Epilepsy & Behavior 24 (2012) 14–22

Contents lists available at SciVerse ScienceDirect

Epilepsy & Behavior

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

Anticonvulsant activity of bisabolene sesquiterpenoids of *Curcuma longa* in zebrafish and mouse seizure models

Adriana Monserrath Orellana-Paucar [a,b], Ann-Sophie K. Serruys [a], Tatiana Afrikanova [a], Jan Maes [a], Wim De Borggraeve [c], Jo Alen [c], Fabián León-Tamariz [b], Isabel María Wilches-Arizábala [b], Alexander D. Crawford [a], Peter A.M. de Witte [a,*], Camila V. Esguerra [a]

[a] *Laboratory for Molecular Biodiscovery, Department of Pharmaceutical & Pharmacological Sciences, University of Leuven, Leuven, Belgium*
[b] *Escuela de Bioquímica y Farmacia, Facultad de Ciencias Químicas, Universidad de Cuenca, Cuenca, Ecuador*
[c] *Molecular Design and Synthesis, Department of Chemistry, University of Leuven, Leuven, Belgium*

ARTICLE INFO

Article history:
Received 20 December 2011
Revised 16 February 2012
Accepted 21 February 2012
Available online 5 April 2012

Keywords:
Curcuma longa
Turmeric
Turmeric oil
Bisabolene sesquiterpenoids
α,β-Turmerone
Ar-turmerone
α-Atlantone
Pentylenetetrazole
Zebrafish PTZ model
Mouse PTZ model

ABSTRACT

Turmeric, obtained from the rhizomes of *Curcuma longa*, is used in South Asia as a traditional medicine for the treatment of epilepsy. To date, *in vivo* studies on the anticonvulsant activity of turmeric have focused on its principal curcuminoid, curcumin. However, poor absorption and rapid metabolism have limited the therapeutic application of curcumin in humans. To explore the therapeutic potential of turmeric for epilepsy further, we analyzed its anticonvulsant activity in a larval zebrafish seizure assay. Initial experiments revealed that the anticonvulsant activity of turmeric in zebrafish larvae cannot be explained solely by the effects of curcumin. Zebrafish bioassay-guided fractionation of turmeric identified bisabolene sesquiterpenoids as additional anticonvulsants that inhibit PTZ-induced seizures in both zebrafish and mice. Here, we present the first report of the anticonvulsant properties of bisabolene sesquiterpenoids and provide evidence which warrants further investigation toward the mechanistic understanding of their neuromodulatory activity.
© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Epilepsy is a widespread neurological disorder that affects approximately 50 million people worldwide [1]. According to the World Health Organization (WHO), about 1% of the total burden of disease corresponds to different forms of epilepsy. Its pharmacological treatment comprises a number of currently available antiepileptic drugs (AEDs). The main problems concerning AEDs are the high incidence of side effects ranging from gastrointestinal distress, hepatotoxicity, depression, cognitive impairment [2–6] and the fact that seizures in about one third of patients suffering from epilepsy remain resistant to these existing treatments [1,2]. Hence, there is a clear need to continue to identify novel AEDs that effectively control pharmacoresistant seizures with minimal or no adverse effects.

Numerous studies point to medicinal plants as an interesting source of novel AEDs [7,8]. One interesting example in this regard is losigamone derived from the kava kava plant and originally used by traditional healers in the South Pacific as an anxiolytic, which is now in early clinical development as a novel AED [9,10]. Likewise, *Curcuma longa*, a perennial herb of the Zingiberaceae family native to South Asia, has been used not only as a condiment and color additive in food but also in traditional medicine against seizures [11]. The major active chemical constituents of turmeric (*C. longa* rhizome powder) are the curcuminoids (3–5%) and turmeric oil (2–7%). Turmeric oil is mainly composed of bisabolene sesquiterpenoids: ar-, α,β-turmerone and α-atlantone, whereas the curcuminoids include curcumin, monodemethoxycurcumin and bisdemethoxycurcumin [11]. Nearly all investigations on the medicinal properties of turmeric have been focused on curcumin, and its anticonvulsant activity has been demonstrated in several rodent models such as the iron-

Abbreviations: AEDs, antiepileptic drugs; DAD, diode array detection; DMSO, dimethyl sulfoxide; dpf, days post-fertilization; ddH_2O, double-distilled water; ESI-MS, electrospray ionization mass spectrometry; i.v., intravenous; i.p., intraperitoneal; LC–MS, liquid chromatography–mass spectroscopy; MES, maximal electroshock seizure; MTC, maximum tolerated concentration; PEG, polyethylene glycol; p.o., "*per os*", orally; PTZ, pentylenetetrazole; RP-HPLC, reverse phase-high performance liquid chromatography; s.c., subcutaneous; TLE, temporal lobe epilepsy.

* Corresponding author at: Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, University of Leuven, Gasthuisberg Campus, Building O&N2, Room 08.4226, Herestraat 49, P.O. Box 824, BE-3000 Leuven, Belgium. Fax: +32 16 323460.
E-mail address: peter.dewitte@pharm.kuleuven.be (P.A.M. de Witte).

1525-5050/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.yebeh.2012.02.020

induced epileptogenesis [12], maximal electroshock [13], kainic acid-induced [14] and pentylenetetrazole-kindling [15] models. Although its anticonvulsant properties have been demonstrated, phase I clinical trials have revealed important pharmacokinetic limitations for curcumin. When administrated *p.o.*, curcumin is poorly absorbed through the gut. Therefore, the amount of curcumin in the circulation is very low. Thus, pharmacokinetic issues have limited its therapeutic applications. For this reason, formulation studies have been performed to enhance curcumin bioavailability [16]. The fact that nearly all the studies have been conducted on curcumin has led to the assumption that the anticonvulsant properties of turmeric are due solely to its activity. However, turmeric oil could also be accountable for its anticonvulsant activity as other essential oils have been shown to be effective in controlling seizure generation [17,18]. Intriguingly, a few studies have reported on the neuroprotective activity of turmeric oil [19–21].

In this paper, we report on the anticonvulsant activity of turmeric methanolic extract and its constituents as assessed in a zebrafish PTZ-induced seizure model. Further confirmation of this activity was carried out in the mouse PTZ model.

The zebrafish (*Danio rerio*) has emerged over the last decade as a valuable model for genetic studies and drug screening. The strength of this *in vivo* model relies on its high genetic, physiologic and pharmacologic homology to humans. Their high fecundity and small size allow for the performance of tests in a medium- to high-throughput fashion using minute (microgram scale) quantities of compound [22,23]. The zebrafish also holds promise as an *in vivo* model for identifying novel neuroactive compounds since the dopaminergic, serotonergic, and GABAergic systems develop early during embryogenesis and are already functional in larvae [24]. Additionally, their rapid development *ex utero* and optical transparency make it possible to easily detect morphological and behavioral effects of test compounds on living embryos and larvae [25]. More recently, zebrafish have also proven useful for the primary screening of potential novel anticonvulsants based on the proconvulsant pentylenetetrazole (PTZ) [26–28]. Likewise, PTZ has been used as a chemoconvulsant in rodent models, and the timed PTZ infusion test has proven competence in enabling the identification of AEDs with different mechanisms of action [29,30].

In this study, we confirmed the reported anticonvulsant properties of turmeric and curcumin. Further testing of turmeric oil and its chromatographic fractions revealed additional constituents capable of suppressing PTZ-induced seizure behaviors in larval zebrafish. Mass spectrometry and NMR analyses of these active purified fractions revealed them to belong to the class of bisabolene sesquiterpenoids: ar-turmerone, α,β-turmerone and α-atlantone. The anticonvulsant activity of turmeric oil, ar-turmerone and α,β-turmerone, identified using the zebrafish PTZ assay, was then confirmed in the equivalent mouse PTZ-induced seizure model.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (99.9%, spectroscopy grade) and the curcuminoid mixture from turmeric (98%; curcumin, demethoxycurcumin and bisdemethoxycurcumin) were procured from Acros Organics. Diethyl ether (99.9%, spectroscopy grade), deuterated chloroform (99.8% D, contains 0.1% (v/v) TMS) and PTZ were obtained from Sigma-Aldrich, methanol (99.8%, reagent grade) and acetonitrile (100%, HPLC grade) from Fisher Scientific. Sodium valproate was obtained from Sanofi-Synthelabo.

2.2. Plant material

Dried rhizome powder of *C. longa* (turmeric) was acquired from a local supplier in Belgium with India as the source of origin. Microscopic authentication according to [31] was completed by R. Ansaloni.

A "voucher specimen" serves as a reliable reference for the identity of botanical samples for further investigations. For this reason, a voucher specimen of turmeric (no. 7850) was deposited in the Herbarium Azuay (HA), Universidad del Azuay, Cuenca, Ecuador.

2.3. Experimental animals

All procedures for animal experiments were performed in accordance with European and National Regulations and approved by the Animal Care and Use Committee of the University of Leuven (Belgium).

2.3.1. Zebrafish (D. rerio)

Adult zebrafish of the Tg (*fli* 1a: EGFP)y1 strain were reared at 28.5 °C on a 14/10-hour light/dark cycle. Eggs were collected from natural breeding and fostered in embryo medium (17 mM NaCl, 2 mM KCl, 1.8 mM $Ca(NO_3)_2$, 0.12 mM $MgSO_4$, 1.5 mM HEPES buffer pH 7.1–7.3 and 0.6 μM methylene blue) in an incubator at 28.5 °C. Zebrafish are considered 'embryos' between 0 and 72 hours post-fertilization (hpf). From 72 hpf to 14 days post-fertilization (dpf), they are referred to as 'early larvae' or 'larvae' [32]. Sorting of zebrafish embryos and larvae and medium refreshment were performed daily until 7 dpf. After completing the experiments, all larvae were sacrificed through administration of an overdose of anesthetic (tricaine).

2.3.2. Mice (Mus musculus)

Male C57Bl/6 mice (20–30 g) at 8 weeks of age were acquired from Charles River Laboratories. The mice were housed in poly-acrylic cages under 12/12-hour light/dark cycle at 28 °C in a quiet room. The animals were fed *ad libitum* with a pellet diet and water until they were 10 weeks old (the age when all assays were carried out).

2.4. Preparation of methanolic extract of turmeric

Turmeric (5 g) was extracted through maceration in methanol (50 ml). The extract was concentrated using a rotary evaporator (Büchi Rotavapor R-114, Germany) to obtain a yield of 0.20 g.

2.5. Preparation of turmeric volatile oil and isolation of major constituents

Volatile oil from turmeric was obtained by subjecting 4×100 g of turmeric to hydro-distillation using a Clevenger-type apparatus for 3 h. A pale yellowish and odiferous oil was obtained (8.56 g). Turmeric oil was dried over anhydrous sodium sulfate and stored at 4 °C until used.

The isolation of some major compounds present in the essential oil was carried out by semi-preparative RP-HPLC, as adapted from the work of He and colleagues [33]. A LaChrom *Elite* HPLC System (VWR Hitachi) equipped with diode array detection (DAD) and an Econosphere 10-μm C18 (250×10 mm) reversed phase column (Grace Davison Discovery Sciences, Belgium) attached to an Econosphere 10 μm C18 (33×7 mm) guard column (Grace Davison Discovery Sciences, Belgium) were used. Amounts of 10-mg volatile oil were repeatedly processed. The column was operated at a flow rate of 5 ml/min at room temperature. The profile of the gradient elution was: double-distilled water (ddH_2O) (A) and acetonitrile (B); 0–15 min, 40–60% B; 15–20 min, 60–100% B. The analytes were monitored with DAD at 260 nm. Eight fractions from turmeric oil were individually collected (Table 1). Solvents from the collected fractions were removed by separation between diethyl ether and ddH_2O. The ether phase was collected and dried over anhydrous sodium sulfate. Ether was removed by passing a slow stream of nitrogen over the sample at room temperature. The concentrated samples were stored at 4 °C until analyzed.

Table 1
Fractions collected from the RP-HPLC analysis of turmeric oil. The collected fractions are referred as percentage (%) of content in turmeric oil.

Fraction no.	%
1	3
2	<1
3	18
4	30
5	15
6	7
7	<1
8	2

Fraction 4: ar-turmerone; fraction 5: α,β-turmerone; fraction 6: α-atlantone.

2.6. Chemical structure elucidation of bisabolene sesquiterpenoids

2.6.1. Nuclear magnetic resonance (NMR) analysis

^{1}H and ^{13}C NMR spectra of fractions 4, 5 and 6 (see below) were obtained from Bruker 300 Avance and Bruker 600 Avance II^{+} equipment using deuterated chloroform as solvent and tetramethylsilane (TMS) as internal standard.

2.6.2. Mass spectroscopy (MS) analysis

The LC–MS analysis was performed on an Agilent 1100 system equipped with degasser, quaternary pump, auto-sampler, UV-DAD detector and thermostated column module coupled to Agilent 6110 single–quadrupole MS. Data acquisition and quantification were obtained from Agilent LC/MSD Chemstation software. Fractions 4, 5 and 6 (see further) were analyzed on a Grace Prevail RP-C18 column 3 μm (150 mm × 2.1 mm) at a flow rate of 0.2 ml/min. The LC gradient comprised two solvents: double-distilled water (ddH_2O) + 0.1% formic acid (A) and acetonitrile (B); 0–17 min, 40–60% B; 17–32 min, 60–100% B.

The ESI-MS analysis was completed in a Thermo Electron LCQ Advantage apparatus with Agilent 1100 pump and injection system coupled to Xcalibur data analysis software.

2.7. Determination of maximum tolerated concentration in zebrafish larvae

The aim of this assay was to determine the range of appropriate concentrations to be tested in zebrafish for the evaluation of anticonvulsant activity. Seven-dpf larvae were placed into a 24-well plate (tissue culture plate, flat bottom, FALCON®, USA), six larvae per well. They were incubated with different concentrations of sample dissolved in 1 ml of embryo medium (at a final DMSO concentration of 1%). The larvae were examined during a period of 24 h in sample and compared to control group to detect the following signs of toxicity: absence of startle response to plate taps, changes in heart rate or circulation, presence of edema, loss of posture, paralysis and death. Thus, the maximum tolerated concentration (MTC) was defined as the highest concentration at which no signs of toxicity were observed in 6 out of 6 zebrafish larvae within 24 h of exposure to sample.

2.8. Evaluation of anticonvulsant activity in the zebrafish PTZ model

Zebrafish larvae from 7 dpf were monitored using the ViewPoint VideoTrack System for Zebrafish™ (Version 2.3.1.0, ViewPoint, France). The system consists of an infrared light source, a high-resolution digital video camera to capture larval movements within a defined time period (30 min in our experimental set-up) and the software to analyze larval locomotor activity.

The highest concentrations of the samples tested correspond to the previously determined MTC values. Zebrafish larvae were placed in a tissue culture, flat bottom 96-well plate (FALCON®, USA); one larva per well. Each stock solution of sample (dissolved in 100% DMSO) was diluted in embryo medium to achieve a final DMSO concentration of 1%. In cases where sample had to be diluted further (*i.e.* more than 1:100), DMSO was added so as to maintain a final concentration of 1%. Each sample was loaded in duplicate adjacent rows. The larvae were then incubated at room temperature under dark and quiet conditions for 1 h. Equal volumes (100 μl) of embryo medium (vehicle) and 40-mM PTZ were then added respectively to the first and second rows of each sample group (resulting in a doubling of the total incubation volume and halving of the final DMSO concentration to 0.5% and PTZ to 20 mM). The total locomotor activity of each larva was video-tracked and assessed in the presence of either vehicle

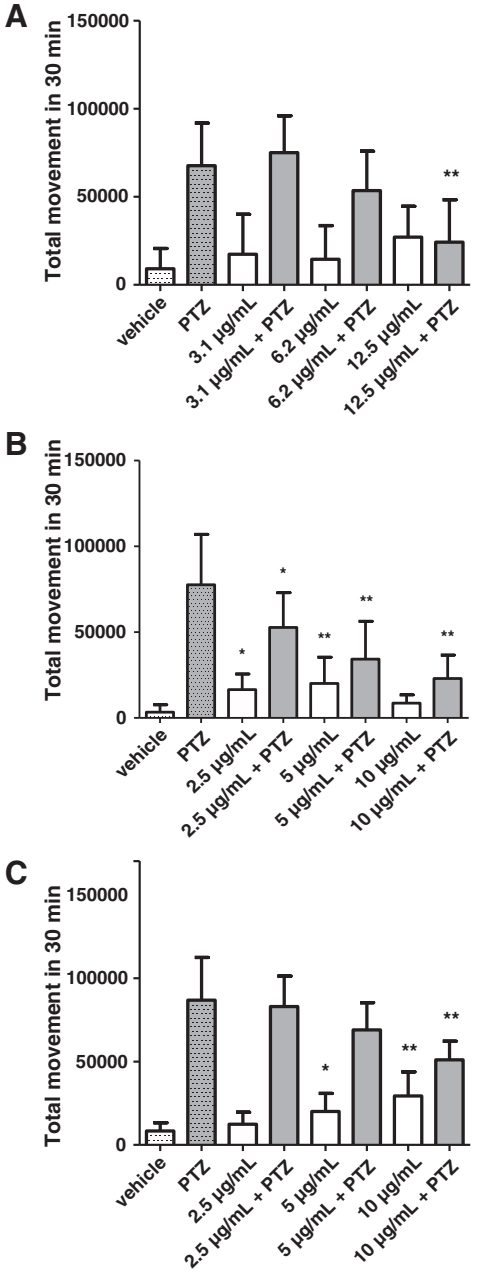

Fig. 1. Evaluation of the anticonvulsant activity of turmeric in the zebrafish PTZ seizure assay. (A) Turmeric methanolic extract; (B) curcuminoids and (C) turmeric oil. Tested concentrations are indicated along the x-axis, and the total gross locomotor activity exhibited by zebrafish larvae within 30 min is displayed along the y-axis. Data are expressed as the mean ± SD (n = 10–12). Statistically significant differences between vehicle-treated and sample-treated (white bars) or PTZ-treated and sample plus PTZ-treated groups (gray bars) are labeled as * for $p<0.05$ and ** for $p<0.01$.

only, vehicle with sample, PTZ only, or PTZ and sample (see Supplementary Fig. S1). Video-tracking of larval movements commenced exactly 5 min after addition of embryo medium or PTZ to the wells and was recorded for 30 min. A total of 8 wells in each plate were left without larvae (medium only) as a negative control, so that each experimental parameter consisted of 10 to 12 larvae. Results were registered as the average value of the total time of larval movement during 30 min.

2.9. EEG recordings

Zebrafish larvae were allowed to swim in 400 μl of either 10 μg/ml turmeric oil or vehicle for 1 h in a well of a 24-well plate. An equal volume of 40-mM PTZ was then added to the well. After a 15-minute exposure, the larvae were embedded in a 2% low-melting-point agarose. A glass electrode filled with artificial cerebrospinal fluid composed of: 124 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose (resistance 1–5 MΩ), was placed into the optic tectum. Recordings were performed in current clamp mode, low-pass filtered at 1 kHz, high-pass filtered at 0.1 Hz, digital gain 10, and sampling interval 10 μs (MultiClamp 700B amplifier, Digidata 1440A digitizer, both Axon Instruments, USA). The recordings started each time exactly 5 min after removal from the proconvulsant bath and were continued for 10 min.

A spike was defined as interictal-like if its amplitude exceeded three times the background and lasted for less than 3 s. Longer discharges were counted as ictal-like ones (Fig. S2). We quantified the number and average duration of either type of electrographic activity as well as the total cumulative duration of all forms of epileptiform discharges during the ten-minute recordings using Clampfit 10.2 software.

2.10. Generation of PTZ-induced seizures in mice: timed i.v. PTZ infusion test

Mice were randomly divided into groups of five animals (vehicle and sample). The animals were isolated into individual cages and pre-warmed in front of an infrared lamp for 10 min to dilate the tail veins. They were then placed in a restrainer, and the lateral tail vein was catheterized with a 1-cm long, 29-gauge needle. After confirming correct placement, the needle was secured to the tail with surgical tape and kept in place until the end of the test. The needle attached to a 0.7-m long polyethylene catheter was connected to two 2.5-ml glass syringes containing: a) sample; heparin 2 UI/ml and b) PTZ (7.5 mg/ml ddH_2O; heparin 2 UI/ml). These syringes were mounted on a motor-driven infusion double pump (ALADOIN-1000 11VDC, 0.75 Å, World Precision Instruments). Thus, 100 μl of control vehicle (PEG200:DMSO 1:1; heparin 2 UI/ml) or sample dissolved in vehicle were i.v. infused at a rate of 50 μl/min for 2 min. Ten minutes later, mice were released from the restrainer and placed in a transparent poly-acrylic cage (32×14×12.5 cm) for observation. Pentylenetetrazole was then infused at a rate of 150 μl/min. Seizure manifestation stages in mice were scored according to the time between the start of PTZ infusion and the following behavioral events: ear, tail and myoclonic twitch, forelimb clonus, falling, tonic hindlimb extension and death [34,35]. Under these conditions, i.v. infused PTZ triggered all aforementioned behavioral parameters, culminating in death of mice treated with vehicle, approximately 3 min after the start of infusion. For this reason, behavior was observed up to a maximum of 5 min of PTZ infusion. Any surviving mice were then sacrificed. Pentylenetetrazole doses were calculated according to the formula: PTZ dose [mg/kg] = (PTZ concentration [mg/ml] × infusion rate [ml/s] × infusion duration [s] × 1000)/mouse weight [g].

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc.). Values were presented as means ± standard deviation (SD). The locomotor activity of zebrafish larvae was evaluated using one-way ANOVA followed by Dunnett's multiple comparison test. Statistically significant differences ($p<0.05$) between a treatment group and the equivalent control groups (vehicle or PTZ) were considered indicative of decrease or increase in locomotor activity of zebrafish larvae. For evaluation of the EEG data and the mouse experiments, significant differences ($p<0.05$) between estimated time intervals prior to above-mentioned seizure stages were calculated using the unpaired Student's *t*-test.

3. Results

3.1. Evaluation of the anticonvulsant activity of turmeric

The analysis of the methanolic extract of turmeric (*C. longa* rhizome powder) revealed anticonvulsant activity in the zebrafish larval PTZ assay (Fig. 1). In order to identify the active constituents present in the methanolic extract of turmeric, the anticonvulsant properties

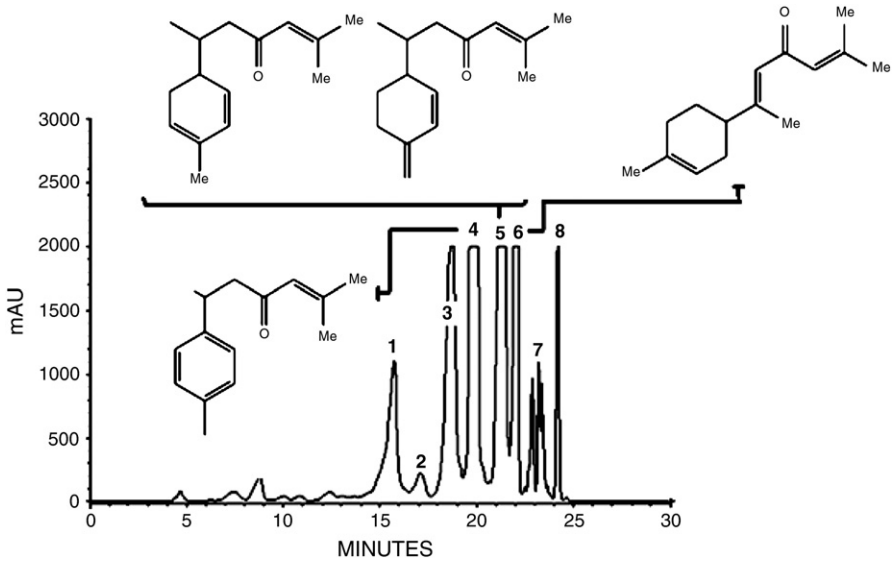

Fig. 2. HPLC chromatogram of turmeric oil and its major constituents. Peak 4 corresponds to ar-turmerone; peak 5 to α,β-turmerone and peak 6 to α-atlantone.

of curcuminoids and turmeric oil were also assessed through video-tracking analysis (Fig. 1). Curcuminoids showed anticonvulsant activity at 2.5 μg/ml ($p<0.05$) and at 5 and 10 μg/ml ($p<0.01$) in our larval PTZ assay. Further analysis uncovered an additional anticonvulsant activity for turmeric oil. The larvae showed a decrease ($p<0.01$) of PTZ-induced convulsions after exposure to turmeric oil (10 μg/ml). Notably, exposure of zebrafish larvae to curcuminoids or turmeric oil alone (*i.e.* in the absence of proconvulsant) also resulted in a slight increase in locomotor activity compared to vehicle-treated controls (Figs. 1B,C). However, no obvious signs of toxicity (as measured by change in heart rate, loss of posture, lack or delay in response to tactile stimuli, or death (see Methods, Section 2.7)) were observed in these larvae.

3.2. Isolation and evaluation of the anticonvulsant activity of bisabolene sesquiterpenoids from turmeric oil

To purify the active constituents in turmeric oil, we performed semi-preparative RP-HPLC analysis. Eight peaks were identified (Fig. 2) and individually collected. From the eight peaks collected, fractions 2 and 7 were not tested in the zebrafish PTZ assay since the collected amounts were not sufficient for carrying out the assay (Table 1). Fractions 1, 3 and 8 did not display any anticonvulsant activity in the zebrafish PTZ assay (data not shown). A significant decrease in the convulsions triggered by PTZ was observed for fractions 4, 5 and 6. Fraction 4 showed anticonvulsant activity at 46 μM ($p<0.05$), fraction 5 at 23 μM ($p<0.01$) and fraction 6 at concentrations of 23 μM ($p<0.05$) and 46 μM ($p<0.01$) (Fig. 3). Sodium valproate, a well-known AED, was included as a positive control for the zebrafish PTZ assay [24,25]. Sodium valproate showed significant activity at 250 μM ($p<0.05$) and 500 μM and 1 mM ($p<0.01$) (Supplementary Fig. S4A).

Fractions 4, 5 and 6 that showed seizure inhibitory activity in the larval zebrafish PTZ assay were further analyzed for chemical structure elucidation. ^{1}H and ^{13}C NMR spectra of fraction 4 are in agreement with reported values for ar-turmerone [36]. Nuclear magnetic resonance analysis indicated fraction 5 as a mixture (1:1) of two isomeric structures identified as α,β-turmerone by 1D- and 2D-NMR analysis [37]. Fraction 6 was identified as α-atlantone (probably the *E*-isomer) based on ^{1}H and ^{13}C NMR spectra [36,38] (data not shown).

Once again, as observed for the curcuminoid and turmeric oil-treated larvae, exposure of zebrafish larvae to α,β-turmerone, ar-turmerone or α-atlantone alone also resulted in a slight increase in locomotor activity compared to vehicle-treated controls (Fig. 3). However, no obvious signs of toxicity were observed. This raised the question as to whether turmeric oil or its individual constituents could perhaps exhibit mild proconvulsant activity.

To determine whether turmeric oil can act as a proconvulsant on zebrafish larvae and to confirm whether the decrease in convulsion-like movements observed in larvae co-treated with turmeric oil and PTZ was truly an inhibition of seizure activity, we carried out open-field recordings from larval brains. The analysis of electrographic activity of larvae showed that exposure to 10 μg/ml of turmeric oil partly protects the larvae from PTZ-induced seizures. Turmeric oil produced no effect on the number and duration of interictal-like spikes compared to controls (Figs. 4A,B,H,I; Supplementary Figs. S3C,D) but significantly reduced both the number and the duration of ictal-like discharges (Figs. 4C,D,H,I). Moreover, the latter were absent in 3 out of five recordings (Supplementary Fig. S3D). In addition, the cumulative duration of all forms of epileptiform discharges quantified was shortened after turmeric oil exposure (Fig. 4E). Importantly, turmeric oil on its own did not induce epileptiform discharges in our experimental set-up (Figs. 4F,G; Supplementary Figs. S3A,B).

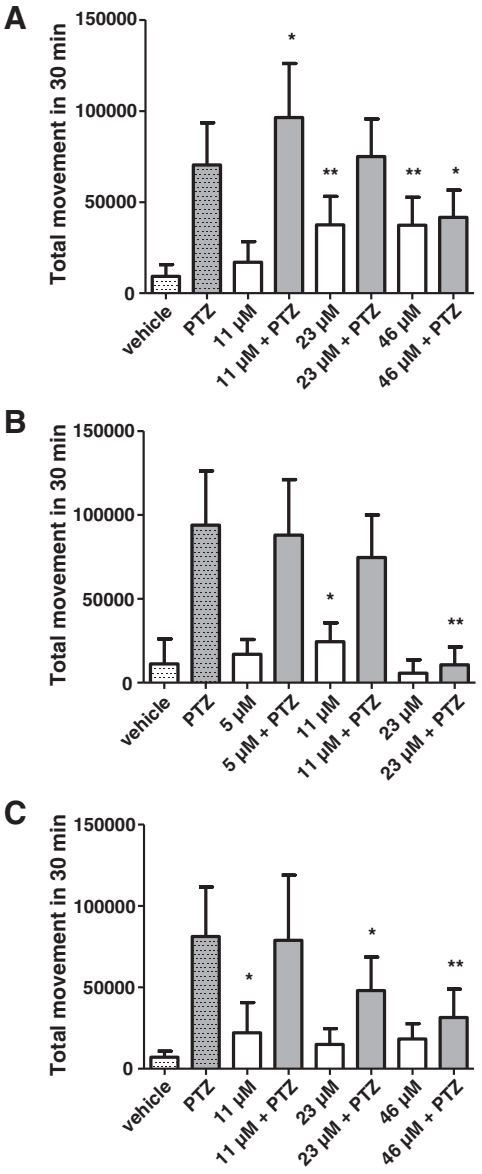

Fig. 3. Evaluation of the anticonvulsant activity of bisabolene sesquiterpenoids in the zebrafish PTZ seizure assay. (A) Ar-turmerone; (B) α,β-turmerone and (C) α-atlantone. The x-axis represents the tested concentration for each one of the sesquiterpenoids. The y-axis indicates the total gross locomotor activity exhibited by zebrafish larvae within 30 min. Data are expressed as the mean ± SD ($n=10–12$). Statistically significant differences between vehicle-treated and sample-treated (white bars) or PTZ-treated and sample plus PTZ-treated groups (gray bars) are labeled as * for $p<0.05$ and ** for $p<0.01$.

3.3. Evaluation of the anticonvulsant activity of turmeric, turmeric oil and bisabolene sesquiterpenoids in the mouse PTZ-induced seizure model

The anticonvulsant activity of curcumin has been described previously in mice [12–15], but no assay for turmeric oil has evaluated its anticonvulsant properties before. Thus, further confirmation of the positive results obtained in zebrafish with turmeric oil and the bisabolene sesquiterpenoids was performed in the mouse assay. Mice treated with turmeric oil (50 mg/kg) showed a significant increase in PTZ doses required to trigger all behavioral endpoints: forelimb clonus, falling and tonic hindlimb extension ($p<0.05$) and ear, myoclonic, tail twitch, and death ($p<0.01$), compared to control group (Fig. 5). Moreover, a dose of 100-mg/kg turmeric oil in the mouse

PTZ assay exhibited significant activity in delaying seizure generation for all seizure parameters and death as compared to control ($p<0.01$) (Fig. 5). Regarding the active bisabolene sesquiterpenoids, ar-turmerone and α,β-turmerone were assessed using the mouse PTZ seizure model (Fig. 6). Mice infused with a dose of 50 mg/kg of ar-turmerone exhibited significant resistance to the generation of seizures leading to an increase in the required dose of PTZ to trigger all assessed events: tonic hindlimb extension ($p<0.05$) and ear, myoclonic and tail twitch, forelimb clonus, falling and death ($p<0.01$). Likewise, the anticonvulsant activity of α,β-turmerone was evaluated, and positive results were also found with a dose of 100 mg/kg for all seizure parameters: forelimb clonus, falling, ear and tail twitch ($p<0.05$) and myoclonic twitch, tonic hindlimb extension and death ($p<0.01$). α-Atlantone was not tested in the mouse model since the collected amount was not sufficient to carry out the assay.

Sodium valproate was included as positive control in our PTZ tail infusion method for AED screening in mice (Supplementary Fig. S4B). Using this assay, sodium valproate (50 mg/kg) was capable of delaying tonic hindlimb extension ($p<0.01$) and death ($p<0.05$).

4. Discussion

Turmeric, the powdered rhizomes of *C. longa*, has been used for centuries as a food condiment but also as an ethnomedical treatment against seizures. We evaluated the anticonvulsant activity of turmeric methanolic extract in the zebrafish PTZ-induced seizure model [27] with positive results. This finding is in line with the anticonvulsant properties of turmeric described in previous studies. In these studies, curcumin has often been cited as the principal substance responsible for the activity of turmeric [12,13,39]. Nevertheless, bioavailability analysis of curcumin evidenced poor absorption, rapid metabolism and excretion impeding its ability to reach the brain in order to exert any potential therapeutic action. Conversely, the other main constituent of turmeric, turmeric oil, has not been evaluated before for potential anticonvulsant properties.

The compounds isolated from turmeric oil through semi-preparative RP-HPLC were individually evaluated in the zebrafish PTZ assay. This assay revealed significant activity for turmeric oil and the major bisabolene sesquiterpenoids: ar-, α,β-turmerone and α-atlantone. All of the aforementioned constituents exhibited

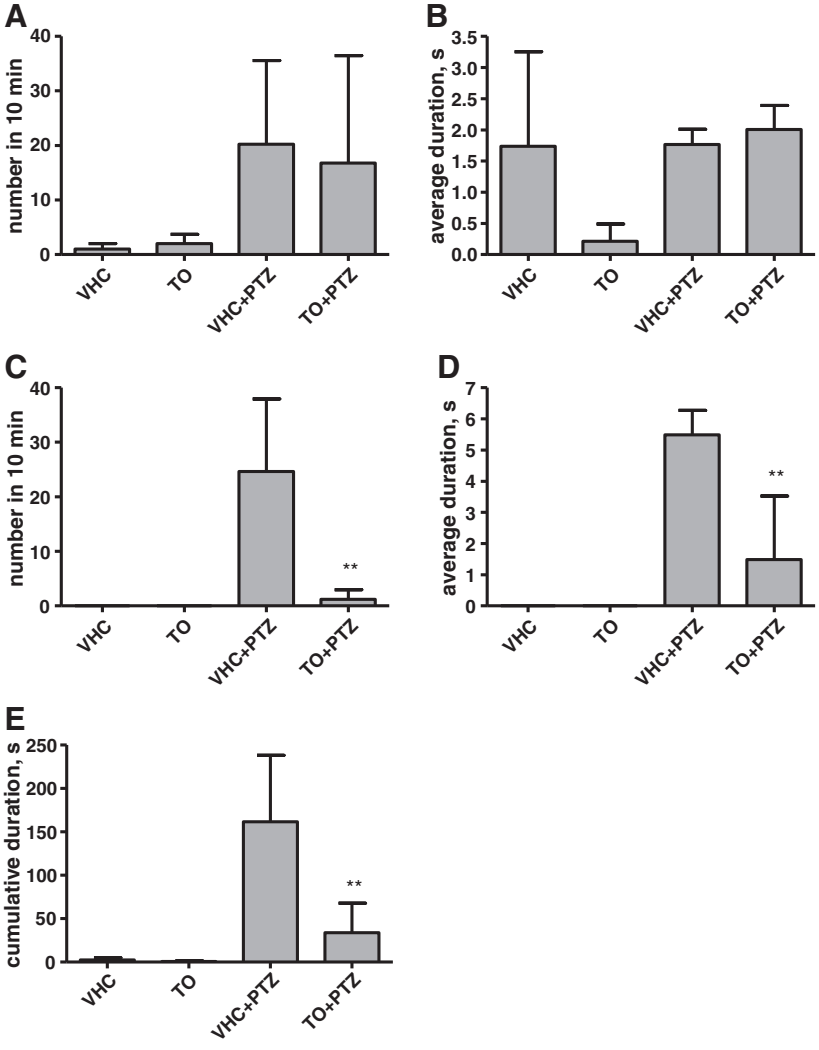

Fig. 4. Electrographic evaluation of the anticonvulsant activity of turmeric oil in the larval zebrafish PTZ seizure model. (A–E) Graphical representation of the changes in seizure parameters analyzed. VHC, vehicle-treated, n = 3; TO, turmeric oil-treated, n = 3; VHC + PTZ, vehicle-treated larvae after PTZ exposure, n = 5; TO + PTZ, turmeric oil-treated larvae after PTZ exposure, n = 5. (A) Number of interictal-like spikes; (B) average duration of interictal-like spikes; (C) number of ictal-like discharges; (D) average duration of ictal-like discharges; (E) cumulative duration of all forms of epileptiform activity. (F–I) Representative 2-minute fragments of electrographic activity recordings. (F) Spontaneous electrical activity in vehicle-treated control; (G) basal activity after exposure to 10-μg/ml turmeric oil; (H) PTZ-induced spiking in vehicle-treated larva; (I) reduced amount of PTZ-induced epileptiform activity after exposure to 10-μg/ml turmeric oil.

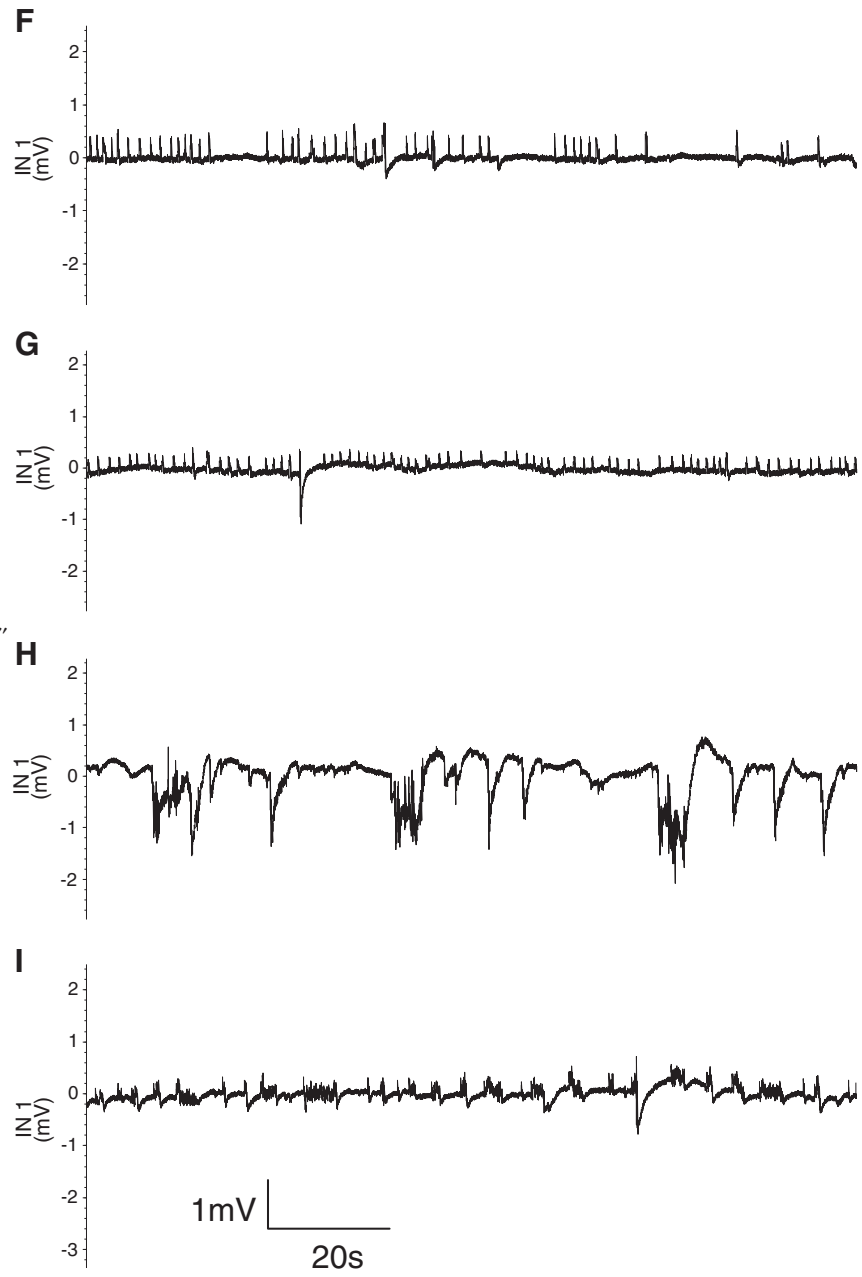

Fig. 4 (*continued*).

anticonvulsant properties at lower concentrations compared to sodium valproate. One possibility for the more potent activity of bisabolene sesquiterpenoids in the zebrafish PTZ assay compared to sodium valproate could be related to their higher lipophilicity (logP). Empirical evidence suggests that small molecules with higher lipophilicity enter zebrafish embryos and larvae more readily than hydrophilic ones (our own unpublished observations). The bisabolene sesquiterpenoids display around one hundred times higher lipophilicity than sodium valproate. Thus, these results are also in line with the neuroprotection studies in rodent models which have shown that turmeric oil and its main bisabolene sesquiterpenoids easily cross the blood-brain barrier, likely due to their lipophilic nature which allows them to easily pass through cell membranes [16,19,20,24]. Turmeric oil and its constituents present better bioavailability and cross biomembranes with less difficulty when compared to curcumin.

The anticonvulsant properties of turmeric oil in the zebrafish model were successfully corroborated in the mouse PTZ model (50 mg/kg, 100 mg/kg). Further analysis leads to the identification of ar-turmerone and α,β-turmerone as the putative compounds responsible for the anticonvulsant activity found for turmeric oil in mice. These findings validate the zebrafish PTZ-induced seizure model as a primary screening tool for identifying novel potential AEDs and reveal the major bisabolene sesquiterpenoids ar-turmerone and α,β-turmerone as anticonvulsant drug candidates to be investigated further. At effective doses, neither turmeric oil nor the tested bisabolene sesquiterpenoids induced sedation, motor impairment or any other sign of toxicity in mice.

Interestingly, anticonvulsant properties have been reported also for other essential oils such as *Laurus nobilis* and *Cymbopogon winterianus* [20,21]. Although their active constituents have not been isolated to identify the responsible compound/s for the anticonvulsant activity, it

Seizure parameters	PTZ dose required to elicit individual seizure parameters (mg/kg)		
	Control (vehicle) n=5	Turmeric oil (50 mg/kg) n=5	Turmeric oil (100 mg/kg) n=5
Ear twitch	47.2 ± 6.3	67.7 ± 12.1	88.7 ± 15.4
Myoclonic twitch	58.9 ± 7.6	90.9 ± 23.4	108.8 ± 19.6
Tail twitch	58.9 ± 7.6	90.9 ± 23.4	101.6 ± 8.6
Forelimb clonus	74.5 ± 15.9	103.0 ± 18.5	147.6 ± 23.5
Falling	61.8 ± 10.7	86.2 ± 23.8	108.0 ± 20.3
Tonic hindlimb extension	105.6 ± 22.7	157.4 ± 35.8	208.4 ± 26.4
Death	128.8 ± 27.1	188.6 ± 36.2	237.8 ± 23.9

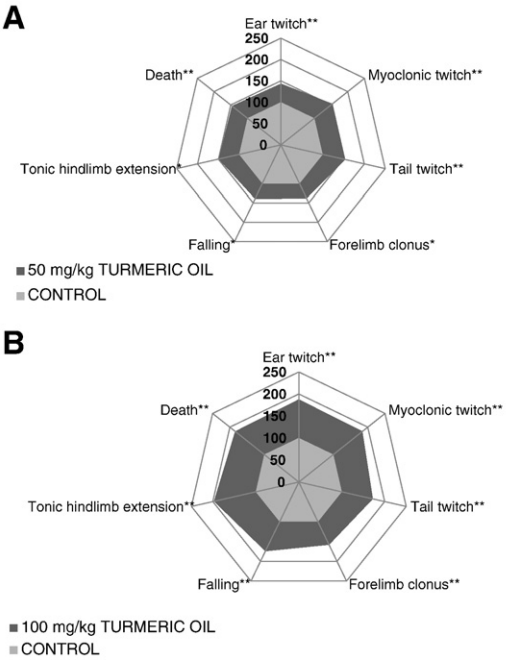

Fig. 5. Evaluation of the anticonvulsant activity of turmeric oil in the mouse PTZ seizure model. Top panel: table listing PTZ dose/s required to elicit the indicated seizure behaviors after treatment with turmeric oil or vehicle only. Data are expressed as the mean ± SD (n = 5). Graphical depiction of tabulated results from (A) turmeric oil at 50 mg/kg and (B) at 100 mg/kg. Results are expressed as relative values compared to control (set as 100%). Statistically significant differences between sample (dark gray) and control group (light gray) are labeled as * for $p<0.05$ and ** for $p<0.01$ (unpaired Student's *t*-test). For sake of clarity, SDs are not depicted in the graphs but are indicated in the tables. However, the coefficient of variation never exceeded 28% (unpaired Student's *t*-test).

Seizure parameters	PTZ dose required to elicit individual seizure parameters (mg/kg)			
	Control A (vehicle) n=5	AB-turmerone (100 mg/kg) n=5	Control B (vehicle) n=5	Ar-turmerone (50 mg/kg) n=5
Ear twitch	56.7 ± 11.4	98.3 ± 30.7	44.6 ± 11.6	70.5 ± 11.8
Myoclonic twitch	59.0 ± 11.2	112.6 ± 36.2	48.3 ± 11.7	74.1 ± 14.2
Tail twitch	58.0 ± 10.3	97.5 ± 30.0	48.3 ± 11.7	73.5 ± 13.9
Forelimb clonus	89.3 ± 12.1	137.4 ± 35.3	61.5 ± 11.0	121.7 ± 15.4
Falling	80.6 ± 17.1	140.2 ± 50.7	57.1 ± 15.8	95.5 ± 21.4
Tonic hindlimb extension	98.7 ± 22.5	215.2 ± 36.0	98.7 ± 23.3	131.5 ± 15.3
Death	122.1 ± 23.2	226.1 ± 30.2	122.1 ± 20.5	195.6 ± 49.2

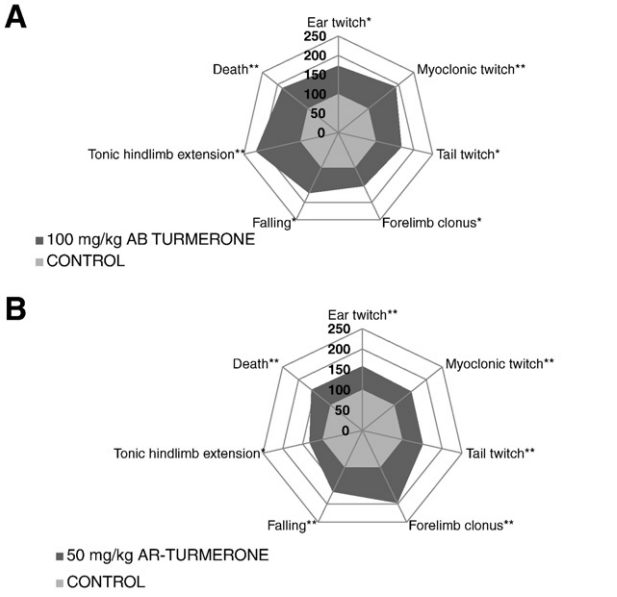

Fig. 6. Evaluation of the anticonvulsant activities of α,β-turmerone and ar-turmerone in the mouse PTZ seizure model. Top panel: table listing PTZ dose/s required to elicit the indicated seizure behaviors after treatment with bisabolene sesquiterpenoid or vehicle only. Graphical depiction of tabulated results from (A) α,β-turmerone at a dose of 100 mg/kg and (B) ar-turmerone at 50 mg/kg. 'Control A' column corresponds to vehicle-treated controls for α,β-turmerone; 'Control B' column corresponds to vehicle-treated controls for ar-turmerone. Data are expressed as the mean ± SD (n = 5). For sake of clarity, SDs are not depicted in the graphs but are indicated in the tables. Results are expressed as relative values compared to control (set as 100%). Statistically significant differences between sample (dark gray) and control group (light gray) are labeled as * for $p<0.05$ and ** for $p<0.01$ (unpaired Student's *t*-test). For the sake of clarity, SDs are not depicted. However, the coefficient of variation never exceeded 28% and 37% in the case of ar-turmerone and α,β-turmerone, respectively.

is known that some of their main constituents are monoterpenes, phenylpropenes and sesquiterpenes. These compounds present in essential oils, including the bisabolene sesquiterpenoids from *turmeric*, show a wide variety of functional groups and differences on their basic carbon skeletons. Since the identified chemical structures of any of the bisabolene sesquiterpenoids are not similar to the structure of the available AEDs, this chemical variability may perhaps increase the chances of identifying compounds acting through different mechanisms of action.

With regard to the mechanism of action of the bisabolene sesquiterpenoids, previous studies on the neuroprotective activity of turmeric oil have shown that these compounds can suppress oxidative DNA damage and lipid peroxidation in rodent models [18,19]. Pentylenetetrazole has been shown to increase glutamatergic transmission that, in turn, leads to an increase in intracellular calcium and cell death [40–42]. Even though the mechanisms of neuroprotection of AEDs have not yet been fully elucidated, several studies have revealed the potential of lamotrigine, levetiracetam, topiramate and zonisamide to limit DNA damage and restrict the extent of neuronal loss [40]. It is, therefore, tempting to speculate that the antioxidant properties of the bisabolene sesquiterpenoids are in some way related to their anticonvulsant activities. Clearly, further experiments are warranted in order to support this hypothesis.

The identification of the pharmacophore/s (functional chemical group/s) responsible for the anticonvulsant activity of ar-turmerone and α,β-turmerone will further lead to their target and the probable mechanisms involved in exerting their activity. It would be of particular interest if these pharmacophores display their action through a novel mechanism since this is the major aim of AED discovery. The main limitation regarding the discovery of novel AEDs is that most of them have been identified using the same models, especially MES [43]. This is probably the main cause for a misguided orientation that has led to the discovery of drugs that act against new-onset epilepsy but not against the refractory types. Therefore, it is currently in our interest to additionally assess the activity of the bisabolene sesquiterpenoids in other model/s of epilepsy such as the 6-Hz seizure test in mice, the hippocampal kindled rat model of temporal lobe epilepsy (TLE), etc.

With regard to its toxicity profile, turmeric has been widely used as a food condiment predominantly in India for centuries and is considered safe for human consumption [44]. Furthermore, toxicity studies performed in healthy human patients [45] and *in silico* analysis [46] have predicted ar-turmerone as a safe potential candidate for

further drug development. The absence of toxic effects was also confirmed in our experiments. Larval zebrafish exposed (for up to 24 h) to either turmeric oil or any one of the bisabolene sesquiterpenoids displayed normal heart rate and showed no signs of motor impairment as evidenced by a normal touch/escape response upon application of a tactile stimulus, no balance defects, and no signs of hypoactivity.

In conclusion, in this study, we demonstrate the usefulness of a zebrafish seizure model for rapid bioactivity-guided fractionation of natural products and their purified compounds for the identification of novel small molecules with anticonvulsant activity *in vivo*. Our findings suggest that the main bisabolene sesquiterpenoids of turmeric could be considered as interesting novel AED candidates that deserve further exploration. Current studies are now underway to test these compounds in additional seizure models and to identify the pharmacophore/s responsible for their anticonvulsant activity through structure–activity relationship (SAR) analysis.

Supplementary materials related to this article can be found online at doi:10.1016/j.yebeh.2012.02.020.

Acknowledgments

The authors wish to thank R. Ansaloni for her assistance regarding the microscopic characterization of turmeric used in the present study and N. Cuzco for her work in preparation of turmeric volatile oil. We are also grateful to I. Smolders and R. Clinckers for sharing their knowledge and experience with regard to performing the mouse PTZ assay and P. Verstreken and R. Habets for providing advice and access to their electrophysiology equipment. AMOP was funded through a fellowship from the Vlaamse Interuniversitaire Raad (VLIR)—"Pharmacological Characterization of Medicinal Plants from the South of Ecuador" Project.

References

[1] World Health Organization. Atlas: epilepsy care in the world. Geneva, Switzerland: WHO Press; 2005. 90 p.
[2] Schmidt D. Drug treatment of epilepsy: options and limitations. Epilepsy Behav 2009;15(1):56–65.
[3] Arroyo S, de la Morena A. Life-threatening adverse events of antiepileptic drugs. Epilepsy Res 2001;47(1–2):155–74.
[4] Carpay JA, Aldenkamp AP, van Donselaar CA. Complaints associated with the use of antiepileptic drugs: results from a community-based study. Seizure 2005;14(3):198–206.
[5] Arif H, Buchsbaum R, Weintraub D, Pierro J, Jr. SRR, Hirsch LJ. Patient-reported cognitive side effects of antiepileptic drugs: predictors and comparison of all commonly used antiepileptic drugs. Epilepsy Behav 2009;14(1):202–9.
[6] Meador KJ. Cognitive and memory effects of the new antiepileptic drugs. Epilepsy Res 2006;68(1):63–7.
[7] Schachter SC. Botanicals and herbs: a traditional approach to treating epilepsy. Neurotherapeutics 2009;6(2):415–20.
[8] Nsour W, Lau CB-S, Wong IC. Review on phytotherapy in epilepsy. Seizure 2000;9(2):96–107.
[9] Malawska B. New anticonvulsant agents. Curr Top Med Chem 2005;5(1):69–85.
[10] Willmore LJ, Losigamone, Dr Willmar Schwabe. Curr Opin Investig Drugs 2001;2(12):1763–6.
[11] World Health Organization. WHO monographs on selected medicinal plants. Geneva, Switzerland: WHO library cataloguing in publication data; 1999. 289 p.
[12] Jyoti A, Sethi P, Sharma D. Curcumin protects against electrobehavioral progression of seizures in the iron-induced experimental model of epileptogenesis. Epilepsy Behav 2009;14(2):300–8.
[13] Jithendra C, Murthy TE, Upadyay L. Protective role of curcumin in maximal electroshock induced seizures, memory impairment and neurotransmitters in rat brain. JPCCR 2008;2(1):35–9.
[14] Sumanont Y, Murakami Y, Tohda M, Vajragupta O, Watanabe H, Matsumoto K. Prevention of kainic acid-induced changes in nitric oxide level and neuronal cell damage in the rat hippocampus by manganese complexes of curcumin and diacetylcurcumin. Life Sci 2006;78(16):1884–91.
[15] Mehla J, Reeta KH, Gupta P, Gupta YK. Protective effect of curcumin against seizures and cognitive impairment in a pentylenetetrazole-kindled epileptic rat model. Life Sci 2010;87(19–22):596–603.
[16] Antony B, Merina B, Iyer VS, Judy N, Lennertz K, Joyal S. A pilot cross-over study to evaluate human oral bioavailability of BCM-95®CG (Biocurcumax™), a novel bioenhanced preparation of curcumin. Indian J Pharm Sci 2008;70(4):445–9.
[17] Sayyah M, Valizadeh J, Kamalinejad M. Anticonvulsant activity of the leaf essential oil of *Laurus nobilis* against pentylenetetrazole- and maximal electroshock-induced seizures. Phytomedicine 2002;9(3):212–6.
[18] Quintans-Júnior LJ, Souza TT, Leite BS, et al. Phytochemical screening and anticonvulsant activity of *Cymbopogon winterianus* Jowitt (Poaceae) leaf essential oil in rodents. Phytomedicine 2008;15(8):619–24.
[19] Dohare P, Varma S, Ray M. Curcuma oil modulates the nitric oxide system response to cerebral ischemia/reperfusion injury. Nitric Oxide 2008;19(1):1–11.
[20] Dohare P, Garg P, Sharma U, Jagannathan NR, Ray M. Neuroprotective efficacy and therapeutic window of curcuma oil: in rat embolic stroke model. BMC Complement Altern Med 2008;8:55.
[21] Rathore P, Dohare P, Varma S, Ray A, Sharma U, Jagannathan NR. Curcuma oil: reduces early accumulation of oxidative product and is anti-apoptogenic in transient focal ischemia in rat brain. Neurochem Res 2008;33(9):1672–82.
[22] Crawford AD, Esguerra CV, de Witte PA. Fishing for drugs from nature: zebrafish as a technology platform for natural product discovery. Planta Med 2008;74(6):624–32.
[23] Tan JL, Zon LI. Chemical screening in zebrafish for novel biological and therapeutic discovery. Methods Cell Biol 2011;105:493–516.
[24] Lockwood B, Bjerke S, Kobayashi K, Guo S. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol Biochem Behav 2004;77(3):647–54.
[25] Zon L, Peterson R. *In vivo* drug discovery in the zebrafish. Nat Rev 2005;4(1):35–44.
[26] Baraban SC, Taylor MR, Castro PA, Baier H. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 2005;131(3):759–68.
[27] Berghmans S, Hunt J, Roach A, Goldsmith P. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res 2007;75(1):18–28.
[28] Winter MJ, Redfern WS, Hayfield AJ, Owen SF, Valentin J-P, Hutchinson TH. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. J Pharmacol Toxicol Methods 2008;57(3):176–87.
[29] Mandhane SN, Aavula K, Rajamannar T. Timed pentylenetetrazol infusion test: a comparative analysis with s.c. PTZ and MES models of anticonvulsant screening in mice. Seizure 2007;16(7):636–44.
[30] Sills GJ, Butler E, Thompson GG, Brodie MJ. Pharmacodynamic interaction studies with topiramate in the pentylenetetrazol and maximal electroshock seizure models. Seizure 2004;13(5):287–95.
[31] Jackson B, Snowdon D. Atlas of microscopy of medicinal plants, culinary herbs and spices. London, U.K.: Belhaven Press; 1990. p. 257 p.
[32] Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Eugene, Oregon: University of Oregon; 2000.
[33] He X-G, Lin L-Z, Lian L-Z, Lindenmaier M. Liquid chromatography–electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa*). J Chromatogr A 1998;818(1):127–32.
[34] Giardina WJ, Gasior M. Acute seizure tests in epilepsy research: electroshock- and chemical-induced convulsions in the mouse. In: Enna SJ, Williams M, Barret JF, Ferkany JW, Kenakin T, Porsolt RD, editors. Current protocols in pharmacology. Hoboken, NJ, USA: Wiley & Sons; 2009. p. 27–30.
[35] Scallier A, Massie A, Loyens E, et al. vGLUT2 heterozygous mice show more susceptibility to clonic seizures induced by pentylenetetrazol. Neurochem Int 2009;55(1–3):41–4.
[36] Kamal A, Shaheer Malik M, Azeeza S, Bajee S, Shaik AA. Total synthesis of (R)- and (S)-turmerone and (7S,9R)-bisacumol by an efficient chemoenzymatic approach. Tetrahedron Asymmetr 2009;20(11):1267–71.
[37] Uehara S-I, Yasuda I, Takeya K, Itokawa H. New bisabolane sesquiterpenoids from the rhizomes of *Curcuma xanthorrhiza* (Zingiberaceae). Chem Pharm Bull 1989;37(1):237–40.
[38] Friesen RW, Blouin M. Preparation of γ,δ-unsaturated β-ketophosphonates from tertiary α-allenic alcohols. The synthesis of (+/−)-(E)-α-atlantone. J Org Chem 1996;61:7202–6.
[39] Bharal N, Sahaya K, Jain S, Mediratta PK, Sharma KK. Curcumin has anticonvulsant activity on increasing current electroshock seizures in mice. Phytother Res 2008;22(12):1660–4.
[40] Ribeiro MCP, de Ãvila DS, Schneider CYM, et al. alpha-Tocopherol protects against pentylenetetrazol- and methylmalonate-induced convulsions. Epilepsy Res 2005;66(1–3):185–94.
[41] Lores Arnaiz S, Travacio M, Llesuy S, Rodríguez de Lores Arnaiz G. Regional vulnerability to oxidative stress in a model of experimental epilepsy. Neurochem Res 1998;23:1477–83.
[42] Willmore LJ. Antiepileptic drugs and neuroprotection: current status and future roles. Epilepsy Behav 2005;7(Suppl. 3):S25–8.
[43] Löscher W, Schmidt D. Modern antiepileptic drug development has failed to deliver: ways out of the current dilemma. Epilepsia 2011;52(4):657–78.
[44] Villegas I, Sánchez-Fidalgo S, Alarcón de la Lastra C. New mechanisms and therapeutic potential of curcumin for colorectal cancer. Mol Nutr Food Res 2008;52:1040–61.
[45] Joshi J, Ghaisas S, Vaidya A, et al. Early human safety study of turmeric oil (*Curcuma longa* oil) administered orally in healthy volunteers. J Assoc Physicians India 2003;51:1055–60.
[46] Balaji S, Chempakam B. Toxicity prediction of compounds from turmeric (*Curcuma longa* L). Food Chem Toxicol 2010;48(10):2951–9.