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Isobolographic analysis of interaction between drugs with nonparallel dose–response relationship curves: a practical application

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Abstract The objective of this study was to characterize the anticonvulsant and acute adverse-effect potentials of topiramate (TPM) and gabapentin (GBP)—two second-generation antiepileptic drugs administered alone and in combination in the maximal electroshock (MES)-induced seizures and chimney test in mice. The anticonvulsant and acute adverse effects of the combination of TPM with GBP at the fixed ratio of 1:1 were determined using the type I isobolographic analysis for nonparallel dose–response relationship curves (DRRCs). To ascertain any pharmacokinetic contribution to the observed interaction between TPM and GBP, total brain concentrations of both drugs were determined. The isobolographic analysis of interaction for TPM and GBP, whose DRRCs were not parallel in both MES and chimney tests, was accompanied with a presentation of all required calculations allowing the determination of lower and upper lines of additivity. The isobolographic analysis revealed that TPM combined with GBP at the fixed-ratio combination of 1:1 interacted supraadditively (synergistically) in terms of suppression of MES-induced seizures, and simultaneously, the combination produced additive interaction with respect to motor coordination impairment (adverse effects) in the chimney test. The evaluation of pharmacokinetic characteristics of interaction for the combination of TPM with GBP revealed that neither TPM nor GBP affected their total brain concentrations in experimental animals, and thus, the observed interaction in the MES test was pharmacodynamic in nature. In conclusion, the combination of TPM with GBP, because of supraadditivity in the MES test and additivity in terms of motor coordination impairment in the chimney test as well as lack of pharmacokinetic interactions between drugs, fulfilled the criterion of a favorable combination, worthy of recommendation in further clinical practice.

Abbreviations

AED	antiepileptic drug
DRRC	dose–response relationship curve
GBP	gabapentin
MES	maximal electroshock seizure test
TPM	topiramate

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Introduction

Relatively recently, there has appeared a trend to characterize interactions among antiepileptic drugs (AEDs) in preclinical studies using isobolographic analysis (Löscher and Wauquier 1996; Deckers et al. 2000; Leach 2000). Indeed, the isobolographic analysis is a valuable method allowing the precise classification of exact types of interactions among drugs, comprehensively evaluating their nature as: supraadditive (synergistic), subadditive (relatively antagonistic), infraadditive (absolutely antagonistic), indifferent or additive (Berenbaum 1989; Greco et al. 1995; Tallarida 2000; Luszczki and Czuczwar 2003, Luszczki et al. 2003a,b, 2006b). 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 (Berenbaum 1989; Porreca et al. 1990; Tallarida 2000). Moreover, the isobolographic analysis is based on some specific presumptions allowing the exact classification of observed interactions in vivo. One of these presumptions is the parallelism of dose–response relationship curves (DRRCs) for drugs administered alone. The test for parallelism provides certainty that the analyzed drug dose ratios are constant for every investigated effect (Tallarida 2000). Sometimes, the DRRCs for drugs are not parallel, and there appears a problem related to the misinterpretation of obtained results and false classification of interactions. In such cases, one should perform the modified isobolographic analysis for different regression line slopes according to Grabovsky and Tallarida (2004) and Tallarida (2006, 2007).

In experimental studies, topiramate (TPM), a newer second generation AED, has been found to be fully effective in suppressing maximal electroshock (MES)-induced seizures in mice (Shank et al. 1994). In contrast, gabapentin (GBP), another second generation AED, has been classified as virtually ineffective in the mouse MES test (Bartoszyk et al. 1986; Dalby and Nielsen 1997), although GBP at doses of 75 and 100 mg/kg significantly elevated the threshold for electroconvulsions in mice (Luszczki et al. 2003a). 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 (White et al. 2002). These controversial results, concerning the antiseizure effects of GBP in the MES test in mice, prompted me to determine thoroughly the anticonvulsant and acute adverse-effect (neurotoxic) profiles for GBP administered alone.

Accumulating experimental evidence indicates that TPM potentiated the anticonvulsant activity of numerous coadministered AEDs. Previously, it has been shown isobolographically that TPM interacted synergistically with phenobarbital, carbamazepine (Shank et al. 1994), lamotrigine (Luszczki et al. 2003c), levetiracetam (Sills et al. 2004; Luszczki et al. 2006a), felbamate, oxcarbazepine (Luszczki and Czuczwar 2004a), and loreclezole (Luszczki et al. 2005b) in the MES test in mice. Similarly, GBP combined with conventional and some newer AEDs (i.e., phenytoin, carbamazepine, phenobarbital, valproate, lamotrigine, talampanel, and oxcarbazepine) exerted synergistic interactions in isobolography, at various fixed-ratios in the MES test in mice (Borowicz et al. 2002; Luszczki et al. 2005a). Considering the above-mentioned combinations and their synergistic interactions in the MES test, it was expected that the combination of TPM with GBP would also be synergistic in this experimental model of epilepsy. It

is widely accepted that the MES test is considered as an experimental model of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in humans (Löscher et al. 1991).

To characterize the type of interactions between TPM and GBP, the two AEDs whose DRRCs were not parallel in the MES-induced seizures in mice, the type I isobolographic analysis for different regression line slopes was used. Additionally, the adverse-effect profiles for the combinations of AEDs were investigated in relation to motor coordination impairment in the chimney test also by using the type I isobolographic analysis for different (nonparallel) regression line slopes. 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 evaluated.

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 $22\pm1°C$, relative humidity of $55\pm5\%$). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of eight mice. Each mouse was used only once. All tests were performed between 0900 and 1400 hours. 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.: 368/2002/349/2002) 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) in saline and were administered intraperitoneally (i.p.), as two separate injections, in a volume of 5 ml/kg 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 before MES, 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 (Luszczki and Czuczwar 2004a,b). 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.

MES test

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 generator (Rodent Shocker, 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). The protective activities of TPM and GBP administered alone were evaluated and expressed as their median effective doses (ED_{50} values in mg/kg) against MES-induced seizures. The animals were administered different drug doses so as to obtain a variable percentage of protection against MES, allowing the construction of a dose–response relationship curve (DRRC) for every AED administered separately. Moreover, to ascertain the effects of TPM and GBP in combination, the animals were administered with the mixture of different drug doses (at a fixed-ratio combination of 1:1) so as to obtain a variable percentage of protection against MES, allowing the construction of a DRRC for the AED combination. The anticonvulsant activity of the fixed-ratio combination of 1:1 was evaluated and expressed as the $ED_{50\ mix}$ value, corresponding to the total dose of the mixture, necessary to protect 50% of mice against tonic hind limb extension in the MES test. Subsequently, the ED_{50} and $ED_{50\ mix}$ values with their 95% confidence limits were calculated according to log-probit method (Litchfield and Wilcoxon 1949). The experimental procedure has been described in more detail elsewhere (Luszczki and Czuczwar 2003, 2004b; Luszczki et al. 2003b,c).

Chimney test

The effects of TPM and GBP administered alone as well as in combination on motor coordination impairment were quantified by the chimney test of Boissier et al. (1960). In this test, animals had to climb backwards up a transparent plastic tube (3 cm inner diameter, 25 cm length), and motor coordination impairment was indicated by the inability of the animals to climb backward up the tube within 60 s. The acute adverse effects of TPM and GBP, administered alone and in combination, were expressed as their median toxic doses (TD_{50} and $TD_{50\ mix}$ values in mg/kg), representing the doses of AEDs, which impaired motor coordination in 50% of the animals tested (Löscher and Nolting 1991). To evaluate each TD_{50} value, at least four groups of animals (each group consisted of eight mice) injected with various doses of GBP, TPM, or their combination at the fixed ratio of 1:1 were challenged with the chimney test. The DRRC

Table 1 Anticonvulsant effects of TPM and GBP administered singly in the MES-induced seizure model

Drug dose (mg/kg)		A/T	E (%)	DRRC analysis
TPM	30	1/8	12.5	ED_{50}=49.55 (39.6–62.0)
	40	3/8	37.5	S=1.582
	50	4/8	50	f_{ED_50}=1.252
	60	5/8	62.5	n=32
	70	6/8	75	SE=5.67
	80	7/8	87.5	q=2.699
GBP	200	1/8	12.5	ED_{50}=638.5 (450.0–906.1)
	300	2/8	25	S=2.922
	400	3/8	37.5	f_{ED_50}=1.419
	500	3/8	37.5	n=72
	600	4/8	50	SE=113.98
	700	4/8	50	p=1.287
	800	5/8	62.5	
	1,000	5/8	62.5	
	1,200	6/8	75	
	1,400	6/8	75	

q/p=2.097
Test for parallelism
Slope function ratio (S.R.)=1.847
f_{STPM}=1.122
f_{SGBP}=1.236
f ratio S.R.=0.105
S.R.>f ratio S.R., the examined two DRRCs are not parallel (Litchfield and Wilcoxon 1949).

Results are presented as median effective doses (ED_{50} values in milligrams per kilogram, with 95% confidence limits in parentheses) of TPM and GBP, administered singly, against MES-induced seizures in mice. Raw data are presented to allow the calculation of the ED_{50} values by using log-probit method according to Litchfield and Wilcoxon (1949). The AEDs were administered systemically (i.p.), 60 min before the MES test. *A*, number of animals protected against MES-induced hind limb extension; *T*, total number of animals per experimental group; *E*, percentage of animals protected against MES; *DRRC*, dose–response relationship curve; *S*, slope function of the DRRC; f_{ED_50}, factor for ED_{50}; *n*, total number of animals used at doses whose expected anticonvulsant effects ranged between 4 and 6 probits (16 and 84%); *SE*, standard error of ED_{50}; *p* and *q*, curve-fitting parameters; *S.R.*, slope function ratio (S_{GBP}/S_{TPM}); $f_{S\ TPM}$, factor for slope function for TPM; $f_{S\ GBP}$, factor for slope function for GBP; f ratio S.R., factor for slope function ratio.

for each AED was fitted on the basis of the percentage of mice showing motor deficits by means of the log-probit method according to Litchfield and Wilcoxon (1949). This experimental procedure has been described in detail elsewhere (Luszczki et al. 2005a, 2006b). Moreover, the protective index for each AED administered alone was calculated by dividing a given TD_{50} value, evaluated in the chimney test, by the respective ED_{50} value determined in the MES test. The protective index is considered an index of the margin of safety and tolerability between anticon-

vulsant doses and doses of AEDs exerting acute adverse effects (e.g., sedation, ataxia, impairment of motor coordination, or other neurotoxic manifestations) in preclinical studies (Löscher et al. 1991).

Measurement of total brain AED concentrations

The animals were given TPM+ vehicle, GBP+ vehicle, or a combination of TPM and GBP. The fixed-ratio combination for estimating brain concentrations of TPM and GBP was

Fig. 1 Log-probit analysis and dose–response relationship curves for GBP and TPM administered alone in the MES-induced seizures (**a**) and chimney test (**b**) in mice. Doses of *TPM* and *GBP* administered alone were transformed into logarithms, whereas the protective effects offered by the AEDs against MES-induced seizures or motor coordination deficits in animals produced by the AEDs in the chimney test were transformed into probits (Litchfield and Wilcoxon 1949). Linear regression equations of DRRCs are presented on the graph, where *y* is the probit of response and *x* is the logarithm (to the base 10) of drug dose. The *dotted lines* represent on the graph median effective doses (*ED_{50}*) or median toxic doses (*TD_{50}*) of TPM and GBP. The test for parallelism of DRRCs (for TPM and GBP) revealed that *lines* are not parallel (Litchfield and Wilcoxon 1949)
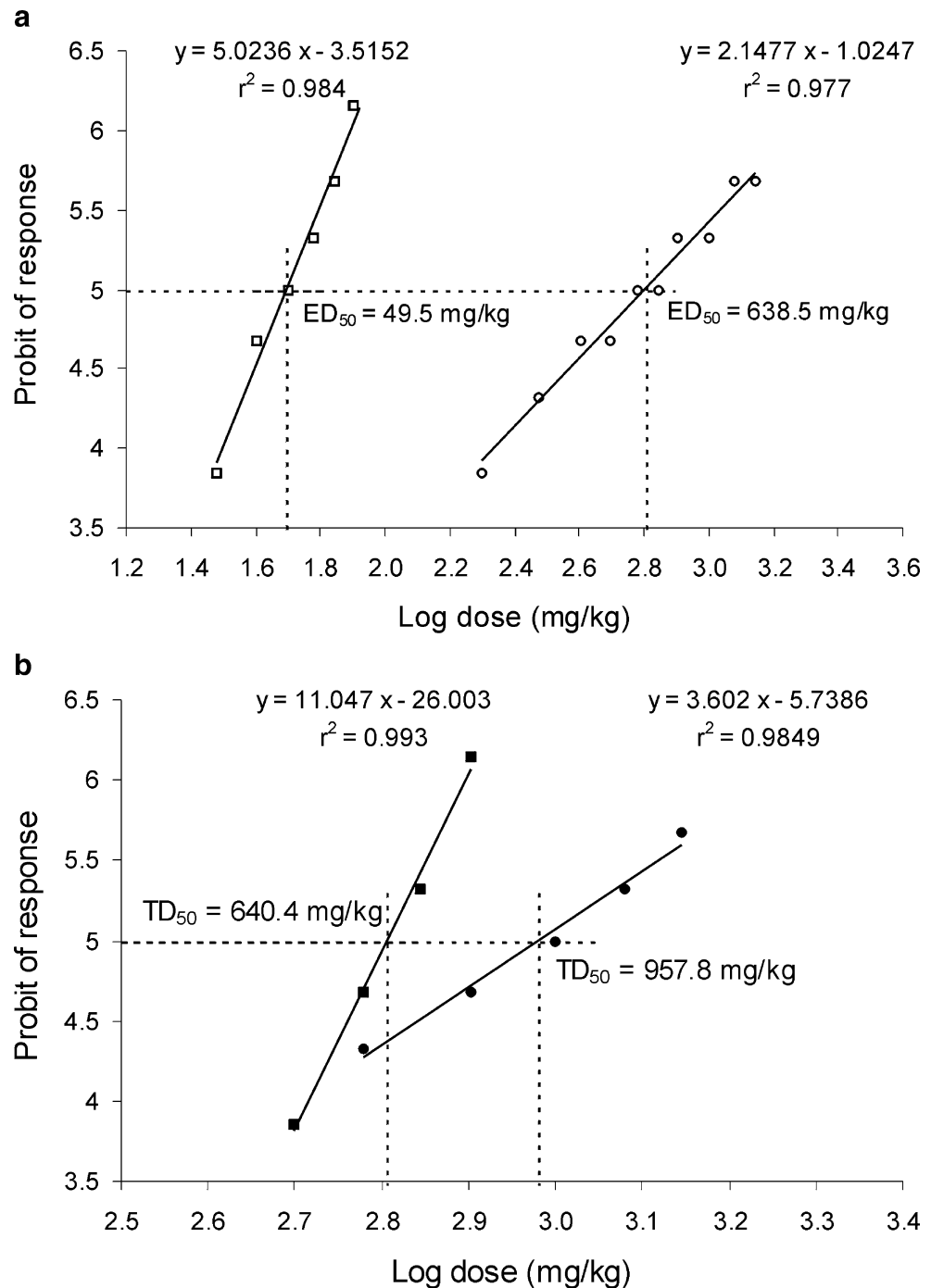
chosen as 1:1 (TPM/GBP) from the MES test. Mice were killed by decapitation at times chosen to coincide with that scheduled for the MES test. The whole brains of the mice were removed from the skulls, weighed, and homogenized using Abbott buffer (2:1 vol/weight) in an Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany). The homogenates were centrifuged at $10,000 \times g$ for 10 min. The supernatant samples (75 µl) containing TPM were analyzed by fluorescence polarization immunoassay using a TDx analyzer and reagents exactly as described by the manufacturer (Seradyn, Indianapolis, IN, USA). Simultaneously, the brain supernatant samples (100 µl) containing GBP were transferred into the high-pressure liquid chromatography (HPLC) technique of drug detection. Details concerning the technique of estimation of GBP concentrations with HPLC have been presented elsewhere (Luszczki et al. 2003a; Luszczki and Czuczwar 2004b). The brain AED concentrations were expressed in micrograms per milliliter of brain supernatants as means±SD of at least eight separate brain preparations.

Isobolographic analysis of interactions

Isobolographic analysis of interactions between GBP and TPM was performed according to the methodology previously detailed (Luszczki et al. 2003a,b, 2006b) and modified according to Grabovsky and Tallarida (2004) and Tallarida (2006, 2007) for drugs with nonparallel DRRCs. The evaluation of ED_{50} and TD_{50} values of AEDs injected alone was performed using log-probit method according to Litchfield and Wilcoxon (1949). Subsequently, based upon these ED_{50} and TD_{50} values, median additive doses of the mixture of GBP with TPM, i.e., doses of the mixture that theoretically should protect 50% of the animals tested against MES-induced seizures ($ED_{50\ add}$) and doses of the mixture that theoretically should impair motor coordination in 50% of the animals tested in the chimney test ($TD_{50\ add}$), were calculated from two equations of additivity presented by Tallarida (2006, 2007). For the lower line of additivity, the equation at a 50% effect is: $y = ED_{50\_TPM} - [ED_{50\_TPM}/(ED_{50\_GBP}/x)^{q/p}]$ for the $ED_{50\ add}$ value, and $y = TD_{50\_TPM} - [TD_{50\_TPM}/(TD_{50\_GBP}/x)^{q/p}]$ for the $TD_{50\ add}$ value, where y is a dose of TPM, x is a dose of GBP, and q and p are curve-fitting parameters (Hill coefficients) for TPM and GBP, respectively. In contrast, for the upper line of additivity, the equation at a 50% effect is: $y = ED_{50\_TPM} [(ED_{50\_GBP} - x)/ED_{50\_GBP}]^{q/p}$ for the $ED_{50\ add}$ value, and $y = TD_{50\_TPM} [(TD_{50\_GBP} - x)/TD_{50\_GBP}]^{q/p}$ for the $TD_{50\ add}$ value. To calculate the curve-fitting parameters (q and p), probits of response for TPM and GBP administered alone were transformed to percent of effect. It is important to note that when two drugs produce maximal effect but are "heterodynamic" (i.e., have nonparallel

DRRCs), the additivity is represented as an area bounded by two defined curves (lower and upper isoboles of additivity). For supraadditivity (synergy), the experimentally derived $ED_{50\ mix}$ or $TD_{50\ mix}$ points are placed below this region bounded by the lower and upper isoboles of additivity, and for subadditivity (antagonism), above the area of additivity (Tallarida 2006, 2007). In isobolography, it is accepted that half the ED_{50}_1 of one drug plus half the ED_{50}_2 of the second drug should be as effective therapeutically as a full dose of either drug administered separately. This concept of adding fractions of the ED_{50}s of drugs is a basic principle of isobolography (Loewe 1953; Berenbaum 1989). Subsequently, proportions of GBP and TPM in the mixture were calculated for the fixed-ratio combination of 1:1, and the mixture of GBP with TPM was administered to animals. The evaluation of the experimen-

Table 2 Acute adverse (neurotoxic) effects of TPM and GBP administered alone in the chimney test

Drug	dose (mg/kg)	I/T	E (%)	DRRC analysis
TPM	500	1/8	12.5	$TD_{50}=640.4$ (554.3–739.9)
	600	3/8	37.5	$S=1.232$
	700	5/8	62.5	$f_{\_TD\_50}=1.155$
	800	7/8	75	$n=16$
				$SE=47.19$
				$q=7.550$
GBP	600	2/8	25	$TD_{50}=957.8$ (723.9–1,267.3)
	800	3/8	37.5	$S=1.895$
	1,000	4/8	50	$f_{\_TD\_50}=1.323$
	1,200	5/8	62.5	$n=40$
	1,400	6/8	75	$SE=136.75$
				$p=2.069$

$q/p=3.649$
Test for parallelism
Slope function ratio (S.R.)=1.538
$f_{STPM}=1.073$
$f_{SGBP}=1.262$
f ratio S.R.=0.105
S.R.>f ratio S.R., the examined two DRRCs are not parallel (Litchfield and Wilcoxon 1949).

Results are presented as median toxic doses of TPM and GBP (TD_{50} values in milligram per kilogram, with 95% confidence limits in parentheses), producing motor coordination deficits in 50% of animals challenged with the chimney test. The TD_{50} values were calculated using log-probit analysis according to Litchfield and Wilcoxon (1949). The AEDs were administered i.p., 60 min before the chimney test. *I*, number of animals showing impairment of motor coordination in the chimney test (i.e., unable to climb backwards up the plastic tube within 60 s); *T*, total number of animals per experimental group; *E*, percentage of animals showing motor coordination impairment; *DRRC*, dose–response relationship curve; *S*, slope function of the DRRC; $f_{\_TD\_50}$, factor for TD_{50}; *n*, total number of animals used at doses whose expected acute neurotoxic effects ranged between 4 and 6 probits; *SE*, standard error of TD_{50}; *p* and *q*, curve-fitting parameters; *S.R.*, slope function ratio (S_{GBP}/S_{TPM}); $f_{S\ TPM}$, factor for slope function for TPM; $f_{S\ GBP}$, factor for slope function for GBP; *f ratio S.R.*, factor for slope function ratio.

Table 3 Isobolographic analysis of interaction between TPM and GBP at the fixed-ratio of 1:1 in the MES-induced seizure model

TPM (mg/kg)	GBP (mg/kg)	Mixture (mg/kg)	A/T	E (%)	DRRC analysis
4.95	63.85	68.80	1/8	12.5	$ED_{50\ mix}$=126.45 (93.81–170.45)
7.43	95.78	103.18	3/8	37.5	S=1.696
9.90	127.70	137.60	4/8	50	f_{ED_50}=1.348
12.38	159.63	172.01	6/8	75	n_{mix}=24
					SE=19.254
$ED_{50\ mix}$ (mg/kg)	n_{mix}	$ED_{50\ add}$[a] (mg/kg)	n_{add}	$ED_{50\ add}$[b] (mg/kg)	n_{add}
126.45±19.25*	24	257.5±81.24	100	430.6±68.46	100

Results are presented as median effective doses (ED_{50}s in milligram per kilogram) for two-drug mixtures, determined either experimentally ($ED_{50\ mix}$) or theoretically calculated ($ED_{50\ add}$) from the equations of additivity (Tallarida 2006, 2007), protecting 50% of the animals against MES-induced seizures. *TPM*, dose of topiramate in the mixture; *GBP*, dose of gabapentin in the mixture; *A*, number of animals protected against MES-induced hind limb extension; *T*, total number of animals per experimental group; *E*, percentage of animals protected against MES; *DRRC*, dose–response relationship curve; *S*, slope function of the DRRC; f_{ED_50}, factor for ED_{50}; *SE*, standard error of $ED_{50\ mix}$; 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 ($n_{add}=n_{\_TPM}+n_{\_GBP}-4$). Statistical evaluation of the data was performed with unpaired Student's *t* test according to Tallarida (2000).
*Significantly different at $P<0.001$ vs. both lower and upper $ED_{50\ add}$ values.
[a] $ED_{50\ add}$ value calculated from the equation for the lower line of additivity
[b] $ED_{50\ add}$ value calculated from the equation for the upper line of additivity.

tally derived $ED_{50\ mix}$ at the fixed ratio of 1:1 was based upon the dose of mixture protecting 50% of animals tested against MES-induced seizures in mice. Similarly, in the chimney test, the $TD_{50\ mix}$ value was isobolographically determined.

To visualize types of the interactions between TPM and GBP, the isobolograms were drawn by plotting the points reflecting the respective doses of GBP on the x-axis and doses of TPM on the y-axis. The lower and upper isoboles of additivity are presented as curves concave downward connecting the ED_{50} or TD_{50} values for GBP and TPM administered alone. The dotted line starting from the point (0,0) corresponds to the fixed ratio of 1:1 for the combination of GBP with TPM. The points A′ and A″ depict the theoretically calculated $ED_{50\ add}$ or $TD_{50\ add}$ values for both lower and upper isoboles of additivity. The point A represents the experimentally derived $ED_{50\ mix}$ (or $TD_{50\ mix}$) value for total dose of the mixture expressed as proportions of GBP and TPM that produced 50% anticonvulsant effects (or 50% impairment of motor coordination) in mice.

Statistics

The ED_{50} and TD_{50} values for TPM and GBP administered alone (with their 95% confidence limits) were calculated by computer log-probit analysis according to Litchfield and Wilcoxon (1949). The respective 95% confidence limits were transformed into standard errors (SE) as described previously (Luszczki et al. 2003b, 2006b). Simultaneously, the test for parallelism of DRRCs of TPM and GBP was performed as an indispensable condition for testing AED interactions with

isobolography, according to the method presented by Litchfield and Wilcoxon (1949). For more details, see Appendix in Luszczki and Czuczwar (2006). The experimentally derived $ED_{50\ mix}$ and $TD_{50\ mix}$ values were statistically compared to their respective $ED_{50\ add}$ and $TD_{50\ add}$ values using unpaired Student's *t* test (Porreca et al. 1990). The SE for additive $ED_{50\ add}$ and $TD_{50\ add}$ values were calculated in approximation according to Tallarida (2006), whereas SE for experimentally derived $ED_{50\ mix}$ and $TD_{50\ mix}$ values were denoted directly from the log-probit analysis. Total brain AED concentrations were statistically analyzed using the unpaired Student's *t* test.

Software used

Microsoft's Excel spreadsheet was used to perform the required calculations and to graph the results in the form of DRRCs and isobolograms. This spreadsheet was programmed to compute all calculations automatically and determine the lower and upper isoboles of additivity. The $ED_{50\ add}$ and $TD_{50\ add}$ values with their SE (for the combination of TPM with GBP at the fixed ratio of 1:1) were also denoted in this program.

Results

Effects of GBP and TPM administered alone on the MES-induced seizures

The anticonvulsant effects of GBP, expressed as the percentage of protection against MES-induced tonic hind limb extension in mice, were ranged between 12.5% for
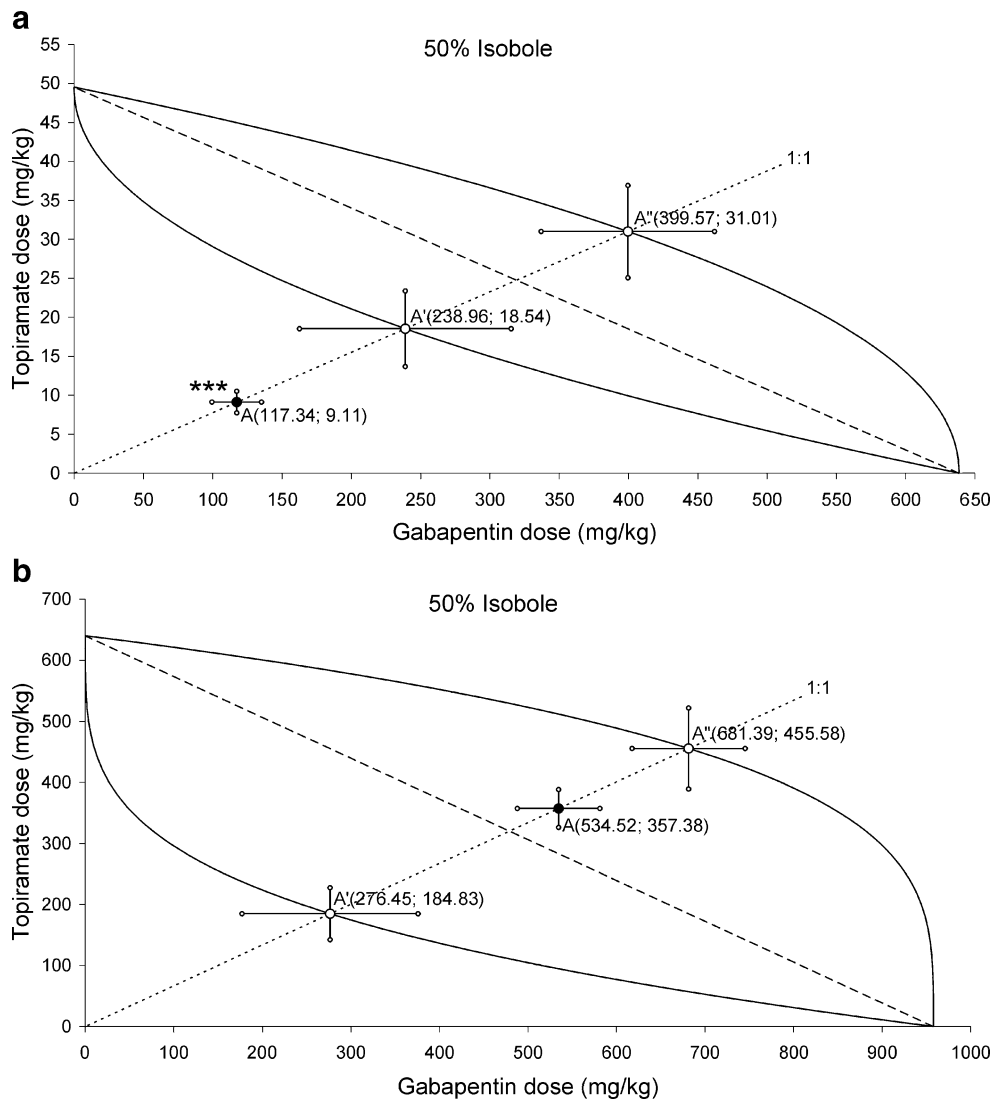
Fig. 2 Isobolograms showing interactions between GBP and TPM against MES-induced seizures (**a**) and chimney test (**b**) in mice. The median effective doses (ED_{50}) and median toxic doses (TD_{50}) for GBP and TPM are shown plotted graphically on the x- and y-axes, respectively. The lower and upper isoboles of additivity represent the *curves* connecting the ED_{50} or TD_{50} values for GBP and TPM administered alone. The *dotted line* starting from the point (0,0) corresponds to the fixed-ratio of 1:1 for the combination of GBP with TPM. The points *A'* and *A''* depict the theoretically calculated $ED_{50\,add}$ or $TD_{50\,add}$ values for both lower and upper isoboles of additivity. The point *A* represents the experimentally derived $ED_{50\,mix}$ or $TD_{50\,mix}$ value for total dose of the mixture expressed as proportions of GBP and TPM that produced 50% anticonvulsant effects in the MES test in mice or impairment of motor coordination in 50% of the animals in the chimney test. The sum of *x* and *y* coordinates, for each point placed on the isobologram (*A*, *A'*, *A''*), corresponds to the respective ED_{50} or TD_{50} values. On the graph, the SE values are presented as *horizontal* and *vertical error bars* for every ED_{50} or TD_{50} value. **a** The $ED_{50\,mix}$ value is placed significantly below the area of additivity bounded by two isoboles of additivity indicating synergistic interactions between GBP and TPM in the MES test in mice ([*triple asterisk*]$P<0.001$, Student's *t* test). **b** The experimentally derived $TD_{50\,mix}$ is placed into the area of additivity suggesting the additive interaction between TPM and GBP in the chimney test in mice

GBP at 200 mg/kg and 75% for GBP at 1,400 mg/kg (Table 1). The log-probit analysis of DRRC for GBP allowed the calculation of its ED_{50} value, which was 638.5 (450.0–906.1) mg/kg (Table 1; Fig. 1a). In contrast, TPM exerted a clear-cut anticonvulsant effect in the MES test, and its ED_{50} value, denoted from the log-probit method, was 49.55 (39.6–62.0) mg/kg (Table 1; Fig. 1a). In this case, TPM at 30 mg/kg produced a 12.5% protection against MES-induced seizures, whereas the drug at 80 mg/kg offered an 87.5% anticonvulsant effect in the MES test (Table 1).

Effects of GBP and TPM administered alone on motor coordination impairment in the chimney test

GBP produced clear-cut acute adverse (neurotoxic) effects with respect to motor coordination impairment in the chimney test, and its TD_{50} value denoted experimentally

Table 4 Isobolographic analysis of interaction between TPM and GBP at the fixed-ratio of 1:1 in the chimney test in mice

TPM (mg/kg)	GBP (mg/kg)	Mixture (mg/kg)	I/T	E (%)	DRRC analysis
256.2	383.1	639.3	1/8	12.5	$TD_{50\ mix}$=891.9 (761.8–1,058.2)
320.2	478.9	799.1	3/8	37.5	S=1.353
384.2	574.7	958.9	5/8	62.5	$f_{\_TD\_50}$=1.186
448.3	670.5	1,118.8	6/8	75	n_{mix}=24
					SE=77.750
$TD_{50\ mix}$ (mg/kg)	n_{mix}	$TD_{50\ add}$[a] (mg/kg)	n_{add}	$TD_{50\ add}$[b] (mg/kg)	n_{add}
891.9±77.75	24	461.3±142.09	52	1,137.0±130.32	52

Results are presented as median toxic doses (TD_{50}s in milligram per kilogram) for two-drug mixtures, determined either experimentally ($TD_{50\ mix}$) or theoretically calculated ($TD_{50\ add}$) from the equations of additivity, impairing motor coordination in 50% of the animals subjected to the chimney test. I, number of animals showing impairment of motor coordination in the chimney test (i.e., unable to climb backwards up the plastic tube within 60 s); T, total number of animals per experimental group. Statistical evaluation of the data was performed with unpaired Student's t test according to Tallarida (2000).

[a] $ED_{50\ add}$ value calculated from the equation for the lower line of additivity

[b] $ED_{50\ add}$ value calculated from the equation for the upper line of additivity

was 957.8 (723.9–1,267.3) mg/kg (Fig. 1b). Noteworthy, GBP at 600 mg/kg produced a 25% impairment of motor coordination in mice, whereas the drug at 1,400 mg/kg exerted a 75% motor deficit in mice challenged with the chimney test (Table 2). In the case of TPM, the drug produced motor coordination deficits in mice at doses ranged between 500 mg/kg (12.5% impairment) and 800 mg/kg (75% impairment); hence, the log-probit analysis of DRRC for TPM allowed the calculation of its TD_{50} value, which was 640.4 (554.3–739.9) mg/kg (Table 2; Fig. 1b). The protective index (as a ratio of TD_{50} and ED_{50} values) for GBP was 1.50, whereas that for TPM was 12.94.

Brain AED concentrations

Total brain AED concentrations were evaluated for TPM and GBP coadministered at the fixed ratio of 1:1 from the MES test. The brain concentrations of GBP, administered singly at 117.4 mg/kg, were 20.95±4.01 μg/ml and did not differ significantly from those evaluated for the mixture of GBP (117.4 mg/kg) with TPM (9.1 mg/kg), which were 23.08±3.85 μg/ml. Similarly, GBP (117.4 mg/kg) coadministered with TPM (9.1 mg/kg) did not affect the brain TPM concentrations. In this case, the brain concentrations of TPM (injected singly at 9.1 mg/kg) were 2.01±0.28 μg/ml, whereas those for the two-drug mixture at the fixed ratio of 1:1 amounted to 2.11±0.26 μg/ml.

Isobolographic assessment of interactions between GBP and TPM

The mixture of GBP and TPM at the fixed ratio of 1:1 exerted supraadditive (synergistic) interaction in the MES test in mice. The $ED_{50\ mix}$ for this fixed-ratio combination was 126.45 mg/kg, whereas the corresponding $ED_{50\ add}$

values were 257.5 mg/kg (for the lower $ED_{50\ add}$) and 430.6 mg/kg (for the upper $ED_{50\ add}$; Table 3). In this case, the $ED_{50\ mix}$ (i.e., protecting a 50% of animals against MES-induced seizures) was substantially reduced by 51% as compared to the theoretically presumed lower $ED_{50\ add}$ (at $P<0.001$; Table 3, Fig. 2a). In contrast, GBP combined with TPM at the fixed ratio of 1:1 exerted additive interaction in the chimney test in mice. The $TD_{50\ mix}$ for the mixture of GBP+TPM at the fixed ratio of 1:1 was 891.9 mg/kg, whereas the $TD_{50\ add}$ values amounted to 461.3 mg/kg (for the lower $TD_{50\ add}$) and 1,137.0 mg/kg (for the upper $TD_{50\ add}$; Table 4, Fig. 2b).

Discussion

The objective of this study was to characterize the anticonvulsant and acute adverse effect (neurotoxic) profiles as well as to determine the exact types of interactions between GBP and TPM in the MES and chimney tests in mice using the type I isobolographic analysis. Because TPM and GBP did not have their DRRCs parallel in both the MES and chimney tests (Fig. 1a,b), the modified isobolographic analysis based on different regression line slopes was used in this study (Tallarida 2006, 2007).

The experimentally derived ED_{50} value of GBP (administered separately, i.p., 60 min before the MES test) was 638.5 mg/kg, and markedly differed from that denoted by White et al. (2002), which was 78.2 mg/kg. At present, it is difficult to explain this discrepancy in the determination of ED_{50} value in the MES test in mice after systemic (i.p.) administration of GBP. The results of this study are partly in agreement with those documented by Dalby and Nielsen (1997), who had reported the lack of the antiseizure activity of GBP up to 300 mg/kg in the MES test. Moreover, it has been previously documented that GBP at 75 and 100 mg/kg

considerably elevated the threshold for electroconvulsions in mice from 7.2 mA (control) to 9.0 mA (GBP at 75 mg/kg) and 9.7 mA (GBP at 100 mg/kg), respectively (Luszczki et al. 2003a). In light of the above-mentioned facts, the ED_{50} value of GBP denoted by White et al. (2002) seems to be incidentally low.

Results presented herein indicate clearly that GBP interacted supraadditively (synergistically) with TPM in terms of suppression of MES-induced seizures in mice. The observed interaction between TPM and GBP against MES-induced seizures is generally in agreement with our previous findings showing that GBP interacts synergistically with a number of conventional and newer AEDs in the MES test in mice (Borowicz et al. 2002; Luszczki et al. 2003a, 2005a). It is worth mentioning that previously performed studies were based on type II isobolographic analysis because GBP was considered virtually ineffective in the MES test. However, as reported herein, it was possible to determine its ED_{50} value against MES-induced seizures. Therefore, the present study was based on the type I isobolographic analysis for nonparallel DRRCs. Moreover, with type I isobolographic analysis for different regression line slopes, it was found that both AEDs exert the additive interaction with respect to their acute adverse effect potentials (motor coordination impairment) in the chimney test. 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 in the MES test. Calculations of protective index values for the AEDs administered alone indicated that there was a narrow range between the doses of GBP offering anticonvulsant protection in the MES test and those producing motor coordination impairment in animals. In the case of TPM, its TD_{50} value (assessed in the chimney test) considerably differed from the ED_{50} value of the drug in the MES test, and thus, TPM displays a wide gap between the anticonvulsant doses and those producing motor coordination deficits in mice. From a preclinical point of view, the AEDs should have their protective index values above 5, which guarantee their safety and tolerability in the clinical setting (Löscher et al. 1991). Nevertheless, the classification of AEDs' efficacy, based on their protective index values in preclinical studies, is not a unique criterion for the clinical application of AEDs.

Another crucial problem deserves more attention and should be discussed here. It is accepted that synergistic interactions offered by AEDs in combinations allow the reduction of drug doses comprised their mixtures (Schmidt 1996). 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 (Perucca 1995). Generally, in the case of synergistic interaction, both AEDs can be given in reduced doses without any loss of their anticonvulsant efficacy, and thus, this modified two-drug therapy may substantially ameliorate the patients' quality of lives by reducing unwanted adverse effects (Perucca 1995; Deckers et al. 2000).

Considering thoroughly preclinical profiles of TPM and GBP administered alone and the synergistic interaction observed in the present study, one could suppose that the combination of TPM with GBP should also be efficacious in epileptic patients refractory to the monotherapeutic use of these AEDs. 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 combined AED therapy in both preclinical and clinical conditions has been presented elsewhere (Stephen and Brodie 2002; Luszczki and Czuczwar 2004a; Luszczki et al. 2005b).

Summing up, synergistic cooperation of both AEDs in suppressing MES-induced seizures, additive interaction with respect to motor coordination impairment in the chimney test, and lack of pharmacokinetic interactions between TPM and GBP make the combination of these AEDs of pivotal importance for patients refractory to the monotherapeutic use of both AEDs. From a preclinical point of view, this profitable AED combination deserves more attention and further clinical verification to provide reliable evidence about its efficacy in epileptic patients. This study, for the first time, describes a practical application of isobolographic analysis of interaction for drugs with different (nonparallel) regression line slopes.

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