VIP Natural Products Very Important Paper

DOI: 10.1002/anie.201305382

Elucidating the Neurotoxicity of the Star Fruit**

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Patients suffering from chronic kidney disease (CKD) were intoxicated after ingestion of *Averrhoa carambola* (star fruit, SF).[1] Additional reports of intoxications justified a human health warning because of a large number of renal patients on dialysis and patients not currently on dialysis were at risk worldwide. Briefly, the main symptoms of intoxication owing to SF are intractable hiccups, mental confusion, prolonged seizures (status epilepticus, SE), and death.[2–4]

We mimicked the intoxication owing to SF in experimental animals using various protocols. First, we induced behavioral and electroencephalogram (EEG) epileptiform activity after giving crude SF extract from fresh commercial fruits by gavage to acute kidney injury (AKI) animals[5] or after application to the brain of control animals from the fraction of SF extract with convulsant activity[1] (see the Supporting Information). Therefore, despite the presence of preliminary protocols showing that crude SF extracts and oxalate-free convulsant-enriched fractions (CEF)[6] induced seizure activity when applied to the brains of rats, the molecular structure of the neurotoxin is still elusive. Thus, we performed a bioguided isolation of the toxin, which resulted in a compound we named caramboxin (Figure 1 A, **1**). This new phenylalanine-like molecule, which contains two carboxylic acid moieties, was characterized by spectroscopic analyses. Comparison of the observed accurate mass spectrum with theoretically calculated formulae for the base peak (signal at

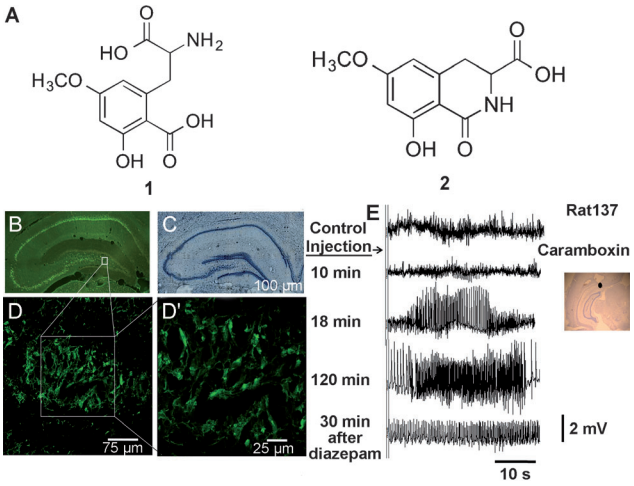

Figure 1. A) Structures of caramboxin (**1**) and inactive analogue **2**. B) Image of histochemical staining with FJ showing strong labeling (FJ+ cells) in the hippocampal formation and subiculum after caramboxin-induced SE. C) Nissl staining reveals the studied region and quality of tissue in a control animal. D) Selective FJ+ cells found in specific areas of the hippocampal formation such as the hilus (D, D′, zoom). E) Illustrative examples of the kinetics of EEG epileptiform events consequent to intracerebral microinjection (hippocampus–cortex transition; black dot in the histological inset) of caramboxin (rat 137). A potent and progressive epileptiform effect, observed over time, is significantly attenuated with the application of diazepam.

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[**] These studies honor the memory of the late Prof. Dr. Joaquim Coutinho-Netto, and were supported by FAPESP (09/51812-0), FAPESP-Cinapce, CNPq, INCT-if, CAPES, and PRONEX.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201305382.

m/z 256.0816) allows for only a few reasonable $[M+H]^+$ ion formulae within a standard deviation of 10 ppm. The ion formula $[C_{11}H_{13}NO_6+H]^+$ had the best mass accuracy (0.7 ppm error) and is in agreement with the NMR data. The MS/MS spectrum of the m/z 256 peak shows an intense ion at m/z 192, in addition to the classical competitive neutral H_2O (m/z 238) and CO_2 (m/z 212) elimination from the carboxylic acid group. In-source dissociation followed by MS/MS analysis revealed that the m/z 192 peak was only obtained from the m/z 238 peak and results from the elimination of CH_2O_2 (46 mass units) as a neutral molecule by ring contraction assisted by the lone electron pair on N (for more details, see the Supporting Information). ^1H and ^{13}C NMR data showed several signals similar to the amino acid phenylalanine with an uncommon aromatic substitution. 2D NMR data from HMQC and HMBC experiments confirmed the entire spectral assignment (see the Supporting Information), and the ^{15}N NMR data are in agreement with

the proposed structure illustrated in Figure 1 A (**1**). Finally, the negative $[\alpha]_{20\,°C/D}$ signal is in agreement with L-phenylalanine. When the toxin was stored at room temperature in water solution, it lost activity. The analysis of the degradation product (inactive compound) by MS indicated the formation of a closed ring analogue (Figure 1 A, **2**). To confirm this data, the chemical synthesis of the caramboxin analogue **2** was performed as reported[7] from dimedone in ten steps with a 7% overall yield. As expected, the closed compound **2** did not act as a neurotoxin like compound **1** (see the Supporting Information).

Crude lyophilized SF extract (20 mg mL^{-1}) and caramboxin (0.1 mg mL^{-1}) solubilized in saline solution were applied intracerebrally to normal animals. Thirty days after SE, animals that got the SF crude extract and those that got caramboxin were perfused, and their brains were removed and processed for histochemical analysis using the Fluoro-Jade (FJ) stain.[8] In Figure 1, the results of histochemical staining with FJ (parts B and D-D′) and Nissl (part C) are presented. See also the EEG effect in control animals microinjected with caramboxin into the hippocampus (Figure 1 E). The microinjection of caramboxin into the hippocampal formation/cortex transition area induced behavioral and strong EEG epileptiform activity in 100% (4/4) of the animals, which confirmed the excitatory effect of the treatment with the crude SF extract and CEF (see the Supporting Information). Figure 1 E illustrates an example of the evolution of the EEG, development of the SE, and the effect of diazepam after 90 min of SE. A similar EEG epileptiform effect was observed when ibotenic acid was applied to the same region (see the Supporting Information) in 100% (4/4) of the animals. Phosphate-buffered saline (PBS; control) microinjection did not induce any EEG or behavioral changes ($p < 0.0001$; Fischer exact test). Additional in vitro characterization of the effect of caramboxin and ibotenic acid confirmed the data from the in vivo studies. Application of CEF or caramboxin to hippocampal slices induced an inward current in pyramidal neurons (see images in the Supporting Information), which was reversed after washout (Figure 2 A) or after application of the ionotropic glutamate AMPA/kainate receptor antagonist DNQX (Figure 2 B). The same effect was observed when the slices were preincubated with the voltage-dependent sodium channel blocker tetrodotoxin (TTX) or the GABAergic ionotropic receptor antagonist bicuculline (Figure 2 C), which showed that the effect was not due to presynaptically released glutamate or glutamate-induced GABA release. When the slices were preincubated in the presence of DNQX, CEF produced only a small reversible inward current and an increase in the frequency of the GABAergic spontaneous inhibitory post-synaptic currents (sIPSCs), which was reverted by application of the *N*-methyl-D-aspartate (NMDA) glutamatergic receptor antagonist DL-AP5 (see Figure 2 D and examples of current recordings (traces) in the Supporting Information). This treatment

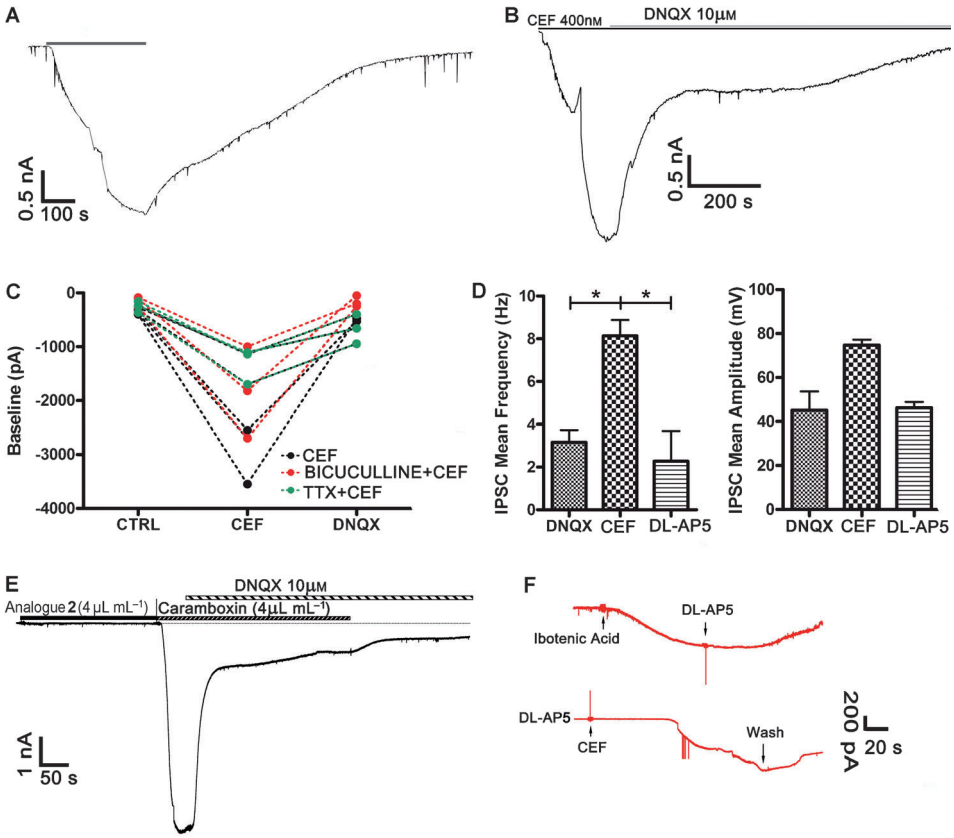

Figure 2. Demonstration of the excitatory effect of CEF (B), caramboxin (A), and **2** (E) in hippocampal slices. In 8 pyramidal neurons held at −70 mV from 5 animals, application of CEF at 400 nM or caramboxin caused an inward deflection of the current, which was reversed after washout (A; the gray line indicates the duration of the treatment with caramboxin) or application of the ionotropic glutamatergic AMPA/kainate receptor antagonist DNQX [10 μM; trace in (B) and black dots in (C)—control: −322.83 ± 25.66 pA, CEF: −2124.33 ± 397.12 pA, and DNQX:−544.00 ± 97.45 pA; P = 0.0006, $n = 6$]. The same effect was observed in the presence of TTX [1 μM; (C)—green dots; control: −150.00 ± 52.09 pA, CEF: −1840.33 ± 490.84 pA, and DNQX: −668.00 ± 157.09 pA; $P = 0.002$, $n = 3$] or with bicuculline [10 μM; (C)—red dots; control: −255.33 ± 60.07 pA, CEF: −1315.33 ± 192.58 pA, and DNQX: −668 ± 157.09 pA; $P = 0.172$, $n = 3$]. In (D), a drastic decrease of the 400 nM CEF effect after incubation with DNQX and recovery of baseline values after CEF washout was observed (DNQX = 3.159 ± 0.57 Hz and 45.12 ± 8.49 pA; CEF = 8.138 ± 0.74 Hz and 74.70 ± 2.50 pA; and DL-AP5 = 2.28 ± 1.41 Hz and 46.23 ± 2.67 pA. $P = 0.0193$ for frequency and 0.793 for amplitude). In (E), caramboxin shows a DNQX-sensitive effect that is not present in analogue **2**. The ibotenic acid effect after DL-AP5 is shown in (F).

shows that caramboxin is also able to activate NMDA receptors. Caramboxin presented a similar DNQX-sensitive effect to CEF, whereas the analogue **2** had no effect (Figure 2E). A similar effect was observed after the application of ibotenic acid (0.4 μM), which is an NMDA analogue and a potent neurotoxin structurally similar to caramboxin. The effect of ibotenic acid was antagonized by DL-AP5. However, when DL-AP5 was applied before CEF, it was not able to antagonize the effect of the toxin (Figure 2F, bottom trace), which confirmed that caramboxin acts on AMPA/kainate receptors. Taken together, the data confirm that the behavioral and EEG epileptiform effects of *Averrhoa carambola* (SF) and its neurotoxic (FJ+) effects cannot be fully associated with the occurrence of high levels of oxalic acid (CEF is devoid of oxalic acid). Therefore, the presence of caramboxin, a new molecule with strong glutamatergic receptor agonist properties, produces the observed brain hyperexcitability.

In conclusion, the structural characterization of the SF toxin, in addition to in vivo and in vitro functional experiments, point towards a glutamatergic ionotropic molecular action of caramboxin (a new nonpeptide amino acid toxin) with potent excitatory, convulsant, and neurodegenerative properties.

Experimental Section

Bioguided isolation and structural elucidation of caramboxin: Star fruit (10 kg) were harvested from trees that were not treated with pesticides, homogenized in distilled water 4:1 (w/v), and clarified by centrifugation before purification. The extract (800 mL) was applied to a DEAE-cellulose classic chromatographic column (5 × 33 cm) washed with ammonium bicarbonate buffer (2.0 L, 0.01 M, pH 7.8), and the active fraction (tested in mice) was eluted with 0.05 M and 0.15 M ammonium bicarbonate buffer (pH 7.8) at a flow rate of 7.0 mL min^{-1}. The active fractions (500 mg) were submitted to CM32-cellulose chromatography (2.5 × 40 cm) and eluted with ammonium bicarbonate buffer (0.2 M, pH 7.8) at a flow rate of 0.4 mL min^{-1}. The convulsant fraction (6 mg) was further fractionated on a reversed-phase HPLC semi-preparative column (Shimadzu, PREP-ODS II; 20 mm × 250 mm, 5 μm) using a linear gradient from 5 to 30% B phase in 20 min at a flow rate of 3.0 mL min^{-1}. The toxin was further purified on an analytical column (Merck, LiChroCART 100 RP-18; 4 mm × 125 mm, 5 μm) using a linear gradient from 10 to 40% B over 20 min at a flow rate of 0.8 mL min^{-1}, which resulted in 2.5 mg of caramboxin. All HPLC steps were performed with the following mobile phase system: Phase A: water and phase B: water/acetonitrile (35:65, v/v). The bioguided isolation protocol described above was applied as many times as necessary to obtain the desired amount of caramboxin. Accurate mass analyses were performed on an UltrOTOF (Bruker Daltonics; Billerica, USA). Solutions were infused into the Analytica ESI source at 100 μL h^{-1}. MS/MS was performed on the isolated protonated molecule ions by CID while applying N_2 as the collision gas. NMR spectra were acquired on a Bruker Avance DRX 400 (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C) equipped with a 5 mm probe head (DUL 13C-1) or on a Bruker Avance DRX 500 (500.13 MHz for ^{1}H and 50.70 MHz for ^{15}N) equipped with a 5 mm probe head (BBI 1H-BB). All NMR analyses were performed using standard pulse sequences supplied by the spectrometer manufacturer. Long-range ^{13}C/^{1}H chemical shift correlations were obtained in experiments with delay values optimized for ^2J (C, H) = 4, 8 and 12 Hz. All experiments were performed at 300 K, and the sample concentrations ranged from 8–12 mg mL^{-1} in CD_3OD, D_2O, or [D_6]DMSO. Nitromethane was used as an internal reference for ^{15}N NMR, and TMS was used as a reference for ^{1}H and ^{13}C NMR. The optical rotation was recorded on a JASCO DIP-370 polarimeter (*c* g mL^{-1}) set to the wavelength of Na (589 nm), and the IR spectra were obtained on a Nicole Protégé 460.

Video-EEG recordings: After SF intragastric and intracerebral treatments, the animals were placed in transparent acrylic observation cages and videotaped with a Handy Cam (Gradiente, Brazil) for six hours to evaluate the resulting behavioral or EEG seizures. Animals in the experimental groups that underwent intragastric and intracerebral treatment with SF crude extract, treatment with CEF and caramboxin, as well as animals that went into continuous seizures without intervals (SE), were rescued with an injection of diazepam (5 mg kg^{-1}) or with a second one, if needed. When the animals survived, they were observed for a longer period, after which they were anesthetized and prepared for histology (see below). For the hippocampal neurotoxin microinjections, Wistar rats were stereotaxically implanted with chemitrodes (cannulas coupled to monopolar electrodes) in the hippocampal formation under general anesthesia [ketamine (0.7 mg kg^{-1} Agener União Saúde Animal—Embu-Guaçu, SP, Brasil) and xylazine (1.0 mg kg^{-1} Bayer Saúde Animal—São Paulo, SP, Brasil)]. After a weeklong recovery, the animals were microinjected with crude SF extract or caramboxin (1.0 μL; 1.0 ug/1.0 μL), and the video-EEG was recorded according to Dutra-Moraes et al.[9]

Video-EEGs were recorded during the control, crude SF extract, CEF, and caramboxin injection protocols employing a signal conditioner (CyberAmp 320; Axon Instruments, CA, USA) coupled to an A/D converter (Biopac Systems Inc. MP100; CA, USA) and a microcomputer with Acqknowledge software (Biopac Systems Inc.; CA, USA). A specific system based on field effect transistors (FET) was used in the recording cable to reduce movement artifacts.[9] The EEG had the following parameters: sampling rate, 500 Hz; amplification, 10000 *x*; and filtering, 0.1 Hz-1 KHz. The signals were band-pass filtered off-line between 1–50 Hz. Initial analysis of the video-EEG was qualitative; however, whenever possible, we highlighted the coupling of behavioral[10] aspects and EEG activity.

Fluoro-Jade staining histochemistry: Animals were perfused after 30 days of SE, and histochemical staining with FJ was performed according to Schmued et al.[8] For better quality and conservation of tissue, the sections were mounted directly inside the cryostat onto gelatin-coated slides. Subsequently, the slides were stored in a freezer at −20 °C. The FJ staining procedure was performed as follows: the tissue was immersed in 100% ethanol for 3 min followed by 1 min in 70% ethanol and 1 min in distilled water. Afterwards, the slides were transferred to a solution of 0.06% potassium permanganate for 15 min and were gently shaken on a rotating platform. The slides were rinsed three times for 1 min in distilled water, transferred to the FJ staining solution and gently shaken for 30 min. The 0.0001% working solution of FJ was prepared by adding 1 mL of the stock FJ solution (0.01%) to 99 mL of 0.1% acetic acid in distilled water. After staining, the sections were rinsed three times (1 min) in distilled water, and the slides were coversliped.

Hippocampal slice preparation and recording: Wistar rats (p14–30) were anesthetized with isofluorane and ketamine 10% (2 g kg^{-1}) and transcardially perfused with ice-cold modified artificial cerebrospinal fluid (aCSF) solution (composition in mM: NaCl 125, KCl 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.25, glucose 25, $CaCl_2$ 0.2, and $MgCl_2$ 3 at pH 7.4 when bubbled with a carbogenic mixture). The animals were killed by decapitation, and the brains were rapidly removed and placed in ice-cold modified aCSF. Hippocampal horizontal slices (200 μm thick) were produced in a vibratome (vibratome VT1000Plus) and incubated in normal aCSF (composition in mM: NaCl 125, KCl 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.25, glucose 25, $CaCl_2$ 2, and $MgCl_2$ 1 at pH 7.4 when bubbled with a carbogenic mixture) at 35 °C for 40 min and at room temperature.

The slices were continuously perfused with normal aCSF at room temperature, and CA1 pyramidal neurons were visualized by IR-DIC using Normarski optics with a 63X water immersion objective (Olympus BX50; Tokyo, Japan). Membrane currents were recorded in whole-cell patch-clamp using borosilicate microelectrodes (3–5 mΩ) filled with a cesium chloride based internal solution (composition in mM: CsCl 140, EGTA 5, and HEPES 10 at pH 7.3 with CsOH). Neurons were held at −70 mV, and currents were acquired at 10 kHz and low-pass (Bessel 8-pole) filtered at 3 kHz using a HEKA EPC-10 patch-clamp amplifier. The fluorescent dye Lucifer Yellow (LY; 50 $\mu g\,mL^{-1}$) was added to the pipette solution. After the experiment, the recorded neuron was visualized with epifluorescence to confirm the morphology of the pyramidal neuron. The slice containing the LY-filled neuron was fixed in 4% paraformaldehyde for 20 min and mounted for further observation under a confocal microscope. The different drugs were applied directly in the bath from 1000× stock solutions. The bath perfusion and suction mechanisms were stopped, and the chamber was directly bubbled with a carbogenic mixture. Drugs were applied using a 2 μL pipette. TTX, bicuculline, and DL-AP5 were from Tocris, and ibotenic acid and 6,7-dnitroquinoxaline-2,3(1*H*,4*H*)-dione (DNQX) were from Sigma.

Received: June 22, 2013
Revised: September 6, 2013
Published online: November 7, 2013

Keywords: natural products · neurological agents · neurotoxins · receptors · star fruit

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