Short Communication

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Future Medicinal Chemistry

Synthesis and biological evaluation of new antiseizure compounds derived from valproic acid

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Background: New hybrid compounds were synthesized by linking the valproic acid (VPA) structure with other anticonvulsant/anti-inflammatory scaffolds. **Materials & methods:** The chemistry involved the incorporation of the linker oxymethyl ester into VPA, followed by reaction with the second scaffold. The antiseizure effects were investigated by the maximal electroshock seizure test, and the most active compound was additionally evaluated in the 6 Hz test and pentylenetetrazol test in mice. **Results:** The compounds showed protection against seizures. The hybrid structure with the butylparaben scaffold exhibited an ED_{50} of 8.265 mg/kg (0.0236 mmol/Kg) in the maximal electroshock seizure test and 50.00 mg/kg (0.147 mmol/kg) in the 6 Hz test. **Conclusion:** The antiseizure activity of the synthesized compounds highlighted the potential of hybrid structures to treat multifactorial diseases such as epilepsy.

Plain language summary: This article focuses on the design of new anticonvulsant compounds that combine the chemical structure of valproic acid with other interesting scaffolds with anticonvulsant or anti-inflammatory properties. These compounds protected against *in vivo* acute seizure models (mice). The results revealed the capacity of combining known scaffolds into a single structure to generate new active compounds with multitarget purposes.

Tweetable abstract: The article focuses on the design and biological evaluation of new anticonvulsant compounds that share the chemical structure of valproic acid linked to other interesting scaffolds with anticonvulsant or anti-inflammatory properties.

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Molecular hybridization is an interesting approach used in drug design that consists of combining different pharmacophoric scaffolds (from different active structures) into a single molecule [1]. Hybrid compounds are expected to preserve the original activities of the parent structures but to have distinct, and hopefully better, pharmacokinetic/pharmacodynamic characteristics (e.g., additive or synergistic activity) and toxicity profiles [1,2]. The design of hybrid compounds usually involves the combination of scaffolds with different pharmacological profiles to promote simultaneous interactions with distinct molecular targets. This goal can also be achieved with the use of rational polytherapy (also known as polypharmacy or combination therapies), but the hybrid approach has the advantage of reducing the probability of drug–drug interactions and increasing patient compliance [3].

The hybridization strategy is particularly valuable for designing active compounds to treat complex multifactorial diseases because it allows the construction of unique structures that can act through multiple mechanisms of action

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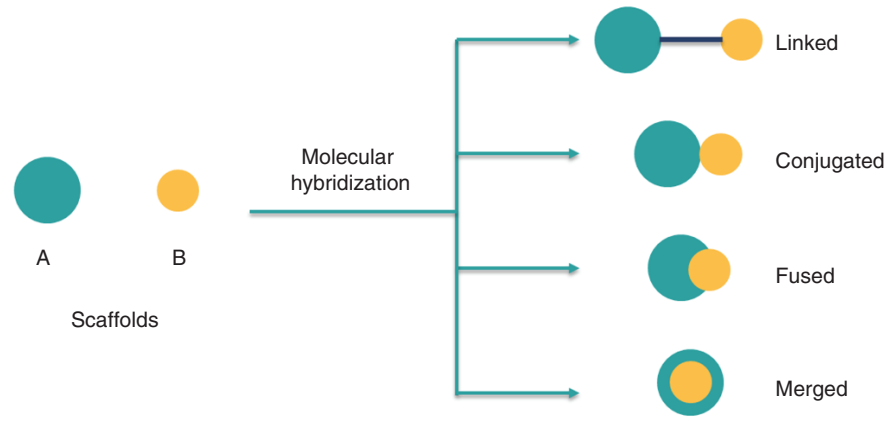

Figure 1. Rational design of hybrid structures from two generic scaffolds, A & B. Molecular hybridization by linking two active scaffolds through a linker group or with different levels of overlap using common functionalities present in pharmacophores.

(MOA) [4,5]. Epilepsies have been included in this category due to their multifactorial origins that may involve a complex interplay between several genetic and environmental factors implied in the etiology of the disease [6].

Antiseizure medications (ASMs) are the primary therapeutic options for treating epilepsy. The development of most ASMs started with the phenotype-based screening of new chemical entities, so their MOAs were investigated after the determination of the antiseizure activity [7]; however, the target-focused approach has also been implemented, as in the cases of brivaracetam, perampanel and other approved ASMs and drug candidates [8–10]. Four "traditional" general MOAs have been defined for ASMs: interaction with voltage-gated ion channels such as certain potassium, sodium or calcium channels; enhancement of inhibition mediated by GABA through interaction with its receptors (GABAA), transporters (GAT1) or catabolic enzymes (GABA transaminase); modulation of synaptic release by binding to SV2A and the α2δ subunit of voltage-gated calcium channels; and prevention of synaptic excitation by inhibition of ionotropic glutamate receptors. The subsequent study of the MOAs also showed that drugs usually act through more than one target, and even other "nontraditional" mechanisms appear to explain, at least in part, their capacity to prevent seizures [7,11,12].

Currently available ASMs are effective in approximately 70% of patients [13]. Reducing the number of pharmacoresistant patients is one of the main challenges faced by the scientific community since this number has not changed over the last few decades despite the persistent introduction of novel ASMs to the market. The multitarget characteristics of many current ASMs support the idea of designing new active compounds with more than one MOA using the molecular hybridization approach.

In terms of chemical structures, hybrid molecules can be classified according to their degree of overlap (Figure 1) [14]: pharmacophores can be integrated (to different degrees) to obtain a new compound that shares the essential molecular features required to interact with two or more pharmacological targets, or they can be combined without interception using a bridging group (linker). Both types of hybrids have been explored to construct structures with antiseizure activity [7,14]. Herein, the use of a linker-based approach for the design of new hybrid derivatives of valproic acid (VPA) is reported. Discovered about 60 years ago, VPA remains one of the most widely prescribed ASMs [15]. It elicits antiseizure activity by interacting with sodium and calcium channels, and possibly with glutamic acid decarboxylase and NMDA receptors [6]. It is worth mentioning that a significant number of compounds have been designed based on the VPA structure with the aim of improving efficacy and reducing serious adverse effects, such as hepatotoxicity and teratogenicity. Structural modifications to VPA include cyclization, conjugation or branching of the VPA hydrocarbon chains, as well as the inclusion of heteroatoms, formation of chiral centers and/or bioisosteric replacement of the acid group by amide [16]. However, these compounds are the result of modifications to the VPA structure and do not include another active scaffold, as proposed in this investigation. To the best of our knowledge, only one structure designed as a combination of VPA connected through a linker group with another anticonvulsant scaffold (phenytoin) has been reported in the literature [17].

The selection of the second scaffold was based on different criteria, in addition to chemical compatibility. The structures of the synthesized molecules are shown in Figure 2. Some of the newly designed compounds (1–4)

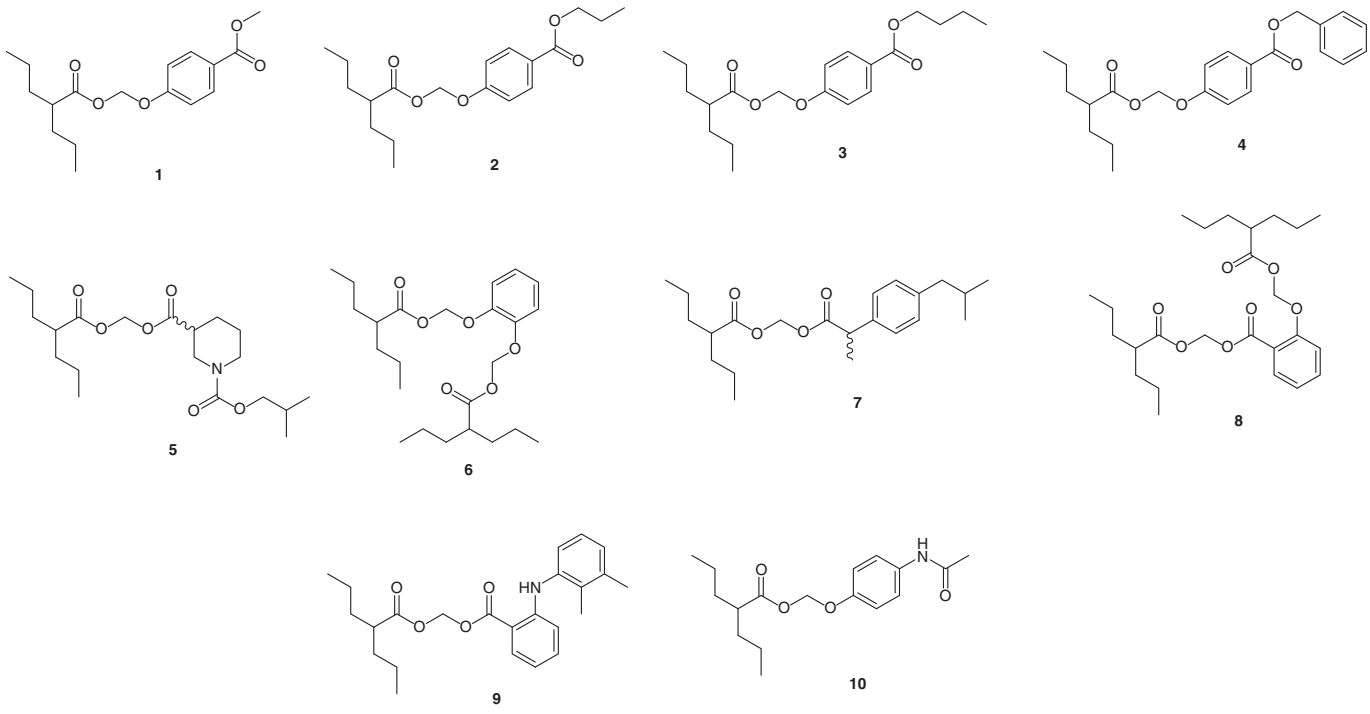

Figure 2. Synthesized compounds.

bridge VPA with members of the paraben family, which are esters of p-hydroxybenzoic acid that are widely used as preservatives in industrial, food, medicinal and cosmetic products. These compounds have shown use-dependent blockade of sodium channels *in vitro* [18] and neuroprotective effects in pilocarpine-induced status epilepticus [19]. Hybrid 5, in contrast, resulted from the combination of VPA and nipecotic acid (NPA). *In vitro* experiments demonstrated that NPA behaves as a potent inhibitor of neuronal and glial GABA uptake [20]. However, due to its hydrophilic nature, it cannot easily cross the blood–brain barrier (BBB) after peripheral administration, limiting its prospects as an ASM [21]. An estimation of logP values using the ALOGPS program yielded -2.36 for NPA and 3.89 for compound 5 [22]. A hybrid compound that includes VPA and an N-protected derivative of NPA may improve BBB permeation by increasing the lipophilicity of the final compound.

Hybrids 6–9 comprise the combination of VPA with structures with anti-inflammatory properties such as catechol, ibuprofen, salicylic acid and mefenamic acid. Epileptic seizures are usually followed by brain tissue inflammation and an increase of inflammatory mediators, which can cause brain damage and predispose patients to new seizures [23,24]. Some authors have suggested the coadministration of anti-inflammatory drugs with ASMs to prevent epileptogenic processes and drug resistance mechanisms [25]. Additionally, some of the chosen anti-inflammatory agents have shown anticonvulsant activity in models of seizures, or neuroprotective properties [26,27]. Finally, paracetamol was included as a scaffold (compound 10), which has shown intrinsic anticonvulsant effects both unchanged and through active metabolites [28–30].

Materials & methods

Drugs

Chemically, VPA has a monofunctional structure that can incorporate linker groups through its carboxylic function. Therefore, the chloroalkyl ester of VPA (Figure 3A) was constructed through the reaction of the acyl chloride derivative of VPA and formaldehyde in the presence of $ZnCl_2$. For most hybrid compounds, the leaving group of the subsequent reaction step was improved by substitution with NaI, to yield the iodoalkyl derivative [31]. The haloalkyl moiety reacts with the second scaffold, which has an acidic function (carboxylic or phenolic group), to obtain the final hybrid compound (Figure 3B). All commercially available reagents were purchased from Sigma-Aldrich (Buenos Aires, Argentina). Amines and solvents were purified prior to use. The synthesized compounds

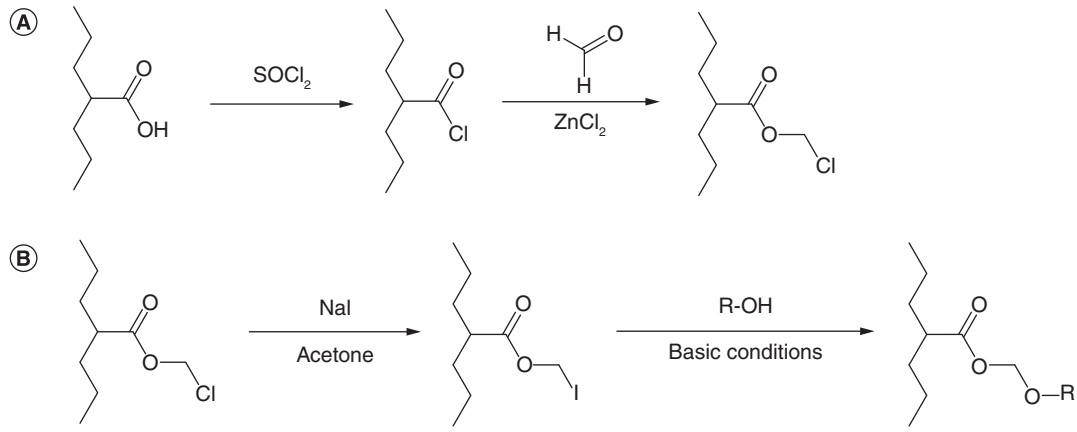

Figure 3. Synthesis of valproic acid hybrids. R-OH represents a scaffold with an acidic group such as phenol or carboxylic acid. **(A)** Synthesis of chloroalkyl ester of VPA. **(B)** Reaction of the haloalkyl derivative with the acidic groups of the second scaffold (carboxylic or phenolic group)

were characterized by ^{1}H NMR, ^{13}C NMR and mass spectrometry. Specific details of the synthetic reactions and spectral data are provided in the Supplementary Materials.

Evaluation of anticonvulsant activity

Animals & drugs

The anticonvulsant activity of the candidates was assessed according to standard protocols provided by the Epilepsy Therapy Screening Program (ETSP) at the National Institute of Neurological Disorders and Stroke [32]. Adult, male, specific-pathogen-free, Swiss albino mice (8 weeks old) provided by the Faculty of Veterinary Medicine, National University of La Plata, Argentina, weighing 20–25 g were used. Following the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines) [33], the list of pathogens from which the animals were declared free, is provided in the Supplementary Materials (list of specific pathogens section). All animals were housed in groups of five in a temperature- and light-controlled environment (12 h light/dark). They were allowed water and food *ad libitum* and placed in cages with an enriched environment (nesting materials and shelters) to reduce anxiety and stress. Physiological acclimatization was performed for 3 days; afterward, animals received 0.1 ml intraperitoneal (ip.) administration of saline once a day for 5 days. All experiments were conducted using naive animals during the light phase. The animal care for all experimental protocols was conducted in accordance with the Ethical Committee of the Faculty of Exact Sciences of the University of La Plata, Argentina. The mice were treated with fresh drug solutions dissolved in DMSO (2 ml/kg, BIOPACK). The administration volume was about 7× lower than the LD_{50} value of DMSO for a single administration [34–36]. Control mice received the vehicle (ip. 2 ml/kg 0.5 h before electrical or chemical stimulus) or the standard anticonvulsant drug selected depending on the test. After each experiment, all animals were euthanized by CO_2 inhalation. Efforts were made to minimize the total number of animals used and their discomfort or suffering. Female experimenters performed all the tests involving laboratory animals.

Maximal electroshock seizure test

Maximal electroshock seizure (MES) tests were carried out as follows: MESs were provoked by delivering a 60 Hz/50 mA electrical stimulus for 0.2 s *via* ear clip electrodes using UGO Basile equipment. To ensure adequate electrical contact, a drop of neutral gel was applied to each ear before electrode placement. Under these conditions, maximal seizures occur in almost all healthy mice. Seizures in the MES test are characterized by a short period of tonic flexion, followed by a longer period of tonic extension of the hind limbs and, finally, a clonic episode [37]. Blockade of the tonic extension of the hind limbs due to drug treatment is taken as the protection criterion. The positive control group received the standard anticonvulsant drug phenytoin (FLUKA; 15 mg/kg ip., 2 h before electrical stimulus). Initially, the anticonvulsant profile of the candidates was evaluated by treating groups of at least two animals per dose (30 or 100 mg/kg body weight) and testing them at 0.5 or 4 h against the MES test. The number of animals tested for the most promising candidates (compounds with full protection at the lower

dose evaluated) was then increased. In addition, quantitative studies were conducted using the MES test. The time of peak effect (TPE) was determined by administering the dose with the best activity in the anticonvulsant profile to five groups of four animals and testing the antiseizure activity at different times. The ED_{50} was determined by treating groups of five mice with different doses at the TPE. More information regarding the TPE and ED_{50} calculations is provided in Supplementary Tables 1 & 2, respectively.

Pentylenetetrazole test

Seizures were chemically induced by subcutaneous (sc.) administration of a fresh 80 mg/kg solution of pentylenetetrazole (PTZ; Siegfried) in 0.9% saline to the midline of the neck in a loose fold of skin, at a 5 ml/kg volume. After administration, the animals were placed into individual observation boxes and their behavior was evaluated for 30 min. Animals without partial or generalized clonic seizures were considered protected against seizures. The control group received the standard drug, phenobarbital (Saporiti; 25 mg/kg ip. 0.5 h before stimulus).

6 Hz test

A low-frequency, long-duration electrical stimulus (6 Hz, 44 mA, 3.0 s [38]) was induced by corneal stimulation, using a UGO Basile pulse generator (ECT 5700, Comerio, Italy). Before discharge, the electrode was wetted with 0.9% saline to provide good electrical conductivity. One drop of 0.5% tetracaine was applied as ocular anesthesia to the eyes of all animals 15 min prior to stimulation. During the current application, animals were manually restrained and, immediately after, released to an observation plexiglass box. Untreated animals exhibit a 60–120 s psychomotor seizure characterized by a stunned stance with rearing and automated behavior, after which they resume exploratory behavior. Seizure protection was considered if normal behavior resumed within 30 s. The control group received levetiracetam (GSK; 30 mg/kg ip. 0.5 h before stimulus). Quantitative studies were conducted using conditions similar to those of the MES test. The calculations of TPE and ED_{50} are provided in Supplementary Tables 3 & 4, respectively.

Rotarod test

The Rotarod test was used to detect motor impairments caused by the tested compound [32]. The test was performed before electrical or chemical stimulation. During the assay, the mice are placed on a horizontal fluted rod, which rotates around its longitudinal axis at 6 rpm, and the ability of the animals to walk forward, stay upright and not fall off the rod is measured. The test was conducted a few min before each test of anticonvulsant activity. Drug-induced motor coordination impairment was considered if the animals fell off the rod three consecutive times in 1 min.

Results & discussion

The results of the anticonvulsant assays (MES test) are shown in Table 1. Evidence of antiseizure activity was observed for all tested structures. Compounds 5 and 7 showed that half of animals were protected at the lowest assayed dose (30 mg/kg), with no signs of neurotoxicity. However, both candidates exhibited mortality in mice after the tests. Compounds 1–2, 4 and 8–10 showed protection at the highest dose tested (100 mg/kg) and passed the Rotarod test, but most exhibited mortality at the lowest dose administered. Hybrid 3 protected against seizures in three of the four animals tested (4 h after administration) and no neurotoxicity or mortality was observed during the assays. Compound 6 was not examined in the MES test due to the signs of neurotoxicity detected *a priori* in the Rotarod test (Table 1). Control mice were included prior to each evaluation. In every assay, the negative control group received the vehicle and exhibited no anticonvulsant activity (0/2 protected animals in each evaluation). The positive control showed protection against seizures (2/2 mice in each evaluation).

The preliminary data shown in Table 1 indicate that compound 3 is the most promising candidate. This hybrid structure includes the paraben derivative with the most hydrophobic alkyl chain of the series. Butylparaben has previously shown an interesting profile as a use- and frequency-dependent sodium channel blocker [15]. In contrast, the incorporation of anti-inflammatory scaffolds yielded compounds with some activity in the MES test. It is worth mentioning that the MES test, despite being one of the most widely used preclinical models for the early identification of new anticonvulsant structures, is considered an acute model of seizures, as it is conducted on healthy animals in which the convulsions are artificially elicited. The recently launched ETSP focuses on detecting not only antiseizure drug candidates but also compounds that can inhibit disease progression and/or that can control the pharmaco-resistant phenotype. Therefore, it would be valuable to test the compounds reported here in other animal models of seizure and epilepsy considered by the ETSP, such as the 6 Hz 44 mA test (which is an acute

Table 1. Antiseizure activity against maximal electroshock seizure test expressed as number of animals protected relative to the total number of animals tested.

Compound	Dose (mg/kg)	MES test[†]		Mortality[‡]		Rotarod test[§]	
		0.5 h	4 h	0.5 h	4 h	0.5 h	4 h
1	30	0/2	0/2	0/2	1/2	0/2	0/2
	100	0/2	1/2	0/2	0/2	0/2	0/2
2	30	0/2	0/2	0/2	1/2	0/2	0/2
	100	0/2	1/2	0/2	0/2	0/2	0/2
3	30	0/2	3/4	0/2	0/2	0/2	0/4
	100	2/4	3/4	0/2	0/2	0/4	0/4
4	30	0/2	0/2	1/2	0/2	0/2	0/2
	100	0/2	1/2	0/2	0/2	0/2	0/2
5	30	1/2	1/2	0/2	1/2	0/2	0/2
	100	0/2	1/2	1/2	1/2	0/2	0/2
6	30	NT	NT	–	–	2/2	2/2
	100	NT	NT	–	–	2/2	2/2
7	30	0/2	1/2	0/2	0/2	0/2	0/2
	100	0/2	1/2	1/2	0/2	0/2	0/2
8	30	0/2	0/2	0/2	0/2	0/2	0/2
	100	0/2	2/2	0/2	0/2	0/2	0/2
9	30	0/3	0/3	0/3	1/3	0/3	0/3
	100	1/2	1/2	0/2	0/2	0/2	0/2
10	30	0/4	0/2	1/4	1/2	0/4	0/2
	100	1/2	1/2	0/2	0/2	0/2	0/2

[†]Number of animals protected relative to total number of animals tested.
[‡]Number of animals that died during experiment relative to total number of animals tested.
[§]Number of animals with motor impairments relative to total number of animals tested.
MES: Maximal electroshock seizure; NT: Not tested.

model of focal seizures that shows resistance to many known ASMs) and the corneal kindled seizure test (a chronic model that better mimics the epileptic brain) [32]. This could be of particular relevance for hybrid compounds based on anti-inflammatory agents since the combination of anti-inflammatory and anticonvulsant activities may interrupt the negative cycle of seizures and brain inflammation and damage. Therefore, it would also be valuable to test the analgesic/anti-inflammatory activity of these molecules. These studies are expected to be conducted in the future.

Quantitative studies in the MES test were conducted for structure 3. The TPE was determined at 6 h. Then, the method of Litchfield and Wilcoxon was used to compute the ED_{50} value [30]. The calculated value was 8.265 mg/kg (95% CI: 0.0201–33.86 mg/kg), which is equivalent to 0.0236 mmol/kg. The comparison with classical antiseizure drugs with high potency against the MES test, such as phenytoin (5.6 mg/kg; 0.0222 mmol/kg), lamotrigine (7.5 mg/kg; 0.0293 mmol/kg) and carbamazepine (7.8 mg/kg; 0.0296 mmol/g) [39] yielded promising results since the potency of the candidate was comparable to that of approved drugs in the same animal model. The calculations of TPE and ED_{50} are provided in Supplementary Tables 1 & 2, respectively.

Based on the activity profiles observed in the MES test, compound 3 was selected for further analysis. Table 2 summarizes the anticonvulsant profile of the candidate against the 6 Hz (44 mA) test and PTZ test. This last chemically induced model of seizures is not included in the current identification phase of the ETSP, but it is a routinely performed adjunctive assay based on the chemical induction of seizures. Moreover, VPA shows better anticonvulsant potency against this assay than in the MES or 6 Hz (44 mA) tests [39].

Compound 3 showed no anticonvulsant activity against PTZ-induced seizures. Conversely, this structure has the ability to protect animals against convulsions elicited by the 6 Hz (44 mA) test, with 2/2 mice protected at the lowest dose 4 h after administration. No signs of motor impairment were observed after drug administration and mouse mortality was detected during the PTZ tests. The quantitative studies for hybrid 3 in the 6 Hz test yielded an ED_{50} value of 50.00 mg/kg (95% CI: 47.87–51.68 mg/kg; TPE: 5 h), which is equivalent to 0.147 mmol/kg. Interestingly, compound 3 showed better potency than the standard drug levetiracetam (ED_{50}

Table 2. Antiseizure activity of compound 3 in 6 Hz 44 mA and pentylenetetrazole tests.

Test	Dose (mg/kg)	Activity[†]		Mortality[‡]		Rotarod test[§]	
		0.5 h	4 h	0.5 h	4 h	0.5 h	4 h
6 Hz test	30	0/2	2/2	0/2	0/2	0/2	0/2
	100	2/2	2/2	0/2	0/2	0/2	0/2
PTZ test	30	0/2	0/2	1/2	0/2	0/2	0/2
	100	0/2	0/2	1/2	0/2	0/2	0/2

[†]Number of animals protected relative to total number of animals tested.
[‡]Number of animals that died during experiment relative to total number of animals tested.
[§]Number of animals with motor impairments relative to total number of animals tested.
PTZ: Pentylenetetrazole.

1089 mg/kg; 6.40 mmol/kg) and VPA (ED_{50} 310 mg/kg; 2.15 mmol/kg) [39]. The calculations of TPE and ED_{50} are provided in Supplementary Tables 3 & 4, respectively.

The results presented in this investigation allowed us to identify a different biological profile for compound 3 compared with VPA. This hybrid lacks activity in the PTZ test (at the time and doses tested) but shows important potency against MES and 6 Hz [44 mA] assays. In contrast, VPA exhibits higher antiseizure activity against the PTZ test than the electrically induced assays mentioned before (ED_{50} 310 mg/kg; 263 mg/kg and 220 mg/kg for 6 Hz [44 mA], MES and PTZ test, respectively [39]).

Another important factor to consider is the metabolic stability of 3 as well as the rest of the tested compounds. We used a random forest-based model available in PredMS server [40] to predict the stability of the candidates in human liver microsomes, and all were predicted as unstable (Supplementary Table 5). While microsomal instability is a wide concept, as the microsomal fraction includes many key drug biotransformation enzymes (e.g., oxidoreductases, flavin-containing monooxygenases, monoamine oxidases, carboxylesterases and glucuronosyltransferase), the presence of the acyloxy moiety in the linker of compounds 1–10 suggests that they could be hydrolyzed by hepatic esterases, at least partially, before reaching the active site(s), thus acting as codrugs. Further predictions using pKCSM [41] suggested that one of the major biotransformation pathways for compounds 1–9 would be metabolism *via* CYP3A4 (results not shown).

Regarding the MOA for structure 3, we think it is premature to propose a mode of action with the available information. Sodium channels could be one of the possible molecular targets, considering the predictions about the ability of 3 to release butylparaben (which was shown to interact with these macrostructures [18]). However, the differences found between hybrid 3 and VPA in the *in vivo* profile (especially in the PTZ test) lead us to infer a more complex MOA than the mere interaction of the two scaffolds with their corresponding targets, and the intrinsic activity of the intact hybrid can not be ruled out at this moment.

Conclusion

In this study, VPA was selected as a common scaffold for the synthesis of hybrid compounds with antiseizure activity. A linker-based approach was used for the construction of the structures, and the second scaffold was chosen following different rational criteria (besides chemical compatibility). All hybrids tested showed evidence of antiseizure activity. Compound 3, the structure with the butylparaben scaffold, exhibited an interesting profile in the two acute seizure models proposed by the ESTP program for the identification of new compounds for the treatment of drug-resistant epilepsy. In terms of potency, the ED_{50} calculated value for the MES test is comparable to that of marketed drugs. Interestingly, the ED_{50} found for the 6 Hz (44 mA) test is lower than the reported for levetiracetam and VPA. Unlike VPA, the compound did not show activity in the PTZ model, suggesting a different mechanism of seizure protection. Undoubtedly, these results are only the starting point for exploring the potential of VPA hybrids to treat epilepsy. Numerous questions arose from this research, for example, how the compounds behave in other seizure models (e.g., chronic seizure models) or the experimental confirmation of the stability of the hybrids before reaching the site of action. These and other questions will be addressed in future research.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fmc-2022-0222

Author contributions

FM Garofalo contributed to the design, interpretation and execution of the synthetic experiments. ML Sbaraglini and EM Barrionuevo contributed to the design, interpretation and execution of the biological assays and wrote part of the manuscript. E Peralta contributed to the execution and interpretation of the biological assays. EL Bonifazi contributed to the execution and interpretation of the spectroscopic experiments. A Talevi contributed to the design and interpretation of the biological assays and wrote part of the manuscript. L Gavernet contributed to the design and interpretation of the synthetic experiments and wrote part of the manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The Animal Care for all experimental protocols was conducted in accordance with the Ethical Committee of the Faculty of Exact Sciences of the University of La Plata. The ethical certificates with the number issued by the committee member for conducting a test on animals were submitted in this manuscript as supporting information.

Summary points

- Ten derivatives of valproic acid were synthesized and tested in the maximal electroshock seizure assay.
- The new structures were designed as hybrid compounds that combine two active scaffolds through a linker.
- The hybrids combined valproic acid with parabens family, nipecotic acid or anti-inflammatory drugs.
- The synthesis involved the construction of the haloalkyl ester of valproic acid followed by reaction with the acidic group of the second scaffold.
- All compounds tested showed signs of antiseizure activity.
- No signs of neurotoxicity were detected by the Rotarod test for most of the structures.
- The derivative with propylparaben showed promising results in terms of potency in the maximal electroshock seizure test.
- The derivative with propylparaben showed promising results in terms of potency in the 6 Hz (44 mA) test.

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