

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 pentylenetetrazole (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.

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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 $21 \pm 1^\circ\text{C}$, relative humidity $55 \pm 3\%$). 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 (ED_{50} 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 ($\text{ED}_{50\text{mix}}$ 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 ($\text{ED}_{50\text{s}}$) 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 Łuszczki [18–20]. Based upon the ED_{50} 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 ($\text{ED}_{50\text{add}}$) – 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: $y = \text{ED}_{50\text{PB}} - [\text{ED}_{50\text{PB}} / (\text{ED}_{50\text{PGB}} / x)^{q/p}]$; where y – is the dose of PB; x – is the dose of PGB; p and q – are 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: $y = \text{ED}_{50\text{PB}} [(\text{ED}_{50\text{PGB}} - x) / \text{ED}_{50\text{PGB}}]^{q/p}$. To calculate the curve-fitting parameters (p and q), probits of response for PB and PGB administered alone were transformed to % effect. Proportions of PGB and PB in the mixture were calculated only for the fixed-ratio combination of 1:1, as recommended earlier [18–20, 22–24, 27, 31], and the mixtures of PGB with PB were administered to animals. The evaluation of the experimentally derived $\text{ED}_{50\text{mix}}$ at the fixed-ratio of 1:1 was based upon the dose of the mixture protecting 50% of animals tested against MES-induced seizures in mice. Finally, to determine the separate doses of PGB and PB in the mixture, the $\text{ED}_{50\text{mix}}$ values were multiplied by the respective proportions of AEDs (denoted for purely additive mixture). Further details regarding these concepts and all required equations allowing the calculation of S.E.M. for $\text{ED}_{50\text{add}}$ values have been published elsewhere [18–20, 22–24, 27, 31].

Measurement of total brain PB concentrations. Total brain concentrations of PB were determined in mice administered with PGB + PB at the fixed-ratio combination of 1:1 from the MES test. Mice were killed by decapitation at times chosen to coincide with that scheduled for the MES test. Whole brains were removed from skulls, weighed, harvested and homogenized using Abbott buffer (2:1 vol/weight; Abbott Laboratories, North Chicago, IL, USA) in an Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany). 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 $\mu\text{g}/\text{ml}$ of brain supernatants as means \pm S.D. of at least 8 separate brain preparations.

Chimney test. The effects of the studied AEDs (PGB and PB) administered alone at their ED_{50} values and in combination (administered at doses corresponding to their $\text{ED}_{50\text{mix}}$ 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 $ED_{50\text{mix}}$ 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 $ED_{50\text{mix}}$ 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 test of Meyer et al. [34]. 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 subsequently, 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 $ED_{50\text{mix}}$ 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 $ED_{50\text{mix}}$ value for the mixture of PGB with PB at the fixed-ratio of 1:1 was statistically compared with their respective theoretically additive $ED_{50\text{add}}$

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 $ED_{50\text{mix}}$ 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 $ED_{50\text{mix}}$ value for this fixed-ratio combination was 28.32 ± 4.26 mg/kg, whereas the additively calculated $ED_{50\text{add}}$ values were 35.90 ± 27.78 mg/kg (for the lower $ED_{50\text{add}}$) and 132.42 ± 15.96 mg/kg (for the upper $ED_{50\text{add}}$; Table 2). Thus, the $ED_{50\text{mix}}$ value did not significantly differ from the $ED_{50\text{add}}$ values (Table 2, Fig. 2).

Total brain PB concentrations. Total brain concentration of PB administered singly at a dose of 4.40 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

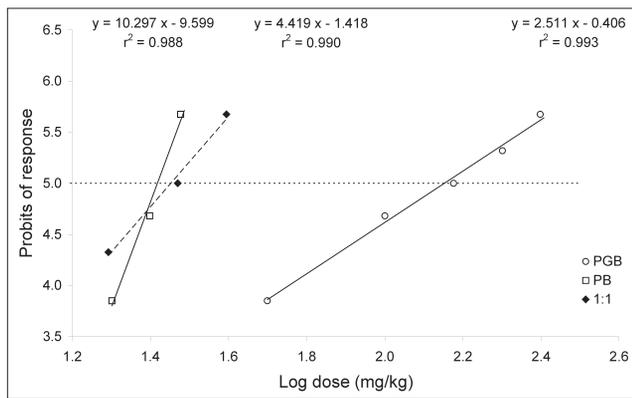


Figure 1 Log-probit dose-response relationship curve (DRRC) analysis of pregabalin (PGB) and phenobarbital (PB) administered alone and in combination against maximal electroshock (MES)-induced seizures in mice. Doses of PGB and PB administered alone and in combination at fixed-ratio 1:1 were transformed to logarithms; protective effects offered by the AEDs against MES-induced seizures were transformed to probits according to Litchfield and Wilcoxon [15]. Linear regression equations of DRRCs for PGB and PB administered alone and in combinations are presented on the graph: y – probit of response; x – logarithm (to the base 10) of an AED dose or a dose of the mixture of PGB with PB; r² – coefficient of determination. Test for parallelism revealed that the experimentally determined DRRC for PGB was non-parallel to that for PB when administered alone. For more details see Table 1.

Table 1 Anticonvulsant effects of pregabalin (PGB) and phenobarbital (PB) administered singly against maximal electroshock (MES)-induced seizures in mice.

Drug	ED ₅₀	n	CFP	q/p
PGB	142.14 ± 32.54	32	1.354 (p)	-
PB	26.17 ± 2.07	16	8.723 (q)	6.442

*Test for parallelism: PGB vs. PB S.R. = 2.000 f ratio S.R. = 1.345 non-parallel S.R. > f ratio S.R., the examined two DRRCs are non-parallel.

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 and p) curve-fitting parameters; q/p – ratio of q and p values; S.R. – slope function ratio for the respective 2-drug combination (i.e., S_{PGB}/S_{PB}), where: S_{PB} and S_{PGB} are slopes for the antiepileptic drugs administered alone; f ratio S.R. – factor for slope function ratio for the respective 2 drug combinations. Test for parallelism of 2 dose-response relationship curves (DRRCs) was performed according to Litchfield and Wilcoxon [15].

* All detailed calculations required to perform the test for parallelism of 2 DRRCs have been presented in the Appendix to the papers by Łuszczki and Czuczwar [23], and Łuszczki et al. [22].

in mice. When PGB and PB were co-administered at doses corresponding to the ED_{50 mix} value at the fixed-ratio of 1:1 from the MES-induced seizure test, motor performance of animals as assessed by the chimney test was unaffected (Table 3). Furthermore, the combination of PGB with PB did not impair long-term memory as determined in the passive avoidance test (Table 3). Similarly, PGB concomitantly administered with PB had no significant impact on skeletal muscular strength of the animals as assessed by the grip-strength test (Table 3). Moreover, it was found that the control (vehicle-treated) mice and animals receiving PGB or PB alone (at doses corresponding to their ED₅₀ values from the mouse MES model) did not

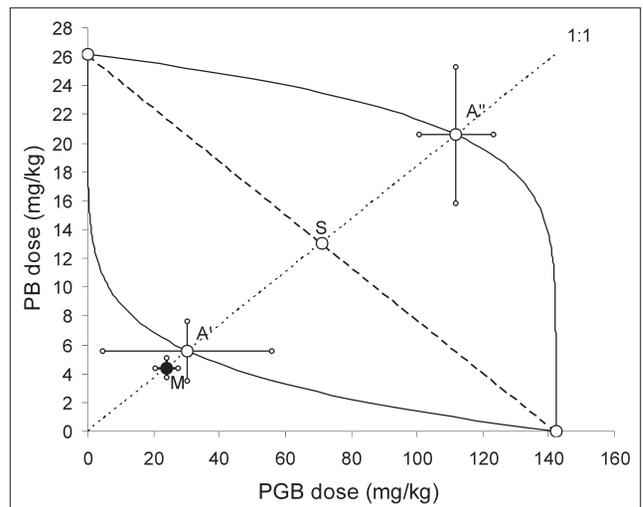


Figure 2 Isobologram showing additive interaction between pregabalin (PGB) and phenobarbital (PB) against maximal electroshock (MES)-induced seizures in mice. The median effective dose (ED₅₀) for PGB is plotted graphically on X-axis; ED₅₀ of PB is placed on Y-axis. The lower and upper isoboles of additivity represent the curves connecting the ED₅₀ values for PGB and PB administered alone. The dotted line starting from the point (0, 0) corresponds to fixed-ratio 1:1 for the combination of PGB with PB. The diagonal dashed line connects the ED₅₀ for PGB and PB on the X- and Y-axes. Closed circle (●) depicts the experimentally derived ED_{50 mix} (± S.E.M.), open circles (○) depict the theoretically calculated ED_{50 add}s (± S.E.M.) for total doses expressed as the proportions of PGB and PB that produced 50% protection of animals against MES-induced seizures. The S.E.M. values are presented as horizontal and vertical error bars for the ED_{50 mix}s and ED_{50 add}s. The points A' and A'' depict the theoretically calculated ED_{50 add} values for both, lower and upper isoboles of additivity. Point M represents the experimentally-derived ED_{50 mix} value for total dose of the mixture expressed as proportions of PGB and PB that produced a 50% anticonvulsant effect (50% isobole) in the mouse MES model. The sum of X and Y coordinates, for each point placed on the isobologram, corresponds to the respective ED₅₀ values. The point S reflects the ED_{50 add} value denoted theoretically from Loewe's equation for fixed-ratio combination 1:1. Experimentally derived ED_{50 mix} value is placed below point A' and close to the lower isobole of additivity, indicating additive interaction between PGB and PB in the mouse MES model. The X- and Y-coordinates for all points presented on the isobologram are as follows: A' (30.32; 5.58), A'' (111.83; 20.59), S (71.07; 13.09), and M (23.92; 4.40).

Table 2 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.

AED combination	ED ₅₀	n	PGB	PB
PGB + PB	ED _{50 mix}	28.32 ± 4.26	23.92	4.40
	#ED _{50 add}	35.90 ± 27.78	30.32	5.58
	&ED _{50 add}	132.42 ± 15.96	111.83	20.59

Data are presented as median effective doses (ED₅₀ values in mg/kg ± S.E.M.) for 2-drug mixtures, determined either experimentally (ED_{50 mix}), or theoretically calculated (ED_{50 add}) 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 ED_{50 mix} and ED_{50 add} values are presented in separate columns.

PGB – dose of PGB in the mixture; PB – dose of PB in the mixture; n – total number of animals used at those doses whose expected anticonvulsant effects ranged between 16% - 84% (i.e., 4 and 6 probits). Total number of animals determined either experimentally (n_{mix}) or theoretically from the equation of additivity (n_{add} = n_{PGB} + n_{PB} - 4); # – ED_{50 add} value calculated from the equation for the lower line of additivity; & – ED_{50 add} value calculated from the equation for the upper line of additivity. Statistical evaluation of data was performed with unpaired Student's t-test.

show any significant signs of impaired motor coordination, long-term memory or muscular skeletal strength, as assessed in the chimney, passive avoidance and grip-strength tests, respectively (Table 3).

Table 3 Effects of pregabalin (PGB), phenobarbital (PB) and their combinations at fixed-ratio 1:1 on motor performance in the chimney test, long-term memory in the passive avoidance task and muscular strength in the grip-strength test in mice.

Treatment (mg/kg)	Motor performance (%)	Retention time (s)	Grip-strength (N)
Vehicle	100	180 (180; 180)	96.5 ± 6.23
PGB (142.14) + vehicle	100	180 (180; 180)	96.3 ± 6.01
PB (26.17) + vehicle	100	180 (180; 180)	97.8 ± 6.44
PGB (23.92) + PB (4.40)	100	180 (175; 180)	94.8 ± 6.25

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 i.p. at times scheduled for the MES test, and at doses corresponding to their ED₅₀ values (when administered alone) and ED₅₀^{mix} 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 in the mouse MES model, one should consider their anticonvulsant mechanisms of action. As mentioned in the Introduction, PGB binds with high affinity and specificity to the $\alpha_2\delta$ subunit of P/Q-type voltage-gated calcium channels and, by decreasing Ca²⁺ 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 radioligand binding sites [9]. PGB 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]. 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 PGB 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 PGB concentrations in experimental animals.

Comparing the nature of interaction between GBP and PGB 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 II 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 ED₅₀ value of 142.14 mg/kg. In experimental studies, type II isobolographic analysis is

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 the mouse MES model (i.e., PB for the combination of GBP 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 (i.e., PGB and PB for the combination of PGB with PB). 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 $ED_{50\text{ add}}$ values for lower and upper isoboles of additivity for the combination were determined and compared to the experimentally derived $ED_{50\text{ mix}}$ 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 $ED_{50\text{ mix}}$ 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 $ED_{50\text{ mix}}$ 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 PGB 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 acute adverse effects at doses corresponding to the $ED_{50\text{ mix}}$ 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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