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Short communication

7-Nitroindazole, but not N^G-nitro-L-arginine, enhances the anticonvulsant activity of pregabalin in the mouse maximal electroshock-induced seizure model

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Abstract:
The objective of this 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 pregabalin (PGB – a third-generation antiepileptic drug) in the maximal electroshock (MES)-induced seizure model in mice.
Electroconvulsions were produced in mice by means of an alternating current (50 Hz, 500 V, 25 mA, ear-clip electrodes, 0.2 s stimulus duration, tonic hindlimb extension taken as the endpoint). The anticonvulsant action of PGB 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 PGB 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.
7NI (50 mg/kg, *ip*) significantly enhanced the anticonvulsant action of PGB by reducing the ED_{50} value of PGB from 145.0 mg/kg to 74.4 mg/kg ($p < 0.01$). Similarly, 7NI at the lower dose of 25 mg/kg also potentiated the anticonvulsant action of PGB by lowering the ED_{50} value of PGB from 145.0 mg/kg to 117.9 mg/kg, although the results did not attain statistical significance. In contrast, NNA (40 mg/kg, *ip*) had no impact on the anticonvulsant effects of PGB. Moreover, none of the examined combinations of PGB 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 and NNA had no effect on the anticonvulsant activity of PGB against MES-induced seizures in mice.

Key words:
7-nitroindazole, N^G-nitro-L-arginine, nitric oxide, pregabalin, maximal electroshock seizure test

Introduction

Pregabalin (PGB; (*S*)-(+)-3-(aminomethyl)-5-methylhexanoic acid or (*S*)-(+)-3-isobutyl GABA) is a third-generation antiepileptic drug (AED) recently licensed as an adjunct therapy for partial (simple and complex) seizures with or without secondary generalization in patients over 18 years of age [9, 14, 16]. Although PGB is a substituted analogue of γ-aminobutyric acid (GABA), the drug is inactive at $GABA_A$, benzodiazepine and $GABA_B$ receptors [13] and does not alter GABA concentration in brain tissue [15]. The drug has no direct action at sodium channels. However, it binds with high affinity and specificity to the $\alpha_2\delta$ subunit of P/Q-type voltage-gated calcium channels, which decreases Ca^{2+} influx at nerve terminals, reducing the release of excitatory neurotransmitters [4, 27, 30]. Experimental evidence indicates that PGB exhibits anticonvulsant activity in maximal electroshock (MES)-induced tonic seizures and pentylenetetrazole (PTZ)-induced clonic seizures in rodents [32]. PGB provided partial protection against seizures induced by picrotoxin or bicuculline [32]. PGB reduced the incidence of sound-induced seizures in DBA/2 audiogenic mice, but it did not alter the incidence of spontaneous absence seizures in genetically susceptible rats (GAERS) [32].

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 [2, 11, 22, 28]. NO is produced by the oxidation of L-arginine by NO synthase (NOS, a Ca^{2+}/calmodulin-dependent enzyme), which exists in three distinct isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [28]. 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, 28].

Experimental evidence indicates that NNA administered systemically (*via* intraperitoneal (*ip*) injection) at a dose of 40 mg/kg had no impact on the anticonvulsant effects of some second-generation AEDs (i.e., lamotrigine (LTG), felbamate (FBM), oxcarbazepine (OXC), loreclezole (LCZ) and topiramate (TPM)) in MES-induced seizures in mice [20, 26]. In contrast, it has been reported that NNA attenuated the anticonvulsant effects of ethosuximide (ETS), OXC and vigabatrin (VGB) in PTZ-induced seizures in mice as indicated by a significant increase in the ED_{50} values of the examined AEDs [10, 23]. In contrast, NNA had no impact on the protective action of tiagabine (TGB), gabapentin (GBP), diazepam (DZP), phenobarbital (PB) and valproate (VPA) in PTZ-induced seizures in mice [10, 23].

Regarding 7NI, the preferential nNOS inhibitor exerted anticonvulsant properties by elevating the threshold for maximal electroconvulsions and suppressing sound-induced seizures in DBA/2 mice [2, 12, 19, 22, 29, 31]. 7NI enhanced the anticonvulsant activity of clonazepam (CZP) and ETS, but not that of PB and VPA in PTZ-induced seizures in mice [7]. 7NI had no impact on the anticonvulsant action of OXC, VGB, TGB and GBP in the PTZ-induced clonic seizure test in mice [24, 25]. Additionally, 7NI potentiated the anticonvulsant action of PB, phenytoin (PHT), VPA, OXC and LCZ, but not that of carbamazepine (CBZ), TPM, LTG and FBM in MES-induced seizures in mice [6, 19, 22, 26]. In DBA/2 mice, 7NI enhanced the anticonvulsant effects of PB, DZP, VPA, CBZ and, to a lesser extent, those of PHT and LTG against audiogenic seizures [12].

Considering the results highlighted above, it was of pivotal importance to evaluate the effects of 7NI and NNA on the anticonvulsant action of PGB in the mouse MES model. Generally, the mouse MES test is considered to be an animal model of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in humans [18]. In addition, the acute adverse-effect potentials of PGB 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 using 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 were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin (license no.: 84/2009).

Drugs

PGB (Lyrica®, Pfizer Ltd., Sandwich, Kent, UK), 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 by *ip* injection in a volume of 5 ml/kg body weight. PGB was administered at 60 min and 7NI and NNA were administered at 30 min before the MES and all behavioral tests. The pretreatment times before testing of PGB, 7NI and NNA were based upon information about their biological activity from the literature and our previous experiments [6–8, 10–12, 19, 20, 22–26, 29, 31, 32].

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 PGB administered alone or in combination with 7NI and NNA was evaluated as its median effective dose (ED_{50} in mg/kg with 95% confidence limits) against MES-induced seizures. The animals received different doses of PGB to obtain a variable percentage of protection against MES, allowing the construction of a dose-effect curve for PGB administered alone or in combination with 7NI and NNA, according to Litchfield and Wilcoxon [17]. Each ED_{50} value represents the dose of PGB required to protect 50% of the animals tested against MES-induced seizures.

Chimney test

Each animal was administered 7NI or NNA with PGB at doses corresponding to the ED_{50} values obtained from the MES test. The effects of combinations of PGB with 7NI and NNA on motor coordination impairment were quantified with the chimney test of Boissier et al. [5]. In this test, animals had to climb backwards up a 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 are 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 [19, 24–26].

Grip-strength test

Each animal was administered 7NI or NNA with PGB at doses corresponding to the ED_{50} values obtained from the MES test. The effects of combinations of PGB with 7NI and NNA 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 N (Newtons) as the means ± SE of at least 8 determinations (8 animals per group). This experimental procedure has been described in detail in our earlier studies [24–26, 35].

Step-through passive avoidance task

Each animal was administered 7NI or NNA with PGB at doses corresponding to the ED_{50} values obtained 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 into 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 for 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, the test may be regarded as a measure of long-term memory [33]. This experimental procedure has been described in detail in our earlier studies [19–26].

Statistics

The ED_{50} values (in mg/kg) with their respective 95% confidence limits were calculated by log-probit analysis [17]. Subsequently, the 95% confidence limits were transformed to SE according to the method described earlier [21]. Statistical analysis of the data was performed either with the log-probit method for single comparison or with a one-way ANOVA followed by the *post-hoc* Tukey-Kramer test for multiple comparisons. Qualitative variables from the chimney test were compared using the Fisher's exact probability test. The results obtained in the passive avoidance task were statistically evaluated using a Kruskal-Wallis nonparametric ANOVA. The results from the grip-strength test were verified with a 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

Effect of 7NI and NNA on the anticonvulsant activity of PGB against MES-induced seizures

PGB administered *via ip* injection 60 min before the test produced a clear anticonvulsant effect and its ED_{50} value was 145.0 ± 18.6 mg/kg (Tab. 1). The combination of PGB with NNA (40 mg/kg) was associated with a slight decrease in the anticonvulsant effect exerted by PGB; the ED_{50} value for PGB increased by 17% to 170.0 ± 19.2 mg/kg (Tab. 1). In contrast, 7NI (50 mg/kg) co-administered with PGB produced a significant (49%) decrease in the ED_{50} value of PGB from 145.0 ± 18.6 mg/kg to 74.4 ± 10.0 mg/kg ($p < 0.01$; Tab. 1). In the case of the combination of PGB with 7NI (25 mg/kg), a slight (19%) reduction of the ED_{50} value of PGB to 117.9 ± 13.6 mg/kg was also observed but the change was not statistically significant (Tab. 1).

Tab. 1. Influence of N^G-nitro-L-arginine (NNA) and 7-nitroindazole (7NI) on the anticonvulsant effects of pregabalin (PGB) in the mouse maximal electroshock (MES)-induced seizure model

Treatment (mg/kg)	ED_{50} (mg/kg)	n
PGB + vehicle	145.0 ± 18.6	32
PGB + NNA (40)	170.0 ± 19.2	32
PGB + vehicle	145.0 ± 18.6	32
PGB + 7NI (25)	117.9 ± 13.6	24
PGB + 7NI (50)	74.4 ± 10.0**	32
F (2;85) = 6.328; p = 0.0027		

Anticonvulsant activity of PGB is presented as its median effective dose (ED_{50} ± SE in mg/kg), protecting 50% of animals tested against tonic hindlimb extension in the MES test. The drugs were administered by *ip* injection as follows: PGB at 60 min and NNA and 7NI at 30 min before the MES test. Statistical analysis of the data was performed either with the log-probit method for single comparison (NNA) or with a one-way ANOVA followed by the *post-hoc* Tukey-Kramer test for multiple comparisons (7NI), n – total number of animals at each dose, whose expected anticonvulsant effects ranged between 4 and 6 probits; F – F-statistics; P - probability. ** $p < 0.01$ *vs.* control group (PGB + vehicle-treated animals)

Effects of 7NI, NNA and their combination with PGB on motor performance, long-term memory and muscular strength of animals as measured by the chimney, step-through passive avoidance and grip-strength tests, respectively

When PGB 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 (Tab. 2). Furthermore, neither PGB with 7NI (50 mg/kg) nor PGB with NNA (40 mg/kg) impaired long-term memory as determined in the passive avoidance test; the median retention times were 180 s (Tab. 2). Likewise, PGB 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 (Tab. 2).

Tab. 2. Effects of N^G-nitro-L-arginine (NNA), 7-nitroindazole (7NI), pregabalin (PGB) 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)	98.28 ± 5.48	0
7NI (50) + vehicle	180 (170; 180)	93.40 ± 5.47	12.5
PGB (74.4) + vehicle	180 (180; 180)	96.53 ± 5.67	0
PGB (74.4) + 7NI (50)	180 (160; 180)	92.33 ± 5.69	0
Control	180 (180; 180)	98.28 ± 5.48	0
NNA (40) + vehicle	180 (165; 180)	91.94 ± 5.74	12.5
PGB (170.0) + vehicle	180 (180; 180)	99.15 ± 5.69	0
PGB (170.0) + NNA (40)	180 (155; 180)	92.81 ± 5.77	25

Results are presented as the following: 1) median retention times (in seconds; with 25^{th} and 75^{th} 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 the data from the passive avoidance task was performed with a nonparametric Kruskal-Wallis ANOVA test, whereas the data from the grip-strength test were analyzed with a one-way ANOVA. The Fisher's exact probability test was used to analyze the results from the chimney test. All drugs were administered by *ip* injection at times scheduled from the MES test and at doses corresponding to the ED_{50} values obtained from MES test (for more details, see the legend to Tab. 1)

Tab. 3. Effect of 7-nitroindazole (7NI) and N^G-nitro-L-arginine (NNA) on the anticonvulsant action of the various AEDs in the mouse maximal electroshock (MES)-induced seizure model

AEDs	7NI	NNA	References
Carbamazepine	0	0	[2, 6, 8, 22]
Felbamate	0	0	[19, 20]
Lamotrigine	0	0	[19, 20]
Loreclezole	↑	0	[26]
Oxcarbazepine	↑	0	[19, 20]
Phenobarbital	↑	↓	[6, 8, 22]
Phenytoin	0 /↑	0	[2, 8, 22]
Pregabalin	↑	0	[present study]
Topiramate	0	0	[19, 20]
Valproate	0 /↑	↓	[2, 6, 8, 22]

0 – no effect; ↑ – increased activity; ↓ – reduced activity

Discussion

Here we showed that 7NI, the preferential nNOS inhibitor, enhanced the protective action of PGB, whereas NNA, the non-selective NOS inhibitor, had no impact on the anticonvulsant action of PGB in mice subjected to the MES test. Our findings are in agreement with those previously documenting that 7NI enhanced the anticonvulsant action of some classical and second-generation AEDs in the mouse MES-induced seizure test (Tab. 3). Similarly, the lack of effect of NNA on the anticonvulsant action of PGB 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 (Tab. 3). The direct comparison of effects produced by 7NI and NNA combined with PGB allowed the evaluation of the effects produced by both NOS inhibitors.

Because 7NI potentiated the anticonvulsant action of PGB by reducing its ED_{50} value and the non-selective NOS inhibitor NNA had no impact on the anticonvulsant action of PGB 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 PGB. In contrast, 7NI could directly interact with some specific binding sites for 7NI, independent of NO pathways, contributing to the enhancement of the anticonvulsant action of PGB in the MES test in mice. Quite recently, it has been suggested that the effects produced by 7NI result from the direct effect of 7NI, which is independent of NO content in the brain [7, 19, 22, 29].

Evaluation of the acute adverse-effect profile for the combination of PGB 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 PGB with 7NI or NNA affected long-term memory in mice in the step-through passive avoidance task or 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 combinations of NNA and 7NI with classical and second-generation AEDs produced no acute adverse effects in the chimney test, step-through passive avoidance task and grip-strength test in animals [19, 20, 22–26].

Another fact is worthy of mentioning while interpreting the results from the present study. We did not measure total brain concentrations of PGB in the experimental animals. Therefore, one could not unequivocally determine the nature of the interaction between PGB and 7NI and NNA in the mouse MES model. However, the existence of a pharmacokinetic interaction between the drugs is less probable because PGB has an ideal pharmacokinetic profile in both preclinical and clinical studies. It has been reported that PGB neither binds to plasma proteins nor replaces the drugs from plasma proteins [3, 30, 34]. PGB undergoes a negligible (2%) metabolic transformation in the liver and the drug is excreted virtually unchanged by the kidneys. PGB neither inhibits nor activates liver enzymes such as cytochrome P450s [3, 30, 34]. Considering the favorable pharmacokinetic profile of PGB, it is unlikely that 7NI and NNA (at doses used in the MES test) would be able to pharmacokinetically affect total brain PGB concentrations in experimental animals. Moreover, it should be stressed that in our previous study, 7NI administered at a dose of 150 mg/kg had no impact on total brain concentrations of CBZ, PHT, PB and VPA in mice [22]. In the present study, 7NI was administered *via ip* injection at a maximal dose of 50 mg/kg, so no pharmacokinetic interaction is expected when combining PGB with 7NI.

Conclusions

The combination of 7NI with PGB 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 PGB seems to be neutral from a preclinical point of view; NNA had no impact on the protective activity of PGB 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 experimental models of epilepsy, the combination of 7NI with PGB could be favorable for epileptic patients as a novel treatment option in refractory epilepsy.

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