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ORIGINAL ARTICLE

7-Nitroindazole does not affect the anti-convulsant action of gabapentin and tiagabine in pentylenetetrazole-induced seizures in mice

Jarogniew J. Łuszczki[1,2], Marcin Szadkowski[1,3], Monika Dudra-Jastrzębska[2], Remigiusz Czernecki[1,4], Damian Filip[1], Marta Misiuta-Krzesińska[1], Beata Barcicka-Kłosowska[1], Jakub Zwoliński[1]

[1] *Department of Pathophysiology, Medical University, Lublin, Poland*
[2] *Department of Physiopathology, Institute of Agricultural Medicine, Lublin, Poland*
[3] *First Department of Internal Medicine with Dialysis Station, County Hospital, Starachowice, Poland*
[4] *Department of Neurology, Neuropsychiatric Hospital, Kielce, Poland*

Abstract: Accumulating experimental evidence indicates that nitric oxide (NO) plays an important role in the pathophysiology of seizures. The purpose of this study was to determine the effect of 7-nitroindazole (7-NI, a preferential neuronal nitric oxide synthase inhibitor) on the anticonvulsant activity of gabapentin (GBP) and tiagabine (TGB) – two newer antiepileptic drugs (AEDs) in the mouse pentylenetetrazole (PTZ)-induced seizure model. The clonic seizures in mice were evoked by subcutaneous injection of PTZ at a dose of 100 mg/kg. The clonic seizure activity was defined as clonus of the whole body lasting over 3 s, with an accompanying loss of righting reflex in mice. The anti-convulsant action of GBP and TGB against PTZ-induced seizures was expressed as median effective doses (ED_{50} values) of the AEDs, protecting 50% of animals tested against PTZ-induced seizures. The acute adverse-effect potentials of GBP and TGB in combination with 7-NI were evaluated by the chimney test (motor coordination). Results indicate that 7-NI administered intraperitoneally at a dose of 50 mg/kg did not significantly affect the anticonvulsant action of GBP and TGB against PTZ-induced seizures. The experimentally-derived ED_{50} values for GBP administered alone and in combination with 7-NI were 289 and 350 mg/kg. Similarly, the ED_{50} values for TGB administered alone and in combination with 7-NI were 0.7 and 0.8 mg/kg, respectively. Moreover, the examined combination of 7-NI (50 mg/kg) with GBP (350 mg/kg) or TGB (0.8 mg/kg) did not affect motor coordination in the chimney test. In conclusion, 7-NI had no impact on the anti-convulsant activity of GBP and TGB in the mouse PTZ-induced seizure model, and did not affect motor coordination of mice challenged with the chimney test.

Key words: 7-Nitroindazole, nitric oxide, gabapentin, tiagabine, pentylenetetrazole-induced seizures, chimney test

INTRODUCTION

Nitric oxide (NO), a gaseous neurotransmitter/neuromodulator synthesized from the amino acid L-arginine by the enzyme NO synthase (NOS), seems to play an important role in a number of physiological and pathophysiological processes in the brain, including neuronal plasticity, cerebral blood-flow, cognitive and behavioral functions, as well as, ischaemia and epilepsy [1-4]. However, the role of NO in the pathophysiology of seizures remains unclear and has not yet been elucidated. In the neurons, NO is produced in response to activation of the N-methyl-D-aspartate (NMDA) receptors by the conversion of L-arginine to L-cytrullline by neuronal NOS [5]. Inhibition of NO synthesis by N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NA) (two non-selective NOS inhibitors of both endothelial and neuronal NOS) or 7-nitroindazole (7-NI) (a preferential inhibitor of neuronal NOS) increases the severity of kindled seizures and aggravates seizures induced by various chemical agents in rodents [6-9]. In contrast, sodium nitroprusside – a NO donor, reduces epileptiform activity elicited by administration of penicillin into the cortex [10]. Moreover, L-arginine, another NO donor, acted as an anticonvulsant agent against kainate-induced seizures [8, 9]. Other reports have shown a pro-convulsant role of NO in epilepsy with an increase of seizure severity induced by L-arginine [11-13] and anti-convulsant properties of NOS inhibitors in models of generalized and focal seizures [14, 15]. Experiments performed using non-selective and preferential NOS inhibitors have produced conflicting results; accordingly, some authors have proposed both pro-and anti-convulsant roles of NO [16].

Experimental studies have revealed that 7-NI exerts anti-convulsant properties by elevating the threshold for electro-

Corresponding author: Dr. Jarogniew J. Łuszczki, Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8, 20-090 Lublin, Poland.
E-mail: jarogniew.luszczki@am.lublin.pl or jluszczki@yahoo.com

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convulsions and suppressing sound-induced seizures in DBA\2 mice [17-22]. 7-NI at 50 mg/kg completely inhibited the pentylenetetrazole (PTZ)-induced increase in NO levels in the hippocampus, but had no behavioural effect on PTZ-induced clonic convulsions in rats [23]. Thus, 7-NI suppressed the development of PTZ-induced kindling without affecting PTZ-induced seizures in rats [23]. Moreover, 7-NI administered systemically (i.p.) at a dose of 50 mg/kg significantly enhanced the anti-seizure activity of clonazepam (CZP) and etosuximide (ETS), but not that of phenobarbital (PB) and valproate (VPA) in PTZ-induced seizures in mice [24]. Additionally, 7-NI potentiated the anti-electroshock action of PB, phenytoin (PHT), VPA, oxcarbazepine (OXC), but not that of carbamazepine (CBZ), topiramate (TPM), lamotrigine (LTG) and felbamate (FBM) in mice [19, 20, 25]. In DBA\2 mice, 7-NI enhanced the anti-seizure effects of PB, diazepam (DZP), VPA, CBZ, and to a lesser extent, those of PHT and LTG against audiogenic seizures [18]. Neurochemical studies have revealed that 7-NI at a dose of 50 mg/kg i.p. enhanced the action of GBP against picrotoxin-induced seizures in rats [26].

Considering the fact that 7-NI enhanced the anti-convulsant action of the various conventional anti-epileptic drugs (AEDs) against PTZ-induced seizures in mice, it was of pivotal importance to assess the influence of 7-NI on the anti-seizure properties of two newer AEDs: (1) gabapentin (GBP), with a main molecular mechanism of action associated with the blockade of $\alpha_2\delta$ subunits of calcium channels in neurons [27, 28]; and (2) tiagabine (TGB), which inhibits GABA re-uptake from synaptic clefts into neurons and glia through the blockade of GABA re-uptake transporter activity [28, 29].

Thus, the question arises whether 7-NI could be able to enhance the anti-convulsant action of GBP and/or TGB against PTZ-induced seizures in mice, or the preferential neuronal NOS inhibitor (7-NI) would have no impact on the anti-convulsant properties of GBP and/or TGB in mice. Generally, it is accepted that PTZ-induced seizures are thought to be an experimental animal model of myoclonic seizures in humans [30]. The potential adverse-effect profiles of GBP and TGB co-administered with 7-NI were determined in the chimney test, which allows assessment of the acute adverse effects produced by AEDs administered alone or in combination, as regards their ability to impair motor coordination in experimental animals [31].

MATERIAL AND METHODS

Animals and experimental conditions. All experiments were performed on adult male Swiss mice weighing 22-26 g. The mice were kept in colony cages with free access to food and tap water, under standardized housing conditions (natural light-dark cycle, temperature 21 ± 1ºC). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups, each group consisting of 8 mice. Each mouse was used only once. All tests were performed between 09.00-14.00. Procedures involving animals and their care were conducted in conformity 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 listed below conformed to the *Guide for the Care and Use of Laboratory Animals* and approved by the Local Ethics Committee at the Medical University in Lublin.

Drugs. The following drugs were used in this study: 7-NI (Sigma, St. Louis, MO, USA), GBP (Neurontin, Parke-Davis, Freiburg, Germany), and TGB (Gabitril, Sanofi Winthrop, Gentilly, France). All drugs were suspended in a 1% aqueous solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered intra-peritoneally (i.p.) in a volume of 0.005 ml/g body weight. Fresh drug solutions were prepared on each day of experimentation and administered as follows: TGB – at 15 min., 7-NI – at 30 min., and GBP – at 60 min., before PTZ-induced seizures and the chimney test. The drug administration times were based on the biological activity, from the literature, and confirmed in our previous experiments [20,21,32]. The times of peak maximum anti-convulsant effects for the drugs were used as reference times in all experiments. PTZ (Sigma, St. Louis, MO, USA) was dissolved in distilled water and administered subcutaneously (s.c.) into a loose fold of skin in the midline of the neck in a volume of 0.005 ml/g body weight.

Pentylenetetrazole (PTZ)-induced convulsions. Clonic convulsions were induced in mice by s.c. administration of PTZ at the doses ranging between 70-120 mg/kg. Following the injection of PTZ, the mice were placed separately into transparent Plexiglas cages (25 × 15 × 10 cm) and observed for 30 min. for the occurrence of clonic seizures. The clonic seizure activity was defined as clonus of the whole body lasting more than 3 s, with an accompanying loss of righting reflex. The number of animals convulsing out of the total number of mice tested was noted for each treatment condition. The convulsive action of PTZ was evaluated as the CD_{50} (the dose of PTZ that produced clonic seizures in 50% of the mice tested). To determine the CD_{50} value, 4 different doses of PTZ were used (8 mice per group). Subsequently, an intensity-response curve was determined from the percentage of mice convulsing according to log-probit method by Litchfield and Wilcoxon [33]. Afterwards, from the equation of intensity-response curve for PTZ, both CD_{50} and CD_{97} values were calculated. It is noteworthy that the CD_{97} value reflected the dose of PTZ required to produce seizures in 97% of animals tested. The anti-convulsant activities of GBP and TGB administered alone and combined with 7-NI against PTZ-induced clonic seizures were determined after s.c. administration of PTZ at its CD_{97} (100 mg/kg). The animals were treated with increasing doses of the AEDs, and the anti-convulsant activity of each drug was evaluated as its ED_{50} value (protecting 50% of mice against PTZ-induced clonic convulsions). To determine the ED_{50} values for the studied AEDs, GBP was administered at doses ranging between 100-600 mg/kg, whereas TGB was administered at doses ranging between 0.5-2 mg/kg. At least 4 groups of animals (8 mice per group) were used to estimate each ED_{50} value for GBP and TGB, calculated from the respective dose-response curves, according to Litchfield and Wilcoxon [33]. This experimental procedure has been described in more detail in our earlier studies [32, 34, 35].

Chimney test. The effect of the combination of 7-NI with GBP and TGB on motor coordination impairment were quantified with the chimney test of Boissier et al. [36]. In this test, mice had to climb backwards up a transparent plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of the mice to climb backward up

the tube within 60 s. Data were presented as a percentage of mice that failed to perform the chimney test.

Statistical analysis. Both CD_{50} and ED_{50} values with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon [33]. Qualitative variables from the chimney test were compared with Fisher's exact probability test. Differences among values were considered statistically significant if $P<0.05$.

RESULTS

Effect of 7-NI on the threshold for PTZ-induced clonic seizures. 7-NI (50 mg/kg) administered i.p., 30 min. before the test, did not alter the threshold for PTZ-induced clonic seizures. The CD_{50} value of PTZ for 7-NI-treated animals was 62.1 (54.6-70.5) mg/kg, and did not differ significantly from the CD_{50} value of PTZ for control animals, which was 73.0 (64.9-82.2) mg/kg (results not shown).

Influence of 7-NI on the anticonvulsant activity of GBP and TGB against PTZ-induced clonic seizures. GBP and TGB displayed definite anti-seizure effects against PTZ-induced clonic seizures in mice. The ED_{50} values are presented in Table 1. The preferential NOS inhibitor – 7-NI administered systemically (i.p.) at a dose of 50 mg/kg had no significant impact on the anti-seizure activity of GBP in the PTZ test; the ED_{50} value for GBP in combination with 7-NI was 350 (261-468) mg/kg (Table 1). Similarly, 7-NI at a dose of 50 mg/kg did not significantly affect the anti-seizure activity of TGB in the PTZ test; the ED_{50} value for TGB in combination with 7-NI was 0.8 (0.4-1.5) mg/kg (Table 1).

Table 1 Effect of 7-nitroindazole (7-NI) on anti-convulsant activity of gabapentin (GBP) and tiagabine (TGB) against pentylenetetrazole (PTZ)-induced clonic seizures in mice.

Treatment (mg/kg)	ED_{50} (mg/kg)	N	SE
GBP + vehicle	289 (215-387)	24	43.15
GBP + 7-NI (50)	350 (261-468)	40	52.04
TGB + vehicle	0.7 (0.4-1.3)	16	0.23
TGB + 7-NI (50)	0.8 (0.4-1.5)	16	0.26

Data are presented as median effective doses (ED_{50}s in mg/kg, with 95% confidence limits in parentheses) of GBP and TGB that protected 50% of mice against PTZ-induced clonic seizures. PTZ-induced seizures were produced by s.c.-injection of PTZ at its CD_{97} (100 mg/kg). GBP was administered i.p. at 60 min., whereas TGB was given i.p. at 15 min. prior to the PTZ test. Statistical evaluation of data was performed with log-probit method according to Litchfield and Wilcoxon [33].

N – total number of animals used at those doses whose expected anticonvulsant effects ranged between 4 and 6 probits.
SE – standard error of ED_{50}.

Influence of 7-NI in combination with GBP and TGB on motor performance in the chimney test. The combination of 7-NI (50 mg/kg) with GBP (350 mg/kg) and TGB (0.8 mg/kg), both AEDs at doses being their ED_{50} values from the PTZ test, did not significantly impair motor coordination in mice subjected to the chimney test (Table 2).

Table 2 Effects of 7-nitroindazole (7-NI), gabapentin (GBP), tiagabine (TGB) and their combinations on motor performance in the chimney test in mice.

Treatment (mg/kg)	I/T	Motor impairment (%)	P
Vehicle	0/8	0	-
7-NI (50) + vehicle	1/8	12.5	1.0
GBP (350) + vehicle	0/8	0	-
GBP (350) + 7-NI (50)	1/8	12.5	1.0
TGB (0.8) + vehicle	0/8	0	-
TGB (0.8) + 7-NI (50)	2/8	25	0.467

Results are presented as percentage of mice showing impairment of motor coordination in the chimney test. The Fisher's exact probability test was used to analyse data from the chimney test. All drugs were administered i.p. at times scheduled from the PTZ test and at doses corresponding to their ED_{50} values against PTZ-induced clonic seizures (for more detail see legend to Table 1).

I – number of animals showing deficits in motor coordination.
T – number of animals in the experimental group.
P – value of probability from the Fisher's test, as compared to control (vehicle-treated animals).

DISCUSSION

Our findings documented that 7-NI at 50 mg/kg did not significantly affect the anti-seizure action of GBP and TGB in mice in the PTZ test. Additionally, it was shown that 7-NI (at 50 mg/kg) did not affect the CD_{50} value of PTZ in mice; this result is consistent with our previous study documenting that 7-NI (50 mg/kg) administered separately had no significant effect on the threshold for PTZ-induced clonic seizures in mice [24]. It is noteworthy that the ED_{50} value of GBP (350 mg/kg) in combination with 7-NI was slightly elevated when compared to its control ED_{50} (289 mg/kg), suggesting that 7-NI alleviated the anti-seizure action of GBP in the PTZ test in mice. In the case of TGB, 7-NI also slightly increased the ED_{50} value of TGB from 0.7 mg/kg- 0.8 mg/kg. Considering the fact that 7-NI is the preferential neuronal NOS inhibitor, one can ascertain that the reduction in NO content in the brain evoked by the application of 7-NI, attenuated the anti-convulsant action of GBP and TGB. It might therefore be hypothesized that NO could possess anti-convulsant properties in the PTZ test. On the other hand, L-NA (the non-selective NOS inhibitor) also did not affect the anti-seizure action of GBP and TGB in the PTZ test in mice [32]. Thus, one can suppose that the inhibitors of NOS activity (7-NI and L-NA) had no impact on the anti-convulsant action of GBP and TGB.

The results obtained in this study are partly in contrast to those reported earlier by Rajasekaran et al. [26], who found that 7-NI at a dose of 50 mg/kg potentiated the anti-convulsant action of GBP against picrotoxin-induced seizures in rats, whereas in our study, 7-NI at 50 mg/kg not only had no impact on the anti-convulsant action of GBP in PTZ-induced seizures, but slightly alleviated the anti-seizure potency of GBP against PTZ-induced clonic seizures in mice. The apparent difference between the experiments can be explained through the different models of seizures (picrotoxin vs. PTZ) and/or different strains of experimental animals used (rats vs. mice). Since picrotoxin and PTZ are considered as non-competitive $GABA_A$ receptor antagonists that induce clonic seizures in rodents, the most likely explanation of the mentioned difference seems to be the strains of animals used experimentally in both studies.

As already mentioned in the Introduction, 7-NI potentiated the anticonvulsant effects of OXC, PB, PHT and VPA in the MES test in mice [19,20], and also enhanced the anti-seizure effects of CZP and ETS in the PTZ test in mice [24]. In contrast, 7-NI did not affect the anti-seizure activities of CBZ in the MES test, and had no impact on the anti-seizure action of PB and VPA in the PTZ-induced seizures in mice [19, 24]. In the case of GBP, the drug synergistically interacted with TGB and OXC [34,35], and exerted additive interactions with FBM and loreclezole (LCZ) in the PTZ test in mice [35]. With respect to TGB, the drug synergistically interacted with GBP and VGB [34, 37], as well as interacted additively with OXC, FBM and LCZ in the PTZ test in mice [35]. All the observed interactions of GBP and TGB with other AEDs were determined by using the isobolographic analysis of interaction. In contrast, the evaluation of the influence of 7-NI on the anticonvulsant action of newer AEDs was performed with the sub-threshold method.

It has been suggested quite recently that 7-NI is able to produce by itself the anti-seizure effects in experimental models of epilepsy in rodents, and that these effects seem to be independent of 7-NI-induced modulation of NO content in the brain [18-24]. If such is the case, the evaluation of the role of NO in seizure phenomena after pre-treatment with 7-NI and newer AEDs might reflect not only the modulation of NO content in the brain, but also a direct anti-seizure action exerted by 7-NI on PTZ-induced seizures in mice. Detailed discussion concerning the role of the 7-NI in seizure phenomena and its influence on the anti-seizure potential of conventional and newer AEDs has been presented elsewhere [4, 17-24].

The evaluation of acute adverse-effect potential for the combination of GBP and TGB with 7-NI revealed that the AEDs, 7-NI and their combination at doses from the PTZ test, had no impact on motor performance in experimental animals.

Based on this pre-clinical study, one can ascertain that 7-NI had no impact on the anti-seizure effects of GBP and TGB in the PTZ test, although a slight increase in the ED_{50} values of both AEDs against PTZ-induced seizures in mice was observed in the presented study. It seems that 7-NI was neutral when combined with GBP and TGB; one can therefore indirectly ascertain that the modulation of NO content in the brain has no impact on the anti-seizure properties of GBP and TGB in experimental animals.

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