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Interactions of tiagabine with some antiepileptics in the maximal electroshock in mice

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Abstract

Tiagabine (TGB), a new potent γ-aminobutyric acid (GABA) uptake inhibitor, is widely applied in adjunctive treatment of partial seizures in humans. Although, polytherapy is not an initial method of epilepsy treatment, clinicians often combine TGB with other antiepileptics as add-on therapy for assuring the anticonvulsant protection in patients with refractory seizures. To evaluate the character of pharmacological interactions between TGB and some antiepileptics, the isobolographic analysis was used as a suitable method for determining the exact types of interactions. Determination of an influence of TGB on the protective effects of diphenylhydantoin (DPH), carbamazepine (CBZ), valproate (VPA), phenobarbital (PB), lamotrigine (LTG), topiramate (TPM), and felbamate (FBM) in maximal electroshock-induced seizures was essential for this study. To exclude or confirm a pharmacokinetic character of observed interactions, the free plasma and brain concentrations of antiepileptic drugs (AEDs) studied were evaluated by using the immunofluorescence or high-pressure liquid chromatography (HPLC).

TGB (up to 2.5 mg/kg) remained ineffective upon the electroconvulsive threshold, whilst the drug in doses of 5 and 10 mg/kg significantly raised the electroconvulsive threshold in mice. According to the isobolography, TGB appears to act synergistically with VPA. The remaining combinations tested exerted additive interactions. A pharmacokinetic character of interaction between TGB and VPA was evidently corroborated either in plasma or brains. Moreover, TGB significantly reduced the plasma and brain concentrations of DPH; however, pharmacokinetic events were not accompanied by any changes in anticonvulsant activity of the latter. Finally, the isobolographic analysis revealed that combinations of TGB with VPA exerted synergistic (supra-additive) interaction resulting from a pharmacokinetic interaction.

Keywords: Tiagabine; Antiepileptic drugs; Isobolographic analysis; Drug interactions; Electroshock maximal; Seizures

1. Introduction

Monotherapy remains the preferred method in epilepsy treatment, albeit some effective strategies of adjunctive or combined treatment of patients with intractable seizures have been developed (Perucca, 1995). However, there is still no accepted consensus on how to efficaciously treat patients with refractory or unsatisfactorily medicated seizures. The rationale for polytherapy in epilepsy treatment, based on animal experimental studies and pharmacological presumptions about mechanisms of action of available antiepileptic drugs (AEDs), allows to predict some drug combinations that might be effective in patients resistant to the applied standard medication (Schmidt, 1996; Deckers et al., 2000).

Novel AEDs, lately introduced into the therapy due to their more specific mechanisms of action, are considered as powerful drugs in the reduction of seizures. It is believed that they may be combined with conventional AEDs or among themselves in order to obtain the spectacular inhibition of seizures in patients with inadequately managed epilepsy (Perucca, 1995; Czuczwar and Patsalos, 2001).

In order to detect some AED combinations, which could be profitable in humans, the animal experiments should

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follow as a first screening, allowing the evaluation of their therapeutic (anticonvulsant) profile (Czuczwar and Borowicz, 2002; Deckers et al., 2000). It is quite clear that, in case of polytherapy, combinations between AEDs may be associated with pharmacokinetic or pharmacodynamic events; therefore, a method permitting to determine advantages of applied drug combinations is needed. At present, only isobolographic analysis is able to correctly classify the observed interactions as additive, supra-additive (synergistic), or infra-additive (antagonistic). There are numerous studies proving the superiority of the isobolographic analysis over the other methods. Therefore, for more detailed information, reviews of Berenbaum (1989), Tallarida (2001), and Gessner (1995) are recommended, which might provide the readers with the basic information necessary to understand the methodology of isobolographic analysis.

Tiagabine [R(-)-N-(4,4-di(3-methyl-thien-2-yl)-but-3-enyl) nipecotic acid, hydrochloride; TGB], a novel AED lately introduced into the therapy of partial seizures in humans, is a potent GABA uptake inhibitor into neurons and glia. TGB blocks the GABA transporter 1 (GAT-1) significantly prolonging the duration of GABA-related inhibitory synaptic potentials (Nielsen et al., 1991; Macdonald and Greenfield, 1997). The net effect of increment in synaptic GABA concentration is evidently related with the reduction of seizure frequency in patients with partial onset seizures (Richens et al., 1995; Sachdeo et al., 1997; Uthman et al., 1998). Moreover, TGB has shown efficacy against refractory partial seizures with or without secondarily generalization (Kalviainen et al., 1998). In experimental epilepsy models, TGB protected against pentylenetetrazol-induced tonic and clonic convulsions, being ineffective in the maximal electroshock test in mice (Rogawski and Porter, 1990; Schachter, 1999). As a matter of fact, it has been experimentally shown that TGB possesses the ability to suppress the maximal electroshock-induced convulsions in mice; however, only at doses above 40 mg/kg, which correspond to the dose range of two to three times higher than that for evoking the impairment of motor coordination in mice (White, 1997).

The purpose of this study was to determine the exact types of interactions between TGB and several AEDs on the basis of isobolographic analysis. In our study, we consciously examined the influence of TGB (at ineffective doses) on the anticonvulsant activity of conventional and some novel AEDs in maximal electroshock-induced seizures in order to detect some potential merits offered by GABA-related mechanism of action of the drug during combined treatment with other AEDs. Furthermore, the influence of TGB on the free plasma and brain concentrations of antiepileptics was evaluated in order to exclude or confirm a pharmacokinetic character of observed interactions. The new drug TGB, tested in our study, is generally applied as adjunctive AED, but the experimental background for its effective combinations with conventional or novel AEDs has not been sufficiently determined. It should be stressed that only combinations of AEDs showing synergistic type of interactions in animal model of epilepsy are recommended to be useful in clinical practice. Therefore, in case of synergy observed in the anticonvulsant activity of combined AEDs, the adverse effects were intended to evaluate for these combinations.

2. Materials and methods

2.1. Animals

The experiments were carried out on male Swiss mice weighing 20–25 g. The animals were housed in colony cages with free access to food (chow pellets) and tap water. After 7 days of acclimatization to standardized laboratory conditions (temperature 21 ± 1 °C, a natural light–dark cycle), the animals were challenged with experimental tests. The tested groups, consisting of 8–10 animals, were chosen by means of a randomized schedule. All experiments were performed between 9:00 a.m. and 2:00 p.m. All experimental procedures listed in this study were approved by the Local Bioethics Committee of the Medical University in Lublin (License No. 161/2000/123/01).

2.2. Drugs

The following AEDs were used in this study: TGB (GABITRIL, Sanofi Winthrop, Gentilly, France); DPH (Polfa, Warsaw, Poland); valproate magnesium (VPA; Polfa, Rzeszów, Poland); carbamazepine (CBZ; Polfa, Starogard, Poland); phenobarbital–*sodium salt* (PB; Sigma, St Louis, MO, USA); lamotrigine (LTG; LAMICTAL, Glaxo Wellcome, Kent, UK); topiramate (TPM; TOPAMAX, Cilag, Schaffhausen, Switzerland), and felbamate (FBM; TALOXA, Schering Plough, Levallois Perret, France). All drugs, except of VPA and PB, were suspended in a 1% solution of Tween 80 (Sigma, St Louis, MO, USA). VPA and PB were dissolved in sterile water. All drugs were administered intraperitoneally in a volume of 10 ml/kg DPH for 120 min; PB, LTG, FBM, and TPM for 60 min; VPA and CBZ for 30 min; and TGB for 15 min before electroconvulsions and the chimney test.

2.3. Electroconvulsions

Electroconvulsions were produced with the use of auricular electrodes and alternating current (50 Hz) delivered by a Hugo Sachs (Type 221, Freiburg, Germany) generator. The stimulus duration was 0.2 s. Tonic hindlimb extension was taken as the endpoint. The electroconvulsive threshold was evaluated as CS_{50}, which is the current strength (in milliamperes; mA) necessary to produce tonic hindlimb extension in 50% of the animals tested. To estimate the electroconvulsive threshold, at least four groups of mice were challenged with electroshocks of various intensities.

Subsequently, an intensity–response curve was calculated on the basis of percentage of mice convulsing. In order to evaluate the respective ED_{50} values (in mg/kg), mice pretreated with different doses of AED were challenged with electroshock of 25 mA. ED_{50} is a 50% effective dose of a respective AED protecting 50% of the animals against electroconvulsions. Again, at least four groups of mice were used to estimate each ED_{50} value. A dose–effect curve was constructed based on the percentage of mice protected.

2.4. Chimney test

The effects of TGB or VPA alone or in combination upon motor performance on mice were determined in the chimney test according to Boissier et al. (1960). In this test, animals had to climb backwards up a plastic tube (25-cm length, 3-cm inner diameter). Motor impairment was indicated by the inability of the animals to perform the test within 60 s. Results were expressed as a percentage of mice failing to perform this test. The tested doses of TGB or VPA correspond to the doses previously denoted in the maximal electroshock test in mice. Moreover, the neurotoxic effects of the drugs administered separately were expressed as their TD_{50} values, which are the doses at which the respective antiepileptics impaired motor coordination in 50% of animals in this test. To evaluate each TD_{50} value, at least four groups of animals injected with various doses of an AED were challenged with the chimney test. A dose–response curve was subsequently calculated on the basis of the percentage of animals showing motor deficits.

2.5. Immunofluorescence estimation of the free plasma and brain concentrations of AEDS

The animals were administered an AED alone or a combination of TGB with the respective AED. The fixed drug ratio combination (TGB: an antiepileptic) for estimating the free plasma and brain concentrations of AEDs was chosen as 1:1 for all antiepileptics except of VPA and TPM for which a fixed-ratio combination was 1:20 and 1:5, respectively. Mice were killed by decapitation at times scheduled for the electroconvulsive test, and samples of blood of approximately 1 ml were collected into original heparinized eppendorf tubes. Simultaneously, brains of mice were removed from skulls and were homogenized with a presence of an original Abbott buffer (2:1 vol/wt) using the Ultra-Turrax T8 homogenizer (Staufen, Germany). The homogenates were centrifuged at $10{,}000 \times g$ (MPW-360 centrifuge; Mechanika Precyzyjna, Warszawa, Poland) for 10 min. Samples of blood were centrifuged at $9350 \times g$ (Abbott centrifuge, Irving, TX, USA) for 5 min, and plasma samples of 300 μl were transferred into system MPS-1 (Amicon, Danvers, USA) for separation of free- from protein-bound microsolutes. Then the MPS-1 tubes were centrifuged at $5000 \times g$ (MPW-360 centrifuge) for 10 min, and either the filtrate samples of 75 μl or the supernatants of 75 μl were put into the Abbott system cartridges. Reagents for the assays of conventional AEDs were purchased from Abbott Laboratories whilst that for TPM were from Oxis International (Portland, OR, USA). Free plasma levels and brain concentrations were estimated by immunofluorescence using an Abbott TDx analyzer and expressed in micrograms per milliliters of plasma or micrograms per grams of wet brain tissue as means ± S.D. of at least eight determinations.

2.6. Chromatographic determination of LTG plasma and brain concentrations

LTG was analyzed quantitatively in plasma and brains of animals at times scheduled for the maximal electroshock-induced seizures in mice. The animals were administered with LTG alone or a combination of TGB with LTG at the fixed ratio of 1:1. Mice were killed by decapitation and samples of blood of approximately 1 ml were rapidly collected into heparinized original eppendorf tubes. Simultaneously, brains of mice were removed from skulls and placed into the deep freeze at −80 °C (Polar 530, Angelantoni, Massa Martana, Italy—sponsored by a KBN grant No. 6P05D 098 21). Samples of blood were centrifuged at $9350 \times g$ (Abbott centrifuge) for 5 min, and plasma samples of 200 μl were stocked into the deep freeze. On the next day, the brains were homogenized with a presence of an original Abbott buffer (2:1 vol/wt), using the Ultra-Turrax T8 homogenizer. The homogenates were centrifuged at $10{,}000 \times g$ (MPW-360 centrifuge) for 10 min. The supernatants were stored again in the deep freeze. All probes (plasma and homogenates) were transferred into the high-pressure liquid chromatography (HPLC) technique of drug detection. The chromatograph (Laboratorij Pristroje, Praha, Czech Republic) was equipped with a 305 micropump (LCP 3001) and an ultraviolet (UV) detector (HP 1050) with a sensitivity setting of 0.1 absorbance units full scale (AUFS) and a time constant of 0.1 s. The Rheodyne 7125 injector valve with a 100 μl sample loop was used for sample injection. For HPLC, a stainless steel HP ODS column (200×4.6 mm) was used at an ambient temperature of 22 °C. The mobile phase was methanol:acetonitrile: citrate buffer (20 mM citric acid/40 mM sodium citrate); 330:90:580 vol/vol/vol (BAKER HPLC grade). The mobile phase flow rate was 1 ml/min. Plasma and brain homogenate samples of 200 μl, after thawing, were added to 200 μl of water, 100 μl of methanol:water solution; 1:1. The solutions were evaporated to dryness under a vacuum system and redissolved in 1 ml of tertbuthyl-methyl ether (HPLC, Aldrich) and again evaporated to dryness under a vacuum system. The remains were redissolved in 4 ml of tertbuthyl-methyl ether; samples of 50 μl were then injected into the chromatograph. LTG concentrations were calculated according to the external standard method using the original Gilson 715 software. The amount of LTG was

determined by comparing their peak area with the peak area of the external standard [7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo(4,5H)-2,3-benzodiazepine]. Stock solutions of LTG serving as internal standards (0.2:0.6:1.2:2.4:4.8 μg/ml) were prepared in mobile phase. They were placed at the beginning and end of each measurement sequence. The wave excitation and emission parameters for the detection of LTG were 270 and 310 nm, respectively. The elution parameter for LTG was 1 ml/min. Plasma levels or brain concentrations of LTG were expressed in micrograms per milliliters of plasma or micrograms per grams of wet brain tissue as means ± S.D. of at least eight determinations.

2.7. Isobolographic analysis

The isobolographic analysis is an experimental method applicable to determine pharmacological interactions among drugs, coadministered in several varying fixed ratio combinations. This method has recently been accepted as the "gold standard" in detecting the drug interactions (Gebhart, 1992; Tallarida et al., 1999). Theoretically, the isobolographic analysis distinguishes four most important types of interactions: pure additivity, supra-additivity (synergy), indifference, and infra-additivity (antagonism) (Berenbaum, 1989; Gessner, 1995; Tallarida et al., 1997). The isobolographic analysis, generally, consists of two alternative experimental procedures for testing the drug interactions. The first procedure is applied when all tested drugs show suitable effects; for instance, in experimental models of epilepsy, all drugs offer a significant protection against experimentally evoked seizures in mice. The second one allows the detection of drug interactions in case of one of the examined AEDs is virtually ineffective in the convulsive test (e.g., against maximal electroshock-induced seizures). The first method of isobolographic analysis was originally elaborated and thoroughly described by Loewe (1953) and subsequently by Tallarida (1992a,b), whereas the second one was precisely described by Porreca et al. (1990). Since TGB in preclinical studies is considered as a virtually ineffective AED in the maximal electroshock test (Rogawski and Porter, 1990), it seemed clear that the second procedure was applied in our study.

Generally, in experimental models of epilepsy, both procedures of the isobolographic analysis comprise of five basic stages as follows:

1. Evaluation of anticonvulsant activity of AEDs tested in an experimental model of epilepsy (e.g., in the maximal electroshock test) and additionally for an AED virtually ineffective evaluation of its efficacy in a convulsive threshold test (e.g., in the electroconvulsive threshold test).
2. Theoretical choice of fixed drug dose ratio combinations followed by calculation of $ED_{50\ add}$s with their S.E.M. for each combination. $ED_{50\ add}$ represents a total additive dose of the drugs, theoretically providing a 50% protection of animals against seizures in an experimental model of epilepsy. It should be stressed that combinations of AEDs, evaluated in preclinical studies, consist usually of two AEDs coadministered.
3. Determination of experimental $ED_{50\ mix}$s with their S.E.M. for the respective, previously chosen, fixed drug dose ratio combinations. $ED_{50\ mix}$ is an experimentally determined total dose of the mixture of two component drugs, which were administered in the fixed ratio combination sufficient for a 50% protective effect against seizures (e.g., electroconvulsions). The experimental $ED_{50\ mix}$ values are determined from the dose–response curves of combined drugs according to Litchfield and Wilcoxon (1949).
4. Statistical comparison of experimentally determined $ED_{50\ mix}$s with theoretically calculated $ED_{50\ add}$s with the use of Student's *t* test.
5. Graphical interpretation of observed interactions in shape of isoboles for each fixed ratio combination tested. All isoboles for the studied two-drug combination create the isobologram displaying the interaction(s) observed between two AEDs studied.

It is important to note that with these two procedures of isobolographic analysis, the different assessment of fixed drug dose ratios for each two-drug combination is closely related. In the first procedure, the equieffective drug doses and their fractions included a mixture that is fundamental and substantial for calculating the additive drug doses, whereas the mass quantities (total amounts) of the drugs in mixture are important for calculating additive values for fixed ratios, in case of one of AEDs tested is virtually ineffective. These two different modes of presentation of the fixed ratio combinations between two AEDs in combination may sometimes lead to some mistakes and misunderstanding. Therefore, in case of one ineffective AED, the calculation of the fixed drug dose ratios is based upon a simple summation of quantity of both drugs applied in mixture. Moreover, in isobolographic analysis, the notation of fixed ratio combinations is based rather upon the natural numbers (1:30, 1:40, 1:16, etc.) than on some fractions of applied AEDs (1:29.8, 1:39.7, etc.). For instance, the description of the combination of TGB + VPA at the fixed ratio of 1:30 means that the drug mixture was composed of TGB (1 mg/kg) added to VPA (30 mg/kg) resulting in the final drug mixture of 31 mg/kg. However, in order to obtain the effective drug dose mixture against maximal electroshock-induced seizures (ED_{50mix} is a measure of the anticonvulsant activity of the drugs in mixture against electroconvulsions), both drugs at the proportionally raised doses were given to animals, and subsequently the dose–response curve was denoted. In other words, the drug doses, at the fixed ratio combination of 1:30, increased proportionally until the total drug dose mixture of 185 mg/kg protected 50% of animals tested. Thus, this mixture consisted of TGB (~6 mg/kg)

Table 1
Influence of TGB upon the electroconvulsive threshold in mice

Treatment (mg/kg)	CS_{50} (mA)
Control	6.6 (5.8–7.5)
TGB (2.5)	7.0 (6.3–7.8)
TGB (5)	8.0 (7.3–8.8)*
TGB (10)	9.6 (8.2–11.2)**

CS_{50} (in mA; 95% confidence limits in parentheses) is the current strength necessary to produce convulsions in 50% of the animals tested. TGB was administered intraperitoneally 15 min prior to the test.
Statistical analysis was performed according to Litchfield and Wilcoxon (1949).
* $P<.01$ versus control group (vehicle-treated animals).
** $P<.001$ versus control group (vehicle-treated animals).

and VPA (179 mg/kg). Similarly, $ED_{50\ mix}$ value for the fixed ratio combination of 1:40 between TGB and VPA was 174.9 mg/kg, composed of TGB (4.3 mg/kg) and VPA (170.6 mg/kg). This mode of description of fixed ratio combinations in shape of natural numbers is widely accepted in the isobolographic analysis (Tallarida, 1992a; Tallarida et al., 1997; Berenbaum, 1989; Gessner, 1995).

In the present study, the mixtures of TGB with an AED were coadministered in a numerous fixed ratio combinations (e.g., 1:1, 1:4, 1:8, 1:16 for CBZ, PB, and DPH; 1:20, 1:30, 1:40, 1:50 for VPA; 1:5, 1:10, 1:20 for TPM and FBM; or 1:1, 1:2, 1:3, 1:4 for LTG). In isobolography, there is a widely accepted rule that an ineffective AED should not outweigh in the two-drug mixture, therefore, the combinations of TGB and AEDs at fixed ratios of 3:1 or 5:1 were not tested. Precise and more detailed description of isobolographic analysis followed by equations showing exactly how to calculate $ED_{50\ add}$ values and their S.E.M., in case of one of the AEDs is ineffective against maximal electroshock, was presented in our previous study (Borowicz et al., 2002).

All results were initially displayed on a graph for ascertaining whether individual responses to applied combinations existed and to determine approximately the strength of obtained interactions (Tallarida, 2002). The isoboles were drawn by plotting the experimentally determined dose of TGB on the *x*-axis and that of an AED on the *y*-axis. The isobolograms showing distinct synergistic or antagonistic interactions are exclusively displayed on the graphs.

2.8. Statistics

CS_{50} values for TGB and ED_{50} or TD_{50} values for AEDs administered alone (and their statistical analysis) were calculated according to Litchfield and Wilcoxon (1949). The free plasma and brain concentrations of AEDs were evaluated with Student's *t* test. Statistical analysis of data from the chimney test was performed with the Fisher's exact probability test.

With isobolography, the experimental $ED_{50\ mix}$ values were statistically compared with the respective theoretical additive $ED_{50\ add}$s by the use of Student's *t* test according to the method presented by Porecca et al. (1990). If the experimental $ED_{50\ mix}$ is not different from the respective theoretical additive $ED_{50\ add}$, then the effect of the drug administration is additive. If the $ED_{50\ mix}$ is statistically lower than the theoretical additive $ED_{50\ add}$ value, a synergistic interaction between drugs is evident.

3. Results

3.1. Effects of TGB on the electroconvulsive threshold in mice

TGB, at the dose of 2.5 mg/kg administered intraperitoneally 15 min before the test, did not affect the electroconvulsive threshold in mice. The drug applied at doses of 5 and 10 mg/kg increased significantly the threshold from 6.6 (5.8–7.5) to 8.0 (7.3–8.8) and 9.6 (8.2–11.2) mA (Table 1).

3.2. Isobolographic analysis of interactions between TGB and studied AEDs in maximal electroshock-induced seizures in mice

The experimentally assessed ED_{50} values ±S.E.M. (Litchfield and Wilcoxon, 1949) for CBZ, DPH, PB, VPA, LTG, FBM, and TPM are presented in Tables 2 and

Table 2
Effects of the combinations of TGB with conventional AEDs (DPH, CBZ, PB, and VPA) against maximal electroshock-induced seizures in mice

Drug combination	F	$ED_{50\ mix}$ (mg/kg)	$ED_{50\ add}$ (mg/kg)
DPH alone		9.8 ± 0.66	–
TGB + DPH	1:1	18.7 ± 1.42	19.6 ± 1.91
	1:4	15.3 ± 0.99	12.3 ± 1.20
	1:8	13.4 ± 0.96	11.0 ± 1.08
	1:16	10.9 ± 0.94	10.4 ± 1.02
CBZ alone		10.7 ± 0.65	–
TGB + CBZ	1:1	18.0 ± 1.31	21.4 ± 1.88
	1:4	14.3 ± 1.20	13.4 ± 1.18
	1:8	11.6 ± 0.89	12.0 ± 1.06
	1:16	11.0 ± 0.84	11.4 ± 1.0
PB alone		14.5 ± 0.97	–
TGB + PB	1:1	25.5 ± 2.28	29.0 ± 2.82
	1:4	20.0 ± 2.24	18.1 ± 1.76
	1:8	17.0 ± 1.55	16.3 ± 1.59
	1:16	16.1 ± 1.21	15.4 ± 1.50
VPA alone		236.1 ± 7.72	–
TGB + VPA	1:20	199.3 ± 9.07*	247.9 ± 11.78
	1:30	185.0 ± 9.39**	244.0 ± 11.59
	174.9 ± 9.41**	242.0 ± 11.50	
	1:50	228.2 ± 10.97	240.8 ± 11.44

Table data are presented as ED_{50} values ± S.E.M.; F, fixed dose ratio (TGB: an antiepileptic drug); $ED_{50\ mix}$, experimental ED_{50} of the respective drug mixture; $ED_{50\ add}$, theoretical additive ED_{50}. TGB, tiagabine; DPH, diphenylhydantoin; CBZ, carbamazepine; PB, phenobarbital; VPA, valproate. Calculations of $ED_{50\ mix}$s were made according to Litchfield and Wilcoxon (1949) and isobolographic $ED_{50\ add}$s were calculated according to Porecca et al. (1990). Statistical analysis was performed by the use of Student's *t* test.
* $P<.01$.
** $P<.001$.

Table 3
Effects of the combinations of TGB with novel AEDs (TPM, FBM, and LTG) against maximal electroshock-induced seizures in mice

Drug combination	F	$ED_{50\ mix}$ (mg/kg)	$ED_{50\ add}$ (mg/kg)
TPM alone		30.5 ± 2.83	–
TGB + TPM	1:5	29.9 ± 3.87	36.6 ± 4.90
	1:10	33.6 ± 4.50	33.6 ± 4.49
	1:20	32.1 ± 4.30	32.0 ± 4.29
FBM alone		39.0 ± 2.99	–
TGB + FBM	1:5	45.7 ± 5.00	46.8 ± 5.18
	1:10	39.9 ± 3.88	42.9 ± 4.75
	1:20	40.0 ± 4.38	41.0 ± 4.53
LTG alone		5.1 ± 0.27	–
TGB + LTG	1:1	8.4 ± 0.78	10.2 ± 0.80
	1:2	7.1 ± 0.68	7.7 ± 0.60
	1:3	6.6 ± 0.65	6.8 ± 0.53
	1:4	6.0 ± 0.59	6.4 ± 0.50

Table data are presented as ED_{50} values ± S.E.M.; TGB, tiagabine; TPM, topiramate; FBM, felbamate; LTG, lamotrigine. Calculations of ED_{50}s were made according to Litchfield and Wilcoxon (1949), and isobolography with statistical analysis were performed according to Porecca et al. (1990). For more details, see also the legend of Table 2 and Materials and methods.

Table 4
Effects of the combination of TGB with VPA upon the motor performance of mice in the chimney test

Treatment (mg/kg)	Number of animals showing motor deficit	Total number of animals tested
Control	0	10
VPA (190)	1	10
TGB (9.3)	1	10
VPA (190) + TGB (9.3)	7*,†	10

Results from the chimney test are presented as a number of animals showing motor deficits in form of inability to climb backwards up the plastic tube within 60 s. Statistical analysis was performed by using the Fischer's exact probability test.
VPA—valproate; TGB—tiagabine.
* $P < .01$ versus control group (vehicle-treated animals) and simultaneously.
† $P < .05$ versus VPA-treated or TGB-treated groups.

3. It was evident that the isobolographically evaluated $ED_{50\ mix}$ values ± S.E.M., for the combinations of TGB with VPA (1:20, 1:30, 1:40), were significantly lower than the theoretically calculated $ED_{50\ add}$ ± S.E.M. for the mixture; thus, strongly indicating the synergistic interaction between the two drugs. However, the drug combination of 1:50 showed merely the additive interaction (Table 2, Fig. 1). The remaining combinations of TGB coadministered with CBZ, DPH, and PB at fixed ratios of 1:1, 1:4, 1:8, 1:16 (Table 2), TPM and FBM (1:5, 1:10, 1:20; Table 3), and LTG (1:1, 1:2, 1:3, 1:4; Table 3) exerted additive interactions.

3.3. Motor performance testing

TD_{50} values for TGB and VPA, administered alone, evaluated in the chimney test were amounted 18.8 (15.2–23.1) and 367.4 (347.3–389.7) mg/kg, respectively. Moreover, the TD_{16} values (16% toxic dose, evoking in 16% of animals tested the impairment of motor coordination) for TGB (12.9 mg/kg) and VPA (332.4 mg/kg) were denoted since they have an essential significance in animal studies.

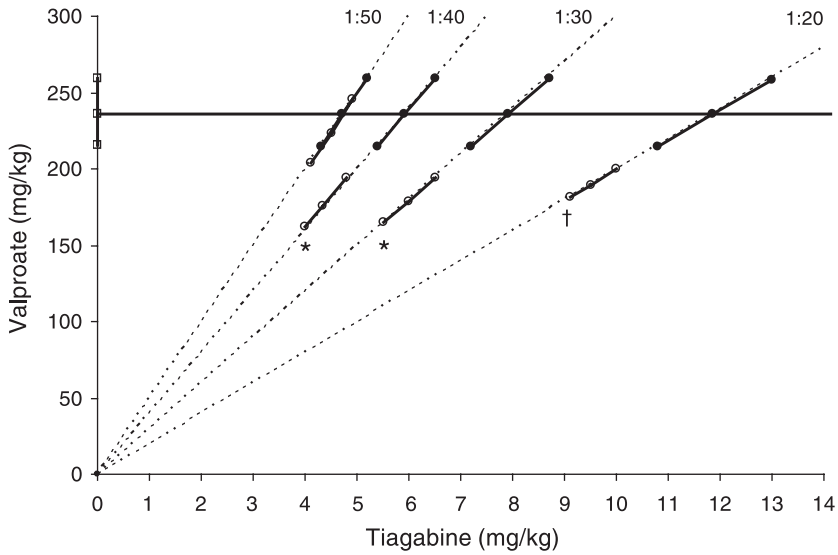

Fig. 1. Isobologram displaying fixed ratio drug interactions between TGB and VPA in the MES test in mice. The ED_{50} value (with 95% confidence limits) for VPA is plotted on the *y*-axis. The heavy line is parallel to the *x*-axis representing the ED_{50} value for VPA and defines the theoretical dose-additive line for a continuum of different fixed dose ratios. The solid sectors (•) intersecting the parallel line of additivity relate to $ED_{50\ add}$s for ratio of drug doses and represent on the graph the 1:20, 1:30, 1:40, and 1:50 fixed dose ratios of TGB and VPA. The open sectors (○) depict the experimentally derived $ED_{50\ mix}$s for total doses expressed as the proportion of TGB and VPA. The experimental sectors for TGB + VPA mixture were found to be significantly below the theoretical additive sectors, indicating supra-additive (synergic) interactions. Interactions between TGB and VPA for the 1:30 and 1:40 fixed ratio combinations are significant at * $P < .001$ and that for 1:20 at † $P < .01$, whilst the fixed ratio combination of 1:50 is not significant.

Table 5
Influence of TGB upon the free plasma and brain concentrations of AEDs

Treatment (mg/kg)	Free plasma level (μg/ml)	Brain concentration (μg/g)
VPA (190)	145.40 ± 18.80	74.94 ± 13.84
VPA (190) + TGB (9.3)	179.32 ± 24.76 *	144.50 ± 17.71 * *
DPH (9.3)	0.37 ± 0.043	0.80 ± 0.08
DPH (9.3) + TGB (9.3)	0.27 ± 0.042***	0.61 ± 0.09***
CBZ (9.0)	1.24 ± 0.20	2.48 ± 0.40
CBZ (9.0) + TGB (9.0)	1.14 ± 0.20	2.14 ± 0.40
PB (12.7)	14.03 ± 0.86	11.87 ± 0.49
PB (12.7) + TGB (12.7)	13.56 ± 0.79	12.30 ± 0.60
TPM (25)	12.92 ± 1.11	5.64 ± 0.78
TPM (25) + TGB (5)	12.80 ± 1.47	6.14 ± 0.80
LTG (4.2)	1.48 ± 0.10	0.55 ± 0.06
LTG (4.2) + TGB (4.2)	1.47 ± 0.12	0.56 ± 0.06

Presented values are the means in micrograms per milliliters of plasma or in micrograms per grams of wet brain tissue ± S.D. of eight determinations. Blood and brain tissue samples were taken at times scheduled for the electroconvulsive test. Plasma and brain levels of CBZ, DPH, PB, VPA, and TPM were evaluated by immunofluorescence and that of LTG by HPLC. Student's *t* test was used for statistical evaluation of the data. See also legend of Tables 2 and 3.

* $P < .05$ versus respective control values.

* * $P < .001$ versus respective control values.

*** $P < .01$ versus respective control values.

TGB (9.3 mg/kg) and VPA (190 mg/kg) administered separately, caused a negligible impairment of motor coordination in mice in the chimney test (Table 4). However, coadministration of TGB (9.3 mg/kg) with VPA (190 mg/kg) at fixed ratio of 1:20 caused a statistically significant (at $P < .01$; Fisher's test) impairment of motor coordination (7 out of 10 mice did not climb backwards up the transparent plastic tube and were classified as unable to perform this test within 60 s). Obviously, all control animals performed the chimney test correctly (Table 4).

3.4. Influence of TGB on the plasma and brain concentrations of antiepileptic drugs

TGB applied at the dose corresponding to the drug in mixture for the fixed ratio combination of 1:1 did not affect the free plasma and brain levels of CBZ, PB, or LTG (Table 5). In contrast, the free plasma and brain concentrations of VPA were significantly elevated by TGB at the dose of 9.3 mg/kg (fixed ratio of 1:20). The free plasma level of VPA was raised by 23%, whilst the brain concentration by 2-fold. On the contrary, both the plasma and brain levels of DPH were significantly reduced by 26% and 23%, respectively, after TGB (9.3 mg/kg) coadministration (Table 5).

4. Discussion

Results obtained in this study showed that TGB weakly influenced the anticonvulsant activity of commonly applied AEDs against maximal electroshock-induced seizures in mice, except of the combination of TGB with VPA. In this case, the observed interactions were evidently synergistic for the fixed ratio combinations of 1:20, 1:30, and 1:40. At other fixed ratio combinations, merely additive interactions were observed.

It is generally accepted that TGB, at therapeutic concentrations, is ineffective in the maximal electroshock seizure test in mice (Rogawski and Porter, 1990). However, most recently, numerous clinical trials have proved the clinical efficacy of TGB in complex partial seizures with or without secondarily generalization in humans (Richens et al., 1995). Little is known as yet about the interactions between TGB and other AEDs.

The maximal electroshock-induced seizure test in mice is considered as an experimental model of generalized tonic–clonic seizures and to a certain degree of partial convulsions in humans (Löscher et al., 1991). It is important to note that GBP, which is also inactive in the maximal electroshock test in mice, when combined with conventional and some novel AEDs (CBZ, VPA, DPH, PB, LTG, and LY 300164) had generally evoked synergistic type of interactions (Borowicz et al., 2002). These interactions have possessed rather a pharmacodynamic character since pharmacokinetic events, which might be responsible for synergy, had been excluded. Furthermore, it should be stressed that TGB combined with GBP (at fixed ratios of 1:3, 1:1, and 3:1) has exerted synergistic interactions in the electroconvulsive threshold test in mice without affecting the free plasma and brain concentrations of GBP (Luszczki et al., unpublished data). Therefore, a question arises whether TGB (as an ineffective drug) is able to interact synergistically (similarly to GBP) with other AEDs in the maximal electroshock test in mice. Considering this fact, we attempted to determine the interactions of TGB with available AEDs in order to create the rationale for the therapeutic choice of effective combinations in which TGB could be applied as add-on therapy for the patients with unsatisfactorily medicated epilepsy.

In our study, some interactions of TGB and AEDs studied, after the isobolographic evaluation in the maximal electroshock test, were verified as regards pharmacokinetic events which might influence the exact character of the observed interactions. Results obtained from pharmacokinetic determinations of AED concentrations evidently showed that the free plasma and brain concentrations of VPA were significantly elevated after coadministration of TGB. However, in contrast to the free plasma level of VPA, which was elevated by TGB by 23%, the brain concentration of VPA after TGB administration reached the level of 2-fold higher than that observed in VPA-alone-treated animals. This pharmacokinetic interaction may be explained through the competition of TGB and VPA to the plasma protein binding. TGB, by displacing VPA from plasma proteins, may cause elevation of the free plasma concentration of VPA. This explanation could, at least in part, confirm the elevation of VPA concentration in the brain. In an in

vitro pharmacokinetic study, it was reported that VPA was able to displace TGB from plasma bound proteins (Perucca, 1999). Therefore, it seems likely that these pharmacokinetic events, especially the increase of VPA concentration in the brain, may explain the synergistic type of interaction observed between these AEDs in the MES test. In contrast, there were no pharmacokinetic events corroborated in clinical practice which might disturb the free plasma levels of VPA after TGB's coadministration (Gustavson et al., 1998). On the other hand, the free plasma level of VPA, which is responsible for anticonvulsant/neurotoxic effects, is about 5-fold higher in mice than in humans; thus, an explanation of the observed discrepancies between our study and clinical reports may be related with the interspecies differences in pharmacokinetics of VPA (Nau, 1986). On the other hand, the influence of TGB on the forced permeability of blood–brain barrier for VPA should not be excluded. At present, one can speculate about the possible mechanism(s) of pharmacokinetic events underlying the interaction of TGB with VPA. Since neither the free plasma nor the brain concentrations of TGB were evaluated, some pharmacokinetic changes in plasma and/or brain concentrations of the drug could be responsible for the observed interactions. However, TGB in the present study was administered at relatively low doses; hence, the occurrence of pharmacokinetic events, masking the pharmacodynamic character of interactions is less likely but not excluded.

It is worth mentioning that the dose ratio may be critical for the final outcome of interactions between AEDs (Luszczki et al., 2003). This is evident from the present results that in some dose ratios, the interactions were simply additive (e.g., TGB:VPA-1:50) and in others, significantly synergistic (1:20, 1:30, and 1:40). Since pharmacokinetic studies were performed for some drug ratios, it is impossible to entirely exclude a possibility of pharmacokinetic interactions for all remaining evaluated drug ratios.

A growing body of evidence suggests that the proper classification of pharmacologic interactions among AEDs should be followed by evaluation of pharmacokinetic events, which may sometimes influence the final effect of observed interactions. There is no doubt that ignoring pharmacokinetic character of interactions among AEDs one can misinterpret the isobolographic types of interactions evaluated in preclinical studies (Cadart et al., 2002). Therefore, it seems clear that the concomitant determination of anticonvulsant efficacy of the two-drug mixture and AED-concentrations within the biophase may give much more insight into the exact character of interactions, which occur among AEDs. This suggestion is generally consistent with the studies of Cadart et al. (2002), Bourgeois (1986, 1988), and Bourgeois and Wad (1984, 1988), who had additionally evaluated a pharmacokinetic character of interactions among AEDs in the brains of animals tested. Considering all these facts, it was evident that all interactions, which would be evaluated in further preclinical studies with isobolographic analysis, should be thoroughly verified as regards the coexistence of some pharmacokinetic interactions, which might mask the exact character of interactions among AEDs. In our opinion, the determination of AED concentrations in the homogenates of animal brain tissue seems the most optimal resolution for the exact determination and classification of two-drug interactions.

Basing on the results from the concomitant administration of VPA with TGB, there appeared another very important problem. Due to the increment of VPA level in the brain, one could observe the enhancement of anticonvulsant (protective) effects but also the neurotoxic effects in terms of the impairment of motor coordination in mice. Considering theoretically the possible mechanism(s) of neurotoxic effects of this VPA + TGB combination, it seems likely that they can be caused by a high concentration of VPA in the brain. With the adverse effects observed in the chimney test for the combination of TGB with VPA at the fixed ratio of 1:20, testifying about the potentiation of impairment of motor coordination in animals, one can explain through the existence of a pharmacokinetic interaction between the drugs in the brains. There is no doubt that 2-fold increment of VPA concentration in the brain tissue has to intensify the side effects of the combination in terms of motor performance of mice. However, a pharmacodynamic interaction between VPA and TGB as regards the side-effect profile of this two-drug combination is also not excluded.

This study demonstrated that pharmacokinetic changes in the brain concentration of VPA may be also the main cause of neurotoxic effects observed between drugs despite the synergistic type of interactions ascertained for this two-drug combination in the maximal electroshock test. In such a case, there is no rationale for combining the drugs in polytherapy for patients with intractable seizures in order to reduce seizure, and thus ameliorate the patients' quality of living.

Furthermore, it was reported that both the free plasma and brain concentrations of DPH were significantly reduced by TGB by 26%, and 23%, respectively. The concentration of DPH in the brains was higher than that detected in plasma, indicating the accumulation of DPH in brain tissue, which was consistent with well-known properties of the drug. However, it has to be highlighted that a pharmacokinetic decrease in the free plasma and brain concentration of DPH masked a pharmacodynamic interaction between these drugs. If pharmacokinetic events had not reduced the plasma and brain DPH levels, the interaction between TGB and DPH would be evidently synergistic. In other words, a possible pharmacodynamic synergy has been overcome by a pharmacokinetic change in the free plasma and brain concentrations of the drug, finally leading to the additivity.

Summing up, it has to be stressed that the observed synergistic interactions between TGB and VPA in mice have a pharmacokinetic background reflected by the increase in VPA-free concentrations in the plasma and brain.

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