Evidence for Involvement of TRPV1 Receptors and Potassium Channels in the Seizures Induced by α -Sanshool

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Key words

Zanthoxylum liebmannianum (Rutaceae), seizures, TRPV1, potassium channels, isobutylamides

received 27.09.2018

revised 28.02.2019

accepted 01.03.2019

Bibliography

DOI <https://doi.org/10.1055/a-0871-2496>

Planta Med Int Open 2019; 6: 1–5

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ISSN 2509-9264

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ABSTRACT

α -Sanshool is an alkamide isolated from the stem bark of *Zanthoxylum liebmannianum*, a Mexican medicinal plant known as Colopahtle. Our research group has reported that the intraperitoneal administration of α -sanshool induces tonic-clonic seizures in mice. In the present study, we investigated the convulsive effect of this alkamide and elucidated its mechanism of action by comparing with well-known convulsive and anticonvulsive drugs in an *in vivo* approach. α -Sanshool showed a potent (ED_{50} [CL 95 %] = 3.06 [2.92–3.22] mg/kg) and immediate (2 ± 2 s) seizure effect after the intraperitoneal administration in mice. The convulsive effect of this alkamide was only observed for intraperitoneal administration; the oral route did not show any effect. α -Sanshool was less potent than strychnine (ED_{50} [CL 95 %] = 1.53 [1.44–1.62] mg/kg), but more effective than bicuculline, 4-aminopyridine, affinin, and pentylenetetrazol, in that order. The seizures induced by α -sanshool were reduced by capsazepine and diazoxide, suggesting the involvement of TRPV1 and potassium channels in the mechanism of action of this compound.

Supporting Information for this article is available online at <http://www.thieme-connect.de/products>.

ABBREVIATIONS

CNS	central nervous system
GC/MS	gas chromatography coupled to mass spectrometry
ICR	Institute of Cancer research
K _{2P}	2-pore domain potassium channels
K _{ATP}	ATP-dependent potassium channels
TRPV1	transient receptor potential vanilloid type 1
TRPA1	transient receptor potential ankyrin type 1

Introduction

Alkamides are common constituents in plants of the *Echinaceae*, *Zanthoxylum*, *Capsicum*, *Piperaceae*, *Spilanthes*, *Acmella*, and *Heliopsis* genera [1]. Several plants of the genus *Zanthoxylum* (Rutaceae) are used in the traditional medicine of native cultures from Africa, America, and Asia [2]. These compounds have a diversity of biological activities, including insecticidal, antibacterial, antifungal, antiparasitic, analgesic, and local anesthetic [3–5]. In the Brazilian Amazon region, *Acmella oleracea* (L.) R.K. Jansen, which contains alkamides, has been reported as an aphrodisiac [6]. Also, some alkamides and the plants that provide them are used as flavoring in foods and beverages due to the unique tingling and numbing orosensations that they produce. For example, the Brazilian alcoholic beverage Cachaça de Jambu contains an extract of *A. oleracea*, in which the alkamide spilanthol is a major metabolite [6]. In recent years, the academic and industrial interest for alkamides has remarkably increased, both for their medical and cosmetic applications and because of their biological mechanisms of action [3]. Sanshools are the predominant alkamides in the *Zanthoxylum* genus [3]. The stimulation of transient receptor potential ankyrin 1 (TRPA1) and TRP vanilloid 1 (TRPV1) channels, the blockage of 2-pore domain potassium channels KCNK3 (TASK-1), KCNK9 (TASK-3) and KCNK18 (TRESK), as well as the antagonism of the cannabinoid receptors 1 (CB1) and 2 (CB2) and the agonist effect on CB2, have been suggested as biomolecular targets for these compounds [3]. In a previous study, we reported the isolation of α -sanshool (► Fig. 1) from the stem bark of *Zanthoxylum liebmannianum* (Engelm.) P.Wilson (Rutaceae) and its anthelmintic effect in naturally infected sheep [4]. In that work, we also noticed that the intraperitoneal injection of this compound induced tonic-clonic seizures in mice. Although low toxicity is generally associated with natural and synthetic alkamides, their convulsive effect in mice was recently reported [7]. This is of great significance due to the growing medical and cosmetic interest in plant extracts containing these compounds, even though little is known about their toxic effects. Accordingly, we were interested in knowing the convulsive potency of α -sanshool, a natural alkamide. In this work, we compared the seizures induced by α -sanshool with those caused by 4 known convulsive drugs: bicuculline (GABA antagonist), 4-aminopyridine (voltage-activated potassium channels KCND2/KCND3 blocker), strychnine (glycine antagonist) and pentylenetetrazol (GABAergic blocker), and also with the effect of affinin (spilanthol), isolated from *Heliopsis longipes* (A. Gray) S.F. Blake (Asteraceae) roots. We also investigated the inhibition of the α -sanshool-induced seizures by diazepam and pentobarbital (positive allosteric modulators of GABA_A receptors), diazoxide (ATP-sensitive potassium channels acti-

vator), ethosuximide (low-threshold T-type Ca²⁺ channels blocker), capsazepine (TRPV1 antagonist), and carbamazepine (voltage-gated sodium channels blocker).

Results

► Fig. 2 shows the dose-response curves for seizures induced by the intraperitoneal administration of increasing doses of the convulsive drugs affinin and α -sanshool. As seen in the plots, the seizure incidence increased with increasing dosage. α -Sanshool was less effective than strychnine (► Table 1) but more potent than bicuculline, 4-aminopyridine, affinin, and pentylenetetrazol, in that

► Fig. 1 Structure of α -sanshool.

► Fig. 2 Dose response curves for seizures induced by the intraperitoneal administration of α -sanshool ($\circ$), strychnine ($\blacktriangle$), bicuculline ($\blacksquare$), 4-aminopyridine ($\bullet$), affinin ($\square$), and pentylenetetrazol ($\blacktriangledown$). The graph represents the decimal logarithm of the doses used for each drug vs. probit. Each symbol represents the probit, derive from the cumulative frequency of animals that present seizures/total of animals (30) for each dose.

order. The latency to the first seizure induced by this alkaloid was extremely short, only 2 s (► **Table 1**), with a mortality of 100 %. Contrastingly, the oral administration of α -sanshool at the same doses than those used for the intraperitoneal injections did not induce seizures. The seizure incidence and the mortality induced by α -sanshool at 9 mg/kg were reduced by 84 % when administering capsazepine (50 mg/kg, s.c.). Diazoxide reduced them by 44 % (at 10 mg/kg, i.p.) and 50 % (at 100 mg/kg, i.p.). Diazepam and pentobarbital were effective (66 % reduction) only at very high doses. Carbamazepine and ethosuximide did not inhibit the α -sanshool-induced seizures (► **Table 2**).

Discussion

α -Sanshool, a compound found in the stem bark of *Z. liebmannianum* [4, 8], showed potent and immediate tonic-clonic seizures in mice. This effect was partially inhibited by capsazepine, diazoxide and high doses of diazepam and pentobarbital (► **Table 2**). The powerful ($ED_{50} = 3$ mg/kg) and immediate (2 s) seizure-inducing effect showed by this compound differs from the pattern of the other convulsive drugs tested (► **Table 1**). This effect, first observed for α -sanshool [3, 4], may be a pharmacological property of alkaloids in general when they are intraperitoneally administered. This is consistent with a recent report for affinin (90 mg/kg, i.p.), capsaicin (90 mg/kg, i.p.) and the synthetic alkaloid *N*-isobutyl-dihydroferuloylamide (180 mg/kg, i.p.) in mice [7]. In that work, the authors found that affinin did not induce seizures at doses lower than 90 mg/kg (i.p.), though we observed its convulsive effect from 20–100 mg/kg in mice ($ED_{50} = 51.74$ mg/kg, i.p., ► **Fig. 2**). It is worth noting that the oral administration of α -sanshool did not induce seizures, suggesting either its poor absorption or fast metabolism. However, in a pilot pharmacokinetic study in humans, 2 alkaloids with a structure similar to that of α -sanshool (hydroxy- α -sanshool and hydroxy- β -sanshool) were absorbed after the oral administration of an extract *Zanthoxylum* spp. fruits, but the rate of absorption for these compounds was not reported [9]. Therefore, additional studies are required to explain the absence of seizure effects in mice when α -sanshool is orally administered.

The seizures and mortality induced by α -sanshool were reduced to only 16 % by the TRPV1 antagonist capsazepine at 50 mg/kg (s.c.) (► **Table 2**). In an additional experiment, the seizures induced by affinin at 80 mg/kg were completely inhibited by capsazepine (50 mg/kg, s.c.). TRPV1 channels, which are considered partially responsible for epileptic seizures and peripheral pain [10], have

been suggested as a biomolecular target for alkaloids [3]. Therefore, the partial inhibition of the α -sanshool-induced convulsions by the TRPV1-antagonist capsazepine suggests that this alkaloid induces the seizures, at least in part, by stimulating the TRPV1 channels (► **Table 2**). It is possible that convulsions induced by affinin, capsaicin, and the synthetic alkaloid reported by de la Rosa et al. [7] also occur via the same mechanism. Additional experimental data are required to assess this statement.

K_{2P} channels have also been suggested as molecular targets for alkaloids [3]. These channels are widely expressed in the central and peripheral nervous system, and their blockage has been associated with local anesthetic-induced seizures [11]. In a previous work, it was reported that the convulsions induced by the local anesthetic bupivacaine (50 mg/kg, i.p.), a blocker of K_{2P} channels, were reduced by intraperitoneal administration of diazoxide (10 and 100 mg/kg), which activates K_{ATP} channels [12]. Here, we found that diazoxide reduced the α -sanshool induced seizures (► **Table 2**) at the same doses than those used to reduce the bupivacaine-induced seizures [12]. Furthermore, seizures induced by 4-aminopyridine, a blocker of voltage-activated potassium channels KCND2/KCND3, were suppressed by capsazepine [13]. Therefore, a cross-effect between the 2-pore domain (K_{2P}) and voltage-activated potassium channels may be possible. These findings suggest that seizures-induced by α -sanshool result from a decrease K^+ efflux from nervous tissue.

Moreover, the doses of diazepam and pentobarbital required to reduce the seizures induced by α -sanshool were very high (► **Table 2**), and the immediate convulsive effect imply a peripheral effect rather than a central one. These results indicate that α -sanshool did not act on the GABA_A receptor. Contrastingly, the participation of GABA_A receptors has been suggested in the antinociceptive effect of the alkaloid affinin [14]. Also, it has been reported that GABA_A receptors are involved in the ototoxicity of local anesthetics because of the efficacy of benzodiazepines when treating central tinnitus, in which K_{2P} channels are implicated [15]. An association between GABA_A receptors and

► **Table 1** Median effective doses 50 (MD50, mg/kg) and 95 % confidence limit (CL) for the evaluated convulsive drugs.

Drug	ED50 (mg/kg)	CL 95 %	Latency of onset (s)
Strychnine	1.53	1.44–1.62	184 ± 32
α -Sanshool	3.06	2.92–3.22	2 ± 2
Bicuculline	4.78	4.55–5.03	76 ± 6
4-Aminopyridine	12.10	11.63–12.50	364 ± 34
Affinin	51.74	47.07–56.33	332 ± 12
Pentylenetetrazol	73.61	70.94–76.37	82 ± 11

► **Table 2** Effect of several anticonvulsant drugs and diazoxide on the seizures induced by intraperitoneal administration of α -sanshool at 9 mg/kg.

Drug	Dose (mg/kg)	N	Seizures		Mortality (%)
			Response/total	Percent	
Diazepam	2.0	6	6/6	100	100
	2.5	6	6/6	100	100
	5.0	6	6/6	100	100
	8.5	6	6/6	100	100
	10.0	6	2/6	33	33
Pentobarbital	42.0	6	6/6	100	100
	63.0	6	2/6	33	33
Diazoxide	10.0	6	4/6	66	66
	100.0	6	3/6	50	50
Capsazepine	50.0	6	1/6	16	16
Carbamazepine	100.0	6	6/6	100	100
	200.0	6	6/6	100	100
Ethosuximide	150.0	6	6/6	100	100

the alkamides acting on potassium channels is likely. However, further experimental support is needed to establish this association.

Based on the above, α -sanshool and other natural or synthetic alkamides can be a useful pharmacological tool to study the seizures induced by the stimulation of TRPV1 or by the blockage K_{2P} and K_{ATP} channels.

In conclusion, we found that intraperitoneally administered α -sanshool induces seizures with high potency ($ED_{50} = 3 \text{ mg/kg}$) and an extremely short latency (2 s). These seizures are reduced by capsazepine and diazoxide, suggesting the involvement of TRPV1 and potassium channels in the convulsant mechanism of this alkamide. Rather than an effect on the CNS, the drug may have a peripheral effect on nervous tissue.

^1H -NMR and GC/MS spectra of α -sanshool, and affinin are available as Supporting Information.

Materials and Methods

General

^1H -NMR (400 MHz) and ^{13}C -NMR (100 MHz) spectra were registered on a Varian VNMRs 400 spectrometer in CDCl_3 with tetramethylsilane as internal standard. GC/MS was performed using an Agilent chromatograph (6890N, Agilent Technologies) equipped with a time-of-flight mass spectrometric detector (LECO) under the following conditions: DB-5MS capillary column (20 m $\times$ 0.18 mm, film thickness 0.18 μm); He as carrier gas (1 mL/min flow rate), injection in split mode (1:400); injector at 300 $^\circ\text{C}$. For the MS, the conditions were as follows: electron impact ionization voltage, 70 eV; ion temperature source, 200 $^\circ\text{C}$; mass scan mode, 20 scan/s; mass range, 45–550 m/z.

Plant material

Dried roots of *H. longipes* were kindly provided by Laboratorios Mixim S.A. de C.V. (Mexico City, Mexico). They were harvested from Mixim's growing fields in July 2015 (Batch number 6702). A voucher specimen (number 2015011) was deposited at the herbarium of the Universidad Autónoma Chapingo, Mexico. The stem bark of *Z. liebmannianum* was collected at Valle de Tehuacán, Puebla, Mexico, in October 2015. A voucher specimen (number 2015126) was also deposited at the herbarium.

Extraction and isolation

Isolation of affinin

Dried roots of *H. longipes* (1012 g) were ground and extracted with a mixture of dichloromethane-acetone (9:1 v/v) in a Soxhlet apparatus for 4 h. The extract was filtered through Whatman paper (No. 2) and the solvent evaporated in a rotatory evaporator under reduced pressure at 40 $^\circ\text{C}$. The solvent-free extract was a dark-yellow semisolid (19.3 g, 1.91 %) and was stored at 4 $^\circ\text{C}$ under nitrogen. Batches of 2 g each were fractionated by open column chromatography using silica gel (40 g, Kieselgel 60 Merck 100–230 mesh, normal phase, 2 $\times$ 100 cm), using hexane:ethyl acetate as eluent (9:1, 8:2, 7:3, and 4:6). Sixty fractions of 10 mL each were collected. The fractions eluted with the 8:2 system was analyzed by TLC; after spraying with acidic anisaldehyde (0.5 mL of anisaldehyde in 50 mL gla-

cial acid and 1 mL 97 % sulfuric acid), the same purple spot was observed for all of them ($R_f = 0.3$, hexane: ethyl acetate 6:4). These fractions (700 mg, yellow oil) were then subjected to a second chromatographic column (10 g, Kieselgel 60 Merck 100–230 mesh, normal phase, 1 $\times$ 100 cm) using hexane:ethyl acetate (9:1). An apparently pure yellow oil (320 mg) was obtained; it had the same R_f than an authentic affinin sample, which was kindly provided by Dr. José Fausto Rivero (Facultad de Química UNAM). The identity of affinin was established by comparing the experimental NMR spectroscopic constants with those reported in the literature [8, 16]. The purity of affinin (97 %) was determined by GC/MS.

Isolation of α -sanshool

Air dried *Z. liebmannianum* stem bark (2 kg) was ground to a fine powder and subjected to extraction by maceration at room temperature ($22 \pm 2^\circ\text{C}$) with either hexane or dichloromethane for 3 d in a 1:4 ratio (w/v). The extracts were filtered, and the solvents evaporated under reduced pressure at 40 $^\circ\text{C}$, yielding 58 (hexane) and 141 (dichloromethane) g of syrupy residues. Part of the dichloromethane extract (31 g) was further fractionated by open column chromatography (300 g, Kieselgel 60 Merck 100–230 mesh, normal phase, 5 $\times$ 100 cm) using dichloromethane:ethyl acetate (9:1, 8:2 and 1:1). Eighty fractions of 100 mL each were collected. Fractions 29–46 (4 g) eluted with the 9:1 system were pooled and re-chromatographed (40 g, Kieselgel 60 Merck 100–230 mesh, normal phase, 2 $\times$ 100 cm) with dichloromethane:ethyl acetate 9:2. Ninety fractions of 20 mL each were collected. From fractions 14 to 65, a yellow oil (635 mg, 0.14 %) was obtained. This oil was identified as α -sanshool by comparison of the NMR spectral data with those reported for this amide [17]. The purity of α -sanshool (94 %) was established by GC/MS.

Animals

All the experiments were performed on adult male ICR mice weighing 25–34 g (60 d old), purchased from Centro UNAM-Harlan (Harlan México, S.A. de C.V.). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. Furthermore, clearance for conducting the studies was obtained from the Ethics Committee for the Use of Animals in Pharmacological and Toxicological Testing (CICUAL/126/16, January 2016), Facultad de Química, UNAM. During the development of the experiments, the animals had free access to water and food, with a 12 h light-dark cycle at room temperature ($22 \pm 2^\circ\text{C}$). Experiments were carried out between 9:00 and 15:00.

Drugs

Pentylentetrazol (P6500, $\geq 99\%$), strychnine (S0532, $\geq 98\%$), 4-aminopyridine (275875, $\geq 99\%$), ethosuximide (E7138, $\geq 98\%$), and diazoxide (D9035, minimum 98 %) were dissolved in saline solution (0.9 %). Bicuculline (14340, $\geq 97\%$) was dissolved in phosphate buffered saline solution (pH = 7.5), carbamazepine (C4024, $\geq 98\%$) in propylene glycol (10 % in saline solution), and capsazepine (C191, $\geq 98\%$) in DMSO (10 % in saline solution). All the drugs were purchased from Sigma Aldrich. A pharmaceutical solution of sodi-

um pentobarbital (Anestesal) for veterinary use was purchased from Pfizer S.A. de C.V. (México). Diazepam (Roche S.A.) and affinin (97 % by GC/MS), isolated from *H. longipes* roots, were suspended in 0.5 % Tween 80 in saline solution. α -Sanshool (94 % by GC/MS), obtained from the stem bark of *Z. liebmannianum*, was dissolved in DMSO (10 % in saline solution). The drugs were freshly prepared each time and intraperitoneally injected in a volume of 0.1 mL/10 g body weight.

Convulsive effect

As a measure of the central nervous system toxicity, we quantified the effect of increasing doses of the drugs on the induction and latency of onset of seizures in individual male ICR mice (n = 30 each). The dosage was chosen based on preliminary data to cover a range that encompassed doses that caused no observable seizure to those that consistently evoked seizures: α -sanshool 1.5–9.0 mg/kg, at 0.5 mg/kg intervals, pentylentetrazol 55–100 mg/kg, at 5 mg/kg intervals, strychnine 0.9–3.0 mg/kg, at 0.1 mg/kg intervals, 4-aminopyridine 10–18 mg/kg, at 1.0 mg/kg intervals, affinin 20–100 mg/kg, at 10 mg/kg intervals, and bicuculline 3.0–7.5 mg/kg, at 0.5 mg/kg intervals. Increasing doses of each drug were injected intraperitoneally; in some cases, mice received only a single dose. The incidence of tonic-clonic seizures and latency at first seizure were recorded. Seizure signs were characterized by whole-body jumps or bursts of running motions (clonic seizure) and rigidity with forelimbs and hindlimbs caudally extended (tonic seizure). A series of quantal dose-response curves were fitted and statistically analyzed using probit analysis [18]. In addition, the ability to inhibit the α -sanshool-induced seizures was determined for the following drugs: diazepam (2.5, 5, 8.5, and 10 mg/kg, i.p.) and pentobarbital (42 and 63 mg/kg, i.p.) doses experimentally determined; carbamazepine (100 and 200 mg/kg, i.p.), ethosuximide (150 mg/kg, i.p.), and capsazepine (50 mg/kg, s.c.) doses based on the work by González-Reyes et al. [13]; and diazoxide (10 and 100 mg/kg, i.p.) doses based on the work by Gantenbein et al. [12]. The drugs were administered 30 min before α -sanshool (9 mg/kg, i.p.) in groups of 6 animals each.

Supporting Information

H-NMR and GC/MS spectra of α -sanshool, and affinin are available as Supporting Information.

Acknowledgments

This work was supported by grants PAIP5000–9143 from Facultad de Química and PAPIIT-IN216516 from Dirección General de Asuntos del Personal Académico (DGAPA), Universidad Nacional Autónoma de México (UNAM).

Conflict of Interest

The authors have no conflicts of interest to declare.

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