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# Synthetic cannabinoid WIN 55,212-2 mesylate enhances the protective action of four classical antiepileptic drugs against maximal electroshock-induced seizures in mice ☆

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ABSTRACT

The aim of this study was to determine the effect of WIN 55,212-2 mesylate (WIN — a non-selective cannabinoid CB1 and CB2 receptor agonist) on the protective action of four classical antiepileptic drugs (carbamazepine, phenytoin, phenobarbital, and valproate) in the mouse maximal electroshock seizure (MES) model. The results indicate that WIN (10 mg/kg, i.p.) significantly enhanced the anticonvulsant action of carbamazepine, phenytoin, phenobarbital and valproate in the MES test in mice. WIN (5 mg/kg) potentiated the anticonvulsant action of carbamazepine and valproate, but not that of phenytoin or phenobarbital in the MES test in mice. However, WIN administered alone and in combination with carbamazepine, phenytoin, phenobarbital and valproate significantly reduced muscular strength in mice in the grip-strength test. In the passive avoidance task, WIN in combination with phenobarbital, phenytoin and valproate significantly impaired long-term memory in mice. In the chimney test, only the combinations of WIN with phenobarbital and valproate significantly impaired motor coordination in mice. In conclusion, WIN enhanced the anticonvulsant action of carbamazepine, phenytoin, phenobarbital and valproate in the MES test. However, the utmost caution is advised when combining WIN with classical antiepileptic drugs due to impairment of motor coordination and long-term memory and/or reduction of skeletal muscular strength that might appear during combined treatment.



## 1. Introduction

Accumulating experimental evidence indicates that naturally occurring ($\Delta^9$-tetrahydrocannabinol — a major constituent of *Cannabis sativa*) and synthetic ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmeth anone mesylate — WIN 55,212-2 mesylate — WIN) cannabinoids possess anticonvulsant activity in various experimental models of epilepsy (Boggan et al., 1973; Corcoran et al., 1973; Cox et al., 1975; Dwivedi and Harbison 1975; Feeney et al., 1973; Karler et al., 1974; Koda et al., 2005; McCaughran et al., 1974; Meldrum et al., 1974; Ten Ham et al., 1975; Wada et al., 1973). In mammals, a number of endogenous cannabinoids (endocannabinoids) have been identified, including 2-arachidonoylglycerol and anandamide (Alger, 2004; Karanian et al., 2007; Monory et al., 2006; Romigi et al., 2010; Sheerin et al., 2004). The endocannabinoid system has been demonstrated to play an important role in regulating seizure activity in brain (Deshpande et al., 2007; Lutz, 2004; Smith, 2005; Wallace et al., 2003) and has been identified as a possible target for controlling status epilepticus (Blair et al., 2006; Wallace et al., 2003).

The anticonvulsant effects of cannabinoids are mediated through activation of the cannabinoid CB1 receptors in the maximal electroshock-induced tonic seizure (MES) model in mice (Wallace et al., 2001, 2002), the pentylenetetrazole-induced clonic seizure model in mice (Bahremand et al., 2009; Gholizadeh et al., 2007; Shafaroodi et al., 2004), the pilocarpine-induced seizure model of temporal lobe epilepsy in rats (Falenski et al., 2007, 2009; Wallace et al., 2003), and penicillin-induced seizure model in rats (Kozan et al., 2009).

*Abbreviations:* MES, maximal electroshock-induced seizure test; WIN, WIN 55,212-2 mesylate.

☆ The results of this study were presented, in part, at the 63rd Annual Meeting of the American Epilepsy Society, held in Boston (MA, USA), on 4–8 December 2009 [abstract available in Epilepsia, 2009; 50 (Suppl. 11): 365].

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Specifically, WIN 55,212-2 mesylate (WIN — a highly potent non-selective cannabinoid type 1 (CB1) and type 2 (CB2) receptor agonist) exerted anticonvulsant effects in the rat pilocarpine-induced seizure model (Wallace et al., 2001). WIN attenuated low-magnesium ($Mg^{2+}$)-induced burst-firing in hippocampal cell cultures (Shen and Thayer, 1998, 1999). Moreover, WIN exerted the anticonvulsant action in an experimental *in vivo* model of complex partial seizures (maximal dentate gyrus activation) in rats (Rizzo et al., 2009). Additionally, WIN attenuated the severity of cocaine-induced convulsive seizures in mice and antagonized L-glutamic acid and N-methyl-D-aspartate (NMDA)-induced convulsions in mice (Hayase et al., 2001).

Although the effects of exogenous and endogenous cannabinoids on epileptogenesis in various experimental models of epilepsy are being extensively examined, little is known about the effects of the cannabinoids on the anticonvulsant action of current frontline and licensed antiepileptic drugs. Quite recently, it has been documented that WIN potentiated the anticonvulsant activity of diazepam (a classical antiepileptic drug) in the mouse MES model (Naderi et al., 2008).

Previously, we have documented that arachidonyl-2′-chloroethylamide (ACEA — a highly selective cannabinoid CB1 receptor agonist) potentiated the anticonvulsant action of phenobarbital and valproate, but not that of lamotrigine, oxcarbazepine, topiramate, phenytoin or carbamazepine in the mouse MES model (Luszczki et al., 2006a, 2010). Moreover, ACEA potentiated the anticonvulsant action of ethosuximide, phenobarbital and valproate, but not that of clonazepam in the mouse pentylenetetrazole-induced seizure model (Czuczwar and Luszczki, 2009).

Considering the above-mentioned facts it was of importance to determine the influence of WIN on the anticonvulsant action of four classical antiepileptic drugs (carbamazepine, phenytoin, phenobarbital and valproate) in the mouse MES model. The MES test is thought to be an experimental model of tonic–clonic seizures and, to a certain extent, of partial seizures with or without secondary generalization (Löscher et al., 1991). Noteworthy, in this experimental test one can readily assess the anticonvulsant potential of agents and compounds possessing the anticonvulsant properties, as well as, to determine their effects on conventional and second-generation antiepileptic drugs, fully effective in suppressing tonic–clonic seizures in humans (Löscher et al., 1991). Therefore, it was appropriate to use the mouse MES model in order to evaluate the effects of WIN on the protective action of carbamazepine, phenytoin, phenobarbital and valproate in this model. Additionally, we investigated the combinations of WIN with classical antiepileptic drugs in relation to impairment of motor coordination, long-term memory and muscular strength by the use of the chimney test, step-through passive avoidance task and grip-strength test, respectively. Finally, total brain antiepileptic drug concentrations were measured with immunofluorescence in order to ascertain whether any observed effects were consequent to a pharmacodynamic and/or a pharmacokinetic interaction.

The aim of this study was to determine whether WIN would enhance the protective action of four classical antiepileptic drugs against tonic–clonic seizures in the mouse MES model. If so, the favorable combinations of WIN with classical antiepileptic drugs from preclinical studies could be transferred to clinical settings for patients with refractory epilepsy. Such combinations would offer the patients with intractable epilepsy a suppression of seizures and thus, WIN in combination with classical antiepileptic drugs would ameliorate the patients' quality of living.

## 2. Materials and methods

### 2.1. Animals and experimental conditions

Adult male Swiss mice (weighing 22–26 g) that were kept in colony cages with free access to food and tap water, under standardized housing conditions (natural light–dark cycle, temperature of 23 ± 1 °C, relative humidity of 55 ± 5%), were used. 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 a.m. and 03.00 p.m. 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 manuscript were approved by the Local Ethics Committee at the Medical University of Lublin and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

### 2.2. Drugs

The following drugs were used: WIN ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmeth anone mesylate; Tocris Bioscience, Bristol, UK), carbamazepine (a gift from Polpharma S.A., Starogard Gdanski, Poland), phenobarbital (Polfa, Krakow, Poland), phenytoin (Polfa, Warszawa, Poland), and valproate (magnesium salt — kindly donated by ICN-Polfa S.A., Rzeszow, Poland). All drugs, except for WIN and valproate, were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water, while WIN and valproate were dissolved in distilled water. All drugs were administered intraperitoneally (i.p.) as a single injection, in a volume of 5 ml/kg body weight. Fresh drug solutions were prepared on each day of experimentation and administered as follows: phenytoin — 120 min, phenobarbital — 60 min, carbamazepine and valproate — 30 min, WIN — 20 min before the initiation of electroconvulsions, motor coordination, grip-strength and long-term memory tests, as well as, before brain sampling for the measurement of antiepileptic drug concentrations. The pretreatment times before testing of the antiepileptic drugs were based upon information about their biological activity from the literature and our previous experiments (Luszczki et al., 2009, 2010). The times to the peak of maximum anticonvulsant effects for all antiepileptic drugs were used as the reference times in all behavioral tests and pharmacokinetic estimation of total brain antiepileptic drug concentrations. The route of i.p. administration of WIN and the pretreatment time before testing of its anticonvulsant effect were based upon information from previous experiments (Naderi et al., 2008).

### 2.3. Maximal electroconvulsions

Electroconvulsions were produced by means of an alternating current (0.2 s stimulus duration, 50 Hz, maximum stimulation voltage of 500 V) delivered via ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs Elektronik, Freiburg, Germany). The electrical system of the stimulator was self-adjustable so that changes in impedance did not result in alterations of current intensity (i.e., the system provides constant current stimulation). The criterion for the occurrence of seizure activity was the tonic hind limb extension (i.e., the hind limbs of animals outstretched 180° to the plane of the body axis). In this experiment, two experimental models of maximal electroconvulsions were used: 1) maximal electroshock seizure threshold test and 2) maximal electroshock seizure test.

#### 2.3.1. Maximal electroshock seizure threshold test

To evaluate the threshold for maximal electroconvulsions, at least 4 groups of mice, consisting of 8 animals per group, were challenged with electroshocks of various intensities to yield 10–30%, 30–50%, 50–70%, and 70–90% of animals with seizures. Then, a

current intensity–response relationship curve was constructed, according to a log-probit method by Litchfield and Wilcoxon (1949), from which a median current strength ($CS_{50}$ in mA) was calculated. Each $CS_{50}$ value represents the current intensity required to induce tonic hindlimb extension in 50% of the mice challenged. Again, after administration of a single dose of WIN to 4 groups of animals, the mice were subjected to electroconvulsions (each group with a constant current intensity). The threshold for maximal electroconvulsions was recorded for 4 different doses of WIN: 2.5, 5, 10 and 15 mg/kg.

#### 2.3.2. Maximal electroshock seizure test

The protective activity of carbamazepine, phenytoin, phenobarbital and valproate was determined as their median effective doses ($ED_{50}$ values in mg/kg) against MES-induced seizures. The animals were administered with different drug doses so as to obtain a variable percentage of protection against MES-induced seizures, allowing for the construction of a dose–response relationship curve for each antiepileptic drug administered alone, according to Litchfield and Wilcoxon (1949). Each $ED_{50}$ value represents the dose of a drug required to protect 50% of the animals tested against MES-induced seizures. Similarly, the anticonvulsant activity of a mixture of an antiepileptic drug with WIN was evaluated and expressed as the $ED_{50}$ value, corresponding to a dose of an antiepileptic drug necessary to protect 50% of mice against tonic hindlimb extension in the MES test. In the present study, carbamazepine was administered at doses of 4, 6, 8, 10, 12, 14 and 16 mg/kg, phenytoin at doses of 4, 6, 8, 10, and 12 mg/kg, phenobarbital at doses of 8, 10, 15, 20, 25 and 30 mg/kg, and valproate at doses of 125, 150, 175, 200, 250 and 275 mg/kg.

### 2.4. Measurement of total brain antiepileptic drug concentrations

Pharmacokinetic evaluation of total brain antiepileptic drug concentrations was performed only for those combinations of WIN with antiepileptic drugs, whose anticonvulsant effect in the MES test was significantly greater than that for control (an antiepileptic drug + vehicle-treated) animals. Thus, the measurement of total brain concentrations of carbamazepine, phenytoin, phenobarbital and valproate was undertaken at the doses, which corresponded to their $ED_{50}$ values from the MES test. Mice were killed by decapitation at times reflecting the peak of maximum anticonvulsant effects for the drugs in the MES test. The whole brains of mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 weight/volume) in an Ultra-Turrax T8 homogenizer (IKA Werke, Staufen, Germany). The homogenates were centrifuged at 10,000 *g* for 10 min. The supernatant samples (75 μl) were analyzed by fluorescence polarization immunoassay for carbamazepine, phenytoin, phenobarbital, and valproate content using a TDx analyzer and reagents exactly as described by the manufacturer (Abbott Laboratories, North Chicago, IL, USA). Total brain antiepileptic drug concentrations were expressed in μg/ml of brain supernatants as means ± S.D. of 8 separate brain preparations.

### 2.5. Grip-strength test

The effects of WIN, classical antiepileptic drugs and their combinations (at the $ED_{50}$ values from the MES test) on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. (1979). 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) comprised a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded as described earlier (Zadrozniak et al., 2009). The mean of 3 measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The muscular strength in mice was expressed in N (newtons) as means ± S.E.M. of 8 determinations.

### 2.6. Step-through passive avoidance task

Each animal was administered an antiepileptic drug either singly (at the $ED_{50}$ values from the MES test) or in combination with WIN on the first day before training. The time before the commencement of the training session (after drug administration) was identical to that for the MES test. Subsequently, animals were placed in an illuminated box (10 × 13 × 15 cm) connected to a larger dark box (25 × 20 × 15 cm) equipped with an electric grid floor. Entrance of animals to the dark box was punished by an adequate electric footshock (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 placed again into the illuminated box and observed 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 the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory (Venault et al., 1986).

### 2.7. Chimney test

The chimney test of Boissier et al. (1960) was used to quantify the adverse effect potential of classical antiepileptic drugs (at the $ED_{50}$ values from the MES test), WIN and their combinations on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 30 cm long), and impairment of motor performance was indicated by the inability of the mice to climb backward up the transparent tube within 60 s. The acute adverse effect potentials for the combinations of classical antiepileptic drugs with WIN were determined for the antiepileptic drugs administered at doses corresponding to their $ED_{50}$ values from the MES test when combined with WIN.

### 2.8. 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 (1949). Subsequently, the respective 95% confidence limits were transformed to S.E.M. as described previously (Luszczki et al., 2006b, 2009). Statistical analysis of data from the maximal electroshock-induced seizure threshold and MES tests was performed with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey–Kramer test for multiple comparisons. Total brain antiepileptic drug concentrations were statistically compared using the unpaired Student's *t*-test. The results from the grip-strength test were verified with one-way ANOVA followed by the post-hoc Bonferroni's test. The results obtained in the step-through passive avoidance task were statistically evaluated using Kruskal–Wallis nonparametric ANOVA followed by the post-hoc Dunn's test. 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).

## 3. Results

### 3.1. Effect of WIN on the threshold for maximal electroshock-induced seizures

WIN administered systemically (i.p., 20 min prior to the test), at doses of 2.5, 5 and 10 mg/kg did not affect the threshold for maximal electroconvulsions in mice (Table 1). In this case, the experimentally derived $CS_{50}$ values for animals receiving WIN did not differ significantly from the $CS_{50}$ value as determined for control animals in the maximal electroshock-induced seizure threshold test in mice (Table 1). In contrast, WIN administered at a dose of 15 mg/kg significantly elevated the threshold for maximal electroconvulsions in mice (Table 1).

### 3.2. Effects of WIN on the protective action of classical antiepileptic drugs in the mouse maximal electroshock seizure model

WIN administered alone at a dose of 15 mg/kg did not protect the animals against MES-induced seizures. In contrast, all studied antiepileptic drugs (carbamazepine, phenobarbital, phenytoin and valproate) administered singly exhibited a clear-cut anticonvulsant activity in the MES test in mice and their $ED_{50}$ values are presented in Table 2. When WIN at a dose of 10 mg/kg was co-administered with carbamazepine, phenytoin, phenobarbital and valproate it significantly enhanced the anticonvulsant action of all antiepileptic drugs in the MES test by reducing their $ED_{50}$ values (Table 2). Similarly, WIN at a dose of 5 mg/kg significantly potentiated the protective action of carbamazepine and valproate against MES-induced seizures (Table 2). In contrast, WIN at a dose of 5 mg/kg did not significantly affect the anticonvulsant action of phenobarbital and phenytoin in the MES test in mice (Table 2). Moreover, WIN at a dose of 2.5 mg/kg had no impact on the anticonvulsant potency of carbamazepine and valproate in the MES test (Table 2).

### 3.3. Effect of WIN on total brain antiepileptic drug concentrations

Fluorescence polarization immunoassay revealed that WIN administered systemically (i.p.) at doses of 5 and 10 mg/kg did not significantly alter total brain concentrations of carbamazepine, phenobarbital, phenytoin or valproate in mice (Table 3).

### 3.4. Effects of WIN in combination with various antiepileptic drugs on muscular strength of animals in the grip-strength test

When WIN (5 mg/kg) was administered in combination with carbamazepine (7.8 mg/kg) a significant impairment of muscular strength in mice was observed ($p<0.05$; Table 4). Similarly, the combination of WIN (5 mg/kg) with valproate (177.5 mg/kg) exerted a significant reduction in muscular strength in mice ($p<0.01$; Table 4). In case of WIN administered alone at the dose of 10 mg/kg, it was found that the non-specific cannabinoid CB1 and CB2 receptor agonist significantly diminished muscular strength in mice ($p<0.05$), as compared to the control (vehicle-treated) animals (Table 4). The animals that received WIN (10 mg/kg) with phenobarbital (10 mg/kg) and phenytoin (4.6 mg/kg) displayed a significant reduction in muscular strength as compared to the control value ($p<0.01$) and to the animals receiving the antiepileptic drugs alone ($p<0.05$; Table 4).

### 3.5. Effects of WIN in combination with various antiepileptic drugs on long-term memory in the step-through passive avoidance task

When WIN (5 mg/kg) was administered in combination with valproate (177.5 mg/kg) a significant impairment of long-term

**Table 1**
Effect of WIN on the threshold for electroconvulsions in mice.

| Treatment (mg/kg) | $CS_{50}$ (mA) | n |
|---|---|---|
| Vehicle | 6.31 ± 0.43 | 16 |
| WIN (2.5) | 6.89 ± 0.51 | 24 |
| WIN (5) | 7.52 ± 0.56 | 24 |
| WIN (10) | 7.93 ± 0.72 | 32 |
| WIN (15) | 9.12 ± 0.43[a] | 24 |
| F (4, 115) = 2.864; P = 0.0264 | | |

Data are presented as median current strengths ($CS_{50}$ values in mA ± S.E.M.) required to evoke seizure activity (tonic hindlimb extension) in 50% of animals tested. The $CS_{50}$ values were calculated according to the log-probit method by Litchfield and Wilcoxon (1949). WIN was administered systemically (i.p.) at 20 min before the initiation of electroconvulsions in mice. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey–Kramer test for multiple comparisons. n — total number of animals tested at those current strength intensities, whose seizure effects ranged between 16% and 84% according to Litchfield and Wilcoxon (1949); WIN — WIN 55,212-2 mesylate; F — F-statistics from one-way ANOVA; P — probability value from one-way ANOVA.
[a] $p<0.05$ vs. the control $CS_{50}$ value for vehicle-treated animals.

**Table 2**
Effect of WIN on the anticonvulsant activity of four classical antiepileptic drugs against maximal electroshock (MES)-induced seizures in mice.

| Treatment (mg/kg) | $ED_{50}$ (mg/kg) | n |
|---|---|---|
| Carbamazepine + vehicle | 13.18 ± 1.20 | 32 |
| Carbamazepine + WIN (2.5) | 11.11 ± 1.62 | 32 |
| Carbamazepine + WIN (5) | 7.82 ± 0.85 [a] | 24 |
| Carbamazepine + WIN (10) | 6.13 ± 0.82 [b] | 16 |
| F (3, 100) = 5.182; P = 0.0023 | | |
| Phenytoin + vehicle | 9.32 ± 1.02 | 24 |
| Phenytoin + WIN (5) | 8.44 ± 0.84 | 16 |
| Phenytoin + WIN (10) | 4.56 ± 0.75 [b] | 16 |
| F (2, 53) = 6.933; P = 0.0021 | | |
| Phenobarbital + vehicle | 26.17 ± 2.07 | 16 |
| Phenobarbital + WIN (5) | 19.12 ± 2.11 | 8 |
| Phenobarbital + WIN (10) | 10.00 ± 2.13 [c] | 16 |
| F (2, 37) = 16.47; P < 0.0001 | | |
| Valproate + vehicle | 244.3 ± 12.74 | 24 |
| Valproate + WIN (2.5) | 224.1 ± 10.70 | 24 |
| Valproate + WIN (5) | 177.5 ± 10.75 [b] | 24 |
| Valproate + WIN (10) | 147.5 ± 15.13 [c] | 32 |
| F (3, 100) = 11.88; P < 0.0001 | | |

Data are presented as median effective doses ($ED_{50}$ values in mg/kg ± S.E.M) of the antiepileptic drugs, protecting 50% of animals tested against MES-induced seizures (tonic hindlimb extension). The $ED_{50}$ values were calculated according to the log-probit method by Litchfield and Wilcoxon (1949). Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey–Kramer test for multiple comparisons. The drugs were administered i.p.: WIN — 20 min, phenytoin — 120 min, phenobarbital — 60 min, carbamazepine and valproate — 30 min prior to the MES test. n — total number of animals tested at those doses whose anticonvulsant effects ranged between 16% and 84% according to Litchfield and Wilcoxon (1949); WIN — WIN 55,212-2 mesylate; F — F-statistics from one-way ANOVA; P — probability value from one-way ANOVA.
[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ vs. the respective control group (an antiepileptic drug + vehicle-treated animals).

**Table 3**
Effect of WIN on total brain concentrations of classical antiepileptic drugs.

| Treatment (mg/kg) | Brain concentration (μg/ml) |
|---|---|
| Carbamazepine (7.8) + vehicle | 1.00 ± 0.26 |
| Carbamazepine (7.8) + WIN (5) | 1.05 ± 0.29 |
| Phenytoin (4.6) + vehicle | 0.79 ± 0.17 |
| Phenytoin (4.6) + WIN (10) | 0.84 ± 0.12 |
| Phenobarbital (10) + vehicle | 4.95 ± 0.29 |
| Phenobarbital (10) + WIN (10) | 5.13 ± 0.24 |
| Valproate (177) + vehicle | 143.1 ± 17.0 |
| Valproate (177) + WIN (5) | 135.7 ± 20.2 |

Data are presented as means ± S.D. of at least 8 separate determinations. Total brain antiepileptic drug concentrations were determined with fluorescence polarization immunoassay. Statistical evaluation of the data was performed using the unpaired Student's *t*-test. The 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 (for more detail see the legend to Table 2). WIN — WIN 55,212-2 mesylate.

**Table 4**
Effects of WIN and its combination with classical antiepileptic drugs on skeletal muscular strength in the grip-strength test, long-term memory in the step-through passive avoidance task and motor performance in the chimney test in mice.

| Treatment (mg/kg) | Grip-strength (N) | Retention time (s) | Motor impairment (%) |
|---|---|---|---|
| Vehicle | 95.63 ± 3.64 | 180 (180; 180) | 0 |
| WIN (5) + vehicle | 89.50 ± 2.82 | 180 (163.5; 180) | 37.5 |
| Carbamazepine (7.8) + vehicle | 91.00 ± 3.04 | 180 (162.5; 180) | 0 |
| Carbamazepine (7.8) + WIN (5) | 81.25 ± 2.60 [a] | 180 (110; 180) | 50 |
| Vehicle | 95.63 ± 3.64 | 180 (180; 180) | 0 |
| WIN (10) + vehicle | 83.00 ± 2.95 [a] | 176.5 (140; 180) | 50 |
| Phenytoin (4.6) + vehicle | 95.13 ± 2.69 | 180 (180; 180) | 0 |
| Phenytoin (4.6) + WIN (10) | 81.50 ± 2.46 [b, d] | 129.5 (97; 180) [a] | 50 |
| Vehicle | 95.63 ± 3.64 | 180 (180; 180) | 0 |
| WIN (10) + vehicle | 83.00 ± 2.95 [a] | 176.5 (140; 180) | 50 |
| Phenobarbital (10) + vehicle | 93.13 ± 3.28 | 180 (180; 180) | 0 |
| Phenobarbital (10) + WIN (10) | 79.63 ± 2.90 [b, d] | 64.5 (39.5; 89.5) [c, f] | 75 [b, e] |
| Vehicle | 95.63 ± 3.64 | 180 (180; 180) | 0 |
| WIN (5) + vehicle | 89.50 ± 2.82 | 180 (163.5; 180) | 37.5 |
| Valproate (177.5) + vehicle | 88.88 ± 2.50 | 162.5 (120.5; 180) | 12.5 |
| Valproate (177.5) + WIN (5) | 78.75 ± 2.99 [b] | 74.5 (45; 156.5) [b] | 75 [b, d] |

The results are presented as: 1) mean muscular strengths (in newtons ± S.E.M.) from the grip-strength test assessing skeletal muscular strength in mice; 2) median retention times (in seconds; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice; and 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 grip-strength test was performed with one-way ANOVA followed by the post-hoc Bonferroni's test for multiple comparisons, whereas the data from the step-through passive avoidance task were analyzed with nonparametric Kruskal–Wallis ANOVA test followed by the post-hoc Dunn's test for multiple comparisons. The 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-induced seizure test and at doses corresponding to their $ED_{50}$ values against MES-induced seizures (for more detail see the legend to Table 2). WIN — WIN 55,212-2 mesylate.
[a]$p<0.05$, [b]$p<0.01$, and [c]$p<0.001$ vs. the respective control group (vehicle-treated animals); [d]$p<0.05$, [e]$p<0.01$, and [f]$p<0.001$ vs. the respective an antiepileptic drug + vehicle-treated animals.

memory in mice was observed in the step-through passive avoidance task ($p<0.01$; Table 4). Similarly, WIN (10 mg/kg) in combination with phenobarbital (10 mg/kg) and phenytoin (4.6 mg/kg) exerted a significant impairment of long-term memory in mice subjected to the passive avoidance task (Table 4). In contrast, neither WIN administered alone at doses of 5 and 10 mg/kg, nor the antiepileptic drugs administered alone at doses corresponding to the $ED_{50}$ values from the MES-induced seizure test, did not significantly affect long-term memory in mice challenged with the passive avoidance task (Table 4). In case of the combination of WIN (5 mg/kg) with carbamazepine (7.8 mg/kg), no significant changes in long-term memory were observed in the experimental animals in the passive avoidance task (Table 4).

### *3.6. Effects of WIN in combination with various antiepileptic drugs on motor performance in the chimney test*

WIN administered alone at doses of 5 and 10 mg/kg produced slight changes in motor coordination in the chimney test, although statistical analysis of data with the Fisher's exact probability test revealed that the drug had no significant impact on motor performance in mice. Only the combinations of WIN (10 mg/kg) with phenobarbital (10 mg/kg) and WIN (5 mg/kg) with valproate (177.5 mg/kg) significantly altered motor coordination in mice, as compared to the control group ($p<0.01$), and the animals receiving the antiepileptic drugs alone ($p<0.05$ and $p<0.01$, respectively; Table 4). In case of the combinations of WIN (5 mg/kg) with carbamazepine (7.8 mg/kg) and WIN (10 mg/kg) with phenytoin (4.6 mg/kg), the animals did not exert any significant motor impairment, as compared to control group with the Fisher's exact probability test (Table 4).

## 4. Discussion

The results presented herein indicate that WIN in a dose-dependent manner elevates the threshold for electroconvulsions in mice. It was found that WIN at a dose of 15 mg/kg significantly increased the threshold for electroconvulsions in mice. Moreover, WIN administered systemically (i.p.) at subthreshold doses of 5 and 10 mg/kg significantly potentiated the anticonvulsant action of four classical antiepileptic drugs (carbamazepine, phenytoin, phenobarbital and valproate) in the mouse MES model. Pharmacokinetic evaluation of total brain antiepileptic drug concentrations with fluorescent polarization immunoassay technique revealed that WIN did not alter total brain concentrations of carbamazepine, phenytoin, phenobarbital and valproate. Thus, the observed enhancement of the anticonvulsant activities of the investigated antiepileptic drugs in the mouse MES model was pharmacodynamic in nature.

In our previous study, we have found that ACEA (a highly selective cannabinoid CB1 receptor agonist) enhanced the anticonvulsant action of valproate in the MES test in mice, although the observed interaction was complicated by a pharmacokinetic increase in free plasma and total brain valproate concentrations in experimental animals (Luszczki et al., 2006a). Moreover, ACEA significantly enhanced the anticonvulsant action of phenobarbital, but not that of carbamazepine and phenytoin in the mouse MES model (Luszczki et al., 2010). In this case, the observed interaction was pharmacodynamic in nature because ACEA did not affect total brain phenobarbital concentration in experimental animals (Luszczki et al., 2010). Since ACEA (the highly selective cannabinoid receptor CB1 agonist) potentiated the anticonvulsant activity of phenobarbital and valproate in the mouse MES model and WIN (the non-selective cannabinoid receptor CB1 and CB2 agonist) enhanced the anticonvulsant action of carbamazepine, phenytoin, phenobarbital and valproate, one can ascertain that WIN due to activation of both CB1 and CB2 cannabinoid receptor demonstrated potentiation of more antiepileptic drugs than ACEA in the mouse MES model.

It is important to note that the combination of WIN with four classical antiepileptic drugs allows the reduction of doses of classical antiepileptic drugs without decreasing the anticonvulsant potential of the drug mixtures that protected the animals against MES-induced seizures. The reduction of antiepileptic drug doses is favorable from a clinical point of view due to the reduction and/or elimination of adverse effects that usually accompany the treatment of epilepsy patients with antiepileptic drugs. In clinical practice, epilepsy patients usually receive antiepileptic drugs in monotherapy with high doses of antiepileptic drugs that are poorly tolerated by the patients.

Therefore, the reduction of side effects related with antiepileptic drugs' therapy is of important for these patients. At present, there exists a tendency to treat the epilepsy patients with rationally established polytherapy based on two or three antiepileptic drugs, applied at low doses that synergistically cooperate in terms of suppression of seizures and produce no or minimal side effects in patients (Deckers et al., 2000). Additionally, several combinations of two and three antiepileptic drugs can provide the epilepsy patients with a state of seizure freedom (Stephen and Brodie, 2002). Thus, the enhancement of the anticonvulsant action of classical antiepileptic drugs by WIN was favorable from a clinical viewpoint due to the reduction of antiepileptic drug doses offering the same protection against tonic–clonic seizures in the mouse MES model. Theoretically, the combinations of WIN with classical antiepileptic drugs were advantageous when considering only their anticonvulsant effects in terms of suppression of MES-induced seizures.

The evaluation of acute adverse-effect profile for WIN administered alone and in combination with the studied antiepileptic drugs, at doses corresponding to their $ED_{50}$ values from the MES test, revealed that WIN at a dose of 10 mg/kg significantly reduced skeletal muscular strength in animals. Moreover, the combinations of WIN with the studied antiepileptic drugs (carbamazepine, phenobarbital, phenytoin, and valproate) also reduced skeletal muscular strength in mice challenged with the grip-strength test. It was observed that muscular strength in animals receiving the combinations of WIN (10 mg/kg) with phenobarbital and phenytoin was diminished, even as compared to the strength in animals receiving the antiepileptic drugs alone. However, none of the antiepileptic drugs administered alone significantly affected skeletal muscular strength in mice.

Additionally, it was reported that WIN combined with phenobarbital, phenytoin and valproate considerably impaired long-term memory in animals challenged with the step-through passive avoidance task. Only the combination of WIN with carbamazepine did not affect long-term memory in animals challenged with the passive avoidance task. As regards the step-through passive avoidance task, we have documented earlier that the combinations of tiagabine with gabapentin or vigabatrin with clonazepam and valproate significantly disturbed long-term memory in mice (Luszczki et al., 2003a, 2005). However, the observed impairment of long-term memory in the step-through passive avoidance task was evoked by the antinociceptive action of the combinations, because the mentioned antiepileptic drug combinations considerably prolonged the latency to the first pain reaction in mice (Luszczki et al., 2003a, 2005). With respect to WIN, the cannabinoid receptor agonist produced antinociceptive action in the hot-plate test in mice (unpublished data). However, considering the fact that WIN 55,212-2 mesylate administered alone did not affect long-term memory in mice, it is less probable that the observed long-term memory deficits in mice receiving the combinations of WIN with classical antiepileptic drugs resulted from the antinociceptive action of WIN. In our opinion, more advanced behavioral studies are required to elucidate the exact nature of acute adverse effects of animals observed after systemic administration of WIN in combination with classical antiepileptic drugs.

Moreover, WIN combined with phenobarbital and valproate significantly impaired motor performance in animals challenged with the chimney test. Only the combinations of WIN with carbamazepine and phenytoin did not significantly impair motor coordination in mice. However, it is worthy of mentioning that statistical analysis of data from the chimney test was performed with the Fisher' exact probability test for 8 animals per group. In such a case, the impairment of motor coordination observed in 4 out of 8 animals (50%), [as observed for WIN (10 mg/kg) and the combinations of WIN with phenytoin and carbamazepine], was not statistically significant as compared to 8 control (vehicle-treated) animals. Quite recently, it has been reported that tiagabine co-administered with valproate produced a significant impairment in motor coordination, as determined in the chimney test (Luszczki et al., 2003b). In the present study, neither WIN, nor the antiepileptic drugs (phenobarbital or valproate) administered alone produced motor coordination impairment in mice. Moreover, in the study by Naderi et al. (2008), it has been reported that WIN, diazepam and their combination did not alter motor coordination in mice challenged with the rotarod test. Additionally, it is important to note that ACEA combined with classical and second-generation antiepileptic drugs did not produce any acute adverse effects in mice challenged with the chimney, passive-avoidance and grip-strength tests. Thus, one can suggest that the selective activation of cannabinoid CB1 receptors produced no acute adverse effects, whereas the non-selective activation of cannabinoid CB1 and CB2 receptors by WIN evoked acute adverse effects. The explanation of such a difference in the acute adverse effect profiles might be associated with selectivity and specificity of ACEA and WIN to cannabinoid CB1 and CB2 receptors. Although this hypothesis is speculative, it could readily explain the observed differences between the drugs in combination with classical antiepileptic drugs in the chimney test, step-through passive avoidance task and grip-strength test in mice. It should be stressed that the above-discussed facts clearly indicate that experimental tests used in the present study to assess acute adverse effects in animals were sensitive enough to detect any significant changes in animals' behavior.

Bearing in mind that WIN potentiated the anticonvulsant action of four classical antiepileptic drugs against tonic–clonic seizures and, simultaneously, produced acute adverse effects in experimental animals, one can conclude that the increased risk of acute side effects in epilepsy patients, despite favorable anticonvulsant properties in suppression of tonic–clonic seizures, votes against the combined treatment of epilepsy patients with classical antiepileptic drugs and WIN.

## 5. Conclusion

The combination of WIN with classical antiepileptic drugs should not be recommended to further clinical settings due to the increased risk of acute adverse effects, although, the application of WIN might occur favorable in certain patients with seizures evoked by metastatic cancer. In such a case, the reduction of antiepileptic drug doses is recommended due to pharmacodynamic interaction between drugs. If the results from this study could be extrapolated into clinical settings, the utmost caution is advised due to the increased risk of acute adverse effects and other signs of neurotoxicity in epileptic patients. Although WIN significantly enhanced the anticonvulsant activity of four classical antiepileptic drugs (carbamazepine, phenytoin, phenobarbital and valproate), it also impaired motor coordination and long-term memory as well as reduced skeletal muscular strength in experimental animals.

## Disclosure of conflicts of interest

Professor S.J. Czuczwar has received support from UCB Pharma and Sanofi-Aventis as a speaker. The remaining authors have no conflicts of interest to disclose.

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