Published in final edited form as:

Acta Trop. 2016 December ; 164: 402–410. doi:10.1016/j.actatropica.2016.09.029.

Ruthenium-Clotrimazole complex has significant efficacy in the murine model of cutaneous leishmaniasis

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Abstract

In previous studies we reported a novel series of organometallic compounds, Ru^{II} complexed with clotrimazole, displaying potent trypanosomatid activity with unnoticeable toxicity toward normal mammalian cells. In view of the promising activity of Ru-clotrimazole complexes against *Leishmania major* (*L. major*), the present work sought to investigate the anti-leishmanial activity of the AM162 complex in the murine model of cutaneous leishmaniasis. In addition, to facilitate the design of new therapeutic strategies against this disease, we investigated the mode of action of two Ru-clotrimazole complexes in *L. major* promastigotes. Overall, we demonstrate that AM162 significantly reduced the lesion size in mice exposed to *L. major* infection. In addition, Ru-clotrimazole compounds are able to induce a mitochondrial dependent apoptotic-like death in the extracellular form of the parasite based on labeling of DNA fragments, mitochondrial depolarization, cell cycle alteration profile and plasma membrane phospholipid externalization. Our findings reveal a promising efficacy of the Ru-clotrimazole AM162 complex for the treatment of cutaneous leishmaniasis, as well as pro-apoptotic activity and thus guarantees further evaluation in pre-clinical studies.

Keywords

Apoptosis; Cutaneous leishmaniasis; Drug discovery; *Leishmania major*; Parasites; Ruthenium-clotrimazole complex

1. Introduction

Trypanosomatid protozoa of the genus *Leishmania* are the causative agents of leishmaniasis. This parasitic disease, which targets mainly macrophages, is transmitted by the bite of an

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infected female phlebotomine sand fly. Currently, this disease is endemic in 98 countries, with more than 2 million new cases per year, and high levels of mortality and morbidity (Alvar et al., 2012). More than 20 species of *Leishmania* affect humans, causing three major different forms of the disease. Cutaneous leishmaniasis (CL), caused by *Leishmania major* (*L. major*), is the most common form of the disease with an estimate of 0.7 to 1.2 million annual cases, characterized by localized cutaneous ulcers (Fortin et al., 2014; Reznickova et al., 2015). Even though CL is considered to be a tropical disease, increased travel and migration within the tropics, subtropics, Middle East and Southern Europe are making this disease a risk for population in regions that were previously unaffected, including the United States, where it is an emerging concern (Blum et al., 2004).

Efforts to discover a reliable human anti-*Leishmania* vaccine remain elusive, and current anti-*Leishmania* chemotherapy is mostly based in pentavalent antimonials developed over 50 years ago and liposomal amphotericin B (amp B) (Navarro et al., 2010). Pentavalent antimonials remain effective and they are the preferred treatment for CL (Sundar, 2015). In addition to their highly toxic side effects, antimonials resistance in *Leishmania* spp. has been reported, restricting limits of their usage (Ait-Oudhia et al., 2011; Navarro et al., 2010). In regions where leishmanial resistance to antimonial is high, amp B deoxycholate is recommended (Sundar and Chakravarty, 2010); nevertheless, its usage has several drawbacks, such as high toxicity with its concomitant low tolerance by patients, especially when long periods of treatment are needed (Murray, 2012). In contrast, due that liposomal amp B is a better-tolerated drug and is widely available in the U.S. (at a dosage of 3 mg/kg, days 1–5, 10 and 21) for CL treatment, the usage of amp B deoxycholate for the treatment of leishmaniasis was already eradicated (Wortmann et al., 2010). However, liposomal amp B is expensive and to be effective requires a long course of high dose treatment. For instance, a total 21 mg/kg dose drug cost for a 70-kg patient is between \$5,000.00-6,000.00, in agreement with FDA-approved guidelines for visceral leishmaniasis (VL); an additional disadvantage is that this drug is not FDA-approved for CL treatment and some health insurance companies will hesitate to cover its cost for CL treatment purposes (Murray, 2012). Additionally, miltefosine is the only effective oral agent for leishmaniasis management (FDA approved). Nonetheless, it is also expensive and can produce several side effects (Dorlo et al., 2012). Thus, there is an urgent need for the development of new chemotherapies against leishmaniasis.

In previous studies we reported a novel series of organometallic compounds containing Ru^{II} and clotrimazole (CTZ) displaying potent leishmanicidal activity without significant toxicity toward normal mammalian cells, most notably [(*p*-cymene)RuCl₂(CTZ)] (**AM160**), and [(*p*-cymene)RuCl(acac)(CTZ)]BF₄ (acac = acetylacetonate) (**AM162**) (Fig. 1) (Martinez et al., 2012). As complex metal compounds, both AM160 and AM162 increased the toxic activity of CTZ on *L. major* promastigotes (after 96 h exposure), most likely due to their ruthenium (Ru^{II})-containing motifs, as evidenced by an LD₅₀ of 0.015 and 0.45 μM, respectively (Martinez et al., 2012). In addition, the effects of both AM160 and AM162 on intracellular proliferation of *L. major* amastigotes in mouse intraperitoneal macrophages was studied after 48 h of treatment by using high-content imaging assay (Nohara et al., 2010). Findings indicate that AM160 caused significant inhibition on the proliferation of intracellular

amastigotes ($IC_{70} = 29 \text{ nM}$), while AM162 displayed anti-leishmanicidal effect at a higher concentration ($IC_{40} = 1 \text{ }\mu\text{M}$) (Iniguez et al., 2013). CTZ is known to act as an sterol biosynthesis inhibitor (SBI), blocking the proliferation of the parasite by inhibiting the action of the enzyme C-14 demethylase, and it has been shown that ruthenium markedly enhances their activity towards *L. major* with undetectable cytotoxic effects towards mammalian cells through a synergistic mechanism (Sanchez-Delgado et al., 2004). Due to these promising *in vitro* findings, in the present study, we sought to determine the efficacy of the Ru-CTZ compounds specifically AM160 and AM162 in BALB/c mice infected with *L. major* metacyclic promastigotes to alleviate CL. Moreover, among various mechanisms of cell death, apoptosis or programmed cell death has been reported in several parasitic protozoans (Lee et al., 2002; Verma and Dey 2004; Sen et al., 2007; Mesquita et al., 2014). Therefore, we investigated the mechanism of cell death inflicted by Ru^{II} -CTZ complexes on *L. major* promastigotes.

2. Materials and methods

2.1. Parasite culture and maintenance

Promastigote forms of *L. major* strain Friedlin clone V1 expressing luciferase gene (Lucas et al., 2012) were grown in $1 \times \text{M199}$ medium supplemented with hemin and 10% heat inactivated fetal bovine serum (FBS). For the *in vivo* infections, *L. major* metacyclic promastigotes were prepared by Ficoll-Paque density gradient centrifugation as previously described (Spath and Beverley, 2001).

2.2. Synthesis of Ru-azole complexes

Ru-azole complexes [$(p\text{-cymene})\text{RuCl}_2(\text{CTZ})$] (**AM160**), and [$(p\text{-cymene})\text{RuCl}(\text{acac})(\text{CTZ})\text{BF}_4$ (acac = acetylacetonate) (**AM162**), the metal-ligand motif (**C3**) and **CTZ** were synthesized and purified by appropriate chemical methods as previously described (Martinez et al., 2012). The Ru-azole compounds were freshly dissolved at the stated concentrations in 50% v/v DMSO/PBS solution and daily administered intraperitoneally for 14 or 10 consecutive days for the pilot or hyper infection experiments, respectively for the *in vivo* studies. For mechanistic *in vitro* studies, compounds AM160, AM162 were dissolved in DMSO.

2.3. Pilot and hyper infection in a murine model

Female BALB/c mice (6–8 weeks old) were acquired from Harlan Laboratory (Indianapolis, IN). Only female mice were utilized for a series of experiments *in vivo* to circumvent gender associated influences (Nunes et al., 2014). For the pilot and hyper infection, 1×10^5 or 1×10^6 *L. major* metacyclic promastigotes in 50 μl volume, respectively, were inoculated into the left hind footpad. Mice were individually treated with AM160 or AM162 at 4 mg/kg/day for the pilot or 6 mg/kg/day for the hyper infection, and monitored for 14 or 21 days post-infection, respectively. Disease progression was examined twice per week by checking body weight and swelling of the *Leishmania* cutaneous lesion (left footpad) with a digital caliper. During the hyper infection experiments, as soon as the CL lesions were visually evident at day 21 post-infection, animals were randomly selected to conform both experimental and control groups without any bias; followed by the measurements of the lesion size. Due to the

high variability of CL lesion sizes between the animal groups, the lesion sizes were normalized as 1 per each group at 21 days post-infection, for comparison purposes with day 34 post-infection (endpoint of experiment). Mice treated with vehicle (50% v/v DMSO/PBS solution) were considered as negative control; naive group, a non-infected mice with *L. major* metacyclic promastigote, but just AM162-treated animals was concurrently analyzed. Furthermore, mice treated with the metal-ligand motif (C3) not containing CTZ, as well as CTZ alone, at a concentration of 4 mg/kg/day, were also included as controls. Animal procedures were performed according to NIH guidelines and the appropriate protocol approved by UTEP's Institutional Animal Care and Use Committee (IACUC).

2.4. Determination of parasite load and cycle threshold using quantitative RT-PCR

Genomic DNA was obtained from BALB/c mice footpads by chloroform-phenol extraction as previously described (Medina-Acosta and Cross, 1993). First, footpad tissue was obtained and dissociated with gentle MACS dissociator (Miltenyl Biotec), centrifuged for 10 min at 3000 rpm, and resuspended with 150 µl of TELT buffer. The same volume 1:1 of chloroform-phenol was added and phases were separated by centrifugation. The aqueous phase was recovered, and the DNA was precipitated with ethanol followed by centrifugation. The DNA pellet was washed with 70% ethanol, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 20 µg/ml of RNase A. Parasite load was determined by the cycle threshold value (Perez-Victoria et al., 2011) with SYBR Green (Bio-Rad, Hercules, CA) and used for product detection and quantification of 120-bp fragment of the minicircle kDNA of *L. major* primers JW11 (forward, 5'-CCTATTTTACACCAACCCCCAGT-3'; and JW12 (reverse, 5'-GGGTAGGGCGTTCTGCGAAA-3')) (Nicolas et al., 2000). Primers were obtained from Integrated DNA Technologies (IDT) (Integrated DNA Technologies, Coralville, IA). Amplification and detection was performed using Step One Real Time PCR System (Life Technologies) as follows: holding step at 95 °C for 15 min, 40 cycles starting in denaturation step at 95 °C for 15 s, annealing at 56 °C for 10 s and extension step at 72 °C for 10 s. Reactions were standardized using a 10-fold serial dilution of promastigotes DNA (50 ng per reaction). Standards, experimental samples and controls were analyzed in triplicates in two independent biological replicates. The results are represented by parasite equivalents.

2.5. Analysis of apoptosis-like pathway via annexin V/PI assay

To quantify cells with phospholipids externalization, Alexa Fluor® 488 annexin V/PI Apoptosis Kit (Invitrogen™-Life technology, Grand Island, NY) was used and monitored by flow cytometer. *L. major* promastigotes (5×10^6) were treated with AM160 or AM162 at 2 µM. After 24 h of exposure, the cells were harvested, washed and resuspended with cold PBS and analyzed *via* flow cytometric protocol following the manufacturer's instructions of annexin V/PI kit. As a positive control, cells treated with 200 µM of hydrogen peroxide (H₂O₂), and as negative control, cells treated with diluent only (DMSO) were analyzed concomitantly. The total percentage of apoptotic cells was obtained with the sum of both early and late apoptotic percentages located in the two right quadrants of the flow cytometric dot plots. For each sample, 10,000 events were collected and analyzed by using a Cytomics FC500 flow cytometer and CXP software (Beckman Coulter, USA).

2.6. Analysis of mitochondrial membrane potential ($\Delta\psi$)

L. major promastigotes (5×10^6) were treated with several concentrations (0.25, 0.50 and 1 μ M) of AM160 or AM162 for 6 h and stained with 2 μ M of the fluorophore 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), following a modified version of manufacturer's instructions (MitoProbe JC-1 Assay Kit, Life technologies, Grand Island, NY). The disruption of mitochondrial membrane potential produces a shift of the JC-1 fluorescence-emitting signal from red to green. For each sample, cells with JC-1 aggregates or monomers, emitting red or green signal, were analyzed with Cytomics FC500 flow cytometer by using FL-2 and FL-1 detectors, respectively. Cells treated with the drug diluent (DMSO) were considered as negative control; whereas cells treated with 50 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used as positive control. Data collection and analysis was performed using CXP software (Beckman Coulter).

2.7. Detections of DNA fragments by terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL) assay

L. major promastigotes (5×10^6) were treated at a concentration of 2 μ M AM160 or AM162 for 6 h. Parasites were harvested and fixed with 4% paraformaldehyde, followed by incubation with 0.2% Triton X-100 in PBS for 5 min at room temperature for permeabilization purposes. DNA fragments were labeled with Click-iT® TUNEL Alexa Fluor® following a modified version of manufacturer's instructions (Invitrogen™-Life technology, Grand Island, NY). Parasites were stained with 10 μ l of propidium iodide (stock 1 mg/ml) and each sample was analyzed with a Beckman Coulter FC500 flow cytometer (Beckman Coulter, USA) (Varela-Ramirez et al., 2011). Parasites were incubated with DNase I as a positive control for producing breaks in the double stranded DNA, and parasites treated with just solvent (DMSO) as a negative control.

2.8. Cell cycle analysis in *L. major* promastigotes

L. major promastigotes, 5×10^6 , were individually treated with 2 μ M of AM160 or AM162 complexes for 24 h at 28 °C and their DNA content was examined *via* flow cytometry. Cell pellets were obtained by centrifugation and fixed with chilled (−20 °C) methanol, and incubated at −20 °C overnight. Then, cells were washed twice with PBS, followed by the addition of 20 μ g/ml of RNase A and incubated for 1 h at 37 °C. Then, cells were labeled with propidium iodide, a fluorescent DNA intercalator, for 20 min at room temperature and analyzed with Beckman Coulter FC500 flow cytometer (Beckman Coulter, USA).

2.9. Statistical analysis

Each data point is presented as average with their corresponding standard deviation and standard error for *in vivo* experiments. To establish statistical significance between two groups two-tailed unpaired Student's *t*-test was performed. To designate whether two groups have statistical significance, a value of $P = 0.05$ (*) was considered significant. Higher significant values $P = 0.01$ (**), $P = 0.001$ (***) and $P = 0.0001$ (****) are also denoted. The graphs were attained using Graph Pad Prism 5 Software (GraphPad Software, Inc., La Jolla, California).

3. Results

3.1. Ruthenium-azole complex exerts anti-leishmanial activity in vivo

In our previous work, AM160 and AM162 exhibited potent *in vitro* leishmanicidal activity with an excellent selectivity index (SI) (Martinez et al., 2012). However, further characterization and study of AM160 and AM162 (Fig. 1) is important to promote its evaluation as clinical candidates against cutaneous leishmaniasis. A pilot experiment was set up to compare the efficacy of CTZ with the Ru-CTZ complexes AM160 and AM162 in BALB/c mice. *L. major* metacyclic promastigotes (1×10^5) were injected in the left hind footpad and mice groups were divided ($n = 3$) as follows: CTZ, C3 (metal control), AM160, AM162 and vehicle control group. After 14 days post-infection, the lesions size were measured and intraperitoneal (i.p.) treatment with experimental compounds was administered at a dosage of 4 mg/kg/day for 14 consecutive days. As observed in Fig. 2A, by day 38 post-infection (endpoint of experiment), the AM160-treated animals exhibited reduction in the size of CL lesions by 26%, as compared with vehicle-treated mice. Moreover, AM162 showed a better leishmanicidal effect than CTZ and reduced the group lesion size by 68% with respect to the group treated with vehicle at the endpoint of experiment with a significant *P* value of 0.00046. Therefore, we continued to investigate the effect of AM162 at a higher concentration (6 mg/kg/day) in a hyper infection (1×10^6) with *L. major*. In this case, mice ($n = 3$) were infected and 21 days post-infection treated with AM162 for 10 consecutive days. Groups included AM162, naive group (AM162-treated but non-infected), and vehicle control. AM162 was given i.p. at 6 mg/kg/day as previously stated. In AM162-treated animals, comparison between day 21 (first day of AM162 treatment) and day 34 (last day of treatment and endpoint of experiment) of lesion sizes increased from 1 to 1.25, respectively. In contrast, by doing the same analysis, but in vehicle control-treated animals, the lesion size increased from 1 to 1.45. Findings revealed that AM162 treatment contributed to growth a smaller CL lesion sizes, as compared with vehicle-treated mice (Fig. 2B). Moreover, analysis of parasite load by identification of the minicircle kDNA of *L. major* by quantitative real-time PCR was performed for the hyper infection experiment. A 10-fold serial dilution with *L. major* promastigotes DNA was used to determine a standard curve and the absolute parasite equivalent in 50 ng of DNA. Subsequently, the parasite load was estimated from the DNA extracted from mice footpads from all groups described. As observed in Fig. 2C, AM162 treatment significantly (*P* = 0.0146) reduced parasite load in comparison with groups treated with vehicle control. Moreover, no significant weight loss was observed after the treatment with the experimental drugs (data not shown). Overall, this data suggests that even though AM162 showed a moderate *in vivo* activity against *L. major* mice infection, the compound was able to reduce the footpad lesions and parasite load by 50% or more, with no significant toxicity.

3.2. Ruthenium-azole complexes induced phospholipid externalization

In early stages of metazoan apoptosis in unicellular organisms several alterations to the plasmatic membrane are observed, including the externalization of phosphatidylserine (PS) (Mackey et al., 2006) from the inside to the outside of the plasma membrane. Recent evidence found through lipid analysis, determined that *Leishmania* promastigotes lack PS (Weingartner et al., 2012). Despite this fact, annexin-V was able to bind other phospholipid

species such as phosphatidylinositol (Weingartner et al., 2012) and phosphatidylethanolamine and cardiolipin in *Leishmania* promastigotes after permeabilization with miltefosine probably as a consequence of alterations or changes in the transbilayer plasma membrane lipid arrangement (Lee et al., 2002). Therefore, annexin-V conjugated with Alexa Fluor 488 was used to detect positive *L. major* promastigotes cells with externalization of phospholipids. In addition, PI was added in order to be able to differentiate between apoptotic cells (annexin-V positive, PI negative), necrotic cells (annexin-V negative, but PI positive), and live cells (negative to both annexin-V and PI). As shown in Fig. 3A, in comparison to the negative control treated with 1% DMSO the percentage of apoptotic cells (annexin-V+, PI-) significantly increased following the treatment with AM160 and AM162 for 24 h at a concentration of 2 μ M for all the compounds. In addition, none of the Ru-azole compounds showed significant necrotic percentages in comparison with the positive control treated with hydrogen peroxide. This data suggests that our compounds produced an apoptotic-like effect in *L. major* promastigotes. These findings indicate that the compounds exert selectively their anti-leishmanial activity primarily *via* apoptosis.

3.3. Ruthenium-azole complexes triggered dissipation of mitochondria membrane potential (ψ_m)

Depolarization of mitochondria transmembrane potential (ψ_m) is an initial biochemical facet implying activation of the intrinsic apoptotic pathway, as the initial triggering signal of apoptosis (Marinho et al., 2014). In addition, it has been proven to be an important feature for cell death in protozoa parasites such as *Leishmania* (Sen et al., 2004). To examine the potential dissipation of ψ_m in cells exposed to the experimental compounds, the membrane-permeant JC-1 reagent (Mitoprobe™ kit; Life Technologies) and flow cytometric protocol were employed. JC-1 is a cationic dye forming aggregates in the mitochondria of healthy cells, emitting red fluorescence signal. In cells with dissipated ψ_m JC-1 is unable to make aggregates, as consequence JC-1 remains as monomer emitting green fluorescent signal. In addition, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) a proton ionophore was concurrently evaluated at 50 μ M as a positive control for dissipation of mitochondrial membrane potential. As depicted in Fig. 4A, cells treated with AM160 or AM162 compounds caused a concentration-dependent loss in ψ_m in comparison to the negative control treated with 1% DMSO. An increase of more than 2-fold (from 30.8% to about 80%) was observed with depolarized mitochondria in promastigotes treated with 0.25 μ M of AM160 or AM162, as compared with the vehicle control (1% DMSO). This depolarized mitochondria percentage incremented with higher concentrations (0.50 and 1 μ M) of both AM160 and AM162. The JC-1 fluorescence emission shift from red (aggregates) to green (monomers) was observed in promastigotes treated with the two AM160 and AM162 Ru-azole compounds, as compared to the negative control (Fig. 4B). Therefore, our findings suggest that Ru-azole compounds are inducing, in a dose-dependent manner, an apoptosis-like cell death in *L. major* promastigotes and that this early event is utilized to initiate the intrinsic pathway evidenced by disruption of ψ_m .

3.4. DNA fragmentation after treatment of Ru-azole compounds

During apoptosis the cleavage patterns of genomic DNA are typical of internucleosomal DNA digestion by an endogenous nuclease, which is considered a late biochemical hallmark of apoptotic cell death (Sen et al., 2004). The terminal deoxynucleotidyl transferase enzyme mediated dUTP end labeling (TUNEL) assay detects the DNA fragmentation at a single-cell level, thereby allowing a better evaluation of the apoptotic cell fraction. In this case the Click-iT® TUNEL Alexa Fluor® assay was used to evaluate DNA fragmentation of promastigotes treated with AM160 and AM162. As observed in Fig. 5, the Ru-CTZ compounds showed a similar pattern of DNA fragmentation as the one observed in the positive control DNase I. Therefore, these data suggest that the Ru-azole complexes were able to induce DNA fragmentation leading to an apoptotic-like death in *L. major* promastigotes.

3.5. Ruthenium-azole compounds alter cell cycle profile

In order to determine if the compounds alter the cell cycle profile of *L. major* promastigotes, flow cytometric analysis was performed after 24 h of incubation with 2 μ M AM160 or AM162. During analysis of cell cycle distribution profile, quantifying cellular DNA content *via* flow cytometry, it is relatively easy to identify a sub-G0/G1 or hypodiploid peak, indicator of the cell population carrying fragmented DNA; a late facet of apoptotic cell death (Robles-Escajeda et al., 2013). Promastigotes treated for 24 h with AM160 or AM162 (2 μ M) exhibited 73.4% or 90.7% of sub-G0/G1 population, respectively; whereas cells treated with solvent control (DMSO) displayed just 7.7% of the same sub-G0/G1 population (Fig. 6). These findings indicate that Ru-azole compounds caused an increase in the percentage of sub-G₀/G₁ peak, which is interpreted as promastigotes carrying fragmented DNA. Concurrently, Ru-azole complexes reduced the percentages of both G0/G1 and S/G2/M population. Thus, Ru-azole compounds alter the cell cycle profile and use the DNA fragmentation pathway as their mechanism to inflict death in *L. major* promastigotes.

4. Discussion

Leishmaniasis is a neglected tropical disease that affects millions of people worldwide. Despite recent advances, poor efficacy and high toxicity of the limited available treatment options demonstrate the need for the development of new chemotherapeutic agents to treat this disease. In an effort to contribute to this concern, novel Ru-azole complexes were synthesized, characterized and tested as chemotherapeutic agents against *L. major* (Martinez et al., 2012; Iniguez et al., 2013). Initially, we used a high-throughput screening approach to evaluate the cytotoxicity of the Ru-CTZ compounds in different mammalian cells and viability of *L. major* promastigotes. To our satisfaction, two compounds displayed potent leishmanicidal activity in both *L. major* promastigotes and intracellular amastigotes with no significant cytotoxicity towards mammalian cells. In addition, we observed that Ru-CTZ compounds improved the anti-leishmanial effect in comparison with CTZ alone *in vitro* (Martinez et al., 2012). Nowadays, as a general rule for a successful drug candidate, *in vivo* experiments are necessary. Therefore, in the present report we sought to study the therapeutic efficacy *in vivo* of the Ru-CTZ compounds in mouse model of CL. Treatment with 4 mg/kg/day of AM162 reduced the lesion size in the murine model of CL by a 68%, a

markedly better effect than CTZ alone or a Ru^{II} complex not containing CTZ (C3). In addition, in a hyper infection experiments and using a dosage of 6 mg/kg/day of AM162, the parasite burden was significantly reduced ($P=0.0146$) (Fig. 2C). Therefore, these findings indicate that AM162 possess efficient anti-leishmaniasis activity *in vivo*, and that the combination of the metal and CTZ in a single molecule is responsible for the anti-parasitic activity.

Additionally, the mechanism of action of current anti-leishmanial drugs remains obscure or unknown. Moreover, with the establishment of the leishmanicidal activity of the Ru-CTZ compounds observed in previous studies is now important to understand their mechanism of action in order to facilitate the design of new chemotherapeutic strategies against leishmaniasis. Apoptosis is considered a feature of multicellular organisms, and it is well defined as a form of programmed cell death in which a series of events leads to self-destruction without inducing inflammatory responses *in vivo*. In contrast with apoptosis, necrosis results from acute tissue injury producing an inflammatory response (Vermes et al., 2000). In *Leishmania* spp., it has also been suggested that apoptosis may be a useful mechanism of cell death to evade an inflammatory response and therefore prevent the establishment of an effective immune response, allowing in this way the intracellular survival form of non-apoptotic parasites (van Zandbergen et al., 2006). It has been reported that several anti-leishmanial drugs such miltefosine (Verma et al., 2007) or artemesinin (Sen et al., 2007) kill the parasite through an apoptotic-like pathway. Therefore, in the present study we sought to investigate the possible mechanism of cell death induced by the AM162 complex in *L. major*.

Phosphatidylserine is mainly situated in the inner leaflet of the plasmatic membrane bilayer in healthy cells and when is translocated to the external membrane leaflet, serves as a sensitive indicator to identify and quantify cells experiencing apoptosis (Mackey et al., 2006). Annexin V protein, conjugated with a fluorophore, is a routinely used reagent due to the fact that it possesses a high affinity for PS and serves to identify cells with externalized PS *via* flow cytometry in a live-cell manner. Promastigotes exposure to AM160 or AM162 for 24 h, exhibited phospholipid externalization after examination by a flow cytometer protocol (Fig. 3), thus suggesting that both Ru-azole complexes use the apoptotic-like pathway to provoke cell death.

Apoptotic cell death can be trigger *via* two main pathways, intrinsic/mitochondrial and extrinsic/death receptor (Elmore, 2007). Previously, CTZ was observed to decrease the mitochondrial membrane potential in *L. major infantum chagasi* promastigotes, in addition to low alteration of their plasma membrane (Mesquita et al., 2014). However, the percentage of mitochondrial membrane potential exerted by CTZ treatment in *L. major infantum chagasi* promastigotes was not significant in comparison with their control (Mesquita et al., 2014). Here, it was explored whether ruthenium-clotrimazole complexes AM160 and AM162 were able to significantly initiate the activation of the intrinsic pathway, which is evidenced by dissipation of the mitochondria membrane potential. *L. major* promastigotes were exposed to AM160 or AM162 and after staining with JC-1 were immediately analyzed *via* flow cytometer. AM160 and AM162 complexes were able to provoke mitochondria

depolarization after 6 h of incubation (Fig. 4), suggesting that the intrinsic pathway is implicated in their mechanism of action to induced cell death.

DNA fragmentation is one of the best-recognized events that occur in the progression of apoptosis through the activation of endonucleases, such as caspase activated deoxiribonuclease and is a late biochemical phase in apoptosis (Robles-Escajeda et al., 2013). To reinforce previous findings, TUNEL assay was performed to investigate if the Ru-CTZ complexes provoke DNA fragmentation to induce cell death. After exposure to AM160 and AM162, promastigotes were processed according with the TUNEL protocol and analyzed by flow cytometer. Results indicate that both AM160 and AM162 provoked DNA fragmentation on promastigotes after treatment exposure (Fig. 5).

Additionally, cell-cycle profile and DNA fragmentation is often measured concomitantly *via* cellular DNA content, using a fluorescence nucleic acids intercalator dye (Weingartner et al., 2012). When utilizing this strategy, a selective increment in the sub G0/G1 (hypodiploid) population, localized to the left of the G0/G1 peak could be identified, which is interpreted as cells carrying fragmented DNA. Promastigotes exposed to both AM160 and AM162 for 24 h exhibited an alteration in the cell cycle profile, proved by decreasing both G0/G1 and S/G2/M populations, concomitantly associated with an increase in the sub-G0/G1 population (Fig. 6). Thus, the results here corroborated our findings obtained from the TUNEL assay, indicating that the two Ru-azole complexes induce DNA fragmentation as their mechanism of action.

Overall, the ruthenium-clotrimazole AM162 complex has demonstrated to be an efficient compound for the treatment of cutaneous leishmaniasis in a murine model, with imperceptible toxicity to the experimental animals. Additionally, both AM160 and AM162 exhibited pro-apoptotic-like properties, implicating phospholipids externalization, mitochondrial depolarization and DNA fragmentation pathway, as its mechanism to induce cell death in *L. major* parasites; encompassing early and late hallmarks of apoptosis. The effects observed of AM162 in the *in vivo* model point us to the synthesis of new chemical formulations, AM162-derived analogs, with the aim to increase its solubility and efficacy against this neglected disease. Furthermore, since the AM162 complex showed the best *in vivo* anti-parasitic activity, new delivery formulations of this compound are currently in development in our laboratory for further pre-clinical studies.

Acknowledgments

Authors thank the NIH for support through Grant No. 5SC1GM089558 to R.A. S-D. Thanks to the Biomolecule Analysis, the Genomic Analysis and the Cytometry, Screening and Imaging Core Facilities at the University of Texas at El Paso supported by NIMHD-NIH Grant No. 5G12MD007592. Thanks to the Border Biomedical Research Center pilot grant to R.A.M, supported by RCMI program Grant No. 2G12MD007592, from the NIMHD-NIH. The RISE Scholars Program at UTEP through NIGMS Grant No R25GM069621-09 supported E.I.

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Fig. 1.
Chemical structures of clotrimazole (CTZ), as well as AM160 and AM162 ruthenium-clotrimazole complexes.

Fig. 2.

In vivo effect of Ru-CTZ complexes on cutaneous leishmaniasis in a murine model. (A) Pilot experiment and lesion size (mm) of mice infected with 10^5 *L. major* metacyclic promastigotes, followed by intraperitoneal treatment for 14 consecutive days of vehicle control (50% v/v DMSO/PBS solution), or AM160, AM162, C3, CTZ at 4 mg/kg/day. Statistical significance was determined using Student's *t*-test. *P* value of 0.00046 was obtained when comparing AM162- with vehicle-treated mice. (B) Hyper infection experiment and lesion size (mm) of mice infected with 10^6 *L. major* metacyclic promastigotes, intraperitoneal treatment for 14 consecutive days was applied: vehicle control

(50% v/v DMSO/PBS solution), or AM162 at 6 mg/kg/day. (C) Quantitative real-time PCR of hyper infection experiment. Parasite equivalents representing the target gene determined by real-time PCR of *L. major* minicircle kDNA present in DNA extracted from footpads (50 ng). Statistical significance was determined using Student's *t*-test. *P* value of 0.0146 was obtained when comparing AM162- with vehicle-treated mice.

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Fig. 3. Ru-CTZ complexes induced phospholipids externalization in *L. major* promastigotes after 24 h of treatment at 2 μ M. (A) Percentage of apoptotic and necrotic parasites treated with the compounds and controls. (B) Representative flow cytometry scatterplots of the compounds and controls, cells were stained with annexin V-Alexa Fluor 488 and propidium iodide. *P* values ranges, *P* < 0.01 (**).

Fig. 4.

Ru-CTZ-mediated cytotoxicity involves ψ m disruption in *L. major* promastigotes. (A) Percentage of JC-1 green fluorescence *L. major* promastigotes treated for 6 h with the Ru-azole compounds. Negative control 1% DMSO and positive control CCCP. (B) Representative flow cytometry plots for JC-1 assay. *P* values ranges, *P* 0.01(**) and *P* 0.001(***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Ru-CTZ complexes induce DNA fragmentation. (A) Flow cytometry scatterplots illustrating the detection of DNA strand breaks in apoptotic *L. major* promastigotes by TUNEL assay. Apoptotic cells characterized by high frequency of DNA strand breaks producing the exponential scale of Y coordinate observed in the positive control treated with DNase I. Apoptosis pattern observed after treatment of AM160 and AM162 at 2 μ M for 6 h.

Fig. 6.

Ru-azole compounds provoked DNA fragmentation in *L. major* promastigotes after 24 h of exposure, evidenced by increment in the sub-G₀/G₁ phase. The percentage distribution of cells for each defined subpopulation of the cell cycle phases are depicted as follow; (A) sub G₀/G₁ (hypodiploid; apoptotic population); (B) G₀/G₁ (diploid); and (C) S/G₂/M (hyperdiploid/tetraploid). (D) Representative flow cytometric histograms of cells treated with the experimental compounds and 1% DMSO. *P* values ranges, *P*_{0.01}(**) and *P*_{0.0001}(****) for the apoptotic population.