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Effect of 7-nitroindazole and N^G-nitro-L-arginine on the protective action of clobazam in the maximal electroshock-induced seizures in mice

Wpływ 7-nitroindazolu i N^G-nitro-L-argininy na ochronne działanie klobazamu w teście maksymalnego wstrząsu elektrycznego u myszy

INTRODUCTION

Clobazam (CLB) is a second-generation antiepileptic drug (AED) licensed as an adjunct therapy for patients with refractory epilepsy [25]. The drug is highly effective as adjunctive therapy for partial and generalized seizures, for intermittent therapy and for controlling non-convulsive status epilepticus [21]. Moreover, CLB is used in pediatric epileptology in treating resistant epilepsies of infancy and childhood [20].

CLB as a 1,5-benzodiazepine binds to γ subunit of the γ-aminobutyric $acid_A$ ($GABA_A$) receptors and thus, potentiates GABAergic inhibition [18]. Experimental evidence indicates that CLB exhibits anticonvulsant activity in various models of epilepsy including: the maximal electroshock (MES)-induced tonic seizures, pentylenetetrazole (PTZ)-induced clonic seizures, bicuculline- and picrotoxin-induced seizures in rodents [19, 24]. CLB is effective in suppressing motor seizures induced by PTZ in immature and developing rats [7, 22]. CLB also suppressed lateral geniculate nucleus-kindled seizures [8], olfactory bulb- and amygdala-kindled seizures in rats [6].

Nitric oxide (NO) as a gaseous molecule possesses neurotransmitter/neuromodulator properties in the brain and plays an important role in the pathophysiology of epilepsy, producing both anti- and pro-convulsant effects in various experimental models of epilepsy in rodents [2, 15, 17]. NO is produced by the oxidation of L-arginine by NO synthase (NOS – a Ca^{2+}/calmodulin-dependent enzyme), existing in three distinct isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [17]. It is generally accepted that N^G-nitro-L-arginine (NNA – a non-selective NOS inhibitor) reduces the activity of both, eNOS and nNOS, to the same extent, whereas 7-nitroindazole (7NI) is considered to be a preferential inhibitor of nNOS activity [1, 17].

Experimental evidence indicates that 7NI, the preferential nNOS inhibitor, exerted the anticonvulsant properties by elevating the threshold for maximal electroconvulsions in mice [2, 11, 15, 23]. 7NI potentiated the anticonvulsant action of phenobarbital (PB), phenytoin (PHT), valproate (VPA), oxcarbazepine (OXC), loreclezole (LCZ), pregabalin (PGB), but not that of carbamazepine (CBZ), topiramate (TPM), lamotrigine (LTG), and felbamate (FBM) in the maximal electroshock (MES)-induced seizures in mice [4, 11, 13, 15, 16].

With regards to NNA administered systemically (i.p.) at a dose of 40 mg/kg, it had no impact on the anticonvulsant effects of some various AEDs (i.e., LTG, FBM, OXC, LCZ, PGB, CBZ, PHT, and TPM) in the mouse MES model [12, 13, 16]. In contrast, NNA significantly reduced the anticonvulsant effect of PB and VPA in the MES-induced seizure test in mice [4, 5, 15]

Considering the above-mentioned fact, it was of pivotal importance to evaluate the effects of 7NI and NNA on the anticonvulsant action of CLB in the mouse MES model. Generally, the mouse MES test is thought to be an animal model of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in human [10]. In this model one can readily evaluate the anticonvulsant effects produced by classical and second-generation antiepileptic drugs in combination with 7NI and NNA, therefore, it was appropriate to use this test in the present study.

Moreover, the acute adverse-effect potentials of CLB in combination with 7NI and NNA were determined in the chimney test (motor performance), step-through passive avoidance task (long-term memory) and the grip-strength test (skeletal muscular strength) in mice.

MATERIAL AND METHODS

Animals and experimental conditions. All experiments were performed on male Swiss mice, kept in colony cages with free access to food and tap water, under standardized housing conditions. The animals were randomly assigned to experimental groups consisting of 8 mice each. All experimental tests were performed between 9.00 a.m. and 2.00 p.m. to minimize confounding effects of circadian rhythms. All experimental procedures described hereupon were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin (license no.: 84/2009).

Drugs. CLB (Frisium®, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany), 7NI (Sigma, St. Louis, MO, USA) and NNA (RBI, Natick, MA, USA) were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in saline and administered intraperitoneally (i.p.) in a volume of 5 ml/kg body weight: CLB, 7NI and NNA – at 30 min before the MES and all behavioral tests. The pretreatment times before testing of CLB, 7NI and NNA were based upon information about their biological activity from the literature and our previous experiments [4, 5, 11-13, 16, 17, 23].

Maximal electroshock – induced seizures. Electroconvulsions were produced by an alternating current (0.2 s stimulus duration, 50 Hz, fixed current intensity of 25 mA, maximum stimulation voltage of 500 V) delivered via ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs, Freiburg, Germany). The criterion for the occurrence of seizure activity was the tonic hindlimb extension. The protective activity of CLB administered alone or in combination with 7NI and NNA was evaluated as its median effective doses (ED_{50} in mg/kg with 95% confidence limits) against MES-induced seizures. The animals received different doses of CLB so as to obtain a variable percentage of protection against MES, allowing the construction of a dose-effect curve for CLB administered alone or in combination with 7NI and NNA, according to Litchfield and Wilcoxon

[9]. Each ED_{50} value represents the dose of CLB required to protect 50% of the animals tested against MES-induced seizures. In the present study, CLB was administered at doses ranging between 8–22 mg/kg. This experimental procedure has been described in detail in our earlier studies [11–16].

Chimney test. The effects of combination of CLB with 7NI and NNA at its ED_{50} values from the MES test on motor coordination impairment were quantified with the chimney test of Boissier et al. [3]. The time before the commencement of the chimney test (after drug administration) was identical to that for the MES test. In this test, animals had to climb backwards up the plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Data were presented as a percentage of animals that failed to perform the chimney test. This experimental procedure has been described in detail in our earlier studies [11–14].

Grip – strength test. The effects of combinations of CLB with 7NI and NNA at its ED_{50} values from the MES test, on muscular strength (tone) 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 mean of 3 measurements for each animal was calculated and, subsequently, the mean maximal force of 8 animals per group was determined. The skeletal muscular strength in mice was expressed in Newtons (N) as means ± SE of at least 8 determinations (8 animals per group). This experimental procedure has been described in detail in our earlier study [13, 27].

Step – through passive avoidance task. Each animal was administered 7NI or NNA co-administered with CLB at doses corresponding to its ED_{50} values 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 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 the ability to acquire the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory [26]. This experimental procedure has been described in detail in our earlier studies [11–16].

Statistics. The ED_{50} values (in mg/kg) with their respective 95% confidence limits were calculated by log-probit analysis [9]. Subsequently, the 95% confidence limits were transformed to SE according to the method described earlier [14]. Statistical analysis of data was performed either with log-probit method for single comparison or with one-way 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. 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 the values were considered statistically significant if $p<0.05$.

RESULTS

EFFECT OF 7NITROINDAZOLE AND N^G-NITRO-L-ARGININE ON THE ANTICONVULSANT ACTIVITY OF CLOBAZAM AGAINST MAXIMAL ELECTROSHOCK-INDUCED SEIZURES

CLB administered i.p. 30 min before the test produced a clear-cut anticonvulsant effect and its ED_{50} value was 15.14 (12.63–18.14) mg/kg. The combination of CLB with NNA (40 mg/kg) was associated with a slight decrease in the anticonvulsant effect exerted by CLB. In such a case, the ED_{50} value for CLB increased by 5%, amounting to 15.97 (12.23–20.86) mg/kg (results not shown). In contrast, 7NI (50 mg/kg) co-administered with CLB produced a significant (by 32%) decrease in the ED_{50} value of CLB from 15.14 (12.63–18.14) mg/kg to 10.23 (8.44–12.40) mg/kg ($p<0.05$; Figure 1). In the case of the combination of CLB with 7NI (25 mg/kg), a slight (by 5%) reduction of the ED_{50} value of CLB was also observed; however, statistical analysis of data with one-way ANOVA followed by the post-hoc Tukey-Kramer test revealed that the observed reduction from 15.14 (12.63–18.14) mg/kg to 14.35 (11.96–17.21) mg/kg did not attain statistical significance (Figure 1).

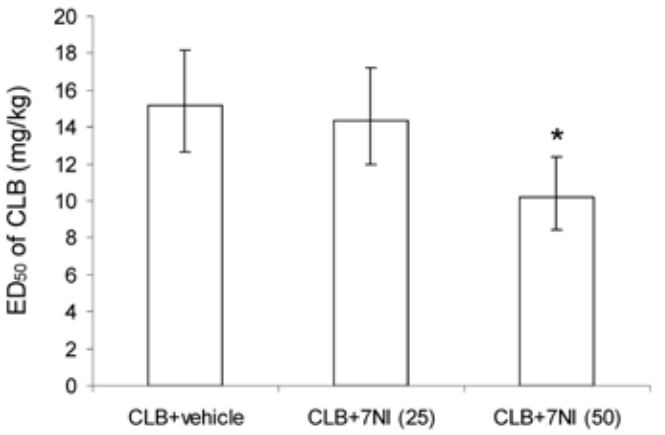

Fig. 1. Effect of 7-nitroindazole (7NI) on the protective activity of clobazam (CLB) against MES-induced seizures in mice

Columns represent median effective doses (ED_{50} in mg/kg with 95% confidence limits as the error bars) of CLB, protecting 50% of animals tested against MES-induced hindlimb extension. CLB and 7NI were administered i.p. at 30 min. prior to the MES test. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. *$p<0.05$ vs. the respective control group (CLB + vehicle-treated animals)

EFFECTS OF 7NITROINDAZOLE, N^G-NITRO-L-ARGININE AND THEIR COMBINATION WITH CLOBAZAM ON MOTOR PERFORMANCE, LONG-TERM MEMORY, AND MUSCULAR STRENGTH OF ANIMALS IN THE CHIMNEY, STEP-THROUGH PASSIVE AVOIDANCE AND GRIP-STRENGTH TESTS

When CLB was administered in combination with 7NI (50 mg/kg) or NNA (40 mg/kg), at doses corresponding to its ED_{50} from the MES test, motor performance as assessed by the chimney test was unaffected (Table 1). Furthermore, none of the combinations of CLB with 7NI (50 mg/kg) or NNA (40 mg/kg) impaired long-term memory as determined in the passive avoidance test, the median retention times being 180 s (Table 1). Likewise, CLB combined with 7NI (50 mg/kg) or NNA (40 mg/kg) had no significant impact on muscular strength of animals as assessed by the grip-strength test (Table 1).

DISCUSSION

Results presented herein indicate that 7NI – the preferential nNOS inhibitor enhanced the protective action of CLB, whereas NNA – the non-selective NOS inhibitor had no impact on the anticonvulsant action of CLB in mice subjected to the MES test. Our findings are in agreement with those documenting earlier that 7NI enhanced the anticonvulsant action of some classical and second-generation AEDs in the mouse MES-induced seizure test [4, 11, 13, 15, 16]. Similarly, the lack of effect of NNA on the anticonvulsant action of CLB was consistent with previous reports showing that NNA did not affect the anticonvulsant action of classical and second-generation AEDs in the mouse MES model [12, 13, 16]. The direct comparison of effects produced by 7NI and NNA combined with CLB allowed the evaluation of effect produced by both NOS inhibitors.

Since 7NI potentiated the anticonvulsant action of CLB by reducing its ED_{50} value and NNA as the non-selective NOS inhibitor had no impact on the anticonvulsant action of CLB in the MES test in mice, one could ascertain that modulation of NO content in the brain of experimental animals by NNA had no effect on the anticonvulsant action of CLB. In contrast, 7NI could directly interact with its specific binding sites, independent on NO pathways, contributing to the enhancement of the anticonvulsant action of CLB in the MES test in mice. Recently, there has appeared a hypothesis suggesting that the effects produced by 7NI resulted from the direct effect of 7NI, which was independent on NO content in the brain [11, 15, 23].

Evaluation of acute adverse-effect profile for the combination of CLB with 7NI or NNA revealed that neither 7NI nor NNA altered motor coordination in animals challenged with the chimney test. Similarly, none of the investigated combinations of CLB with 7NI and NNA affected long-term memory in mice in the step-through passive avoidance task as well as altered skeletal muscular strength in mice subjected to the grip-strength test. These findings are also in agreement with the results from our previous studies documenting that the combinations of NNA and 7NI with classical and second-generation AEDs produced no acute adverse effects in the chimney, step-through passive avoidance and grip-strength tests in mice [11–13, 15, 16].

Table 1. Effects of 7-nitroindazole (7NI), NG-nitro-L-arginine (NNA), clobazam (CLB) and their combinations on long-term memory, skeletal muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Motor coordination impairment (%)
Control	180 (180; 180)	108.98 ± 4.58	0
7NI (50) + vehicle	180 (165.8; 180)	106.40 ± 4.87	12.5
CLB (10.23) + vehicle	180 (180; 180)	107.05 ± 4.77	0
CLB (10.23) + 7NI (50)	180 (122.3; 180)	105.33 ± 4.69	12.5
Control	180 (180; 180)	108.98 ± 4.58	0
NNA (40) + vehicle	180 (163.8; 180)	106.44 ± 5.00	12.5
CLB (15.97) + vehicle	180 (180; 180)	104.15 ± 4.79	0
CLB (15.97) + NNA (40)	175 (135.3; 180)	107.11 ± 4.87	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 ± SE) 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. Each experimental group consisted of 8 animals. 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 MES test and at doses corresponding to the ED50 values against MES-induced seizures

CONCLUSIONS

The combination of 7NI with CLB deserves more clinical attention due to its favorable effects in terms of suppression of MES-induced seizures and lack of any significant acute adverse effects in experimental animals. The combination of NNA with CLB seems to be neutral from a preclinical point of view, because NNA had no impact on the protective activity of CLB against MES-induced seizures and NNA did not exert any acute adverse effects in mice. If the results from this study could be extrapolated into clinical settings and additionally confirmed in different various experimental models of epilepsy, the combination of 7NI with CLB would occur favorable for epileptic patients as a novel treatment option in refractory epilepsy.

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Disclosure of conflicts of interest. The authors have no disclosures to declare.

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SUMMARY

The aim of this study was to determine the effect of 7-nitroindazole (7NI – a preferential neuronal nitric oxide synthase [NOS] inhibitor) and N^G-nitro-L-arginine (NNA – a non-selective NOS inhibitor) on the anticonvulsant action of clobazam (CLB – a second-generation antiepileptic drug) in the maximal electroshock (MES)-induced seizure model in mice. Electroconvulsions were produced in adult male Albino Swiss mice by means of an alternating current (50Hz, 500V, 25mA, ear-clip electrodes, 0.2s stimulus duration). The anticonvulsant action of CLB in the MES test was expressed as median effective doses (ED_{50} values) of the drug, protecting 50% of animals tested against MES-induced seizures. The acute adverse-effect potentials of CLB in combination with 7NI and NNA were evaluated in the chimney test (motor coordination), passive avoidance task (long-term memory), and grip-strength test (skeletal muscular strength) in mice. Results indicate that 7NI (50 mg/kg; i.p.) significantly enhanced the anticonvulsant action of CLB by reducing its ED_{50} value from 15.14 mg/kg to 10.23 mg/kg ($p<0.05$). Similarly, 7NI at the lower dose of 25 mg/kg also potentiated the anticonvulsant action of CLB, although the results did not attain statistical significance. In contrast, NNA (40 mg/kg; i.p.) had no impact on the anticonvulsant effect of CLB. Moreover, none of the examined combinations of CLB with 7NI and NNA affected motor coordination, long-term memory, and skeletal muscular strength in mice. Based on this preclinical study, one can conclude that 7NI significantly enhanced, whereas NNA had no effect on the anticonvulsant activity of CLB against MES-induced seizures in mice.

Keywords: 7-Nitroindazole, N^G-nitro-L-arginine, clobazam, nitric oxide, maximal electroshock seizure test

STRESZCZENIE

Celem pracy była ocena wpływu 7-nitroindazolu (7NI – preferencyjnego inhibitora neuronalnej syntazy tlenku azotu [NOS]) i N^G-nitro-L-argininy (NNA – nieselektywnego inhibitora NOS) na przeciwdrgawkowe działanie klobazamu (CLB – leku przeciwpadaczkowego drugiej generacji) w modelu maksymalnego wstrząsu elektrycznego (MES) u myszy. Drgawki elektryczne były wywoływane u dorosłych samców myszy Albino Swiss poprzez prąd zmienny (50Hz, 500V, 25mA, elektrody uszne, 0,2s czas trwania impulsu). Przeciwdrgawkowe działanie CLB w teście MES wyrażono jako mediany dawek skutecznych (wartości ED_{50}) leku, chroniące 50% zwierząt przed drgawkami wywoływanymi elektrycznie. Ostre potencjalne działania niepożądane CLB w kombinacji z 7NI i NNA oceniono w teście komina (koordynacja ruchowa), teście biernego unikania (pamięć długotrwała) i teście chwytania (siła mięśni szkieletowych) u myszy. Wyniki wskazują, że 7NI (50 mg/kg; i.p.) istotnie nasilał przeciwdrgawkowe działanie CLB poprzez zmniejszenie jego wartości ED_{50} z 15,14 mg/kg do 10,23 mg/kg ($p<0,05$). Podobnie 7NI w niższej dawce 25 mg/kg nasilał przeciwdrgawkową aktywność CLB, chociaż wyniki nie uzyskały istotności statystycznej. Przeciwnie, NNA (40 mg/kg; i.p.) nie miała żadnego wpływu na przeciwdrgawkowe działanie CLB. Ponadto żadna z badanych kombinacji CLB z 7NI i NNA nie wpływała na koordynację ruchową, pamięć długotrwałą i siłę mięśni szkieletowych u myszy. W oparciu o to badanie przedkliniczne można stwierdzić, że 7NI istotnie nasilał, podczas gdy NNA nie miała wpływu na przeciwdrgawkowe działanie CLB w teście MES u myszy.

Słowa kluczowe: 7-Nitroindazol, N^G-nitro-L-arginina, klobazam, tlenek azotu, maksymalny wstrząs elektryczny