



Short communication

Gabapentin synergistically interacts with topiramate in the mouse maximal electroshock seizure model: an isobolographic analysis

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Abstract:

The anticonvulsant effects produced by topiramate (TPM) and gabapentin (GBP) – two second-generation antiepileptic drugs, in numerous fixed-ratio combinations of 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 were examined by isobolographic analysis in the mouse maximal electroshock seizure (MES) model.

Results indicate that the combinations of TPM and GBP at the fixed-ratios of 2:1, 1:1, 1:2, 1:4 and 1:8 resulted in supra-additive (synergistic) interaction against MES-induced seizures. Moreover, the combinations of TPM and GBP (at their median effective doses) did not affect motor performance of animals challenged with the chimney test and had no impact on neuromuscular tone in the grip-strength test. Additionally, GBP had no impact on total brain TPM concentrations, and simultaneously, TPM did not alter brain GBP concentrations, indicating that the interaction between drugs was pharmacodynamic in nature.

In conclusion, supra-additive interaction of TPM with GBP against MES-induced seizures, lack of motor coordination and neuromuscular tone impairments as well as lack of pharmacokinetic interactions between TPM and GBP in preclinical study, strongly support the combined application of both antiepileptic drugs in patients with refractory partial epilepsy.

Key words:

topiramate, gabapentin, maximal electroshock, isobolographic analysis, pharmacodynamic interaction

Abbreviations: AED – antiepileptic drug, AMPA – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, DRRC – dose-response relationship curve, ED₅₀ – median effective dose, GABA – γ -aminobutyric acid, GBP – gabapentin, MES test – maximal electroshock seizure test, MEST test – maximal electroshock seizure threshold test, NMDA – N-methyl-D-aspartic acid, PTZ – pentetrazole, TID₂₀ – threshold increasing dose by 20%, TID₅₀ – threshold increasing dose by 50%, TPM – topiramate

Introduction

In spite of ten newer (second-generation) antiepileptic drugs (AEDs), lately approved for the treatment of

patients with epileptic attacks, there are around 30% of patients, who inadequately respond to the monotherapeutic use of currently available AEDs and continue to have seizures [22]. Consequently, the rational combination of two or more AEDs may provide these patients with a state of seizure freedom. In fact, several two-AED combinations have occurred advantageous for patients with refractory epilepsy, considerably suppressing their seizures over 1 year [43]. Unfortunately, ~14% of epileptic patients remain still resistant to the rationally prescribed AED combinations [22], and some novel therapeutic strategies (for instance, combinations of newer and/or novel AEDs) for suppressing epileptic attacks are urgently needed

[26]. Although direct evaluation of the antiseizure efficacy and tolerability of new AED combinations in the clinical setting is impossible due to methodological and ethical issues, preclinical studies in animals can help to preselect the most promising combinations offering supra-additive (synergistic) interactions in terms of seizure protection, devoid of any deleterious adverse effects. Importantly, seizure models in laboratory animals provide usually an invaluable means allowing for identification of potentially useful AED polytherapy regimens, which could subsequently be evaluated in patients [10, 26].

Relatively recently, there has appeared a new trend to characterize interactions among AEDs in preclinical studies using isobolographic analysis [10, 23]. Indeed, the isobolographic analysis allows for precise classification of exact types of interactions between drugs, evaluating their nature as: supra-additive (synergistic), sub-additive (relatively antagonistic), infra-additive (absolutely antagonistic), indifferent or additive [2, 18, 28, 46]. Generally, favorable combinations proved isobolographically in preclinical studies on animals merit further prospective investigations in clinical conditions in order to establish the algorithms of treatment in patients with intractable seizures [10, 23, 31].

Overwhelming clinical evidence indicates that topiramate (TPM) and gabapentin (GBP) are of pivotal importance in controlling partial convulsions with or without secondary generalization (GBP and TPM) and/or tonic-clonic seizures (only TPM) in epileptic patients [6]. Obviously, both AEDs can be used separately in adults and children with newly diagnosed and/or refractory epilepsy as adjunctive therapy with conventional AEDs. Additionally, TPM can be successfully used for reducing drop attacks in patients with the Lennox-Gastaut syndrome [6]. In experimental studies, TPM has been found to be fully effective in suppressing maximal electroshock (MES)-induced seizures in mice [40]. In the case of GBP, the AED has been classified as virtually ineffective in the mouse MES test [7], although, GBP at doses of 75 and 100 mg/kg significantly elevated the threshold for electroconvulsions in mice [36]. On the other hand, one experimental report has documented that GBP produced a clear-cut anticonvulsant effect against MES-induced seizures in mice and its median effective dose (ED_{50}) was 78.2 (46.6–127) mg/kg [51]. These controversial results, concerning the antiseizure effects of GBP in the MES test in mice, prompted us

to determine thoroughly the anticonvulsant profile for GBP administered alone.

Accumulating experimental evidence indicates that TPM potentiated the anticonvulsant activity of numerous co-administered AEDs. Previously, it has been shown isobolographically that TPM interacted synergistically with phenobarbital, carbamazepine [40], lamotrigine [32], levetiracetam [27, 41], felbamate, oxcarbazepine [31], and lorcetazepine [34] in the MES test in mice. Additionally, TPM (at a subthreshold dose of 2.5 mg/kg) potentiated the antiseizure effects of diazepam, phenobarbital, valproate, phenytoin and lamotrigine, but not those of carbamazepine and felbamate against the clonic phase of sound-induced seizures in DBA/2 mice [8]. Moreover, the combinations of TPM with clobazam, lamotrigine and levetiracetam offered the essential anticonvulsant protection against pentetrazole (PTZ)-induced clonic seizures in mice, although the constituent compounds were virtually inactive in this test [41]. More recently, it has also been found that TPM synergistically interacted with budipine (a low-affinity N-methyl-D-aspartate (NMDA)-receptor antagonist) in reducing the seizure activity in the rat perforant path-stimulated self-sustaining status epilepticus (a model of refractory status epilepticus) [12]. Similarly, GBP combined with conventional and some newer AEDs (i.e., phenytoin, carbamazepine, phenobarbital, valproate, lamotrigine and talampanel) exhibited synergistic interactions in isobolography, at various fixed-ratios in the MES test in mice [5]. Isobolographic analysis revealed also that GBP interacted synergistically with tiagabine and oxcarbazepine against MES and PTZ-induced clonic seizures in mice [29, 30, 34, 36]. Moreover, GBP (at a subthreshold dose of 2.5 mg/kg) potentiated the anticonvulsant effects produced by diazepam, phenobarbital, valproate, felbamate and phenytoin, but not those by carbamazepine and lamotrigine in DBA/2 mice against the clonic phase of sound-induced seizures [9].

Up to date, neither clinical nor experimental reports have described the anticonvulsant profile of a combination between TPM and GBP. We sought, therefore, to characterize the type of interactions between these AEDs against MES-induced seizures in mice using the isobolographic analysis. It is widely accepted that the MES test is considered to be an experimental model of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in humans [25]. Hence, it

was considered appropriate to use this test for assessing the exact characteristics of interaction between TPM and GBP in our study. Additionally, the adverse-effect profiles for the combinations of AEDs were investigated in relation to motor coordination impairment in the chimney test and neuromuscular tone deficits in the grip-strength test. To ascertain whether the observed anticonvulsant effects for the combination of GBP with TPM were consequent to a pharmacodynamic and/or a pharmacokinetic interaction, total brain TPM and GBP concentrations were measured.

Materials 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 9.00 a.m. and 2.00 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 animals' 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 nr: 368/2002/349/02) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

The following AEDs were used in this study: GBP (Neurontin; Parke-Davis, Freiburg, Germany) and TPM (Topamax; Cilag AG, Schaffhausen, Switzerland). Both drugs were suspended in a 1% solution of Tween 80 (Sigma, USA) in saline and administered

intraperitoneally (*ip*), as two separate injections, in a volume of 5 ml/kg of body weight. The control animals received adequate amounts of vehicle (1% solution of Tween 80 in saline). Fresh drug solutions were prepared on each day of experimentation and administered 60 min prior to MES test, chimney test and brain sampling for the measurement of AED concentrations. This pretreatment time before testing of the AEDs was based on information about their biological activity from the literature and our previous experiments [29, 31]. The time of peak maximum anticonvulsant effects for TPM and GBP (60 min) was used as reference time in all experimental tests and pharmacokinetic estimation of brain AED concentrations.

Maximal electroconvulsions

Electroconvulsions were produced by means of an alternating current (0.2 s stimulus duration, 50 Hz, maximum stimulation voltage of 500 V) delivered *via* ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs Elektronik, Freiburg, Germany). The electrical system of the stimulator was self-adjustable so that changes in impedance did not result in alterations of current intensity (i.e., the system provides constant current stimulation). The criterion for the occurrence of seizure activity was the tonic hind limb extension (i.e., the hind limbs of animals outstretched 180° to the plane of the body axis). In this experiment, two experimental models of maximal electroconvulsions were used: 1) threshold for maximal electroshock seizures (MEST) and 2) MES.

Maximal electroshock seizure threshold (MEST) test

To evaluate the threshold for electroconvulsions, at least 4 groups of mice, consisting of 8 animals per group, were challenged with electroshocks of various intensities to yield 10–30%, 30–50%, 50–70%, and 70–90% of animals with seizures. Then, a current intensity-response relationship line was constructed, according to a log-probit method of Litchfield and Wilcoxon [24], from which a median current strength (CS_{50} in mA) was calculated. Each CS_{50} value represents the current intensity required to induce tonic hindlimb extension in 50% of the mice challenged. Again, after administration of a single dose of GBP to 4 groups of animals, the mice were subjected to elec-

troconvulsions (each group with a constant current intensity). The threshold for electroconvulsions was recorded for 5 different doses of GBP: 50, 75, 100, 125 and 150 mg/kg. Subsequently, the percentage of increase in CS_{50} values for animals injected with increasing doses of GBP over the control (vehicle-treated animals) was calculated and plotted graphically in rectangular coordinates of the Cartesian plot system. From least-squares linear regression analysis according to Glantz and Slinker [16], threshold increasing doses by 20% and 50% (TID_{20} and TID_{50}) were determined as recommended by Löscher et al. [25]. The experimental procedure has been described in more detail in our earlier studies [27, 29, 30, 33, 34].

Maximal electroshock seizure (MES) test

The anticonvulsant activity of TPM administered alone was evaluated and expressed as its median effective doses (ED_{50} values in mg/kg with their 95% confidence limits), required to protect 50% of the animals tested against MES-induced seizures (at fixed current intensity of 25 mA). The ED_{50} values were calculated using log-probit method according to Litchfield and Wilcoxon [24]. Moreover, the animals were administered with the mixture of TPM with GBP (at various fixed drug dose-ratio combinations) so as to obtain a variable percentage of protection against MES, allowing the construction of a dose-response relationship curve (DRRC) for every AED combination. The anticonvulsant activities of numerous fixed-ratio combinations of TPM with GBP were evaluated and expressed as their $ED_{50\text{ mix}}$ values, corresponding to the total doses of mixtures necessary to protect 50% of mice against tonic hindlimb extension in the MES test. The experimental procedure has been described in more detail in our earlier studies [28, 29, 33, 35, 45].

Isobolographic analysis

To perform the isobolographic analysis of interactions between TPM and GBP (as regards their anticonvulsant activity against MES-induced seizures) mixtures of both AEDs in numerous fixed-ratio combinations of 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8 were administered to animals. Subsequently, the experimentally-derived $ED_{50\text{ mix}}$ values for the mixtures of TPM with GBP (with their corresponding 95% confidence lim-

its) were determined using log-probit method according to Litchfield and Wilcoxon [24]. The obtained 95% confidence limits were transformed into SE as described previously [5, 33]. In isobolography, the $ED_{50\text{ mix}}$ values (\pm SE) were statistically compared with their respective theoretically-additive $ED_{50\text{ add}}$ values (\pm SE) by the use of unpaired Student's *t*-test according to Porreca et al. [39] and Tallarida [46]. Noteworthy, the theoretically-additive $ED_{50\text{ add}}$ values (\pm SE) were calculated from the equation presented by Porreca et al. [39], as follows: $ED_{50\text{ add}} = ED_{50\text{ drug } 1} / P_1$; where, P_1 – is the proportion of the first drug (fully effective against MES-induced seizures, that is, TPM) in the total amount of the drug mixture. For two-drug mixture the equation presented above is true when: $P_1 + P_2 = 1$; where, P_2 – is the proportion of the second drug (virtually ineffective in the MES test, that is, GBP). It is worth mentioning that the proportions of AEDs are based on a mass quantity of AEDs in the mixture, and thus, the fixed-ratio combination of 1:1 comprised equal amounts of TPM and GBP. This particular kind of isobolographic analysis (so-called type II) allows the acceptance of mass quantity of drugs in the mixture as the basis to construct the notation of fixed-ratio combinations (i.e., 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8). A more detailed description and the theoretical background concerning isobolographic analysis followed by equations showing how to calculate $ED_{50\text{ add}}$ values and their SE, in a case if one of the investigated AEDs is virtually ineffective against MES, has been presented in our previous studies [5, 27, 36]. To visualize the types of interactions between GBP and TPM, the isoboles were drawn by plotting the points reflecting the respective doses of GBP (on the X-axis), and doses of TPM on the Y-axis in the Cartesian plot system. The straight line, parallel to the X-axis represents the theoretical line of additivity. When the experimentally-derived points reflecting combinations of various fixed-ratios fall significantly below this line, the two component drugs act supra-additively (synergistic).

Measurement of total brain AED concentrations

The animals were given TPM + vehicle, GBP + vehicle or a combination of TPM + GBP. The fixed-ratio combination for estimating brain concentrations of TPM was chosen as 1:1 (TPM : GBP). Mice were killed by decapitation at times chosen to coincide with that scheduled for the MES test. The whole brains of

mice were removed from skulls, weighed, and homogenized using Abbott buffer (2:1 v/w) in an Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany). The homogenates were centrifuged at $10,000 \times g$ for 10 min. The supernatant samples (75 μ l) containing TPM were analyzed by fluorescence polarization immunoassay using a TDx analyzer and reagents exactly as described by the manufacturer (Opus Diagnostics, Fort Lee, NJ, USA). Simultaneously, the brain supernatant samples (100 μ l) containing GBP were transferred into the high pressure liquid chromatography (HPLC)-system of drug detection. Details concerning the technique of estimation of GBP concentrations with HPLC have been presented in our earlier reports [29, 36]. The brain AED concentrations were expressed in μ g/ml of brain supernatants and μ g/g of wet brain tissue as the means \pm SD of at least 8 separate brain preparations. Total brain AED concentrations were statistically analyzed using the unpaired Student's *t*-test.

Chimney test

The effects of TPM and GBP in combinations (at doses corresponding to the $ED_{50 \text{ mix}}$ values for all fixed-ratios tested) on motor performance impairment were quantified with the chimney test of Boissier et al. [4]. In this test, animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length), and motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test.

Grip-strength test

The effects of TPM combined with GBP (at doses corresponding to their $ED_{50 \text{ mix}}$ values for all fixed-ratio tested from the MES test) on neuromuscular strength in mice were quantified by the grip-strength

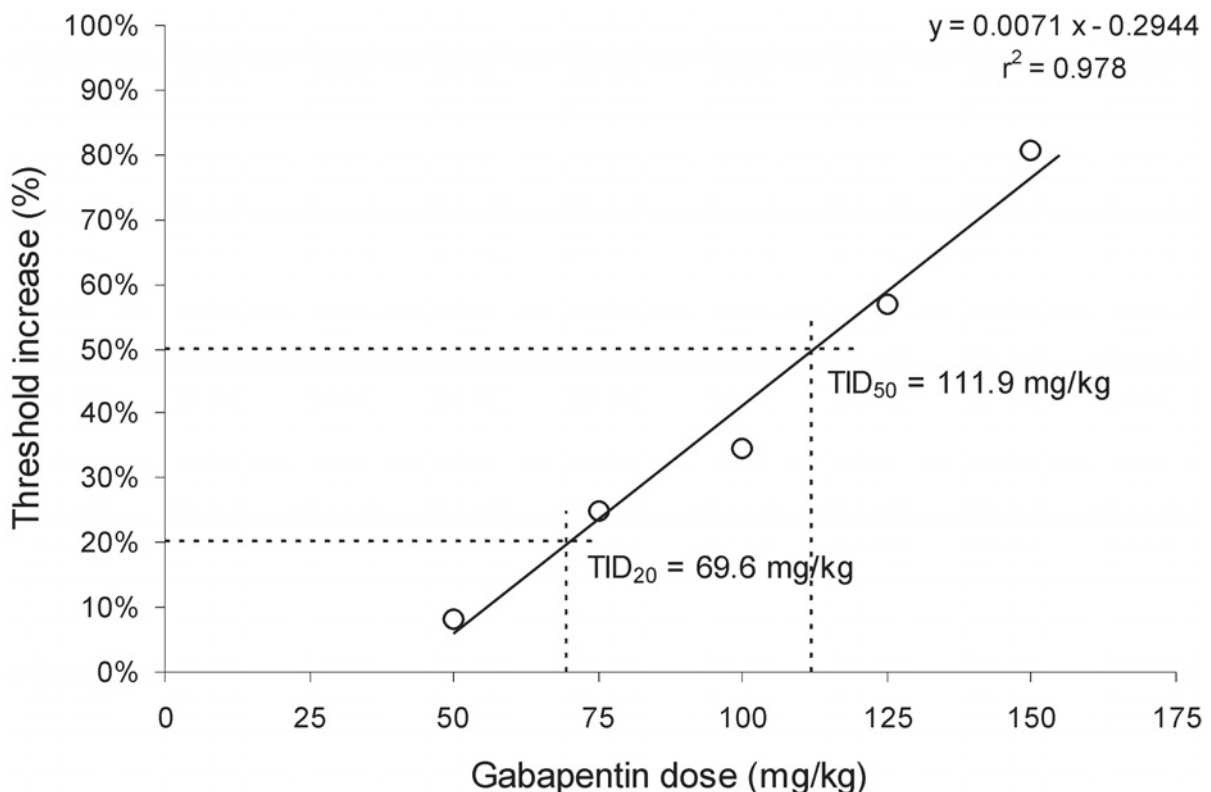


Fig. 1. Linear regression dose-response relationship curve (DRRC) analysis for gabapentin (GBP) in the maximal electroshock seizure threshold (MEST) test in mice. Doses of GBP are plotted against the percentage of increase in the electroconvulsive threshold in mice. Analysis of data was performed with least-squares linear regression according to Glantz and Slinker [16]. The equation of DRRC for GBP evaluated in the MEST test was $y = 0.0071x - 0.2944$ ($r^2 = 0.978$); where *y* – % threshold increase, *x* – GBP dose, and r^2 – coefficient of determination. Test for homogeneity revealed that points forming the line are homogenous and good-to-fit. The TID_{20} and TID_{50} values (threshold increasing doses by 20% and 50%), calculated directly from the equation for GBP were 69.6 mg/kg and 111.9 mg/kg, respectively

Tab. 1. Isobolographic analysis of interactions between topiramate (TPM) and gabapentin (GBP) in the maximal electroshock (MES)-induced seizures

FR	TPM	+ GBP	= ED _{50 mix} (mg/kg)	N _{mix}	ED _{50 add} (mg/kg)	= TPM	+ GBP	N _{add}
8:1	43.1	5.4	48.5 ± 4.3	24	52.4 ± 5.0	46.6	5.8	30
4:1	36.8	9.2	46.0 ± 7.0	24	58.1 ± 5.5	46.6	11.5	30
2:1	29.4	14.7	44.1 ± 6.2**	24	69.6 ± 6.6	46.6	23.0	30
1:1	21.6	21.6	43.2 ± 8.0***	24	93.2 ± 8.8	46.6	46.6	30
1:2	14.1	28.2	42.3 ± 10.6***	24	139.8 ± 13.2	46.6	93.2	30
1:4	12.2	48.8	61.0 ± 10.3***	24	233.0 ± 22.0	46.6	186.4	30
1:8	9.1	72.6	81.7 ± 12.2***	24	419.4 ± 39.6	46.6	372.8	30

Results are presented as median effective doses (ED₅₀ ± SE in mg/kg) for drug mixtures, determined either experimentally (ED_{50 mix}) or theoretically calculated (ED_{50 add}) from the equation of additivity. FR – fixed-ratio combination; TPM – dose of topiramate in the mixture; GBP – dose of gabapentin in the mixture; N_{mix} – 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_{add} – total number of animals calculated for the additive mixture of the drugs examined. Statistical evaluation of the data was performed with unpaired Student's *t*-test according to Porecca et al. [38] and Tallarida [44]. ** – Significantly different at *p* < 0.01; and *** – at *p* < 0.001 vs. the respective ED_{50 add}.

test. 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 tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The grip-strength test was used to determine neuromuscular strength in mice, which was expressed in Newtons as the means ± SD of at least 24 determinations (3 measurements for each of 8 animals per group). Statistical evaluation of data from the grip-strength test was performed with one-way ANOVA followed by the *post-hoc* Bonferroni's test.

Results

Effect of GBP on the threshold for electroconvulsions

GBP administered systemically (*ip*) at doses ranging between 50 and 150 mg/kg raised dose-dependently the threshold for electroconvulsions in mice (Fig. 1). The linear trend between the increasing doses of GBP and their resultant percentage of threshold increase over the control group (vehicle-treated animals) in the

MEST test in mice allowed for the determination of the TID₂₀ and TID₅₀ values for GBP, which were 69.6 and 111.9 mg/kg, respectively (Fig. 1).

Effect of TPM administered alone on the MES-induced seizures

TPM exerted a clear-cut anticonvulsant effect in the MES test and its ED₅₀ value, derived from log-probit method was 49.5 (39.6–62.0) mg/kg. As for GBP, the drug at the dose up to 200 mg/kg did not protect the animals against MES-induced seizures in mice.

Isobolographic characterization of interactions between TPM and GBP in the MES test in mice

TPM combined with GBP at fixed-ratio combinations of 2:1, 1:1, 1:2, 1:4, and 1:8 exhibited supra-additive (synergistic) interaction in seizure suppression in the MES test (Tab. 1, Fig. 2). The experimentally-derived ED_{50 mix} values for the fixed-ratios of 2:1, 1:1, 1:2, 1:4 and 1:8 were significantly lower than the theoretically calculated ED_{50 add} values (*p* < 0.01 and *p* < 0.001), indicating supra-additive interaction between TPM and GBP (Tab. 1, Fig. 2). Only two fixed-ratio combinations of 8:1 and 4:1 were classified as additive, although a trend towards supra-additivity was observed (Fig. 2).

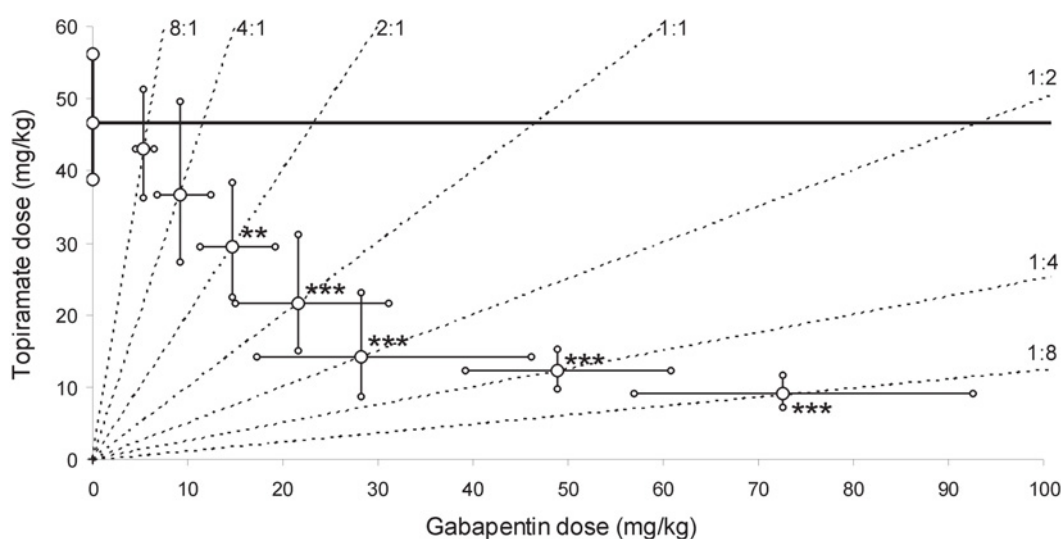


Fig. 2. Isobologram showing interactions between gabapentin (GBP) and topiramate (TPM) against maximal electroshock (MES)-induced seizures in mice. Doses of GBP and TPM are shown plotted graphically on the X- and Y-axes, respectively. The heavy line is parallel to the X-axis, representing the ED_{50} value for TPM, and defines the theoretical dose-additive line for a continuum of different fixed dose ratios. The dotted lines correspond to the fixed drug-dose ratio combinations. The open points (O) depict the experimentally-derived $ED_{50\text{ mix}}$ values for total doses of mixtures expressed as proportions of GBP and TPM that produced median anticonvulsant effects. All 95% confidence limits of the experimentally determined $ED_{50\text{ mix}}$ values are presented horizontally and vertically in the form of crosses. The $ED_{50\text{ mix}}$ values for the mixture of TPM with GBP at the fixed-ratios of 2:1, 1:1, 1:2, 1:4 and 1:8 are significantly below the theoretical line of additivity, indicating supra-additive (synergistic) interactions (** $p < 0.01$ and *** $p < 0.001$). In contrast, the $ED_{50\text{ mix}}$ values at the fixed-ratios of 8:1 and 4:1 are close to the line of additivity displaying additive interactions with a trend towards supra-additivity in the MES test

Brain AED concentrations

Total brain AED concentrations were evaluated for TPM and GBP co-administered at the fixed-ratio of 1:1 from the MES test. The brain concentration of GBP, administered alone at 21.6 mg/kg, was $3.09 \pm 0.28 \mu\text{g/ml}$ of supernatant ($7.32 \pm 0.66 \mu\text{g/g}$ of wet brain tissue), and did not differ significantly from that evaluated for the mixture of GBP (21.6 mg/kg) with TPM (21.6 mg/kg), which was $3.21 \pm 0.29 \mu\text{g/ml}$ ($7.45 \pm 0.67 \mu\text{g/g}$). Similarly, GBP (21.6 mg/kg) co-administered with TPM (21.6 mg/kg) did not affect the brain TPM concentrations. In this case, the brain concentration of TPM (injected singly at 21.6 mg/kg) was $2.07 \pm 0.22 \mu\text{g/ml}$ ($5.02 \pm 0.53 \mu\text{g/g}$), whereas that for the two-drug mixture at the fixed-ratio of 1:1 amounted to $2.22 \pm 0.26 \mu\text{g/ml}$ ($5.12 \pm 0.60 \mu\text{g/g}$).

Effect of the combinations of TPM and GBP on motor coordination in the chimney test and neuromuscular strength in the grip-strength test

The combinations of TPM with GBP at doses corresponding to their $ED_{50\text{ mix}}$ values for all fixed-ratios studied (i.e., 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8) did

Tab. 2. Influence of the combinations of topiramate (TPM) and gabapentin (GBP) on motor performance of the animals in the chimney tests and grip-strength

FR	AED combination (mg/kg)	Mice impaired (%)	Neuromuscular strength (N)
–	Vehicle	0	99.8 ± 14.2
8:1	TPM (43.1) + GBP (5.4)	0	96.5 ± 13.8
4:1	TPM (36.8) + GBP (9.2)	0	94.9 ± 13.4
2:1	TPM (29.4) + GBP (14.7)	0	95.3 ± 13.9
1:1	TPM (21.6) + GBP (21.6)	0	95.3 ± 14.4
1:2	TPM (14.1) + GBP (28.2)	0	96.9 ± 13.5
1:4	TPM (12.2) + GBP (48.8)	12.5	95.8 ± 14.0
1:8	TPM (9.1) + GBP (72.6)	0	94.4 ± 13.7

Results from the chimney test are expressed as the percentage of animals showing motor impairment, whereas the data from the grip-strength test are presented as means of neuromuscular strength (in Newtons \pm SD of 24 determinations). Each experimental group consisted of 8 animals. The AEDs were administered *ip* 60 min before the chimney and grip-strength tests. Statistical evaluation of the data from the chimney test was performed with Fisher's exact probability test, whereas the results from the grip-strength test were statistically analyzed with one-way ANOVA followed by the *post-hoc* Bonferroni's

not affect motor performance of animals in the chimney test (Tab. 2). Furthermore, none of the combinations studied (TPM with GBP) impaired the neuromuscular strength as determined in the grip-strength test in mice (Tab. 2).

Discussion

The objective of this study was to characterize precisely the anticonvulsant profile of GBP administered alone in the MEST test in mice, as well as to determine the exact type of interactions between GBP and TPM in the MES model using the isobolographic analysis. In the present study, it was found that GBP increased the threshold for electroconvulsions in a dose-dependent manner in mice.

As already mentioned, experimental evidence provides some contradictory results indicating that GBP is either virtually ineffective (up to a dose of 300 mg/kg) in the MES test [5, 7] or produces clear-cut anticonvulsant effects with its ED₅₀ value amounting to 78.2 (46.6–127) mg/kg [51]. Previously, we have also documented that GBP at doses of 75 and 100 mg/kg significantly increased the threshold for electroconvulsions [29, 36]. Results presented herein seem to confirm the observation that GBP increases the threshold for electroconvulsions and is virtually ineffective in the mouse MES model. Therefore, our results are generally in agreement with those documented by Dalby and Nielsen [7] and Borowicz et al. [5], and simultaneously, are in contrast with those reported by White et al. [51]. As for TPM, its ED₅₀ value, evaluated in our study, was 49.5 (39.6–62.0) mg/kg and did not differ considerably from that estimated by White et al. [51], who have reported an ED₅₀ for TPM of 33.0 (16.3–58.1) mg/kg.

From a methodological point of view, to perform the conventional (type I) isobolographic analysis of interactions between two drugs, both drugs have to display parallel DRRCs, otherwise one cannot precisely calculate proportions of these drugs in the mixture at the respective fixed-ratio combinations. In such a case, the respective doses of the first drug are too high, whereas the doses of the second drug in the mixture are too low, producing *per se* supra- or sub-additive interactions instead of additivity [29, 46]. Since GBP is virtually ineffective in the MES test and

has no DRRC in this test, the type II isobolographic analysis had to be used in this study. Noteworthy, the type II isobolographic analysis assumes *a priori* that one of the AEDs in the mixture is virtually ineffective. It was additionally assumed that doses of GBP in the mixture did not exceed its TID₅₀ value (i.e., the dose of GBP increasing the threshold for electroconvulsions by 50%). In other words, doses of GBP in the mixture with TPM could not produce *per se* the anticonvulsant effects (i.e., GBP doses administered alone need not offer any protection against MES-induced seizures). Noteworthy, in this study, GBP at a dose of 200 mg/kg exerted no seizure suppression in the MES test in mice. Bearing in mind the above-mentioned assumptions and conducting the experiment using type II isobolographic analysis, it was shown that GBP synergistically interacted with TPM in the MES test at doses lower than 100 mg/kg (Tab. 1, Fig. 2). This is why the upper limit of fixed-ratio combinations tested in our study was 8:1 (but not 16:1, 32:1 or higher), in order not to exceed the TID₅₀ value for GBP, which was 111.9 mg/kg. With isobolography, supra-additive (synergistic) interactions were observed between GBP and TPM at the fixed-ratios of 1:2, 1:1, 2:1, 4:1 and 8:1 (Fig. 2).

The synergistic interaction between GBP and TPM one can explain taking into account their molecular mechanisms of action. Electrophysiological and neurochemical studies have indicated that TPM possesses multiple diverse mechanisms of action. For instance, the drug: 1) inhibits voltage-sensitive Na⁺ channels [47]; 2) potentiates γ -aminobutyric acid (GABA)-mediated inhibitory neurotransmission through binding to a novel site on the GABA_A-receptor complex [50]; 3) blocks excitatory neurotransmission through a negative modulatory effect on Ca²⁺-permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate subtypes of glutamate receptors [14]; 4) inhibits neuronal L-type high-voltage-activated Ca²⁺ channels [52]; 5) inhibits GABA_A-receptor mediated depolarizing responses in pyramidal neurons in the CA1 region of the rat hippocampus enhancing simultaneously the conductance of some types of K⁺ channels [20]; 6) weakly inhibits the carbonic anhydrase isoenzymes CA II and CA IV, and through the modulation of pH, the drug influences voltage- and receptor-gated ion channels [11], and 7) selectively inhibits GluR5 kainate receptors [19]. Moreover, TPM binds to phosphorylation sites on AMPA/kainate receptors thereby exerting an al-

losteric modulatory effect on channel conductance [1, 40]. In the case of GBP, the exact mechanisms of action of the drug are still unknown; however, several potential mechanisms may account largely for its anticonvulsant activity. Despite its chemical similarity to GABA, GBP does not act directly through GABA_A or GABA_B receptors [21]. At therapeutically relevant concentrations, the drug does not interact with receptors for glutamate, glycine or dopamine [48] and does not affect voltage-gated Na⁺ channels [42]. It has been reported that GBP inhibits Ca²⁺ voltage-gated channels through interaction with the $\alpha_2\delta$ subunit [13]. The drug interacts also with several enzymes of the inextricably linked metabolic pathways of GABA (activates glutamate decarboxylase [GAD] and weakly inhibits GABA transaminase [GABA-T]; increasing GABA concentration) and metabolic pathways of glutamate (slightly activates glutamate dehydrogenase [GDH] and potently inhibits branched-chain amino acid aminotransferase [BCAA-T]; decreasing glutamate concentration) [17]. GBP competes with transport of branched-chain amino acids (L-leucine, L-valine, L-phenylalanine), and thus, some pharmacological properties of GBP may arise from changes in cytosolic concentrations of endogenous amino acids in neurons [46]. Moreover, the drug increases the conductance of hyperpolarization-activated cation currents (I_h) contributing to the protection of neurons against excessive synaptic or intrinsic activity, and stabilizing neuronal network within the hippocampus [44]. More recently, it has been found that GBP selectively activates presynaptic GABA_B heteroreceptors (but not GABA_B autoreceptors) decreasing neurotransmitters' release by reducing Ca²⁺ conductance in neurons of the CNS [37]. Considering the above-mentioned molecular mechanisms of action of the examined AEDs, one can hypothesize that GBP (through the blockade of voltage-gated Ca²⁺ channels) and TPM (by inhibiting Na⁺ and L-type Ca²⁺ channels, blocking AMPA/kainate receptors and enhancing the conductance of K⁺ channels) cooperate and enhance their anticonvulsant activity in the MES test. It is possible that their complementary mechanisms of action are responsible for the observed synergy in the MES test.

Another crucial problem deserves more attention and should be discussed here. It is accepted that synergistic interactions offered by AEDs in combinations allows for the reduction of drug doses comprising the drug mixtures [38]. Undoubtedly, any decrease in

both drug doses in the mixture may considerably reduce or even eliminate undesired side effects, closely associated with two-AED therapy in the clinical setting. Generally, in case of synergistic interaction, both AEDs can be given at reduced doses without any loss of their anticonvulsant efficacy, and thus, the reduced two-drug therapy may substantially ameliorate the patients' quality of lives [10, 38].

Moreover, our results indicated that the combinations of TPM with GBP, administered at various fixed-ratios, produced no acute adverse effects with respect to motor coordination deficits in the chimney test and neuromuscular strength impairment in the grip-strength test in mice. Additionally, no significant changes in total brain concentrations of both AEDs were identified in this study, demonstrating the lack of pharmacokinetic interaction between GBP and TPM. It is worth mentioning that a fixed-ratio of 1:1 was chosen for pharmacokinetic examination because the mixture at this combination consisted of maximal doses of both AEDs (21.6 mg/kg), which corresponded to the ED_{50 mix} value from the MES test. Considering pharmacokinetic profiles of GBP and TPM in humans, any changes in concentrations of these drugs when used in combination are unlikely. It has been reported that GBP neither binds to nor displaces other drugs from plasma proteins [49]; hence, it is impossible that the drug would be able to interact with TPM, whose binding to plasma proteins is minimal (13–17%) [3]. Noticeably, GBP is eliminated by renal excretion as unchanged drug and does not undergo liver metabolic transformation [48], whereas TPM is partly metabolized in the liver to 7 various metabolites and, finally, 40–70% of the drug is excreted in urine intact [3]. An experimental study, using human liver microsomes *in vitro*, has indicated that TPM interacts with the cytochrome P450 isoenzyme CYP2C19 [15], while GBP neither induces nor inhibits any hepatic CYP450 isoforms [49]. In the light of the above-mentioned facts, it is improbable that both AEDs would be able to interact pharmacokinetically in patients with epilepsy. Since no changes in brain AED concentrations were observed in this study, it may be concluded that the investigated interaction between TPM and GBP in the MES test was pharmacodynamic in nature.

Considering thoroughly preclinical profiles of TPM and GBP administered alone, their combinations with other AEDs as well as the synergistic interaction observed in the present study, one can suggest that the

combination of TPM with GBP should also be efficacious in refractory epileptic patients. Generally, the combinations found to be synergistic in the MES test were also synergistic in humans, protecting refractory patients against seizure attacks. More detailed discussion concerning the efficacy of the combined AED therapy in both preclinical and clinical conditions has been presented elsewhere [31, 33, 43].

Summing up, synergistic cooperation of both AEDs in suppressing MES-induced seizures, lack of potentially acute adverse effects and no pharmacokinetic interactions between these AEDs, make the combination of TPM and GBP of a pivotal importance for patients with refractory epilepsy. From a preclinical point of view, this beneficial AED combination deserves more attention and further clinical verification to provide reliable evidence about its efficacy in epileptic patients.

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