Naunyn-Schmiedeberg's Arch Pharmacol (2006) 374:51–64
DOI 10.1007/s00210-006-0100-3

ORIGINAL ARTICLE

Biphasic characteristic of interactions between stiripentol and carbamazepine in the mouse maximal electroshock-induced seizure model: a three-dimensional isobolographic analysis

Jarogniew J. Luszczki · Stanislaw J. Czuczwar

Received: 13 June 2006 / Accepted: 17 July 2006 / Published online: 14 September 2006

Abstract The anticonvulsant effects produced by stiripentol (STP), carbamazepine (CBZ), and their combination in the maximal electroshock (MES)-induced seizures in mice were investigated using three-dimensional (3D) isobolographic analysis. With 3D isobolography, the combinations of both drugs at the fixed-ratios of 1:3, 1:1, and 3:1 for 16%, 50% and 84% antiseizure effects, respectively, were examined in order to evaluate the preclinical characteristics of the interactions between STP and CBZ. Additionally, to characterize precisely the types of interactions observed in the MES test, free plasma and total brain CBZ concentrations were estimated for all fixed-ratios tested.

The 3D isobolographic analysis showed that STP and CBZ combined at the fixed-ratio of 1:3 produced supra-additive (synergistic) interactions in the MES test for the anticonvulsant effects ranging between 16% and 84%. In contrast, the combination of STP with CBZ at the fixed-ratio of 3:1 exerted sub-additive (antagonistic) interactions in 3D isobolography for all antiseizure effects examined in the MES test. Only the combination of STP and CBZ at the fixed-ratio of 1:1 was additive for the investigated effects (16%, 50% and 84%) in 3D isobolography. Pharmacokinetic evaluation of CBZ concentrations revealed that STP increased both free plasma and total brain CBZ concentrations for all fixed-ratio combinations tested (1:3, 1:1 and 3:1).

In conclusion, the 3D isobolographic findings suggest that the combination of STP with CBZ exerted biphasic characteristics of interactions in the MES test, despite the pharmacokinetic increase in CBZ content in plasma and brains of experimental animals.

Keywords Three-dimensional isobolographic analysis · Stiripentol · Carbamazepine · Electroshock maximal · Pharmacokinetic interaction

Abbreviations

3D	three-dimensional
AED	antiepileptic drug
CBZ	carbamazepine
MES	maximal electroshock seizure test
STP	stiripentol

Introduction

From a pharmacological point of view, certain combinations of two fully-active drugs evoke interactions whose nature may be pharmacodynamic, pharmacokinetic or mixed. Therefore, a detailed knowledge of the potential interactions among drugs in vivo is still a challenging issue for researchers today. The progress in this field requires a meticulous examination of the type and strength of interactions in preclinical studies on animals.

Epilepsy is one of the central nervous system disorders that sometimes requires a combined therapy, especially for patients with refractory seizures inadequately controlled with monotherapy. The combined therapy with two or more antiepileptic drugs (AEDs) for patients with refractory epilepsy is rationally preselected based on both theoretical considerations about the mechanisms of action of combined AEDs and the observed effects exerted by these drugs in preclinical studies. Generally, the AED combinations,

J. J. Luszczki (✉) · S. J. Czuczwar
Department of Pathophysiology, Medical University of Lublin,
Jaczewskiego 8,
20-090 Lublin, Poland
e-mail: jarogniew.luszczki@am.lublin.pl

exerting supra-additivity (synergy) with respect to the anticonvulsant activity and producing minimal or no side effects in animals, are considered as beneficial and may be recommended to clinical practice (Perucca 1995; Perucca and Levy 2002; Deckers et al. 2000).

To date, several methods analyzing the effects produced by a combined treatment with two or more drugs in preclinical experiments have been published (for review see Berenbaum 1989; Greco et al. 1995). Of these methods, the most commonly used has been an isobolographic analysis proposed by Loewe (1953). This principal method has been modified by Tallarida (1992), who had evaluated pharmacological characteristics of interactions among drugs co-administered in several varying fixed-ratio combinations. Theoretically, one can distinguish five types of interactions with isobolography: additivity, supra-additivity (synergy), indifference, sub-additivity (relative antagonism), and infra-additivity (absolute antagonism) (Loewe 1953; Berenbaum 1989; Tallarida et al. 1999).

Generally, when two drugs are combined and investigated in preclinical studies, three variables always exist: the doses of each drug and their resultant biological effect. In conventional (two-dimensional; 2D) isobolographic analysis, one variable must always be constant, so 2D isobolography is usually performed for median (50%) effects (Berenbaum 1989; Greco et al. 1995). However, with three-dimensional (3D) isobolographic analysis, one can detect and identify any subtle changes in the existing relationship between administered doses of two drugs and their resultant pharmacological effects (Berenbaum 1989; Greco et al. 1995). Relatively recently, a trend for investigating two-drug combinations and their interactions at several various effect levels with 3D response-surface analysis has appeared (Prichard et al. 1991, 1993; Kanzawa et al. 1997; Tallarida et al. 1999; Tallarida 2001, 2006). For epilepsy research studies, this modern approach (3D isobolographic analysis) is of pivotal importance allowing the determination of existing relationship between AEDs. Undoubtedly, the 3D isobolography might contribute to the preselection of AED combinations whose synergistic anticonvulsant effects in animals would be recommended and further adopted to rational bi-therapy in patients refractory to monotherapy.

In the present study, the anticonvulsant effects produced by stiripentol (STP {4,4-dimethyl-1-[3,4-(methylenedioxy)-phenyl]-1-penten-3-ol} - a novel AED), carbamazepine (CBZ, a conventional AED), and their combination at three fixed-ratios of 1:3, 1:1 and 3:1 were determined in maximal electroshock (MES)-induced seizures in mice using 3D isobolographic analysis. The rationale for testing the combination of STP with CBZ in the MES test in mice was based on previous clinical studies showing the efficacy of both AEDs in add-on therapy in patients with refractory partial epilepsy (Loiseau et al. 1990; Renard et al. 1993; Tran et al. 1996; Perez et al. 1999). Generally, it is accepted that the MES test in rodents is considered as an experimental model of tonic-clonic seizures and, to a certain extent, of partial convulsions in humans (Löscher et al. 1991; White et al. 2002). In the clinical settings, CBZ is effective in epileptic patients with partial and tonic-clonic seizures in humans (Brodie and Schachter 2001), whereas STP suppresses partial seizures and severe myoclonic epilepsy in infants (Chiron et al. 2000). Therefore, it was appropriate to use the MES test to evaluate the antiseizure effects produced by the combination of STP with CBZ in mice. Additionally, free plasma and total brain CBZ concentrations were estimated in this study to ascertain whether the observed effects result from a pharmacodynamic and/or a pharmacokinetic interaction.

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, temperature of 21±1°C, relative humidity of 55±3%). 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 to minimize confounding effects of circadian rhythms. Procedures involving animals and their care were conducted in conformity with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and Polish legislation on animal experimentation. 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 above were conformed to the Guide for the Care and Use of Laboratory Animals and approved by the Local Ethics Committee at the Medical University of Lublin (License no. 420/2003/446/2003).

Drugs

In this study: STP (a kind gift from Dr. Jean Vincent, Biocodex Laboratoires, Gentilly, France) and CBZ (a kind gift from Polfa, Starogard Gdanski, Poland) were suspended in a 1% aqueous solution of Tween 80 (Sigma, St. Louis, Mo., USA) and administered intraperitoneally (i.p.), as two separate injections, in a volume of 5 ml/kg body

weight. Fresh drug solutions were prepared on each day of experimentation and administered: STP at 60 min and CBZ at 30 min before the electroconvulsions and brain sampling for the measurement of AED concentrations. The route of i.p. administration and pre-treatment time of the AEDs were based on information about their biological activity from the literature and our previous experiments (Luszczki et al. 2003a,b). The time to the peak of maximum anticonvulsant effects for STP and CBZ was used as the reference time in pharmacokinetic estimation of free (non-protein bound) plasma and total brain CBZ concentrations.

Maximal electroshock seizure (MES) test

Electroconvulsions were produced by means of an alternating current (0.2 s stimulus duration, fixed current intensity of 25 mA, maximum stimulation voltage of 500 V, current frequency of 50 Hz) delivered via ear-clip electrodes by a generator (Rodent Shocker, Type 221; Hugo Sachs, Freiburg, Germany). The criterion for the occurrence of seizure activity was the tonic hindlimb extension (i.e., the hind limbs of animals outstretched 180° to the plane of the body axis). The protective activities of STP and CBZ were evaluated as their effective doses (ED_{16}, ED_{50}, and ED_{84} in mg/kg) against MES-induced seizures. Different drug doses were administered in order to obtain a variable percentage of protection of animals against MES, allowing the construction of a dose-response relationship curve (DRRC) for each AED administered alone, according to Litchfield and Wilcoxon (1949). Subsequently, the ED_{16}, ED_{50}, and ED_{84} values with their 95% confidence limits were calculated. Each of the ED_{16}, ED_{50} and ED_{84} values represents a drug dose required to protect 16%, 50%, and 84% of animals against MES, respectively. Similarly, the anticonvulsant activity of the mixture of STP and CBZ was evaluated and expressed as $ED_{16\ mix}$, $ED_{50\ mix}$, and $ED_{84\ mix}$, corresponding to doses of the mixture of two component drugs necessary to protect 16%, 50%, and 84% of animals against tonic hindlimb extension in the MES test. This test has been described in more detail in our earlier studies (Luszczki and Czuczwar 2003, 2004a,b).

Measurement of free (non-protein bound) plasma and total brain CBZ concentrations

The measurement of free plasma and total brain concentrations of CBZ after co-administration of STP was undertaken at doses of both AEDs, which corresponded to their $ED_{50\ mix}$ values for all fixed-ratio combinations (1:3, 1:1 and 3:1) in the MES-induced seizures. Mice were killed by decapitation at the time chosen to coincide with that scheduled for the MES test, and samples of blood of approximately 1 ml were collected into heparinized Eppendorf tubes. Simultaneously, the whole brains of mice were harvested, weighed, and homogenized in distilled water (2:1 vol/weight) in an Ultra-Turrax T8 homogenizer (Staufen, Germany). The homogenates were centrifuged at 10,000 *g* for 10 min. Blood samples were centrifuged at 5,000 *g* for 5 min and plasma (300 μl) were transferred into an Amicon Centrifree Micropartition System (MPS-1; Amicon, Danvers, USA) for the separation of free (non-protein bound) CBZ concentrations from those that are protein bound. The MPS-1 tubes were centrifuged at 5,000 *g* at 25°C for 10 min and the filtrate samples (70 μl) or the supernatant samples (70 μl) containing CBZ were analyzed by fluorescence polarization immunoassay (FPIA) using a TDx analyzer and reagents exactly as described by the manufacturer (Abbott Laboratories, North Chicago, Ill., USA). The free plasma and total brain CBZ concentrations were expressed in μg/ml of plasma or brain supernatants as means±SD of at least 8 determinations (separate plasma and brain preparations). Statistical evaluation of data was performed with the unpaired Student's *t*-test.

Isobolographic analysis of interactions

Isobolographic analysis of interactions between STP and CBZ was performed according to the methodology previously detailed in our earlier studies (Luszczki et al. 2003a–c, 2006; Luszczki and Czuczwar 2003, 2004a,b, 2005). In the present study, 3D isobolographic analysis comprised 5 basic stages as follows:

1. Evaluation of the anticonvulsant effects of STP and CBZ followed by the determination of their dose-response relationship curves (DRRCs) by means of log-probit linear regression analysis according to Litchfield and Wilcoxon (1949). Generally, linear log-probit analysis yields DRRC with parameter values for median effective dose (ED_{50}), slope function (S), equation of DRRC and coefficient of determination (r^2). The effective doses of STP and CBZ [ED_xs (x=16, 50 or 84) with their 95% confidence limits or SE] were directly calculated from the respective DRRC equations according to Litchfield and Wilcoxon (1949). It is worth of note that the examined DRRCs for AEDs administered alone have to be parallel in 3D isobolographic analysis and therefore the test for parallelism was performed in this study (see Appendix). It is important to note that log-probit transformation of AED doses and their resultant anticonvulsant effects reduces some problems associated with (a)symmetry of non-

linear DRRCs for drugs administered alone. Relatively recently, there has appeared a trend to fit all experimentally derived non-linear data to both symmetrical and asymmetrical DRRC models so as to chose the best-fit equation describing the existing relationship between drugs and their biological effects (Van der Graaf and Schoemaker 1999; Giraldo et al. 2002).

2. Theoretical choice of three fixed drug dose ratio combinations of the examined AEDs (1:3, 1:1 and 3:1) associated with calculations of the additive effective doses ($ED_{x\ add}$s) with their SE. The $ED_{x\ add}$ represents a total additive dose of the mixture, theoretically providing an x-% (x=16, 50 or 84) effect, i.e., protection of animals against MES-induced seizures. The additive doses in the mixture of STP and CBZ ($ED_{x\ add}$s) were calculated from the general equation of additivity presented by Loewe (1953), as follows: $a/A+b/B=1$; where a and b are doses of STP and CBZ, co-administered in the mixture that exerted a desired effect (an initially established reference point). A and B are doses of the drugs administered separately, which also exerted the same desired effect (ED_{16}, ED_{50} or ED_{84}). In isobolography, it is widely accepted that half the ED_x of one drug added to half the ED_x of another drug should be as theoretically effective as one ED_x of either drugs administered singly (where x is an effect ranging between 16% and 84%). This basic tenet of isobolography is, however, closely related to linear dose-response analysis for drugs administered alone.
3. Experimental determination of the effective doses ($ED_{x\ mix}$s) with their SE for the respective fixed-ratio AED combinations (1:3, 1:1 and 3:1). The $ED_{x\ mix}$ is an experimentally determined total dose of the mixture of two component drugs, at the respective fixed-ratio combination, sufficient for an x-% (x=16, 50 and 84) protective effect against MES-induced seizures. The experimentally-derived $ED_{x\ mix}$ values (with their 95% confidence limits) were calculated from the respective DRRC equations of combined drug mixtures according to Litchfield and Wilcoxon (1949) and, subsequently, their 95% confidence limits were transformed to SE, according to the method previously presented in our studies (Luszczki et al. 2003a, 2006).
4. Statistical comparison of the experimentally-derived $ED_{x\ mix}$s with their corresponding theoretical additive $ED_{x\ add}$s by the use of unpaired Student's t-test, according to Porreca et al. (1990) and Tallarida (2000).
5. Graphical illustration of the examined interactions as 2D and 3D isobolograms. To display types of interactions and determine their strength, 2D isobolograms were drawn by plotting the points reflecting the respective ED_x (x=16, 50, and 84) of STP on the X-axis and these of CBZ on Y-axis. The straight line, connecting ED_x values for these drugs, represents the theoretical isobole for an additive effect. If the experimentally-derived data points are placed on this line, the two-drug mixture exerts purely additive interaction (Loewe 1953). When the experimentally-derived points reflecting combinations of various fixed-ratios fall significantly below this line, the two component drugs act synergistically. Conversely, antagonism may be recognized if these points are localized above the additive isobole. In case of 3D isobologram, doses of STP and CBZ are plotted graphically on X- and Y-axes, whereas their resultant anticonvulsant effects on Z-axis. The convex curve on the graph represents supra-additive (synergistic) interaction between STP and CBZ. The concave curve reflects sub-additive (antagonistic) interaction observed between the AEDs in the MES test.

To simplify the notation and nomenclature of interactions in isobolography, the drug doses were administered at the fixed-ratio combinations (e.g., 1:3, 1:1, and 3:1). The fixed drug dose ratios are usually presented in form of natural numbers (1:3, 1:1, 3:1) and they reflect fractions of ED_{50} values denoted for the drugs used separately. For instance, the mixture at the fixed-ratio of 1:3 is consisted of ¼ of the ED_{50} of the first drug and ¾ of the ED_{50} of the second drug. Thus, the isobolographic notation of fixed-ratio combinations contains only numerators of fractions of ED_{50} values for AEDs used in the mixture. In the present study, the ED_{50} values for STP and CBZ administered alone in the MES test were 277.7 mg/kg and 11.5 mg/kg, respectively (Table 1). Hence, the mixture of STP with CBZ at the fixed-ratio of 1:3 was composed of STP at (¼ of 277.7 mg/kg=69.43 mg/kg) and CBZ (¾ of 11.5 mg/kg= 8.63 mg/kg). In this two-drug mixture, CBZ prevailed over STP with respect to its pharmacological activity against maximal electroconvulsions, but it did not exceed quantitatively in the mixture (Table 3). Analogously, the two-drug mixture for the combination of 1:1 in the MES test consisted of STP (½ of 277.7 mg/kg=138.85 mg/kg) and CBZ (½ of 11.5 mg/kg=5.75 mg/kg), where the drugs were combined in equi-effective (iso-effective) doses (Table 3). Likewise, the fixed-ratio combination of 3:1 was a simple notation of the mixture composed of STP (¾ of 277.7 mg/kg=208.28 mg/kg) and CBZ (¼ of 11.5 mg/kg=2.88 mg/kg). In this case, there is no doubt that STP prevailed over CBZ in the mixture (Table 3). All the above-mentioned drug doses for the respective fixed-ratio combinations were primarily considered as additive because they were directly calculated from the equation of additivity presented by Loewe (1953).

Table 1 The anticonvulsant activity of stiripentol (STP) and carbamazepine (CBZ) in the maximal electroshock-induced seizures in mice

Drug	P/T		DRR analysis
STP (mg/kg)			
225	0/8	ED_{50}=277.7 (254.9−302.5)	y=15.204x−32.151
250	2/8	S=1.164	r^2=0.969
275	4/8	f=1.089	*df*=2
300	5/8	*n*=24	χ^2_E=0.930
325	7/8	SE=12.125	χ^2_T=5.991
350	8/8	$f_{\_S}$=1.056	*F*=62.173
CBZ (mg/kg)			
8	0/8	ED_{50}=11.5 (10.1−13.1)	y=12.368x−8.138
10	2/8	S=1.205	r^2=0.961
12	4/8	f=1.138	*df*=1
14	7/8	*n*=16	χ^2_E =1.641
16	8/8	SE=0.759	χ^2_T=3.841
		$f_{\_S}$=1.082	*F*=24.917

PR ($ED_{50\ STP}/ED_{50\ CBZ}$)=24.056

Test for parallelism: SR (S_{CBZ}/S_{STP})=1.035; $f_{\_ratio\_SR}$=1.100

Since SR< $f_{\_ratio\_SR}$, the examined two DRR lines are parallel (Litchfield and Wilcoxon 1949)

Raw data for each AED administered alone allow the calculation of their dose-response relationship (DRR) lines. *P* number of animals protected against electroconvulsions; *T* total number of animals challenged with the MES test.

ED_{50}-median effective dose of an AED protecting 50% of animals tested, calculated according to log-probit method by Litchfield and Wilcoxon (1949); S slope function of DRR line; f-factor for ED_{50}; *n* total number of animals used between 4 and 6 probits of expected anticonvulsant effects; SE standard error of ED_{50}; $f_{\_S}$ factor for slope function; y effect in probits; x logarithm of a drug dose to the base 10; r^2 coefficient of determination for DRR line; *df* degrees of freedom from DRR analysis (i.e., number of analyzed points-2); χ^2_E value of χ^2 (Chi-square) goodness-of-fit test, determined experimentally by means of DRR analysis; χ^2_T tabular χ^2 value for the respective *df*, according to Glantz and Slinker (2001); *F* value of *F*-distribution statistic, experimentally calculated from the DRR analysis. PR potency ratio, is a quotient of the examined ED_{50} values; SR slope function ratio, is a quotient of calculated slope functions of the examined DRR lines; $f_{\_ratio\_SR}$ factor ratio for slope function ratio. As the DRR analysis was performed according to the log-probit method by Litchfield and Wilcoxon (1949), the original notation of DRR parameters was applied in the present study. For more details see the Appendix

Interaction index

Interaction index for all fixed-ratio combinations was calculated as a ratio of the respective $ED_{x\ mix}$ and $ED_{x\ add}$ values from the MES test. This isobolographic parameter describes the strength and magnitude of interactions between two drugs in the mixture (Berenbaum 1989; Tallarida et al. 1999; Tallarida 2002).

Software used

Microsoft's Excel spreadsheet was used to perform calculations and to graph the results in form of 2D isobolograms. This spreadsheet was programmed to compute all calculations automatically and determine the DRR lines of AEDs administered alone from the log-probit linear regression analysis according to Litchfield and Wilcoxon (1949). The theoretically additive interactions at the fixed-ratio combinations of 1:3, 1:1 and 3:1 for various effect levels (i.e., $ED_{16\ add}$, $ED_{50\ add}$, and $ED_{84\ add}$) were also calculated with this program. The 3D isobolograms for the experimentally-derived dose-response surfaces were constructed with commercially available program Statistica.

Results

Anticonvulsant effects of STP and CBZ administered singly in the MES test in mice

STP and CBZ exerted clear-cut anticonvulsant activities against MES-induced seizures in mice (Fig. 1) and their ED_{50} values are presented in Table 1. Log-probit linear regression analysis according to Litchfield and Wilcoxon (1949) followed by χ^2 (Chi-square) test revealed that the data points creating the lines of DRRs for STP and CBZ are good-to-fit. As the experimentally determined value of χ^2_E for STP (0.930) was considerably lower than the critical value of χ^2_T for 2 degrees of freedom (*df*) (5.991) at $P<0.05$ (Table 1), one can conclude that the DRR line for STP was good-to-fit (Litchfield and Wilcoxon 1949). Similarly, the experimentally calculated value of χ^2_E for CBZ (1.641) was lower than the critical value of χ^2_T for 1 *df* (3.841) at $P<0.05$ (Table 1), hence, the DRR line for CBZ was best-fit, too (Litchfield and Wilcoxon 1949). Additionally, to detect variance influencing the homogeneity of data points of DRRs for STP and CBZ, the *F*-distribution statistic was performed according to the method described by Glantz and Slinker (2001). The calculated *F*-value for STP ($F_{1,3}$=62.173) was greater than the critical *F*-value of 10.13, at $P<0.05$ (Table 1). Likewise, the calculated *F*-value for CBZ ($F_{1,2}$=24.917) differed significantly from the critical *F*-value of 18.51, at $P<0.05$ (Table 1). So, the *F*-distribution statistic revealed that the log-probit lines for STP and CBZ are good-to-fit (Fig. 1). Moreover, the coefficient of determination (r^2) for both DRRs (STP and CBZ) was determined. The r^2 for the DRR line of STP was 0.969; hence, the straight DRR line (at the equation of y=15.204x−32.151) describes 96.9% of the existing relationship between the doses of STP and the observed anticonvulsant effects in the MES test (Table 1; Fig. 1). Similarly, the r^2 for the DRR line of CBZ was 0.961 and, thus, the linear regression equation (y=12.368x−8.138) describes 96.1% of the relationship between CBZ doses and the observed antiseizure effects in the MES test in mice (Table 1; Fig. 1). Finally, the linear regression analysis followed by the test for parallelism

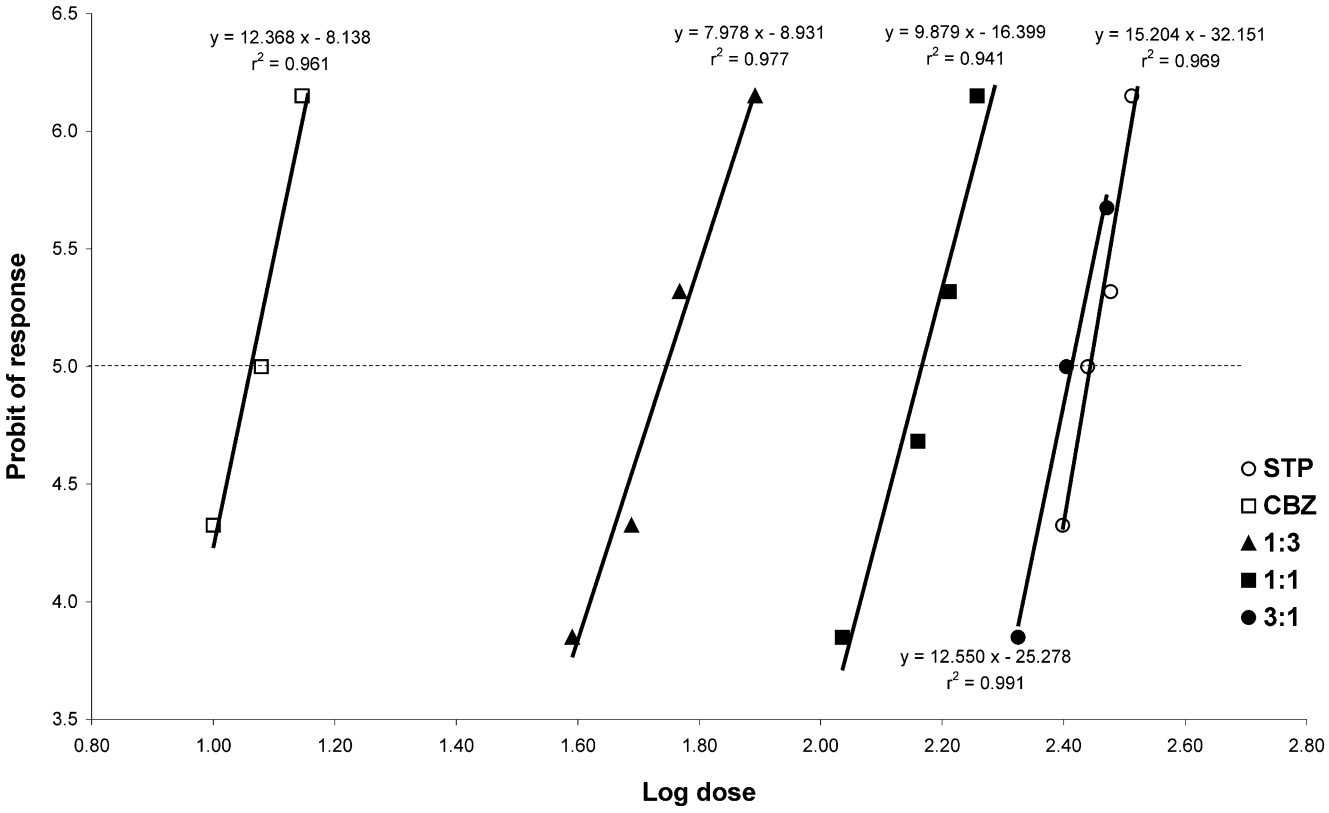


Fig. 1 Log-probit analysis of dose-response relationship (DRR) lines for stiripentol (STP), carbamazepine (CBZ) and their combinations at three fixed-ratios of 1:3, 1:1 and 3:1 in the maximal electroshock (MES)-induced seizures in mice. Drug doses of STP and CBZ injected alone were transformed into logarithms to the base 10 and plotted on a log scale (abscissa, x), whereas their corresponding protective effects were transformed to probits and plotted on a probit scale (ordinate, y). The equation of DRR lines for STP in the MES test, following log-probit analysis, was: y=15.204x−32.151 (r^2=0.969), whereas that for CBZ was y=12.368x−8.138 (r^2=0.961). The equations of DRR lines for the fixed-ratios of 1:3, 1:1 and 3:1 in the MES test were: y=7.978x−8.931 (r^2=0.977), y=9.879x−16.399 (r^2=0.941), and y=12.55x−25.278 (r^2=0.991), respectively. Test for homogeneity of data points for denoted DRR lines, performed with χ^2 (Chi-square) goodness-of-fit test and *F*-distribution statistic, revealed that all data points are good-to-fit. Test for parallelism of the DRR lines for STP and CBZ revealed that the denoted lines are parallel (Litchfield and Wilcoxon 1949). For more details see Tables 1 and 2

indicated that the examined DRR lines for STP and CBZ fulfilled the criterion of parallelism (Table 1). The slope function ratio (SR) for STP and CBZ in the MES test (SR=1.035) was lower than the factor ratio for slope function ratio ($f_{\_ratio\_SR}$=1.100; Table 1). Noteworthy, since the test for parallelism of two log-probit DRR lines was performed according to Litchfield and Wilcoxon (1949), the original notation for SR and $f_{\_ratio\_SR}$ was applied in the present study.

Anticonvulsant activity of combinations of STP and CBZ in the MES test in mice

The mixture of STP and CBZ at the fixed-ratio of 1:3 exerted a potent anticonvulsant activity in the MES test. In this case, log-probit analysis allowed the determination of DRR equation for the mixture of both AEDs (y=7.978x−8.931; r^2=0.977). Since, the calculated χ^2_E value (2.455) was considerably lower, and *F*-distribution statistic ($F_{1,3}$=83.058) greatly exceeded the corresponding critical values (at P<0.05), one can conclude that the DRR for the mixture of STP and CBZ at the fixed-ratio of 1:3 was good-to-fit (Tallarida 2000; Glantz and Slinker 2001). So, the $ED_{50\ mix}$ for the mixture at the fixed-ratio of 1:3 was 55.7 (45.6–68.0) mg/kg (Table 2; Fig. 1). Similarly, the DRR equation for the mixture of STP with CBZ at the fixed-ratio of 1:1 was y=9.878x−16.399 (r^2=0.941). The experimentally-derived χ^2_E for 2 *df* was 3.667, and $F_{1,3}$=32.061, indicating that the DRR for the mixture at the fixed-ratio of 1:1 was good-to-fit. The $ED_{50\ mix}$ for this fixed-ratio was 146.6 (128.5–167.2) mg/kg (Table 2; Fig. 1). The log-probit analysis allowed the calculation of DRR equation for the AEDs in the mixture at the fixed-ratio of 3:1 (y=12.550x−25.278; r^2=0.991). As the calculated χ^2_E value (0.474) was considerably lower, and *F*-distribution statistic ($F_{1,2}$=106.909) greatly exceeded the corresponding critical values (at P<0.05), the DRR for the mixture of STP and CBZ at the fixed-ratio of 3:1 was good-to-fit. In this case, the $ED_{50\ mix}$ was 258.6 (227.8–293.7) mg/kg (Table 2; Fig. 1).

Table 2 Effects of stiripentol (STP) and carbamazepine (CBZ) in combination on the maximal electroshock-induced seizures in mice

FR	STP (mg/kg)	CBZ (mg/kg)	Mixture (mg/kg)	P/T		DRR analysis
1:3	26.0	3.2	29.2	0/8	ED_{50mix}=55.7 (45.6−68.0)	y=7.978 x−8.931
	34.7	4.3	39.0	1/8	S=1.335	r^2=0.977
	43.4	5.4	48.8	2/8	f=1.221	*df*=2
	52.1	6.5	58.6	5/8	*n*=16	χ^2_E=2.455; χ^2_T=5.991
	69.4	8.6	78.0	7/8	SE=5.679	*F*=83.058
	78.1	9.7	87.8	8/8		
1:1	69.4	2.9	72.3	0/8	ED_{50mix}=146.6 (128.5−167.2)	y=9.879x−16.399
	104.1	4.4	108.5	1/8	S=1.262	r^2=0.941
	138.8	5.8	144.6	3/8	f=1.141	*df*=2
	156.2	6.6	162.8	5/8	*n*=24	χ^2_E=3.667; χ^2_T=5.991
	173.5	7.3	180.8	7/8	SE=9.852	*F*=32.061
	208.2	8.7	216.9	8/8		
3:1	166.6	2.3	168.9	0/8	ED_{50mix}=258.6 (227.8−293.7)	y=12.550x−25.278
	208.3	2.9	211.2	1/8	S=1.201	r^2=0.991
	250.3	3.5	253.8	4/8	f=1.135	*df*=1
	291.6	4.1	295.7	6/8	*n*=16	χ^2_E=0.474; χ^2_T=3.841
	312.5	4.4	316.9	8/8	SE=16.757	*F*=106.909

STP + CBZ = Mixture

FR fixed-ratio combination; $ED_{50\ mix}$ median effective dose of the two-drug mixture, which protects 50% of animals tested against MES-induced seizures. For more details see the legend to Table 1

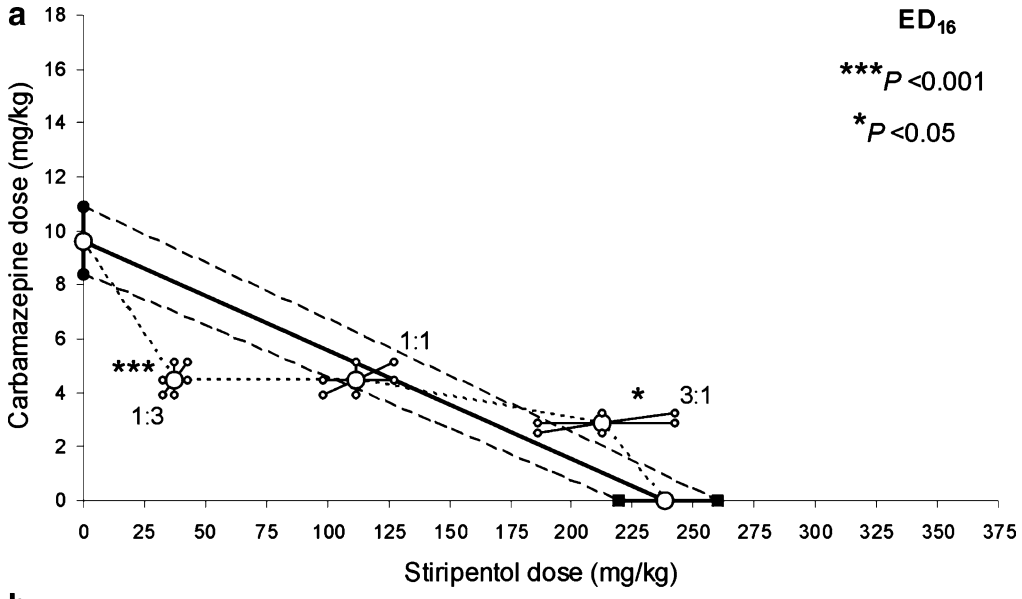
Isobolographic analysis of interactions between STP and CBZ in the MES test in mice

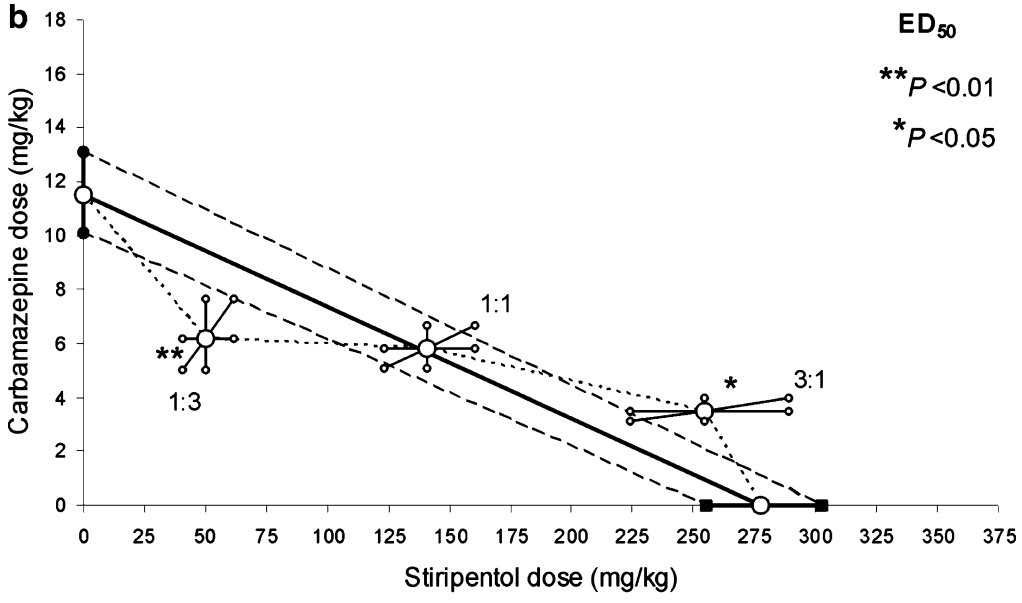
Isobolographic analysis revealed that the mixture of STP and CBZ at the fixed-ratio of 1:3 exerted supra-additivity (synergy) for all examined effects (ED_{16}, ED_{50} and ED_{84}) in the MES test (Fig. 2a–c; Fig. 3a,b). The experimentally-derived $ED_{16\ mix}$ was 41.8±4.26 mg/kg and significantly differed from the theoretically calculated $ED_{16\ add}$, which was 66.8±3.08 mg/kg (P<0.001; Table 3; Fig. 2a). Similarly, the $ED_{50\ mix}$ was 55.7±5.68 mg/kg, whereas the $ED_{50\ add}$ was 78.1±3.60 mg/kg (P<0.01; Table 3; Fig. 2b). The $ED_{84\ mix}$ value (74.4±7.58 mg/kg) was considerably lower than the $ED_{84\ add}$ value (91.2±4.21 mg/kg) at P<0.05, indicating a supra-additive interaction between STP and CBZ at the fixed-ratio of 1:3 (Table 3; Fig. 2c). The mixture of STP and CBZ at the fixed-ratio of 1:1 displayed additive interactions in the MES test for all drug dose-effects investigated (Table 3; Fig. 2a–c; Fig. 3a,b). The experimentally denoted $ED_{16\ mix}$, $ED_{50\ mix}$, and $ED_{84\ mix}$ values did not differ significantly from their corresponding theoretically calculated $ED_{16\ add}$, $ED_{50\ add}$, and $ED_{84\ add}$ values (Table 3; Fig. 2a–c). Moreover, the mixture of STP and CBZ at the fixed-ratio of 3:1 exerted sub-additivity (antagonism) for all examined effects (ED_{16}, ED_{50} and ED_{84}) in the MES test (Fig. 2a–c; Fig. 3a,b). In this case, the experimentally-derived $ED_{16\ mix}$ was 215.3±13.95 mg/kg, and considerably differed from the theoretically additive $ED_{16\ add}$ (181.4±7.97 mg/kg) at P<0.05 (Table 3; Fig. 2a). Analogously, the $ED_{50\ mix}$ (258.6±16.76 mg/kg) was significantly greater than the theoretically additive $ED_{50\ add}$ (211.1±9.28) at P<0.05 (Table 3; Fig. 2b). Similarly, the experimentally-derived $ED_{84\ mix}$ was 310.7±20.13 mg/kg, and significantly differed from the $ED_{84\ add}$ (245.8± 10.81 mg/kg) at P<0.01 (Table 3; Fig. 2c), indicating sub-additive interaction between STP and CBZ at the fixed-ratio of 3:1.

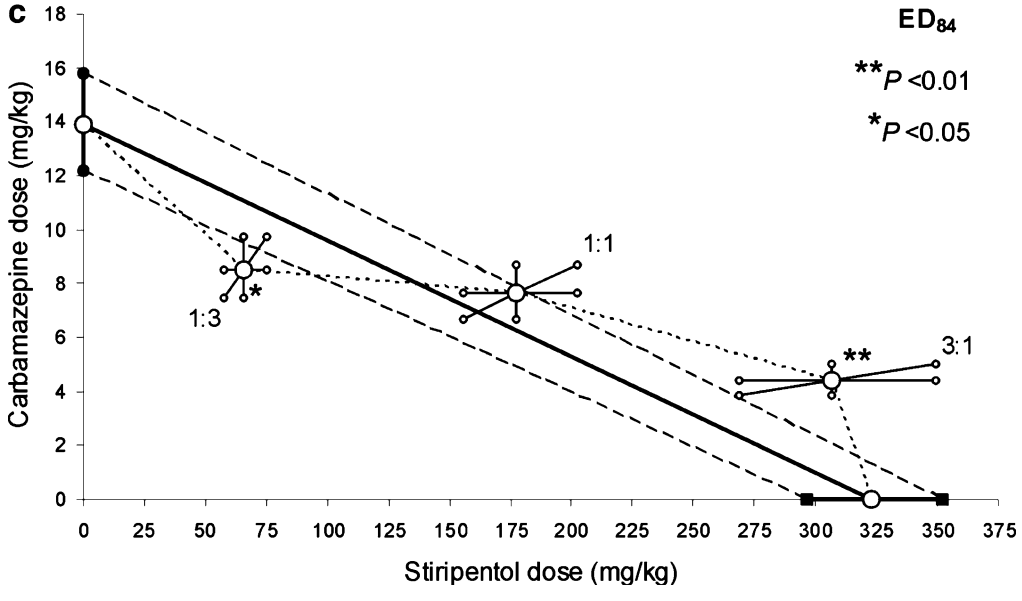
The interaction index values for the fixed-ratio combination of 1:3 increased from 0.63 (ED_{16}) to 0.82 (ED_{84}) and, thus, supra-additivity decreased by 0.19 (19%) along with the increase of examined anticonvulsant effects from ED_{16} to ED_{84} (Table 3). Similarly, the interaction index values for the fixed-ratio of 1:1 increased from 0.94 (ED_{16}) to 1.10 (ED_{84}) (Table 3). The fixed-ratio combination of 3:1 displayed also an increase in interaction index value, which was associated with increment of observed anticonvulsant effects. In this case, the interaction index values were ranged between 1.19 (ED_{16}) and 1.26 (ED_{84}), hence a 7% increase in sub-additivity (antagonism) was observed (Table 3).

Free plasma and total brain CBZ concentrations

The estimation of free plasma and total brain concentrations of CBZ was performed for all fixed-ratio combinations tested (1:3, 1:1 and 3:1) at doses corresponding to their $ED_{50\ mix}$ values from the MES test. The co-administration of STP (49.5 mg/kg) and CBZ (6.2 mg/kg) at the fixed-ratio


a
ED16
***P <0.001
*P <0.05
Carbamazepine dose (mg/kg)
Stiripentol dose (mg/kg)
1:1
1:3
3:1
***
*


b
ED50
**P <0.01
*P <0.05
Carbamazepine dose (mg/kg)
Stiripentol dose (mg/kg)
1:1
1:3
3:1
**
*


c
ED84
**P <0.01
*P <0.05
Carbamazepine dose (mg/kg)
Stiripentol dose (mg/kg)
1:1
1:3
3:1
*
**

◀ **Fig. 2** Two-dimensional isobolograms displaying the interactions observed for the combination of stiripentol (STP) with carbamazepine (CBZ) in the maximal electroshock (MES)-induced seizure test in mice. Doses of STP and CBZ for various anticonvulsant effects (ED_{16}, ED_{50} and ED_{84}) are shown plotted graphically (**a**–**c**), on the X- and Y-axes, respectively. The *solid lines* on the axes represent the 95% confidence limits for the AEDs administered alone. The straight diagonal lines connecting the respective ED_x values on each graph represent the theoretical lines of additivity for a continuum of different fixed dose ratios ($ED_{x\ add}$s). The *open points* (o) depict the experimentally-derived $ED_{x,\ mix}$ values for a total dose of the mixture of STP and CBZ that produced a desired anticonvulsant effect (16%, 50% and 84%) with 95% confidence limits. The *dashed lines* represent the theoretical additive 95% confidence limits for $ED_{x\ add}$ values. **a** Interactions between STP and CBZ for a 16% anticonvulsant effect (ED_{16}) in the MES test in mice. The experimentally-derived $ED_{16\ mix}$ for the mixture of STP and CBZ, at the fixed-ratio of 1:3 is significantly below the theoretical line of additivity indicating supra-additive (synergistic) interaction (***P<0.001). In contrast, the $ED_{16\ mix}$ at the fixed-ratio of 3:1 is significantly above the theoretical line of additivity, displaying sub-additive (antagonistic) interaction (*P<0.05). Only the $ED_{16\ mix}$ for the fixed-ratio of 1:1 is close to the line of additivity, showing additive interaction in the MES test. **b** Interactions between STP and CBZ for a 50% antiseizure effect (ED_{50}) against electroconvulsions in mice. The experimental $ED_{50\ mix}$ for the mixture of STP and CBZ at the fixed-ratio of 1:3 is significantly below the theoretical line of additivity, and thus indicating supra-additive interaction (**P<0.01). In contrast, the $ED_{50\ mix}$ at the fixed-ratio of 3:1 is significantly above the theoretical line of additivity, displaying sub-additive interaction (*P<0.05). The $ED_{50\ mix}$ for the fixed-ratio of 1:1 is placed on the line of additivity, showing additive interaction in the MES test. **c** Interactions between STP and CBZ for an 84% anticonvulsant effect (ED_{84}) in the MES test. The $ED_{84\ mix}$ for the mixture of STP and CBZ at the fixed-ratio of 1:3 is significantly below the line of additivity, and thus displaying supra-additive interaction (*P<0.05). Inversely, the $ED_{84\ mix}$ at the fixed-ratio of 3:1 is significantly above the theoretical line of additivity, showing sub-additive interaction (**P<0.01). Only, the $ED_{84\ mix}$ for the fixed-ratio of 1:1 is close to the line of additivity, showing additive interaction in the MES test

of 1:3 was associated with a significant 21% increase in free plasma CBZ concentrations when compared to animals administered CBZ alone (P<0.05; Table 4). Similarly, total brain CBZ concentrations increased by 25% following the i.p. administration of STP at 49.5 mg/kg (P<0.05; Table 4). STP (at 140.7 mg/kg) co-administered with CBZ (5.9 mg/kg) at the fixed-ratio of 1:1 produced a significant 29% increase in free plasma CBZ concentration (P<0.01; Table 4). Similarly, STP (140.7 mg/kg) co-administered with CBZ (5.9 mg/kg) significantly elevated (by 33%) total brain CBZ concentration (P<0.01; Table 4). The combination of STP (255.1 mg/kg) with CBZ (3.5 mg/kg) was associated with a 39% increase in free plasma CBZ concentrations (P<0.001; Table 4). Simultaneously, total brain concentrations of CBZ administered at 3.5 mg/kg considerably increased (by 48%) following the i.p. administration of STP at 255.1 mg/kg (P<0.001; Table 4).

Discussion

Results presented herein indicate clearly that STP combined with CBZ produced a biphasic characteristic of interactions in the MES test in mice, exerting both supra-additivity (synergy) and sub-additivity (antagonism) depending on the dose ratio of STP and CBZ used in the mixture. When the effects produced by CBZ in the mixture prevailed over those offered by STP (at the fixed-ratio of STP:CBZ=1:3), the interaction was synergistic. In contrast, when the effects exerted by STP exceeded those produced by CBZ (at the fixed-ratio of 3:1), antagonism was observed. The 3D isobolographic study revealed that supra-additivity was reduced along with the increases in anticonvulsant effects and simultaneously, the sub-additive interaction was strengthened in the MES test. Noteworthy, the interaction index, assessing the strength of observed interactions between STP and CBZ in 3D isobolography, increased along with the increment of the anticonvulsant effects for all fixed-ratio combinations tested.

Relatively recently, a similar biphasic characteristics of interactions between oxcarbazepine (OXC) and clonazepam (CZP) has been reported in the MES test in mice (Luszczki and Czuczwar 2003). The mixtures of OXC and CZP at the fixed-ratios of 2:1 and 1:1 exerted supra-additive interactions, whereas the anticonvulsant effects produced by OXC and CZP at the fixed-ratios of 1:3, 1:4 and 1:7, were sub-additive in the MES test (Luszczki et al. 2003a). The transition of the type of interactions from synergy to antagonism for the combination of OXC and CZP has been denoted isobolographically at the fixed-ratio of 1:2 (Luszczki and Czuczwar 2003). In the present study, the transition of interactions from synergy to antagonism was determined at the fixed-ratio combination of 1:1. Moreover, with 3D isobolography, it has been shown that CZP produced synergistic interactions when combined with lamotrigine (LTG) - an AED with Na^+ channel blocker properties (Luszczki and Czuczwar 2004b). Similarly, synergistic interactions have been documented experimentally for the other Na^+ channel blockers (i.e., phenytoin [PHT] and CBZ) combined with CZP against MES-induced seizures in mice (Luszczki et al. 2003a).

Considering the above-mentioned facts, one can ascertain that there exists a synergistic relationship between CZP and Na^+ channel blockers (PHT, CBZ, LTG and OXC) in the mouse MES test. Exceptionally, only some fixed-ratio combinations of OXC with CZP were antagonistic, whereas all fixed-ratios of CZP with LTG, CBZ and PHT were synergistic or additive with a tendency towards synergy in the MES test (Luszczki et al. 2003a; Luszczki and Czuczwar 2004b). In light of this fact and results presented in this study, one can hypothesize that the combinations of STP with Na^+ channel blockers may also produce similar

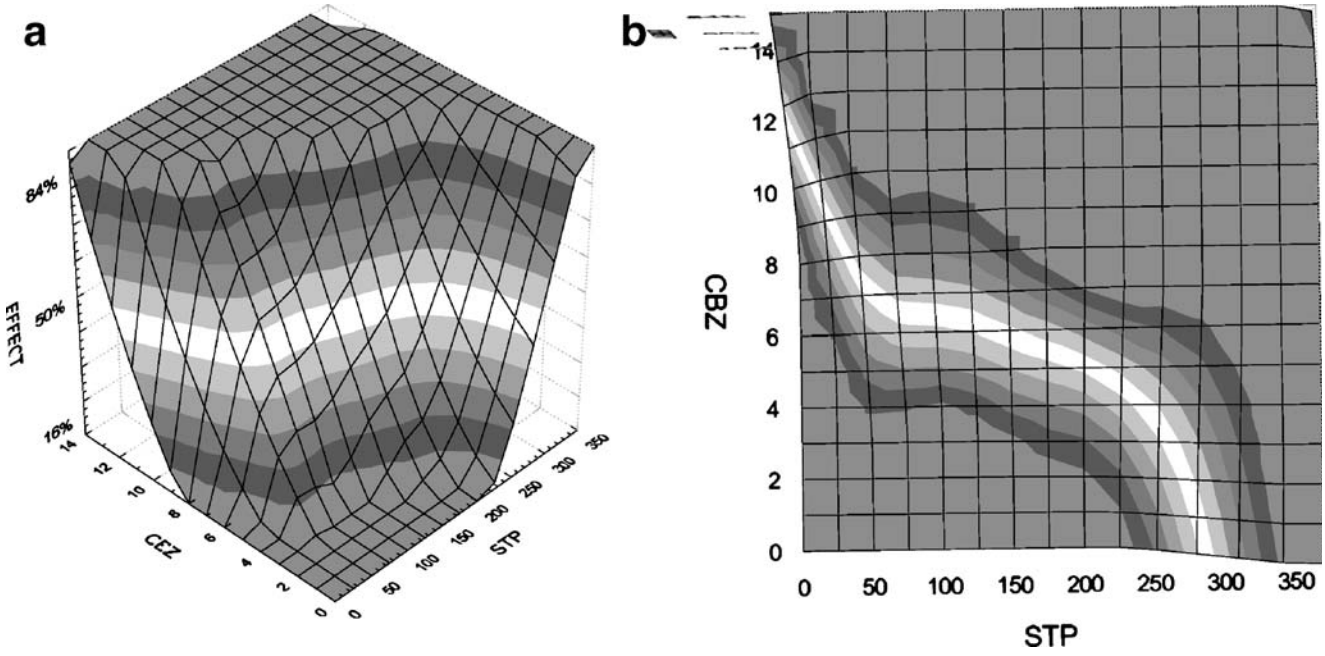


Fig. 3 Three-dimensional (3D) isobolographic analysis for interactions between stiripentol (STP) and carbamazepine (CBZ) in the maximal electroshock (MES)-induced seizure test in mice. **a** Doses of STP and CBZ are plotted graphically on X- and Y-axes, whereas their resultant anticonvulsant effects on Z-axis. The *convex curve* on the graph represents supra-additive (synergistic) interaction between STP and CBZ. The *concave curve* reflects sub-additive (antagonistic) interaction observed between the AEDs in the MES test. To facilitate the interpretation of 3D isobolograph the effects produced by the mixture of STP and CBZ were separated by greyscale layers. **b** 3D isobolograph was vertically rotated to elucidate the transition of interaction from synergy to antagonism on the graph. For more details see the legend to Table 2

interactions as the combinations of CZP with Na^+ channel blockers. This hypothesis needs, however, experimental verification whether LTG and OXC will interact synergistically with STP in the MES test in mice. Hence, further isobolographic studies are required to confirm or reject this hypothesis concerning synergistic interactions between STP and AEDs with Na^+ channel blocker properties in the mouse MES model.

It is worthy of note that the general principle in isobolography is based on the equation of additivity and

Table 3 Isobolographic analysis of interactions between stiripentol (STP) and carbamazepine (CBZ) in the MES-induced seizures in mice

ED_x	FR	STP + CBZ		$ED_{x,\ add}$	N_{add}	$ED_{x,\ mix}$	STP + CBZ		N_{mix}	II
ED_{16}	1:3	59.4	7.4	66.8±3.08	36	41.8±4.26***	37.2	4.6	16	0.63
	1:1	119.1	5.0	124.1±5.53	36	116.1±7.80	111.5	4.6	24	0.94
	3:1	178.9	2.5	181.4±7.97	36	215.3±13.95*	212.4	2.9	16	1.19
ED_{50}	1:3	69.43	8.63	78.1±3.60	36	55.7±5.68**	49.5	6.2	16	0.71
	1:1	138.85	5.75	144.6±6.44	36	146.6±9.85	140.7	5.9	24	1.01
	3:1	208.28	2.88	211.2±9.28	36	258.6±16.76*	255.1	3.5	16	1.23
ED_{84}	1:3	81.1	10.1	91.2±4.21	36	74.4±7.58*	66.1	8.3	16	0.82
	1:1	161.8	6.7	168.5±7.51	36	185.1±12.44	177.7	7.4	24	1.10
	3:1	242.4	3.4	245.8±10.81	36	310.7±20.13**	306.5	4.2	16	1.26

Results are presented as effective doses (ED_xs in mg/kg±SE), protecting x-% (x=16, 50, and 84) of animals in the MES test. The ED_{16}, ED_{50} and ED_{84} values were either experimentally determined from various mixtures of two AEDs ($ED_{x,\ mix}$) or theoretically calculated from the equation of additivity ($ED_{x,\ add}$). Statistical evaluation of data was performed by using unpaired Student's *t*-test. FR-fixed-ratio of drug dose combinations; N_{add}-total number of animals calculated for the additive mixture of the drugs examined (i.e., $N_{add}=N_1+N_2-4$; where N_1 and N_2 are the total number of animals used between 4 and 6 probits for drugs administered alone). N_{mix}-total number of animals used between 4 and 6 probits for the experimental mixture; II-interaction index (as a ratio of the respective $ED_{x,\ mix}$ and $ED_{x,\ add}$ values) determines the strength and magnitude of interactions between STP and CBZ in the MES test. Its values close to 1 indicate additive interaction, whereas the values lower than 1 or greater than 1 indicate supra-additive (synergistic) or sub-additive (antagonistic) interactions, respectively (Loewe 1953; Berenbaum 1989)

*P<0.05, **P<0.01, and ***P<0.001 versus the respective $ED_{x,\ add}$

Table 4 Free (non-protein bound) plasma and total brain concentrations of carbamazepine (CBZ) administered singly or in combination with stiripentol (STP)

Treatment	Plasma concentration		Brain concentration	
(mg/kg)	(μg/ml)	% increase[a]	(μg/ml)	% increase[a]
CBZ (6.2) + vehicle	1.846±0.219		1.605±0.243	
CBZ (6.2) + STP (49.5)	2.240±0.387*	21	2.009±0.315*	25
CBZ (5.9) + vehicle	1.985±0.293		1.805±0.266	
CBZ (5.9) + STP (140.7)	2.570±0.332**	29	2.393±0.312**	33
CBZ (3.5) + vehicle	1.409±0.172		1.164±0.159	
CBZ (3.5) + STP (255.1)	1.960±0.230***	39	1.725±0.201***	48

Results are presented as means±SD of 8 determinations. Statistical evaluation of data was performed using the unpaired Student's *t*-test. The AEDs (CBZ and STP) were administered at doses corresponding to their $ED_{50\ mix}$ values from the MES test for all fixed-ratio combinations tested (1:3, 1:1 and 3:1)

[a] Increase in free plasma and total brain CBZ concentrations with respect to the adequate control groups (CBZ + vehicle-treated animals)

*P<0.05, **P<0.01, and ***P<0.001 versus the respective control (CBZ administered alone) group

presumptions that the half the ED_x of the first drug co-administered with the half the ED_x of the second drug are as therapeutically effective as the ED_x of both AEDs administered alone (Loewe 1953). As already mentioned, this general rule is true if both AEDs produce clear-cut anticonvulsant effects and have their DRRCs parallel. It should be emphasized that 3D isobolography requires the parallelism of DRRCs of AEDs administered separately because the test for parallelism provides researchers with a certainty that proportions of drugs in the mixture do not change during the evaluation of various effective doses (i.e., $ED_{16\ mix}$, $ED_{50\ mix}$, $ED_{84\ mix}$; Sühnel 1992, 1998). In other words, if the DRRCs are parallel, the proportions of drugs in the mixture are constant for every investigated effect ranging between 16% and 84%. The effects lower than 16% and/or higher than 84% are estimated with approximation and therefore they do not precisely reflect the existing relationship between the drug doses and their corresponding biological effects (Finney 1971). This is why, in the present study, only the effects ranging between 16% and 84% were evaluated. In contrast, the lack of parallelism between the analyzed DRRCs makes the above-mentioned isobolographic principle true only for median doses (ED_{50} values). In this case, one can apply the axiomatic theory of parallelism between two non-parallel DRRCs, assuming that two non-parallel DRRCs are exceptionally parallel only for one fixed point-effect in the Cartesian plot system (the point reflecting ED_{50} for both drugs). With this oversimplification, one can conduct the isobolographic analysis of interactions between drugs, but only for the fixed-ratio of 1:1, when the effects produced by drugs are equi-effective (iso-effective). The isobolographic analysis of interactions at the fixed-ratio combinations different from 1:1 (especially, at the border fixed-ratios of 1:5, 1:3, 3:1, or 5:1, etc.) generates a priori sub- or supra-additive interactions because proportions of both drugs in the mixture (theoretically calculated from the equation of additivity for non-parallel DRRCs) are inadequately determined. Thus, drug doses in the mixture may be too low or too high, producing finally the isobolographic interactions, whose nature can be erroneously classified as supra- or sub-additive. Generally, the isobolographic analysis based on log-probit linear transformation of data is devoid of problems related to non-linear DRRCs of drugs. In our opinion, the replacement of non-linear DRRCs by linear log-probit DRRCs eliminates some oversimplifications related to asymmetry of DRRCs during 3D response surface analysis. For instance, each sigmoidal DRRC should be verified with regard to its asymmetry (Van der Graaf and Schoemaker 1999; Giraldo et al. 2002).

Relatively recently, there has appeared a trend to evaluate interactions between two drugs in the clinical setting by the use of a pharmacokinetic-pharmacodynamic (PK/PD) model based on 3D response surface analysis (Minto and Schnider 1998; Minto et al. 1997, 2000, 2003; Short et al. 2002). This model allows the determination of mathematical equations for interactions between drugs and, thus, it can predict some advantageous or unfavorable combinations, based on the drug dose-ratios (Minto et al. 2000). Generally, the PK/PD mathematical models are created to describe the interactions between drugs with firmly established (well-known) molecular mechanisms of action (Groten et al. 2001; Jonker et al. 2005). In case of AEDs, the drugs exert their antiseizure effects through various sometimes unknown as yet mechanisms of action. Therefore, the determination of mathematical equations describing PK/PD interactions between AEDs, in our opinion, seems to be premature. The detailed description of PK/PD models applied during the detection of drug interactions has lately been presented by Jonker et al. (2005).

It is noteworthy that the 3D isobologram can be used to predict combination effects for any dose combinations

within the dose range used for the fitting procedure (Groten et al. 2001). Moreover, 3D isobologram provides a quantitative measure of the intensity of interaction and, thus, it has a potential to explain characteristics of complex interactions between drugs. On the other hand, the 3D isobologram reflects the experimentally derived values (plotted in the Cartesian plot system). However, in contrast to 3D response surface analysis, the 3D isobologram does not require the knowledge of molecular mechanisms of action between the investigated drugs (Tallarida et al. 1999).

Another fact is worthy of mentioning while performing the 3D isobolographic analysis. Although DRRCs for AEDs used separately have to be parallel, the experimentally derived DRRCs for the mixture of AEDs at various fixed-ratios need not fulfill the criterion of parallelism. In such a case, a significant difference may appear between the interactions evaluated experimentally for the ED_{16} and ED_{84} values (Loewe 1953).

In this study, it was confirmed that the log-probit DRRCs of STP and CBZ (administered alone) were parallel. This is why the detailed presentation of respective equations for the test for parallelism (according to Litchfield and Wilcoxon 1949) was presented in the Appendix. It is noteworthy that the straightforward procedures required to properly analyze DRRCs, homogeneity and parallelism of DRRCs, slope functions and their influence on the steepness of DRRCs are presented in some statistical textbooks (Finney 1971; Pöch 1993; Tallarida 2000; Glantz and Slinker 2001).

It should be stressed that the isobolographic analysis displays the final effect resulting from pharmacodynamic and/or pharmacokinetic interactions between drugs. Relatively recently, distinct discrepancies during pharmacokinetic evaluation of AED concentrations in plasma and biophase of experimental animals (i.e., brain homogenates or cerebrospinal fluid) have been elicited (Cadart et al. 2002; Luszczki et al. 2003c). Therefore, the isobolographically-denoted interactions from the present study were verified to identify any contribution consequent to a pharmacokinetic interaction between STP and CBZ. The evaluation of CBZ concentrations provided evidence that STP increased both free plasma and total brain CBZ concentrations for all fixed-ratio combinations examined in this study. Comparing the isobolographically determined interactions between STP and CBZ in the MES test with their pharmacokinetic profiles (at the fixed-ratios of 1:3, 1:1 and 3:1), one could surprisingly ascertain that the combination at the fixed-ratio of 3:1 was antagonistic despite the significant increase in both free plasma and total brain CBZ concentrations.

It is important to mention that STP administered alone displays in vivo pharmacokinetics of the Michaelis–Menten type (Levy et al. 1984). Therefore, it might be suggested that the isobolographically derived fixed-ratio combinations and fractions of AEDs in the mixture, as well as the respective $ED_{16\ add}$, $ED_{50\ add}$, and $ED_{84\ add}$ values, could not be exactly and correctly calculated and, thus, sub- and supra-additivity would occur. This suggestion, concerning erroneous calculations of $ED_{x\ add}$ values, is less probable in the present study because: (1) it was found experimentally that the DRRCs of STP and CBZ administered alone were parallel, and (2) the combinations of STP with other conventional AEDs (phenobarbital and valproic acid) in the MES test did not produce any biphasic interactions with isobolography (unpublished data). These facts may indirectly testify that the isobolographic calculations were performed correctly, despite the non-linear kinetics of STP.

Based on this preclinical study, one can conclude that STP combined with CBZ exerted both synergistic and antagonistic interactions depending on the fixed drug dose ratio used in the MES test. The pharmacokinetic increase in free plasma and total brain CBZ concentrations for all fixed ratios tested in the MES test provides evidence that the combination of STP with CBZ should be used with caution in clinical practice. In our opinion, the test for parallelism of DRRCs for AEDs tested with 3D isobolography should be implemented as a standard procedure in order to avoid methodological errors associated with false determination of AED proportions in the mixture for various fixed-ratio combinations. Further biochemical and pharmacokinetic studies are required to elucidate the exact nature of biphasic characteristics of interactions between STP and CBZ in the MES test in mice.

Acknowledgments This study was supported by a grant (KBN 2P05D 051 26) from the State Committee for Scientific Research, Warszawa, Poland. The authors express their thanks to Dr. Jean Vincent from Biocodex Laboratoires (Gentilly, France) for the kind supply of stiripentol. Moreover, a kind gift of carbamazepine from Polfa (Starogard Gdanski, Poland) is greatly appreciated.

Appendix

Test for parallelism of two DRR log-probit lines according to Litchfield and Wilcoxon (1949) comprises three calculations, as follows:

1. The determination of the slope ratio (SR), as a quotient of slope functions for the respective DRR lines. $SR=S_1/S_2$ where S_1 and S_2 are the slopes of the DRR lines for the first and second drug. Generally, $SR \geq 1$.
2. The calculation of the f ratio for the slope ratio ($f_{\_ratio\_SR}$), as follows: $f_{\_ratio\_SR} = \mathrm{sqrt}\{[\log(f_{\_ratio\_SR1})]^2 + [log(f_{\_ratio\_SR2})]^2\}$

where, $f_{\_ratio\_S1}$ and $f_{\_ratio\_S2}$ are the f ratios for the slope function for the first and second drug, respectively; sqrt is the square root of the expression in parentheses {}; log is the logarithm to the base 10.

Noticeably, the $f_{\_ratio\_S1}$ is calculated, as follows: $f_{\_ratio\_S1} = A^{2.77/sqrt(N')}$

where $A=10^a$

$a=1.1\times(logS_1)^2/logR$

R=(largest dose)/(smallest dose of a drug used).

Hence, $A = 10^{1.1\times[(\log S1)\,\wedge\,2]/\log(\text{largest/smallest dose})}$, where N' is the total number of animals at those doses whose expected anticonvulsant effects ranged between 4 and 6 probits; ^2 is the power of 2. Transforming the above-mentioned equations, one can obtain: $f_{\_ratio\_S1} = \{10^{1.1\times[(logS1)\wedge2]/log(largest/smallest\ dose)}\}^{2.77/sqrt(N'1)}$ and, analogously, $f_{\_ratio\_S2} = \{10^{1.1\times[(logS2)\wedge2]/log(largest/smallest\ dose)}\}^{2.77/sqrt(N'2)}$

Finally, one calculates $f_{\_ratio\_SR}$, as presented above: $f_{\_ratio\_SR} = sqrt\{[log(f_{\_ratio\_S1})]^2 + [log(f_{\_ratio\_S2})]^2\}$

3. The comparison of the SR with $f_{\_ratio\_SR}$. Noticeably, two DRR lines are parallel if the calculated SR< $f_{\_ratio\_SR}$, otherwise the two DRR lines are convergent.

References

Berenbaum MC (1989) What is synergy? Pharmacol Rev 41:93–141. Erratum in (1990) Pharmacol Rev 41:422

Brodie MJ, Schachter SC (2001) Fast Facts-Epilepsy, 2nd edn. Health Press, Oxford, UK

Cadart M, Marchand S, Pariat C, Bouquet S, Couet W (2002) Ignoring pharmacokinetics may lead to isoboles misinterpretation: illustration with the norfloxacin-theophylline convulsant interaction in rats. Pharm Res 19:209–214

Chiron C, Marchand MC, Tran A, Rey E, d'Athis P, Vincent J, Dulac O, Pons G (2000) Stiripentol in severe myoclonic epilepsy in infancy: a randomised placebo-controlled syndrome-dedicated trial. STICLO study group. Lancet 356:1638–1642

Deckers CL, Czuczwar SJ, Hekster YA, Keyser A, Kubova H, Meinardi H, Patsalos PN, Renier WO, Van Rijn CM (2000) Selection of antiepileptic drug polytherapy based on mechanisms of action: the evidence reviewed. Epilepsia 41:1364–1374

Finney DJ (1971) Probit analysis. 3rd edn. Cambridge University Press, Cambridge, UK

Giraldo J, Vivas NM, Vila E, Badia A (2002) Assessing the (a) symmetry of concentration-effect curves: empirical versus mechanistic models. Pharmacol Ther 95:21–45

Glantz SA, Slinker BK (2001) Primer of applied regression and analysis of variance. 2nd edn. MacGraw-Hill, New York

Greco WR, Bravo G, Parsons JC (1995) The search for synergy: a critical review from response surface perspective. Pharmacol Rev 47:331–385

Groten JP, Feron VJ, Sühnel J (2001) Toxicology of simple and complex mixtures. Trends Pharmacol Sci 22:316–322

Jonker DM, Visser SA, van der Graaf PH, Voskuyl RA, Danhof M (2005) Towards a mechanism-based analysis of pharmacodynamic drug-drug interactions in vivo. Pharmacol Ther 106:1–18

Kanzawa F, Nishio K, Fukuoka K, Fukuda M, Kunimoto T, Saijo N (1997) Evaluation of synergism by a novel three-dimensional model for the combined action of cisplatin and etoposide on the growth of a human small-cell lung-cancer cell line, SBC-3. Int J Cancer 71:311–319

Levy RH, Loiseau P, Guyot M, Blehaut HM, Tor J, Moreland TA (1984) Michaelis-Menten kinetics of stiripentol in normal humans. Epilepsia 25:486–491

Litchfield JT, Wilcoxon F (1949) A simplified method of evaluating dose-effect experiments. J Pharmacol Exp Ther 96:99–113

Loewe S (1953) The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3:285–290

Loiseau P, Levy RH, Houin G, Rascol O, Dordain G (1990) Randomized double-blind, parallel, multicenter trial of stiripentol added to carbamazepine in the treatment of carbamazepine resistant epilepsies. An interim analysis. Epilepsia 31(Suppl. 5): 618–619

Löscher W, Fassbender CP, Nolting B (1991) The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. II. Maximal electroshock seizure models. Epilepsy Res 8:79–94

Luszczki JJ, Czuczwar SJ (2003) Isobolographic and subthreshold methods in the detection of interactions between oxcarbazepine and conventional antiepileptics-a comparative study. Epilepsy Res 56:27–42

Luszczki JJ, Czuczwar SJ (2004a) Isobolographic profile of interactions between tiagabine and gabapentin: a preclinical study. Naunyn-Schmiedeberg's Arch Pharmacol 369:434–446

Luszczki JJ, Czuczwar SJ (2004b) Three-dimensional isobolographic analysis of interactions between lamotrigine and clonazepam in maximal electroshock-induced seizures in mice. Naunyn-Schmiedeberg's Arch Pharmacol 370:369–380

Luszczki JJ, Czuczwar SJ (2005) Isobolographic characterisation of interactions among selected newer antiepileptic drugs in the mouse pentylenetetrazole-induced seizure model. Naunyn Schmiedeberg's Arch Pharmacol 372:41–54

Luszczki JJ, Borowicz KK, Swiader M, Czuczwar SJ (2003a) Interactions between oxcarbazepine and conventional antiepileptic drugs in the maximal electroshock test in mice: an isobolographic analysis. Epilepsia 44:489–499

Luszczki JJ, Czuczwar M, Kis J, Krysa J, Pasztelan I, Swiader M, Czuczwar SJ (2003b) Interactions of lamotrigine with topiramate and first-generation antiepileptic drugs in the maximal electroshock test in mice: an isobolographic analysis. Epilepsia 44:1003–1013

Luszczki JJ, Swiader M, Czuczwar M, Kis J, Czuczwar SJ (2003c) Interactions of tiagabine with some antiepileptics in the maximal electroshock in mice. Pharmacol Biochem Behav 75:319–327

Luszczki JJ, Ratnaraj N, Patsalos PN, Czuczwar SJ (2006) Isobolographic analysis of interactions between loreclezole and conventional antiepileptic drugs in the mouse maximal electroshock-induced seizure model. Naunyn Schmiedeberg's Arch Pharmacol 373:169–181

Minto C, Schnider T (1998) Expanding clinical applications of population pharmacodynamic modelling. Br J Clin Pharmacol 46:321–333

Minto CF, Schnider TW, Shafer SL (1997) Pharmacokinetics and pharmacodynamics of remifentanil. II. Model application. Anesthesiology 86:24–33

Minto CF, Schnider TW, Short TG, Gregg KM, Gentilini A, Shafer SL (2000) Response surface model for anesthetic drug interactions. Anesthesiology 92:1603–1616

Minto CF, Schnider TW, Gregg KM, Henthorn TK, Shafer SL (2003) Using the time of maximum effect site concentration to combine pharmacokinetics and pharmacodynamics. Anesthesiology 99:324–333

Perez J, Chiron C, Musial C, Rey E, Blehaut H, d'Athis P, Vincent J, Dulac O (1999) Stiripentol: efficacy and tolerability in children with epilepsy. Epilepsia 40:1618–1626

Perucca E (1995) Pharmacological principles as a basis for polytherapy. Acta Neurol Scand Suppl 162:31–34

Perucca E, Levy RH (2002) Combination therapy and drug interactions. In: Levy RH, Mattson RH, Meldrum BS, Perucca E (eds) Antiepileptic drugs, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 96–102

Pöch G (1993) Combined effects of drugs and toxic agents. Modern evaluation in theory and practice. Springer, Berlin Heidelberg Wien

Porreca F, Jiang Q, Tallarida RJ (1990) Modulation of morphine antinociception by peripheral [Leu5]enkephalin: a synergistic interaction. Eur J Pharmacol 179:463–468

Prichard MN, Prichard LE, Baguley WA, Nassiri MR, Shipman C (1991) Three-dimensional analysis of the synergistic cytotoxicity of gancyclowir and zidovudine. Antimicrob Agents Chemother 35:1060–1065

Prichard MN, Prichard LE, Shipman C (1993) Strategic design and tree-dimensional analysis of antiviral drug combinations. Antimicrob Agents Chemother 37:540–545

Renard F, Musial C, Tor J, Dulac O (1993) Single-blind add-on trial of stiripentol in epileptic children. Epilepsia 34(Suppl 6):74

Short TG, Ho TY, Minto CF, Schnider TW, Shafer SL (2002) Efficient trial design for eliciting a pharmacokinetic-pharmacodynamic model-based response surface describing the interaction between two intravenous anesthetic drugs. Anesthesiology 96:400–408

Sühnel J (1992) Zero interaction response surfaces, interaction functions and difference response surfaces for combinations of biologically active agents. Arzneimittelforschung 42:1251–1258

Sühnel J (1998) Parallel dose-response curves in combination experiments. Bull Math Biol 60:197–213

Tallarida RJ (1992) Statistical analysis of drug combinations for synergism. Pain 49:93–97

Tallarida RJ (2000) Drug synergism and dose-effect data analysis. Chapman & Hall/CRC, Boca Raton

Tallarida RJ (2001) Drug synergism: its detection and applications. J Pharmcol Exp Ther 298:865–872

Tallarida RJ (2002) The interaction index: a measure of drug synergism. Pain 98:163–168

Tallarida RJ (2006) An overview of drug combination analysis with isobolograms. J Pharmcol Exp Ther (in press) DOI 10.1124/jpet.106.104117

Tallarida RJ, Stone DJ, McCary JD, Raffa RB (1999) Response surface analysis of synergism between morphine and clonidine. J Pharmcol Exp Ther 289:8–13

Tran A, Vauzelle-Kervroedan F, Rey E, Pous G, d'Athis P, Chiron C, Dulac O, Renard F, Olive G (1996) Effect of stiripentol on carbamazepine plasma concentration and metabolism in epileptic children. Eur J Clin Pharmacol 50:497–500

Van der Graaf PH, Schoemaker RC (1999) Analysis of asymmetry of agonist concentration-effect curves. J Pharmacol Toxicol Methods 41:107–115

White HS, Woodhead JH, Wilcox KS, Stables JP, Kupferberg HJ, Wolf HH (2002) Discovery and preclinical development of antiepileptic drugs. In: Levy RH, Mattson RH, Meldrum BS, Perucca E (eds) Antiepileptic drugs, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 36–48