

Influence of 7-nitroindazole and N^G-nitro-L-arginine on the anticonvulsant activity of loreclezole in maximal electroshock-induced seizures in mice

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Abstract: The aim of the study was to determine the effects 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 loreclezole (LCZ - an innovative antiepileptic drug) in maximal electroshock-induced seizures (MES) in mice. Electroconvulsions were produced in mice by means of an alternating current (50 Hz, 500 V, 25 mA, administered by ear-clip electrodes, 0.2-s stimulus duration, tonic hind limb extension taken as the end point). The anticonvulsant action of LCZ in the MES test was expressed as the median effective dose (ED₅₀ value) of the drug, protecting 50% of animals tested against MES-induced seizures. The acute adverse-effect potentials of LCZ in combination with 7NI and NNA were evaluated in the chimney test (motor coordination), step-through 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 LCZ by reducing its ED₅₀ value from 108.9 mg/kg to 60.5 mg/kg (P<0.05). Similarly, 7NI at the lower dose of 25 mg/kg also enhanced the antiseizure action of LCZ by lowering the ED₅₀ value of LCZ from 108.9 mg/kg to 82.7 mg/kg, although the results did not attain statistical significance. In contrast, NNA (40 mg/kg; i.p.) attenuated the anticonvulsant effects of LCZ by increasing its ED₅₀ value from 108.9 mg/kg to 137.4 mg/kg; however, statistical analysis of the data revealed no significance between both ED₅₀ values. Moreover, none of the examined combinations of LCZ with 7NI and NNA affected motor coordination, long-term memory, or skeletal muscular strength in the mice. Based on this preclinical study, one may conclude that 7NI significantly enhanced, whereas NNA attenuated the antiseizure effects of LCZ against MES-induced seizures in mice.

Key words: 7-Nitroindazole, N^G-nitro-L-arginine, nitric oxide, loreclezole, maximal electroshock seizure test, acute adverse-effect profile

INTRODUCTION

Overwhelming evidence indicates that nitric oxide (NO), a gaseous molecule possessing neurotransmitter/neuromodulator properties in the brain, plays an important role in the pathophysiology of epilepsy, producing both anti- and pro-convulsant effects in various experimental models of epilepsy in rodents [1-5]. NO is produced by the oxidation of L-arginine (L-arg) by NO synthase (NOS - a Ca²⁺/calmodulin-dependent enzyme), existing in three distinct isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [3]. Considerable progress has been made in the examination of the role of NO in the brain during seizures after discovery of synthetic L-arg analogues inhibiting the NOS activity in the brain [3]. 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 [3, 6].

Experimental evidence indicates that NNA administered systemically (i.p.) at a dose of 40 mg/kg attenuated the anticonvulsant effects of some newer antiepileptic drugs (AEDs) (lamotrigine [LTG], felbamate [FBM] and topiramate [TPM]) in maximal electroshock (MES)-induced seizures in mice [7]. Moreover, it has been reported that NNA attenuated the anticonvulsant effects of ethosuximide (ETS), oxcarbazepine (OXC) and vigabatrin (VGB) in pentylenetetrazole (PTZ)-induced seizures in mice, manifested by a significant increase in the ED₅₀ values of the examined AEDs [8, 9]. With regards to 7NI, the preferential nNOS inhibitor exerted the anticonvulsant properties by elevating the threshold for maximal electroconvulsions and suppressing sound-induced seizures in DBA/2 mice [4, 7, 10-13]. 7NI administered systemically (i.p.) at a dose of 50 mg/kg significantly enhanced the antiseizure activity of clonazepam (CZP) and ETS, but not that of phenobarbital (PB) and valproate (VPA) in PTZ-induced seizures in mice [14]. Additionally, 7NI enhanced the antiseizure action of PB, phenytoin (PHT), VPA, OXC, but not that of carbamazepine (CBZ), TPM, LTG and FBM in the MES-induced seizures in mice [7, 11, 15]. In DBA/2 mice, 7NI enhanced the antiseizure effects of PB, diazepam (DZP), VPA, CBZ and, to a lesser extent, those of PHT and LTG against audiogenic seizures [10].

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Loreclezole (LCZ) is a novel AED that acts specifically at two separate allosteric regulatory sites on GABA_A receptors. LCZ potentiates GABA_A receptor-mediated Cl⁻ currents through a site present on the β2 and β3 (but not β1) subunits of GABA_A receptors [16]. It has been observed that low doses of LCZ potentiate GABA receptor currents, increasing inhibitory neurotransmission, whereas at high concentrations, the drug attenuates the effectiveness of inhibitory neurotransmission by reducing the duration of post-synaptic GABA receptor activity [17]. Experimental evidence indicates that LCZ possesses a broad spectrum of anticonvulsant activity suppressing PTZ- and MES-induced seizures in mice [18, 19], as well as amygdala-kindling seizures in rats [20].

The objective of this study was to evaluate the effects of 7NI and NNA on the anticonvulsant action of LCZ in the mouse MES model. Moreover, the acute adverse effect potentials of LCZ 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. All experiments were performed on male Swiss mice, kept in colony cages with *ad libitum* 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 09.00 - 14.00 to minimize confounding effects of circadian rhythms. All experimental procedures described herein were approved by the Local Ethics Committee at the Medical University of Lublin.

Drugs. LCZ (Janssen Research Foundation, Beerse, Belgium), 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: LCZ at 60 min., whereas 7NI and NNA - at 30 min. before the MES and chimney tests.

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 hind limb extension. The protective activity of LCZ administered alone or in combination with 7NI and NNA was evaluated as its median effective dose (ED₅₀ in mg/kg with 95% confidence limits) against MES-induced seizures. The animals received different doses of LCZ in order to obtain a variable percentage of protection against MES, allowing the construction of a dose-effect curve for LCZ administered alone or in combination with 7NI and NNA, according to Litchfield and Wilcoxon [21]. Each ED₅₀ value represented the dose of LCZ required to protect 50% of the animals tested against MES-induced seizures.

Chimney test. The effect of the combination of LCZ with 7NI and NNA on motor coordination impairment were quantified with the chimney test of Boissier et al. [22]. In this

test, the animals had to climb backwards up a plastic tube (inner diameter: 3 cm, length: 25 cm). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within a period of 60 s. Data were presented as the percentage of animals that failed to perform the chimney test. The adverse effect potentials of LCZ co-administered with 7NI and NNA were determined for LCZ administered at doses corresponding to its ED₅₀ values from the MES test. This experimental procedure has been described in detail in our earlier studies [7, 23].

Grip-strength test. The effects of the combinations of LCZ with 7NI and NNA at its ED₅₀ 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 cm × 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 each 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 N (Newtons) as means ± SD of at least 24 determinations (3 measurements for each of 8 animals per group). This experimental procedure has been described in detail in our earlier study [23].

Step-through passive avoidance task. Each animal was administered 7NI or NNA co-administered with LCZ at doses corresponding to its ED₅₀ 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, the animals were placed in an illuminated box (10 cm × 13 cm × 15 cm) connected to a larger dark box (25 cm × 20 cm × 15 cm) equipped with an electric grid floor. Entry of the animals into the dark box was punished by an adequate electric shock to the paw (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation. On the following day (24 h later), the pre-trained animals were again placed in the illuminated box and observed for up to 180 s. Mice that avoided the dark compartment for 180 s were considered as having remembered the task. The time 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 provided information about ability to perform the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measurement of long-term memory [24]. This experimental procedure has been described in detail in our earlier studies [7, 23, 25].

Statistics. The ED₅₀ values (in mg/kg) with their respective 95% confidence limits and SE were calculated by log-probit analysis [21]. Statistical analysis of data was performed either by the 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, whereas the results obtained in the passive avoidance task were

statistically evaluated using Kruskal-Wallis non-parametric 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 7NI and NNA on the anticonvulsant activity of LCZ against MES-induced seizures. LCZ administered i.p. 60 min. before the test produced a clear-cut anticonvulsant effect and its ED₅₀ value was 108.9 mg/kg (Table 1). The combination of LCZ with NNA (40 mg/kg) was associated with a slight decrease in the antiseizure effect exerted by LCZ. In such a case, the ED₅₀ value for LCZ increased by 26%, amounting to 137.4 mg/kg (Table 1). In contrast, 7NI (50 mg/kg) co-administered with LCZ produced

a significant (by 44%) decrease in the ED₅₀ value of LCZ from 108.9 mg/kg to 60.5 mg/kg - $P < 0.05$ (Table 1). In the case of the combination of LCZ with 7NI (25 mg/kg), a slight (by 24%) reduction of the ED₅₀ value of LCZ was also observed (Table 1). However, statistical analysis of data with one-way ANOVA followed by the *post-hoc* Tukey-Kramer test revealed that the observed reduction from 108.9 mg/kg to 82.7 mg/kg did not attain statistical significance (Table 1).

Effects of 7NI, NNA and their combination with LCZ on motor performance, long-term memory, and muscular strength of animals in the chimney, step-through passive avoidance and grip-strength tests. When LCZ was administered in combination with 7NI (50 mg/kg) or NNA (40 mg/kg), at doses corresponding to its ED₅₀ from the MES test, motor performance as assessed by the chimney test was unaffected (Table 2). Furthermore, none of the combinations of LCZ 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 2). Likewise, LCZ 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 2).

Treatment (mg/kg)	ED ₅₀ (mg/kg)	N	SE
LCZ + vehicle	108.9 (88.0-134.8)	16	11.86
LCZ + NNA (40)	137.4 (118.5-159.3)	24	10.36
LCZ + vehicle	108.9 (88.0-134.8)	16	11.86
LCZ + 7NI (25)	82.7 (62.3-109.9)	16	11.98
LCZ + 7NI (50)	60.5 (39.6-92.6) *	24	13.12

F (2;53) = 3.67; P = 0.0323

Anti-convulsant activity of loreclezole (LCZ) presented as its median effective dose (ED₅₀ in mg/kg), protecting 50% of mice tested against tonic hind limb extension in the MES test (with 95% confidence limits in parentheses). Drugs were administered i.p.: LCZ – at 60 min., NNA and 7NI – at 30 min. before MES test. Statistical analysis of data was performed either with log-probit method for single comparison (NNA) or with one-way ANOVA, followed by the *post-hoc* Tukey-Kramer test for multiple comparisons (7NI).

N – total number of animals at those doses, whose expected anticonvulsant effects ranged between 4-6 probits.
SE – standard error of the ED₅₀ values; F – F-statistics; P – probability.
* $P < 0.05$ vs. control group (LCZ + vehicle-treated animals).

DISCUSSION

Results presented in this study indicate that 7NI (the preferential nNOS inhibitor) enhanced the antiseizure action of LCZ, whereas NNA (the non-selective NOS inhibitor) attenuated the antiseizure action of LCZ in mice subjected to the MES test. Our findings are in agreement with those documenting that 7NI enhanced the anti-seizure action of some conventional and newer AEDs in the MES-induced seizure test in mice [7, 11, 15]. Similarly, the reduction of the anti-seizure action of LCZ after co-administration of NNA was consistent with previous reports showing that NNA was able to attenuate the anticonvulsant action of conventional and newer AEDs in experimental seizure models in rodents [8, 23]. The direct comparison of the effects produced by 7NI and NNA combined with LCZ allowed the exact evaluation of the action produced by both NOS inhibitors. Quite recently, there

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Motor coordination impairment (%)
Control	180 (180; 180)	82.38 ± 5.32	0
7NI (50) + vehicle	180 (170; 180)	78.50 ± 5.27	12.5
LCZ (60.5) + vehicle	180 (180; 180)	81.63 ± 5.47	0
LCZ (60.5) + 7NI (50)	180 (160; 180)	79.43 ± 5.67	12.5
Control	180 (180; 180)	82.38 ± 5.32	0
NNA (40) + vehicle	180 (165; 180)	78.50 ± 5.27	12.5
LCZ (137.4) + vehicle	180 (180; 180)	80.25 ± 5.30	0
LCZ (137.4) + NNA (40)	180 (155; 180)	78.01 ± 5.41	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 ± SD) 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 non-parametric 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 (for more detail, see legend to Table 1).

has appeared a suggestion that the effects produced by 7NI resulted from the direct activity of 7NI, which independently affects NO pathways in the brain, and thus enhances the anticonvulsant action of several conventional and newer AEDs [7, 11, 12, 14]. Although this hypothesis is highly speculative, it can readily explain the effects observed in this study. It is noteworthy that 7NI enhanced the anti-seizure action of LCZ by reducing its ED₅₀ value, whereas NNA attenuated the anticonvulsant effects of LCZ by elevating its ED₅₀ value in the MES-induced seizure test in mice. This apparent difference in the action of both NOS inhibitors can be explained by a mechanism which is independent of NO content in the brain of experimental animals. Another fact worth mentioning while interpreting the results of this study is that since NNA attenuated the anti-seizure action of LCZ in the MES test in mice, one could ascertain that the non-selective NOS inhibitor reduced the anti-seizure action of LCZ; therefore, NO has anti-convulsant properties when interacted with LCZ. It is highly likely that NO can modulate the affinity of LCZ to GABA_A receptors. Similarly, 7NI might directly interact with its specific binding sites, contributing to the enhancement of the anti-seizure action of LCZ in the MES test in mice.

Evaluation of acute adverse effect profile for the combination of LCZ 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 LCZ with 7NI and NNA affected long-term memory in mice in the step-through passive avoidance task, nor did it alter skeletal muscular strength in mice subjected to the grip-strength test.

Finally, one may conclude that the combination of 7NI with LCZ deserves more clinical attention due to its favourable effects in terms of suppression of MES-induced seizures, and lack of any significant acute adverse-effect potential in experimental animals. 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 LCZ would be favourable for epileptic patients as an innovative treatment option in refractory epilepsy.

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