Pharmacological Reports 71 (2019) 299–305

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

New derivative of 1,2,4-triazole-3-thione (TP427) potentiates the anticonvulsant action of valproate, but not that of carbamazepine, phenytoin or phenobarbital in the mouse tonic-clonic seizure model

Jarogniew J. Łuszczki[a,b,*], Paweł Marzeda[a], Agata Gut-Lepiech[a], Maria W. Kondrat-Wróbel[a], Paula Wróblewska-Łuczka[a], Sławomir Karwan[c], Tomasz Plech[d]

[a] Department of Pathophysiology, Medical University, Lublin, Poland
[b] Isobolographic Analysis Laboratory, Institute of Rural Health, Lublin, Poland
[c] Regional Specialized Children's Hospital, Olsztyn, Poland
[d] Department of Pharmacology, Medical University, Lublin, Poland

ARTICLE INFO

Article history:
Received 7 August 2018
Received in revised form 8 November 2018
Accepted 4 January 2019
Available online 6 January 2019

Keywords:
Antiepileptic drugs
Tonic-clonic seizures
Pharmacokinetic/pharmacodynamic interaction
Isobolographic transformation

ABSTRACT

Background: To assess the effects of 5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (TP427) on the protective anticonvulsant action of four classical antiepileptic drugs (carbamazepine, phenobarbital, phenytoin and valproate) in the tonic-clonic seizure model in mice, an isobolographic transformation of data was used.

Methods: Electrically-induced tonic-clonic seizures were experimentally evoked in adult male albino Swiss mice. The anticonvulsant effects of TP427, when used singly, were determined by the calculation of the threshold increasing the dose by 20% (TID_{20} value). The influence of TP427 on the anticonvulsant potency of four various classical antiepileptic drugs was determined with a subthreshold method. Types of interactions between drugs were determined using the isobolographic transformation of data. Additionally, total brain antiepileptic drug concentrations were measured.

Results: TP427, when administered separately, significantly increased the threshold for electroconvulsions. The experimentally determined TID_{20} value for TP427 was 11.71 mg/kg. Moreover, TP427 (10 mg/kg) significantly increased the anticonvulsant activity of valproate ($p < 0.01$), but not that of carbamazepine, phenobarbital or phenytoin in the mouse tonic-clonic seizure model. Isobolographic transformation of data confirmed that the interaction between TP427 and valproate was synergistic. Pharmacokinetic study revealed that TP427 increased total brain valproate concentrations, and had no impact on total brain concentrations of carbamazepine, phenobarbital or phenytoin in mice.

Conclusion: The synergistic interaction between TP427 and valproate in the mouse tonic-clonic seizure model might occur favorable for epilepsy patients in future. The combinations of TP427 with carbamazepine, phenobarbital and phenytoin were additive in the mouse tonic-clonic seizure model and also deserves clinical attention.

Introduction

Epilepsy is one of the most serious neurological disorders affecting approx. 1% of the human population worldwide [1,2]. Despite progress in the treatment of patients with epilepsy, due to the introduction of several novel (third-generation) antiepileptic drugs, there are still a number of epilepsy patients who are refractory to the currently available antiepileptic drugs [3,4]. There is a substantial need to create novel more efficacious therapeutic strategies in order to offer these patients a significant reduction of seizures and/or a state of freedom from seizures [5]. Nowadays, researchers and clinicians focus their studies on the design and discovery of novel therapeutic agents possessing a wide therapeutic (anticonvulsant) spectrum with no or minimal side-effects [5].

Abbreviations: TP427, 5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione.

* Corresponding author.
E-mail address: jarogniew.luszczki@umlub.pl (J.J. Łuszczki).

https://doi.org/10.1016/j.pharep.2019.01.003
1734-1140/

Relatively recently, a novel drug structure has become of interest in medicinal chemistry as the structure for promising novel anticonvulsant agents. Experimental evidence indicates that 1,2,4-triazole-3-thione derivatives possess the anticonvulsant properties in *in vivo* preclinical studies. Several 1,2,4-triazole-3-thione derivatives produced the anticonvulsant effects in the mouse tonic-clonic seizure model [6–11]. It is widely accepted that the antiepileptic drugs, which suppress tonic hind limb extension in mice in the mouse tonic-clonic seizure model are also active in protecting epilepsy patients with tonic-clonic seizures and partial onset convulsions with or without secondary generalization [12,13]. Moreover, some 1,2,4-triazole-3-thione derivatives (TP4 and TP10) enhanced the anticonvulsant potency of some classical antiepileptic drugs in the mouse tonic-clonic seizure model [6,7].

Considering the above-mentioned facts, it seems interesting and necessary to continue experiments with 5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (TP427 – a novel promising 1,2,4-triazole-3-thione derivative (Fig. 1), in order to determine its anticonvulsant properties in combination with four classical antiepileptic drugs (carbamazepine, phenobarbital, phenytoin and valproate) in the mouse tonic-clonic seizure model. The anticonvulsant effects exerted by the combination of TP427 with classical antiepileptic drugs were determined with a subthreshold method, and subsequently transformed isobolographically to precisely characterize the nature of interaction between the investigated drugs. Finally, to verify whether the observed anticonvulsant interactions of TP427 with classical antiepileptic drugs were pharmacodynamic, pharmacokinetic or both, in nature, total brain antiepileptic drug concentrations were measured.

Materials and methods

Animals

In this study, 456 adult (8–9 week-old) male albino Swiss mice were used. After a week of adaptation to laboratory conditions, the mice (weighing 24 ± 3 g) were randomly assigned to experimental groups. Each group consists of 8 mice. All experiments complied with the ARRIVE guidelines and were conducted in strict accordance with the EU Directive 2010/63/EU for animal experiments.

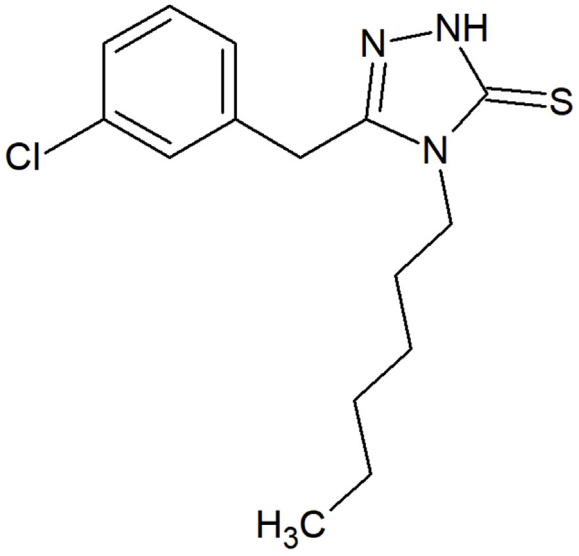

Fig. 1. Structural formula of 5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (TP427).

Drugs

We used: TP427 (5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione) (Fig. 1), carbamazepine (Polpharma, Starogard Gdanski, Poland), phenobarbital (Polfa, Krakow, Poland), phenytoin and valproate (both from Sigma-Aldrich, Poznan, Poland). The studied drugs were administered *intraperitoneally* (*ip*) as follows: phenytoin - 120 min, phenobarbital - 60 min, carbamazepine and valproate - 30 min, and TP427 - 15 min, before tonic-clonic seizures and brain sampling for the estimation of total antiepileptic drug concentrations, as reported earlier [9,14,15].

Threshold for tonic-clonic seizures

Tonic-clonic seizures in mice were induced by means of an alternating current (50 Hz; 500 V) delivered *via* ear-clip electrodes, with stimulus duration of 0.2 s. In this seizure model, 3 groups of control mice were challenged with current, which intensities ranged between 5–8 mA. The threshold for tonic-clonic seizures was expressed as the median current strength value (CS_{50} in mA). Similarly, the threshold for tonic-clonic seizures was determined for 3 increasing doses of TP427 (5, 10 and 20 mg/kg) and current intensities ranged from 6 to 10 mA. The CS_{50} values were calculated from the log-probit method [16]. Next, for each CS_{50} value for TP427 (in doses of 5, 10 and 20 mg/kg), a percentage of threshold increase over the threshold for control animals was calculated. Linear regression analysis of doses of TP427 and their threshold increases allowed calculation of the threshold increasing doses by 20% (TID_{20} values), as recommended earlier [12,17,18].

Tonic-clonic seizures in animals

The tonic-clonic seizures in mice were induced by an alternating current (50 Hz; 500 V) with a fixed current intensity of 25 mA, delivered *via* ear-clip electrodes with stimulus duration of 0.2 s. In this model, 3 groups of mice were treated with increasing doses of the antiepileptic drug alone or in combination with TP427, and subjected to the electrically-evoked seizures. The protection of the animals from electrically-induced tonic-clonic seizures was expressed as median effective dose (ED_{50}) of the antiepileptic drug, according to the log-probit method [16].

Isobolographic transformation of data

Isobolographic transformation of data was performed in strict accordance to the procedure described for the two-drug mixture by Loewe [19]. In this study, the ED_{50} fractions for the respective classical antiepileptic drugs and TP427 were calculated from the equation of additivity by Loewe. Summing the fractions of ED_{50} of two drugs (*i.e.*, TP427 and the classical antiepileptic drug), it was possible to approximately determine the isobolographic characteristic of interaction for the analyzed two-drug combination, as reported elsewhere [20].

Measurement of total brain antiepileptic drug concentrations

Pharmacokinetic estimation of total brain concentrations of classical antiepileptic drugs was performed by fluorescence polarization immunoassay in mice that received the antiepileptic drugs either alone or in combination with TP427 (10 mg/kg), in doses reflecting their ED_{50} values from the tonic-clonic seizure model. Preparation of the brain tissue has been described elsewhere [6,7]. Brain concentrations of classical antiepileptic drugs were expressed in μg/ml of brain supernatants as means ± SEM of 8 separate brain preparations.

Statistical analysis

The CS_{50} and ED_{50} values (± SEM) were statistically analyzed with one-way ANOVA followed by the *post-hoc* Tukey-Kramer test. The unpaired Student's *t*-test was used to statistically compare the ED_{50exp} and ED_{50add} values for each combination of an antiepileptic drug with TP427 and total brain concentrations of classical antiepileptic drugs.

Results

Effect of TP427 on the threshold for tonic-clonic seizures in mice

TP427 administered singly (*ip*, 15 min before the test) in doses of 5, 10 and 20 mg/kg elevated the threshold for tonic-clonic seizures in mice (Table 1). Moreover, TP427 in a dose-dependent manner increased the threshold for tonic-clonic seizures over the threshold for control animals by 6%, 18% and 36%, respectively (Table 1; Fig. 2). Linear regression of doses of TP427 and their corresponding threshold increases allowed the calculation of TID_{20} value that amounted to 11.71 mg/kg (Fig. 2).

Effect of TP427 on the anticonvulsant activity of various classical antiepileptic drugs in the tonic-clonic seizure model in mice

In the mouse tonic-clonic seizure model, TP427 (10 mg/kg) considerably potentiated the anticonvulsant action of valproate by reducing its ED_{50} value by 34%, from 320.1 mg/kg to 211.0 mg/kg ($p < 0.01$) (Fig. 3D). In contrast, TP427 (10 mg/kg) had no significant impact on the anticonvulsant action of carbamazepine, phenobarbital or phenytoin in the tonic-clonic seizure test in mice (Fig. 3A–C). Similarly, TP427 (5 mg/kg) did not affect the anticonvulsant action of all the tested antiepileptic drugs in this seizure test in mice (Fig. 3A–D).

Isobolographic transformation of interactions of TP427 with classical antiepileptic drugs in the tonic-clonic seizure model in mice

The ED_{50exp} values for the combinations of TP427 (10 mg/kg) with carbamazepine, phenobarbital and phenytoin did not differ from the theoretical additive ED_{50add} values (Table 2; Fig. 4A–C). Only the ED_{50exp} value for the combination of TP427 (10 mg/kg) with valproate significantly differed from the theoretical additive ED_{50add} value ($p < 0.01$) (Table 2; Fig. 4D). Isobolographic transformation revealed that the interaction for the mixtures of TP427 with carbamazepine, phenobarbital and phenytoin was additive (Fig. 4A–C). Only the interaction for the combination of TP427 with valproate was synergistic in the mouse model of tonic-clonic seizures. In this case, the ED_{50exp} value was placed significantly below the ED_{50add} value ($p < 0.01$) (Fig. 4D).

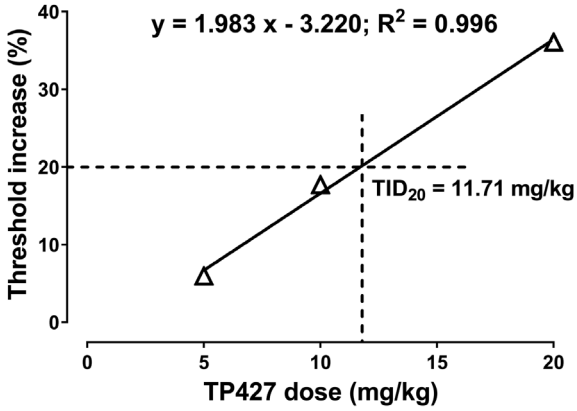

Fig. 2. Doses of TP427 and their corresponding threshold increases in the mouse tonic-clonic seizure threshold test. Points illustrate threshold increasing doses of TP427 from the mouse tonic-clonic seizure threshold test. Doses of TP427 were linearly related to the threshold increases. The dashed lines indicate the TID_{20} (threshold increasing doses by 20%) value for TP427.

Effect of TP427 on total brain antiepileptic drug concentrations

TP427 (10 mg/kg) considerably elevated (by 21%; $p < 0.05$) the total brain concentration of valproate (211 mg/kg) (Fig. 5D). In contrast, TP427 (10 mg/kg) had no significant impact on total brain concentrations of carbamazepine, phenobarbital and phenytoin in experimental animals (Fig. 5A–C).

Discussion

Results presented in this study revealed that TP427 significantly potentiated the anticonvulsant potency of valproate in the mouse model of tonic-clonic seizures. In contrast, the anticonvulsant effects of carbamazepine, phenobarbital or phenytoin remained almost unchanged after co-administration of TP427 in this seizure model in mice. To characterize the nature of interaction occurring between TP427 and classical antiepileptic drugs, we used the isobolographic transformation allowing for the proper classification of interaction between drugs, when one of these drugs is used in a fixed-drug dose (*i.e.*, TP427 in the constant dose of 10 mg/kg). Isobolograms revealed that only the combination of TP427 with valproate produced synergistic interaction in the mouse model of tonic-clonic seizures. The other studied combinations (*i.e.*, TP427 with carbamazepine, phenobarbital and phenytoin) exerted additivity in this seizure model.

In this study, we determined the influence of TP427 on the anticonvulsant activity of four classical antiepileptic drugs by using the subthreshold method, and subsequently, the results from the subthreshold method were isobolographically transformed in order to characterize the nature of interaction between TP427 and classical antiepileptic drugs. The isobolographic transformation of

Table 1
Isobolographic analysis of interaction between TP427 and four classical antiepileptic drugs in the tonic-clonic seizures in mice.

Drug combination	ED_{50exp} (mg/kg)	n_{exp}	ED_{50add} (mg/kg)	n_{add}	I.I.
Carbamazepine + TP427	22.92 ± 1.50	16	23.32 ± 1.12	52	0.98
Phenobarbital + TP427	30.80 ± 3.11	24	31.95 ± 1.45	52	0.96
Phenytoin + TP427	20.43 ± 1.41	16	21.72 ± 1.23	52	0.91
Valproate + TP427	221.0 ± 21.0**	16	285.7 ± 10.7	44	0.80

All ED_{50exp} and ED_{50add} values (± SEM) were calculated by using log-probit analysis.
n_{exp} and n_{add} – total numbers of animals used at those doses, whose expected antiseizure effects ranged between 4–6 probits.
I.I. – interaction index (a ratio of ED_{50exp} and ED_{50add} values).
** $p < 0.01$ *vs.* respective ED_{50add} value (unpaired Student's *t*-test).

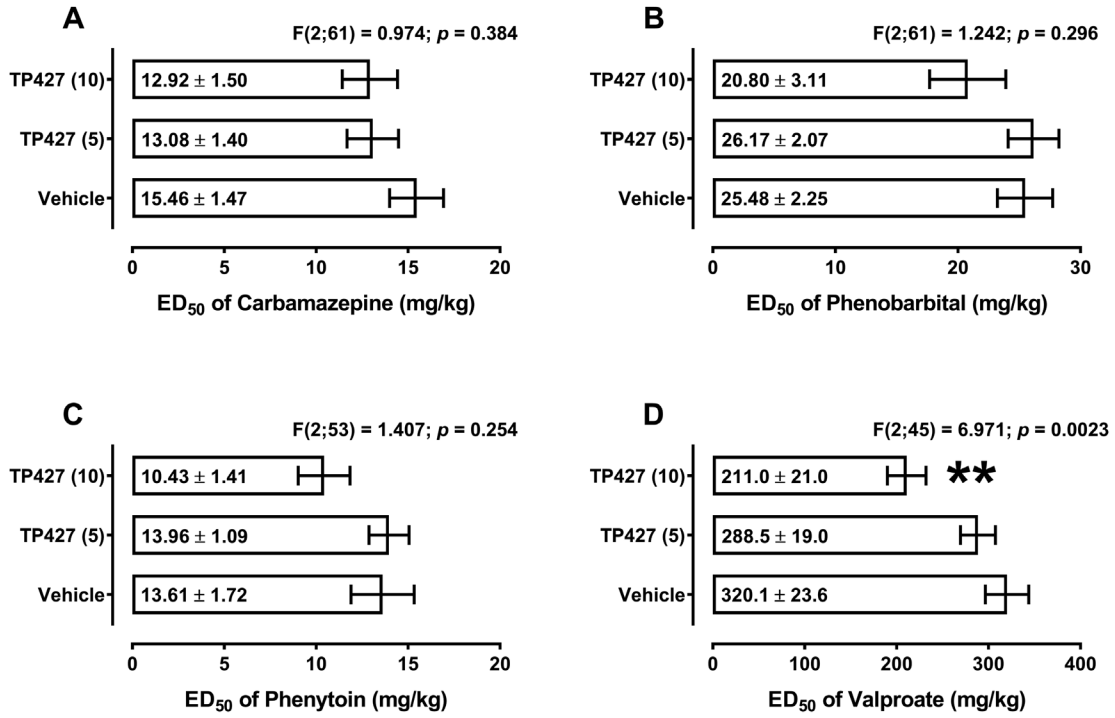

Fig. 3. A–D. Effects of TP427 on the anticonvulsant potency of carbamazepine, phenobarbital, phenytoin and valproate in the tonic-clonic seizure model in mice. Columns represent median effective doses (ED_{50} in mg/kg ± SEM) of antiepileptic drugs [carbamazepine (A), phenobarbital (B), phenytoin (C) and valproate (D)] that protected half of the tested mice from tonic-clonic seizures. The log-probit method was used for calculating the ED_{50} values.
$^{**}p < 0.01$ *vs.* control (valproate + vehicle-treated) animals (one-way ANOVA and *post-hoc* Tukey-Kramer test).

data from the subthreshold method is a widely accepted method in preclinical studies, which has been described in more detail elsewhere [20–24].

We also measured total brain concentrations of all classical antiepileptic drugs to exclude or confirm a pharmacokinetic contribution to the observed interaction to properly classify the nature of the interaction. With fluorescence polarization immunoassay, TP427 significantly elevated total brain concentrations of valproate, but not those of carbamazepine, phenobarbital or phenytoin in experimental animals. Taking into account both the pharmacokinetic increase in total brain valproate concentrations and potentiation of the antiseizure effects of valproate by TP427, one can ascertain that the pharmacokinetic (21%) increase in total brain valproate concentrations cannot be exclusively responsible for a 34% enhancement of the anticonvulsant effects of valproate in mice challenged with the mouse model of tonic-clonic seizures. It is likely that both pharmacokinetic and pharmacodynamic interactions between TP427 and valproate are responsible for the observed synergistic effects in the mouse tonic-clonic seizure model.

To explain the nature of pharmacodynamic interaction between TP427 and valproate, one should consider the fact that TP427, like other 1,2,4-triazole-3-thione derivatives, inhibits voltage-gated sodium channels. More specifically, in a radioligand binding assay it was reported that a chlorophenyl derivative of 4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (TP315) inhibited the binding of [^{3}H]batrachotoxin to sodium channels [8]. Since TP427 is a chlorobenzyl derivative of 4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione, one can accept that its molecular mechanisms of action are almost identical to those of TP315. Of note, both compounds produce strong anticonvulsant effects in the mouse model of tonic-clonic seizures [8,9]. Moreover, molecular studies confirmed that 1,2,4-triazole-3-thione derivatives neither directly, nor allosterically modulate $GABA_A$ receptors [9]. On the other hand, TP427 as a sodium channel blocker, may compete with phenytoin and carbamazepine (two classical antiepileptic drugs with firmly established sodium channel blocker properties) in their affinity towards voltage-gated sodium channels. This could be the main reason that TP427, when combined with phenytoin and carbamazepine, produced a barely additive interaction. In the case of the interaction with phenobarbital, TP427 did not affect the $GABA_A$ receptor-mediated inhibition of neurotransmission evoked by phenobarbital, and thus the voltage-gated sodium channel blockade evoked by TP427 was insufficient to significantly potentiate the antiseizure effects of phenobarbital. Since valproate possesses multiple molecular mechanisms of anticonvulsant action, it is highly likely that TP427, through the inhibition of voltage-gated sodium channels, contributes to the enhanced anticonvulsant potency of valproate. Molecular studies confirmed that valproate affects voltage-gated sodium channels [25]. It seems that the affinity of TP427 to voltage-gated sodium channels is higher than that of valproate and thus, TP427 potentiates the antiseizure action of valproate in the mouse model of tonic-clonic seizures. Although this explanation is highly speculative, it is very probable that TP427 enhances the blockade of sodium channels in neurons, contributing to the potentiation of the antiseizure effects of the two-drug mixture in the mouse model of tonic-clonic seizures. However, this needs experimental confirmation in further neurochemical studies.

The isobolographic transformation of data for the combinations of TP427 with classical antiepileptic drugs was conducted similar to that described in more detail for the combinations of MRZ 2/576 with second-generation antiepileptic drugs in the mouse model of tonic-clonic seizures [21]. To properly classify the observed interactions between drugs we calculated fractions of the ED_{50} values for drugs comprising the mixtures. According to general isobolographic rules, drugs co-administered in specific fractions exerted the effect that finally is a sum of effects produced by

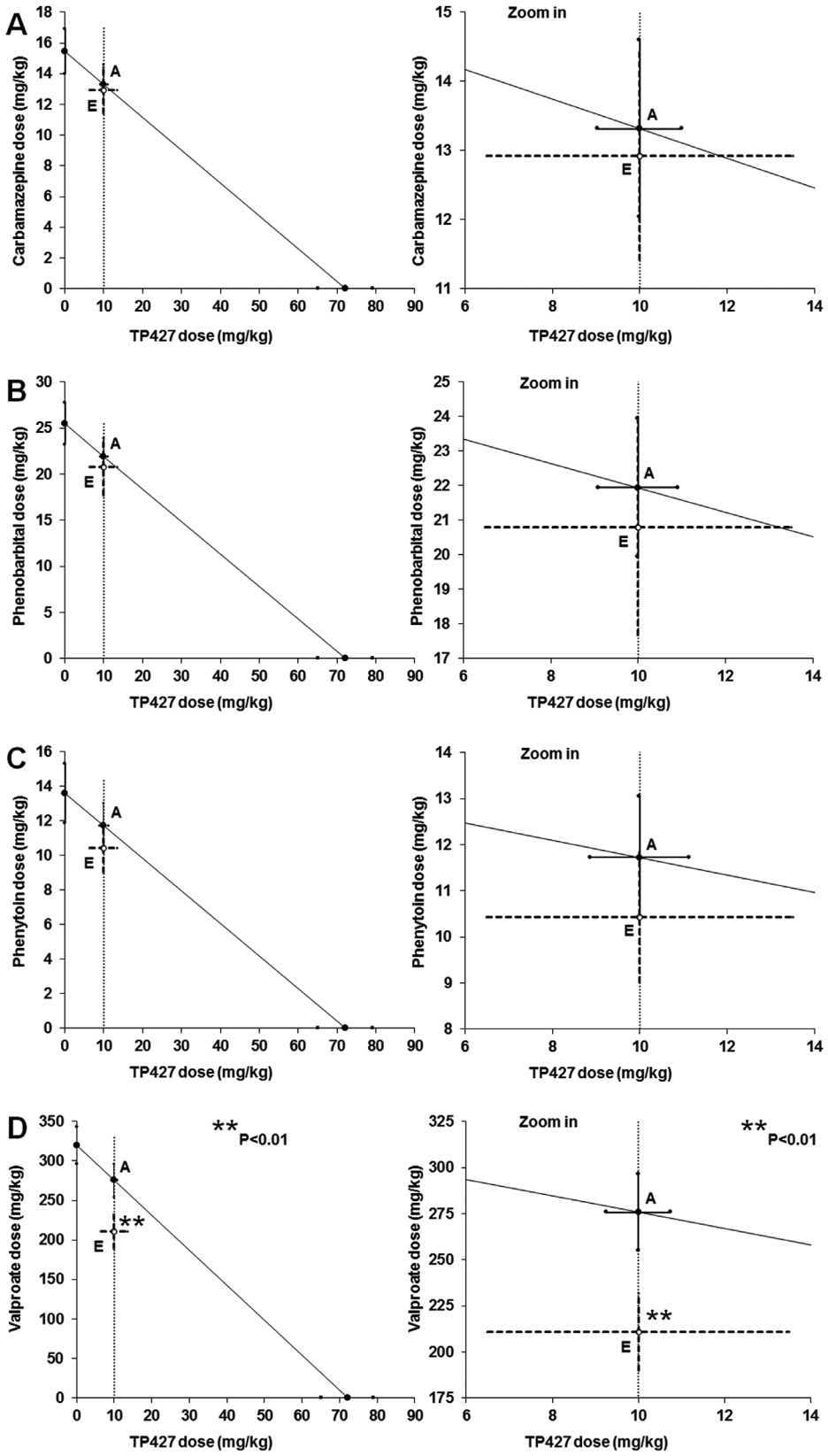

Fig. 4. A–D. Isobolographic transformation of anticonvulsant effects of TP427 in combination with carbamazepine, phenobarbital, phenytoin and valproate in the tonic-clonic seizure model in mice. The ED_{50} values for TP427 and classical antiepileptic drugs [carbamazepine (A), phenobarbital (B), phenytoin (C), and valproate (D)] are plotted graphically on the X- and Y-axes, respectively. The solid lines on the X and Y axes represent the SEM for the ED_{50} values for the studied compounds when administered separately. The dotted line starting from the point (10; 0) and parallel to the Y-axis corresponds to the fixed dose of TP427. Point A depicts the theoretical additive ED_{50add} value. Point E represents the experimentally-derived ED_{50exp} value for total dose of the mixture expressed as proportions of TP427 and classical antiepileptic drugs. Point E on graphs A–C is placed close to Point A, indicating additive interaction between TP427 and carbamazepine, phenobarbital and phenytoin, respectively. In contrast, Point E on graph D is placed significantly below Point A, indicating synergistic interaction between TP427 and valproate in the tonic-clonic seizure model in mice.
$^{**}p < 0.01$ *vs.* the ED_{50add} value (the unpaired Student's *t*-test).

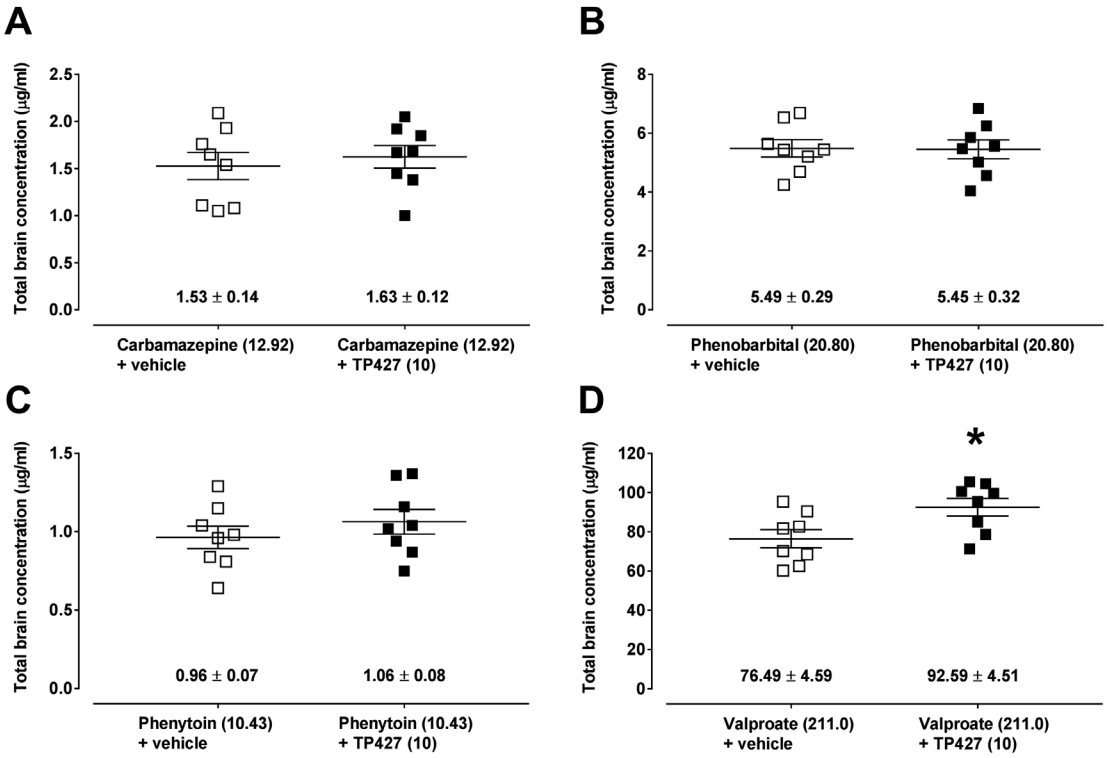

Fig. 5. A–D. Influence of TP427 on total brain concentrations of classical antiepileptic drugs in mice. Scatter plots represent total brain concentrations of carbamazepine, phenobarbital, phenytoin and valproate in μg/ml (as means ± SEM, as the error bars) (n = 8 mice/group).
*$p < 0.05$ *vs.* control (valproate + vehicle-treated) animals (unpaired Student's *t*-test).

particular drugs used in mixture [19]. By calculating fractions for classical antiepileptic drugs and TP427 we could determine the interaction index that, in this study, ranged from 0.8 (for the combination of TP427 with valproate) to 0.98 (for the combination of TP427 with carbamazepine) in the mouse model of tonic-clonic seizures (Table 1). Additionally, to properly classify the interactions between TP427 and classical antiepileptic drugs, we statistically compared the ED_{50exp} and ED_{50add} values with their SEMs by using the Student's *t*-test, as recommended elsewhere [23]. In this study, the interaction index value for the combination of TP427 with valproate was 0.8 and thus, according to the classification based on the interaction index value, the nature of interaction should be additive [20,26–28]. On the contrary, statistical analysis of data with Student's *t*-test revealed that both, ED_{50exp} and ED_{50add} values significantly differed ($p < 0.01$), confirming synergistic interaction between TP427 and valproate in the mouse model of tonic-clonic seizures.

At present, the isobolographic analysis is thought to be the method of choice in the classification and characterization of types of interactions between drugs [22,23,26–28]. Bearing all the above in mind, we combined two methods (isobolographic analysis and the subthreshold method) to compare the results for 1,2,4-triazolo-3-thione derivative from the tonic-clonic seizure model, to correctly classify the interactions between drugs and not to repeat experiments in animals. In this study, we created a new protocol allowing classification of the interactions between drugs, even if the investigated drug was applied in subthreshold doses. This unique method of combining the subthreshold method with isobolographic transformation is in strict accordance with the 3Rs principles (Replacement, Reduction and Refinement) when conducting experiments on animals. It is worth mentioning that we obtained additional information from isobolographic analysis without performing additional experiments on mice.

It is noteworthy that in this study we used TP427 in doses which were considered to be inactive *(per se)* in the mouse model of tonic clonic seizures. Both doses of TP427 (*i.e.*, 5 and 10 mg/kg) were lower than the experimentally derived TID_{20} value for TP427 that amounted to 11.41 mg/kg. Also of note is that calculation of TID_{20} values for drugs and agents possessing the anticonvulsant properties is a prerequisite in preclinical studies.

Conclusions

Potentiation of the anticonvulsant effects of valproate by TP427, despite the pharmacokinetic increase in valproate concentrations, renders the combination of valproate with TP427 of crucial importance for further clinical practice.

Disclosure of conflicts of interest

The authors have no disclosures to declare.

Acknowledgement

This study was supported by the National Science Centre, Poland [Sonata funding, UMO-2013/11/D/NZ7/01170].

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