ORIGINAL ARTICLE

Journal of Pre-Clinical and Clinical Research, 2008, Vol 2, No 2, 141-146
www.jpccr.eu

Interaction of tiagabine with clonazepam in the mouse pentylenetetrazole-induced seizure model: a type I isobolographic analysis for parallel log-probit dose-response relationship lines

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Abstract: Isobolographic analysis was used to characterize the interaction between tiagabine (TGB) and clonazepam (CZP) against pentylenetetrazole (PTZ)-induced clonic seizures in mice. The anticonvulsant effects of TGB in combination with CZP at 3 fixed-ratios of 1:3, 1:1 and 3:1, were evaluated in the PTZ test in mice using type I isobolographic analysis for parallel dose-response relationship lines. Acute adverse-effect profiles for the combinations were determined by use of the step-through passive avoidance task (long-term memory), grip-strength (muscular strength), and chimney (motor coordination) tests. All combinations of TGB with CZP (at fixed-ratios 1:3, 1:1 and 3:1) were additive in terms of clonic seizure suppression in the PTZ test. Additionally, none of the examined combinations of TGB with CZP (at their median effective doses from the PTZ test) affected long-term memory, muscular strength and motor coordination in mice. Based on the isobolographic analysis of interaction, the combinations of TGB with CZP at the fixed-ratios 1:3, 1:1 and 3:1 displayed additivity in terms of suppression of PTZ-induced seizures and appear to be neutral combinations from a clinical viewpoint.

Key words: tiagabine, clonazepam, additivity, interactions, isobolographic analysis, pentylenetetrazole-induced seizures

INTRODUCTION

Despite significant progress in recent years in the characterization of seizure phenomena and the understanding of pathophysiological causes of seizure initiation, amplification and propagation, there are approx. 30% of patients with epilepsy who still have seizures [1, 2]. Consequently, in these patients, polytherapy with anti-epileptic drugs (AEDs) in combination is administered to enhance seizure control. To date, only some two-drug combinations have proved to be effective against specific forms of epilepsy [3]. However, the application of two or more drugs is always associated with interactions between drugs, whose nature may be pharmacodynamic, pharmacokinetic, or mixed [4, 5]. Unfortunately, the direct testing of anticonvulsant efficacy of all two-drug combinations in patients with refractory epilepsy is not possible for ethical reasons and/or methodological limitations. Nonetheless, such combinations may be more readily identified and selected in preclinical studies on animals, and only those whose anti-convulsant effects offer optimal protection against seizures and, simultaneously, which are devoid of any serious neurotoxic side effects [6], can be further investigated and verified in the clinical setting. To evaluate pharmacodynamic interactions between drugs in preclinical studies on animals, the isobolographic analysis of interactions can be used, which is accepted as the 'gold standard' in the detection of interactions between drugs [7].

Accumulating evidence indicates that tiagabine (TGB – a second-generation AED) has been approved for use as add-on therapy in refractory partial epilepsy [8]. TGB reduces seizure activity, especially, by blocking GABA re-uptake from synaptic clefts [9]. Therefore, it can be considered appropriate to evaluate its preclinical profile in combination with clonazepam (CZP – a conventional AED), prescribed for patients with myoclonic seizures [8]. Experiments were performed in the mouse PTZ-induced clonic seizure model, a model of myoclonic seizures in humans [10], and the data analyzed using isobolographic analysis. The chimney test (a measure of motor performance impairment), the step-through passive avoidance task (a measure of long-term memory deficits), and the grip-strength test (a measure of muscular strength impairment) were used to evaluate acute adverse-effect potential of TGB in combination with CZP.

MATERIAL AND METHODS

Animals and experimental conditions. All experiments were performed on adult male 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 (12 h light-dark cycle, temperature 21 ± 1°C). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned

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Received: 1 October 2008, accepted: 19 November 2008

to experimental groups consisting of 8 mice. Each mouse participated only in one experiment. All tests were performed between 09:00–14:00 to minimize confounding effects of circadian rhythms. Procedures involving animals and their care were conducted in accordance 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 described in this study were approved by the Local Ethics Committee at the Medical University in Lublin.

Drugs. The following AEDs were used in the study: TGB (Sanofi Winthrop, Gentilly, France) and CZP (Polfa, Warsaw, Poland). The drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in saline, and administered by intraperitoneal (i.p.) injection in a volume of 0.005 ml/g body weight. Fresh drug solutions were prepared on each day of experimentation, and both AEDs were administered 15 min. before seizures and all behavioural tests. This pretreatment time was chosen based on information about their biological activity from the literature and our previous studies [11, 12]. PTZ (Sigma, St. Louis, MO, USA) was dissolved in distilled water and administered subcutaneously (s.c.) into a loose fold of skin in the midline of the neck in a volume of 0.005 ml/g body weight.

Pentylenetetrazole-induced convulsions. The anticonvulsant activities of TGB and CZP against PTZ-induced clonic seizures were determined after s.c. administration of PTZ at its CD_{97} (convulsive dose 97, i.e., the dose of PTZ that produced clonic seizures in 97% of mice, which in this study was 100 mg/kg). Following PTZ administration, mice were placed separately into transparent Plexiglas cages (25 × 15 × 10 cm) and observed for 30 min. for the occurrence of clonic seizures. Clonic seizure activity was defined as clonus of the whole body lasting for over 3 s, with an accompanying loss of righting reflex. The number of animals convulsing out of the total number of mice tested was noted for each treatment regimen. The animals were administered with increasing doses of the AEDs, and the anticonvulsant activity of each drug was evaluated as the ED_{50} (median effective dose of an AED, protecting 50% of mice against clonic convulsions). At least 4 groups of animals were used to estimate each ED_{50} value calculated from the respective log-probit dose-response relationship line according to Litchfield and Wilcoxon [13]. The anticonvulsant activity of TGB administered alone was studied at doses of 0.25, 0.5, 1, 2 and 3 mg/kg, whereas that of CZP alone at doses of 0.01, 0.025, 0.05, and 0.075 mg/kg against the clonic phase of PTZ-induced seizures in mice. Similarly, the anticonvulsant activity of a mixture of TGB with CZP was evaluated and expressed as $ED_{50\ mix}$, corresponding to the dose of a mixture of both drugs required to protect 50% of animals tested against PTZ-induced clonic convulsions. This experimental procedure has been described in more detail in our earlier studies [12, 14-16].

Isobolographic analysis of interactions. Interactions between TGB and CZP against PTZ-induced clonic seizures were analyzed according to methodology previously described in our earlier studies, where precise descriptions of the theoretical background with the respective equations showing how to undertake isobolographic calculations have been presented [14, 16-19].

In the present study, the isobolographic analysis comprised of 6 stages, as follows:

1. Determination of ED_{50} values for TGB and CZP (administered singly) by means of log-probit linear regression analysis according to Litchfield and Wilcoxon [13].
2. Calculation of purely additive $ED_{50\ add}$ values ± S.E.M. for a mixture of the examined AEDs, which is associated with the choice of at least 3 fixed drug-dose ratio combinations (usually, 1:3, 1:1 and 3:1). The $ED_{50\ add}$ represents a total additive dose of the drugs in the mixture, providing theoretically a 50% protection against PTZ-induced clonic seizures.
3. Experimental determination of the $ED_{50\ mix}$ values ± S.E.M. for the corresponding fixed-ratio AED combinations. $ED_{50\ mix}$ is an experimentally determined total dose of a mixture of two component drugs, which was administered at a fixed-ratio combination, sufficient for 50% protective effect against PTZ-induced seizures. To determine the $ED_{50\ mix}$ value, both drugs in the mixture (at proportionally increased doses) were administered to the animals, and a dose-response relationship for the mixture (at the fixed-ratio) was denoted using the log-probit method.
4. Statistical comparison of the experimentally-derived $ED_{50\ mix}$ values with their corresponding theoretically additive $ED_{50\ add}$ values was undertaken by use of the unpaired Student's *t*-test, according to Porreca et al. [20] and Tallarida [7].
5. Graphical illustration of the examined interactions as an isobologram, which is a simple form of visualization of interactions.
6. Finally, to determine the separate doses of TGB and CZP in the mixture, the $ED_{50\ mix}$ values were multiplied by the respective proportions of AEDs (denoted for purely additive mixture).

In isobolography, it is accepted that half of the effective dose of a first drug, plus half of the effective dose of a second one, should be as therapeutically effective as a full dose of each drug administered separately. This concept of adding fractions of the effective doses of AEDs is a principal, fundamental and crucial rule underlying the isobolographic analysis [7, 21, 22]. To simplify the notation and nomenclature of interactions in isobolography, the drug doses were administered in fixed-ratio combinations (e.g. 1:3, 1:1, and 3:1). The additive doses of drugs tested in various combinations were calculated from the 'equation of additivity', presented by Loewe [21] as follows:

$x/X + y/Y = 1$; where x and y are respectively the doses of a first and the second drug, co-administered in the mixture, exerting a 50% protection against PTZ-induced seizures; X and Y are respectively the doses of the drugs administered separately in order to obtain the same effect (50% suppression of PTZ-induced seizures).

The fixed drug dose ratios are usually presented in the form of natural numbers (1:3, 1:1, 3:1) and reflect fractions of ED_{50} values denoted for the drugs used separately. For example, the mixture at the fixed-ratio of 1:3 was comprised of ¼ of the ED_{50} of TGB and ¾ of the ED_{50} of CZP. Thus, the isobolographic notation of fixed-ratio combinations comprised of numerators of fractions of ED_{50} values for AEDs used in the mixture. For instance, in the present study, the ED_{50} values for TGB and CZP administered alone in the PTZ test were 0.989 mg/kg

and 0.023 mg/kg, respectively. Hence, the mixture of TGB with CZP at the fixed-ratio of 1:3 comprised of TGB at (¼ of 0.989 mg/kg = 0.248 mg/kg) and CZP (¾ of 0.023 mg/kg = 0.017 mg/kg). Thus, the $ED_{50\ add}$ value for the fixed-ratio of 1:3 was 0.265 mg/kg (Table 1).

It is noteworthy that in this two-drug mixture, CZP prevailed over TGB with respect to its pharmacological (antiseizure) activity against PTZ-induced clonic seizures, but it did not exceed quantitatively in the mixture. Analogously, the two-drug mixture for the combination of 1:1 in the PTZ test comprised of TGB (½ of 0.989 mg/kg = 0.495 mg/kg) and CZP (½ of 0.023 mg/kg = 0.012 mg/kg), where the drugs were combined in equi-effective (iso-effective) doses. Thus, the $ED_{50\ add}$ value for the fixed-ratio combination of 1:1 was 0.507 mg/kg (Table 1). Likewise, the fixed-ratio combination of 3:1 comprised of TGB (¾ of 0.989 mg/kg = 0.742 mg/kg) and CZP (¼ of 0.023 mg/kg = 0.006 mg/kg), producing the $ED_{50\ add}$ of 0.748 mg/kg (Table 1). In this combination, TGB clearly prevailed over CZP in the mixture.

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. Further details regarding these concepts have been published elsewhere [14-19].

Chimney test. The effects of combinations of TGB with CZP (at the fixed-ratios of 1:3, 1:1 and 3:1 from the PTZ test) on motor coordination impairment were quantified with the chimney test of Boissier et al. [23]. In this test, the animals have to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Data were presented as a percentage of animals that failed to perform the chimney test. This experimental procedure has been described in detail in our earlier studies [11, 19].

Grip-strength test. The effects of combinations of TGB with CZP (at fixed-ratios of 1:3, 1:1 and 3:1 from the PTZ test) on muscular strength in mice were quantified by the grip-strength test. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the PTZ test. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 3 measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The skeletal muscular strength in mice was expressed in N (Newtons) as means ± S.E.M. of at least 8 determinations. This experimental procedure has been described in detail in our earlier studies [24, 25].

Step-through passive avoidance task. Each animal was administered TGB in combination with CZP at the fixed-ratios of 1:3, 1:1 and 3:1 from the PTZ test on the first day before training. The time before the commencement of the training session (after drug administration) was identical to that for the PTZ test. Subsequently, animals were placed in an illuminated box (10 × 13 × 15 cm) connected to a larger dark box (25 × 20 × 15 cm) equipped with an electric grid floor. Entrance of animals to the dark box was punished by an adequate electric foot shock (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation. On the following day (24 h later), the pre-trained animals were again placed in the illuminated box and observed for up to 180 s. Mice that avoided the dark compartment for 180 s were considered to have rememberd the task. The time that the mice took to enter the dark box was noted and the median latencies (retention times) with 25th and 75th percentiles were calculated. The step-through passive avoidance task gives information about ability to acquire the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory [26]. This experimental procedure has been described in detail in our earlier studies [11, 12].

Statistics. The percent protection of animals against PTZ-induced clonic seizures per dose of the AEDs, and subsequently dose-response relationship lines were fitted using log-probit linear regression analysis according to Litchfield and Wilcoxon [13]. The ED_{50} values with their 95% confidence limits were calculated by computer-assisted log-probit analysis according to Litchfield and Wilcoxon [13]. To precisely analyze the experimental data, the test for homogeneity of data points creating the dose-response relationship lines and the test for parallelism of the dose-response relationship lines for TGB and CZP were presented as indispensable conditions for testing AED interactions with isobolography. The obtained 95% confidence limits were transformed to standard errors of the means (S.E.M.) as described previously [18, 19]. Statistical evaluation of isobolographic interactions was performed by the use of Student's *t*-test in order to detect the differences between the experimentally derived ($ED_{50\ mix}$) and theoretical additive ($ED_{50\ add}$) values, according to Porreca et al. [20] and Tallarida [7]. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test. The results from the step-through passive avoidance task were statistically analyzed using Kruskal-Wallis non-parametric ANOVA test, followed by the *post-hoc* Dunn's test. The data from the grip-strength test were statistically analyzed using one-way ANOVA, followed by the *post-hoc* Bonferroni's test. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences among values were considered statistically significant if $P<0.05$.

RESULTS

Log-probit dose-response relationship line analysis for TGB and CZP against PTZ-induced clonic seizures in mice. In the PTZ-induced clonic seizure test, TGB was administered separately at increasing doses of 0.5, 1, 2, and 3 mg/kg, respectively, and the percent protection offered by these drug doses was 25, 50, 75 and 87.5, respectively. Subsequently, log-probit transformation of data allowed to denote the equation of dose-response relationship for TGB administered alone ($y = 2.3232\ x + 5.0104$ [$r^2 = 0.9987$]; where y – probit of response, x – logarithm of the drug dose to the base 10, and r^2 – coefficient of determination). To analyze homogeneity of data, the test of the line for 'goodness of fit' was conducted using Chi-squared analysis according to Litchfield and Wilcoxon [13]. In this test, the χ^2 [$(Chi)^2$] value

Table 1 Isobolographic characterization of interactions between tiagabine (TGB) and clonazepam (CZP) in the mouse pentylenetetrazole (PTZ)-induced clonic seizure model.

AED-combination	FR	TGB	+	CZP	=	$ED_{50\,mix}$ (mg/kg)	n_{mix}	$ED_{50\,add}$ (mg/kg)	=	TGB	+	CZP	n_{add}
TGB + CZP	1:3	0.140		0.010		0.150 ± 0.036	24	0.265 ± 0.077		0.248		0.017	44
TGB + CZP	1:1	0.379		0.009		0.388 ± 0.076	16	0.507 ± 0.145		0.495		0.012	44
TGB + CZP	3:1	0.569		0.004		0.573 ± 0.112	16	0.748 ± 0.214		0.742		0.006	44

Data are presented as median effective doses (ED_{50}s ± S.E.M.) protecting 50% of animals tested against PTZ-induced clonic seizures. The clonic phase of PTZ-induced seizures was produced by the s.c.-injection of PTZ at its CD_{97} (100 mg/kg). The ED_{50}s were either experimentally determined from the mixture of two AEDs ($ED_{50\,mix}$) or theoretically calculated from the equation of additivity ($ED_{50\,add}$). Additionally, the actual doses of TGB and CZP that comprised the mixtures, at all fixed-ratio combinations and for both $ED_{50\,mix}$ and $ED_{50\,add}$ values, are presented in separate columns. Statistical evaluation of the data was performed by using unpaired Student's *t*-test according to Porecca et al. [20] and Tallarida [7]. FR – fixed-ratio of drug dose combinations; n – total number of animals used at those doses whose expected anticonvulsant effects ranged between 4 and 6 probits, denoted for the experimental mixture of drugs (n_{mix}) and theoretically calculated (n_{add}) from the equation of additivity.

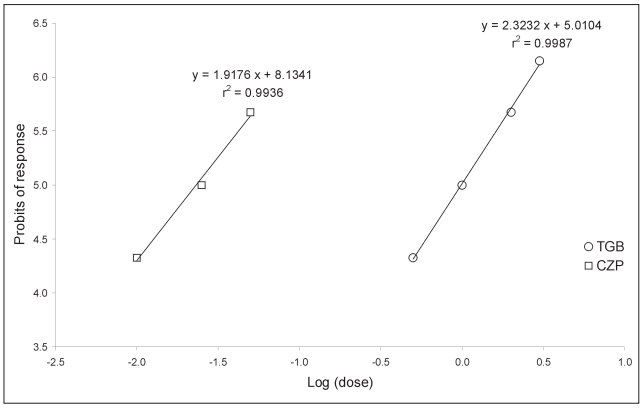

Figure 1 Log-probit dose-response relationship lines for tiagabine and clonazepam administered alone in the mouse pentylenetetrazole-induced clonic seizure model.
Doses of tiagabine (TGB) and clonazepam (CZP) were transformed to logarithms, whereas the protective effects offered by the AEDs administered alone against PTZ-induced seizures were transformed to probits of response [13]. Equations of dose-response-relationship lines for TGB and CZP are presented on the graph, as follows: y = 2.3232 x + 5.0104 (r^2 = 0.9987) for TGB, and y = 1.9176 x + 8.1341 (r^2 = 0.9936) for CZP, where y – probit of response, and x – logarithm to the base 10 of drug doses. The test for parallelism of two dose-response relationship lines revealed that both dose-response-relationship lines were parallel. For more details see Material and method section.

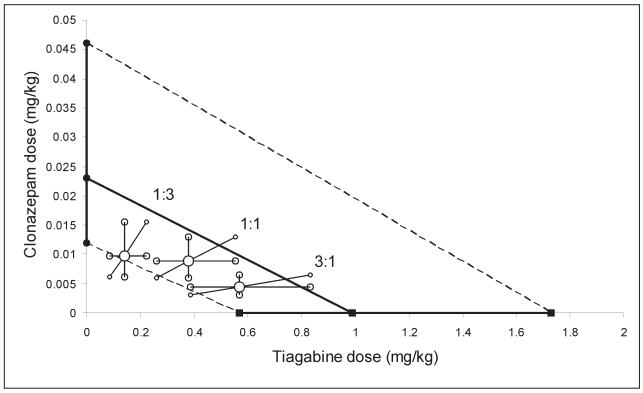

Figure 2 Isobologram showing additive interactions between tiagabine and clonazepam against pentylenetetrazole-induced clonic seizures in mice.
Median effective dose (ED_{50}) for tiagabine is plotted graphically on the X-axis. ED_{50} value of clonazepam is plotted on the Y-axis. The solid lines on the X and Y axes represent the 95% confidence limits (CLs) for the AEDs administered alone. The straight line connecting these two ED_{50} values on the axes represents the theoretical line of additivity for a continuum of different fixed dose ratios. The dashed lines represent the theoretical additive 95% CLs of $ED_{50\,add}$s. Open circles (o) depict the experimentally derived $ED_{50\,mix}$s (with 95% CLs as the error bars) for the total dose expressed as the proportion of tiagabine and clonazepam that produced a 50% anticonvulsant effect. Alternatively, all 95% CLs of the experimental $ED_{50\,mix}$s are presented horizontally and vertically in the shape of a cross. The experimental $ED_{50\,mix}$s of the mixture of tiagabine with clonazepam for the fixed-ratios of 1:3, 1:1 and 3:1 are placed below the line of additivity, although they did not attain statistical significance, and thus displaying additivity with the tendency towards supra-additivity.

for experimentally-derived points for TGB was 0.0331, whereas the χ^2 tabular (for 2 degrees of freedom at the 95% level of significance for P<0.05) was 5.99. Because the experimentally-derived χ^2 value was lower than the tabular χ^2 value, data points comprising the line were not heterogeneous, i.e., the log-probit line for TGB was good-to-fit [13]. Thus, the experimentally-derived ED_{50} value for TGB was 0.989 (0.565 – 1.733) mg/kg.

CZP was given at doses of 0.01, 0.025, 0.05, and 0.075 mg/kg, respectively, and the protection (in %) against PTZ-induced clonic seizures was 25, 50, 75, and 100%, respectively. The equation of dose-response relationship for CZP was y = 1.9176 x + 8.1341 [r^2 = 0.9936]. The experimentally denoted χ^2 for CZP was 0.1628, whereas the tabular χ^2 (for 1 degree of freedom at the 95% level of significance for P<0.05) was 3.84. Since the experimental χ^2 value was lower than the tabular χ^2 value, data points were not heterogeneous; therefore, the log-probit line for CZP was 'good-to-fit' [13]. The ED_{50} for CZP was 0.0232 (0.0118 – 0.0458) mg/kg.

The test for parallelism of two log-probit dose-response relationship lines revealed that slope function ratio for these lines (S.R. = 1.233) was lower than the factor for slope function ratio (f ratio S.R. = 1.922), indicating that the lines were parallel to one another (Figure 1). For more details concerning the calculation of S.R. and f ratio S.R. see Appendix in [18].

Isobolographic characteristic of interaction between TGB and CZP against PTZ-induced clonic convulsions. Statistical evaluation of data revealed that the combinations of TGB with CZP for all fixed-ratios of 1:3, 1:1 and 3:1 were additive in suppressing PTZ-induced clonic seizures (Table 1; Figure 2). The experimentally derived $ED_{50\,mix}$ for the fixed-ratio of 1:3 was 0.150 mg/kg, whereas the $ED_{50\,add}$ calculated from the line of additivity was 0.265 mg/kg (Table 1; Figure 2). Likewise, the $ED_{50\,mix}$ for the combination of TGB with CZP at the fixed-ratio of 1:1 was 0.388 mg/kg, while the $ED_{50\,add}$ amounted to 0.507 mg/kg (Table 1; Figure 2). Similarly, the $ED_{50\,mix}$ for the fixed-ratio of 3:1 was 0.573 mg/kg, whereas the $ED_{50\,add}$ value was 0.748 mg/kg (Table 1; Figure 2).

Effects of TGB administered alone and in combinations with CZP on long-term memory, motor coordination and muscular strength in mice. When TGB was co-administered with CZP at the fixed-ratios of 1:3, 1:1 and 3:1, motor coordination in mice was unaffected. Furthermore, none of the studied combinations impaired long-term memory as determined in the passive avoidance task, the median retention times being 180 s. Similarly, these combinations had no effect on muscular strength, as assessed by the grip-

strength test. Moreover, TGB and CZP administered alone at doses of 0.989 mg/kg and 0.0232 mg/kg, being their ED_{50} values from the PTZ test, did not significantly affect long-term memory, muscular strength and motor performance in animals (results not shown).

DISCUSSION

The objective of this study was to evaluate the type of interaction between TGB and CZP in the mouse PTZ-induced clonic seizure model using type I isobolographic analysis. Results presented in this study indicate that TGB interacted additively with CZP in terms of suppression of PTZ-induced clonic seizures in mice. However, a slight tendency towards supra-additivity was observed for the combination of TGB with CZP for all fixed-ratios tested (1:3, 1:1 and 3:1). This was because the isoboles, reflecting the $ED_{50\,mix}$ values on the graph, were placed below the line of additivity. Nevertheless, statistical evaluation of data with unpaired Student's t-test revealed no synergistic interactions between TGB and CZP in the PTZ test in mice.

It has been reported recently that two variants of type I isobolographic analysis of interactions could be used in preclinical studies. The first variant for two drugs whose dose-response relationship lines were non-parallel [27, 28], and the second variant for two drugs with parallel dose-response relationship lines [7, 22, 29]. This is why in this study, the analysis of log-probit dose-response relationship lines for TGB and CZP was included as an integral part of the isobolographic analysis, which allows to properly classify interactions between drugs. In this study, the analysis of log-probit dose-response relationship lines for TGB and CZP, according to the method of Litchfield and Wilcoxon [13], revealed that the lines for both drugs were parallel to each other.

Previously, it has been documented with isobolographic analysis that TGB synergistically (supra-additively) interacted with vigabatrin [24], and exerted additive interactions when combined with oxcarbazepine, loreclezole, felbamate and gabapentin in the PTZ test in mice [14, 30]. The combination of TGB with gabapentin in the mouse maximal electroshock-induced seizure threshold (MEST) test was supra-additive in nature [11]. Moreover, with type II isobolographic analysis it has been reported that TGB interacted additively with numerous conventional and newer AEDs (carbamazepine, phenytoin, phenobarbital, topiramate, felbamate and lamotrigine) in the mouse maximal electroshock-induced seizure (MES) model [31]. Only the combination of TGB with valproate was supra-additive in the MES test, although this combination was complicated by a pharmacokinetic increase in total brain valproate concentrations in experimental animals, indicating the pharmacokinetic nature of the interaction between TGB and valproate [31]. As regards CZP, it has been reported that with isobolographic analysis the drug interacted additively with felbamate, vigabatrin, loreclezole, and stiripentol, against PTZ-induced seizures in mice [12, 15, 32, 33]. In contrast, CZP interacted supra-additively (synergistically) with LTG in the MES test [34], and the drug produced biphasic interaction when combined with oxcarbazepine in the MES test in mice, by exerting supra-additive (synergistic), additive and sub-additive (antagonistic) interactions depending on drug doses used in the mixture [16]. Our findings, showing that the combination of TGB with CZP (at all fixed-ratios tested) was additive in the PTZ test in mice, confirm that CZP has a similar pattern of interaction with vigabatrin, felbamate, loreclezole and stiripentol.

In considering the molecular mechanisms of the anticonvulsant action of TGB and CZP, it can be ascertained that the blockade of GABA reuptake from synaptic clefts to neurons and glia evoked by TGB, slightly enhanced the effect of CZP on $GABA_A$-benzodiazepine-chloride ionophore receptor complex. This is because the observed interaction between TGB and CZP was additive with a tendency towards supra-additivity. On the other hand, it is highly likely that CZP, as a partial agonist of $GABA_A$-benzodiazepine-chloride ionophore receptor complex, is able to change its affinity to specific receptor binding sites within the $GABA_A$-benzodiazepine-chloride ionophore receptor complex in the presence of increased concentration of GABA – a natural agonist of this receptor complex [35]. In such a case, CZP could exert agonistic and/or antagonistic properties, especially if the GABA content in synaptic clefts was increased due to the blockade of GABA reuptake evoked by TGB.

There is another fact worth mentioning while discussing the results of this study. It is widely accepted that TGB is able to aggravate myoclonic seizures or to induce non-convulsive status epilepticus in children with refractory partial epilepsy [36-39]. Therefore, the results obtained from this study, based on PTZ-induced clonic seizures, cannot be directly extrapolated to clinical practice. Nevertheless, the combinations of TGB with CZP at the fixed-ratios of 1:3, 1:1 and 3:1 appear to be neutral AED combinations in terms of suppression of PTZ-induced seizures in mice.

The combination of TGB with CZP should be investigated in additional preclinical models of epilepsy, before the clinical application of this combination in epilepsy patients becomes an option.

ACKNOWLEDGEMENT

This study was supported by a Grant from Medical University of Lublin.

REFERENCES

1. Kwan P, Brodie MJ: Early identification of refractory epilepsy. *New Eng J Med* 2000, **342,** 314-319.
2. Kwan P, Brodie MJ: Epilepsy after the first drug fails: substitution or add-on? *Seizure* 2000, **9,** 464-468.
3. Stephen LJ, Brodie MJ: Seizure-freedom with more than one antiepileptic drug. *Seizure* 2002, **11,** 349-351.
4. Patsalos PN, Fröscher W, Pisani F, van Rijn CM: The importance of drug interactions in epilepsy therapy. *Epilepsia* 2002, **43,** 365-385.
5. Patsalos PN, Perucca E: Clinically important drug interactions in epilepsy: general features and interactions between antiepileptic drugs. *Lancet Neurol* 2003, **2,** 347-356.
6. Löscher W, Wauquier A: Use of animal models in developing guiding principles for polypharmacy in epilepsy. *Epilepsy Res* 1996, **Suppl. 11,** 61-65.
7. Tallarida RJ: Drug synergism and dose-effect data analysis. Chapman & Hall/CRC, Boca Raton, 2000.
8. Brodie MJ, Schachter SC: Fast Facts. Epilepsy (2nd ed.). Health Press, Oxford 2001.
9. Czuczwar SJ, Patsalos PN: The new generation of GABA enhancers. Potential in the treatment of epilepsy. *CNS Drugs* 2001, **15,** 339-350.
10. Löscher W, Honack D, Fassbender CP, Nolting B: The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. III. Pentylenetetrazole seizure models. *Epilepsy Res* 1991, **8,** 171-189.

11. Łuszczki JJ, Świąder M, Parada-Turska J, Czuczwar SJ: Tiagabine synergistically interacts with gabapentin in the electroconvulsive threshold test in mice. *Neuropsychopharmacology* 2003, **28,** 1817-1830.
12. Łuszczki JJ, Wójcik-Ćwikła J, Andres MM, Czuczwar SJ: Pharmacological and behavioral characteristics of interactions between vigabatrin and conventional antiepileptic drugs in pentylenetetrazole-induced seizures in mice: an isobolographic analysis. *Neuropsychopharmacology* 2005, **30,** 958-973.
13. Litchfield JT, Wilcoxon F: A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 1949, **96,** 99-113.
14. Łuszczki JJ, Czuczwar SJ: Isobolographic profile of interactions between tiagabine and gabapentin: a preclinical study. *Naunyn-Schmiedebergs Arch Pharmacol* 2004, **369,** 434-446.
15. Łuszczki JJ, Ratnaraj N, Patsalos PN, Czuczwar SJ: Pharmacodynamic and/or pharmacokinetic characteristics of interactions between loreclezole and four conventional antiepileptic drugs in pentylenetetrazole-induced seizures in mice: an isobolographic analysis. *Epilepsy Behav* 2005, **7,** 639-651.
16. Łuszczki JJ: Isobolographic analysis of interaction between oxcarbazepine and valproate in pentylenetetrazole-induced seizures in mice. *JPCCR* 2008, **2,** 40-45.
17. Łuszczki JJ, Czuczwar SJ: Isobolographic and subthreshold methods in the detection of interactions between oxcarbazepine and conventional antiepileptics - a comparative study. *Epilepsy Res* 2003, **56,** 27-42.
18. Łuszczki JJ, Ratnaraj N, Patsalos PN, Czuczwar SJ: Isobolographic analysis of interactions between loreclezole and conventional antiepileptic drugs in the mouse maximal electroshock-induced seizure model. *Naunyn-Schmiedebergs Arch Pharmacol* 2006, **373,** 169-181.
19. Łuszczki JJ, Borowicz KK, Swiader M, Czuczwar SJ: Interactions between oxcarbazepine and conventional antiepileptic drugs in the maximal electroshock test in mice: an isobolographic analysis. *Epilepsia* 2003, **44,** 489-499.
20. Porreca F, Jiang Q, Tallarida RJ: Modulation of morphine antinociception by peripheral [Leu5]enkephalin: a synergistic interaction. *Eur J Pharmacol* 1990, **179,** 463-468.
21. Loewe S: The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung* 1953, **3,** 285-290.
22. Berenbaum MC: What is synergy? *Pharmacol Rev*, 1989, **41,** 93-141. *Erratum* in: *Pharmacol Rev* 1990, **41,** 422.
23. Boissier JR, Tardy J, Diverres JC: Une nouvelle méthode simple pour explorer l'action tranquilisante: le test de la cheminée. *Med Exp* (Basle) 1960, **3,** 81-84.
24. Łuszczki JJ, Czuczwar SJ: Isobolographic characterization of interactions between vigabatrin and tiagabine in two experimental models of epilepsy. *Prog Neuropsychopharmacol Biol Psychiatry* 2007, **31,** 529-538.
25. Łuszczki JJ, Zadrożniak A, Wlaź A, Andres-Mach M, Dudra-Jastrzębska M, Zwoliński J, Misiuta-Krzesińska M, Sielski M: Characterization of acute adverse-effect profile of carbamazepine and valproate in the grip-strength test in mice. *JPCCR* 2008, **2,** 46-48.
26. Venault P, Chapouthier G, de Carvalho LP, Simiand J, Morre M, Dodd RH, Rossier J: Benzodiazepines impair and beta-carbolines enhance performance in learning and memory tasks. *Nature* 1986, **321,** 864-866.
27. Tallarida RJ: Interactions between drugs and occupied receptors. *Pharmacol Ther*, 2007, **113,** 197-209.
28. Łuszczki JJ: Isobolographic analysis of interaction between drugs with nonparallel dose-response relationship curves: a practical application. *Naunyn-Schmiedebergs Arch Pharmacol* 2007, **375,** 105-114.
29. Greco WR, Bravo G, Parsons JC: The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev* 1995, **47,** 331-385.
30. Łuszczki JJ, Czuczwar SJ: Isobolographic characterisation of interactions among selected newer antiepileptic drugs in the mouse pentylenetetrazole-induced seizure model. *Naunyn Schmiedebergs Arch Pharmacol* 2005, **372,** 41-54.
31. Łuszczki JJ, Świąder M, Czuczwar M, Kiś J, Czuczwar SJ: Interactions of tiagabine with some antiepileptics in the maximal electroshock in mice. *Pharmacol Biochem Behav* 2003, **75,** 319-327.
32. Borowicz KK, Łuszczki JJ, Czuczwar SJ: Isobolographic and subthreshold analysis of interactions among felbamate and four conventional antiepileptic drugs in pentylenetetrazole-induced seizures in mice. *Epilepsia* 2004, **45,** 1176-1183.
33. Łuszczki JJ, Ratnaraj N, Patsalos PN, Czuczwar SJ: Characterization of the anticonvulsant, behavioral and pharmacokinetic interaction profiles of stiripentol in combination with clonazepam, ethosuximide, phenobarbital, and valproate using isobolographic analysis. *Epilepsia* 2006, **47,** 1841-1854.
34. Łuszczki JJ, Czuczwar SJ: Three-dimensional isobolographic analysis of interactions between lamotrigine and clonazepam in maximal electroshock-induced seizures in mice. *Naunyn Schmiedebergs Arch Pharmacol* 2004, **370,** 369-380.
35. Haefely W: Benzodiazepines: mechanisms of action. In: Levy R, Mattson M, Meldrum BS, eds. Antiepileptic drugs. (3rd ed.). Raven Press, New York 1989, 721-734.
36. Kellinghaus C, Dziewas R, Ludemann P: Tiagabine-related non-convulsive status epilepticus in partial epilepsy: three case reports and a review of the literature. *Seizure*, 2002, **11,** 243-249.
37. Genton P: When antiepileptic drugs aggravate epilepsy. *Brain Dev* 2000, **22,** 75-80.
38. Perucca E, Gram L, Avanzini G, Dulac O: Antiepileptic drugs as a cause of worsening seizures. *Epilepsia* 1998, **39,** 5-17.
39. Thomas P, Valton L, Genton P: Absence and myoclonic status epilepticus precipitated by antiepileptic drugs in idiopathic generalized epilepsy. *Brain* 2006, **129,** 1281-1292.