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ORIGINAL ARTICLE

No effect of 3-(N-p-isopropoxyphenyl-succinimidomethylamino)-cinnamic acid on anticonvulsant action of different classical antiepileptic drugs in mouse maximal electroshock-induced seizure model

Jarogniew J. Łuszczki[1,2], Tadeusz Marczewski[2], Ewa Marzęda[1], Dariusz Durmowicz[1], Dominika Podgórska[2], Sergey L. Kocharov[3], Magdalena Florek-Łuszczki[4]

[1] *Isobolographic Analysis Laboratory, Institute of Rural Health, Lublin, Poland*
[2] *Department of Pathophysiology, Medical University, Lublin, Poland*
[3] *Mndjoyan's Institute of Fine Organic Chemistry, National Academy of Sciences, Yerevan, Republic of Armenia*
[4] *Department of Public Health, Institute of Rural Health, Lublin, Poland*

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Abstract

Introduction and objective: The aim of the study was to determine the effects of 3-(N-p-isopropoxyphenylsuccinimido-methylamino)-cinnamic acid (IPPSMA-CA – a new succinimide derivative) on the protective action of 4 classical antiepileptic drugs (AEDs): carbamazepine [CBZ], phenobarbital [PB], phenytoin [PHT] and valproate [VPA]), against maximal electroshock (MES)-induced tonic seizures in mice.

Materials and methods: Tonic hind limb extension (seizure activity) was evoked in adult male albino Swiss mice by a current (sine-wave, 25 mA, 500 V, 50 Hz, 0.2 s stimulus duration) delivered via auricular electrodes. Acute adverse-effect profiles of the combination of IPPSMA-CA and 4 classical AEDs (CBZ, PB, PHT and VPA) with respect to motor performance, long-term memory and skeletal muscular strength were measured in the chimney, passive avoidance and grip-strength tests, respectively.

Results: IPPSMA-CA administered at 150 mg/kg (i.p.) significantly elevated the threshold for electroconvulsions in mice ($p<0.01$). IPPSMA-CA at doses of 50 and 100 mg/kg, however, had no significant impact on the threshold for electroconvulsions in mice. Nor did IPPSMA-CA (100 mg/kg) significantly affect the anticonvulsant activity of CBZ, PB, PHT and VPA in the MES test in mice. None of the examined combinations of IPPSMA-CA (100 mg/kg, i.p.) with CBZ, PB, PHT and VPA (at their ED_{50} values from the MES-induced seizure test) affected motor coordination in the chimney test, long-term memory in the passive avoidance task, and muscular strength in the grip-strength test in mice. This indicates no possible acute adverse effects in animals.

Conclusions: IPPSMA-CA elevated the threshold for electroconvulsions in mice in a dose-dependent manner. However, IPPSMA-CA at a sub-protective dose of 100 mg/kg did not affect the anticonvulsant action of various classical AEDs in the mouse MES model. Thus, the combinations of IPPSMA-CA with CBZ, PB, PHT and VPA are neutral from a preclinical viewpoint.

Key words

antiepileptic drugs, maximal electroshock-induced seizures, pharmacokinetic/pharmacodynamic interaction, p-isopropoxyphenylsuccinimide derivative

INTRODUCTION

In spite of advances in our knowledge on pathophysiological processes underlying seizure attacks and numbers of antiepileptic drugs (AEDs) licensed and approved for the treatment of epilepsy, there still remain approx. 30% of epilepsy patients inadequately medicated with currently available AEDs. For these patients, some novel AEDs should be created and developed that would protect the patients against seizures, thereby providing them with the state of freedom from seizures. There is undoubtedly a pressing need for developing some novel drugs possessing anticonvulsant properties in both experimental and clinical studies [1].

Experimental *in vivo* studies indicate that several succinimide derivatives possess anticonvulsant properties in rodents [2, 3, 4, 5, 6, 7, 8]. Previously, it has been reported that 3-cyclohexyl-succinimides [2], N-morpholinemethyl derivative of m-bromophenyl-succinimide [3], N-pyridyl-substituted succinimides [5], N-(anilinomethyl)-p-isopropoxyphenylsuccinimide (AMIPPS) [4], p-isopropoxyphenylsuccinimide monohydrate (IPPS) [6], N-(ortho-carboxyanilinomethyl)

Address for correspondence: Jarogniew J. Łuszczki, Isobolographic Analysis Laboratory, Institute of Rural Health, Jaczewskiego 2, 20-090 Lublin, Poland.
E-mail: jluszczki@yahoo.com/jarogniew.luszczki@gmail.com

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-p-isopropoxyphenylsuccinimide (o-CAMIPPS), N-(meta-carboxyanilinomethyl)-p-isopropoxyphenylsuccinimide (m-CAMIPPS), and N-(para-carboxyanilinomethyl) -p-isopropoxyphenylsuccinimide (p-CAMIPPS) [7], and N-hydroxymethyl-p-isopropoxyphenylsuccinimide (HMIPPS) [8], exhibited potent anticonvulsant effects in the maximal electroshock-induced seizure (MES) test, recognized as the most widely employed animal seizure model for early identification of candidate anticonvulsant drugs.

In our pilot study, it was found that 3-(N-p-isopropoxyphenylsuccinimidomethylamino)-cinnamic acid (IPPSMA-CA) exerted anticonvulsant properties by suppressing tonic-clonic seizures in the mouse MES test (unpublished data). We sought, therefore, to evaluate the effect of IPPSMA-CA on the threshold for electroconvulsions, and to assess its influence on the protective activity of 4 classical AEDs (carbamazepine (CBZ), phenobarbital (PB), phenytoin (PB), and valproate (VPA)) in the mouse MES-induced seizure model. The test evaluating the threshold for electroconvulsions and the MES test in mice are both thought to be experimental models of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in humans [9]. In these experimental tests, one can readily determine the anticonvulsant potential of agents and compounds having anticonvulsant properties and evaluate their effects on various classical AEDs, which are effective in suppression of tonic-clonic seizures in humans [9]. Additionally, acute side effects of IPPSMA-CA administered alone and in combination with the 4 classical AEDs were investigated regarding impairment of motor coordination, long-term memory and muscular strength utilizing the chimney test, step-through passive avoidance task, and grip-strength test, respectively.

MATERIALS AND METHODS

Animals and experimental conditions. Adult male Swiss mice (weighing 22-26 g) were used, kept in colony cages with free access to food and tap water, housed under standardized housing conditions (natural light-dark cycle, temperature of 23 ± 1°C, and relative humidity of 55 ± 5%). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups each comprised of 8 mice. Each mouse was used only once and all tests were performed between 08:00-15:00. Procedures involving animals and their care were conducted in accordance 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 described in this study were approved by the First Local Ethics Committee at the Medical University in Lublin (License No.: 18/2006) and the Second Local Ethics Committee at the University of Life Sciences in Lublin (License Nos.: 79/2009, 15/2012), and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs. The following drugs were used:
- 3-(N-p-isopropoxyphenylsuccinimidomethylamino)-cinnamic acid (IPPSMA-CA – $C_{23}H_{24}N_2O_5$ – molecular weight = 408.440, synthesized by Dr. S.L. Kocharov, Mndjoyan's Institute of Fine Organic Chemistry of the National Academy of Sciences in Yerevan, Republic of Armenia);
- carbamazepine (CBZ – a gift from Polpharma, Starogard Gdański, Poland);
- phenobarbital (PB – Polfa, Kraków, Poland);
- phenytoin (PHT – Polfa, Warsaw, Poland);
- valproate (VPA – magnesium salt (donated by ICN-Polfa SA, Rzeszów, 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 directly dissolved in distilled water. All drugs were administered *intraperitoneally* (*i.p.*), in a volume of 5 ml/kg body weight, as follows: PHT – 120 min, PB and IPPSMA-CA – 60 min, CBZ and VPA – 30 min before electroconvulsions, motor coordination, grip-strength and long-term memory tests. The pretreatment times before testing the AEDs were based on information concerning their biological activity from the literature, and from own previous experiments [4, 6, 7, 8]. The times to the peak of maximum anticonvulsant effects for all AEDs were used as the reference times in all behavioural tests. The pretreatment time (60 min.) before testing IPPSMA-CA was established in a pilot study as its peak time of maximum anticonvulsant activity (unpublished data).

Maximal electroshock seizure threshold (MEST) test. The MEST test was used first to assess the anticonvulsant effects of IPPSMA-CA administered alone. Electroconvulsions were induced by applying an alternating current (50 Hz; 500 V) *via* ear-clip electrodes from a rodent shocker generator (type 221; Hugo Sachs Elektronik, Freiburg, Germany). The stimulus duration was 0.2 s and tonic hind limb extension was used as the endpoint. In this test, at least 4 groups of control mice, each consisting of 8 animals, were challenged with currents of varying intensities ranging between 5-8 mA, so that 10-30%, 30-50%, 50-70% and 70-90% of animals exhibited the endpoint. After establishing the current intensity-effect curve (i.e. current intensity in mA *vs.* percentage of mice convulsing) for each dose of IPPSMA-CA tested, the electroconvulsive threshold was calculated according to the log-probit method of Litchfield and Wilcoxon [10]. The electroconvulsive threshold was expressed as the median current strength value (CS_{50} in mA) predicted to produce tonic hind limb extension in 50% of the animals tested.

This experimental procedure was performed for various increasing doses of IPPSMA-CA (50, 100 and 150 mg/kg), until the threshold for electroconvulsions of IPPSMA-CA-injected animals was statistically different from that of the control animals. Only doses of IPPSMA-CA that did not significantly affect the seizure threshold in the MEST test were selected for testing in combination with the 4 classical AEDs in the MES test (see below). This approach allowed the ruling out any contribution of the intrinsic anticonvulsant efficacy of IPPSMA-CA in the effects observed in combination with the AEDs in the MES test.

Maximal electroshock seizure (MES) test. Electroconvulsions were induced by applying an alternating current (50 Hz; 500 V) *via* ear-clip electrodes from a rodent shocker generator (type 221; Hugo Sachs Elektronik, Freiburg, Germany). The stimulus duration was 0.2 s and

tonic hind limb extension was used as the endpoint. In the MES test, mice were challenged with a current of the fixed intensity (25 mA) that was 4-5-fold higher than the CS_{50} value in vehicle-treated control mice [9]. These parameters of stimulation (maximal electroshock) typically result in all mice responding with tonic hind limb extension immediately after stimulation.

The AEDs administered alone and their combination with IPPSMA-CA were tested for their ability to increase the number of animals not responding with tonus (i.e. protected from tonic hind limb extension) after stimulation. Again, at least 4 groups of mice, each consisting of 8 animals and treated with a different dose of the AEDs alone or in combination with IPPSMA-CA, were challenged with a current of 25 mA to yield 10-30%, 30-50%, 50-70% and 70-90% of animals protected from tonic seizures. After constructing a dose-effect curve (i.e. dose in mg/kg *vs.* percentage of mice protected), the protective median effective dose (ED_{50}) value of the AED tested was calculated according to a log-probit method [10]. Each ED_{50} value represented a dose of the AED (in mg/kg) predicted to protect 50% of mice tested against MES-induced extension of the hind limbs. IPPSMA-CA was tested for its ability to affect the anticonvulsive potency of AEDs. As mentioned earlier, IPPSMA-CA was administered in a dose of 100 mg/kg that *per se* had no effect on seizure threshold in the MEST test. In this experimental protocol, an increase in the anticonvulsant potency of the AED tested in combination with IPPSMA-CA would be reflected by a lower ED_{50} value of the test AED (i.e. lower dose of the test drug was necessary to protect 50% of mice challenged). In the present study, CBZ and PHT were administered at doses ranging between 8-14 mg/kg, PB at doses ranging between 15-30 mg/kg, and VPA at doses ranging between 200-300 mg/kg.

Step-through passive avoidance task. The effects of IPPSMA-CA (100 mg/kg) applied alone or in combination with classical AEDs (at doses equal to their ED_{50} values against MES-induced tonic seizures) on long-term memory were evaluated in the step-through, passive avoidance task. Each animal was administered an AED, either alone or in combination with IPPSMA-CA, on the first day before training. The pretreatment time of drugs in the passive avoidance task was identical to that of the MES test. Subsequently, the animals were placed in an illuminated box (10 × 13 × 15 cm) connected to a larger dark box (25 × 20 × 15 cm) equipped with an electric grid floor. Entrance of pretreated animals to the dark box was punished by an adequate electric shock to the foot (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation.

On the following day (24 h later), the pre-trained animals were again placed 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. The time that the mice took to enter the dark box was noted and the median latencies (retention times) with 25th and 75th percentiles were calculated. The step-through passive avoidance task gives information about ability to acquire a task (learning) and to recall a task (retrieval). Therefore, it may be regarded as a measure of long-term memory [11]. This experimental procedure has been described in detail in our earlier studies [4, 6, 7, 8].

Grip-strength test. The effects of combinations of IPPSMA-CA (100 mg/kg) with classical AEDs at doses corresponding to their ED_{50} values from the MES test on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. [12]. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the MES test. The grip-strength apparatus (BioSeb, Chaville, France) was equipped with a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of three measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The muscular strength in mice is expressed in N (Newtons) as means ± S.E. of at least 8 determinations. This experimental procedure has also been described in detail in our earlier studies [4, 6, 7, 8].

Chimney test. Potential adverse effects of combinations of classical AEDs administered at doses corresponding to their ED_{50} values from the MES test with IPPSMA-CA (100 mg/kg) were evaluated using the chimney test of Boissier et al. [13]. In this test, the mice were placed at the bottom of a transparent plastic tube (30 cm long, 3 cm inner diameter,) and had to climb backwards up to the top of the tube. Impairment of motor performance was indicated by the inability of the mice to climb out of the tube within 60 s., and the results were expressed as the percentages of animals that failed to perform the task. This experimental procedure, too, has been described in detail in our earlier studies [4, 6, 7, 8].

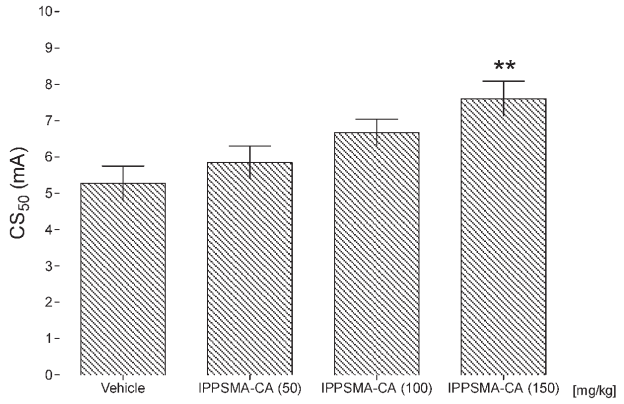
Statistics. Both CS_{50} and ED_{50} values with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon [10]. Statistical analysis of data from the MEST test was performed with one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey-Kramer test for multiple comparisons among 4 CS_{50} values. Statistical analysis of data from the MES test was performed with log-probit analysis according to Litchfield and Wilcoxon [10] for 2 ED_{50} values.

The results obtained in the step-through passive avoidance task were statistically evaluated using Kruskal-Wallis nonparametric ANOVA. The results from the grip-strength test were verified with one-way ANOVA. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test. Differences among values were considered statistically significant if $p<0.05$. All statistical tests were performed using commercially available GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Influence of 3-(N-p-isopropoxyphenylsuccinimido-methylamino)-cinnamic acid (IPPSMA-CA) on the threshold for electroconvulsions. IPPSMA-CA administered systemically (i.p. 60 min. prior to the MEST test) at a dose of 150 mg/kg significantly elevated the threshold for electroconvulsions in mice from 5.27 ± 0.45 mA – 7.60 ± 0.49 mA ($p<0.01$) (Fig. 1). The experimentally-derived CS_{50}

values for animals receiving IPPSMA-CA at doses of 50 and 100 mg/kg did not significantly differ from that for control animals subjected to the MEST test (Fig. 1).



Columns represent median current strengths (CS_{50} values in mA ± S.E.) required to produce tonic hind limb extension in 50% of animals tested in the MEST test. IPPMA-CA was administered *i.p.* 60 min. before the test. Statistical evaluation of the data was performed with log-probit method and one-way ANOVA, followed by the *post-hoc* Tukey-Kramer test for multiple comparisons.
**p<0.01 *vs.* the control (vehicle-treated) animals.

Figure 1. Effect of 3- (N-p-isopropoxyphenylsuccinimidomethylamino) -cinnamic acid (IPPSMA-CA) on the threshold for electroconvulsions in mice

Effects of IPPSMA-CA on the protective action of carbamazepine, phenobarbital, phenytoin and valproate in the mouse maximal electroshock seizure model. All investigated classical AEDs (CBZ, PB, PHT and VPA) administered alone exhibited a clear-cut anticonvulsant activity in the MES test in mice (Tab. 1). When IPPSMA-CA (100 mg/kg) was co-administered with CBZ, PB, PHT and VPA, it did not significantly enhance the anticonvulsant action of the AEDs in the MES test. The experimentally-derived ED_{50} values for the AEDs in combination with IPPSMA-CA did not considerably differ from those ED_{50} values as documented for the AEDs administered separately (Tab. 1).

Table 1. Effect of 3- (N-p-isopropoxyphenylsuccinimidomethylamino) -cinnamic acid (IPPSMA-CA) on the protective activity of 4 classical AEDs against MES-induced seizures in mice

Treatment (mg/kg)	ED_{50} (mg/kg)
CBZ + vehicle	10.8 (9.1-12.8)
CBZ + IPPSMA-CA (100)	9.0 (8.2-9.8)
PB + vehicle	20.1 (17.2-23.4)
PB + IPPSMA-CA (100)	21.8 (17.6-27.0)
PHT + vehicle	11.9 (10.2-13.9)
PHT + IPPSMA-CA (100)	10.1 (8.8-11.7)
VPA + vehicle	266.0 (243.5-290.4)
VPA + IPPSMA-CA (100)	235.8 (213.9-260.0)

Results are presented as median effective doses (ED_{50} in mg/kg, with 95% confidence limits in parentheses) of AEDs, protecting 50% of animals tested against MES-induced hind limb extension. All AEDs were administered *i.p.*: PHT – 120 min., PB – 60 min., CBZ and VPA – 30 min. prior to the MES test. IPPSMA-CA was administered *i.p.* 60 min. before the MES test. Statistical analysis of data was performed with log-probit method according to Litchfield and Wilcoxon [9].

CBZ – carbamazepine, PB – phenobarbital, PHT – phenytoin, and VPA – valproate.

Effects of IPPSMA-CA in combination with classical antiepileptic drugs on motor performance, long-term memory, and skeletal muscular strength of animals in the chimney, step-through passive avoidance and grip-strength tests. IPPSMA-CA administered alone at the dose of 100 mg/kg did not alter long-term memory in animals challenged with the step-through passive avoidance task (Tab. 2). Similarly, IPPSMA-CA at the dose of 100 mg/kg neither affected muscular strength in mice in the grip-strength test, nor disturbed motor coordination in mice subjected to the chimney test (Tab. 2). IPPSMA-CA (100 mg/kg, i.p.) administered in combination with CBZ, PB, PHT and VPA at doses equal to their ED_{50} values from the MES test did not impair long-term memory as determined in the passive avoidance test (Tab. 2). Additionally, IPPSMA-CA (100 mg/kg, i.p.) co-administered with classical AEDs had no significant impact on skeletal muscular strength of the animals, as assessed by the grip-strength test (Tab. 2). Furthermore, none of the combinations studied affected motor performance in mice subjected to the chimney test (Tab. 2). Regarding the AEDs administered alone at doses corresponding to their ED_{50} values from the MES test, the AEDs had no significant impact on long-term memory, muscular strength and motor performance in mice (results not shown).

Table 2. Effects of 3- (N-p-isopropoxyphenylsuccinimidomethylamino) -cinnamic acid (IPPSMA-CA) and its combinations with 4 classical AEDs on long-term memory, muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Motor coordination impairment (%)
Vehicle	180 (180; 180)	0.986 ± 0.050	0
IPPSMA-CA (100) + vehicle	180 (180; 180)	0.999 ± 0.053	0
CBZ (9.0) + IPPSMA-CA (100)	180 (180; 180)	0.990 ± 0.050	0
PB (21.8) + IPPSMA-CA (100)	180 (180; 180)	0.977 ± 0.058	0
PHT (10.1) + IPPSMA-CA (100)	180 (180; 180)	0.990 ± 0.053	0
VPA (235.8) + IPPSMA-CA (100)	180 (155.8; 180)	0.970 ± 0.056	25

Results are presented as: 1) median retention times (in seconds (s); 25th and 75th percentiles (in parentheses) from the passive avoidance task, assessing long-term memory in mice. 2) mean grip-strengths (in newtons (N) ± S.E.) from the grip-strength test, assessing muscular strength in mice. 3) percentage (%) of animals showing motor coordination impairment in the chimney test in mice. Each experimental group consisted of 8 mice. Statistical analysis of data from the passive avoidance task was performed with nonparametric Kruskal-Wallis ANOVA test; those from the grip-strength test were analyzed with one-way ANOVA; Fisher's exact probability test was used to analyze the results from the chimney test. All drugs were administered *i.p.* at times scheduled from the MES test, and at doses corresponding to their ED_{50} values against MES-induced seizures in mice (for more details see Table 1 legend).

DISCUSSION

The results of the presented study indicate that IPPSMA-CA elevated in a dose-dependent manner the threshold for electroconvulsions in mice. IPPSMA-CA at the sub-protective dose of 100 mg/kg (i.e. the dose that by itself did not significantly affect the threshold for electroconvulsions) had no impact on the protective action of CBZ, PB, PHT and VPA against MES-induced tonic seizures in mice, thus indicating neutral interactions between these drugs in the mouse MES model.

Comparing the effects produced by IPPSMA-CA with those reported earlier for AMIPPS, IPPS, o-CAMIPPS, m-CAMIPPS, p-CAMIPPS, and HMIPPS, it can be ascertained that IPPSMA-CA had no impact on the anticonvulsant properties of the 4 classical AEDs. Previously, we have documented that p-isopropoxyphenylsuccinimide monohydrate (IPPS) potentiated the anticonvulsant action of PHT and VPA, but not that of CBZ and PB [6]. Moreover, AMIPPS and HMIPPS significantly enhanced the

anticonvulsant action of PB and VPA, but not that of CBZ and PHT in the mouse MES model [4, 8].

On the other hand, o-CAMIPPS attenuated the anticonvulsant action of CBZ and had no significant impact on the protective action of PHT, PB and VPA against MES-induced seizures in mice [7]. With regards to m-CAMIPPS and p-CAMIPPS, both compounds had no impact on the protective action of CBZ, PHT, PB and VPA in the mouse MES model [7]. Of note, the anticonvulsant profile of IPPSMA-CA when combined with classical AEDs is similar to that reported earlier for m-CAMIPPS and p-CAMIPPS in the mouse MES model.

There is another fact worth mentioning while explaining the results obtained in the presented study. It was found that IPPSMA-CA administered either alone or in combination with classical AEDs, at doses corresponding to their ED_{50} values from the MES test, did not affect the motor coordination of mice in the chimney test. Additionally, IPPSMA-CA had no impact on long-term memory and muscular strength in mice subjected to the step-through passive avoidance task and grip-strength test, respectively. Since IPPSMA-CA had no impact on the acute adverse-effect profile of classical AEDs, the tested succinimide derivative seems to be neutral in preclinical studies.

Although IPPSMA-CA significantly raised the threshold for maximal electroconvulsions in mice, it did not affect the protective action of different classical AEDs in the mouse MES model. Thus, one can ascertain that IPPSMA-CA has an anticonvulsant action against electrically-evoked tonic seizures in experimental animals. This action, however, is too weak to enhance the protective activity of different classical AEDs in the mouse MES-induced tonic seizure model. Perhaps IPPSMA-CA will be effective in suppression of clonic or limbic seizures in other experimental models of epilepsy. To confirm or reject this hypothesis, more advanced studies are required.

CONCLUSION

Finally, based on the results from this study, one can ascertain that the co-administration of IPPSMA-CA with various classical AEDs (CBZ, PB, PHT and VPA) was neutral in the mouse MES model. Additionally, IPPSMA-CA had no impact on acute adverse effect profiles of classical AEDs, as determined in the passive avoidance, grip-strength and chimney tests in mice.

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