by AMPA receptors [13]. Thus, one can hypothesize that the blockade of the calcium channels in neurons exerted by PGB additively cooperated with activation of GABA-ergic neurotransmission in the brain evoked by PB.

While considering the results from this study, another important fact should be noted. Since PGB is a structural analogue of the inhibitory neurotransmitter GABA with a pharmacological profile similar to that of gabapentin (GBP – a second-generation AED), one can therefore suggest that the interaction between PGB and PB should be identical or similar to that denoted for GBP with PB in the mouse MES test. Experimental studies have revealed that the interaction of GBP with PB at the fixed-ratios of 1:1 and 3:1 was additive, whereas the combination of GBP with PB at the fixed-ratios of 5:1, 7:1, and 10:1 exerted supra-additive (synergistic) interactions in the mouse MES model [4].
used if one of the investigated drugs in the mixture is virtually ineffective. Since GBP was considered as a virtually ineffective drug, type II isobolographic analysis of interaction was used to analyze the characteristics of interaction between GBP and PB in the mouse MES model [4]. Moreover, the fixed-ratio combinations in type II isobolographic analysis are based on doses of the drug fully effective in suppressing seizures in animals (i.e., PGB and PB for the combination of PGB with PB). In contrast, in type I isobolographic analysis for parallel and non-parallel DRRCs, the fixed-ratio combinations are based on proportions of ED$_{50}$ values of the drugs fully effective in suppressing seizures in animals. Since both types I and II isobolographic analysis considerably differ from one another, the fixed-ratios for the combinations of PGB with PB and GBP with PB also differ. This is why the combination of GBP with PB was investigated at several fixed-ratios of 1:1, 3:1, 5:1, 7:1, and 10:1, whereas the combination of PGB with PB was examined only at a fixed-ratio of 1:1.

Because the DRRCs for PGB and PB were not parallel to one another, type I isobolographic analysis for non-parallel DRRCs was used in this study. This is why 2 ED$_{50}$add values for lower and upper isoboles of additivity for the combination were determined and compared to the experimentally derived ED$_{50}$mix value at the fixed-ratio of 1:1 in the mouse MES model. If DRRCs are non-parallel to each other, one cannot precisely calculate proportions of 2 AEDs in the mixture, except for the proportion of 1:1, in which both AEDs are combined in equi-effective doses. In other fixed-ratio combinations (i.e., 3:1, 5:1, 7:1 and 10:1), the respective doses of the first and second AEDs in the mixture would be inappropriately selected and thus, the experimentally derived ED$_{50}$mix values for the combinations of 3:1, 5:1, 7:1, and 10:1 could be erroneously calculated. This is why only the fixed-ratio combination of 1:1 was tested for the mixture of PGB with PB, whose DRRCs were not parallel to one another. Details concerning the isobolographic background have been presented elsewhere [18-20, 23, 26, 35].

Evaluation of potential acute adverse effects for the combinations of PGB with PB at doses corresponding to their ED$_{50}$mix value from the MES test revealed that the combination of PGB with PB did not alter motor coordination in animals, as assessed in the chimney test. Moreover, the combination neither impaired skeletal muscular strength in the grip-strength test in mice, nor disturbed long-term memory in experimental animals challenged with the step-through passive avoidance task.

Based on this preclinical study, one can conclude that the combination of GBP with PB can offer an additive interaction against MES-induced seizures in mice. Moreover, the lack of pharmacokinetic interactions between PGB and PB at the fixed-ratio of 1:1, and no adverse acute effects at doses corresponding to the ED$_{50}$mix values from the MES test, make the combination of particular importance for consideration during the selection of PGB combinations in further clinical settings. If the results from this study could be extrapolated into clinical trials, the combination of PGB with PB would be beneficial for patients remaining refractory to currently available AEDs.

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