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Anti-Trypanosomatid Activity of Ceragenins

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Abstract

Cationic steroid antibiotics (CSAs), or ceragenins, are amphiphilic compounds consisting of cholic acid backbone that is attached to several cationic amines. In this study, we tested the hypothesis that CSAs possess anti-parasitic activities with minimal to no effects on mammalian cells, and thus could be used as potential therapeutic agents against pathogenic trypanosomatids. To investigate this, we synthesized CSAs and determined their trypanocidal and leishmanicidal activities in vitro. The 3 ceragenins, i.e., CSA-8, CSA-13, and CSA-54, assayed showed several degrees of parasitocidal activity. CSA-13 was the most effective compound against *Leishmania major* promastigotes and *Trypanosoma cruzi* trypomastigotes, LD₅₀ 4.9 and 9 μM, respectively. The trypanocidal activities of these ceragenins were also assessed by infectivity experiments. We found CSA-8 was more effective on *T. cruzi* intracellular amastigotes, when the infected host cells were treated during 24 hr (LD₅₀ 6.7 μM). Macrophages and LLC-MK₂ (treated for 72 hr) showed relative low susceptibility to these compounds. Our results suggest that ceragenins are indeed promising chemotherapeutic agents against trypanosomatids, but require further investigation.

The trypanosomatid protozoan parasites *Leishmania* spp. and *Trypanosoma cruzi* are of tremendous medical importance because they affect millions of people worldwide (Banuls et al., 2007; Tarleton et al., 2007). *Leishmania* spp. are transmitted to humans primarily via sandflies. The infection is initiated when an infected phlebotomid sandfly takes a blood meal and simultaneously injects metacyclic promastigote forms of the parasite into the host. The promastigotes are phagocytosed by macrophages and transform into the amastigotes, which proliferate by binary fission inside phagolysosomes. After a few days, the heavily infected cell bursts, releasing the amastigotes, which then infect new macrophages, or are ingested by a sandfly during the blood meal, or both. Over 20 species and subspecies of *Leishmania* can infect humans, each causing different type of symptoms. However, current treatments are expensive and require long-term follow up. More recently, drug-resistant forms of *Leishmania* spp. have also been identified (Polonio and Efferth, 2008).

Trypanosoma cruzi causes Chagas disease (or American trypanosomiasis), which is prevalent in Latin America. Reports indicate that Chagas disease is also a potential public health problem in the US (Kirchhoff and Pearson, 2007) and Europe (Dobbaro et al., 2008). Human infection with this protozoan parasite begins when metacyclic trypomastigote forms, present in insect-vector (triatomine) feces, invade the host blood circulation through the insect bite wound or exposed mucosal tissues. Immediately after infecting a variety of host cells, the metacyclic forms transform into amastigotes, which proliferate and finally differentiate into

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trypomastigotes. These forms are finally released into the extracellular space, reaching the blood stream and invading other cells and tissues leading to chronic infection. The parasite is also transmitted by blood transfusion, organ transplant, oral ingestion of contaminated food, and congenitally. Infection may result in permanent disability or death (WHO, 2002).

Nifurtimox and benznidazole are the 2 drugs currently available for the treatment of Chagas disease (Urbina, 2002). These drugs have been successfully used for the treatment of the acute phase of the disease. However, their use during the chronic phase is still controversial, because of their toxicity, potential carcinogenic properties, and variable efficacy (Marin-Neto et al., 2009; Wilkinson and Kelly, 2009). Therefore, it is paramount the development of new, improved drugs for the treatment of Chagas disease. To treat the infection by *Leishmania* spp., pentavalent antimonials have been recommended and used for over half a century. However, these drugs are extremely toxic with severe side effects and their frequent use can generate drug-resistant trypanosomatids (Murta and Romanha, 1998). Therefore, more effective drugs are also needed for leishmaniasis chemotherapy.

Ceragenins are facially amphiphilic compounds consisting of a sterol backbone appended with multiple cationic amine groups and other groups attached to them (Epanand et al., 2008). The ceragenins were designed to mimic amphiphilic characters of anti-microbial peptides (AMPs); however, they are not peptide-based and cannot be digested by proteases (Savage et al., 2002; Ding et al., 2004). Earlier studies suggested that ceragenins display broad-spectrum antibacterial and anti-viral activities (Chin et al., 2007). Furthermore, the large-scale synthesis of ceragenins is not at all expensive. In the present study, we aimed to test our hypothesis assaying *in vitro* the potential trypanocidal and leishmanicidal activity of the ceragenins CSA-8, CSA-13, and CSA-54.

Materials and Methods

Trypanosomatid cultures

Trypomastigote forms of *T. cruzi* Y strain were obtained from infected BALB/c mice by cardiac puncture 4 days following the intraperitoneal infection with 10^5 parasites. The procedure was performed minimizing the distress and pain for the animals following the NIH guidance and animal protocol approved by UTEP's Institutional Animal Care and Use Committee (IACUC). Cell-derived trypomastigotes were initially obtained by infecting Green monkey kidney-derived LLC-MK₂ cells (American Type Culture Collection, Rockville, MD) as previously described (Andrews and Colli, 1982). Briefly, semi-confluent host cell monolayers were maintained in DMEM medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (DMEM-10% FBS), at 37 C, in 5% CO₂ humidified atmosphere. Cells were infected with trypomastigotes at 1:10 host cell/parasite ratio. Four days following the infection, trypomastigotes were harvested from the culture supernatant, centrifuged in 50-ml sterile, endotoxin-free conical polypropylene tubes (Fisher Scientific) (15 min, 3,000×g, 4 C), washed twice in 5 ml fresh DMEM-10% FBS, resuspended in the same medium, and used in the assays described below. To maintain the trypomastigote virulence, a maximum of nine *in vitro* passages (infections) were performed.

LLC-MK₂ and macrophage cultures

Rhesus monkey LLC-MK₂ (ATCC # CCL-7) epithelial and mouse macrophage (Raw 264.7) (American Type Culture Collection, Manassas, Virginia) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% inactivated FBS, along with 1% of 10,000 units/ml penicillin and 10 mg/ml streptomycin, in 0.9% sodium chloride.

Ceragenins

CSA-8, CSA-13, and CSA-54 were synthesized as described (Savage et al., 2002; Chin et al., 2007). The structures of the ceragenins are showed in the Figure 1.

In vitro viability assay

Cells were seeded into 96-well flat-bottom microplates (Invitrogen, Carlsbad, California) at a density of 1×10^5 cells/200 μ l in the presence of CSA-8, CSA-13, or CSA-54, serially diluted to concentrations from 100 to 1 μ M, and incubated for 72 hr at 37 C in a humidified atmosphere of 5% CO₂. Wells without drug served as controls. Twenty μ l of 10% Alamar Blue® (AbD Serotec, Raleigh, North Carolina) were added to wells after 56 hr, and fluorescence was determined at the 72-hr point using a Fluoroskan Ascent FL microplate fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) with a 530-nm excitation and 590-nm emission wavelengths. The viability of *T. cruzi* trypomastigotes, *L. major* promastigotes, and mammalian host macrophages and LLC-MK₂ cells was assessed using this assay.

In vitro infection experiments

The in vitro effect of ceragenins on *T. cruzi*-infected host cells was tested by treating infected host cells (macrophages and LLC-MK₂ cells). Briefly, sterile 12-mm coverslips (Thermo Fisher Scientific) were placed into a 24-well microplate. In each well, 5×10^4 LLC-MK₂ cells were cultured for 24 hr in DMEM supplemented with 10% iFBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml), and incubated at 37 C under 5% CO₂ humid atmosphere. A monolayer of LLC-MK₂ cells was infected with trypomastigotes (1:20, host cell/parasite ratio), for 1 hr at 37 C. The non-adherent parasites were removed by five consecutive washes with 1 ml phosphate-buffered saline (PBS). The infected cells were treated with the specific ceragenin (CSA-8, CSA-13, or CSA-54) at 100, 50, 25, 10, and 1 μ M for 24 hr at 37 C. The medium was removed and the cells were fixed with methanol for 30 min (300 μ l/well), followed by 2 washes with ice-cold PBS and 2-hr incubation at room temperature (rt) with PBS. Cells were also stained with 300 μ l of DAPI (4',6-diamidino-2-phenylindole, 1 μ g/ml) solution for 5 min at rt. After incubation, cells were washed twice with PBS and additional 500 μ l PBS were added while slides were prepared. The cover slips were mounted onto the slides using 10 μ l Vectorshield (Vector Laboratories, Burlingame, California) mounting solution. Both host cell and parasite nuclei were stained with DAPI and cells were analyzed by microscopy using an LSM5 Pascal Zeiss confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, New York). A total of 100 cells (infected and uninfected) were analyzed.

Statistical analysis

The statistical significance of the cytotoxicity for each of the 3 ceragenins in the parasites and mammalian cells, as well as in the in vitro infectivity of LLC-MK₂ cells by *T. cruzi* was calculated using multivariate analysis of variance (MANOVA), analysis of variance (ANOVA), and Tukey Grouping. The LD₅₀ was also calculated for all assays. These tests and calculations were incorporated in the Graph Pad Prism Software (GraphPad Software, Inc., La Jolla, California) for display.

Results

First, we tested the cytotoxicity of ceragenins CSA-8, CSA-13, and CSA-54 on host cells, i.e., LLC-MK₂ epithelial cells and RAW 264.7 macrophages. Higher levels of cytotoxicity were demonstrated by CSA-8- and CSA-13-treated LLC-MK₂ cells when compared to cytotoxicity levels on macrophages (Fig. 2, Table I). The ANOVA for each individual drug showed a *P*-value of <0.0001.

Next, we tested in vitro the effect of ceragenins CSA-8, CSA-13, and CSA-54 on *L. major* promastigotes and *T. cruzi* trypomastigotes. We found that the 3 ceragenins were more effective against promastigote forms of *L. major* (LD₅₀ 19.4, 4.9, and 12.4 μ M, respectively) than against trypomastigote forms of *T. cruzi* (LD₅₀ 61, 9, and 99 μ M, respectively). In the promastigote forms of *L. major*, it is clear that CSA-13 had the lowest LD₅₀ (4.9 μ M; $P < 0.0001$) of the compounds tested (Fig. 3, Table I). In *T. cruzi*, CSA-13 also had the highest parasitocidal activity on trypomastigote forms (LD₅₀, ~ 9 μ M) and CSA-8 and CSA-54 were less toxic (61 and 99 μ M, respectively, P -value < 0.0001) (Table I).

The infectivity experiments with *T. cruzi* trypomastigotes showed a differential effect of the ceragenins between the number of infected cells and the amount of parasite per LLC-MK₂ cell. CSA-13 decreased the number of infected cells, whereas CSA-8 was more efficient in affecting the proliferation of amastigotes (Fig. 4, Table I). CSA-54 was similarly efficient in the clearance of infected cells and the inhibition of intracellular amastigote proliferation. Based on the results of the in vitro infectivity experiments, we suggest a time-dependent activity of the ceragenins, or a differential toxicity, or both, in *T. cruzi* amastigotes and trypomastigotes. Our results showed that ceragenins decrease the infection in the mammalian cells invaded by the parasites (Fig. 4). Of the 3 ceragenins tested, CSA-8 affected the amastigote proliferation in LLC-MK₂ cells more efficiently and had the lowest cytotoxicity activity in mammalian cells (Figs. 2, 4).

Discussion

The plasma membranes of both the protozoan parasite and mammalian cell play a critical role in the interaction during parasite invasion of host cells. For instance, the integrity of the plasma membrane is essential in order to maintain the biophysical properties and viability of the trypanosomatid. In this regard, to target the plasma membrane several drugs have been studied, some interact directly with the membrane, like amphotericin B and AMPs, and others are inhibitors of biosynthetic enzymes from the lipid metabolism, e.g., ketoconazole (Maldonado et al., 1993; Urbina et al., 1993; Petit et al., 1999).

It has been proposed that the mechanism of action of the ceragenins is similar to the AMPs (Epanand et al., 2008). AMPs are small amphiphilic cationic molecules that are part of innate immune system, and their antimicrobial activity has generally been attributed to disruption of the integrity of the membrane. Recently, several studies have been published showing the trypanocidal and leishmanicidal activity of some AMPs (Silva et al., 2000; McGwire et al., 2003; Alberola et al., 2004; Singh and Sivakumar, 2004). McGwire et al. (2003) determined the trypanocidal activity of α - and β -defensins, and cathelicidins. They showed both in vitro and in vivo that cathelicidins were the most effective AMPs for controlling *T. brucei* infection. Boulanger et al. (2002) have also proposed that the innate immune response of the insect vector through the production of AMPs may control the infection by *T. brucei rhodesiense*. In that study, the authors identified a new AMP of 42 amino acids, named stomoxyn, constitutively expressed and secreted exclusively in the anterior midgut of *Stomox calcitrans* exhibits trypanolytic activity to *T. brucei rhodesiense*. It was suggested that stomoxyn activity may help to explain why *S. calcitrans* is not a vector of trypanosomes causing African sleeping sickness and nagana. Recently, several groups have been working in the characterization of AMPs as anti-leishmanial alternative (Alberola et al., 2004). In *L. donovani*, it has been shown that the indolicidin and 2 peptides derived from seminalplasmin induced 1, or more, pathways for autophagic cell death in addition to their effects on the leishmanial membrane (Bera et al., 2003).

The aim of our study was to determine whether ceragenins could be used as alternative drugs to treat parasitic infections by *T. cruzi* and *L. major*. We suggest that the differential toxicity

observed here might be due to the membrane potentials (or voltage across the membrane) as well as the structural and functional properties of ceragenins. The membrane potential is an indicative of the electronegativity of the membrane. In this regard, the resting membrane potential of promastigote forms of *Leishmania* spp. is -113 ± 4 mV, and in the case of *T. cruzi* it is -107 ± 6 mV for trypomastigotes (Diaz-Achirica et al., 1998). Thus, the plasma membrane of *Leishmania* spp. promastigote is more negatively charged than that of *T. cruzi* trypomastigote, and, perhaps for that reason, the ceragenins are more effective, i.e., lower LD₅₀, on the first (Table I). The higher toxicity observed in LLC-MK₂ cells with respect to macrophages could also be due to differences on their resting membrane potential. Macrophages have a resting potential of -13 mV, whereas the resting potential of LLC-MK₂ is -77 mV (Olesen et al., 1988). Thus, we speculate that a higher electrostatic attraction between the ceragenins and the LLC-MK₂ cells might occur because these cells are more negatively charged than macrophages.

When compared to other eukaryotic cells, the plasma membranes of *T. cruzi* and *Leishmania* spp. are strongly negatively charged (Ince et al., 1984; Ferguson, 1999; Guha-Niyogi et al., 2001; Acosta-Serrano et al., 2007). In the case of *Leishmania* spp., the plasma membrane is covered with high levels of lipophosphoglycan (LPG), found in all species of *Leishmania* that infect humans (Turco and Descoteaux, 1992; Mendonca-Previato et al., 2005). The strong negatively charged membrane is due to an anionic polysaccharide that covers more than 60% of the whole surface (Turco and Descoteaux, 1992). The negatively charged membrane of *T. cruzi* is due to the presence of mucins, which in epimastigotes totals about 9% in absolute terms and approximately 24% in terms of surface density. When comparing trypomastigotes to epimastigotes, the former contains a slightly higher density of mucins with about 47% in absolute terms and 127% in terms of surface density (Pereira-Chioccia et al., 2000). Another important characteristic of the plasma membrane of *T. cruzi* and *Leishmania* spp. is that their lipid composition is characterized by a slightly higher percentage of anionic phospholipids than the standard mammalian membrane (Wassef et al., 1985a), in addition to presence of ergosterol instead of cholesterol as in mammalian cell membranes (Wassef et al., 1985b; Urbina, 1997; Roberts et al., 2003; Duschak and Couto, 2007). The differences in the electronegativity of mammalian cells and parasites used in this study are compatible with the degree of toxicity of ceragenins found here. In bacteria, it has been shown that CSA-8, CSA-54, and CSA-13 appear to have 2 distinct mechanisms of action. The first happens through pore formation due to the electrostatic interactions of the ceragenins with the membrane, whereas the second is insensitive to the structure of the ceragenin or even to their overall charge (Epan et al., 2008). The latter authors suggested that it seems to be a relationship between the antimicrobial activity of these compounds and the phospholipid composition of the membrane. Here, we speculate that most likely similar phenomena may occur with *Leishmania major* and *T. cruzi*. Further studies are needed to address this issue.

In the case of the in vitro infectivity experiments performed with CSA-13, we speculate that the compound might have cleared the infection in the cells invaded by few parasites, explaining the lower number on infected cells in comparison to CSA-8 (~20% less). Another possibility is that due to the invasion process, the cytoskeleton and the membrane stability are altered, and treatment of the cells just after infection made them more susceptible to lysis by CSA-13; consequently, less infected cells were detected.

The differences in cytotoxicity of ceragenins with host cells and parasites suggest that ceragenins may find use in treating and/or preventing infections. However, the use of proper drug delivery system of the drugs such as liposomes may increase the efficacy of CSA molecules in treating infections by *T. cruzi* and *L. major*.

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FIGURE 1.

Survival of mammalian cells treated with CSAs. Rhesus LLC-MK₂ epithelial cells and mouse Raw 264.7 macrophages were treated for 72 hr with several concentrations (1, 10, 25, 50, and 100 μ M) of CSA-8, CSA-13, and CSA-54. The experiments were performed 3 times, in triplicate.

FIGURE 2.

Viability assays of *L. major* promastigotes treated with 100, 33, 11, 3.3, and 1.25 μM CSA-8, CSA-13 or CSA-54. Surviving cells were detected by incubation with Alamar Blue and expressed as a percent of survived cells. Bars represent means $\pm$ standard errors of 3 independent experiments. Controls represent parasites incubated only with culture media.

FIGURE 3.

T. cruzi-Infected LLC-MK₂ cells treated with CSAs. (A) Percent of cells infected by *T. cruzi* after 24-hr treatment with CSA-8, CSA-13, or CSA-54. B) Number of intracellular *T. cruzi* amastigotes in 100 cells after 24-hr CSA treatment. Surviving cells were detected by incubation with Alamar Blue and expressed as a percent of survived cells. Bars represent means $\pm$ standard errors of 3 independent experiments. Control represents LLC-MK₂ cells incubated only with culture media.

FIGURE 4.

Table I

LD₅₀ of the mammalian cell lines and parasites treated with CSAs.

CELL TYPE	CSA-8 LD ₅₀ μ M (p-value)	CSA-13 LD ₅₀ μ M (p-value)	CSA-54 LD ₅₀ μ M (p-value)
LLC-MK ₂	79.9 (<0.0001) ^c	31.1 (<0.0001)	69.2 (<0.0001)
Raw 264.7 macrophages	90.4 (< 0.002) ^c	25.8 (<0.0001)	89.8 (<0.0002)
<i>T. cruzi</i> intracellular amastigotes	6.7 (<0.0001) ^b	38.2 (<0.0001)	20 (<0.0002)
<i>T. cruzi</i> trypomastigotes	>100 ^a 61 (<0.0001) ^b	>100 9 (<0.0001)	>100 99 (<0.0001)
<i>L. major</i> promastigotes	19.2 (< 0.0001) ^c	4.9 (< 0.0001)	12.4 (< 0.0001)

^a, ^b, ^c 1-, 24-, and 72-hr incubation, respectively.