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Amlodipine enhances the anticonvulsant effect of oxcarbazepine in the maximal electroshock-induced seizure test in mice

A large body of evidence indicates that calcium ions (Ca^{2+}) play a crucial role in the pathophysiology of epilepsy because changes in both extracellular and intracellular calcium concentrations are usually observed prior to the onset of seizure activity (1). Moreover, experimental evidence indicates that some calcium channel antagonists reduce the incidence of seizures and possess anticonvulsant properties in various experimental seizure models [2–5). For instance, it has been found that some dihydropyridine derivatives were effective in the maximal electroshock seizure (MES), pentylenetetrazole, picrotoxin, N-methyl-D-aspartic acid, pilocarpine, amygdala-kindling, and sound-induced seizure models in rodents (6–11). Interestingly, some calcium channel antagonists (amlodipine, diltiazem, flunarizine, and nimodipine), enhance the protective activity of some antiepileptic drugs (AEDs) in both preclinical studies on animals (3, 4, 12, 13), and clinical settings in humans (14–18). Specifically, flunarizine, cinnarizine, and nimodipine have been reported to be beneficial as add-on treatment in epileptic patients (14–18). Generally, it is thought that the blockade of high voltage-activated (L-, N-, P/Q-type) calcium channels is associated with control of partial seizures with or without secondary generalization (1, 5, 8).

Considering the fact that seizure activity depends on calcium ions and some AEDs interfere with calcium ion fluxes (1, 5, 8, 14–18), the objective of this study was to determine the effects of amlodipine (a calcium channel antagonist) on the protective activity of oxcarbazepine (a second-generation AED) in the mouse MES model. Noteworthy, oxcarbazepine has been licensed as add-on treatment for adults with refractory epilepsy and as monotherapy in newly diagnosed epilepsy, especially, in patients with generalized tonic-clonic seizures and partial convulsions with or without secondary generalization (19). It is widely accepted that the MES test is considered as an experimental animal model, allowing to select drugs that are effective in suppression of generalized tonic-clonic seizures and, to a certain extent, of partial seizures with or without secondary generalization (20). Thus, it was appropriate to examine the anticonvulsant effects of oxcarbazepine administered alone and in combination with amlodipine in the mouse MES model. Moreover, the acute adverse-effect potentials of oxcarbazepine in combination with amlodipine were determined in the chimney test (motor performance), step-through passive avoidance task (long-term memory) and grip-strength test (muscular strength) in mice. To confirm or exclude pharmacokinetic characteristics of interactions between oxcarbazepine and the calcium channel antagonists, total brain oxcarbazepine concentrations were measured with high-performance liquid chromatography (HPLC).

MATERIAL AND METHODS

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, the 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 First Local Ethics Committee in Lublin (License no. 516/2005/550/2005) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Guide to the Care and Use of Experimental Animals.

Drugs. The following drugs were used in this study: amlodipine (Adamed, Pienkow, Poland) and oxcarbazepine (Novartis Pharma AG, Basel, Switzerland). The drugs were suspended in 1% aqueous solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered intraperitoneally (i.p.), in a volume of 5 ml/kg body weight. Fresh drug solutions were prepared on each day of experimentation and administered as follows: amlodipine – 120 min, and oxcarbazepine – 30 min before electroconvulsions, motor coordination, muscular strength and long-term memory evaluation, as well as, before brain sampling for the measurement of oxcarbazepine concentrations. These pretreatment times were based upon information about their biological activity from the literature and our previous studies (3, 4, 12, 13, 21). The time to the peak of maximum anticonvulsant effects for oxcarbazepine was used as the reference time in all behavioral tests and pharmacokinetic estimation of brain oxcarbazepine concentrations. In this study, oxcarbazepine was administered at doses ranging between 6 to 16 mg/kg.

Maximal electroshock-induced seizures. Electroconvulsions were produced by a current (sine-wave impulse, fixed current intensity of 25 mA, 500 V, 0.2 s stimulus duration) delivered via ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs Elektronik, Freiburg, Germany). 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). The protective activity of oxcarbazepine was determined as its median effective dose (ED_{50} value 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, allowing construction of a dose-response relationship curve for oxcarbazepine administered alone, according to Litchfield and Wilcoxon (22). The ED_{50} value represents the dose of a drug required to protect half of the animals tested against MES. Similarly, the anticonvulsant activity of the mixtures of oxcarbazepine with the calcium channel antagonist amlodipine was evaluated and expressed as ED_{50}, corresponding to the dose of oxcarbazepine necessary to protect 50% of mice against tonic hindlimb extension in the MES test. This experimental procedure has been described in detail in our earlier studies (12, 13, 23–26).

Chimney test. The chimney test of Boissier et al. (27) was used to quantify the adverse effect potential of oxcarbazepine administered in combination with amlodipine on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 30 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 adverse-effect potential of oxcarbazepine co-administered with amlodipine were determined for drugs administered at doses corresponding to

their ED_{50} values from the MES test. This experimental procedure has been described in detail in our earlier studies (12, 13, 24, 26).

Grip-strength test. The effects of the combination of oxcarbazepine with amlodipine at doses corresponding to the ED_{50} value from the MES test, on muscular strength in mice were quantified by the grip-strength test. 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. The muscular strength in mice was expressed in N (newtons) as means ± S.E. of 8 animals per group. This experimental procedure has been described in detail in our earlier studies (12, 13, 28).

Step-through passive avoidance task. Each animal was administered oxcarbazepine with amlodipine at doses corresponding to the ED_{50} value from the MES test 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 did not receive any treatment and 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 25^{th} and 75^{th} 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 (30).

Measurement of total brain oxcarbazepine concentration. The measurement of total brain concentration of oxcarbazepine was undertaken at a dose, which corresponded to its ED_{50} value from the MES test for the combination of oxcarbazepine with amlodipine. Mice were killed by decapitation at times chosen to coincide with that scheduled for the MES test and the whole brains of mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 w/v) in an Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany). The homogenates were centrifuged at 10,000 × g for 10 min. The supernatant samples (400 µl) were analyzed by HPLC for oxcarbazepine content. The chromatograph (Laboratorij Pristroje, Praha, Czech Republic) was equipped with a 305 micropump (LCP 3001) and an ultraviolet (UV) detector (HP 1050) with a sensitivity setting of 0.1 AUFS (absorbance units full scale) and a time constant of 0.1 s. The Rheodyne 7125 injector valve with a 100 µl sample loop was used for sample injection. For HPLC, a stainless-steel Hypersil ODS column (200 × 4.6 mm) was used at an ambient temperature of 22°C. The mobile phase was methanol: acetonitrile: acetate buffer (5 mM acetic acid/ 50 mM sodium acetate); 15:15:70 vol/vol/vol (Baker HPLC grade). The mobile phase flow rate was 1 ml/min. Brain supernatants of 400 µl were added to 400 µl of distilled water and shaken. Subsequently, the external standard of 0.15 µg of carbamazepine in 150 µl of methanol:water solution (1:1) was added. Again, the samples were shaken and to each sample, a volume of 4 ml of tertbuthyl-methyl ether was added and centrifuged for 4 min at 2,000×g. The samples were evaporated to dryness under a vacuum system and redissolved in 1 ml of naphthyl ether (HPLC, Aldrich), and again evaporated to dryness under a vacuum system. The remains were redissolved in 100 µl of the mobile phase; samples of 50 µl were then injected into the chromatograph. Oxcarbazepine concentrations were calculated according to the

external standard method using the original Gilson 715 software. The amount of oxcarbazepine was determined by comparing their peak area with the peak area of the external standard (carbamazepine). The wave excitation and emission parameters for detection of oxcarbazepine were 220 and 310 nm, respectively. The limit of detection of the method was 0.01 μg/ml and the within-batch and between-batch precisions were <5% and <6% respectively. Total brain concentrations of oxcarbazepine were expressed in μg/ml of brain supernatants as means ± standard deviations (S.D.) of 8 determinations (8 separate brain preparations).

Statistical analysis. The ED_{50} values for oxcarbazepine with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon (22). Subsequently, the respective 95% confidence limits were transformed to standard errors (S.E.), as described previously (24, 26). Statistical analysis of data from the MES model 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 the 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. Total brain oxcarbazepine concentrations were statistically compared using the unpaired Student's *t*-test. Differences among values were considered statistically significant if $P<0.05$. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

EFFECT OF AMLODIPINE ON THE PROTECTIVE ACTION OF OXCARBAZEPINE IN THE MOUSE MAXIMAL ELECTROSHOCK-INDUCED SEIZURE MODEL

Oxcarbazepine administered alone (i.p.) produced clear-cut anticonvulsant effects against MES-induced seizures in mice and its ED_{50} value is presented in Table 1. Amlodipine co-administered with oxcarbazepine enhanced, in a dose-dependent manner, the antielectroshock action of oxcarbazepine by reducing its ED_{50} value in the MES test. One-way ANOVA followed by the *post-hoc* Tukey-Kramer test for multiple comparisons revealed that amlodipine at a dose of 20 mg/kg significantly decreased (by 33%) the ED_{50} value of oxcarbazepine from 14.25 to 9.52 mg/kg ($P<0.01$; Table 1). Amlodipine at lower doses of 5 and 10 mg/kg also reduced the ED_{50} value of oxcarbazepine, however, the statistical analysis of data did not attain significance with the Tukey-Kramer *post-hoc* test (Table 1).

EFFECT OF OXCARBAZEPINE IN COMBINATION WITH AMLODIPINE ON MOTOR PERFORMANCE, LONG-TERM MEMORY, AND MUSCULAR STRENGTH OF ANIMALS IN THE CHIMNEY, STEP-THROUGH PASSIVE AVOIDANCE AND GRIP-STRENGTH TESTS

When oxcarbazepine was administered in combination with amlodipine at doses corresponding to its ED_{50} value from the MES test, motor performance as assessed by the chimney test was unaffected (Table 2). Furthermore, the combination of oxcarbazepine with the calcium channel antagonist amlodipine did not impair long-term memory as determined in the passive avoidance test, and the median retention times being approximately 180 s (Table 2). Likewise, oxcarbazepine combined with amlodipine had no significant impact on the muscular strength of animals as assessed by the grip-strength test (Table 2).

Table 1. Effect of amlodipine on the anticonvulsant activity of oxcarbazepine in the mouse maximal electroshock seizure model

Treatment (mg/kg)	ED_{50} (mg/kg)	n	S.E.
Oxcarbazepine + vehicle	14.25 (12.79–15.89)	16	0.790
Oxcarbazepine + Amlodipine (5)	12.24 (10.78–13.90)	16	0.793
Oxcarbazepine + Amlodipine (10)	11.58 (10.07–13.31)	24	0.824
Oxcarbazepine + Amlodipine (20)	9.52 (8.15–11.12) **	16	0.756
F (3;68) = 5.110; P = 0.0030			

Results are presented as median effective doses (ED_{50} in mg/kg; with 95% confidence limits in parentheses) required to protect 50% of animals tested against maximal electroshock-induced seizures. The ED_{50} values were calculated by the use of log-probit method (22), followed by the method transforming 95% confidence limits to S.E. (26). Amlodipine and oxcarbazepine were suspended in 1% aqueous solution of Tween 80 and administered systemically (i.p.), as follows: amlodipine at 120 min and oxcarbazepine 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 – number of animals at those doses, whose anticonvulsant effects ranged between 16% and 84% (4 and 6 probits); S.E. – standard error of the ED_{50} values
**P<0.01 vs. the respective control group (Oxcarbazepine + vehicle-treated animals)

Table 2. Effect of amlodipine, oxcarbazepine and their combination on long-term memory, skeletal muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Motor coordination impairment (%)
Vehicle	180 (180; 180)	82.01 ± 4.58	0
Amlodipine (20) + vehicle	180 (170; 180)	78.95 ± 4.27	12.5
Oxcarbazepine (9.5) + vehicle	180 (180; 180)	80.63 ± 4.47	0
Oxcarbazepine (9.5) + Amlodipine (20)	180 (180; 180)	79.63 ± 4.67	25

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 the ED_{50} value against maximal electroconvulsions (for more detail see the legend to Table 1).

INFLUENCE OF AMLODIPINE ON TOTAL BRAIN CONCENTRATION OF OXCARBAZEPINE

The HPLC method revealed that total brain concentration of oxcarbazepine administered alone at a dose of 9.5 mg/kg was 1.260 ± 0.424 μg/ml of brain supernatant and did not differ significantly from that for the combination of oxcarbazepine (9.5 mg/kg) with amlodipine (20 mg/kg), which was 1.428 ± 0.458 μg/ml of brain supernatant.

DISCUSSION

Results clearly indicate that amlodipine significantly enhanced the antiseizure action of oxcarbazepine in the mouse MES test. These findings are in agreement with those observed previously, showing that amlodipine potentiated the anticonvulsant action of carbamazepine, lamotrigine,

phenobarbital, topiramate, and valproate, but not that of phenytoin in the MES test in mice (4, 12, 13). It is important to note that doses of amlodipine used in this study did not significantly affect the threshold for electroconvulsions in mice. Experimental evidence indicates that amlodipine (up to 20 mg/kg) had no significant impact on the threshold for maximal electroconvulsions in mice (12, 13), and these findings are partially consistent with those documented earlier (3, 4).

To scientifically explain the appearance of the favorable interaction between oxcarbazepine and amlodipine in the MES test, one should consider molecular mechanisms of action of these drugs. With respect to amlodipine, the drug belongs to the 1,4-dihydropyridine class of calcium channel antagonists (31), and it blocks N- and P/Q-type calcium channels, showing high affinity for both these channels (32, 33). As regards the anticonvulsant effect of oxcarbazepine, it has been documented that the drug acts at voltage-dependent sodium channels to decrease the presynaptic release of the excitatory neurotransmitter glutamate (34, 35). Oxcarbazepine blocks high voltage activated N-type calcium channels (36). Thus, it seems that amlodipine enhances the antiseizure action of oxcarbazepine due to the similar mechanisms of action associated with the blockade of N-type calcium channels in terms of the reduction of seizure activity in the MES test in mice. Nevertheless, more advanced biochemical and electrophysiological studies are required to confirm or reject this hypothesis and to elucidate the nature of observed interactions between drugs.

Furthermore, it was found that amlodipine did not affect acute adverse-effect potential of oxcarbazepine in animals challenged with the chimney test, passive avoidance task, and grip-strength test. These observations are partially in contrast to the results shown by Kaminski et al. (4), who have found that amlodipine potentiated the impairment of motor coordination of the animals receiving carbamazepine, phenytoin, phenobarbital and valproate in the chimney test. The lack of effect of amlodipine on acute adverse-effect potential of oxcarbazepine, one can explain through more favorable safety and tolerability profiles of oxcarbazepine in comparison to conventional AEDs used in preclinical studies.

With respect to the combination of oxcarbazepine with amlodipine, the calcium channel antagonist did not alter total brain oxcarbazepine concentration and thus, the observed interaction was pharmacodynamic in nature. Previously, it has been documented that amlodipine significantly increased free plasma concentration of carbamazepine, but not that of valproate, phenobarbital and phenytoin in mice (4). It is worth mentioning that pharmacokinetic evaluation of total brain oxcarbazepine concentrations in this study provided the exact insight into the nature of observed interaction between drugs in the MES test. Relatively recently, it has been demonstrated that only total brain concentrations of AEDs precisely characterize pharmacokinetic interactions between drugs, influencing central nervous system (37, 38). It is highly likely that some drugs can markedly change total brain concentrations of AEDs, having no impact on free (non-protein bound) plasma concentrations and inversely, some agents can considerably alter free plasma concentrations without any significant changes in total brain concentrations of AEDs. For instance, it has been reported that 2-phosphonomethyl-pentanedioic acid significantly elevated total brain valproate concentrations, having had no impact on the free plasma concentrations of valproate (25). Similarly, tiagabine combined with valproate markedly increased total brain concentrations of the latter drug, having had little effect on the free plasma valproate concentrations (38). In contrast, loreclezole co-administered with valproate significantly increased the free plasma concentrations of valproate, exerting simultaneously, no changes on total brain valproate concentrations (26). This is why, in the present study, total brain concentrations instead of the free plasma oxcarbazepine concentrations were evaluated with HPLC.

In conclusion, amlodipine enhanced the anticonvulsant action of oxcarbazepine, produced no acute adverse effects when combined with oxcarbazepine and had no impact on total brain

concentrations of oxcarbazepine in experimental animals. If the results from this study can be extrapolated to the clinical settings, a novel therapeutic option in the management of epilepsy might be created for epileptic patients. Thus, amlodipine deserves more attention from a clinical point of view, as a potentially favorable drug that could be applied in patients treated with oxcarbazepine, who additionally required a calcium channel antagonist treatment for conditions other than epilepsy.

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SUMMARY

To assess the influence of amlodipine (a calcium channel antagonist) on the anticonvulsant action of oxcarbazepine (a second-generation antiepileptic drug), the mouse maximal electroshock seizure (MES) model was used. Tonic hindlimb extension (seizure activity) was evoked in Albino Swiss mice by using electroconvulsions (25 mA, 500 V, 0.2-s stimulus duration) delivered via auricular electrodes. Pharmacokinetic estimation of total brain oxcarbazepine concentrations was performed with high-performance liquid chromatography. Amlodipine (20 mg/kg, i.p.) significantly enhanced the anticonvulsant action of oxcarbazepine in the MES test in mice, by reducing its ED_{50} value from 14.25 to 9.52 mg/kg ($P<0.01$). In contrast, amlodipine (5 and 10 mg/kg) had no significant impact on the antiseizure action of oxcarbazepine in the MES test in mice. Chromatographic evaluation of oxcarbazepine concentrations revealed that amlodipine did not significantly alter total brain oxcarbazepine concentrations in experimental animals, indicating a pharmacodynamic nature of interaction between the tested drugs. The favorable combination of oxcarbazepine with amlodipine deserves more attention from a clinical viewpoint because of the enhanced antiseizure action of oxcarbazepine and lack of the pharmacokinetic interaction between drugs.

Amlodypina nasila przeciwdrgawkowe działanie okskarbazepiny w teście maksymalnego wstrząsu elektrycznego u myszy

Aby określić wpływ amlodypiny (antagonisty kanałów wapniowych) na przeciwdrgawkowe działanie okskarbazepiny (leku przeciwpadaczkowego drugiej generacji), zastosowano test maksymalnego wstrząsu elektrycznego (MES) u myszy. Toniczny wyprost kończyn tylnych (aktywność drgawkową) wywołano u myszy Albino Swiss przy użyciu elektrowstrząsów (25 mA, 500 V, 0,2-s czas trwania stymulacji) dostarczanych przez elektrody uszne. Farmakokinetycznej oceny całkowitego mózgowego stężenia okskarbazepiny dokonano wysokosprawną chromatografią cieczową. Amlodypina (20 mg/kg, i.p.) istotnie nasilała przeciwdrgawkowe działanie okskarbazepiny w teście MES u myszy, zmniejszając jej wartość ED_{50} z 14,25 do 9,52 mg/kg ($P<0,01$). Przeciwnie, amlodypina (5 i 10 mg/kg) nie miała istotnego wpływu na przeciwdrgawkowe działanie okskarbazepiny w teście MES u myszy. Chromatograficzna ocena stężeń okskarbazepiny ujawniła, że amlodypina nie zmieniała istotnie całkowitego mózgowego stężenia okskarbazepiny u zwierząt doświadczalnych, wykazując farmakodynamiczną naturę interakcji pomiędzy badanymi lekami. Należy wnioskować, że korzystna kombinacja okskarbazepiny z amlodypiną zasługuje na większą uwagę z klinicznego punktu widzenia ze względu na nasilone przeciwdrgawkowe działanie okskarbazepiny i brak interakcji farmakokinetycznej pomiędzy lekami.