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Effect of osthole on the protective activity of carbamazepine and phenobarbital against maximal electroshock-induced seizures in mice

Wpływ ostolu na ochronne działanie karbamazepiny i fenobarbitalu w teście maksymalnego wstrząsu elektrycznego u myszy

INTRODUCTION

Osthole [7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one] – a natural coumarin derivative is extracted from many medicinal plants, such as *Angelica pubescens, Cnidium monnieri* and *Peucedanum ostruthium* [2, 3, 5, 6, 24, 28]. Previous experimental studies have shown that osthole exerts a broad spectrum of pharmacological activities due to its anti-platelet [8], anti-tumor [35], anti-allergic [4, 22], anti-apoptotic [26], anti-oxidative [27, 30, 31], anti-proliferative [5, 7], estrogen-like [9], anti-osteoporotic [9, 10, 33], hepatoprotective [34], antidiabetic [11], and anti-inflammatory [25] effects. In addition, osthole is a potential antioxidant eliminating oxygen free radicals and inhibiting lipid peroxidation [27, 30, 31].

Accumulating evidence indicates that imperatorin [9-(3-methylbut-2-enyloxy)-7H-furo[3,2-g] chromen-7-one] – another natural coumarin derivative, possesses the anticonvulsant activity in preclinical studies by elevating the threshold for electroconvulsions [17] and enhancing the anticonvulsant action of carbamazepine, phenobarbital, phenytoin [16], and lamotrigine [21] in the mouse maximal electroshock-induced seizure model. Moreover, it has been found that both natural coumarin derivatives (imperatorin and osthole) exerted a clear-cut anticonvulsant activity against maximal electroshock-induced seizures in mice [20].

Since osthole and imperatorin exerted the antielectroshock action in mice when administered alone, and imperatorin potentiated the anticonvulsant action of some classical antiepileptic drugs, it was of importance to determine whether osthole enhances the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model. It is widely accepted that the maximal electroshock seizure test is considered as an experimental model of tonic-clonic seizures and, to a certain extent, of partial seizures with or without secondary generalization [13]. Moreover, this experimental model of epilepsy is widely used for an investigation of the new drugs and for selection of the agents with antiseizure activity in vivo [13]. In this model one can readily evaluate the anticonvulsant effects produced by classical antiepileptic drugs in combination with osthole, therefore, it was appropriate to use this test in the present study.

Additionally, we investigated the combinations of osthole 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.

MATERIALS AND METHODS

A n I m a l s a n d e x p e r i m e n t a l . 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 ± 1ºC, relative humidity of 55 ± 5%), were used. After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups comprising of 8 mice. Each mouse was used only once and all tests were performed between 08.00 and 15.00 hours. 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 Second Local Ethics Committee at the University of Life Sciences in Lublin (License no. 78/2009) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

D r u g s . The following antiepileptic drugs were used in this study: carbamazepine (a gift from Polfa, Starogard, Poland) and phenobarbital (Polfa, Krakow, Poland). All drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and administered intraperitoneally (i.p.) as a single injection, in a volume of 5 ml/kg body wt. Fresh drug solutions were prepared on each day of experimentation and administered as follows: phenobarbital - 60 min and carbamazepine - 30 min, before electroconvulsions, motor coordination, grip-strength and long-term memory tests. The pretreatment times before testing of the antiepileptic drugs were based upon information about their biological activity from the literature and our previous experiments [14-16]. The times to the peak of maximum anticonvulsant effects for all antiepileptic drugs were used as the reference times in all behavioral tests. Osthole ([7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one]; $C_{15}H_{16}O_3$; 244.29 MW; chemical purity >97%) was extracted from roots of *Peucedanum ostruthium* (L.) Koch, which were collected from plants in September 2007, in Karpacz Gorny (Sudetes, Poland). The air-dried and powdered roots (920 g) were extracted exhaustively

(~120 h) in the Soxhlet extractor with petroleum ether. After extraction and cooling procedure osthole was crystallized and drained off. The petroleum ether extract was concentrated in a rotary vacuum evaporator. The remains were dissolved in methanol and left for final osthole precipitation (40 g of remains were dissolved in 200 ml of boiling methanol). Osthole obtained from the methanol extract was added to that from petroleum ether and recrystallized by obtaining 10 g of pure osthole. The identity of osthole was confirmed by TLC and ^{1}H-NMR analyses. Osthole was suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and administered i.p. at 30 min before the initiation of electroconvulsions and behavioural tests.

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.

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 [12], 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 osthole 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 osthole: 50, 100, 150 and 200 mg/kg. The experimental procedure has been described in more detail in our earlier studies [14-16, 18, 20, 21].

Maximal electroshock seizure test. The protective activity of two classical antiepileptic drugs (carbamazepine and phenobarbital) was determined as their median effective doses (ED_{50} values in mg/kg) against maximal electroshock-induced seizures (fixed current intensity of 25 mA). The animals were administered with different drug doses so as to obtain a variable percentage of protection against maximal electroshock seizures, allowing the construction of a dose-response relationship curve for each antiepileptic drug administered alone, according to Litchfield and Wilcoxon [12]. Each ED_{50} value represents the dose of a drug required to protect half of the animals tested against maximal electroshock seizures. Similarly, the anticonvulsant activity of a mixture of an antiepileptic drug with osthole was evaluated and expressed as ED_{50} corresponding to the dose of an antiepileptic drug necessary to protect 50% of mice against tonic hindlimb extension in the maximal electroshock seizure test. In the present study, carbamazepine was administered at doses ranging between 4–12 mg/kg and phenobarbital at doses ranging between 10–25 mg/kg. This experimental procedure has been described in detail in our earlier studies [14-16, 18, 20, 21].

Chimney test. The chimney test of Boissier et al. [1] was used to quantify the adverse effect potential of classical antiepileptic drugs administered in combination with osthole on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length), and motor performance impairment was indicated by the inability of the mice to climb backward up the transparent tube within 60 s. The acute adverse effect potentials of classical antiepileptic drugs administered in combination with osthole were determined for antiepileptic drugs administered at doses corresponding to their ED_{50} values from the maximal electroshock seizure test when combined with osthole at a dose of 200 mg/kg. This experimental procedure has been described in detail in our earlier studies [14, 16, 18-21].

Grip – strength test. The effects of combinations of osthole with classical antiepileptic drugs at their ED_{50} values from the maximal electroshock seizure test, on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. [23]. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the maximal electroshock seizure test. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 x 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 3 measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The neuromuscular strength in mice was expressed in Newtons (N) as means ± S.E. of at least 8 determinations. This experimental procedure has been described in detail in our earlier study [32].

Light – dark, step – through passive avoidance task. Each animal was administered an antiepileptic drug either singly or in combination with osthole on the first day before training. The time before the commencement of the training session (after drug administration) was identical to that for the maximal electroshock seizure test. Subsequently, animals were placed in an illuminated box (10 x 13 x 15 cm) connected to a larger dark box (25 x 20 x 15) 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 [29]. This experimental procedure has been described in detail in our earlier study [19].

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 [12]. Subsequently, the respective 95% confidence limits were transformed to standard errors (S.E.), as published earlier [14]. Statistical analysis of data from the electroconvulsive tests was performed with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey/Kramer test for multiple comparisons. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test, whereas, the results obtained in the passive avoidance task were statistically evaluated using Kruskal-Wallis nonparametric ANOVA. The results from the grip-

strength test were verified with one-way ANOVA. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences among values were considered statistically significant if $p<0.05$.

RESULTS

INFLUENCE OF OSTHOLE UPON THE THRESHOLD FOR ELECTROCONVULSIONS

Osthole (administered alone, i.p., 30 min prior to the test) dose-dependently raised the CS_{50} values, necessary to produce tonic hindlimb extension in 50% of animals. In this case, osthole at a dose of 200 mg/kg significantly elevated the CS_{50} value from 6.40 mA to 9.05 mA (by 41%; $P<0.01$; Table 1). In contrast, the CS_{50} values for osthole, administered at doses of 50, 100 and 150 mg/kg, did not reach statistical significance, although a slight (dose-dependent) increase in the CS_{50} values was observed in the maximal electroshock seizure threshold test in mice (Table 1).

Table 1. Effect of osthole on the threshold for maximal electroconvulsions in mice

Treatment (mg/kg)	CS_{50} (mA)	n
Vehicle	6.40 ± 0.47	16
Osthole (50)	6.51 ± 0.41	24
Osthole (100)	6.79 ± 0.37	16
Osthole (150)	7.21 ± 0.36	32
Osthole (200)	9.05 ± 0.45 **	16
F (4; 99) = 5.446; P = 0.0005		

Results are presented as median current strengths (CS_{50} in mA ± S.E.) required to produce tonic hindlimb extension in 50% of animals tested. The CS_{50} values were calculated using the log-probit method [12], followed by the method transforming 95% confidence limits to S.E. [14]. Osthole was administered i.p., at 30 min before maximal electroconvulsions. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey/Kramer test for multiple comparisons. n – number of animals at those current strengths, whose convulsant effects ranged between 16% and 84%; F – F-statistics from one-way ANOVA; P – probability value from one-way ANOVA; **p<0.01 vs. control group (vehicle-treated animals)

EFFECTS OF OSTHOLE ON THE PROTECTIVE ACTION OF CARBAMAZEPINE AND PHENOBARBITAL IN THE MOUSE MAXIMAL ELECTROSHOCK-INDUCED SEIZURE MODEL

The investigated classical antiepileptic drugs (carbamazepine and phenobarbital) administered alone exhibited a clear-cut anticonvulsant activity in the maximal electroshock seizure test in mice and their ED_{50} values are presented in Table 2. When osthole at doses of 50, 100 and 150 mg/kg was co-administered with carbamazepine, it did not significantly enhance the anticonvulsant effect of the latter drug against maximal electroshock-induced seizures (Table 2). In the case of phenobarbital,

osthole at doses of 50, 100 and 150 mg/kg did not significantly affect the antielectroshock action of phenobarbital in the mouse maximal electroshock-induced seizure model (Table 2). In all cases, the osthole-induced reduction in ED_{50} values of classical antiepileptic drugs did not attain statistical significance with one-way ANOVA (Table 2).

Table 2. Effect of osthole on the protective activity of carbamazepine and phenobarbital against maximal electroshock-induced seizures in mice

Treatment (mg/kg)	ED_{50} (mg/kg)	n
Carbamazepine + vehicle	8.87 ± 0.87	16
Carbamazepine + osthole (50)	8.18 ± 0.80	16
Carbamazepine + osthole (100)	7.65 ± 0.84	8
Carbamazepine + osthole (150)	6.89 ± 0.71	32
F (3; 68) = 1.210; P = 0.3127		
Phenobarbital + vehicle	18.17 ± 1.80	16
Phenobarbital + osthole (50)	17.72 ± 1.83	24
Phenobarbital + osthole (100)	15.31 ± 2.04	16
Phenobarbital + osthole (150)	12.35 ± 2.32	24
F (3; 76) = 1.792; P = 0.1558		

Results are presented as median effective doses (ED_{50} in mg/kg ± S.E.) of antiepileptic drugs, protecting 50% of animals tested against maximal electroshock-induced hindlimb extension. The antiepileptic drugs were administered i.p.: phenobarbital – 60 min., and carbamazepine – 30 min. prior to the maximal electroshock-induced seizure test. Osthole was administered i.p. at 30 min. before electroconvulsions. 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 used at those doses whose anticonvulsant effects ranged between 4 and 6 probits. F – F-statistics from one-way ANOVA; P – probability value from one-way ANOVA.

EFFECTS OF OSTHOLE IN COMBINATION WITH CARBAMAZEPINE AND PHENOBARBITAL ON MOTOR PERFORMANCE, LONG-TERM MEMORY, AND MUSCULAR STRENGTH OF ANIMALS IN THE CHIMNEY, STEP-THROUGH PASSIVE AVOIDANCE AND GRIP-STRENGTH TESTS

When osthole was administered in combination with carbamazepine and phenobarbital at doses corresponding to their ED_{50}s from the maximal electroshock seizure test, motor performance as assessed by the chimney test was unaffected (Table 3). Furthermore, none of the combinations studied impaired long-term memory as determined in the passive avoidance test, the median retention times being approximately 180 s (Table 3). Likewise, osthole combined with two classical antiepileptic drugs had no significant impact on muscular strength of animals as assessed by the grip-strength test (Table 3).

Table 3. Effects of osthole and its combinations with carbamazepine and phenobarbital on long-term memory, muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Impairment of motor coordination (%)
Control	180 (180; 180)	104.38 ± 4.88	0
Osthole (150) + vehicle	180 (180; 180)	98.50 ± 4.27	12.5
Carbamazepine (6.9) + vehicle	180 (180; 180)	105.15 ± 4.59	0
Carbamazepine (6.9) + osthole (150)	180 (180; 180)	99.69 ± 4.47	0
Phenobarbital (12.4) + vehicle	180 (180; 180)	102.31 ± 4.68	0
Phenobarbital (12.4) + osthole (150)	180 (180; 180)	100.25 ± 4.30	0

Results are presented as: 1) median retention times (in seconds; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice; 2) mean grip-strengths (in Newtons ± S.E.) from the grip-strength test, assessing muscular strength in mice; and 3) percentage of animals showing motor coordination impairment in the chimney test in mice. Statistical analysis of data from the passive avoidance task was performed with nonparametric Kruskal-Wallis ANOVA test, whereas those from the grip-strength test were analyzed with one-way ANOVA. 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 maximal electroshock seizure test and at doses corresponding to their ED50 values against maximal electroconvulsions (for more details see the legend to Table 2)

DISCUSSION

Results presented in this study indicate that osthole dose-dependently increased the threshold for electroconvulsions in mice. The natural compound administered systemically (i.p.) at a dose of 200 mg/kg significantly elevated the threshold for electroconvulsions in mice. It has been reported that osthole at doses ranging between 50 and 150 mg/kg also increased the threshold, although statistical analysis of data revealed no significance between the threshold in mice receiving osthole as compared to control animals. This finding is consistent with our previous results reporting that osthole administered i.p. exerted a clear-cut anticonvulsant action in the mouse maximal electroshock-induced seizure model [20].

Moreover, we found that osthole administered systemically (i.p.) at doses up to 150 mg/kg did not significantly affect the anticonvulsant action of two classical antiepileptic drugs (carbamazepine and phenobarbital) in the mouse maximal electroshock-induced seizure model. In contrast, imperatorin (a natural coumarin derivative) possessed a clear-cut anticonvulsant action and enhanced the anticonvulsant action of carbamazepine, phenobarbital, phenytoin and lamotrigine, but not that of valproate in the mouse maximal electroshock-induced seizure model [16, 21].

As regards the acute adverse-effect potentials of osthole in combination with classical antiepileptic drugs, one can conclude that osthole, similarly to imperatorin, did not affect long-term memory, motor performance or muscular strength in mice. Thus, one can suggest that the combined therapy of osthole with classical antiepileptic drugs is devoid of any acute side effects. Previously, we have reported that WIN 55,212-2 mesylate (WIN – a synthetic cannabinoid CB1 and CB2 receptor agonist) significantly impaired long-term memory in animals receiving WIN in combination with classical antiepileptic drugs [15]. Moreover, WIN disturbed muscular strength in mice receiving the combination of WIN with classical antiepileptic drugs [15]. Additionally, we have reported that the combination of tiagabine with valproate significantly impaired motor coordination in mice challenged with the chimney test [18]. The above-mentioned facts suggest that all behavioral tests applied in this study (chimney, passive avoidance and grip-strength tests) were sensitive enough to detect any changes in normal behavior in mice. Thus, one can ascertain that since osthole in combination with classical antiepileptic drugs is devoid of any acute adverse effects in animals, the further therapy based on these drugs might be safe and tolerable by patients. However, more advanced studies are required to establish the influence of osthole on seizure activity in various experimental models of epilepsy. It is noteworthy that osthole was tested in the present study at doses up to 200 mg/kg, whereas the ED_{50} value for this compound, as determined in the mouse maximal electroshock-induced seizure model was 253 mg/kg [20].

CONCLUSIONS

The results presented herein indicate that osthole can be used as a supplement of diet in epileptic patients. However, the coumarin derivatives exerted no significant enhancement of the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model. Perhaps, the combinations of osthole with these classical antiepileptic drugs could be

advantageous in other epilepsy models, however, to confirm this hypothesis more advanced studies are required in various experimental models.

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SUMMARY

The aim of this study was to determine the effect of osthole on the anticonvulsant activity of two classical antiepileptic drugs (carbamazepine and phenobarbital) in the mouse maximal electroshock seizure model. Electroconvulsions were evoked in adult Albino Swiss mice by a current (50Hz, 500V, 0.2s stimulus duration) delivered via auricular electrodes. Acute adverse-effect profiles of the combination of osthole with carbamazepine and phenobarbital were measured in the chimney test (motor performance), passive avoidance task (long-term memory) and grip-strength test (skeletal muscular strength) in mice. Results indicate that osthole administered intraperitoneally (i.p.) at a dose of 200 mg/kg significantly elevated (by 41%; $p<0.01$) the threshold for electroconvulsions in mice. Osthole at lower doses of 50, 100 and 150 mg/kg had no significant impact on the threshold for electroconvulsions in mice. Osthole (50, 100 and 150 mg/kg, i.p.) did not significantly affect the protective action of carbamazepine and phenobarbital in the maximal electroshock-induced seizures in mice. Moreover, osthole in combination with carbamazepine and phenobarbital did not alter motor performance, long-term memory or skeletal muscular strength in experimental animals. The present study demonstrates that osthole, although elevated the threshold for electroconvulsions, had no significant effect on the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model.

Keywords: Osthole, carbamazepine, phenobarbital, maximal electroshock seizure test

STRESZCZENIE

Celem pracy była ocena wpływu ostolu na przeciwdrgawkowe działanie dwóch klasycznych leków przeciwpadaczkowych (karbamazepiny i fenobarbitalu) w modelu maksymalnego wstrząsu elektrycznego u myszy. Drgawki elektryczne były wywoływane u dorosłych samców myszy Albino Swiss prądem (50Hz, 500V, 0,2s czas trwania impulsu) przez elektrody uszne. Profil ostrych działań niepożądanych kombinacji ostolu z karbamazepiną i fenobarbitalem oceniono w teście komina (koordynacja ruchowa), teście biernego unikania (pamięć długotrwała) i teście chwytania (siła

mięśni szkieletowych) u myszy. Wyniki wskazują, że ostol podany dootrzewnowo (i.p.) w dawce 200 mg/kg istotnie podnosił (o 41%; $p<0,01$) próg pobudliwości drgawkowej. Ostol w niższych dawkach 50, 100 i 150 mg/kg nie miał istotnego wpływu na próg dla drgawek elektrycznych u myszy. Ostol (50, 100 i 150 mg/kg; i.p.) nie wpływał istotnie na ochronne działanie karbamazepiny i fenobarbitalu w teście maksymalnego wstrząsu elektrycznego. Ponadto ostol w kombinacji z karbamazepiną i fenobarbitalem nie zmieniał koordynacji ruchową, pamięci długotrwałej i siły mięśni szkieletowych u badanych zwierząt. Bieżące badanie wykazało, że ostol chociaż podnosił próg pobudliwości drgawkowej, to nie miał istotnego wpływu na przeciwdrgawkowe działanie karbamazepiny i fenobarbitalu w modelu maksymalnego wstrząsu elektrycznego u myszy.

Słowa kluczowe: Ostol, karbamazepina, fenobarbital, maksymalny wstrząs elektryczny