

STUDY ON THE INFLUENCE OF POTENT INHIBITORS OF NEURONAL NITRIC OXIDE SYNTHASE ON THE ANTINOCICEPTIVE AND ANTICONVULSANT ACTIVITY OF BENZODIAZEPINES IN MICE

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Study on the influence of potent inhibitors of neuronal nitric oxide synthase on the antinociceptive and anticonvulsant activity of benzodiazepines in mice. S. FIDECKA, Pol. J. Pharmacol., 2003, 55, 193–201.

The influence of 1-(2-trifluoromethylphenyl)imidazole (TRIM) and 3-bromo-7-nitroindazole (3-Br-7-NI), potent and relatively selective inhibitors of neuronal nitric oxide (NO) synthase, on the antinociceptive and anticonvulsive effects of diazepam and clonazepam was investigated in mice. The effects were assessed in the writhing test and pentetrazole-induced seizures, respectively. The antinociceptive effects of the threshold doses of diazepam (1 mg/kg) and clonazepam (0.0375 mg/kg) were significantly increased by TRIM (7.5 mg/kg) but not by 3-Br-7-NI (10 mg/kg). L-arginine (125 mg/kg) was able to reverse the effects produced by co-administration of TRIM (7.5 mg/kg) with diazepam (1 mg/kg), and also of TRIM (7.5 mg/kg) with clonazepam (0.0375 mg/kg). Protective efficacy of the threshold dose (0.05 mg/kg) of diazepam against pentetrazole-induced tonic convulsions and death was significantly increased by TRIM (25 mg/kg) but not by 3-Br-7-NI (10 and 100 mg/kg). TRIM (25 mg/kg) intensified the protective efficacy of the threshold dose (0.005 mg/kg) of clonazepam, but the effect was not reversed by L-arginine (125 mg/kg). The present results seem to confirm, at least partly, participation of NO in antinociceptive and anticonvulsant effects of benzodiazepines, and point to TRIM as a better tool than 3-Br-7-NI for examination of the role of NO in behavioral studies.

Key words: diazepam, clonazepam, 1-(2-trifluoromethylphenyl)imidazole (TRIM), 3-bromo-7-nitroindazole (3-Br-7-NI), nociception, seizures, mice

INTRODUCTION

The benzodiazepines have occupied a prominent place in medicine, primarily as sedative-anxiety drugs. A large number of them have broad antiseizure properties. Clinical observations suggest that the benzodiazepines have meaningful analgesic properties in most clinical circumstances [8]. Some of them may be used for abolishing acute muscle spasm, concomitant chronic pain and anxiety, and neuropathic pain [33]. A major, but not the only, mechanism of action of benzodiazepines is connected with the intensification of inhibitory neurotransmission activated by γ -aminobutyric acid (GABA).

Nitric oxide (NO), a free radical gas, is known to play a role in different physiological systems [26]. The literature data point to relationship between L-arginine : NO : cGMP pathway and GABA-mediated neurotransmission. There are numerous proofs for co-localization of NO synthase with GABA in the cerebral cortex [48] and spinal cord neurons [47], and release of NO by the rat cerebral cortex [46]. Some data suggest that NO plays a modulatory role in the release or uptake of neurotransmitters, including GABA, in the brain. Segovia et al. [39] have reported that the NO donor 3-morpholino-sydnonimine induces the release of GABA in the striatum and hippocampus, and these results are in contrast with the findings of Semba et al. [40], which indicated that the inhibition of NO synthesis increased extracellular GABA contents. Moreover, it has also been postulated that NO can modulate the activity of GABA_A receptors [50] and/or have direct influence on these receptors [16].

It has also been found that NO may be involved in the modulation of seizure activity (reviewed in [32]) and pain perception [11]. Additionally, in the previous studies [42] we have shown that antinociceptive and antiepileptic effects of benzodiazepines were potentiated by N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NOARG) and 7-nitroindazole (7-NI), NO synthase inhibitors.

The aim of this study was to estimate the influence of new potent and relatively selective neuronal NO synthase inhibitors, 3-bromo-7-nitroindazole (3-Br-7-NI) and 1-(2-trifluoromethylphenyl)imidazole (TRIM), on the antinociceptive and anticonvulsant effects of diazepam and clonazepam, the benzodiazepines used in clinical practice. 3-Br-7-NI

exhibits 4-fold greater potency than 7-NI as an inhibitor of neuronal NO synthase *in vitro* [4]. TRIM is a relatively selective inhibitor of neuronal and inducible isoform of NO synthase with very low activity against endothelial NO synthase [18]. The selected dose of TRIM and 3-Br-7-NI was based on our preliminary experiments and results presented by other authors [4, 12, 18]. The threshold doses of diazepam and clonazepam were chosen on the basis of our previous study [43, 44]. In behavioral experiments, L-arginine given systematically was efficient at the doses ranging from 50 to 600 mg/kg [5–7, 18, 29, 30]. In the present experiments, the dose of L-arginine has been chosen on the basis of our preliminary study [15, 43, 44].

Some results presented in this paper have been published previously in abstract form [14].

MATERIALS and METHODS

Animals

The experiments were carried out on male Albino Swiss mice (20–27 g) (Farm of Laboratory Animals, Szostak, Warszawa, Poland). The animals were housed in groups of 8–10 to a cage, at room temperature of 21°C with a 12 h light-dark cycle. Standard food (LSM, Bacutil-Motycz, Poland) and water were available *ad libitum*. All experiments were carried out between 9.00 a.m. and 3.00 p.m.

The experiments were performed in accordance with the ethical requirements.

Drugs

The following drugs were used: diazepam (Relanium, Polfa, Poland), clonazepam (Rivotril, Hoffmann-La Roche, Germany), 1-(2-trifluoromethylphenyl)imidazole (TRIM, RBI, USA), 3-bromo-7-nitroindazole (3-Br-7-NI, Tocris Cookson, UK), pentetrazole (Cardiazolum, Polfa, Poland). Diazepam and clonazepam were diluted to appropriate concentration, 3-Br-7-NI was suspended in Tween 80 and TRIM was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany). Drug solutions or suspension were prepared before each experiment. The control animals were injected with an appropriate volume of the solvent at the respective time before the test. All drugs were administered in a volume of 10 ml/kg.

Experimental procedures

The nociceptive reactions in mice were investigated in the writhing test according to Koster et al. [22]. The number of writhing episodes was counted during a 10 min period, starting 5 min after the intraperitoneal (*ip*) administration of 10 ml/kg of 0.6% acetic acid solution. Other compounds were administered subcutaneously (*sc*) 30 min before the acid: diazepam at 1 mg/kg, clonazepam at 0.0375 mg/kg, TRIM at 7.5–30 mg/kg and 3-Br-7-NI at 5–20 mg/kg. L-arginine (125 mg/kg) was given *sc* 35 min before the acid. The absolute mean values of writhing episodes in control groups ranged from 20.9 ± 3.05 to 33.75 ± 3.9 (mean \pm SEM) and were considered as 100%. Abdominal constrictions were not observed in saline-treated mice. The experimental groups consisted of 8–10 animals each.

The anticonvulsant activity of the tested drugs was studied in pentetrazole (110 mg/kg, *sc*)-induced seizures in mice. The animals were observed for 60 min after pentetrazole administration, and the number of mice developing clonic seizures, tonic convulsions and dead animals was recorded in that period. Benzodiazepines (diazepam 0.05 mg/kg, clonazepam 0.005 mg/kg) and TRIM (10, 25 and 50 mg/kg) or 3-Br-7-NI (10 and 100mg/kg) were injected *ip* 30 min and L-arginine (125 mg/kg) 35 min before pentetrazole. Dose of the used benzodiazepines was chosen on the basis of pilot experiments. Benzodiazepines given at lower doses had no permanent, repeatedly protective efficacy.

Statistics

Statistical significance of the obtained data was evaluated using Student's *t*-test (writhing test) and χ^2 test with Yates correction (pentetrazole-induced seizures). The nociceptive reactions are presented as mean \pm SEM (data in Figures 1–4). Differences with *p* value > 0.05 are reported as not significant (NS).

RESULTS

The antinociceptive effects of TRIM and 3-Br-7-NI in the writhing test in mice

TRIM and 3-Br-7-NI, given at lower doses (7.5 and 5 mg/kg, respectively), decreased the number of writhing episodes, but these results did not reach the level of significance. Significant, but not dose-

dependent reduction in the number of the episodes was induced by higher doses of TRIM (15 and 30 mg/kg) and 3-Br-7-NI (10 and 20 mg/kg) (Fig. 1).

The influence of TRIM on effects of diazepam and clonazepam in the writhing test in mice

Co-administration of TRIM (7.5 mg/kg) with diazepam or clonazepam (at the threshold doses of 1 mg/kg or 0.0375 mg/kg, respectively) resulted in

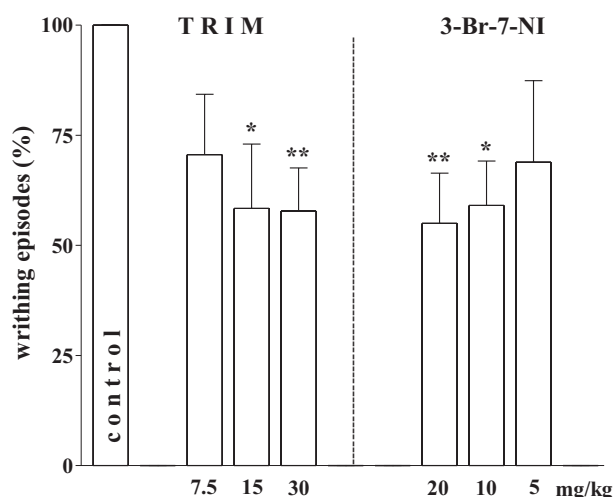


Fig. 1. The antinociceptive activity of 1-(2-trifluoromethylphenyl)imidazole (TRIM) and 3-bromo-7-nitroindazole (3-Br-7-NI) in the writhing test in mice. Each bar represents the mean \pm SEM for a group of 10 mice. The data are expressed as per cent of control group. The absolute mean values of writhing episodes in control groups were 22.6 ± 3.8 and 25.01 ± 1.83 (\pm SEM). * *p* < 0.05 , ** *p* < 0.02 vs. control

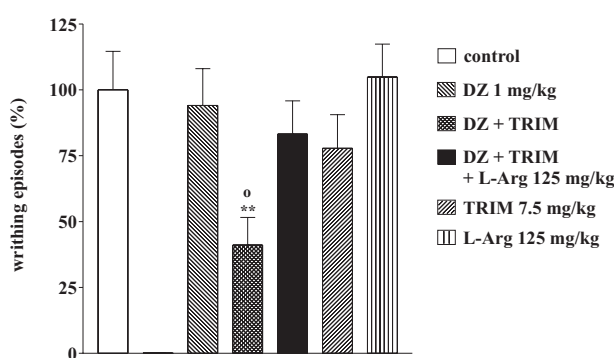


Fig. 2. The influence of 1-(2-trifluoromethylphenyl)imidazole (TRIM) on effects of diazepam (DZ) in the writhing test in mice. Each bar represents the means \pm SEM for a group of 10 mice. The data are expressed as per cent of control group. The absolute mean values of writhing episodes in control group was 23.1 ± 3.4 (\pm SEM). ** *p* < 0.01 vs. DZ, o *p* < 0.05 vs. TRIM, • *p* < 0.05 vs. DZ + TRIM + L-Arg

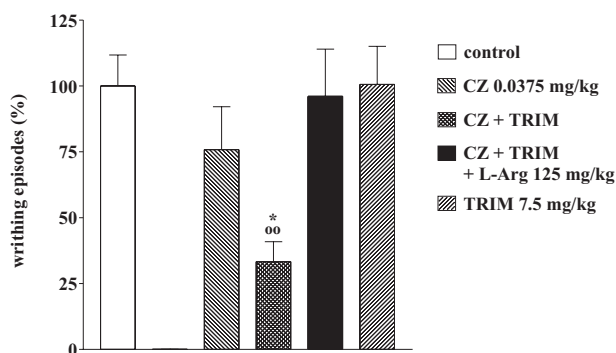


Fig. 3. The influence of 1-(2-trifluoromethylphenyl)imidazole (TRIM) on effects of clonazepam (CZ) in the writhing test in mice. Each bar represents the mean \pm SEM for a group of 10 mice. The data are expressed as per cent of control group. The absolute mean value of writhing episodes in control group was 28.75 ± 3.4 (\pm SEM). * $p < 0.05$ vs. CZ, oo $p < 0.01$ vs. TRIM, ** $p < 0.01$ vs. CZ + TRIM + L-Arg

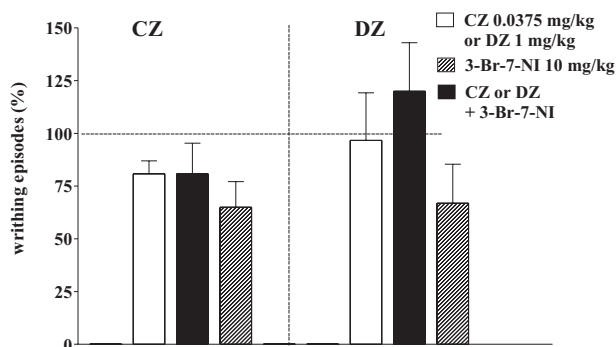


Fig. 4. The influence of 3-bromo-7-nitroindazole (3-Br-7-NI) on effects of clonazepam (CZ) and diazepam (DZ) in the writhing test in mice. The results are expressed as means \pm SEM of groups of 10 mice. The absolute mean values of a number of writhing episodes in the respective control groups, 20.9 ± 3.05 and 33.75 ± 3.9 (\pm SEM), were assumed to be 100%

significant antinociception and the effects were reversed by L-arginine (125 mg/kg) (Fig. 2 and 3). L-arginine (125 mg/kg), given alone, did not influence the acetic acid-induced writhing in mice, as shown in Figure 2.

The influence of 3-Br-7-NI on effects of clonazepam and diazepam in the writhing test in mice

The effects of clonazepam and diazepam, given at the doses of 0.0375 and 1 mg/kg, respectively, were not changed by 3-Br-7-NI (10 mg/kg) (Fig. 4).

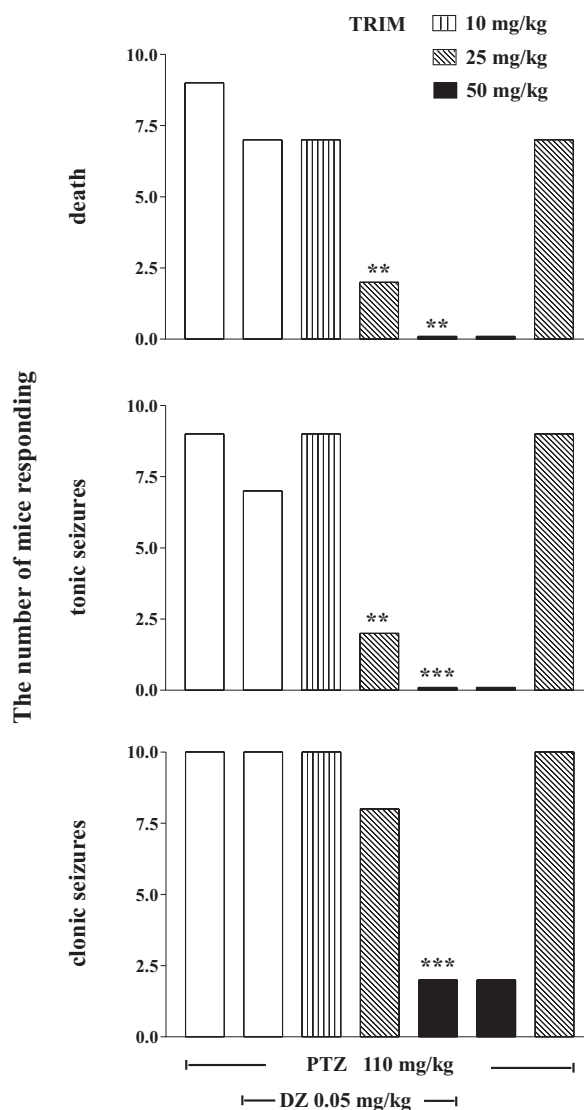


Fig. 5. The influence of 1-(2-trifluoromethylphenyl)imidazole (TRIM) on activity of diazepam (DZ) in pentetrazole (PTZ)-induced seizures in mice. *** $p < 0.001$, ** $p < 0.01$ vs. PTZ + DZ; ooo $p < 0.001$, o $p < 0.01$ vs. PTZ + TRIM; $\phi\phi\phi$ $p < 0.001$ vs. PTZ (χ^2 test with the Yates correction)

The influence of TRIM and 3-Br-7-NI on activity of diazepam in pentetrazole-induced seizures in mice

Pentetrazole (110 mg/kg) produced clonic seizures in all control mice, and tonic convulsion and death of most animals. The effect of the threshold dose of diazepam (0.05 mg/kg) was significantly intensified by TRIM, given at the dose of 25 mg/kg, which was evidenced by a decrease in the number of mice with tonic convulsions and dead animals.

Co-administration of diazepam (at the threshold dose) with TRIM (at the higher dose of 50 mg/kg) resulted in potent, significant anticonvulsive effect, but the identical results were observed when TRIM (at the same dose) was given alone (Fig. 5).

The activity of diazepam (0.05 mg/kg) in pentetrazole-induced seizures was not changed by 3-Br-7-NI given at the doses of 10 and 100 mg/kg (data not shown).

The influence of TRIM on activity of clonazepam in pentetrazole-induced seizures in mice

TRIM, given at the dose of 25 mg/kg, increased the protective efficacy of the threshold dose (0.005 mg/kg) of clonazepam, significantly reducing the number of mice responding with tonic convulsions, and mortality. L-arginine (125 mg/kg) was ineffective in changing the protection produced by combinations of clonazepam and TRIM (Fig. 6).

The anticonvulsant activities of TRIM and 3-Br-7-NI in pentetrazole-induced seizures in mice

TRIM, given at the dose of 50 mg/kg, protects completely against tonic convulsions and death, and significantly decreases the clonic phase of pentetrazole-induced seizures in mice (Fig. 5). Only slight reduction of mortality was produced by lower dose (25 mg/kg) of TRIM (Fig. 5 and 6). 3-Br-7-NI, given at the dose of 100 mg/kg, was able to slightly attenuate only clonic phase of the seizures (data not shown).

DISCUSSION

It is well documented that NO, a small, highly reactive gaseous molecule, can participate in nociceptive transmission. Enhancement of NO activity, e.g. by sodium nitroprusside, results in hyperalgesia [21]. On the other hand, NO synthase inhibitors prevent the thermal hyperalgesia [21] and attenuate the nociceptive responses in mice when tested using the writhing assay [23] or the formalin test [2, 3, 23, 27, 29]. In the present studies, we have used writhing test in mice to evaluate the antinociceptive activity of TRIM and 3-Br-7-NI, a potent non-arginine NO synthase inhibitors, and

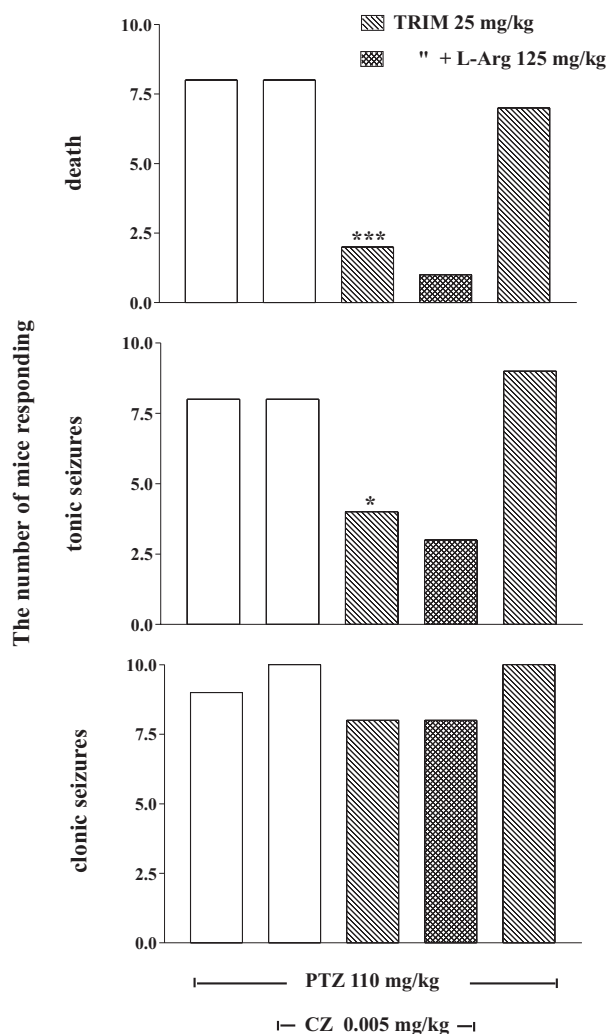


Fig. 6. The influence of 1-(2-trifluoromethylphenyl)imidazole (TRIM) on activity of clonazepam (CZ) in pentetrazole (PTZ)-induced seizures in mice. *** $p < 0.001$, * $p < 0.05$ vs. PTZ + CZ; oo $p < 0.01$, o $p < 0.05$ vs. PTZ + TRIM (χ^2 test with the Yates correction)

their influence on antinociceptive effects of the threshold dose of diazepam and clonazepam.

The writhing test is a simple screening method recommended by many investigators to evaluate both central and peripheral analgesics. Although the test has a low specificity, it is sensitive and shows a good correlation between analgesic effects in animals and humans [24]. Some authors [23, 27, 29] used the acetic acid-induced writhing assay for its high degree of sensitivity to NO synthase inhibitors.

Antinociceptive effects of both TRIM and 3-Br-7-NI observed in this study were significant but not dose-dependent. Antinociceptive potencies of

TRIM and 3-Br-7-NI were comparable with that of morphine, given at the doses of 0.5–1.0 mg/kg (our unpublished results). The antinociceptive effects of TRIM were also demonstrated by suppression by this compound of the second phase of hindpaw licking behavior induced by formalin injection in mice [18] and inhibition of carrageenan-induced mechanical and thermal hyperalgesia in rats [17].

The analgesic effects of benzodiazepines are controversial. A direct antinociceptive activity of benzodiazepines has been demonstrated using the hot plate test [13], tail flick test [49] and writhing test [41] but other findings do not support these observations [37, 38]. Despite that, the benzodiazepines are accepted as adjuvant analgesics in the management of several specific disorders associated with pain [33].

A vast body of evidence pointing to an interaction between NO and GABA systems [36, 40, 50], prompted researchers to carry out studies on the participation of NO in antinociceptive effects of benzodiazepines.

Our previous study [43] demonstrated intensification of the antinociceptive effects of benzodiazepines by L-NAME, a nonselective inhibitor of neuronal NO synthase [34] and 7-NI, a selective [2] inhibitor of this enzyme. The results of the present study show that TRIM, but not 3-Br-7-NI, is able to enhance the antinociceptive effects of diazepam and clonazepam. Moreover, the effect evoked by co-administration of TRIM with clonazepam was blocked by L-arginine, endogenous donor of NO. Although 3-Br-7-NI is more potent inhibitor of neuronal NO synthase than 7-NI *in vitro* [4], its behavioral activity is not stronger than that of 7-NI. The reason of the lack of influence of 3-Br-7-NI on antinociceptive effects of benzodiazepines is not clear. It is more likely that observed differences between TRIM and 3-Br-7-NI in their influence on benzodiazepines effects may be due to different pharmacokinetic characteristics (absorption, protein binding, penetration in the brain, biotransformation *in vivo*) or different pharmacodynamic properties of indazole (3-Br-7-NI)- and imidazole (TRIM)-derivatives. TRIM exhibited selectivity for inhibition of neuronal NO synthase *in vitro* and *in vivo* [18], and 3-Br-7-NI is more potent inhibitor of the synthase *in vitro* than 7-NI but less specific [4]. Regarding the specificity of the inhibitors, an involvement of some additional non-specific effects unrelated to synthesis of the NO synthase, in their

activities, could not be excluded. For example, it has been demonstrated that 7-NI is able to promote the release of both noradrenaline and dopamine from hippocampal and striatal neurons (reviewed by [28]), and additionally, inhibit monoamine oxidase [10]. Nevertheless, the results on the activity of TRIM in writhing assay in mice seem to confirm participation of NO in antinociceptive effects of the tested benzodiazepines.

Although the importance of NO to epileptogenesis has been demonstrated by several authors, its role in the expression of seizures remains unclear. Some studies have suggested that NO acts as an endogenous anticonvulsant [31, 32] while the others showed its proconvulsant properties [9, 12, 30]. These discrepancies may result from various experimental conditions, and above all, from different models of epilepsy used [1, 19, 20, 32]. In addition, the drugs used to modify the NO metabolism might contribute to conflicting results. NO synthase inhibitors are the most frequently used tools in those studies.

Our previous study [42, 44] showed that L-NAME and L-NOARG possessed some protective activity against seizures evoked by pentetrazole, and 7-NI was effective against electroshock-induced tonic hindlimb extension in mice. The results of the present study show that only TRIM, but not 3-Br-7-NI, was able to protect against pentetrazole-induced seizures in mice. Anticonvulsant activity of TRIM, 3-Br-7-NI, and also S-methyl-L-thiocitrulline (S-Me-TC), a potent, non-arginine NO synthase inhibitor, was demonstrated in the pilocarpine-induced seizures in mice by Dzoljic et al. [12]. Although all three NO synthase inhibitors were able to prolong the latencies of seizures and status epilepticus, and delay mortality, only 3-Br-7-NI and TRIM decreased the frequency of status epilepticus, and mortality. TRIM, however, was the only one to significantly reduce the incidence of seizures.

The anticonvulsant efficacy of benzodiazepines is partially but not fully mediated by α_1 subtype of GABA receptor [25]. It is not yet possible to rule out the participation of another system in their antiepileptic activity. The evidence pointing to an interaction between NO and GABA systems (see Introduction) prompted us to study the influence of NO system on the antiepileptic effects of benzodiazepines. We used the pentetrazole model to examine this relationship. The selected model of seizures has proved to be a good predictor of clinical effi-

cacy against generalized spike-wave epilepsies of the absence type [35].

In our previous study [44], we showed that NO synthase inhibitors, L-NAME and 7-NI, and also methylene blue, an inhibitor of guanylate cyclase, potentiated the protective action of diazepam and clonazepam against pentetrazole-induced seizures. The enhancing effect of L-NAME on antiepileptic activity of diazepam was reversed by L- but not D-arginine. Additionally, 7-NI increased the protective activities of diazepam and clonazepam against electroconvulsions measured as duration of hindlimb extension phase. These observations seem to confirm the involvement of NO in seizure mechanisms. In the present study, protective efficacy of the threshold dose of diazepam against pentetrazole-induced tonic seizures and death was enhanced by TRIM given at the dose of 25 mg/kg. TRIM given at the higher dose of 50 mg/kg indeed powerfully increased the antiepileptic effect of diazepam, but when given alone at this dose, it showed an equally potent, protective activity against pentetrazole-induced seizures. Similarly, TRIM intensified the antiepileptic activity of the threshold dose (0.005 mg/kg) of clonazepam, reducing the tonic seizures and mortality. Again, L-arginine (125 mg/kg) did not reverse the TRIM-induced potentiation. 7-NI-induced potentiation of the protective action of clonazepam against pentetrazole-induced convulsions in mice was also not sensitive to L-arginine both at 125 mg/kg [42, 44] and 500 mg/kg [6]. In addition, L-arginine (500 mg/kg) remained ineffective in changing the anticonvulsant action of the combinations of 7-NI and clonazepam against amygdala-kindled seizures in rats [5]. The results seem to indicate that interactions of non-arginine NO synthase inhibitors (i.e. 7-NI and TRIM) with clonazepam in some models of epilepsy are independent of NO system. Moreover, molsidomine, a donor of NO, remained without effect upon the anticonvulsive efficacy of clonazepam [45], and N^G-nitro-L-arginine, an inhibitor of NO synthase, was ineffective against protective effects of diazepam [7] in pentetrazole-induced seizures. The studies seem to suggest the lack of involvement of NO in the mechanism of the anticonvulsive efficacy of clonazepam and diazepam. On the other hand, reversal of the anticonvulsant effects of the combination of L-NAME, an arginine-derivative NO synthase inhibitor, with diazepam by L-arginine [44] suggests its NO-dependent mechanism of action.

Summing up, both NO synthase inhibitors, TRIM and 3-Br-7-NI, produced antinociceptive effects in the writhing test in mice, but only TRIM was able to intensify the analgesic activity of diazepam and clonazepam. Also TRIM, but not 3-Br-7-NI, potentiated antiepileptic effects of the tested benzodiazepines. L-arginine, an endogenous NO donor, reversed intensifying effects of TRIM on antinociceptive, but not anticonvulsive action of clonazepam.

Finally, the presented results seem to confirm, at least in part, participation of NO in antinociceptive and anticonvulsant effects of benzodiazepines, and point to TRIM as a better tool than 3-Br-7-NI for examination of the role of NO in behavioral studies.

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