REVIEW

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An update of 4-aminopyride as a useful model of generalized seizures for testing antiseizure drugs: *in vitro* and *in vivo* studies

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Aminopyridines constitute a drug family with the ability to enhance synaptic transmission. In particular, 4-aminopyridine (4-AP) has been used as a model of generalized seizures. 4-AP is a K^+ channel blocker, but its mechanism of action has not yet been fully described; some evidence has shown that it acts on the K^+ channel types Kv1.1, Kv1.2, Kv1.4 and Kv4, which are localized in the axonic terminals of pyramidal neurons and interneurons. When 4-AP blocks the K^+ channels it triggers depolarization and prolongs the action potential in the neuron, which causes nonspecific neurotransmitter release. Among these neurotransmitters, glutamate is the principal excitatory neurotransmitter released in the hippocampus. Once glutamate is released, it reaches its ionotropic and metabotropic receptors continuing the neuronal depolarization chain and propagation of hyperexcitability. This brief review is focused on the use of 4-AP as an effective seizure model for testing antiseizure drugs in relevant *in vitro* and *in vivo* studies.

Key words: 4-aminopyridine, antiseizure drugs, hyperexcitability, K^+ channels, seizures

INTRODUCTION

Epilepsy is a neurological disease with a worldwide incidence of 70 million people (Thijs et al., 2019; Löscher et al., 2020). Epilepsy is considered one of the most common neurological disorders with 80% of epilepsy patients living in developing countries (Angus-Leppan, 2008). Approximately five million people with epilepsy live in Latin America, of which more than 3 million remain untreated. In Mexico, an epidemiological study on neurological diseases reported a prevalence of 3.9 cases per 1,000 habitants (Yemadje et al., 2011).

Epilepsy is a recurrent cerebral dysfunction that is characterized by the sustained and synchronous discharge of a group of brain neurons. Temporal lobe epilepsy (TLE) is one of the most frequent and drug-resistant forms of epilepsy, and the hippocampus (HIP) is the most important structure in the generation of discharges in TLE (Ben-Ari, 1985; Lévesque et al., 2012; Löscher et al., 2020); however, there is evidence that other structures also participate, such as the entorhinal cortex (EC) (Bartolomei et al., 2005; Tolner et al., 2005). The EC has extensive reciprocal connections within itself and with the HIP and other brain areas, which makes it a potential candidate for the generation and propagation of discharges in this type of epilepsy (Bartolomei et al., 2005). It has been suggested that the deep layers of the EC are responsible for the onset of epileptiform activity; in this regard, there is evidence of neuronal loss in layer III of the EC, both in patients with TLE and in the animal model of this type of epilepsy (Fountain et al., 1998; Tolner et al., 2005).

4-aminopyridine (4-AP) was produced in the 1960s as an avicide (Avitrol®) and is a K^+ channel blocker that has been used in research for decades due to its ability to generate generalized seizures (Spyker et al., 1980;

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Pasantes-Morales and Arzate, 1981; Tapia and Sitges, 1982; Glover, 1982; Pasantes-Morales et al., 1987; Mihály et al., 1990). The convulsive effect of 4-AP is due to its prolongation of the depolarization phase of action potentials, increasing the neuronal firing rate (Nisenbaum et al., 1994). There is also evidence of the release of various neurotransmitters, primarily glutamate also neurotransmitters such as catecholamines, after the administration of 4-AP in the EC or HIP (Medina Ceja et al., 2000; Mora and Tapia, 2005; Morales-Villagrán et al., 2008a; 2008b; Sadeghnia et al., 2017), in *in vitro* preparations (brain slices, neuromuscular junctions or synaptosomes) and in *in vivo* experiments (Morales-Villagrán and Tapia, 1996; Rothman, 2009; Alpdogan et al., 2020). Additionally, 4-AP increases Ca^{2+} influx and subsequently increases interneuron and neuromuscular synaptic transmission.

This review focuses on the use of 4-AP as a model of seizures to test antiseizure drugs both *in vitro* and *in vivo*. The first section of this review explains the probable mechanism of action of 4-AP. The second section describes the use of 4-AP to induce epileptiform activity in *in vitro* studies to test antiseizure drugs. The third section compiles *in vivo* studies in which 4-AP is used to induce generalized seizures to test antiseizure drugs.

Action mechanism of 4-aminopyridine

The pattern of convulsive seizures induced by 4-AP administration intraperitoneally (i.p.) in the rat is very similar to that produced by the intracerebral (i.c.) administration of kainic acid (Ben-Ari, 1985). Seizures induced by 4-AP are characterized by the appearance of a long seizure period, with an initial stage of hyperexcitation, followed by clonic and tonic-clonic seizures, including death during a tonic seizure in some cases, while the animals that survive have clonic movements frequently alternating with tonic contractions of medium intensity (Fragoso-Veloz et al., 1990). Similarly, 4-AP exerts a seizure effect when administered in the lateral cerebral ventricle (Gandolfo et al., 1989) or into the HIP of rats (Fragoso-Veloz et al., 1990; Medina-Ceja et al., 2000). The seizures induced by 4-AP are associated with neuronal damage in the CA1 and CA3 regions of the HIP and correlate with an increase in the concentration of extracellular glutamate (Medina-Ceja et al., 2008; 2010). 4-AP induces highly rhythmic theta oscillations (6-11 Hz) in CA3 and the EC before the onset of seizures (Lévesque et al., 2012). In turn, deep electrode recordings in rats treated with systemic 4-AP have shown generalized tonic seizures that were associated with epileptiform discharges that occurred in the HIP, amygdala and neocortex (Fragoso-Veloz et al., 1990). The mechanism of action through which 4-AP induces epileptiform activity and seizures has not been described in detail; however, relevant information from *in vitro* studies has revealed some process aspects.

It is known that 4-AP blocks voltage-activated K^+ channels in a wide variety of cells, including neurons and heart muscle, skeletal muscle and smooth muscle cells (Gillespie and Hunter, 1975; Kenyon and Gibbons, 1979; Hara et al., 1980). Electrophysiological studies have shown that 4-AP blocks the transient current of K^+ (I_{to}) and slow inactivation ($l_{to,\ slow}$), which leads to the extension of the action potential through a delay in the repolarization phase, a phenomenon that keeps the voltage-sensitive Ca^{2+} channels open for a long time (Rogawski and Barker, 1983; Armstrong and Loboda, 2001; Boiko et al., 2013), which could explain the increase in neurotransmitter release (Fig. 1). Although the release of neurotransmitters is non-specific, several studies have shown that glutamate is the main excitatory neurotransmitter released (Tapia et al., 1999; Medina-Ceja et al., 2000). 4-AP acts on the cytoplasmic side of the K^+ channels, as the 4-AP is protonated when it passes through the membrane, and the electrostatic potential distribution of the protonated 4-AP shows that an approaching nucleophile will be oriented toward the N-H (protonated) bond. This protonated site interacts with the carboxylic residue by an H bond of the α-subunit that is in the internal part of the K^+ channels (Kirsch et al., 1993; Armstrong and Loboda, 2001; Muñoz-Caro and Niño, 2002), and some studies have postulated that 4-AP preferentially binds to the open state of the K^+ rectifier channels (Kirsch et al., 1993, Armstrong and Loboda, 2001). Moreover, 4-AP increases the Ca^{2+} current (Agoston et al., 1983; Gibson and Manger, 1988; Boiko et al., 2013).

In addition, recent studies have shown that 4-AP blocks voltage-sensitive K^+ channels, specifically those that contain the Kv2.1 and Kv2.2 α-subunits, probably because 4-AP binds to the central part of the channel (Muñoz-Caro and Niño, 2002; Stas et al., 2015; Page et al., 2018); these K^+ channels are expressed in almost all tissues, and the current of these subunits increases when interacting with the Kv5, Kv8 and Kv9 subunits, which are electrically silent (Bocksteins, 2016). Consequently, the interaction of the Kv2.1 and Kv2.2 α-subunits with the Kv5, Kv8 and Kv9 subunits, makes them a more desirable pharmacological and therapeutic target due to their unique biophysical properties. In addition, 4-AP, in a single dose of 10 mg in mouse and human tissues, potentiated Kv6.4 currents in the K^+ channels (Bocksteins et al., 2014; Stas et al., 2015; Taranto-Montemurro et al., 2017).

4-aminopyridine as a model to induce epileptiform activity to test antiseizure drugs: *In vitro* studies

4-AP has been used in numerous *in vitro* studies as a model of the induction of epileptiform activity, as shown in HIP and cortex slice experiments (Tapia et al., 1999, Mikroulis et al., 2018; Alcantara-González et al., 2019; Chen et al., 2020). *In vitro* studies have shown that nonspecific release of neurotransmitters occurs, independent of the mediating substance, the type of synapse or species (Thesleff, 1980; Chen et al., 2019; 2020). In a recent study, 4-AP (1 mmol/L) was used to block other voltage-activated channels – such as ATP channels (sensitive to glibenclamide), the K^+ channel activated by calcium (sensitive to clotrimazole) and the internal rectifier K^+ channel (Kir) (sensitive to $BaCl_2$) – in order to include most types of K^+ channels and study the antihypertensive effect of this drug, as well as its relaxing effect on rat organ tissue vessels (Chen et al., 2019).

The 4-AP model is being used in studies to assess the antiseizure properties of different drugs (Table 1), such as lacosamide, zonisamide and levetiracetam (Heuzeroth et al., 2019). In addition, the number of generalized seizures induced by 4-AP was increased with amyloid-beta, and these pro-epileptogenic effects were related to a reduction in synaptic coupling in Schaffer collateral synapses in CA1 (Yamamoto et al., 2011; Alcantara-González et al., 2019).

4-amynopiridine as a model of generalized seizures to test antiseizure drugs: *In vivo* studies

4-AP induced seizures in different animal species when administered i.p. or i.c. (Spyker et al., 1980; Pas-

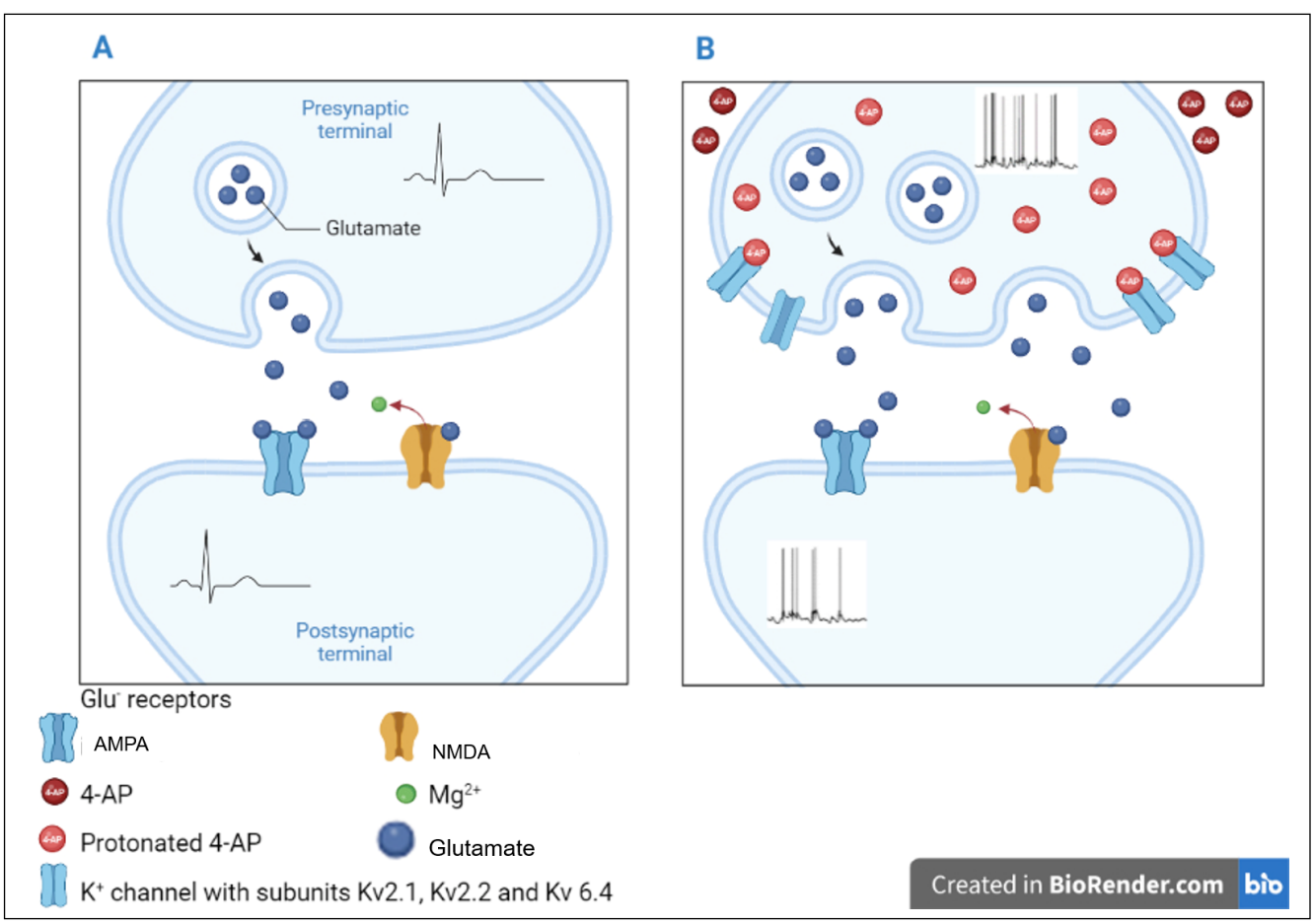

Fig. 1. (A) Under physiological conditions, electrical stimulation of the presynaptic terminal triggers a glutamate release and activation of ionotropic glutamate receptors AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid). This activation produces depolarization and releases Mg^{2+} blocking from the NMDA receptors (N-methyl-D-aspartate). (B) When 4-AP is administered, it crosses the neuronal membrane, then it is protonated in the cytoplasm and blocks K^+ channels, delaying the repolarization phase and maintaining depolarization for a period of time, which leads to the release of neurotransmitters, particularly glutamate. This neurotransmitter activates ionotropic and metabotropic postsynaptic receptors that facilitate hyperexcitability and, subsequently, epileptiform activity.

Table 1. *In vitro* studies using the 4-AP model to assess the antiseizure effects of different drugs.

4-AP doses	Treatment protocol	Main results	References
100 μM	Human hippocampal slices resected from patients suffering from intractable mesial temporal lobe epilepsy; EEG recordings in different subregions of the hippocampus in the presence of high-potassium (8 mM) / low-magnesium (0.25 mM).	Propagation of epileptiform activity throughout the intrinsic circuitry of the hippocampus. Provided insights into seizure control and prevention and a platform to develop novel, antiseizure therapeutics.	Hsiao et al., 2015
2.5 mM	Transverse hippocampal brain slices from mice (4–7 weeks old). Patch-clamp recording was performed using borosilicate glass electrodes (4–7 MΩ). Carisbamate (300 μM) was tested.	Carisbamate blocked epileptiform discharges.	Kim et al., 2017
50 μM	Brain slices from Sprague-Dawley rats (8–16 days). Voltage-activated calcium current was recorded in CA-3, whole-cell voltage-clamp mode in the presence of SR95531 (10 μM), DNQX (10 μM), AP-5 (50 μM) and fructose-1,6-bisphosphate (F1, 6BP, 2.5-5 mM).	Bath application of F1,6BP blocked epileptiform population bursts, but F1,6BP did not block spontaneous intrinsic firing of the CA3 neurons when synaptic transmission was eliminated with DNQX, AP-5 and SR95531.	Shao et al., 2018
1-100 μM	Hippocampal tissue slices were acutely isolated from Sprague-Dawley rats (2–4 months) and nonhuman primates. Extracellular recordings with an electrode placed within the CA1 in the *stratum pyramidale*. Diazepam (10–500 μM) and lidocaine (1–500 μM).	With both drugs, population spike activation was repressed at high concentrations. Population spikes were evoked through the stimulation of the CA3 Schaffer collateral pathway.	Accardi et al., 2018
50 μmol/L	Brain slices from C57Bl6 mice (4–6 weeks old). Patch-clamp recordings from layer 2/3 pyramidal neurons. Slices were treated with sulfasalazine (250 μmol/L).	In voltage-sensitive dye (VSD) recordings, sulfasalazine decreased VSD optical signals induced by 4-AP.	Alcoreza et al., 2019
100 μM	Brain slices (hippocampal-entorhinal cortex) from Wistar rats; Extracellular local field potential recordings with a glass electrode (150 mM NaCl, electrode resistance 1–2 MΩ). Lacosamide (10, 33 and 100 μM), zonisamide (33, 100 and 300 μM) and levetiracetam (33, 100 and 300 μM).	Lacosamide (100 μM) and zonisamide (300 μM) decreased seizure-like events, and the effect of levetiracetam was markedly reduced.	Heuzeroth et al., 2019
100 μM/L	Subicular neurons of acute hippocampal slices from rat. Patch clamp recordings. Lactate (6 mmol/L).	Lactate reduced the spike frequency and hyperpolarized the subicular neurons.	Jorwal and Sikdar, 2019
10 μmol/L	Slices of immature C57Bl/6 mouse (postnatal days 4–7) hippocampus. Field potential recordings. Muscimol at 0.1, 0.5 and 5 μmol/L; taurine at 0.1 and 0.5 mmol/L.	Taurine at concentrations between 0.1 and 0.5 mmol/L induced a proconvulsive effect, but upon co-application significantly augmented the anticonvulsive effect of moderate muscimol doses (0.5–1 μmol/L).	Winkle et al., 2019.
0.1 mM	Brain slices from Wistar rats (3-week-old) treated with low-frequency electrical stimulation and the blockade of GABAB. Paired-pulse test and stimulation with bipolar electrode in the radial layer of CA1.	Low-frequency electrical stimulation increased the interval between ictal discharges in the entorhinal cortex. Under the blockade of GABA B receptors, low-frequency electrical stimulation became entirely ineffective, indicating that the activation of GABA B receptors underlies the main low-frequency electrical stimulation antiepileptic effect.	Smirnova et al., 2020
100 μM	Brain slices from rats (3 week-old). Action potentials were evoked using rectangular current steps of 300 pA lasting 2 or 50 s. Treated with capsaicin 2 μM and 60 μM.	Capsaicin abolished long ictal events evoked in zero magnesium solution containing 4-AP.	Pasierski and Szulczyk, 2020
100 μM	Brain slices were prepared from 12- to 16-day-old juvenile Sprague-Dawley rats. A microelectrode (1–2 M) filled with 3 M NaCl was placed caudal to the CA1 region of the hippocampus and deep into the layers (V/VI) of the visual cortex. Treated with oil from *Melissa officinalis* 0.1 mg/ml.	Oil from *M. officinalis* (lemon balm) reversibly blocked spontaneous ictal-like discharges and secondary spikes from sustained repetitive firing, suggesting anticonvulsant effects and voltage-gated sodium channel blockade.	Chindo et al., 2021
2 mM	Brain slices from Wistar rats (20 to 30 day-old). Membrane currents and voltages were measured with the single-electrode patch-clamp technique. Treated with carbenoxolone 300 μM.	Carbenoxolone reduced the epileptiform activity induced by 4-AP in the medium of hippocampal slices.	Volnova et al., 2022

Table 2. *In vivo* studies using the 4-AP model to assess the antiseizure effects of different drugs.

4-AP Doses	Treatment protocol	Main results	References
500 µM Intra-hippocampal	Male Wistar rats (250–300 g). Standard Krebs Ringer HEPES with levetiracetam (250 µM), carbamazepine (250 µM), phenytoin (250 µM) or valproate (250 µM). Standard Krebs Ringer HEPES with lamotrigine (250 µM), oxcarbazepine (250 µM), topiramate (250 µM), vinpocetine (25 µM) and/or sertraline (25 µM).	Carbamazepine, phenytoin, lamotrigine and oxcarbazepine inhibited the increase in Ca2+ concentration induced by 4-AP. Topiramate was unable to inhibit the Ca2+ response to 4-AP. The course of the rise in Ca2+ induced by 4-AP depolarization was also unchanged by valproate, topiramate or levetiracetam at high concentrations. In contrast, the exposure of the 4-AP-predepolarized nerve endings to 25 µM vinpocetine returned the Ca2+ concentration level to baseline values.	Sitges et al., 2015
12 mg/kg i.p.	ICR mice received *Pseudospondias microcarpa* extract (30, 100 or 300 mg kg, p.o.), vehicle or the standard drug carbamazepine (30, 100 or 300 mg kg, p.o.). One hour after drug administration, animals were treated with a single injection of 4-AP.	Pretreatment of animals with *Pseudospondias microcarpa* extract caused a significant delay in the latency to both clonic and tonic seizures. Carbamazepine produced effects analogous to the extract in the 4-AP-induced seizures, but the effects increased with increasing dose.	Adongo et al., 2017
15 mg/kg i.p.	Wistar rats treated with saline or berberine (50, 100 and 200 mg/kg, i.p.) 40 min before 4-AP administration.	Berberine attenuated seizures, decreasing hippocampal aspartate and glutamate release in rats.	Sadeghnia et al., 2017
3 mg/kg i.p.	Adult male rats. Short-term evaluation of fluoxetine (10 mg/kg, for 7 days).	Fluoxetine increased the latency of seizures and reduced the markers of hippocampal damage induced by 4-AP.	Shiha et al., 2017
5 mM i.p.	2-deoxy-glucose solution (200 mg/kg) was injected in mouse 15 min after the first seizure.	The duration and amplitude of seizures were reduced; however, epileptiform activity was still present one hour after 2-deoxy-glucose administration.	Bazzigaluppi et al., 2017
10 mg/kg i.p.	Two groups of Wistar rats were fed with a ketogenic diet and a normal diet for 35 days to determine the antiepileptic effect on acute epileptic models.	The ketogenic diet increased animal resistance to induced seizures by 4-AP and showed higher latency and shorter seizures than the normal diet after proconvulsive induction.	Sanya et al., 2017
10 mg/kg i.p.	ICR mice were randomized into seven groups and pretreated with *Psydrax subcordata* extract (30, 100 or 300 mg/kg) or vehicle (normal saline; 10 ml/kg, p.o.). Thirty minutes (i.p.) or 1 h (p.o.) after the treatments, mice were injected with 4-AP.	*P. subcordata* extract pretreatment significantly delayed the onset of seizures and improved survival.	Daanaa et al., 2018
7 µg/5 µl Intra-hippocampal	Pretreatment with metyrapone (150 mg/kg, i.p.) in male adult Sprague-Dawley rats weighting 373.6±5.8 g (85–90 days old).	Metyrapone reduced ictal hypermetabolism, as well as all markers of brain damage, except for microglia-mediated neuroinflammation.	Garcia-Garcia et al., 2018
10 mg/kg Subcutaneous	Thalidomide (100, 200 and 400 mg/kg) was administered in male CD1 mice (25-30 g) one hour before 4-AP.	Thalidomide did not elicit an anticonvulsant effect in mice at any of the doses. However, thalidomide at 200 mg/kg produced a significantly greater latency to convulsions and a shorter total duration of convulsions; the protection was marginal.	Islas-Espinoza et al., 2018
10 mg/kg	The neuropharmacological activity was tested with the open field test and elevated plus maze in mice with acute convulsions with 4-AP, ortho and para bis-isoindoline-1,3-dione, phthaloylglycine (nonchiral) (562.3 mg/kg) and N-substituted with aspartate or glutamate (chiral) (316 mg/kg).	The activity of chiral phthalimide molecules N-substituted with aspartate or glutamate (S-TGLU, S-TASP and R-TASP) lowered the convulsion and death rate.	Campos-Rodríguez et al., 2019
13 mg/kg i.p.	Thirty minutes after treatment with rosmarinic acid (8 and 16 mg/kg), male mice (30–40 g, 2–3 months old) received injections of 4-AP.	Rosmaniric acid could not prevent seizures, nor did it enhance the latency time to the first seizure at the tested doses.	Luft et al., 2019
150 µM	Focal *status epilepticus* was induced by unilateral application of a pledget soaked with 4-AP and GABAzine to the dura overlying the sensorimotor cortex in FVB mice. Two hours after the behavioral seizure activity began, the pledget was removed, the cortex was washed with saline solution, and diazepam was administered (i.p. 5–10 mg/kg).	Diazepam completely suppressed behavioral seizures in 8 animals within 5 to 10 min, while contralateral behavioral jerking persisted in the other 78 mice for up to 2.5 h after the diazepam injection.	Perez-Ramirez et al., 2020
15 mg/kg i.p.	*Canarium schweinfurthii* (0.01, 0.03, 0.1, 0.3, 1, 1.2, 3, 6, 10, 11.9, 30, 60, 100, 150, 200, and 300 mg/kg, p.o.); phenobarbital (0.01, 0.03, 0, 1, 0.3, 1, 3, 10, 25, 30, 60, 100, 120, 160, 300, and 500 mg/kg, i.p.). One hour later, the voltage-gated K+ and Ca2+ channel blocker 4-AP was administered in male Swiss albino mice (19–29 g, 37–48 days of age). Behavior was observed for 30 min for the appearance of motor seizures (tonic and clonic seizures).	*C. schweinfurthii* protected 50% of mice against the tonic-clonic seizures induced by 4-AP.	Kandeda et al., 2021
25 mM, 2 µL intracortical (Group 1) 50 mM, 4 µL intracortical (Group 2)	Intracortical saline or carbenoxolone administration 30 min before 4-AP in rats.	Rats from Group 1 showed more severe forms of epileptiform activity than rats from Group 2.	Volnova et al., 2022

4-Aminopyridine (4-AP), intraperitoneal (i.p.), per oral (p.o.), Institute of Cancer Research (ICR), Friend Virus B (FVB). *ICR: Institute of Cancer Research. Produced in 1947 by Hauschka at the Institute of Cancer Research, Fox Chase. Derived from Swiss mice of the Rockefeller Institute and now widely distributed (Chia 2005 and Rice, 1980).

ante-Morales and Arzate, 1981; Tapia and Sitges, 1982; Glover, 1982; Pasantes-Morales et al., 1987; Mihaly et al., 1990; Medina-Ceja et al., 2010, Salam et al., 2017; Wang et al., 2018). Rats receiving injections of 4-AP into the substantia nigra, HIP, cerebral ventricles and cerebral cortex showed seizures, as demonstrated by electroencephalogram (EEG) recordings from these animals (Tapia et al., 1999; Medina-Ceja et al., 2015; Guo et al., 2016; Myers et al., 2018; Liou et al., 2018). In addition, 4-AP produced ictal events *in vivo* in mouse and human tissues (Chang et al., 2019).

The 4-AP model of seizures has been used to study possible neuroprotective and antiseizure treatments with different drugs; a summary of these studies is shown in Table 2 (Sitges et al., 2015; Sitges et al., 2016; Shiha et al., 2017; Bazzigaluppi et al., 2017; García-García et al., 2018).

CONCLUSION

4-AP is a convulsive drug that has been used for more than 50 years that is known primarily for its capacity to induce seizures *in vitro* and *in vivo*. This model helps researchers study different antiseizure drugs and to examine the best dosage for reducing or eliminating seizures. Thanks to these studies, we know more about the properties of some promising antiseizure drugs, and new possibilities for seizure treatment have been opened.

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