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Pharmacological and Behavioral Characteristics of Interactions between Vigabatrin and Conventional Antiepileptic Drugs in Pentylenetetrazole-Induced Seizures in Mice: An Isobolographic Analysis

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To characterize the anticonvulsant effects and types of interactions exerted by mixtures of vigabatrin (VGB) and conventional antiepileptic drugs (valproate (VPA), ethosuximide (ESM), phenobarbital (PB), and clonazepam (CZP)) in pentylenetetrazole (PTZ)-induced seizures in mice, the isobolographic analysis for three fixed-ratio combinations of 1 : 3, 1 : 1, and 3 : 1 was used. The adverse-effect profile of the combinations tested, at the doses corresponding to their median effective doses (ED_{50}) at the fixed-ratio of 1 : 1 against PTZ-induced seizures, was determined by the chimney (motor performance), step-through passive avoidance (long-term memory), pain threshold (pain sensitivity), and Y-maze (general explorative locomotor activity) tests in mice. Additionally, the observed isobolographic interactions were verified in terms of a pharmacokinetic interaction existence. VGB combined with PB or ESM exerted supra-additive (synergistic) interactions against the clonic phase of PTZ-induced seizures, which was associated with the increment of PB or ESM concentrations in the brains of examined animals. The remaining combinations tested (ie VGB + VPA and VGB + CZP) occurred additive in the PTZ test, which was associated with no significant changes in the brain concentrations of VPA and CZP. None of the examined combinations exerted motor impairment in the chimney test in mice. In the standard variant of passive avoidance task (current of 0.6 mA; 2 s of stimulus duration), the combinations of VGB + CZP and VGB + VPA significantly affected long-term memory in mice. Moreover, VGB in a dose-dependent manner lengthened the latency to the first pain reaction in the pain threshold test in mice. The modified variant of step-through passive avoidance task (current of 0.6 mA; stimulus duration based on the latency from the pain threshold test) revealed no significant changes in the long-term memory of animals for the combinations of VGB + VPA and VGB + CZP; so the observed effects in the standard variant of passive avoidance task were a result of the antinociceptive effects produced by VGB. In the Y-maze test, VGB also, in a dose-dependent manner, increased the general explorative locomotor activity of the animals tested. Similarly, the total number of arm entries in the Y-maze was significantly increased for the combinations of VGB + CZP and VGB + ESM, but not for VGB + PB and VGB + VPA. The application of VGB in combination with PB, ESM, CZP, and VPA suppressed the clonic phase of PTZ-induced seizures, having no harmful or deleterious effects on behavioral functioning of the animals tested, which might be advantageous in further clinical practice.
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INTRODUCTION

Rational polytherapy is accepted as a treatment of choice in patients with refractory seizures, whose epileptic attacks are resistant to the applied current front-line antiepileptic drugs (AEDs) in monotherapy. The problem of refractoriness of epileptic patients on the available medication with monotherapy still concerns around 30% of patients worldwide. In such cases, the clinicians are expected to adequately combine some AEDs in order to provide the patients with a state of seizure freedom. To date, several two-drug combinations have been approved as efficacious against specific forms of epileptic attacks (Stephen and Brodie, 2002). However, with the advent of newer (second generation) and some novel (third generation) AEDs, recently introduced to the therapy of epilepsy, a number of possible

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combinations exponentially increase. For instance, 10 AEDs may provide 45 various two-drug combinations, while 20 AEDs generate 190 diverse two-drug combinations. It is expected that several of them would be favorable in clinical practice. However, the direct testing of anticonvulsant efficacy of all two-drug combinations in patients with refractory epilepsies is impossible for ethical reasons and/or methodological difficulties. Nonetheless, such combinations may be easily preselected in preclinical studies on animals, and only those that display synergistic interactions, in terms of the antiseizure effects, can be further analyzed and verified in clinical conditions. As it is known, the anticonvulsant activities of all conventional and some newer AEDs (except for phenobarbital (PB)) have been first discovered in animals and, subsequently, these drugs proved efficacious in patients with epilepsy. Considering this fact, chances are next to none that some ineffective AED combinations in animal models of epilepsy prove advantageous in patients with epileptic attacks.

Vigabatrin (VGB; ((±)-4-amino-hex-5-enoic acid; gamma-vinyl-GABA)) is a newer AED with a specific mechanism of action. The drug consists of a racemic mixture of R(−)- and S(+)-enantiomers in equal proportions. The R(−)-enantiomer is completely inactive, but full pharmacological and toxic effects are produced by the S(+)-enantiomer (Haegele and Schechter, 1986; Rey *et al*, 1990). VGB binds to neuronal and glial γ-aminobutyrate-α-oxoglutarate aminotransferase (GABA-transaminase; GABA-T; EC 2.6.1.19), and irreversibly inhibits the enzyme, thus increasing GABA levels and enhancing GABA-ergic neurotransmission in the brain (Lippert *et al*, 1977; Jung *et al*, 1977; Abe and Matsuda, 1983). In preclinical studies, VGB exerted a significant anticonvulsant effect in amygdala-kindling rats (Myslobodsky *et al*, 1979; Kalichman *et al*, 1982; Shin *et al*, 1986), in photosensitive baboons (Meldrum, 1984), in mice exposed to strychnine or pentylenetetrazole (PTZ)-induced seizures (Mirski and Ferrendelli, 1986; Bernasconi *et al*, 1988; Stuchlik *et al*, 2001; Mares and Slamberova, 2004), as well as in bicuculline and picrotoxin seizure models (Kendall *et al*, 1981; Holland *et al*, 1992; Dalby and Nielsen, 1997). Experimental studies in the maximal electroshock-induced seizures (MES) in rodents have indicated some contradictory results showing that VGB is active against MES (Iadarola and Gale, 1981; Bonhaus and McNamara, 1988) or entirely ineffective in this test (Bernasconi *et al*, 1988; Holland *et al*, 1992). Moreover, it has been documented that VGB increased the absence seizure frequency and duration in lethargic mice (*lh/lh*)—a model of experimental absence seizures (Hosford and Wang, 1997)—and significantly increased spike–wave discharges in GAERS (the genetic absence epilepsy rat from Strasbourg) (Marescaux *et al*, 1992). These findings are consistent with the pro-absence effects of VGB observed in humans with myoclonic, tonic, and absence convulsions (Murphy and Delanty, 2000; Panayiotopoulos *et al*, 1997; Perucca *et al*, 1998; Guerrini *et al*, 1998).

At present, the drug is prescribed only as an add-on treatment for patients with partial epilepsy with or without secondary generalization, refractory to other available AEDs, as well as in children with infantile spasms, where VGB is indicated as the drug of choice and may be used in monotherapy (Bialer *et al*, 2001; Brodie and Schachter, 2001). The clinical application of VGB has been drastically limited after the observation that the drug irreversibly restricts the peripheral visual field in patients receiving VGB (Lawden *et al*, 1999; Wild *et al*, 1999). Experimental studies on rats have indicated that VGB preferentially accumulated in the retina reaching concentrations five-fold higher than in the brain, which was additionally associated with a three-fold increase in GABA levels in the retina (Sills *et al*, 2001).

This study was aimed at determining the characteristic of interactions between VGB and some conventional AEDs (ethosuximide (ESM), valproate sodium (VPA), clonazepam (CZP), and PB) in PTZ-induced seizures in mice. It is widely accepted that PTZ test is considered as an experimental model of epilepsy, in which the AEDs effective against myoclonic and to a certain extent absence seizures in humans protect also the experimental animals against the clonic phase of PTZ-induced seizures (Löscher and Schmidt, 1988; Löscher *et al*, 1991). To provide unequivocal evidence of efficacy of combinations between VGB and conventional AEDs, the evaluation of interactions was performed using the isobolographic analysis—an eligible method applied for the experimental determination and classification of observed interactions as supra-additive (synergistic), additive, subadditive (antagonistic), or indifferent. This method takes into consideration final effects observed for mixtures of two drugs applied at three fixed-ratio combinations of 1 : 3, 1 : 1, and 3 : 1. Additionally, to determine a preclinical pharmacological profile of interactions, the combinations at the fixed-ratio of 1 : 1 for all examined drugs in the PTZ test were evaluated in the chimney (motor performance), step-through passive avoidance (long-term memory), pain threshold (pain sensitivity), and Y-maze (rudimentary locomotor activity) tests. Furthermore, total brain concentrations of conventional AEDs were estimated in order to confirm or exclude the existence of any pharmacokinetic events that might affect the observed interactions between VGB and conventional AEDs.

MATERIALS AND METHODS

Animals and Experimental Conditions

All experiments were performed on adult male albino Swiss mice weighing 22–26 g. The mice were kept in colony cages with free access to food and tap water *ad libitum*, under standardized housing conditions (natural light–dark cycle, temperature was 21 ± 1°C). 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. Procedures involving animals and their care were conducted in conformity with current European Community 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 hereupon were approved by the Local Ethics Committee at the Medical University of Lublin and confirmed with the *Guide for the Care and Use of Laboratory Animals* (license no. 403/2003/432/03).

Drugs

The following AEDs were used in the study: VGB (SABRIL, Marion Merrell SA, Puteaux, France), VPA (kindly donated by ICN-Polfa SA, Rzeszów, Poland), PB (Polfa, Cracow, Poland), ESM (Sigma, St Louis, MO, USA), and CZP (Polfa, Warsaw, Poland). All drugs, except for VPA, were suspended in a 1% solution of Tween 80 (Sigma, St Louis, MO, USA) in distilled water, while VPA was dissolved in distilled water. The drugs were injected intraperitoneally (i.p.) in a volume of 0.005 ml/g body weight to avoid effects of overhydration and hypertension in animals. Fresh drug solutions were prepared *ex tempore* each day of the experiments and administered before seizures, motor coordination, long-term memory, pain threshold, and locomotor activity evaluations, as well as before brain level measurements as follows: VGB, 240 min; PB, 60 min; ESM, 45 min; and VPA and CZP, 30 min.

Route of i.p. administration and pretreatment times before testing of AEDs were based upon information about their biological activity from the literature (Löscher *et al*, 1989). All experimental procedures on animals were performed at times corresponding to the peak of maximum anticonvulsant effects of tested AEDs. It is widely accepted that VGB exhibited maximum protective activity 4 h after single-dose administration, although GABA-T activity is maximally inhibited at 24 h following the drug administration (Engelborghs *et al*, 1998). This is why the effects of VGB after 4 h of i.p. injection of the drug were evaluated in the present study.

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. Since the anesthetic and/or analgetic drugs may interfere with free plasma and brain concentrations of AEDs, the animals did not receive such drugs in our study.

In order to minimize the variability of animal behavioral response to the mild stress produced by handling and i.p. injections, each mouse was given two consecutive injections of vehicle (1% solution of Tween 80 in distilled water) or respective AEDs. For the mixture of VGB with a conventional AED, the animals received both drugs in two separate injections; however, when one of the mixture component drugs was tested alone, the animals were coadministered with an adequate amount of vehicle as the second injection. Similarly, the control animals in our study were given two consecutive injections of vehicle: the first one at 4 h before the testing procedure (that imitates the injection of VGB) and the second one at the time corresponding to a conventional AED tested. This procedure of two consecutive vehicle injections is a principle of behavioral studies, investigating the effects of two coinjected drugs influencing the central nervous system (Irwin, 1968). Briefly, to minimize the variance in animal behavioral responses among the examined groups, the animals were subjected to the same experimental conditions in our study.

Pentylenetetrazole-Induced Convulsions

Clonic convulsions were induced in mice by s.c. administration of PTZ at the doses ranging between 70 and 120 mg/kg. Following the injection of PTZ, mice were placed separately into transparent Plexiglas cages ($25 \times 15 \times 10$ cm) and observed for 30 min for the occurrence of clonic seizures. The clonic seizure activity was defined as the clonus of whole body lasting 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 condition. The convulsive action of PTZ was evaluated as CD_{97} (convulsive dose 97, ie the dose of PTZ that produced the clonic seizures in 97% of the mice). To determine CD_{97}, four or five various doses of PTZ were used (eight mice per group) and, subsequently, an intensity–response curve was calculated from the percentage of mice convulsing according to the log-probit method by Litchfield and Wilcoxon (1949). This experimental procedure has been described in more detail in our earlier study (Luszczki and Czuczwar, 2004a).

The anticonvulsant activity of VGB and conventional AEDs (ESM, CZP, VPA, and PB) against the clonic phase of PTZ-induced seizures was determined after s.c. administration of PTZ at its CD_{97} (110 mg/kg). The animals were treated with increasing doses of AEDs, and the anticonvulsant activity of each drug was evaluated as ED_{50} (median effective dose of an AED, protecting 50% of mice against clonic convulsions). At least four groups of animals were used to estimate each ED_{50} value calculated from the respective dose–response curves (DRCs), according to Litchfield and Wilcoxon (1949). Similarly, the anticonvulsant activity of a mixture of VGB with an AED 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 generalized clonic convulsions in mice.

Isobolographic Analysis of Interactions

Isobolographic analysis of interactions was performed according to the method detailed in our earlier studies (Luszczki *et al*, 2003a–c; Luszczki and Czuczwar, 2003, 2004a). It is widely accepted that isobolography allows the determination of equieffective doses of AEDs administered in various proportions in combination and, simultaneously, the adequate classification of observed interactions as supra-additive (synergistic), subadditive (antagonistic), indifferent, or additive (Berenbaum, 1989; Gessner, 1995; Tallarida *et al*, 1997; Luszczki and Czuczwar, 2003). The evaluation of median effective doses (ED_{50}s with 95% confidence limits) for each AED injected alone was performed by using the log-probit analysis according to Litchfield and Wilcoxon (1949). DRCs for all AEDs in the PTZ test were fitted by using linear regression analysis based upon the method of Litchfield and Wilcoxon (1949). The lines of best fit of DRCs for VGB and conventional AEDs were analyzed using χ^2 test and the test for parallelism, as described in our previous study (Luszczki and Czuczwar, 2004a). Subsequently, based upon these ED_{50} values, the median additive doses of mixtures of VGB with each conventional AED ($ED_{50\,add}$s), that is, doses of the drug mixtures that theoretically should protect 50% of the animals tested against convulsions for three fixed-ratio combinations of 1 : 3, 1 : 1, and 3 : 1, were calculated from the equation of additivity presented by Loewe (1953).

Subsequently, proportions of drugs in mixtures were evaluated and the mixtures of VGB with each studied AED were administered to the animals. The evaluation of experimental median drug doses in mixtures ($ED_{50\,mix}$s) for three fixed-ratios of 1 : 3, 1 : 1, and 3 : 1 was based upon the doses protecting 50% of animals tested against PTZ-induced seizures. The precise descriptions of theoretical background concerning the isobolographic analysis with the respective equations showing how to isobolographically analyze data have been presented in our previous studies (Luszczki *et al*, 2003a–c; Luszczki and Czuczwar, 2003, 2004a).

Measurement of Total Brain AED Concentrations

The animals were administered with an AED + vehicle or a combination of VGB with the respective AED. The fixed-ratio combination for estimating the total brain concentrations of AEDs was chosen as 1 : 1 for VGB and a conventional AED in combination. Mice were killed by decapitation at times chosen to coincide with that scheduled for the PTZ test and the whole brains of mice were removed from skulls, weighted, and homogenized using Abbott buffer (2 : 1 vol/wt) in a Ultra-Turrax T8 homogenizer (Staufen, Germany). The homogenates were centrifuged at 10 000 × g (MPW-360 centrifuge; Mechanika Precyzyjna, Warszawa, Poland) for 10 min. The supernatants of 75 μl were put into Abbott system cartridges, which were subsequently put into a carousel for up to 20 samples. Control samples of a conventional AED were placed at the beginning and end of each carousel for verification of the calibration. The total brain AED concentrations were analyzed by fluorescence polarization immunoassay using a TDx analyzer and reagents exactly as described by the manufacturer (Abbott Laboratories, North Chicago, IL, USA). In the case of estimation of CZP concentrations, an original Abbott reagent for 'Benzodiazepine' was used. The AEDs analyzed were ESM, VPA, PB, and CZP, and total brain concentrations were expressed in μg/ml except for brain CZP concentrations expressed in ng/ml of brain supernatants as means ± SD of at least eight determinations.

Chimney Test

The effects of VGB and conventional AEDs alone or in combinations on motor impairment were quantified with the chimney test of Boissier *et al* (1960). In this test, the animals had to climb backwards up the 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. The combinations of VGB with a conventional AED at the drug doses corresponding to the respective $ED_{50\,mix}$ were challenged with the chimney test and motor performance of the animals was determined.

Light–Dark, Step-Through Passive Avoidance Task

The test was performed as described by Venault *et al* (1986). The apparatus consisted of a two-compartment chamber with an illuminated box (10 × 13 × 15 cm) connected to a large darkened box (25 × 20 × 15) by a guillotine door. The darkened compartment is equipped with an electric grid floor (stainless steel rods through which an electric footshock is delivered). The entrance of animals to the darkened box was punished by an electric footshock (0.6 mA; facilitation of acquisition). During the training session, the mouse was placed in the illuminated compartment turned back from the guillotine door. After the mice entered the darkened compartment, the door was closed and an electric footshock was delivered. The mice that did not enter the dark compartment were excluded from the experiment. The next day (24 h later), the pretrained animals were again put into the illuminated box and observed for up to 180 s. Mice that avoided the dark compartment for 180 s were considered to remember the task. Time at which the mice entered the dark box was noted and subsequently, the medians with 25 and 75 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, may be regarded as a measure of long-term memory (Venault *et al*, 1986). The animals were administered with AEDs either singly or in combinations on the first day before training. The time before the commencement of the training session (after the drug administration) was identical to that for the PTZ test (ie VGB, 240 min; PB, 60 min; ESM, 45 min; VPA and CZP, 30 min). The next day (24 h later), the mice (without any treatment) were challenged with the test and the retention was measured. In the present study, the influence of AEDs, administered alone or in combinations, on long-term memory was evaluated in two variants as follows:

(1) Standard variant—performed as described above in which the time duration of an electric footshock was constant for all groups of animals tested and established on a standard value of 2 s (Venault *et al*, 1986).
(2) Modified variant—identically performed as the standard variant except for the time duration of an electric footshock, which was based upon the median latency to the first pain reaction of animals subjected to the pain threshold test. Each group of mice that received the adequate drug combination was challenged with an electric stimulation, whose duration exceeded twice the time required to evoke the first pain reaction in animals. More details have been described in our earlier study (Luszczki *et al*, 2003c).

Pain Threshold Test

The pain threshold was assessed as the minimal exposing time to an electrical stimulation required to induce a pain reaction in animals. This test was performed under conditions identical to the experiment testing of the step-through passive avoidance task. The animals were placed separately on the grid surface connected with a current generator. Afterwards, each mouse was exposed to a direct current (0.6 mA), and the time to induce the first pain reaction in animals was measured and expressed in seconds as the latency. For ethical reasons, the animals were exposed to the current impulse up to 8 s. In the event that the mouse did not express any reaction to the stimulus of 8 s, the test was terminated and the animal was assigned this cutoff

latency. It has to be stressed that the first pain reaction was considered as the end point and after displaying some reactions the animals were immediately set free from the stimulation. To evaluate the latency to pain reaction, the testing took place at times chosen to coincide with that scheduled for the PTZ test. The first pain reaction of animals observed in our study was expressed as jerks of forelimbs or the whole body with or without squeaking, violent seeking of escape with a tendency of jumping out, and wild running. The reaction of control animals to an electric stimulus was instantaneous and the latency did not exceed 2 s. All testing was performed in unanesthetized mice.

Spontaneous Locomotor Activity of Animals in the Y-Maze

Spontaneous locomotor activity in the Y-maze test is based on the rodents' innate curiosity to explore novel areas and to enter arms of the Y-maze. The Y-maze apparatus consisted of three identical arms (three compartments of $15 \times 15 \times 10$ cm) with the connector ($10 \times 6 \times 10$ cm) radiating out from the center. The walls of each arm of the Y-maze test were made of a diversely colored plastic: the first arm was black, the second white, and the third was striped in a black-and-white vertical patterning. The floor of each arm was covered by paper towels, which were changed at each testing in order to eliminate olfactory stimuli. To minimize stress occurring when animals are placed in new environmental conditions, the maze was placed in a sound-attenuated room under dim illumination (70 lx). Mice were placed separately at the end of one of the arms (starting arm), their head pointing away from the center of the maze, and they were allowed to freely traverse the apparatus for 5 min. The entrance of the animal was considered to be complete when the hind paws of the mouse had entirely entered the arm. The series of arm entries were manually recorded by an investigator who was not aware of the treatments. The total number of arm entries within 5 min reflects inquisitive behavior of animals (locomotor activity of animals at the rudimentary level). In the present study, the Y-maze was used only for locomotor and general exploration measurement (as presented by Hodges, 1996; D'Mello and Steckler, 1996).

During the locomotor activity testing in the Y-maze test, the new control groups of mice were determined each day of experiments; so, four separate controls were obtained. Noticeably, each mouse was used once and there were no repeated measures of locomotor activity in mice challenged with the Y-maze test. Since the Y-maze test is a time-consuming procedure, only one combination of AEDs (VGB + an AED) was investigated daily. Every combination of AEDs comprised four groups of animals (eight mice per group) as follows: control (vehicle + vehicle); VGB + vehicle; an AED + vehicle; and a mixture of both AEDs (VGB + an AED). Each experimental day, each mouse received two injections at the times corresponding to the peak of maximum anticonvulsant activity of AEDs tested: VGB or vehicle (as the first injection) and subsequently an AED or vehicle (as the second injection). Since each dose of combination was tested in eight animals and each mouse participated only in one experimental session, a total of 16 groups and 128 observations were made in the Y-maze test. It is noteworthy that all animals were tested in the same Y-maze apparatus; so, to test the animal behavioral response, at times of peak maximum anticonvulsant activities, the mice were sequentially injected with a 5-min period of delay between each other. Obviously, each mouse was branded to avoid mistakes and methodological errors. To confirm or exclude the existence of differences between locomotor patterns of control animals, the controls (as reference values) were evaluated each day of the experiment and compared to values obtained for the respective AEDs. Moreover, variance among control groups for 4 consecutive days of experiments was analyzed to confirm that there is no difference in variance in the Y-maze test for control animals.

Statistical Analysis

Median effective doses of AEDs (ED_{50}) with their 95% confidence limits were calculated by computer log-probit analysis (Litchfield and Wilcoxon, 1949). The 95% confidence limits obtained were transformed to standard errors of the mean (SEM) as described previously (Luszczki *et al*, 2003a, b). Statistical analysis of the observed interactions was performed by the use of Student's *t*-test to evaluate the difference between experimental ($ED_{50\,mix}$) and theoretical additive ($ED_{50\,add}$) values. Total brain AED concentrations administered alone or in combinations with VGB were statistically compared using unpaired Student's *t*-test. Since there is still no consensus on how to present the results from isobolographic analysis, all necessary statistical indicators (ie *t*-test values, the corresponding degrees of freedom, *P*-values, and numbers of animals tested for each additive and experimental group) were presented in order to facilitate the interpretation of such data by readers. Qualitative variables from the chimney test were compared with Fisher's exact probability test, whereas the results obtained in the passive avoidance task were statistically evaluated using Kruskal–Wallis nonparametric ANOVA followed by *post hoc* Dunn's test. Spontaneous locomotor activity (general exploration) of the mice evaluated in the Y-maze test was analyzed by one-way ANOVA followed by *post hoc* Bonferroni or Dunnett tests.

RESULTS

Anticonvulsant Effects of Examined AEDs against the Clonic Phase of PTZ-Induced Seizures in Mice

All antiepileptics studied, that is, VGB, PB, ESM, VPA, and CZP, displayed clear-cut antiseizure effects against the clonic phase of PTZ-induced seizures in mice. The ED_{50} values for all AEDs, calculated from their DRCs according to the log-probit method, are presented in Table 1.

Isobolographic Assessment of Interactions between VGB and Conventional AEDs

The mixture of VGB and ESM at the fixed-ratio of 1 : 1 exerted supra-additive (synergistic) interaction in the PTZ test in mice. $ED_{50\,mix}$ for this fixed-ratio combination was 221.2 mg/kg, whereas the corresponding $ED_{50\,add}$ was 376.5 mg/kg (Table 2). In this case, $ED_{50\,mix}$ (ie protecting

50% of the animals against the clonic phase of PTZ-induced seizures) was substantially reduced by 41% than theoretically presumed $ED_{50\,add}$ (at $P<0.05$; Table 2, Figure 1a). The remaining fixed-ratios tested for the mixture of VGB + ESM (ie 1 : 3 and 3 : 1) were additive in isobolography (Table 2, Figure 1a). Similarly, VGB combined with PB at the fixed-ratios of 1 : 3 and 1 : 1 exerted supra-additive (synergistic) interactions in the PTZ-test in mice. $ED_{50\,mix}$ for the mixture of VGB + PB at the fixed-ratio of 1 : 3 was 101.1 mg/kg, while $ED_{50\,add}$ amounted to 161.7 mg/kg. Thus, a significant reduction of a dose mixture by 37% was observed for this combination (at $P<0.05$; Table 2, Figure 1b). Likewise, the fixed-ratio of 1 : 1 for the mixture of VGB + PB was supra-additive in isobolography. In this case, $ED_{50\,mix}$ (186.4 mg/kg) was significantly lower than the corresponding $ED_{50\,add}$ (314.0 mg/kg), at $P<0.01$; hence, reduction of the dose mixture reached 41% (Table 2, Figure 1b). Only the mixture of VGB + PB at the fixed-ratio of 3 : 1 was additive in isobolography (Table 2, Figure 1b). Moreover, all fixed-ratio combinations, examined in the present study for the mixtures of VGB + VPA and VGB + CZP, were additive in the PTZ test in mice (Table 2, Figure 1c and d).

Table 1 Anticonvulsant Activity of Vigabatrin and Conventional AEDs Administered alone against PTZ-Induced Seizures in Mice

Drug	ED_{50} (mg/kg)	N	SEM
VGB	618.5 (482.0–793.7)	32	78.650
PB	9.4 (7.4–11.8)	24	1.122
ESM	134.4 (113.4–159.2)	32	11.627
VPA	145.5 (110.0–192.4)	40	20.739
CZP	0.011 (0.007–0.019)	16	0.003

Results are presented as median effective doses (ED_{50} in mg/kg; 95% confidence limits in parentheses) protecting 50% of animals tested against PTZ-induced convulsions. *N* : total number of animals tested at the doses whose expected anticonvulsant effects were between 16 and 84%, according to Litchfield and Wilcoxon (1949); SEM: standard error of the means. The antiepileptics were administered i.p.: vigabatrin (VGB) 240 min, phenobarbital (PB) 60 min, ethosuximide (ESM) 45 min, valproate (VPA) and clonazepam (CZP) 30 min, prior to the PTZ test. Clonic convulsions were evoked by the s.c. administration of PTZ at the dose of CD_{97}, which was 110 mg/kg.

Effect of VGB on the Total Brain Concentrations of Conventional AEDs

VGB administered singly at the dose of 181.7 mg/kg (corresponding to the dose of a mixture at the fixed-ratio of 1 : 1 from the PTZ test) elevated by 39% the total brain concentrations of ESM (39.5 mg/kg) from 1.80 to 2.51 μg/ml ($P<0.01$; Table 3). Similarly, VGB at 183.6 mg/kg raised by 15% the total brain concentrations of PB (2.8 mg/kg) from 1.72 to 1.98 μg/ml ($P<0.01$; Table 3). In contrast, VGB at a higher dose of 299.4 mg/kg did not affect the total brain concentrations of VPA (Table 3). Furthermore, the total brain concentrations of CZP (injected exceptionally at the dose of 5.6 mg/kg) were not changed following i.p. injection of VGB at the dose of 308.9 mg/kg (Table 3).

Influence of VGB Alone and Its Combinations with Conventional AEDs on Motor Performance in the Chimney Test in Mice

VGB up to the dose of 3000 mg/kg (injected singly, i.p., 4 h prior to the test) did not significantly impair the motor

Table 2 Interactions between Vigabatrin and Conventional AEDs against PTZ-Induced Seizures in Mice—An Isobolographic Analysis

Combination	FR	$ED_{50\ mix}$ (mg/kg)	N_{mix}	$ED_{50\ add}$ (mg/kg)	N_{add}	t	df	*P*	*I*
VGB+ESM	1 : 3	192.0 ± 20.5	24	255.4 ± 28.4	64	1.810	83	0.074	A
	1 : 1	221.2 ± 39.8*	32	376.5 ± 45.1	64	2.582	89	0.012	S
	3 : 1	539.3 ± 68.0	32	497.5 ± 61.9	64	0.418	94	0.677	A
VGB+VPA	1 : 3	206.0 ± 22.8	8	263.8 ± 35.2	72	1.378	51	0.174	A
	1 : 1	369.8 ± 23.2	24	382.0 ± 49.7	72	0.222	91	0.825	A
	3 : 1	629.0 ± 47.6	16	500.3 ± 64.2	72	1.610	70	0.112	A
VGB+PB	1 : 3	101.1 ± 17.3*	32	161.7 ± 20.5	56	2.259	84	0.027	S
	1 : 1	186.4 ± 24.5**	32	314.0 ± 39.9	56	2.725	83	0.008	S
	3 : 1	485.8 ± 44.3	24	466.2 ± 59.3	56	0.265	76	0.792	A
VGB+CZP	1 : 3	102.4 ± 19.3	32	154.6 ± 19.7	48	1.810	78	0.074	A
	1 : 1	308.9 ± 42.9	24	309.3 ± 39.3	48	0.006	70	0.995	A
	3 : 1	579.6 ± 38.0	16	463.9 ± 59.0	48	1.649	61	0.104	A

Results are presented as median effective doses (ED_{50} in mg/kg) ± SEM of a drug mixture, determined either experimentally ($ED_{50\ mix}$) or theoretically calculated ($ED_{50\ add}$) from the line of additivity. FR: fixed-ratio combination; N_{mix} and N_{add}: total number of animals tested at the doses whose expected anticonvulsant effects were between 16 and 84%, determined experimentally (N_{mix}) or calculated theoretically (N_{add}); *t*: Student's *t*-test value; df: degrees of freedom; *P*: probability; *I*: isobolographic characteristic of interaction (A: additivity; S: supra-additivity (synergy)). Statistical evaluation of data was performed with unpaired Student's *t*-test according to Porreca *et al* (1990). *Significantly different at $P<0.05$ and **$P<0.01$ *vs* the respective $ED_{50\ add}$. The clonic phase of PTZ-induced seizures was produced by the s.c. injection of PTZ at its CD_{97} (110 mg/kg).

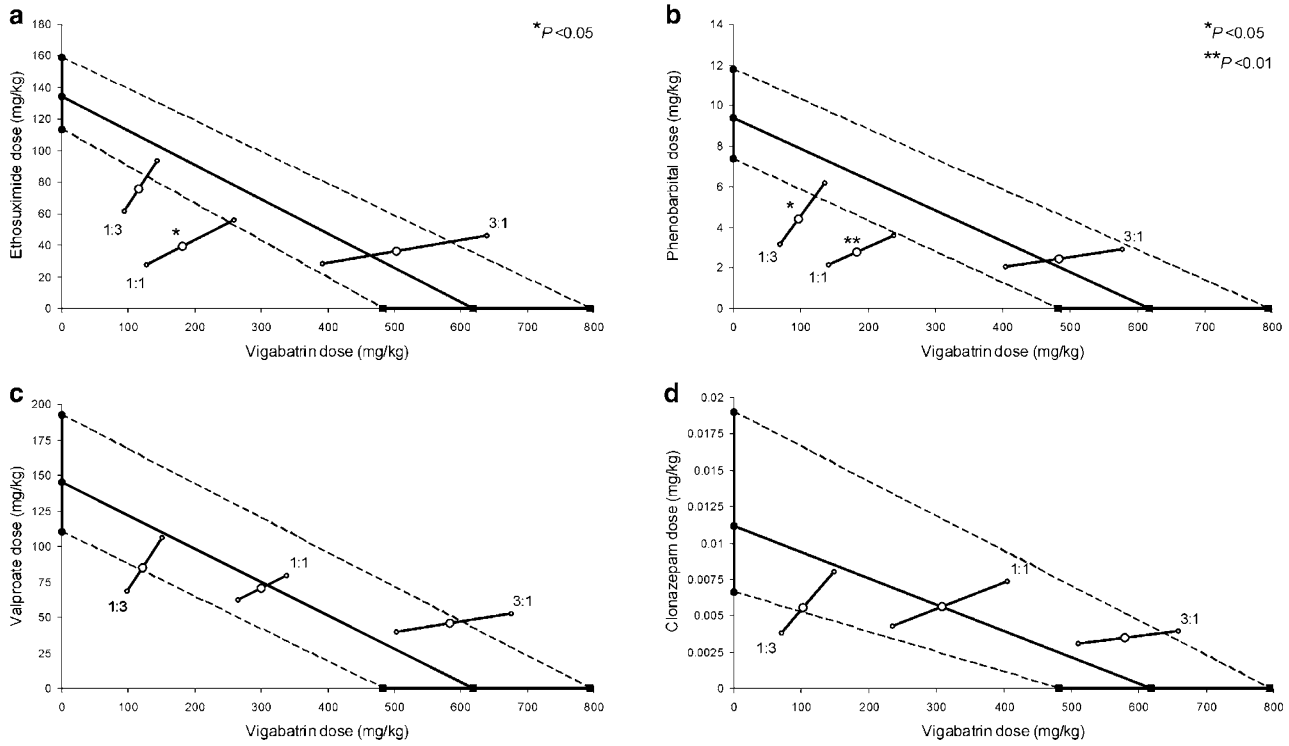

Figure 1 The median effective doses (ED_{50}) for VGB and ESM, PB, VPA, and CZP are shown plotted graphically (a–d, respectively). The solid line on the axes represents the 95% confidence limits (CLs) for the AEDs administered alone. The straight line connecting these two ED_{50} values on each graph represents the theoretical line of additivity for a continuum of different fixed-dose ratios. The open points (o) depict the experimentally derived $ED_{50\,mix}$s (with 95% CLs as the error bars) for total dose expressed as the proportion of VGB and a conventional AED that produced a 50% anticonvulsant effect. The dashed lines represent on each isobologram the theoretical additive 95% CLs of $ED_{50\,add}$s. (a) Interactions between VGB and ESM. The experimental $ED_{50\,mix}$s of the mixture of VGB + ESM for the fixed-ratio of 1 : 1 is significantly below the theoretical line of additivity, indicating supra-additive (synergistic) interaction at $P<0.05$. $ED_{50\,mix}$ for the fixed-ratio of 1 : 3 is close to the line of additivity, thus displaying a tendency toward supra-additivity. $ED_{50\,mix}$ for the fixed-ratio of 3 : 1 displays additivity. (b) Interactions between VGB and PB. The experimental $ED_{50\,mix}$s for the mixtures of VGB + PB at the fixed-ratios of 1 : 3 and 1 : 1 are significantly below the theoretical isobole of additivity, indicating supra-additive (synergistic) interactions at $P<0.05$ and $P<0.01$, respectively. Only, the fixed-ratio combination of 3 : 1 is close to the line of additivity, thus displaying pure additive interaction. (c) Interactions between VGB and VPA. The experimentally derived $ED_{50\,mix}$s for the mixtures of VGB + VPA at the fixed-ratios of 1 : 3 and 3 : 1 displayed a trend toward supra-additivity (for the fixed-ratio of 1 : 3) and subadditivity (for the fixed-ratio of 3 : 1), although their values did not reach statistical significance. $ED_{50\,mix}$ for the fixed-ratio of 1 : 1 is close to the line of additivity, suggesting pure additive interaction. (d) Interactions between VGB and CZP. The experimental $ED_{50\,mix}$s for the mixture of VGB + CZP at the fixed-ratios of 1 : 3 and 3 : 1 are placed near the line of additivity displaying a slight trend toward supra- and subadditivity, respectively. For the fixed-ratio of 1 : 1, pure additivity is noticeable.

coordination in animals tested. It was observed that two mice out of 10 in the experimental group (injected with VGB at 3000 mg/kg) did not correctly perform the chimney test. Moreover, none of the examined combinations of VGB with ESM, PB, VPA, and CZP, applied at the doses corresponding to $ED_{50\,mix}$s at the fixed-ratio combination of 1 : 1 from the PTZ test, produced motor deficits in the chimney test in mice (results not shown).

Pain Threshold Testing

Median latency to the first pain reaction for control animals was 1 s (Table 4). The increased doses of VGB applied alone lengthened the latency to the first pain reaction in a dose-dependent manner in mice. It was shown that VGB at the dose of 300 mg/kg significantly increased the pain latency from 1 to 2.25 s (at $P<0.01$; Table 4). Lower doses of VGB studied, that is, 100 and 200 mg/kg, lengthened the latency to 1.5 and 1.75 s, respectively. The median latency for VGB applied alone at the dose of 400 mg/kg increased to 2.2 s (at $P<0.01$), whereas the latency for VGB (500 mg/kg) was 2.5 s (at $P<0.01$; Table 4).

The combinations of VGB with conventional AEDs at the doses corresponding to their $ED_{50\,mix}$s also lengthened the latency to the first pain reaction in mice. Median latency for the combination of VGB + VPA was increased from 1 to 2.25 s (at $P<0.01$; Table 5). Likewise, the latency to the first pain reaction for the combination of VGB + CZP was considerably lengthened from 1 to 2.5 s (at $P<0.01$; Table 5). The remaining combinations tested (ie VGB + ESM and VGB + PB) in the pain threshold test did not significantly alter the pain latency in the animals tested (Table 5).

Dark Avoidance Acquisition and Retention Testing

VGB (299.4 mg/kg) co-administered with VPA (70.4 mg/kg) significantly altered long-term memory in mice challenged with the conventional variant of passive avoidance task. The median retention time of animals spent in the light compartment was significantly shortened from 180 to

Table 3 Influence of Vigabatrin (VGB) upon the Total Brain Concentrations of Conventional AEDs in Mice

Treatment (mg/kg)	Brain concentrations (μg/ml) or (ng/ml)
ESM (39.5)+vehicle	1.80±0.30
ESM (39.5)+VGB (181.7)	2.51±0.46**
PB (2.8)+vehicle	1.72±0.15
PB (2.8)+VGB (183.6)	1.98±0.17**
VPA (70.4)+vehicle	59.82±5.58
VPA (70.4)+VGB (299.4)	63.25±5.61
CZP (5.6)+vehicle	35.74±1.69
CZP (5.6)+VGB (308.9)	37.65±1.97

Results are presented as means±SD of at least eight determinants and expressed as μg/ml (or ng/ml for CZP) of brain supernatants. Statistical evaluation of data was performed with unpaired Student's *t*-test. Since the immunofluorescence assay was not sensitive enough to detect the concentration of CZP at 0.0056 mg/kg, the drug at the dose of 5.6 mg/kg (ie 1000-fold higher) was subjected to immunofluorescence determination. **Significant difference at $P<0.01$ *vs* the respective AED-alone-treated group. ESM: ethosuximide, VGB: vigabatrin; PB: phenobarbital; VPA: valproate; CZP: clonazepam.

Table 4 Effect of Increasing Doses of VGB on the Latency to the First Pain Reaction in Mice

Treatment (mg/kg)	Latency (s)
Control	1.0 (0.63–1.0)
VGB (100)	1.5 (1.0–2.0)
VGB (200)	1.75 (1.0–2.75)
VGB (300)	2.25 (2.0–2.88)**
VGB (400)	2.2 (2.0–2.5)**
VGB (500)	2.5 (2.0–3.38)**

Results are presented as median latencies (in seconds; 25 and 75 percentiles in parentheses) of 10 determinants. Statistical analysis of data was performed with Kruskal–Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons *post hoc* test. **$P<0.01$ *vs* the control group (vehicle-injected animals).

Table 5 Effect of VGB in Combination with Conventional AEDs on the Latency to the First Pain Reaction in Mice

Treatment (mg/kg)	Latency (s)
Control	1.0 (0.63–1.0)
VGB (181.7)+ESM (39.5)	2.0 (1.63–2.0)
VGB (183.7)+PB (2.8)	1.75 (1.23–2.0)
VGB (299.4)+VPA (70.4)	2.25 (1.63–3.0)**
VGB (308.9)+CZP (0.0056)	2.5 (1.5–3.0)**

Results are presented as median latencies (in seconds; 25 and 75 percentiles in parentheses) of 10 determinants. Statistical analysis of data was performed with Kruskal–Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons *post hoc* test. **Significantly different at $P<0.01$ *vs* the control group (twice vehicle-injected animals).

Table 6 Effect of VGB alone or Combined with Conventional AEDs on Long-Term Memory in the Conventional Variant of Step-Through Passive Avoidance Task in Mice

Treatment (mg/kg)	Retention (s)
Control	180 (180–180)
ESM (39.5)+vehicle	180 (173.8–180)
VGB (181.7)+vehicle	180 (176.8–180)
VGB (181.7)+ESM (39.5)	180 (131.5–180)
PB (2.8)+vehicle	180 (180–180)
VGB (183.6)+vehicle	180 (174.3–180)
VGB (183.6)+PB (2.8)	180 (162.8–180)
VPA (70.4)+vehicle	180 (147.5–180)
VGB (299.4)+vehicle	150 (120–180)
VGB (299.4)+VPA (70.4)	115.5 (83.5–128.3)[a]
CZP (0.0056)+vehicle	180 (173.8–180)
VGB (308.9)+vehicle	140 (120–180)
VGB (308.9)+CZP (0.0056)	97 (71–135.8)[a,b]

Results are presented as median retention time (in seconds; 25 and 75 percentiles in parentheses) of mice that avoided the entrance to the dark compartment. Statistical evaluation of data was performed with Kruskal–Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons *a posteriori* test.
[a]$P<0.01$ *vs* control (twice vehicle-treated animals).
[b]$P<0.05$ *vs* CZP-injected mice.
VGB: vigabatrin, ESM: ethosuximide, PB: phenobarbital, VPA: valproate, CZP: clonazepam.

115.5 s (at $P<0.01$; Table 6). Similarly, VGB (308.9 mg/kg) combined with CZP (0.0056 mg/kg) drastically reduced the retention time of mice from 180 (control) to 97 s (VGB + CZP) at $P<0.01$ (Table 6). Moreover, a slight reduction in time retention was observed for animals injected with VGB (299.4 or 308.9 mg/kg) alone or administered the combination of VGB + ESM; however, this reduction did not reach statistical significance (Table 6). All remaining combinations and separate component drugs examined did not influence long-term memory in mice challenged with the conventional variant of step-through passive avoidance task (Table 6).

In the modified variant of step-through passive avoidance task, none of the examined combinations of VGB with conventional AEDs, at the doses corresponding to their $ED_{50\,mix}$s from the PTZ-test, affected long-term memory in mice (Table 7). It should be noted that the time duration of an electric stimulus was two-fold higher than median latencies determined in the pain threshold test in mice.

Spontaneous Locomotor Activity Testing

In the Y-maze test, VGB administered alone (i.p., 4 h before the test) increased locomotor activity of tested animals in a dose-dependent manner. The mean number of arm entries for the control (vehicle-treated) animals was 21.5, whereas VGB at the doses of 100 and 200 mg/kg has no significant impact on spontaneous locomotor activity of animals. The

Table 7 Influence of VGB alone or in Combination with Conventional AEDs on the Long-Term Memory in Mice Challenged with the Modified Variant of Step-Through Passive Avoidance Task

Treatment (mg/kg)	Retention (s)
Control	180 (180–180)
VGB (181.7)+ESM (39.5)	180 (180–180)
VGB (183.7)+PB (2.8)	180 (180–180)
VGB (200)+vehicle	180 (180–180)
VGB (299.4)+VPA (70.4)	180 (180–180)
VGB (300)+vehicle	180 (176.5–180)
VGB (308.9)+CZP (0.0056)	180 (170–180)

Results are presented as median retention time (in seconds; 25 and 75 percentiles in parentheses) of eight determinants. Each group of animals was exposed to an electric stimulus, the duration of which was previously established in the pain threshold test. Statistical evaluation of data was performed with Kruskal–Wallis nonparametric ANOVA test followed by Dunn's *post hoc* test. For detailed information, see also the legend to Table 6.

Table 8 Effect of VGB on Spontaneous Locomotor Activity of Animals in the Y-Maze Test

Treatment (mg/kg)	Locomotor activity score
Control	21.50 ± 2.35
VGB (100)	22.25 ± 1.83
VGB (200)	25.50 ± 2.76
VGB (300)	31.50 ± 2.23*
VGB (400)	33.88 ± 2.37**
VGB (500)	35.25 ± 2.99**

Results of locomotor activity are presented as mean numbers of arm entries (±SEM) of eight mice challenged with the Y-maze test within 5 min of observation. Statistical evaluation of data was performed with one-way ANOVA followed by Dunnett's *post hoc* test. *$P<0.05$; **$P<0.01$ *vs* control (vehicle-treated animals). VGB: vigabatrin was injected i.p. 4 h before the commencement of the training session.

mean numbers of arm entries within the 5 min observational period were 22.25 and 25.5, respectively (Table 8). In contrast, VGB at the dose of 300 mg/kg significantly raised the mean number of arm entries from 21.5 (control) to 31.50 (VGB 300 mg/kg) at $P<0.05$ (Table 8). Likewise, VGB at 400 and 500 mg/kg considerably increased the spontaneous locomotor activity of animals in the Y-maze test. The mean numbers of arm entries were 33.88 and 35.25, respectively (both at $P<0.01$; Table 8).

In the Y-maze test, CZP injected alone at the dose of 0.0056 mg/kg significantly increased the number of arm entries from 19.63 (control) to 26.88 (CZP-injected mice) at $P<0.05$ (Table 9). Similarly, VGB at the dose of 308.9 mg/kg markedly increased locomotor activity of animals from 19.63 (control I) to 32.75 (VGB 308.9 mg/kg) at $P<0.01$. The combination of both AEDs (VGB + CZP) considerably increased the mean number of arm entries from 19.63 (control I) to 38.50 (VGB + CZP) at $P<0.001$ (Table 9). One-way ANOVA followed by Bonferroni's *post hoc* test

Table 9 Influence of VGB alone or in Combination with Conventional AEDs on Spontaneous Locomotor Activity of Animals in the Y-Maze Test

Treatment (mg/kg)	Locomotor activity score
Control I	19.68 ± 1.44
CZP (0.0056)+vehicle	26.88 ± 2.32*
VGB (308.9)+vehicle	32.75 ± 1.69**
CZP (0.0056)+VGB (308.9)	38.50 ± 3.31***,[a]
Control II	21.13 ± 1.88
VPA (70.4)+vehicle	18.75 ± 1.68
VGB (299.4)+vehicle	26.25 ± 1.33
VPA (70.4)+VGB (299.4)	26.88 ± 1.36[b]
Control III	20.38 ± 1.70
ESM (39.5)+vehicle	20.88 ± 1.27
VGB (181.7)+vehicle	25.13 ± 1.75
ESM (39.5)+VGB (181.7)	27.02 ± 1.62*
Control IV	20.88 ± 2.17
PB (2.8)+vehicle	28.14 ± 2.76
VGB (183.6)+vehicle	28.50 ± 2.38
PB (2.8)+VGB (183.6)	28.25 ± 3.43

Locomotor activity of animals is expressed as mean numbers of arm entries (±SEM) of eight mice challenged with the Y-maze test within 5 min of observation. Statistical evaluation of data was performed with one-way ANOVA followed by Bonferroni's *post hoc* test. Each combination was statistically compared to its own control since the observations were performed each day for 4 consecutive days. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ *vs* the respective control (twice vehicle-treated animals).
[a]$P<0.01$ *vs* CZP-treated animals.
[b]$P<0.05$ *vs* VPA-injected animals.
VGB: vigabatrin; CZP: clonazepam; VPA: valproate; ESM: ethosuximide; PB: phenobarbital.

revealed that the combination of VGB + CZP potentiated locomotor activity of animals when compared to CZP-injected alone (at $P<0.01$; Table 9). In the Y-maze test, VPA (70.4 mg/kg) slightly decreased locomotor activity of animals from 21.13 (control II) to 18.75 (VPA-injected animals) (Table 9). In such a situation, one-way ANOVA followed by Bonferroni *post hoc* test revealed a significant increase in locomotor activity of animals injected with VGB at 299.4 mg/kg. The mean number of arm entries was 18.75 (for VPA alone) and 26.88 (for VGB + VPA) at $P<0.05$ (Table 9). The combination of VGB + ESM markedly enhanced locomotor activity of animals when compared to the respective control animals. The mean number of arm entries was considerably elevated from 20.38 (control III) to 27.0 (VGB + ESM) at $P<0.05$ (Table 9). Furthermore, no significant changes in locomotor activity of animals were observed for the combination of VGB (183.6 mg/kg) with PB (2.8 mg/kg) (Table 9). Additionally, one-way ANOVA revealed no significant differences among the control groups tested each consecutive day for 4 days; so, the interday variance as to the spontaneous locomotor activity of animals was not significant.

DISCUSSION

The results presented herein indicate clearly that the mixture of VGB + PB exerted supra-additive (synergistic) interactions at the fixed-ratios of 1 : 3 and 1 : 1, whereas the mixture of both AEDs at the fixed dose ratio of 3 : 1 showed pure additive interaction against the clonic phase of PTZ-induced seizures in isobolography. Likewise, a mixture of VGB + ESM at the fixed-ratio of 1 : 1 displayed supra-additivity (synergy) in terms of the anticonvulsant activity in the PTZ test, while the remaining fixed-ratios tested between VGB and ESM (ie 1 : 3 and 3 : 1) were additive in this test. Furthermore, the drug mixtures of VGB + CZP or VGB + VPA for all fixed-ratio combinations tested (1 : 3, 1 : 1, and 3 : 1) occurred additive against the clonic phase of PTZ-induced seizures in mice. Additionally, it was observed that VGB combined with the examined conventional AEDs at the fixed-ratio of 3 : 1 (ie when the antiseizure effects exerted by VGB prevailed over those produced by conventional AEDs) displayed a tendency toward subadditivity (antagonism) in PTZ-induced seizures in mice. This fact is generally consistent with some experimental data obtained by other authors, who had demonstrated that VGB administered at high doses may paradoxically act as a pro-convulsive substance and induce seizures in the examined animals (Löscher *et al*, 1989; Stuchlik *et al*, 2001; Mares and Slamberova, 2004).

Pharmacokinetic estimation of conventional AEDs concentrations in whole brain homogenates of tested animals was performed only for the mixtures of the AEDs at the fixed-ratio of 1 : 1, where both AEDs (ie VGB and an evaluated conventional AED) were administered at the equieffective doses. The results from our pharmacokinetic study revealed a substantial increase in total brain concentrations of PB and ESM following the i.p. administration of VGB. Hence, it seems probable that the increased brain concentrations of both conventional AEDs (in biophase) are responsible for the observed supra-additive (synergistic) interactions in the PTZ test in mice. In contrast, VGB did not affect the total brain concentrations of VPA or CZP, whereby the isobolographic characteristic of these interactions was additive. In our previous study, it has been found that VGB administered at a subthreshold dose of 250 mg/kg did not affect the free plasma levels of VPA or CZP, but considerably elevated (by two-fold) the total plasma concentrations of ESM (Swiader *et al*, 2003). It has to be clearly stated that all concentrations of conventional AEDs in the present study were measured in biophase (ie in whole brain homogenates), since some distinct discrepancies between plasma and brain concentrations of conventional AEDs had been elicited in some isobolographic experiments (Cadart *et al*, 2002; Luszczki *et al*, 2003c). Additionally, pharmacokinetic effects of VGB on the conventional AEDs concentrations were evaluated in the present study following 4 h of VGB injection, while in our previous experiment, the concentrations of AEDs were measured after 1 h of VGB administration (Swiader *et al*, 2003). In spite of different time periods to the measurement of conventional AED concentrations and various tissues undergoing the examination, both pharmacokinetic evaluations are in strict agreement, revealing a pharmacokinetic nature of interactions between VGB and conventional AEDs. From a pharmacokinetic point of view, any changes in VGB concentrations (in biophase), after acute administration of the drug in combination with conventional AEDs, are improbable. As known, VGB possesses a favorable pharmacokinetic profile, since the drug does not bind to plasma proteins and is minimally metabolized by liver enzymes, being eliminated by renal excretion as unchanged drug (Patsalos and Sander, 1994; Patsalos and Perucca, 2003). Therefore, considering a pharmacokinetic profile of VGB, the drug interactions between VGB and other AEDs should not theoretically occur. However, in clinical practice, VGB decreased (by 20%) plasma phenytoin concentrations (Rimmer and Richens, 1989; Rey *et al*, 1992; Battino *et al*, 1995; Guberman *et al*, 2000). Additionally, it has been documented that VGB slightly reduced the plasma concentrations of PB or primidone in humans (Browne *et al*, 1989; Guberman *et al*, 2000), and conversely the drug increased plasma CBZ concentrations (Jedrzejczak *et al*, 2000). The exact causes of these pharmacokinetic interactions are unknown as yet. Perhaps, VGB is a potent inhibitor and/or inducer of some hepatic enzymes, which itself does not undergo a metabolic degradation, but substantially influences the metabolic pathways of coadministered AEDs. This hypothesis may theoretically explain the existence of observed pharmacokinetic interactions, although some possible changes in pharmacological profiles of VGB and concomitantly administered AEDs during the chronic treatment should be borne in mind. Possible explanations should also include the enhanced permeability of the blood–brain barrier alleviating the transportation of conventional AEDs to the brain after VGB administration.

Considering theoretically molecular mechanisms of action of the examined AEDs, it became clear that VGB may potentiate the antiseizure effects of some conventional AEDs tested. With respect to ESM's mechanism(s) of action, the advanced molecular and neurochemical studies have provided evidence that the drug preferentially binds to the inactivated state of low-threshold T-type Ca^{2+} channels and selectively inhibits pathological firing without any effect on normal neuronal activity (Coulter *et al*, 1989; Gomora *et al*, 2001). Moreover, it has been found that ESM decreases the Ca^{2+}-activated K^+ current in thalamo-cortical neurons (Coulter *et al*, 1989) and partially reduces the noninactivating Na^+ current (Leresche *et al*, 1998). All these changes in Na^+, K^+, and Ca^{2+} currents following the ESM administration are responsible for disrupting thalamo-cortical synchronized activity of neurons during spike and wave discharges *in vivo* (Crunelli and Leresche, 2002). So, taking into account the separate mechanisms of action of VGB and ESM, it is important to note that VGB (through the irreversible inhibition of enzymatic GABA degradation and indirect increase in GABA level within synaptic clefts) and ESM (by blocking low-threshold T-type Ca^{2+} channels in the thalamo-cortical neurons) should synergistically interact, reducing PTZ-induced convulsions in mice. As for the interaction of VGB and ESM, our results showed that a 39% increase in total brain ESM concentrations was associated with a significant decrease in ED_{50} value for the mixture of VGB with ESM at the fixed-ratio of 1 : 1. It was found that $ED_{50\,mix}$ (221.2 mg/kg) was lower (by 41%) than $ED_{50\,add}$ (376.5 mg/kg) (Table 2). In this case, the

increment of total brain ESM concentrations and reduction of ED_{50} were similar, suggesting that the observed supra-additive interaction of VGB and ESM is pharmacokinetic in origin. So, despite the existing theoretical presumptions as regards a possibility of pharmacodynamic potentialization of anticonvulsant effects offered by VGB and ESM (due to their complementary mechanisms of action), the experimentally derived synergistic effects produced by these AEDs in our study resulted from a pharmacokinetic interaction.

Another fact should be borne in mind and discussed here. It has recently been reported that the same two-AED combinations might differently interact exerting either supra-additivity or pure additivity, depending on the experimental models of epilepsy used. The example perfectly illustrating this phenomenon is the combination of PB with loreclezole (LCZ; a novel broad-spectrum AED, potentiating $GABA_A$ receptor-mediated Cl^- currents through interaction with an allosteric modulatory site within the supramolecular $GABA_A$ receptor/benzodiazepine receptor/chloride ionophore complex). With isobolography, it has been found that PB combined with LCZ at the fixed-ratio of 1 : 1 exerted supra-additivity in the MES test in mice (Luszczki and Czuczwar, 2004b) and, simultaneously, the same combination at the fixed-ratio of 1 : 1 exerted additivity in the PTZ test in mice (Luszczki *et al*, 2002). A similar situation was observed for the combinations of topiramate (TPM) with PB or VPA. In the amygdala-kindling model, TPM combined with PB or VPA significantly potentiated the anticonvulsant effects of coadministered AEDs, whereas the former drug in the PTZ test was without any effect on the antiseizure action of PB or VPA against PTZ-induced seizures (Borowicz *et al*, 2003). The above-mentioned facts indicate clearly that the final outcome of AED combinations is dependent on the experimental model of epilepsy used. Perhaps, diverse various molecular mechanisms of action of applied AEDs are responsible for the anticonvulsant activity of the drugs in various experimental models of epilepsy, displaying either synergistic or additive interactions. The same holds true for the observed efficacy of AEDs administered alone or in combination against various seizure types and epileptic attacks in patients. So, it is highly probable that various mechanisms of action offered by AEDs are involved in the anticonvulsant activity of the drugs against various seizure manifestations in epileptic patients. Considering that the same two-drug combination may differently act in diverse experimental models of epilepsy, theoretical presumptions concerning molecular mechanisms of action of applied AEDs cannot provide unequivocal evidence that the examined AED combinations will produce synergistic effects. In light of these facts, it seems that only experimental verification of theoretically beneficial combinations in animal models of epilepsy can yield overwhelming evidence on the efficacy of two-drug combinations for further clinical practice. To date, little is known about theoretical presumptions as to the combining of AEDs in order to obtain supra-additive (synergistic) effects against seizures in clinical practice since all clinically efficacious two-drug combinations have been empirically tested in clinical trials, retrospectively providing information about their further application in epileptic patients. In our study, the combination of VGB and ESM, in spite of theoretical presumptions about the synergistic cooperation of both AEDs in reducing PTZ-induced seizures, produced pure additive pharmacodynamic interaction, which was secondarily masked by a pharmacokinetic event leading to the increase in total brain ESM concentrations, resulting finally in supra-additivity observed with isobolography.

Similarly, in the case of PB combined with VGB, the drugs should also synergistically interact due to the complementary mechanisms of action. It is accepted that PB exerts its anticonvulsant effect by facilitating GABA-mediated inhibition through the allosteric modulation of neuronal postsynaptic $GABA_A$ receptors (Rogawski and Porter, 1990). The drug enhances the activation of $GABA_A$ receptors by increasing the mean channel open duration, having simultaneously no impact on open frequency and channel conductance (Barker and McBurney, 1979; MacDonald *et al*, 1989). The resultant increase in Cl^- flux through the supramolecular $GABA_A$ receptor/benzodiazepine receptor/chloride ionophore complex hyperpolarizes the postsynaptic neuronal cell membrane, thus disrupting the epileptiform transmission (Twyman *et al*, 1989). Moreover, PB is able to activate directly the $GABA_A$ receptors in the absence of GABA (Rho *et al*, 1996). Electrophysiological and biochemical studies have provided evidence that PB, at relatively low concentrations, inhibits responses mediated by excitatory amino-acid (non-NMDA) receptors (Miljkovic and MacDonald, 1986; Frandsen *et al*, 1990; Ko *et al*, 1997). Hence, the increment of GABA concentration into synaptic clefts after the inhibition of GABA degradation by VGB associated with the prolongation of time channel opening of postsynaptic $GABA_A$ receptors and inhibition of excitatory amino-acid-mediated neurotransmission by PB may contribute to the appearance of synergy between these AEDs with respect to their anticonvulsant activities in the PTZ-induced seizure model in mice. In our study, it was observed that a 15% increase in total brain PB concentrations was associated with a considerable (41%) reduction of ED_{50} for the mixture of VGB + PB at the fixed-ratio of 1 : 1 from 314.0 mg/kg ($ED_{50\,add}$) to 186.4 mg/kg ($ED_{50\,mix}$) (Table 2). Hence, the observed synergistic interaction, as regards the anticonvulsant activity of VGB and PB in the PTZ test, probably cannot be exclusively explained through the existence of pharmacokinetic alterations in total brain PB concentrations. It is highly likely that the interaction between VGB and PB is also pharmacodynamic in nature, and finally both pharmacokinetic and pharmacodynamic interactions may be responsible for supra-additive effects exerted by the combination of AEDs in the PTZ test in mice.

As for VPA, generally, the drug increases GABA in the whole brain and nerve terminals, although its precise molecular mechanisms of action are still unknown (Löscher, 2002). The drug inhibits enzymes involved in GABA degradation including GABA-T and succinic semialdehyde dehydrogenase (Löscher, 1980, 2002; Larsson *et al*, 1986; Phillips and Fowler, 1982). In addition, VPA increases the activity of glutamic acid decarboxylase, the enzyme responsible for GABA synthesis (Phillips and Fowler, 1982; Nau and Löscher, 1982; Luder *et al*, 1990). Additionally, VPA has been shown to block Na^+ channels in a voltage-dependent manner (McLean and Macdonald, 1986). In both

in vivo and *in vitro* experiments, VPA considerably suppressed *N*-methyl-D-aspartate (NMDA)-induced excitation in rat neocortical neurons (Zeise *et al*, 1991) and antagonized systemic and intracerebroventricular NMDA-induced convulsions in mice (Czuczwar *et al*, 1985; Turski *et al*, 1990). Moreover, it has been reported that VPA reduced convulsive activities induced by several α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor agonists in rodents (Turski *et al*, 1990; Steppuhn and Turski, 1993). Also, VPA (in clinically relevant concentrations) inhibited [^{3}H]AMPA binding to human post-mortem hippocampus (Künig *et al*, 1998). The drug has been shown to reduce the level and release of the excitatory amino-acid aspartate in rat and mouse brains (Chapman *et al*, 1984). Additionally, VPA (at high concentrations) activates K^+ conductance and blocks low-threshold T-type Ca^{2+} channels in peripheral ganglion neurons (Kelly *et al*, 1990). As for the combination of VGB with VPA, it should be clearly stated that VPA, due to its multiple mechanisms of action, whose main antiseizure effects are closely related with the enhancement of GABA neurotransmission within the brain, can exert additivity when combined with VGB because of the activation of similar mechanisms of action.

A growing body of evidence indicates that CZP interacts specifically with a benzodiazepine receptor site to modulate allosterically the efficiency of GABA at the $GABA_A$ receptors (Haefely, 1989; Macdonald, 2002). Moreover, CZP (such as all benzodiazepines) at high concentrations blocks Na^+ channels in a voltage-dependent manner, reducing high-frequency repetitive firing in cultured mammalian neurons (McLean and Macdonald, 1988). Considering the separate mechanisms of action of VGB and CZP, one can suppose that these AEDs should also synergistically interact and potentiate the GABA inhibitory neurotransmission. Nevertheless, results in our study indicated that both AEDs exerted barely additive interaction. In this case, the observed additivity between VGB and CZP might be largely accounted for by the administration of CZP at very low doses ranging from 0.003 to 0.008 mg/kg, which could be insufficient to potentiate the antiseizure effects offered by VGB (Figure 1d). In this case, no pharmacokinetic alterations in total brain CZP concentrations were detected. Moreover, effects of VGB combined with diazepam (DZP, another benzodiazepine ligand similar to CZP) on spike and wave discharges have recently been analyzed isobolographically in rats (Bouwman *et al*, 2004). However, the authors have examined the combination of VGB and DZP only in one fixed-ratio of 25 : 1 (VGB : DZP). Unfortunately, this fixed-ratio combination was inadequately preselected for isobolographic analysis and the authors had failed to display any interaction between these AEDs (Bouwman *et al*, 2004). In their study, DZP was administered at very high doses (up to 5 mg/kg), which entirely masked the effects produced by VGB (15–125 mg/kg), so, as the authors themselves have stated, no conclusions could be provided for further clinical practice from such an experiment (Bouwman *et al*, 2004). No doubt exists that the adequate (optimal) preselection of fixed-ratio combinations for testing interactions between AEDs is the first step in isobolography (a principle of isobolography; Gessner, 1995; Luszczki and Czuczwar, 2004a). Being aware of this fact, the isobolographic analysis of interaction between VGB and conventional AEDs in our study was performed for three fixed-ratio combinations, although, sometimes, many more fixed dose ratios would be required to characterize precisely the interactions between two AEDs (Gessner, 1995; Luszczki and Czuczwar, 2003; Luszczki *et al*, 2003a–c). Briefly, the lack of adequate determination of fixed drug–dose ratio combinations may become a main cause of fatal methodological errors that entirely destroy the isobolographic experiments, providing no additional information and conclusions as for the examined combinations.

The standard screening tests, estimating the adverse-effect profile of VGB coadministered with conventional AEDs, were insufficient to detect all possible effects produced by the drugs in the present study. It was shown that VGB up to 3000 mg/kg did not significantly impair motor performance of the examined animals in the chimney test. Similarly, the combinations of VGB with conventional AEDs at the doses corresponding to $ED_{50\,mix}$ for the fixed-ratio of 1 : 1 did not affect motor coordination in tested animals. However, VGB at the doses of 308.9 and 299.4 mg/kg alone or combined with CZP and VPA substantially altered long-term memory in mice challenged with the standard variant of passive avoidance task. It was observed that the mice treated with VGB or its combinations with CZP and ESM spent less time in the light compartment in the standard variant of step-through passive avoidance task as compared to control, but not in the modified variant of step-through passive avoidance task, which considered the latency for the first pain reaction, showing that the antinociceptive effects of VGB prevented the adequate acquisition of step-through passive avoidance task. The results from the pain threshold test indicated evidently that VGB, in a dose-dependent manner, lengthened the time to the first pain reaction in animals subjected to the pain threshold evaluation. Hence, the alterations in long-term memory in the conventional variant of step-through passive avoidance test was falsely evoked by the lack of animal response to an electric stimulation, being a necessary factor permitting the mice to acquire the memory related to this aversive stimulus. No doubt exists that the animals will not remember the task if they do not experience the aversive (punitive) stimulus and they do not learn the task at the beginning. Considering that in the step-through passive avoidance test the animals have to acquire (learn) the task, based on an aversive (punitive) stimulus as a negative reinforcement, it is widely accepted that if this stimulus will be attenuated by an antinociceptive effect of a drug, the animals cannot properly learn and, consequently, associate stimuli and finally form a memory for this event. Our modified variant of step-through passive avoidance test, in which the time duration of stimulation was based upon the latency to the first pain reaction from the pain threshold test, revealed that neither VGB alone nor in combinations with conventional AEDs affected long-term memory. Hence, the time duration of an electric stimulus required to induce an aversive memory pattern in animals should be first evaluated in the pain threshold test and, subsequently, used in the step-through passive avoidance task. More detailed discussion concerning the role of antinociception, its detection, and impact on long-term memory in experimental animals has been presented in our previous study,

where tiagabine and gabapentin also exerted the antinociceptive effects and altered long-term memory in animals tested (Luszczki *et al*, 2003c).

Quite recently, in *in vivo* experiments, it has been found that the direct microinjection of VGB into the rostral agranular insular cortex (RAIC) in rats resulted in a clear and consistent analgesia (Jasmin *et al*, 2003). This VGB-mediated antinociceptive effect was reversed by coinjection with the $GABA_A$ receptor antagonist bicuculline, providing evidence that the observed analgesic effect is dependent on the increased GABA concentration (Jasmin *et al*, 2003). Moreover, it has been observed that systemic (i.p.) administration of VGB suppressed the pain sensation in experimental animals challenged with the formalin test (Czuczwar *et al*, 2001). So, there is no doubt that by increasing GABA concentration within the RAIC or other parts of the brain, one can obtain the increment of pain threshold and the resultant analgesia. The additional antinociceptive effects produced by VGB may be of similar significance for patients with epilepsy, who similar to normally healthy people suffer from incidental pains (headaches, migraine, odontalgias, algomenorrhoeas, etc). In such situations, the epileptic patients suffering from pain take additionally some commonly available analgesics, which may interact pharmacokinetically with applied AEDs changing their plasma levels, which consequently may provoke some neurotoxic effects or even evoke seizures. There is no doubt that by eliminating incidental pains and the associated analgesic medications, the risk of loss of adequate anticonvulsant control in epileptic patients is reduced, which evidently contributes to amelioration of patients' quality of living. It has to be emphasized that these additional properties of VGB would be clinically valuable to treat adequately the patients with seizures. However, more advanced clinical trials are required to assess the antinociceptive effect offered by VGB. It should be noted that tiagabine and gabapentin (the drugs influencing GABA-ergic neurotransmission in the brain) potently suppress neuropathic pain in both experimental and clinical conditions (Bennett and Simpson, 2004; Backonja, 2002; Laughlin *et al*, 2002).

To further clarify and elicit any subtle adverse effects produced by VGB alone or in combinations with conventional AEDs, the Y-maze test was used. In this test, another unexpected phenomenon was ascertained. It was found that VGB combined with ESM or CZP, at the doses corresponding to $ED_{50\,mix}$ at the fixed-ratio of 1 : 1 from the PTZ test, increased locomotor activity of the examined mice. Additionally, it was shown that VGB in a dose-dependent manner enhanced locomotor activity of animals tested, raising the mean number of arm entries within 5 min of the observational period. It should be clearly stated that the Y-maze test in our study was used only to evaluate the influence of AEDs on general locomotor activity. Since it was observed that VGB enhanced dose-dependently locomotor activity of mice, the further determination of learning and working memory processes in the Y-maze could not be performed. No doubt exists that the animals with increased locomotor activity may paradoxically display improvement and amelioration of learning and working memory processes, because they can quickly perform the test as compared to the control animals. On the other hand, the agitation (hyperlocomotor activity) may be associated with a high frequency of chaotic movements in animals, which additionally votes against the use of the Y-maze for testing alternation and working memory in such a situation. Briefly, after corroborating hyperlocomotor activity in mice challenged with the Y-maze, the testing procedure was terminated in our study. It should be emphasized that the results from the locomotor activity testing are generally consistent with those observed in clinical practice, since hyperkinesias and agitation, in children receiving high doses of VGB as an adjunctive AED, have been reported (Brodie and Schachter, 2001). This increased locomotor activity can be explained through the increase in GABA concentration within the brain (Schechter *et al*, 1977; Petroff *et al*, 1996; Preece *et al*, 1994). In such a situation, GABA released from presynaptic terminals binds to $GABA_A$ receptors clustered at the postsynaptic neuronal membranes and activates inhibitory postsynaptic currents (IPSCs). Moreover, the increased GABA concentration stimulates the binding to $GABA_B$ autoreceptors present on presynaptic membranes, where their activation reduces the release of GABA (Olsen and Avoli, 1997; Treiman, 2001; Overstreet and Westbrook, 2001). Thus, it is possible that drugs enhancing the GABA-ergic neurotransmission in the brain may paradoxically evoke excitation instead of inhibition, especially if the drugs are administered for the first time at high doses. Moreover, a slight improvement in cognitive performances in patients taking VGB has been clinically reported (Provinciali *et al*, 1996).

Considering all above-mentioned facts, VGB combined with conventional AEDs might occur advantageous in patients with intractable seizures. No deleterious side effects were associated with the combinations of VGB and conventional AEDs. So, the pharmacological preclinical profile of such combinations is beneficial, although some additional 'adverse effects' such as the enhancement of locomotor activity and antinociceptive effects of VGB were detected. Moreover, it was ascertained that conventional screening tests, commonly used in preclinical studies, were insufficient to detect, examine, and elicit the adverse-effect profile of VGB alone or combined with conventional AEDs. Therefore, much more advanced experiments (associated with evaluation of the pain threshold) were required to provide evidence on pharmacological characteristic of interactions between VGB and conventional AEDs. The pain threshold test should be included in preclinical screening tests in order to analyze properly the effects of the drugs on long-term memory in animals challenged with the step-through passive avoidance task as well as to elicit some additional antinociceptive properties offered by AEDs.

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