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Research Article

Benzylidene Barbituric Acid Derivatives Shown Anticonvulsant Activity on Pentylenetetrazole-Induced Seizures in Mice: Involvement of Nitric Oxide Pathway

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ABSTRACT

Background: Barbituric acid derivatives have long been used as central nervous system (CNS) suppressants, such as sedatives, hypnotics and anticonvulsants. In addition, previous studies have implicated the involvement of nitric oxide (NO) in the anticonvulsive effects of barbiturates in CNS. Therefore, the purpose of this study was to figure out the effects of a novel class of barbituric acid derivatives on pentylenetetrazole (PTZ)-induced seizures in male mice.

Methods: Thirteen synthesized barbituric acid derivatives (a-m) and phenobarbital were administered intraperitoneally (i.p.) 30 min before induction of seizures by PTZ administration. The mechanisms of PTZ-induced seizures in the mice was evaluated using a non-selective nitric oxide synthase (NOS) inhibitor, selective inducible NOS (iNOS) inhibitor, a selective neuronal NOS (nNOS) inhibitor, and NO substrate.

Results: Administration of most of the above mentioned derivatives significantly increased the seizures threshold ($P<0.001$). The most potent derivative (compound **a**), was chosen in order to investigate the mechanism of action involving in anticonvulsant activity. Administration of a non-selective NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) and a selective nNOS inhibitor, 7-nitroindazole (7-NI) reversed anticonvulsant activity of compound **a**. However, injection of the nitric oxide precursor, L-arginine (L-Arg) and a selective iNOS inhibitor, aminoguanidine (AG), did not change anticonvulsant activity of the mentioned compound.

Conclusion: These results indicated that the NO system, specifically nNOS may contribute to the anticonvulsant activity of benzylidene barbituric acid derivative **a**. Therefore, this compound is a good candidate in order to designing new anticonvulsant medications.

Introduction

Epilepsy is one of the most frequent neurological disorders which is resulted from the unusual neurons firing in the brain.[1-3] This disrupts the normal working of the parts of the brain, resulted in clinical symptoms. The mentioned disorder is controlled by several mechanisms including cellular changes such as transformation in ion channel function, neurotransmitter receptor function or neurotransmitter level which could lead to seizures.[4-8]

Most of approved antiepileptic medications have demonstrated dose-related adverse drug reactions.[9] Muscle stiffness, repetitive movements and loss of consciousness are some seizure's diverse symptoms.[10] 5, 5-disubstituated barbituric acid derivatives (such as phenobarbital, ...) are the group of medicines which are well-known as central nervous system (CNS) depressants.[11] This class of compounds has long been used in clinic as a sedative and hypnotic agent. *Phenobarbital*, a long-acting *barbiturate, is used* in the treatment of epilepsy[12] and thiobarbiturate in intravenous anesthesia induction.[13] Furthermore, barbituric acids, a large and diverse class of barbiturates are used as hypnotic, sedative and anticonvulsant agents.[11] Despite the expanding use of phenobarbital, it has some adverse side effects such as double vision, nausea, drowsiness and dizziness.

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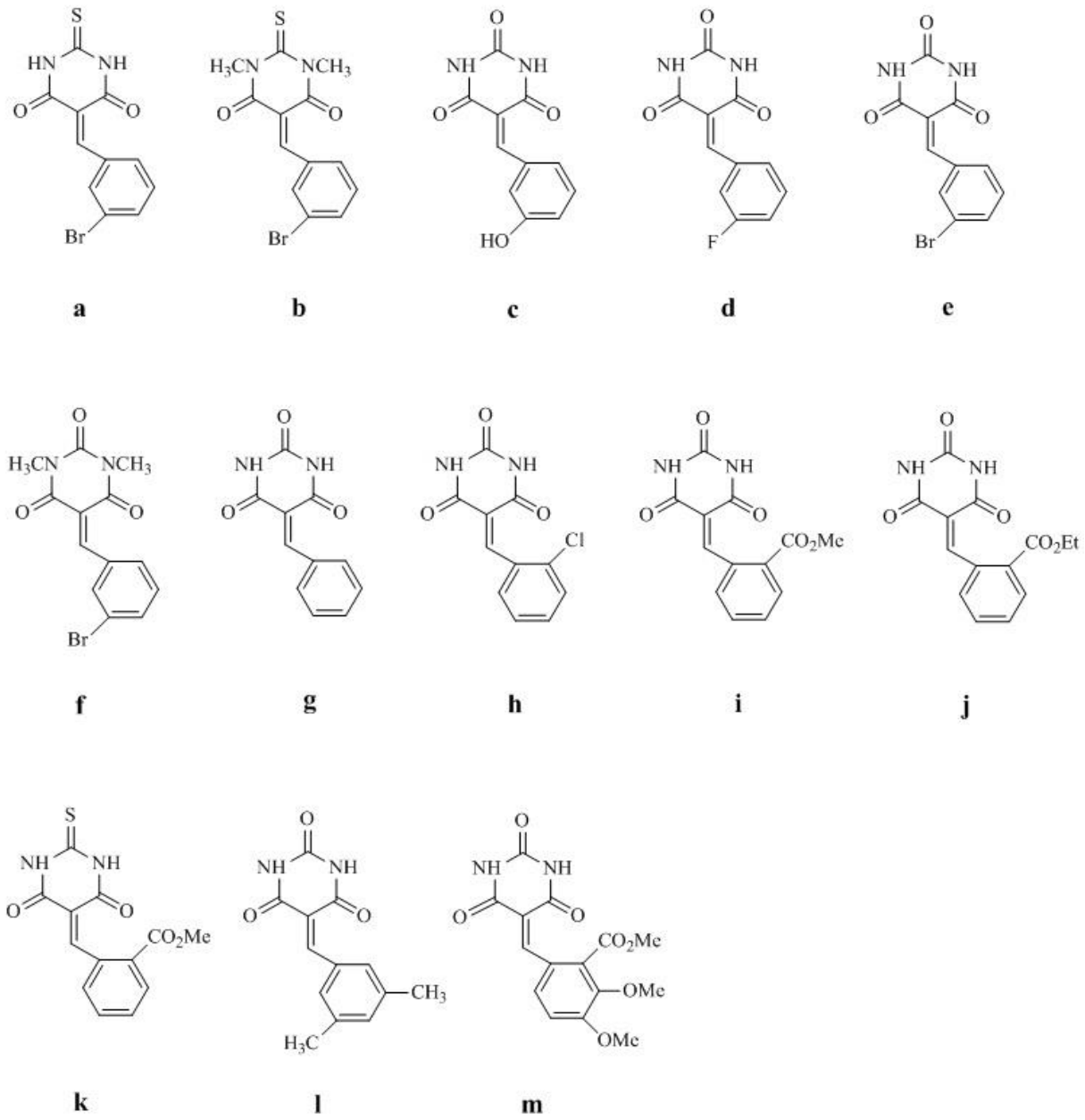

Figure 1. The chemical structure of benzylidene barbituric acid derivatives **a-m**.

Nitric oxide (NO) is considered to be a gaseous free radical synthesized from L-arginine (L-Arg) by different nitric oxide synthase (NOS) forms.[14] It seems to operate as a neurotransmitter in the brain[15] and play a fundamental role in numerous processes including seizure induction and progression.[16] Regarding the presence of the three kinds of NOS isoforms, including endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)[17,18] and also the potential side effects of barbiturate antiepileptic drugs, the present study was designed to evaluate anticonvulsant activity of 13 barbituric acid derivatives (Figure 1) in order to represent a potent anticonvulsant agent in comparison with phenobarbital. Moreover, the correlation of NOS/NO with the effects of the mentioned derivative on pentylenetetrazole (PTZ)-induced seizures in the mice, was evaluated using a non-selective NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), selective iNOS inhibitor, aminoguanidine (AG), a selective nNOS inhibitor, 7-nitroindazole (7-NI) and NO substrate, L-Arg.

Materials and Methods

Experimental animals

The experiments were performed on male NMRI mice weighing 23–30 g.[19,20] The animals were housed in standard cages, in groups of 5–6, in a room with normal light (12 h light/12 h dark cycle) and temperature (22 ± 1 °C), while they had access to food and water except for a short time before the experiments. All behavioral experiments were done between 09:00 and 13:00. Each mouse just used once and each treatment group comprised of 6-8 animals. Moreover, efforts were made to reduce

animal suffering and to perform experiments in accordance with Tehran University of Medical Sciences guidelines for animal care.

Chemicals

PTZ, L-NAME, 7-NI, AG, L-Arg and dimethyl sulfoxide, (DMSO) were purchased from Sigma Co. (St. Louis, MO, USA). Barbituric acid derivatives have been synthesized based on a Knoevenagel condensation[21] and also through the reaction of 2-formylbenzoic acids with (thio)barbituric acids in the presence of p-toluenesulfonic acid (p-TsOH) in MeOH/EtOH. [22] The mentioned compounds, (**a-m)** solution were prepared in saline (0.9%)/DMSO (90:10, v/v), to provide the appropriate concentrations. PTZ was dissolved in saline (0.9%) in order to provide the 5 mg/ml (0.5%) concentration. Except PTZ, which was administered intravenously (i.v.), all other drugs were administered i.p. at required doses. Proper controls were used for each experiment.[23]

Determination of seizure threshold

The PTZ threshold was determined by infusion of PTZ (0.5%) solution into the tail vein of mice through a 30-gauge butterfly needle. The needle was then fixed on the tail by a piece of adhesive tape. The PTZ solution (0.5%) was infused into the mouse tail vein at a constant rate (1 ml/min)[19,20] employing an infusion pump (NE 1000, New Era Pump System, Inc.). Infusion was paused as soon as forelimb clonus and then full body clonus was observed. The minimal essential dose of PTZ (mg/kg mice weight) to induce clonic seizure, was used as the clonic seizure threshold index.[24,25]

Treatment

Experiment 1

In order to determine the optimum required time, Barbituric acid derivatives (equivalent doses to phenobarbital (20 mg/kg, i.p.))[26] was administered 30, 60 and 90 min before determination of PTZ-induced seizure threshold). Control animals received saline (0.9 %)/DMSO (90:10, v/v) solution at the same procedure.

Experiment 2

Animals received acute i.p. injections of phenobarbital (20 mg/kg, i.p.) and equivalent doses of barbituric acid derivatives 30 min before determination of PTZ-induced seizure threshold. Control animals received saline/DMSO solution at the same procedure.

Experiment 3

The nonspecific NOS inhibitor, L-NAME (10 mg/kg) and also NO precursor, L-Arg (60 mg/kg), were acutely administered 15 min before saline and barbituric acid derivative **a** (the compound which induced the highest seizure threshold in experiment 2) (26.8 mg/kg, i.p.), and 45 min before induction of seizure by PTZ. In addition, in order to assess the different NOS roles, the specific nNOS inhibitor 7-NI (30 mg/kg, i.p.) was administered 15 min before saline and barbituric acid derivative **a** (26.8 mg/kg, i.p.) as well as the specific iNOS inhibitor, AG (100 mg/kg, i.p.). The results were compared to the control group.

The doses and times of injection of L-NAME, L-Arg, AG and 7-NI were chosen based on previously published studies.[20,27]

Control animals received the same volume of the solution (saline (0.9%)/DMSO) in all experiments.

Statistical analysis

Data are expressed as mean ± S.E.M. of clonic seizure threshold in each experimental group . The one-way ANOVA followed by Tukey's post hoc were used to analyze the data where appropriate. Tests of homogeneity of variance were used to ensure normal distribution of the data. The P value of less than 0.05 was considered statistically significant.

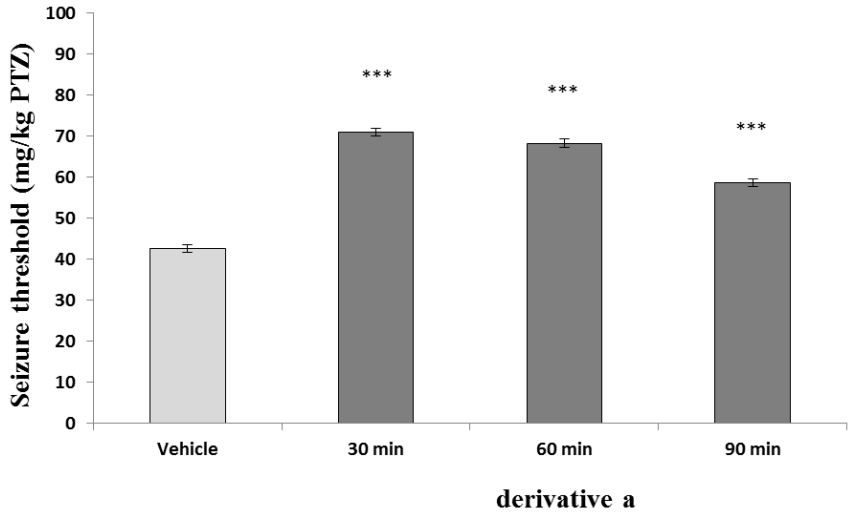

Figure 2. Determination the optimum required time to develop the maximum effect of barbituric acid derivative **a** on the PTZ-induced seizure threshold. The mentioned compound was administered 30, 60 and 90 min before PTZ and the effects compared with those of a control sample at the same time course. Data are expressed as means ± SEM of the seizure threshold in each group. ***P<0.001 compared with the control group.

Results

Effect of time course in barbituric acid derivatives treatment on the PTZ-induced seizure threshold

Figure 2 shows the effect of time course of barbituric acid derivative **a** (as an example among those derivatives which were randomly selected to test) on the seizure threshold. As illustrated, intraperitoneal administration of derivative **a** (26.8 mg/kg) significantly increased the PTZ-induced seizure threshold (P<0.01) 30, 60 and 90 min after administration, compared with saline-treated control animals [(t (7) = -21.548, P<0.001; t (7) = -18.108, P<0.001; t (7) = -13.393, P<0.001); respectively]. However, the seizure threshold enhancement at 30 min was higher than that of 60 and 90 min.

Effect of different barbituric acid derivatives on the seizure threshold

Figure 3 illustrates the effect of acute intraperitoneal administration of derivatives **a-m** on the PTZ-induced seizure threshold, which were administered 30 min prior to PTZ. Most of these derivatives increased the PTZ-induced seizure threshold (P<0.01) compared to saline-treated control animals (F (14, 105) = 33.720, P<0.001). Interestingly, compounds **a** and **e** significantly increased the PTZ-induced seizure threshold in comparison with phenobarbital (20 mg/kg, i.p.; P<0.001).

Effect of L-NAME, L-Arg, 7-NI and AG on the anticonvulsant effect of barbituric acid derivative a

Figure 4 depicts effect of NO pathway modulator on anticonvulsant activity of derivative **a**.

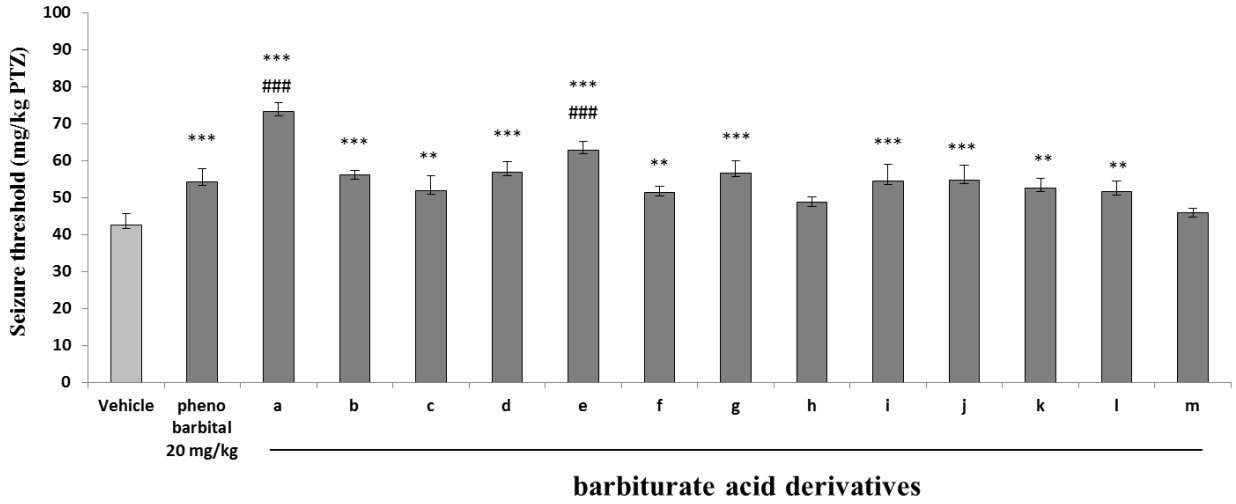

Figure 3. Effect of barbituric acid derivatives on the PTZ-induced clonic seizure threshold in mice. Barbituric acid derivatives were administered 30 min before PTZ, and their effects were compared with that of a control sample and also phenobarbital at the same time. Data are expressed as means ± SEM of the seizure threshold in each group. **P <0.01 and ***P <0.001 compared with the corresponding saline group. $^{###}$P<0.001 compared with phenobarbital-treated group.

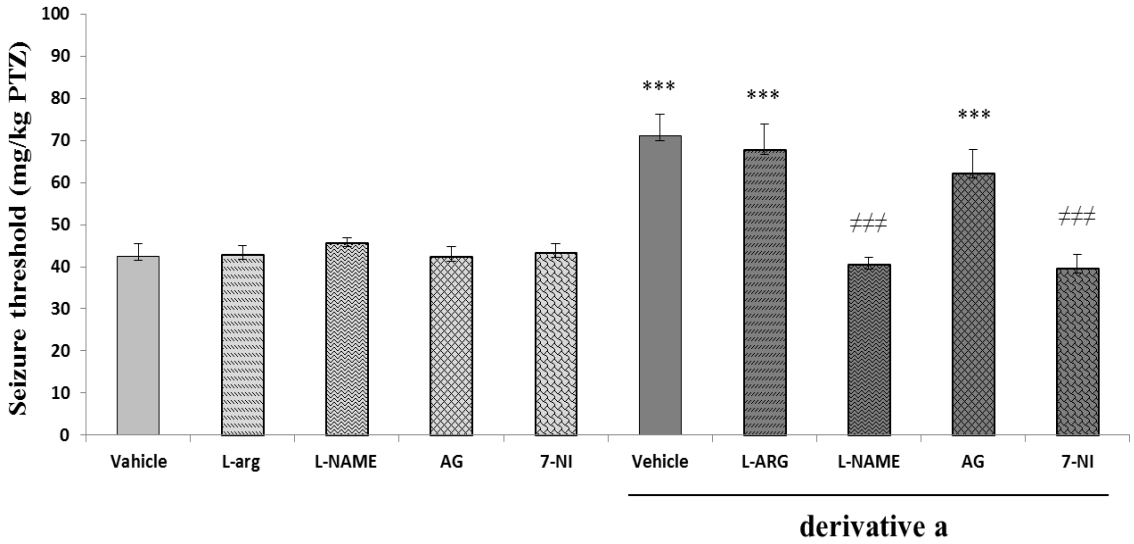

Figure 4. Effect of L-NAME, L-Arg, 7-NI and AG on anticonvulsant effect of barbituric acid derivative **a**. L-NAME (10 mg/kg, i.p.), L-Arg (60 mg/kg, i.p.), 7-NI (30 mg/kg, i.p.) and AG (100 mg/kg, i.p.) were administered 15 min before barbituric acid derivative **a** (26.8 mg/kg, i.p.). Data are expressed as means ± SEM. ***P<0.001 compared with vehicle group, $^{###}$P<0.001 compared with derivative **a** treated group.

As illustrated, acute administration of non-selective NOS inhibitor, L-NAME (10 mg/kg, i.p.) and selective nNOS inhibitor, 7-NI (30 mg/kg, i.p.) significantly reversed the anticonvulsant activity of derivative **a** ((F(3, 28)=144.638, P<0.001; (F(3, 28)=111.902, P<0.001); respectively). Whereas, neither administration of precursor of NO, L-Arg (60 mg/kg, i.p.), nor administration of iNOS inhibitor, AG (100 mg/kg, i.p.) alter the anticonvulsant activity of derivative **a**. L-NAME, 7-NI, L-Arg and AG were administered 15 min before derivative **a** and 45 min before induction of seizure.

Discussion

In the current study, we demonstrated anticonvulsant effect of a novel class of barbituric acid derivatives on pentylenetetrazole (PTZ)-induced seizures in male mice.

We also demonstrated that the anticonvulsant effect of the mentioned derivatives were completely reversed by acute pretreatment of L-NAME (a nonselective nitric oxide synthase inhibitor) and 7-NI (a selective neuronal nitric oxide synthase inhibitor). Moreover, aminoguanidine (a selective inducible nitric oxide synthase inhibitor) did not change the anticonvulsant effect of barbituric acid derivatives. Therefore, we propose contribution of the nitric oxide (NO) pathway and especially nNOS pathway in the anticonvulsive effects of barbiturates in CNS.

In the mentioned class of barbituric acid derivatives, different substituents at the aromatic ring demonstrated a considerable influence on the anticonvulsant activity by regulating the lipophilicity and facilitating permeation across biological membrane (Table 1) and also through the interaction with the active site.

Physiochemical features of CNS drugs such as molecular weight, lipophilicity, and hydrogen bond donor and acceptor are related to their ability to penetrate the blood-brain barrier affinity and exhibit CNS activity. The optimal activity is observed at LogP = 2.[28] Frequently, the logP (the octanol/water partitioning coefficient—a measure of lipophilicity) of a compound can be used as a quick and simple prediction of its permeability and can be either measured or calculated. Replacement of oxygen by Sulphur at carbon-2 (thiobarbiturates) in compound a and b increases lipid solubility.

The compound a with LogP=1.95 showed a good effect. In compound b and f despite of the presence of relatively high lipophilicity, methyl substituents on nitrogen atoms decrease potency in comparison to other derivatives. The LogP value of the compound d is low but the replacement of the fluorine atom increases the potency in barbituric acid derivatives. The compound of g without any substituent on aromatic ring showed a moderate effect. Compound c and m have less lipophilicity than other derivatives which reduces their potency.

Therefore, structure activity relationship (SAR) studies which demonstrated the correlation of the electronic nature of the substituent group and the anticonvulsant activity, could be useful in order to optimization and generation a more potent compound with lower side effects.

Table 1. Log P values of benzylidene barbituric acid derivatives (**a-m**).

benzylidene barbituric derivatives	Log P
a	1.95
b	2.42
c	0.19
d	0.73
e	1.4
f	1.88
g	0.58
h	1.13
i	0.4
j	0.73
k	0.94
l	1.55
m	0.14

High lipophilicity and in turn high permeability across biological membrane such as blood brain barrier and also the presence of bromine, an electron withdrawing group on phenyl ring, resulted in the most potent anticonvulsant compound in this study, barbituric acid derivative **a.** The presence of bromine, an electron withdrawing group on phenyl ring, increases the potency of the compound **e** compared to compound having no substitution on phenyl ring **g**. The bromo substitution increases lipophilicity, due to increased permeability across blood brain barrier. SAR studies in this series of barbiturate acid derivatives, indicated that the lipophilic and electron withdrawing substituent on meta position over the phenyl ring **d**, exerted significant anticonvulsant activity, whereas lipophilic and electron withdrawing substituent on ortho position showed less anticonvulsant activity, **h**.

In general mono-substituted N-methyl derivatives of barbituric acid are more potent than the 1,3-dimethyl barbiturates (**b** and **f**) and also than non-methylated derivatives (**a** and **e**) in anticonvulsant test. The difference in potency between N-methyl substituted compounds and non-methylated NH derivatives is because of the increased lipophilicity and acidity, and as a result the increased permeability across biological membrane. N-methyl derivatives are the most potent compounds, regarding the tautomerization effects. For compound **m**, low lipophilicity and high steric hindrance led to a decreased anticonvulsant activity. Presence of ester group indicated that the mentioned compound is subjected to metabolizing in the biological environment.

Overall, it was found that there is not a direct correlation between log P value of compounds and the *in vivo* anticonvulsant activity. The steric hindrance of the substituents on phenyl ring, also play a remarkable role in anticonvulsant activity.

Conclusion

In conclusion, besides to introducing the effects of a novel class of barbituric acid derivatives on pentylenetetrazole (PTZ)-induced seizures in male mice, this study indicated that derivatives **a** and **e** are more potent anticonvulsant agents than phenobarbital (the standard anticonvulsant medication). The mentioned derivatives significantly increased the PTZ-induced seizure threshold versus phenobarbital (20 mg/kg, i.p.; P<0.001). Therefore, the

mentioned compounds could be subjected for further optimization and modification to generate anticonvulsant agents with lower toxicity and fewer side effects due to the promising anticonvulsant activity.

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Conflict of interests

The authors claim that there is no conflict of interest.

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