Epilepsy Research 139 (2018) 171–179

Contents lists available at ScienceDirect

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

Teratogenic and anticonvulsant effects of zinc and copper valproate complexes in zebrafish

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ARTICLE INFO

Keywords:
Valproate complex
Zebrafish
Teratogenic
Anticonvulsant
Pentylenetetrazole

ABSTRACT

Valproic acid (VPA) is an antiepileptic drug (AED) that has the broadest spectrum across all types of seizures and epileptic syndromes. Unfortunately, approximately 30% of epileptic patients are refractory to the classical AED. Metal ions have been frequently incorporated into pharmaceuticals for therapeutic or diagnostic purposes and research. In this preliminary study, we assess the embryo toxicity and the anticonvulsant activity of 4 novel metallodrugs, with Zn^{+2} and Cu^{+2}, a derivative of valproic acid and the *N*-donor ligand in an adult zebrafish epileptic seizure model induced by pentylenetetrazole. The most toxic complex was [$Cu(Valp)_2Bipy$], in which the LC_{50} was 0.22 µM at 48 h post fertilization (HPF) and 0.12 µM at 96 HPF, followed by [$Zn(Valp)_2Bipy$] (LC_{50} = 10 µM). These same metallodrugs ([$Cu(Valp)_2Bipy$] 10 mM/kg and [$Zn(Valp)_2Bipy$] 30 mM and 100 mM/kg) displayed superior activity, thus reducing the seizure intensity by approximately 20 times compared to sodium valproate (175 mM/kg). Overall, [$Cu(Valp)_2Bipy$] showed the best anticonvulsant effects. However, because of the toxicity of copper, [$Zn(Valp)_2Bipy$] is considered the most promising anticonvulsant for future studies.

1. Introduction

Valproic acid (VPA) is an antiepileptic drug (AED) that has the broadest spectrum across all types of seizures and epileptic syndromes (Belcastro and Striano, 2012). Unfortunately, approximately 30% of epileptic patients are refractory to classical pharmacological treatments (Langer et al., 2011). The development of new AEDs poses a great challenge, demanding screening for new compounds and/or chemical modifications of classical AEDs, such as VPA.

Metal ions play a vital role in the life cycle by serving as essential cofactors and fulfilling cellular functions that cannot be achieved by organic molecules (Thompson and Orvig 2003); they are often incorporated into pharmaceuticals for therapeutic purposes (Barry and Sadler, 2013; Lemire et al., 2013). The choice of metal ions with different oxidation states and coordination environments is a crucial factor, which may render new metallodrugs with different spectra of activity, such as in the treatment of cancer, epilepsy (Zhao et al., 2014), Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), diabetes, inflammatory states, and cardiovascular diseases (Chohan et al., 2005).

No organometallic drug has been recently used in epilepsy treatment. However, new complex entities of valproic acid with divalent copper ions have demonstrated therapeutic effects that are much more effective than the original drug (Sylla-Iyarreta et al., 2009). The valproate compound bis(1,10-phenanthroline) copper (II) was effective in preventing minimal clonic convulsions (ED_{50} 8 mM/kg) and the compound bis(1,10-phenanthroline) copper (II) displayed

Abbreviations: Zn^{+2}, zinc; Cu^{+2}, copper; HPF, hours post fertilization; FET, zebrafish toxicity test; $Cu(Valp)_2Phen$, bis(2-propil-pentanoate)(1,10-phenanthroline)copper(II); $Cu(Valp)_2Bipy$, bis(2-propil-pentanoate)(2,2-bipyridine)copper(II); $Zn(Valp)_2Phen$, bis(2-propil-pentanoate)(1,10-phenanthroline)zinc(II); $Zn(Valp)_2Bipy$, bis(2-propil-pentanoate)(2,2-bipyridine)zinc(II); VPA, sodium valproate (2-propil-pentanoate); Phen, phenanthroline; Bipy, bipyridine; PTZ, pentylenetetrazole; LD_{50}, lethal dose$_{50}$; AED, antiepileptic drug; GABA, gama-aminobutyric acid

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https://doi.org/10.1016/j.eplepsyres.2018.01.005
Received 21 February 2017; Received in revised form 5 December 2017; Accepted 3 January 2018
Available online 19 January 2018
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anticonvulsant activity in the maximal electroshock model (MEM) (Sylla-Iyarreta et al., 2009). The synthesis of valproic acid derivatives with Zn, 2,2 – bipyridine and phenanthroline as potential ligands presented an LD_{50} of 409 μg/mL in a brine shrimp toxicity model.

Addressing the need for novel experimental models and the search for a new AED, zebrafish offer a reasonable compromise between physiological complexity and throughput results. The zebrafish model can be utilized in screening for a wide range of anticonvulsants, offering potential advantages in comparison with mice (Berghmans et al., 2007). Embryos of zebrafish have been employed in toxicological studies due to their sensitivity to environmental changes and their physiological characteristics such as other vertebrates (Pamanji et al., 2015). The lack of a systematic approach to study the toxicity of metal compounds and general structural information on membrane transporters hampers the understanding of the complex mechanism of drug absorption and excretion, particularly in medicinal inorganic chemistry (Spreckelmeyer et al., 2014). The acute seizures induced in wild-type zebrafish by 4-aminopyridine and pentylenetetrazole or heat closely resemble those induced in mammals, both physiologically and behaviorally (Baraban et al., 2005; Hunt et al., 2012). The same is observed in a more complex system regarding adult zebrafish (Mussulini et al., 2013) with potential i.p. injection compounds (Alfaro et al., 2011). The rules for the initiation and termination of neuronal electrical seizures in zebrafish, mice and humans are similar (Jirsa et al., 2014).

In this study, we assess the embryo toxicity and the anticonvulsant activity of bis(2-propil-pentanoate) (1,10-phenanthroline) copper(II); bis(2-propil-pentanoate)(2,2-bipyridine)copper(II); bis(2-propil-pentanoate)(1,10-phenanthroline)zinc(II); and bis(2-propil-pentanoate) (2,2-bipyridine)zinc(II) compared to sodium valproate (2-propil-pentanoate) in an adult zebrafish epileptic seizure model induced by pentylenetetrazole.

2. Material and methods

2.1. Chemicals

All chemicals utilized in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained from a Milli-Q system (Millipore Corporate®, Billerica, MA, USA). Ethanol was purchased from MERCK (Darmstadt, Germany).

2.2. Preparation of the zinc and copper valproate complexes

The chemical synthesis was performed according to Santos et al. (2015) and Sylla-Iyarreta et al. (2009). The chemical structures (Fig. 1) were characterized by UV–vis FTIR, High Resolution Mass Spectrometry HRMS (ESI-QTOF), Nuclear Magnetic Resonance NMR for ^{1}H and ^{13}C and X-ray. Metal concentrations were determined by Atomic Absorption Spectrophotometry Flame (FAAS).

2.3. Animals

Embryos and adult shortfin wild-type zebrafish (*Danio rerio*) were obtained from the university's fish facility. Zebrafish embryos were obtained from pair-wise breeding and maintained in embryo medium (NaCl 5.03 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM, $MgSO_4$ 0.33 mM, methylene blue 0.1%, pH 7.5 ± 0.5, 28 ± 1° C) while awaiting the tests. Adult zebrafish were maintained in a recirculating housing system (ZebTech®, Tecniplast, Italy) at the following water quality parameters: 28 ± 1° C, pH 7.5 ± 0.5, conductivity 500 uS/cm, and a 14 h/10 h dark/light cycle. The animals were housed in 3- and 8-L tanks at 5 animals/L. The fish were fed three times a day with commercial fish food (once) and *Artemia* sp. (twice). All animals were experimentally naive, healthy and disease-free. All procedures were performed according to the Brazilian law for the care and use of laboratory animals (Law 11794/2008) and were previously approved by the Research Ethical Committee from the Federal University of Rio Grande do Sul (number #27725).

2.4. Embryo toxicity assay

The fish embryo toxicity (FET) test was performed according to the OECD Guidelines for the Testing of Chemicals (2013). Toxicity was observed only when fertilization was ≥80%. Eggs fertilized within 1.5 h post-fertilization (HPF) (4–16-cell stage) were placed in a 24-well plate at one egg/well. Eggs were treated during 96 HPF with 1 mL of the following solutions: embryo medium (control group), ethanol 0.01% (vehicle group), sodium valproate (0.008–5000 μM) or organometallic complexes (0.00037–100 μM). Each solution was changed every 24 h. Lethality and morphological alterations (malformation of the head, tail, or heart; scoliosis, deformity of the yolk, and growth retardation) were assessed every 24 h using a Nikon SMZ 800 inverted stereomicroscope (Kimmel et al., 1995). According to the OECD Guidelines for the Testing of Chemicals (2013), the hatching rate of zebrafish embryos was evaluated at 72 HPF. The sample size was 20–24 embryos per group.

2.5. PTZ-induced seizure

Adult animals were carefully weighed and measured to select the ones of a similar weight and size (350 ± 20 mg and 4 ± 0.5 cm, respectively) to avoid putative variations of pharmacodynamic and pharmacokinetic drugs. Animals were randomly handled from their home tanks and individually transferred to beakers filled with 160 μg/mL tricaine. Then, the animals were anesthetized and injected intraperitoneally (i.p.) with ethanol 0.01%, sodium valproate (0.875 and 1.75 mM/kg), [Cu(Valp)$_2$Phen]/[Cu(Valp)$_2$Bipy] (0.1 and 0.3 mM/kg), or [Zn(Valp)$_2$Phen]/[Zn(Valp)$_2$Bipy] (0.1, 0.3 and 1.0 mM/kg). The volume of injection was 10 μL/g. Thirty min after i.p. injection, the animals were immersed in PTZ solution (10 mM) or vehicle for 20 min. Seizures were scored according to Mussulini et al. (2013): (0) short

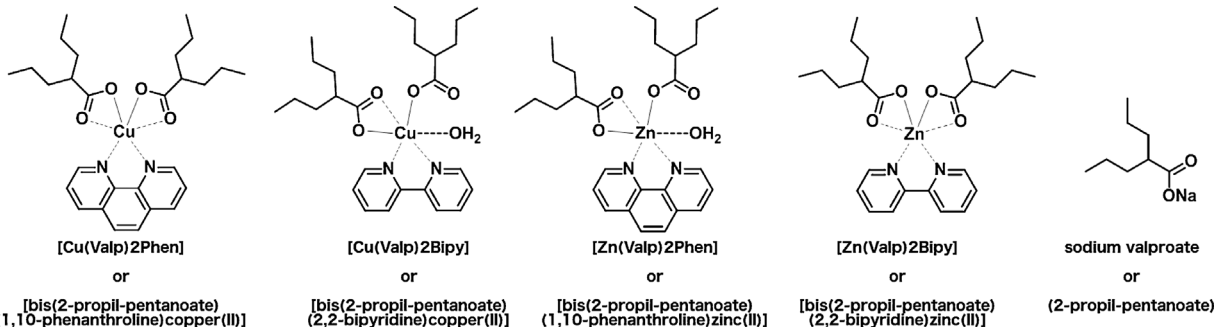

Fig. 1. Chemical structures of the tested compounds: **(1)** [bis(2-propil-pentanoate)(1,10-phenanthroline)copper(II)] or [Cu(Valp)$_2$Phen]; **(2)** [bis(2-propil-pentanoate)(2,2-bipyridine) copper(II)] or [Cu(Valp)$_2$Bipy]; **(3)** [bis(2-propil-pentanoate)(1,10-phenanthroline)zinc(II)] or [Zn(Valp)$_2$Phen], **(4)** [bis(2-propil-pentanoate)(2,2-bipyridine)zinc(II)] or [Zn (Valp)$_2$Bipy]; and **(5)** sodium valproate (2-propil-pentanoate).

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