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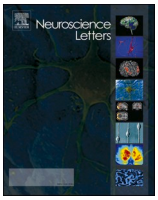

Azoles display promising anticonvulsant effects through possible PPAR-α activation

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ABSTRACT

Azoles such as nafimidone, denzimol and loreclezole are known for their clinical efficacy against epilepsy, and loreclezole acts by potentiating γ-aminobutyric acid (GABA)-ergic currents. In the current study, we report a series of azole derivatives in alcohol ester and oxime ester structure showing promising anticonvulsant effects in 6 Hz and maximal electro shock (MES) models with minimal toxicity. The most promising of the series, **5f**, was active in both 6 Hz and MES tests with a median effective dose (ED_{50}) of 118.92 mg/kg in 6 Hz test and a median toxic dose (TD_{50}) twice as high in mice. The compounds were predicted druglike and blood–brain barrier (BBB) penetrant *in silico*. Contrary to what was expected, the compounds showed no *in vitro* affinity to $GABA_A$ receptors ($GABA_ARs$) in radioligand binding assays; however, they were found structurally similar to peroxisome proliferator-activated receptors alpha (PPAR-α) agonists and predicted to show high affinity and agonist-like binding to PPAR-α in molecular docking studies. As a result, **5f** emerged as a safe azole anticonvulsant with a wide therapeutic window and possible action through PPAR-α activation.

1. Introduction

Epilepsy is a neurological chronic disorder characterized with recurrent seizures affecting more than 50 million people worldwide. Epilepsy treatment requires long-term drug use exposing patients to certain side effects. In addition, one third of the cases are nonresponsive to current medications, thus antiepileptic drug design is an urgent topic [1].

To date, several major pathways for pathophysiology of epilepsy, including γ-aminobutyric acid (GABA) receptors, glutamate receptors, sodium and potassium channels, and other targets affecting concentrations of neurotransmitter were associated with epileptic seizures [2]. Targets such as carbonic anhydrases, gap junctions, serotonin receptors, and peroxisome proliferator-activated receptors-α (PPAR-α) are also of interest for antiepileptic drug design [3–6].

Azoles are one of the antiseizure compound classes known for clinical candidates nafimidone, denzimol and loreclezole (Fig. 1) [7]. Loreclezole is a known positive allosteric modulator of type A GABA receptors ($GABA_ARs$) [8]. Enhanced GABA receptor action triggers influx of chloride in the central nervous system, which leads to increased inhibitory post-synaptic potential hence seizure threshold [9].

Previously we reported a set of azole derivatives in alcohol ester and oxime ester structure with protection against seizures induced by 6 Hz, a model for therapy-resistant partial seizures that tests the ability of a compound to block psychomotor seizures induced by long-duration, low frequency (6 Hz) stimulation, in addition to maximal electroshock (MES), a model for generalized tonic-clonic seizures and provides insights into a compound's ability to prevent seizure spread when all neuronal circuits in the brain are maximally active [10–14]. Compounds with anticonvulsant effects of these studies possessed mostly imidazole as azole ring, chlorinated benzene as aryl group and lipophilic moieties as the ester group in their structures. Therefore, in this study, we tested a series of carboxylic acid esters of 1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol, 1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanone oxime, and 2-(1*H*-imidazol-1-yl)-1-phenylethanone oxime (**5a-l**, Fig. 1) for their anticonvulsant properties using 6 Hz and MES models in mice to

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identify promising hits, especially against therapy-resistant seizures. Median effective and toxic doses (ED_{50} and TD_{50}, respectively) of the most promising derivative (**5f**) were determined in 6 Hz model. The title compounds were also evaluated *in silico* regarding druglikeness and pharmacokinetic properties including blood–brain barrier (BBB) penetration. To understand their anticonvulsant mechanism of action, *in vitro* $GABA_A$Rs binding and *in silico* modelling studies were performed.

2. Materials and methods

2.1. Synthesis of the compounds

The title compounds were previously synthesized using literature methods and reported (see the Supplementary Information file for details) [15,16].

2.2. Anticonvulsant activity tests

Anticonvulsant activity neurotoxicity tests were performed *in vivo* by the National Institute of Neurological Disorders and Stroke (NINDS) Epilepsy Therapy Screening Program (ETSP) in compliance with animal welfare laws and policies overseen by National Institutes of Health's Office of Laboratory Animal Welfare (OLAW).

For anticonvulsant identification, 6 Hz Psychomotor and MES tests were applied on CF1 mice via intraperitoneal route. Rotarod test was performed for toxicity. For anticonvulsant quantification, ED_{50} and TD_{50} of **5f** were determined at the time to peak effect (TPE) in 6 Hz and rotarod test, respectively. TPE was determined by testing animals at 0.25, 0.5, 1.0, 2.0, and 4.0 h. Groups of 4–8 animals were tested at various doses at the TPE until at least two points were established between 0 to full protection. The ED_{50}, TD_{50}, 95 % confidence interval, slope of the regression line, and standard error values were calculated using Probit analysis [17]. See the Supplementary Information file for details.

2.3. Molecular modelling

The title compounds were modelled using MacroModel (2021–2, Schrödinger LLC, New York, NY) according to the Optimized Potentials for Liquid Simulations 4 (OPLS4) forcefield parameters and conjugate gradient method [18]. Molecular descriptors were calculated using QikProp (2021–2, Schrödinger LLC, New York, NY), SwissADME (www.swissadme.ch) [19], vNN-ADMET (https://vnnadmet.bhsai.org) [20], CBligand (https://www.cbligand.org/BBB) [21] and LightBBB (http://bioanalysis.cau.ac.kr:7030) [22]. Molecular docking was performed using human PPAR-α crystallographic structure (PDB code: 3KDU [23] resolution 2.07 Å) and Glide (2021–4, Schrödinger LLC, New York, NY) [24]. See the Supplementary Information file for details.

3. Results

3.1. Title compounds display seizure protection in both models of epilepsy

The identification test was performed at two time points (0.5 and 2 h) and three doses (30, 100, and 300 mg/kg). Compound **5b**, **5d**, **5f-i**, and **5k** were protective in both 6 Hz and MES tests (Table 1). **5a** and **5j** were activity MES-selective, while **5l** was active only in 6 Hz-induced seizures. Only **5d**, **5f** and **5g** showed seizure protection at 100 mg/kg and

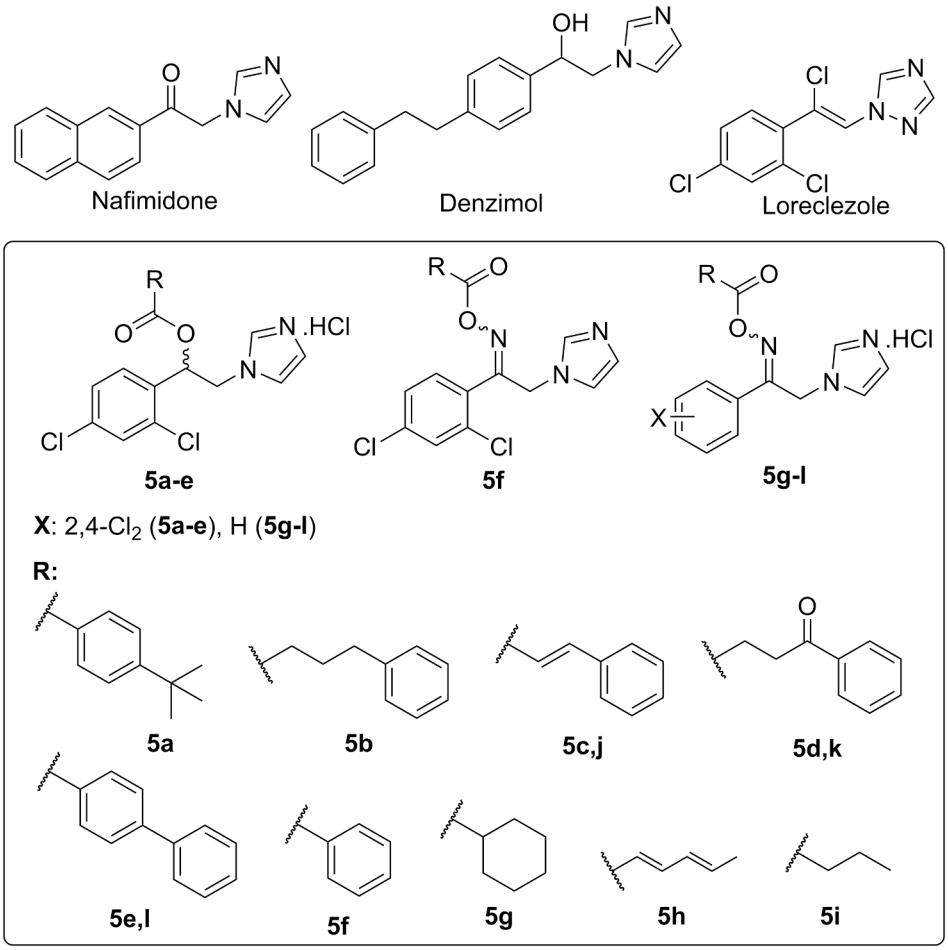

Fig. 1. Antiepileptic azoles and the title compounds of the current study (**5a-l**).

Table 1
Anticonvulsant identification results [a] of the title compounds.

Test	Time (h)	Dose (mg/kg)	Compound											
			5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5 g**	**5 h**	**5i**	**5j**	**5 k**	**5 l**
6 Hz	0.5	30	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		100	0/4	0/4	0/4	**3/4**	0/4	**3/4**	**3/4**	0/4	0/4	1/4	0/4	0/4
		300	0/4	**2/4**	0/4	**4/4**	0/4	**4/4**	**4/4**	**4/4**	**3/4**	1/4	**4/4**	0/4
	2	30	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4
		100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		300	0/4	0/4	0/4	**4/4**	0/4	**2/4**	**2/4**	0/4	0/4	0/4	**2/4**	**3/4**
MES	0.5	30	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		100	0/4	1/4	1/4	**3/4**	0/4	1/4	**2/4**	1/4	1/4	0/4	0/4	0/4
		300	**3/4**	**4/4**	**3/4**	**4/4**	0/4	**2/4**	**4/4**	**4/4**	**4/4**	**3/4**	**4/4**	0/4
	2	30	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		300	**4/4**	**4/4**	**2/4**	**4/4**	0/4	0/4	1/4	**3/4**	1/4	0/4	0/4	0/4
Toxicity	0.5	30	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
		100	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
		300	0/8	3/8	0/8	**8/8**	0/8	2/8	**8/8**	**8/8**	**8/8**	0/8	**5/8**	0/8
	2	30	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
		100	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
		300	0/8	0/8	0/8	2/8	0/8	0/8	2/8	0/8	3/8	0/8	0/8	0/8

[a] Data in each cell represents the number of mice observed to be protected or suffer toxic effects/the number of mice tested. Data indicating activity or toxicity for at least 50% of the tested animals is highlighted as bold.

these compounds maintained their effects up to 2 h when administered at 300 mg/kg, however only **5f** was nontoxic at this dose. None of the compounds were active at 30 mg/kg and no activity was observed for **5e**.

3.2. Compound *5f* has a wide therapeutic window

Compound **5f** was selected for further quantification tests in 6 Hz model due to its promise in anticonvulsant identification tests. In order to determine the ED_{50} and TD_{50} vales of **5f** in 6 Hz model in mice, the TPE was determined between 0.25 and 4 h. Seizure protection of **5f** peaked at 0.5 h and maintained at 1 h, then diminished at 2 h (see Table S1 of the Supplementary Information file). Toxicity was parallel to the anti-6 Hz activity but at a higher dose. Thus, 0.5 h was selected as TPE for ED_{50} and TD_{50} quantification. ED_{50} of **5f** was determined as 118.92 mg/kg at 0.5 h in 6 Hz model in mice, while its TD_{50} was 241.70 mg/kg (Table 2).

3.3. Compound *5d* and *5f* do not bind to $GABA_ARs$

The most promising compounds of the series, **5d** and **5f**, were tested for the orthosteric site and BZ binding site of $GABA_A$ receptors however no affinity for $GABA_A$ receptors was observed (see Table S2 of the Supplementary Information file for details).

3.4. Title compounds are predictably druglike and BBB penetrant

Molecular descriptors of the title compounds relevant to orally available druglike chemical space, namely molecular weight (MW), number of rotatable bonds (RB), hydrogen bond donor and acceptor counts (HD and HA), logP, and polar surface area (PSA) were calculated and found to be within the value limits defined for these descriptors, although some compounds were found to exceed the LogP limit defined by Lipinski et al. (see Table S3 of the Supplementary Information file for details).

Table 2
ED_{50} and TD_{50} values of **5f** in mice at 0.5 h regarding 6 Hz test.

Median dose type	Dose (mg/kg)	95 % Confidence interval	Slope	Standard error
ED_{50}	118.92	85.01 – 167.39	4.86	1.54
TD_{50}	241.70	175.22 – 303.28	6.62	2.12

We used several programs and web servers applying different methods to predict whether the title compounds were BBB penetrant or not. Among them QikProp uses forcefield-based algorithms and improves predictions by similarity calculations using normalized Euclidean functional form. SwissADME relies on Monte Carlo optimization of logP and PSA values of a 660-molecule training set to predict BBB permeation. vNN-ADMET uses variable nearest neighbor (vNN) method, while CBligand support vector machine algorithm. LightBBB uses LigthGBM as a machine learning algorithm. According to the results, most of the compounds were predicted BBB permeant by the majority of the utilized methods. **5a** and **5e** were the compounds predicted non-BBB permeant by three methods, while **5g** was predicted BBB permeant by all the methods (see Table S4 of the Supplementary Information file for details).

3.5. Title compounds predictably act through PPAR-α activation

In addition to descriptor calculation, QikProp identifies drug molecules with similar to the query compounds. In our study, QikProp suggested ronifibrate and fenofibrate as similar drugs regarding **5d** and **5f**, respectively, with similarity above 85 %. Fibrates are PPAR-α agonists and clinically used for the treatment of hypercholesterolemia. PPARs are nuclear receptor transcription factors that take part in cellular differentiation and metabolism through regulation of the related genes' expression. In the central nervous system (CNS), PPAR-α regulates neuronal activity through modulation of nicotinic acetylcholine receptors, which are involved in seizure mechanisms. Selective PPAR-α agonists such as fibrates (e.g., fenofibrate) palmitoylethanolamide were reported to display potent antiseizure activities [3,25].

In this respect, we docked the title compounds to PPAR-α active site to predict their affinity and binding modes and optimized the obtained ligand-receptor complexes through MM-GBSA calculations applying sidechain flexibility to the interacting residues. MM-GBSA is a method that provides more precise free binding energy (ΔG) values by combining molecular mechanics and continuum solvent approach [26]. Prior to this, we tested the reliability of the molecular docking method by redocking the co-crystallized agonist (NKS) of the PPAR-α structure used in this study to the ligand binding site. When compared to the original co-crystallized conformation, Glide predicted a highly similar binding mode for NKS (RMSD: 0.29 Å). The title compounds were predicted to show high affinity with docking scores, and especially ΔG values, comparable to that of NKS (see Table S5 of the Supplementary Information file for details).

The ligand binding site of PPAR-α is located in the extranuclear ligand binding domain and surrounded by helices and β-sheet to assume a T shape. A H bonding pattern network made up of H bond interactions with critical residues for functionality of the receptor, including Ser280, Tyr314, His440, and Tyr464, is conserved among the PPAR-α agonists [23,27]. The co-crystallized agonist NKS reportedly makes strong H bonds with these residues via its carboxylate moiety [23], as well as with Thr279 through water and Cys276 (Fig. 2A) The aromatic moieties at both sides of this carboxylate engage in hydrophobic interactions. The predicted binding modes of **5d**, **5f**, and **5g** (Fig. 2B-D, respectively) also feature electrostatic interactions with Ser280, Tyr314, His440. The phenyl or 2,4-dichlorophenyl of these derivatives occupies the lipophilic pocket of the active site just like the 4-methylphenoxy of NKS.

4. Discussion

The title compounds share a common scaffold comprising of imidazole and an aryl ring (phenyl or 2,4-dichlorophenyl) connected by an ethylene linker, to which various aromatic and aliphatic moieties are attached via alcohol or oxime ester functionality. According to the anticonvulsant identification results, the aryl ring (phenyl or 2,4-dichlorophenyl), as well as the type of the ester functionality (alcohol or oxime ester) did not appear to have an effect on the activity, while the moieties attached via ester did. Compounds with 4-phenylbenzoyl were those with the poorest activity, which probably made the compounds too lipophilic as found in the ADMET predictions. The cinnamoyl group, however, led to poor, MES-specific activity. Aromaticity of this group did not have considerable effect on the activity, either. **5d**, **5f**, and **5g** were the most promising compounds in the series. **5f** and **5g** possess a phenyl or cyclohexane ring directly attached to the ester carbonyl, which are of similar steric properties. **5g** has a phenyl ring 3-C away from the ester carbonyl. However, unlike **5b** and **5c**, the alkyl linker of **5g**'s tail bears a carbonyl group putting it in a tolerable lipophilic range. Toxicity was usually parallel to the anticonvulsant activity, i.e., those compounds with better activity profiles also showed toxicity to an extent, with **5f** setting a good exception.

Anticonvulsant quantification was performed for **5f** and its anticonvulsant effect in 6 Hz model peaked at 0.5 h. TD_{50} of **5f** was value more than twice of its ED_{50} value, suggesting that **5f** is safe *in vivo* with a wide therapeutic window.

A number of ligand binding sites have been identified for $GABA_A$Rs. One of them is the orthosteric site, aka GABA recognition site, muscimol site or active site, where agonists and antagonists of $GABA_A$Rs bind. The BZ binding site is known for binding of positive allosteric modulators of $GABA_A$ receptors, such as benzodiazepines. Given that the title compounds share common structural features with loreclezole, we considered that they might display affinity for $GABA_A$ receptors. However, the *in vitro* tests suggested that **5d** and **5f** did not show affinity to orthosteric BZ binding site of $GABA_A$R. Thus, it is plausible that the compounds exert their pharmacological effects through a different mechanism, which will be assessed in our following studies.

The title azoles were predicted druglike indicating a low possibility for ADMET issues during development of such compounds into drug candidates. Among other ADMET properties, ability to penetrate BBB is crucial for CNS-acting drugs. The compounds were predicted mostly BBB-penetrant by a number of *in silico* tools, which use different methods. This finding was partly in agreement with the *in vivo* results showing that the ability of the compounds to prevent seizures might be predominantly related to their ability to penetrate the BBB.

As the title azoles were found similar to some fibrates, which are

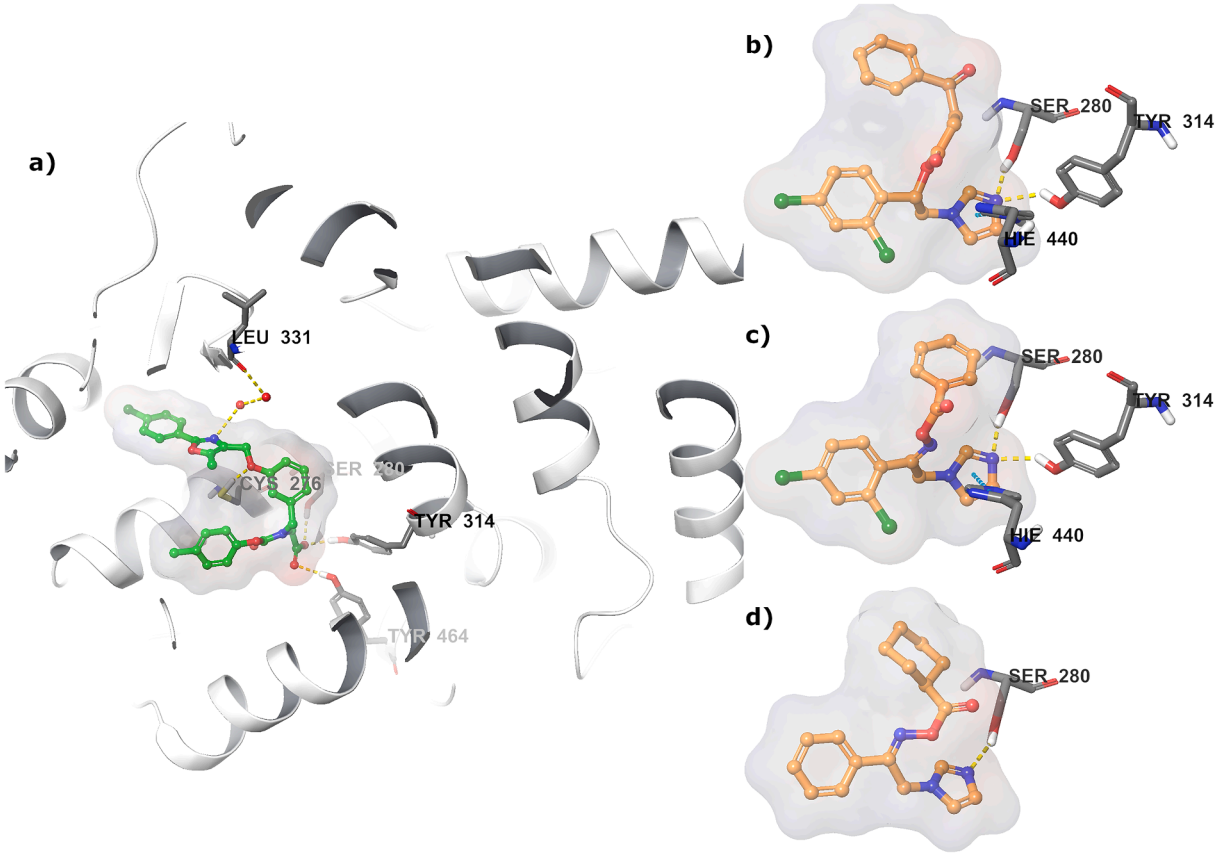

Fig. 2. Co-crystallized binding of KDS, a selective PPAR-α agonist (A), and the predicted binding of **5d** (B), **5f** (C), and **5g** (D) in PPAR-α active site. Ligands are represented as colour ball-and-stick model, amino acid residues as grey sticks, protein backbone as white ribbons and electrostatic interactions as dashed lines. Ligand molecular surface is rendered.

PPAR-α agonists, they were docked to PPAR-α active site. The compounds were predicted to show high affinity the PPAR-α active site interacting with the key residues and assuming very similar binding modes to those of PPAR-α agonists.

5. Conclusions

Taken together, current study identifies a number of azoles with promising anticonvulsant activity. Among these compounds **5f** stood out with dual anti-MES and anti-6 Hz activity as well as negligible toxicity. In vivo quantification studies showed that **5f** had a wide therapeutic range with an ED_{50}/TD_{50} ratio of ~ 0.49. The compounds were predicted to have balanced physicochemical properties placing them in the druglike chemical space. Their anticonvulsant effects were somewhat related to their ability of BBB penetration according to molecular modelling. In vitro studies singled out a possible $GABA_AR$ binding for, however molecular modelling suggested possible PPAR-α inhibition responsible for their anticonvulsant action. These results may help to expand the chemical space of azole derivatives and warrants their further evaluation as potential anticonvulsant agents.

6. Significance statement

Azoles are able to prevent seizures triggered by 6 Hz psychomotor method, a model for therapy-resistant partial seizures, in mice with a wide therapeutic window.

CRediT authorship contribution statement

Suat Sari: Writing – original draft. **Sibel Yurtoğlu:** Writing – original draft. **Merve Zengin:** Writing – original draft. **Monika Marcinkowska:** Writing – original draft. **Agata Siwek:** Writing – review & editing. **Selma Saraç:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2024.137750.

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