CIENCIA 17(1), 25 - 32, 2009
Maracaibo, Venezuela

Activity of acnistins against *Leishmania mexicana, Trypanosoma cruzi* and *Plasmodium falciparum*

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Recibido: 25-01-08 Aceptado 20-02-09

Abstract

Acnistin A and E, two withanolides with a bicyclic side-chain at C-17, isolated from the leaves of *Acnitus arborescens* (L) Schlecht that grows in the Venezuelan Andes inhibited the growth of Leishmania mexicana and Trypanosoma cruzi *in cultured medium. On the other* hand *acnistins A, E,* and L, also inhibited the growth of a chloroquine-resistant strain and a chloroquine-sensitive strain of Plasmodium falciparum in cultured medium. Data indicated that acnistins exerted a higher inhibitory activity upon these parasites that ketoconazole, mevastatin and lapachol in the same conditions of assay.

Key words: acnistins, withanolides, L*eishmania mexicana, Trypanosoma cruzi, Plasmodium falciparum.*

Actividad de acnistinas contra *Leishmania mexicana, Trypanosoma cruzi* y *Plasmodium falciparum*

Resumen

Acnistina A y E, dos withanólidos con una cadena bicíclica en C-17, aislados de las hojas del *Acnistus arborescens* (L) Schlecht que crece en Los Andes venezolanos inhibió el crecimiento de *Leishmania mexicana* y *Trypanosoma cruzi* en medio de cultivo. Por otra parte, las acnistinas A, E y L también inhibieron el crecimiento de una cepa cloroquina-resistente y de una cepa cloroquina-sensible de *Plasmodium falciparum* en medio de cultivo. Nuestros datos indicaron que las acnistinas ejercieron una mayor actividad inhibitoria sobre esos parásitos que ketoconazol, mevastatina y lapachol en las mismas condiciones de ensayo.

Palabras clave: acnistinas, withanólidos, *Leishmania mexicana, Trypanosoma cruzi, Plasmodium falciparum.*

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Introduction

The protozoan hemoflagellates belonging to the order kinetoplastida, which comprises the genera *Trypanosoma* and *Leishmania* are the causative agents of a number of important diseases in humans, such as sleeping sickness in tropical Africa, Chagas´ disease in South America, and the different clinical manifestations of leishmaniasis in almost all tropical and subtropical parts of the world. Together with malaria, it constitutes a public health problem around the world. Even when several drugs have been developed such as the nitrofuran Nifurtimox and nitroimidazoles as benznidazole for Chagas´ disease, the pentavalent antimonials sodium stibogluconate and N-methylglucamine antimonite for leishmaniasis and artemisin and analogs for malaria, most of them are toxic or lethal, requiring prolonged treatment (1).

The withanolides are a group of natural steroidal lactones, isolated from several species of *Solanaceae* (2), characterized by a 28 carbon skeleton with a 9 carbon atom side chain and a 6-member lactone ring. Using the approach of testing of different natural natural antitripanocidal, antileishmanicidal and antimalarial compounds, the analysis of the effects of acnistins, whitanolide-type lactones, was proposed because some whitanolides (steroidal lactones derived of ergostane) show biological activity of medicinal value (3). There are evidences that withanolides show antitumoral (4), anti-inflammatory (5), cytotoxic (6), immunosuppresive (7), antihyperglicemic (8) and antifungal (9) activities. Also, it has been demonstrated that some of them elicits humoral and cell-mediated inmune response (10), inhibits cell proliferation and induces apoptosis (11) and exhibits antileishmanial and trypanocidal activity (12). Acnistin A **(1)**, E **(2)** and L **(3)**, are three withanolide-type lactones, with a bicyclic side-chain at C-17 that have been isolated from *Acnitus arborescens* (L) Schlecht and *Dunalia solanacea* Kunth (13-15). In the present study, the anti-parasite activity of acnistins A and E against *Leishmania mexicana*, strain AZV and acnistins A, E and L against *Trypanosoma cruzi*, strain EP, and *Plasmodium falciparum* F32 and FcM29 strains *in vitro* is reported. The activity of acnistins upon these parasites was compared to those of ketoconazole, an antimycotic agent, lapachol and mevastatin which have been reported as active antiparasite compounds (16-21).

Materials and methods

General experimental procedures

Melting points were measured on a Fisher-Johns hot stage, and they are uncorrected. Specific rotations $[\alpha]_D$ were measured at the sodium-D line using a Jasco electropolarimeter model DIP-370; the concentration c, is given in g/100 mL. ^1H and ^{13}C-NMR measurements were performed on a Bruker Avance DRX-400. For TLC Merck 60 F_{254} plates were used and as solvent a mixture of C_6H_6: EtOAc (1:1) was employed. Columns for vacuum chromatography were packed with TLC grade Merck Silica gel 60 H and eluted with C_6H_6 and C_6H_6/EtOAc mixtures. All the chemicals and reagents used in the present studies were of an analytical grade. Ketoconazole, lapachol and mevastatin were obtained from Sigma Biochemical Company. Organic solvents were freshly distilled before use.

Extraction and purification of acnistin A, E and L

Acnistin A **(1)**, E **(2)** and L **(3)** were isolated from the leaves of *Acnistus arborescens* according to the method used by Usubillaga *et al.* (13). Dry ground leaves (3.5 kg) were extracted with 8.0 L of methanol at room temperature. The methanolic extract was concentrated to half its original volume, diluted with an equal volume of water and extracted several times with hexane to eliminate chlorophyll. After hexane treatment the aqueous methanolic phase was shaken with diethyl ether. The diethyl ether layer

was evaporated to dryness to yield 62 g of crude extract and chromatographed in an open column over silica gel. Elution started with benzene followed by benzene: ethyl acetate mixtures. Acnistin A (345 mg, **1**) was eluted with C_6H_6:EtOac (1:1) and subsequently the same solvent eluted a 5.42 g mixture from which acnistins E (3.87 g, **2**) and L (260 mg, **3**) were separated by vacuum chromatography over silica gel. The identity of acnistin A, E, and L was established by direct comparison with authentic control samples (TLC, mp and NMR spectra) (13,14). The chemical structure of acnistins A, E and L is shown in figure 1.

Parasites

Leishmania mexicana, strain AZV, and *Trypanosoma cruzi (Schizotrypanum)*, strain EP, were both growth as promastigotes and epimastigotes, in a modified liquid liver-infusion tryptose medium (LIT) containing 7.5% heat inactivated fetal calf serum and 20 μg/mL hemine at 27°C. The cultured *Leishmanias* and *Trypanosomes* had all characteristics of the *in vivo* forms including morphology, antigenic variation, infectivity and glucose metabolism. A chloroquine-resistant strain FcM29 of *Plasmodium falciparum* and a nigerian chloroquine-sensitive F32 strain were cultured continuously (22).

Biological assays *in vitro*

Culture and maintenance of *Leishmania mexicana*. Promastigote forms of *Leishmania Mexicana* were grown at 28°C in liver infusion tryptose medium (LIT) containing 10% heat inactivated (56°C for 30 min) fetal bovine serum. Promastigote cultures in the logarithmic phase of growth were maintained by transferring 10^6 cells per mL. Acnistin A and E, ketoconazole, lapachol and mevastatin were added to the medium in variable concentrations. The activity of the compounds was evaluated every day during 9 days. Parasites were counted in a haemocytometer every day (23) and the inhibition of *Leishmania mexicana* growth was calculated from the percentage of inhibition of growth determined to each concentration of compounds used in the assay. The percentage of inhibitory growth activity was calculated with respect to the control without assay compounds. The 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentration were chosen for the comparison of susceptibili-

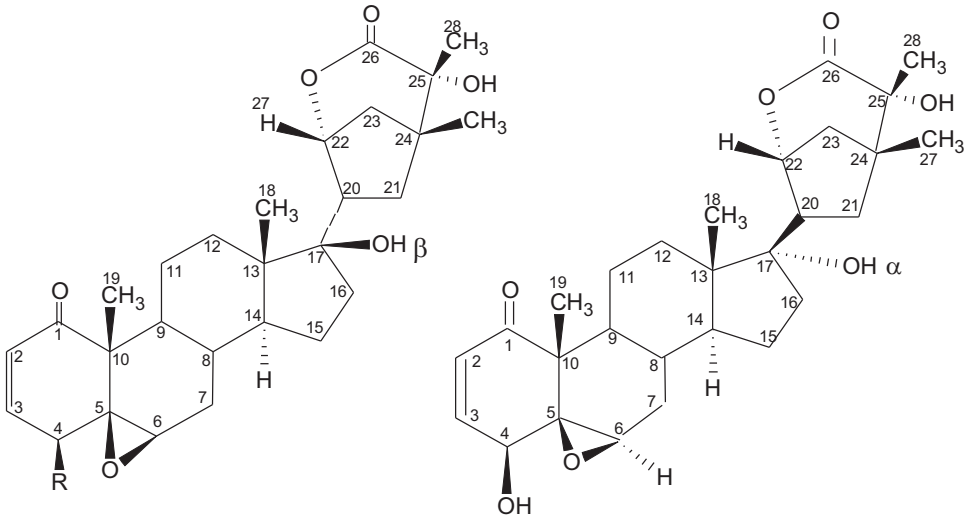

Figure 1. Structure of acnistins A (1), E (2), and L (3).

ties of the strains to drugs tested. Ketoconazole, an inhibitor of sterol biosynthesis in *Leishmania mexicana* (16), *Trypanosoma cruzi (17)* and *Plasmodium falciparum* (18) was used as reference drug and lapachol and mevastatin, which act both upon *Leishmanias* (19), *Trypanosomes* and *Plasmodium* (20, 21) were used as comparison drugs for measuring the efficacy of the acnistins. Each assay was performed in triplicate.

Culture and maintenance of *Trypanosoma cruzi*. *Trypanosoma cruzi* epimastigotes were maintained in continuous exponencial growth in liver infusion tryptose medium supplemented with 10% fetal calf serum at 28°C. The cultures were initiated with a cell density of 1×10^6 epimastigotes/mL and the compounds were added at a cell density of 1×10^7 epimastigotes/mL. Cell densities were measured by direct counting with a hemocytometer. Cell viability was examined by trypan blue exclusion using light microscopy (24). Acnistins A, E, L and ketoconazole, were added to parasites at varying concentrations and the activity was evaluated every day during 9 days. The viable and non viable number of parasites were compared to the controls grown without drug as described for *Leishmania mexicana*. Ketoconazole was used for comparison. Each assay was performed by triplicate.

Antiplasmodial activity. The antiplasmodial activity assays of the compounds were carried out *in vitro*, in triplicate, using the radioactive micromethod of Valentine *et al.* (22).

Statistical analysis

The number of parasites taken from cells grown in the absence of drugs were taken as 100% cell survival (control). Ketoconazole, was used as a positive control (16-18). For each acnistin, mevastatin and lapachol, the percentage of decrease in the number of parasites values were analyzed by Dunnett´s one-tailed test (16). The dose-response lines were converted to probit and fitted using least-squares linear regression and IC_{50} and IC_{90} (concentration required to reduce viability by 50% and 90%, respectively) against the parasite cells and its 95% confidence intervals were calculated. Data are shown as mean standard deviation (s.d.).

Results

Isolation of acnistins

Acnistin A, E and L isolated from *Acnitus ramiflorum* showed structural differences in the group attached to C-4 and quirality of the hydroxyl group in C-17 (figure 1), which could explain the differences in activity of such a compounds toward the flagellates parasites. In fact, Cardona *et al.* (25) suggest certain correlation between bioactivity and some steric and electrostatic characteristics around the A and B rings of the steroidal skeleton of withajardins and acnistins.

Activity of acnistin A and E upon growth of *Leishmania mexicana*

The inhibitory activity of the acnistin A, E, ketoconazole, mevastatin and lapachol on the growth of *Leishmania mexicana* promastigote forms is shown in table 1. The activity of acnistins upon *Leishmania* increases with the time. The data shown indicate that the inhibitory concentration (IC_{50} and IC_{90}) values for acnistin A were lower than for acnistin E whatever the day. The inhibitory activity of ketoconazole, lapachol and mevastatin, at different periods of time showed that ketoconazole appears to be more effective in its inhibitory activity than lapachol and this compound was more effective than mevastatin at days 8 and 9., but lapachol was more active than ketoconazole and mevastatin in the period between days 4 and 7. The inhibition concentration IC_{50} of ketoconazole, between days 8 and 9, is in the order of 2.5 to 1.8 μg/mL. In contrast, for the same period of time, it is about 3.5 μg/mL and 8-9 μg/mL for lapachol and mevastatin, respectively.

Table 1

Calculated IC_{50} and IC_{90} values for acnistin A, E, ketoconazole, lapachol and mevastatin upon *Leishmania mexicana*.

Time of contact between the parasites andtested compounds* (day)	acnistin IC_{50}	A IC_{90}	acnistin E IC_{50}	E IC_{90}	Ketoconazole IC_{50}	IC_{90}	Lapachol IC_{50}	IC_{90}	IC_{50}	Mevastatin IC_{90}
9	1.2±0.2	4.0±0.1	1.6±0.1	12.9±0.1	1.8±0.1	59.5±0.6	3.4±0.1	6.2±0.1	7.7±0.1	23.9±0.3
8	1.3±0.2	4.2±0.1	1.8±0.1	12.5±0.2	2.5±0.1	86.0±1.0	3.5±0.1	6.5±0.1	8.9±1.0	28.0±0.4
7	1.6±0.5	10.0±0.1	2.2±0.2	15,7±0.2	6.1±0.1	114,0±1.0	3.6±0.1	6.7±0.1	10.6±0.2	34.4±0.6
6	2.2±0.4	16.5±0.5	2.7±0.1	17.1±0.1	10.3±0.7	129.0±2.0	4.1±0.1	9.5±0.2	15.8±0.2	68±2.0
5	2.4±0.1	23.0±0.3	3.8±0.2	30.9±0.1	13.6±0.2	175.4±2.0	5.1±0.1	12.4±0.2	26.0±2.0	184±2.0
4	3.7±0.1	22.7±0.3	5.7±0.1	48.6±0.4	24.0±1.0	269.0±2.0	6.3±0.1	16.4±0.2	449±2	758±4.0

*Each compound at different concentrations was added at day 3 after initiation of the culture. IC_{50} and IC_{90} values are expressed in µg/mL.

Activity of acnistin A, E and L upon growth of *Trypanosoma cruzi*

The analysis of the effects of acnistins against *Trypanosoma cruzi* showed that acnistin A and E also inhibited the growth of this parasite (table 2). The susceptibility of *Trypanosoma cruzi* was higher with acnistin A than acnistin E or L.

Activity of acnistin A,E and L upon growth of *Plasmodium falciparum*

Acnistins showed a high activity against the two *Plasmodium falciparum* strains (table 3). The activity of acnistin A upon these parasites was higher than acnistins L and E on the strain FcM29 chloroquine-resistant. However, acnistin L was more active than acnistin A and E in the strain F32 chloroquine-sensitive. In both strains, ketoconazole showed a lesser activity against these parasites than acnistin A and L and close to the values obtained for acnistin E.

Discussion

The literature contains numerous reports on the biological activity of the withanolides. For instance, 20-deoxywithanolide D is active against gram-positive bacteria (26), withanolides from *Tubocapsicum anomalum* are cytotoxic (6) and immunosuppressive (7) and withaferin A exhibits antimicrobial, cytotoxic and inmunostimulating activities (9, 27). All these compounds present a steroidal skeleton, an α,β unsaturated carbonil group, a 17β hydroxyl and some of them an epoxi moiety, as it is the case for acnistin A and E. The cytotoxic properties of these compounds have been attributed to the presence of the epoxi moiety and to the 17β hydroxyl group (2,7). On the other hand, the immunossupressive effect has been attributed to the lactone ring and to their structural similarity with cortisol (2). This paper report the biological activity of acnistin A and acnistin E against *Leishmania mexicana*, and acnistin A,E and L against *Trypanosoma cruzi* and *Plasmodium falciparum*. A report of an extract of whitanolides from *Dunalia brachyacantha* (12) also indicated moderate antileishmanial and antitrypanosomal activities to other withanolides as 18-acetoxywithenolide D and 18-acetoxy-5,6-deoxy-5-withenolide D which reveal the potential of withanolides as antiparasitary compounds. The inhibitory activities of acnistin A and E against *Leishmania mexicana* and *Trypanosoma cruzi* is dose-dependent. The effect appears to be more pronounced with acnistin A than with acnistin E. This could indicate that the presence of a hydroxyl group at C-4 which increased the polar character of the compound could also diminish its activity. It was

Table 2
Calculated inhibition concentration (IC_{50} and IC_{90}) values for acnistins A, E, and L upon *Trypanosoma cruzi*.

Time (day)*	Acnistin A		Acnistin E		Acnistin L		Ketoconazole	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
9	0.7±0.1	7.1±0.1	3.3±0.1	36.9±0.1	5.7±0.1	37.3±0.1	4.8±0.1	10.6±0.1
8	1.3±0.2	8.6±0.2	3.9±0.1	46.6±0.2	8.4±0.2	67.5±0.2	6.2±0.2	10.8±0.2
7	1.6±0.1	10.3±0.1	5.1±0.1	72.3±0.1	20.0±0.5	199,1±0.5	6.5±0.1	11.0±0.1
6	1.9±0.2	10.9±0.2	6.3±0.2	99.3±0.1	29.1±0.3	216,2±0.3	6.7±0.1	11.2±0.1
5	2.5±0.1	15.8±0.1	9.5±0.2	189.2±0.5	ND	ND	7.1±0.1	11.6±0.1
4	3.8±0.3	21.6±0.3	13.3±0.3	234.8±0.6	ND	ND	7.3±0.1	11.7±0.1

*Acnistins and ketoconazole at different concentrations were added at day 3. IC_{50} and IC_{90} values are expressed in µg/mL. ND: non determined.

Table 3
IC_{50} (µg/mL) values of acnistin A, E, L and ketoconazole against *Plasmodium falciparum* chloroquine-sensitive (F32) and chloroquine-resistant strain FcM29.

Compounds tested	*Plasmodium falciparum F32* (chloroquine-sensitive)		*Plasmodium falciparum FcM 29* (chloroquine-resistant)	
	Time (hours)			
	24	72	24	72
IC_{50} Acnistin A (µg/mL)	3.3 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
IC_{50} Acnistin E (µg/mL)	4.5 ± 0.1	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.1
IC_{50} acnistin L (µg/mL)	2.6 ± 0.1	0.9 ± 0.1	2.4 ± 0.2	2.0 ± 0.2
Ketoconazole (µg/mL)	3.4 ± 0.2	6.5 ± 0.1	2.9 ± 0.1	3.7 ± 0.1

interesting to observe the difference in activity of acnistin A and acnistin L against *Plasmodium falciparum*. In the strain F32 chloroquine-sensitive of *Plasmodium falciparum*, acnistin L showed a higher activity than acnistin A, but the effect is reverted in the strain FcM29 chloroquine-resistant. Even acnistin A or acnistin L showed more activity than acnistin E. The only difference between these two compounds is the stereochemistry of the hydroxyl at C-17, which is beta oriented in acnistin A while in acnistin L it is alfa oriented. On the other hand acnistin L has an hydroxyl at C-4 while C-4 is not substituted on acnistin A. Comparison of the activity of acnistins with ketoconazol, mevastatin and lapachol, indicated that acnistins had a higher inhibitory effect compared with these compounds. We propose a mechanism of action of acnistins based on the fact that the protozooas synthesize sterols by a biochemical pathway different to the mechanism present in vertebrates (24) and ketoconazole as an inhibitor of sterol biosynthesis in fungi and parasites. The steroidal frame of acnistins could facilitate the interaction of these compounds with cholesterol or other neutral lipids present in the parasite membrane (1) acting as ketoconazole, an inhibitor of sterol biosynthesis in *Leishmania mexicana* (16), *Trypanosoma cruzi* (17) and *Plasmodium falciparum* (18).

Conclusions

Acnistin A and E exerted an inhibitory activity upon *Leismania mexicana* and acnistins A, E and L upon *Trypanosoma cruzi and Plasmodium falciparum*. The effects observed apparently depends on the structure of each acnistin under study: the presence of a hydroxyl group at C-4 in acnistin E and L, absent in acnistin A and the hydroxyl at C-17 in acnistin L which it is alfa oriented while in acnistin A is beta oriented. Acnistin A showed to be more active than acnistin E to inhibit the growth of *Leismania Mexicana* and *Trypanosoma cruzi.* It was interesting to observe the difference in activity of acnistin A and acnistin L against *Plasmodium falciparum*. In the strain F32 chloroquine-sensitive of *Plasmodium falciparum*, acnistin L showed a higher activity than acnistin A, but the effect is reverted in the strain FcM29 chloroquine-resistant.

Acknowledgements

The present work has been supported by funds from CDCHT-ULA C-1196-03-03-A. The authors also thanks to MWR-Mérida for providing funds throught the program LOCTI: “El estudio de las propiedades medicinales de plantas venezolanas sobre bacterias; hongos; parásitos flagelados y líneas cancerosas. Aplicabilidad en la preparación de cremas cosméticas y lociones para la piel”.

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