

# Interaction of tiagabine with valproate in the mouse pentylenetetrazole-induced seizure model: an isobolographic analysis for non-parallel dose-response relationship curves

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## ABSTRACT

**Purpose:** To characterize the interaction between tiagabine (TGB) and valproate (VPA) – two antiepileptic drugs in the mouse pentylenetetrazole (PTZ)-induced clonic seizure model, type I isobolographic analysis for non-parallel dose-response relationship curves (DRRCs) was used.

**Material and Methods:** Clonic seizures were evoked in albino Swiss mice by subcutaneous injection of PTZ at its  $CD_{97}$  (100 mg/kg). To ascertain the nature of interaction between TGB and VPA administered in combination, total brain concentrations of TGB and VPA were estimated by using high-performance liquid chromatography (HPLC) and fluorescence polarization immunoassay (FPIA).

**Results:** TGB and VPA produced clear-cut anticonvulsant effects against PTZ-induced clonic seizures in mice and their DRRCs were not parallel to one another. The type I isobolographic analysis for non-parallel DRRCs revealed that the combination of TGB with VPA at the fixed-ratio of 1:1 exerted additive interaction against PTZ-induced clonic seizures in mice. With FPIA, it was found that TGB did not affect total brain VPA concentrations in experimental animals. Moreover, VPA had no significant impact on total brain concentrations of TGB in mice, as measured with HPLC.

**Conclusion:** The additive interaction between TGB and VPA at the fixed-ratio of 1:1 in the mouse PTZ model was pharmacodynamic in nature.

**Key words:** tiagabine, valproate, pentylenetetrazole, isobolographic analysis, pharmacodynamic/pharmacokinetic interaction, dose-response relationship curve

## INTRODUCTION

Overwhelming evidence indicates that the isobolographic analysis is a valuable method allowing the precise classification of exact types of interactions among drugs, comprehensively evaluating their nature as: supra-additive (synergistic), sub-additive (relatively antagonistic), infra-additive (absolutely antagonistic), indifferent or additive [1-8]. There are two types of isobolographic analysis: type I – used if all examined drugs are fully active, and type II – if one of the drugs produces no effect and is considered as virtually ineffective in an experimental model [1,9]. Quite recently, a novel variant of isobolography

for non-parallel dose-response relationship curves (DRRCs) of drugs administered separately, has been introduced into experimental studies [10-14]. The isobolographic analysis has been successfully applied in experimental epileptology to characterize interactions among antiepileptic drugs (AEDs) in preclinical studies [6,15-19].

Previously, it has been documented that tiagabine (TGB – a second-generation AED) had its DRRC parallel to those of oxcarbazepine, lorclezole, felbamate and gabapentin in the pentylenetetrazole (PTZ)-induced seizure test in mice and produced additive interaction with type I isobolographic analysis for parallel DRRCs [18, 20]. Moreover, TGB had its DRRC parallel to that of vigabatrin in the PTZ-induced seizure

test in mice and the combination produced supra-additive (synergistic) interaction with type I isobolographic analysis for parallel DRRCs [21]. In contrast, TGB had its DRRC non-parallel to that of ethosuximide in the PTZ-induced seizure test in mice and the drugs produced additive interaction with the isobolographic analysis for non-parallel DRRCs [12].

Therefore, it was of pivotal importance to characterize the interaction profile between TGB and valproate (VPA – a conventional AED), prescribed in patients with idiopathic generalized epilepsies including tonic-clonic, partial, absence, and myoclonic seizures [22]. It is widely accepted that the PTZ-induced seizure test is considered as an experimental model of myoclonic seizures in man [23]. Hence, it was considered appropriate to use this test for assessing the characteristics of interaction between TGB and VPA in this study. To ascertain whether the observed anticonvulsant effects for the combination of TGB with VPA were consequent to a pharmacodynamic and/or a pharmacokinetic interaction, total brain concentrations of VPA and TGB were evaluated using fluorescence polarization immunoassay (FPIA) and high performance liquid chromatography (HPLC).

## MATERIAL AND METHODS

### Animals and experimental conditions

All experiments were performed on adult male albino 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, ambient temperature of  $22 \pm 1^\circ\text{C}$ , relative humidity of  $55 \pm 5\%$ ). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of 8 mice. Each mouse was used only once. All tests were performed between 09.00 a.m. and 03.00 p.m. Procedures involving animals and their care were conducted in accordance with current European Community and Polish law on the experimentation and protection of animals. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures listed were approved by the Local Ethics Committee at the Medical University of Lublin (License no. 425/2003/451/2003) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

### Drugs

The following AEDs were used in this study: TGB (Gabitril®, Sanofi Winthrop, Gentilly, France) and VPA (magnesium salt - kindly donated by ICN-Polfa S.A., Rzeszow, Poland). TGB was suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water, while VPA was dissolved in distilled water and both AEDs were administered intraperitoneally (i.p.), as two separate injections, in a volume

of 5 ml/kg body weight. The control animals received adequate volume of vehicle (1% solution of Tween 80 in distilled water). The AEDs were administered as follows: TGB – 15 min, and VPA – 30 min. prior to the PTZ and before the brain sampling for the measurement of AED concentrations. The route of systemic (i.p.) administration and pretreatment times before testing of the AEDs were based on information about their biological activity from the literature and our previous experiments [24]. PTZ (Sigma, St. Louis, MO, USA) was dissolved in sterile saline and administered subcutaneously (s.c.) into a loose fold of skin in the midline of the neck in a volume of 5 ml/kg body weight.

### Pentylenetetrazole-induced convulsions

The anticonvulsant activities of TGB and VPA against PTZ-induced clonic seizures were determined after s.c. administration of PTZ at its  $CD_{97}$  (convulsive dose 97, i.e., the dose of PTZ that produced clonic seizures in 97% of mice, which in this study was 100 mg/kg). Following PTZ administration, mice were placed separately into transparent Plexiglas cages (25×15×10 cm) and observed for 30 min for the occurrence of clonic seizures. Clonic seizure activity was defined as clonus of whole body lasting for over 3 s, with an accompanying loss of righting reflex. The number of animals convulsing out of the total number of mice tested was noted for each treatment regimen. The animals were administered with increasing doses of the AEDs, and the anticonvulsant activity of each drug was evaluated as the  $ED_{50}$  (median effective dose of an AED, protecting 50% of mice against clonic convulsions). At least 4 groups of animals were used to estimate each  $ED_{50}$  value calculated from the respective log-probit DRRC according to Litchfield and Wilcoxon [25]. The anticonvulsant activity of TGB administered alone was studied at doses of 0.5, 1, 2 and 3 mg/kg, whereas that of VPA administered alone at doses of 125, 150, 175, and 200 mg/kg against the clonic phase of PTZ-induced seizures in mice. Similarly, the anticonvulsant activity of a mixture of TGB with VPA was evaluated and expressed as  $ED_{50\text{mix}}$ , corresponding to the dose of the mixture of both drugs required to protect 50% of animals tested against PTZ-induced clonic convulsions. This experimental procedure has been described in more detail in our earlier studies [18,20,21].

### Measurement of total brain AED concentrations

The animals were administered VPA + vehicle, TGB + vehicle or the combination of VPA + TGB, at doses corresponding to the  $ED_{50\text{mix}}$  value at the fixed-ratio of 1:1 from the PTZ test. Mice were killed by decapitation at times chosen to coincide with that scheduled for the PTZ test and the whole brains of mice were removed from skulls, weighed, harvested and homogenized with Abbott buffer (1:2 weight/vol) in an Ultra-Turrax T8 homogenizer (IKA Werke, Staufen, Germany). The brain homogenates were centrifuged at 10,000 g for 10 min and the supernatant samples (100 µl) were analyzed for VPA content by FPIA using a TDx analyzer and reagents exactly

as described by the manufacturer (Abbott Laboratories, North Chicago, IL, USA). Simultaneously, the identically prepared supernatant samples (200 µl) containing TGB were analyzed using an automated Gilson HPLC system (Anachem Ltd., Bedfordshire, UK), consisting of Gilson 234 autosampler and Gilson 306 pumps. The measurement of total brain TGB concentrations was performed according to the method, as described earlier [12]. The concentrations of VPA were expressed in µg/ml, while those of TGB were expressed in ng/ml of brain supernatant as means ± S.D. of at least 8 determinations (separate brain preparations).

### Isobolographic analysis of interactions

The isobolographic analysis has been recommended as the method of choice for evaluation of characteristics of interactions between drugs. However, the original isobolographic analysis has a fundamental presumption requiring the parallelism of two DRRCs of the investigated drugs administered separately. Recently, a novel isobolographic approach has been developed to analyze the interactions between drugs whose DRRCs are not parallel [3-5, 10-14, 26]. The isobolographic analysis of interactions between TGB and VPA was performed for drugs with non-parallel DRRCs. The percent protection of animals against PTZ-induced clonic seizures per dose of an AED administered alone and the DRRC for each investigated AED in the PTZ-induced clonic seizure model were fitted using log-probit linear regression analysis according to Litchfield and Wilcoxon [25]. Subsequently, from the respective linear equations the median effective doses ( $ED_{50}$ s) of AEDs administered alone were calculated. Subsequently, based upon these  $ED_{50}$  values, median additive doses of the mixture of TGB with VPA – i.e., doses of the mixture, which theoretically should protect 50% of the animals tested against PTZ-induced clonic seizures ( $ED_{50\text{ add}}$ ) were calculated from two equations of additivity presented by Tallarida [13, 14]. In isobolography, it is accepted that if the interaction is additive, then half the  $ED_{50}$  of TGB plus half the  $ED_{50}$  of VPA drug should be as effective therapeutically as the  $ED_{50}$  of either drug administered separately. This concept of adding fractions of the  $ED_{50}$  values of drugs is a basic principle of isobolography [1, 27]. Subsequently, proportions of TGB and VPA in the mixture were calculated for the fixed-ratio combination of 1:1, and the mixture of TGB with VPA was administered to animals. The evaluation of the experimentally-derived  $ED_{50\text{ mix}}$  at the fixed-ratio of 1:1 was based upon the dose of the mixture protecting 50% of animals tested against PTZ-induced seizures in mice. Statistical comparison of the experimentally-derived  $ED_{50\text{ mix}}$  value with its corresponding theoretically additive  $ED_{50\text{ add}}$  values was undertaken by use of the unpaired Student's *t*-test, according to Tallarida [8]. Finally, to determine the separate doses of TGB and VPA in the mixture, the  $ED_{50\text{ mix}}$  value was multiplied by the respective proportions of AEDs (denoted for purely additive mixture). Further details regarding these concepts have been published elsewhere [3-5, 11-14].

### Statistics

The percent protection of animals against PTZ-induced clonic seizures per dose of the AEDs and the DRRCs for VPA and TGB administered alone and in combination at the fixed-ratio of 1:1 were fitted using log-probit linear regression analysis according to Litchfield and Wilcoxon [25]. The  $ED_{50}$  values with their 95% confidence limits were calculated by computer-assisted log-probit analysis according to Litchfield and Wilcoxon [25]. To precisely analyze the experimental data, the test for parallelism of the DRRCs for TGB and VPA was presented as indispensable conditions for testing AED interactions with isobolography. The obtained 95% confidence limits were transformed to S.E.M. as described previously [7]. Statistical evaluation of isobolographic interactions was performed by the use of Student's *t*-test to detect the differences between the experimentally-derived ( $ED_{50\text{ mix}}$ ) and theoretical additive ( $ED_{50\text{ add}}$ ) values, according to Tallarida [8]. Total brain concentrations of VPA and TGB were statistically analyzed using the unpaired Student's *t*-test. 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

### Effects of TGB and VPA administered alone on PTZ-induced clonic seizures

TGB produced a clear-cut anticonvulsant effect in the PTZ-induced clonic seizure test in mice and thus, its  $ED_{50}$  value was 0.99 (0.57 – 1.73) mg/kg (*Tab. 1; Fig. 1*). Similarly, VPA exerted clear-cut anticonvulsant effects in the PTZ test in mice and its  $ED_{50}$  value, as denoted from the log-probit method was 142.37 (119.41 – 169.74) mg/kg (*Tab. 1; Fig. 1*). The test of parallelism of two DRRCs for TGB and VPA administered alone in the PTZ test revealed that both AEDs had their DRRCs non-parallel to one another (*Tab. 1; Fig. 1*).

### Isobolographic assessment of interaction between TGB and VPA

The  $ED_{50\text{ mix}}$  for the mixture of TGB and VPA at the fixed-ratio of 1:1 was 66.82 mg/kg, whereas the corresponding  $ED_{50\text{ add}}$  values were 45.23 mg/kg (for the lower  $ED_{50\text{ add}}$ ) and 98.18 mg/kg (for the upper  $ED_{50\text{ add}}$ ; *Tab. 2*). In this case, the  $ED_{50\text{ mix}}$  (i.e., protecting a 50% of animals against PTZ-induced clonic seizures) did not significantly differ from the  $ED_{50\text{ add}}$  values and thus, indicating additive interaction between drugs (*Tab. 2, Fig. 2*).

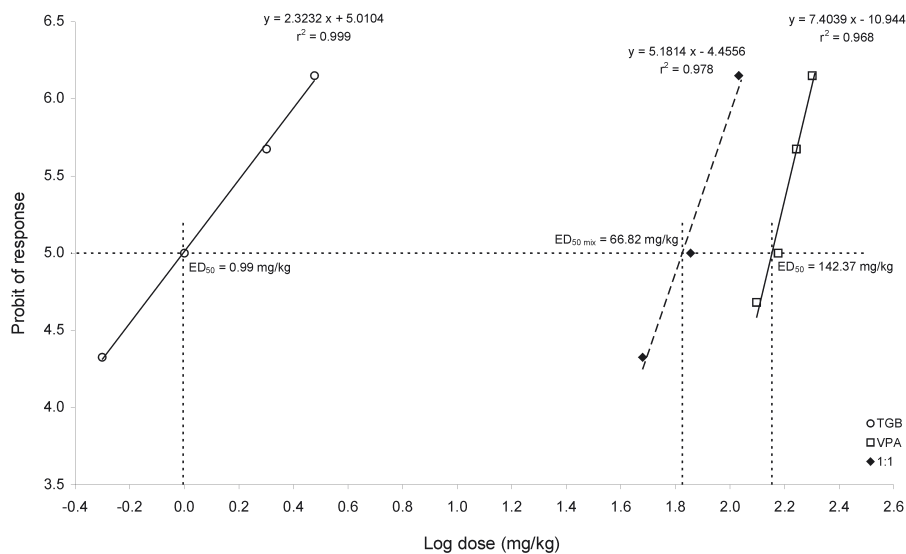
### Total brain AED concentrations

Total brain AED concentrations were evaluated for VPA and TGB administered at doses corresponding to the  $ED_{50\text{ mix}}$  at the fixed-ratio of 1:1 from the PTZ test. With FPIA technique,

**Table 1. Anticonvulsant effects of tiagabine (TGB) and valproate (VPA) administered singly against pentylenetetrazole (PTZ)-induced clonic seizures in mice.**

Drug	ED <sub>50</sub>	n	CFP	q/p
TGB	0.99 ± 0.283	24	1.229 (p)	-
VPA	142.37 ± 12.767	24	3.740 (q)	3.043
Test for parallelism:	TGB vs. VPA	S.R. = 1.974	f ratio S.R. = 1.439	
S.R. > f ratio S.R., the examined two DRRCs are not parallel [25].				

Results are presented as median effective doses (ED<sub>50</sub> values in mg/kg ± S.E.M. in parentheses) of TGB and VPA administered singly against PTZ-induced clonic seizures in mice. The AEDs were administered systemically (i.p.), as follows: TGB – 15 min and VPA – 30 min before the PTZ seizure initiation. *n* – total number of animals used at doses whose expected anticonvulsant effects ranged between 4 and 6 probits (16% and 84%); S.E.M. – standard error of the mean of ED<sub>50</sub>; CFP – (*q* and *p*) curve-fitting parameters; *q/p* – ratio of *q* and *p* values; S.R. – slope function ratio for the combination; f ratio S.R. – factor for slope function ratio for the combination. Test for parallelism of two DRRCs was performed according to Litchfield and Wilcoxon [25]. All detailed calculations required to perform the test for parallelism of two DRRCs were presented in the Appendix to the paper by Luszczycki and Czuczwar [19].

**Figure 1. Log-probit analysis and dose-response relationship curves (DRRCs) for tiagabine (TGB) and valproate (VPA) administered alone and their combination at the fixed-ratio of 1:1 in the pentylenetetrazole (PTZ)-induced seizure test in mice.****Table 2. Isobolographic analysis of interaction between tiagabine (TGB) and valproate (VPA) at the fixed-ratio of 1:1 against pentylenetetrazole (PTZ)-induced clonic seizures in mice.**

ED <sub>50 mix</sub>	n <sub>mix</sub>	TGB	VPA	#ED <sub>50 add</sub>	n <sub>add</sub>	TGB	VPA	§ED <sub>50 add</sub>	n <sub>add</sub>	TGB	VPA
66.82 ± 10.49	16	0.46	66.36	45.23 ± 15.72	44	0.31	44.92	98.18 ± 28.21	44	0.68	97.50

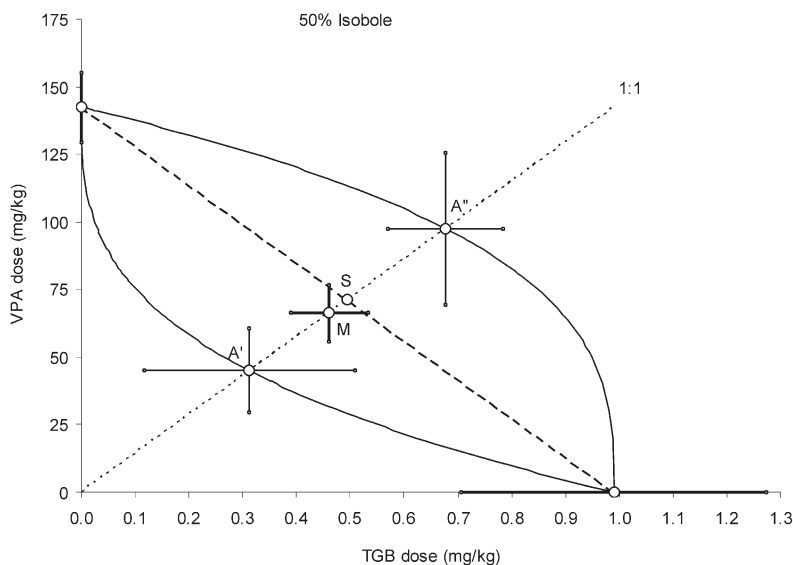
Results are presented as median effective doses (ED<sub>50</sub> values in mg/kg ± S.E.M.) for two-drug mixtures, determined either experimentally (ED<sub>50 mix</sub>) or theoretically calculated (ED<sub>50 add</sub>) from the equations of additivity [13,14], protecting 50% of the animals against PTZ-induced clonic seizures. The actual doses of TGB and VPA that comprised the mixtures at the fixed-ratio of 1:1 for the ED<sub>50 mix</sub> and ED<sub>50 add</sub> values are presented in separate columns. TGB – dose of TGB in the mixture; VPA – dose of VPA in the mixture; n<sub>mix</sub> – total number of animals used at those doses whose expected anticonvulsant effects ranged between 16% and 84% (i.e., 4 and 6 probits) for the experimental mixture; n<sub>add</sub> – total number of animals calculated for the additive mixture of the drugs examined (n<sub>add</sub> = n<sub>TGB</sub> + n<sub>VPA</sub> - 4); # – ED<sub>50 add</sub> value calculated from the equation for the lower line of additivity; § – ED<sub>50 add</sub> value calculated from the equation for the upper line of additivity. Statistical evaluation of data was performed with unpaired Student's t-test [8,9].

total brain concentration of VPA for the mixture of VPA (66.82 mg/kg) with TGB (0.46 mg/kg) did not considerably differ from that evaluated for VPA administered singly at 66.82 mg/kg (Tab. 3). Similarly, with HPLC technique, the total brain concentration of TGB, administered singly at 0.46 mg/kg did not significantly differ from that evaluated for the mixture of TGB (0.46 mg/kg) with VPA (66.82 mg/kg) (Tab. 3).

## DISCUSSION

Results indicate that the combination of TGB with VPA at the fixed-ratio of 1:1 exerted additive interaction against PTZ-induced clonic seizures in mice. It is noteworthy that the isobolographic analysis of interaction was performed in this study only for the fixed-ratio of 1:1 because of the lack of parallelism of two DRRCs for the drugs administered alone.

**Figure 2. Isobologram showing additive interaction between tiagabine (TGB) and valproate (VPA) against pentylenetetrazole (PTZ)-induced clonic seizures in mice.**



The median effective doses ( $ED_{50}$ ) for TGB and VPA are shown plotted graphically on the X- and Y-axes, respectively. The solid lines on the X and Y axes represent the S.E.M. for the  $ED_{50}$ s of AEDs administered alone. The lower and upper isoboles of additivity represent the curves connecting the  $ED_{50}$  values for TGB and VPA administered alone. The dotted line starting from the point (0,0) corresponds to the fixed-ratio of 1:1 for the combination of TGB with VPA. The points A' and A'' depict the theoretically calculated  $ED_{50\text{ add}}$  values for both, lower and upper isoboles of additivity. The point M represents the experimentally-derived  $ED_{50\text{ mix}}$  value for total dose of the mixture expressed as proportions of TGB and VPA that produced 50% anticonvulsant effects in the PTZ test in mice. The point S on the graph reflects the  $ED_{50\text{ add}}$  value denoted theoretically from the Loewe's equation for the fixed-ratio combination of 1:1. On the graph, the S.E.M. values are presented as horizontal and vertical error bars for every  $ED_{50}$  value. The  $ED_{50\text{ mix}}$  value is placed close to the point S and within the area bounded by two isoboles of additivity, indicating additive interaction between TGB and VPA in the PTZ test in mice. The sum of X and Y coordinates, for each point placed on the isobologram (M, A', A'', S), corresponds to the respective  $ED_{50}$  values that are as follows: A' (0.31; 44.92), A'' (0.68; 97.50), S (0.49; 71.18), and M (0.46; 66.36).

**Table 3. Total brain concentrations of the studied AEDs when administered singly or in combination.**

Treatment [mg/kg]	Total brain AED concentrations <sup>a</sup>	
	[ $\mu\text{g/ml}$ ]	[ng/ml]
TGB (0.46) + vehicle		56.94 $\pm$ 9.05
TGB (0.46) + VPA (66.36)		61.47 $\pm$ 9.87
VPA (66.36) + vehicle	78.93 $\pm$ 18.80	
VPA (66.36) + TGB (0.46)	89.52 $\pm$ 19.64	

Data are presented as means ( $\pm$  S.D.) of at least 8 determinations. <sup>a</sup>Concentrations relate to that AED shown first in the respective treatment column. Statistical evaluation of data was performed by the use of the unpaired Student's *t*-test. The AEDs were administered systemically (i.p.), as follows: TGB – 15 min and VPA – 30 min before the brain sampling for the measurement of AED concentrations.

Other fixed-ratio combinations, such as: 1:3 and 3:1, etc., could not be evaluated appropriately with this method due to changing proportions of the drugs in the mixture along with the increase in AED doses [11-14].

It should be stressed that the additive interaction, documented for the combination of TGB with VPA against PTZ-induced clonic seizures, is consistent with that reported earlier for the combinations of TGB with oxcarbazepine, lorcetazepam, felbamate, ethosuximide and gabapentin in the PTZ test in mice [12,18,20]. Moreover, with respect to the combination of TGB

with VPA, experimental studies have reported that the two-drug mixture produced additive interaction in the amygdala-kindled rats [28], and supra-additive (synergistic) interaction in the mouse maximal electroshock-induced seizure model [18,24]. However, the observed supra-additive interaction between TGB and VPA in the mouse maximal electroshock-induced seizure model was complicated by a significant increase in total brain VPA concentrations in experimental animals [24]. The pharmacokinetic evaluation of total brain TGB and VPA concentrations in this study revealed that neither TGB nor VPA significantly altered total brain AED concentrations in experimental animals. Thus, the observed additive interaction between TGB and VPA in the PTZ test was pharmacodynamic in nature. The results from the pharmacokinetic study presented herein are partly in contrast to those reported earlier for the combination of TGB with VPA in the mouse maximal electroshock-induced seizure model. The distinct difference in the pharmacokinetic profile of the combination of TGB with VPA may probably be related to doses of TGB and VPA co-administered in the mixture because, in the present study, TGB (0.46 mg/kg) was co-administered with VPA (66.36 mg/kg), whereas in the maximal electroshock-induced seizure model, TGB (9.3 mg/kg) was co-administered with VPA (190 mg/kg) [24].



It is worthy of mentioning that the pharmacokinetic profile of interaction of TGB with VPA in this study was verified bidirectionally, because the total brain concentrations of two AEDs (TGB and VPA) were measured. The bidirectional estimation of total brain AED concentrations allowed the proper classification of the interaction from the PTZ test as the pharmacodynamic in nature. During the pharmacokinetic evaluation of AED concentrations, total brain concentrations are of pivotal importance because they precisely reflect the exact characteristics of interaction between AEDs. As documented earlier, only total brain AED concentrations provided valuable information about the exact nature of interactions between drugs in experimental animals [24,29].

It should be stressed that the AEDs administered alone and in combination at the fixed-ratio of 1:1, at doses corresponding to their ED<sub>50</sub> values from the PTZ test, produced no acute adverse effects in the chimney test (evaluating motor performance), passive avoidance task (examining long-term memory) or grip-strength test (assessing skeletal muscular strength) in mice (results not shown). Since the investigated AEDs in combination exerted no acute adverse effects in preclinical study, one can ascertain that the tested combination of TGB with VPA would be safe and devoid of acute side effects in epilepsy patients, receiving these AEDs. Additionally, a substantial reduction of doses of both AEDs in combination is expected to decrease the risk of side effects in epilepsy patients.

Moreover, it is worth mentioning that the combination of VPA with TGB in the PTZ test has some limitations because TGB is not a drug used clinically in patients with myoclonic seizures. Accumulating evidence indicates that the drug can aggravate myoclonic seizures in epileptic patients or even, it can evoke non-convulsive status epilepticus [22]. Although the PTZ test in rodents is considered as an animal model of myoclonic seizures in humans [23], the combination of TGB with VPA cannot be recommended for patients with myoclonic seizures, unless for patients with several epileptic syndromes and/or various complex seizure types for which the combination of VPA with TGB might appear favorable. On the other hand, the additive interaction between TGB with VPA could occur favorably in clinical settings because this combination could offer epileptic patients a substantial reduction of acute adverse effects associated with the treatment of patients with an AED at high effective, but poorly tolerated doses. Therefore, the application of two-drug combination may be beneficial due to the reduction of adverse effects and better tolerance of the applied AEDs in epileptic patients without losing the anticonvulsant effects [6,15].

Finally, based on this preclinical study, one can ascertain that the combination of TGB with VPA exerted additive interaction in the mouse PTZ model and the nature of this interaction was pharmacodynamic.

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## Disclosure of conflicts of interest

The authors have no conflicts of interest to disclose.

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