Journal of Coordination Chemistry

ISSN: 0095-8972 (Print) 1029-0389 (Online) Journal homepage: http://www.tandfonline.com/loi/gcoo20

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To cite this article: Esteban Rodríguez Arce, Cynthia Sarniguet, Tania S. Moraes, Marisol Vieites, A. Isabel Tomaz, Andrea Medeiros, Marcelo A. Comini, Javier Varela, Hugo Cerecetto, Mercedes González, Fernanda Marques, M. Helena García, Lucía Otero & Dinorah Gambino (2015) A new ruthenium cyclopentadienyl azole compound with activity on tumor cell lines and trypanosomatid parasites, Journal of Coordination Chemistry, 68:16, 2923-2937, DOI: 10.1080/00958972.2015.1062480

To link to this article: http://dx.doi.org/10.1080/00958972.2015.1062480

Journal of Coordination Chemistry, 2015
Vol. 68, No. 16, 2923–2937, http://dx.doi.org/10.1080/00958972.2015.1062480

A new ruthenium cyclopentadienyl azole compound with activity on tumor cell lines and trypanosomatid parasites

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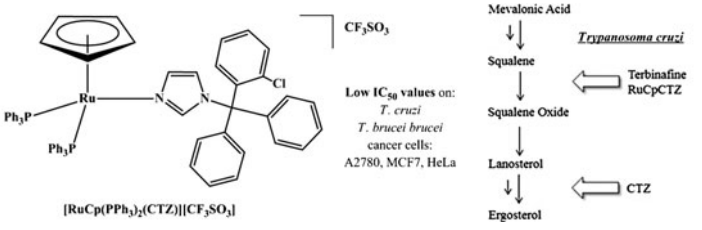
(*Received 3 March 2015; accepted 22 May 2015*)



As part of our efforts to develop organometallic ruthenium compounds bearing activity on both trypanosomatid parasites and tumor cells, a new Ru(II)–cyclopentadienyl clotrimazole complex, $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$, where Cp = cyclopentadienyl, CTZ = clotrimazole, was synthesized and characterized. The compound was evaluated *in vitro* on *T. cruzi* (Y strain), the infective form of *T. brucei brucei* strain 427 (cell line 449), on three human tumor cell lines with different sensitivity to cisplatin (A2780, ovary; MCF7, breast; HeLa, cervix) and on J774 murine macrophages as mammalian cell model. The new compound is more cytotoxic on *T. cruzi* and on the tumor cell lines than the reference drugs (Nifurtimox and cisplatin, respectively). In addition, complexation of the bioactive CTZ to the $\{RuCp(PPh_3)\}$ moiety leads to significant increase of the antiparasitic and antitumoral activity. To get insight into the potential "dual" mechanism of antiparasitic action emerging from the presence of Ru(II) and CTZ in a single molecule, the inhibitory effect of this new complex on the biosynthesis of *T. cruzi* sterols of membrane and the interaction with DNA were studied. Although the tested complex does not affect DNA, it affects the *T. cruzi* biosynthetic pathway of

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conversion of squalene to squalene oxide. According to the results reported here, [RuCp$(PPh_3)_2$(CTZ)][CF_3SO_3] could be considered a prospective antiparasitic and/or antitumoral agent that deserves further evaluation.

Keywords: Organometallic ruthenium compound; Clotrimazole; Cancer; Trypanosomes

1. Introduction

American Trypanosomiasis (Chagas disease) and Human African Trypanosomiasis (Sleeping sickness), diseases caused by the genetically related trypanosomatid parasites *Trypanosoma cruzi* and *Trypanosoma brucei* (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*), respectively, constitute major health concerns in the developing world [1–6], although in the context of the current global society the impact of these diseases is not strictly geographically confined [7, 8]. The available chemotherapeutics are decades old and suffer from limited efficacy, undesirable collateral effects and development of resistance. Therefore, novel strategies for the development of more efficient and less toxic agents, that could also circumvent drug resistance, are urgently needed. Based on the recognition of common targets in both parasites, drugs that are active against both protozoa could offer an innovative approach for drug discovery [4].

In a not very different context, cancer is the second largest cause of death in developed countries, and the number of worldwide deaths from this disease is projected by WHO to rise to over 13.1 million people in 2030 [9]. Metabolic pathways of highly proliferative cells, such as tumor cells and parasites, are expected to have resemblances that could lead to a correlation between antiparasitic and antitumor activities. In fact, several antitumoral drugs also show significant antiparasitic activity and *vice versa* [10].

The development of bioactive metal-based compounds through inorganic medicinal chemistry strategies has been recognized as a promising and attractive approach in the search for antitumoral and antiparasitic drugs [11–27].

In particular, ruthenium complexes have been the most widely studied non-platinum, metal-based anticancer candidates and hold potential as successful alternatives in cancer treatment [10, 13, 16, 18, 28, 29]. In this perspective, ruthenium–cyclopentadienyl complexes presenting N-heteroaromatic ligands have exhibited excellent antitumoral properties and are very promising agents for cancer therapy [30–34]. In addition, ruthenium classical coordination compounds and organometallic compounds have been recognized as prospective agents against trypanosomatid parasites [35–42]. The design of antiparasitic compounds which combine ligands bearing antitrypanosomal activity and pharmacologically active metals in a single chemical entity could provide drugs capable of modulating multiple targets simultaneously. Our group has been working on the development of ruthenium complexes with different families of trypanosomicidal ligands [10, 21, 38–42]. In particular, Ru-*p*-cymene compounds with this type of ligands were developed and studied in depth [39–41].

Based on a similar approach, Sánchez-Delgado's group performed leading research in the field by developing ruthenium and other metal complexes with the bioactive azole derivative clotrimazole (CTZ, figure 1) as ligand [35, 37, 43–45]. CTZ is a well-known antifungal agent, acting, such as other azole derivatives, as a sterol biosynthesis inhibitor in fungi. In particular, it inhibits the cytochrome P-450 dependent C(14) demethylation of lanosterol to ergosterol. The sterol biosynthetic pathways of trypanosomatids are similar to those of

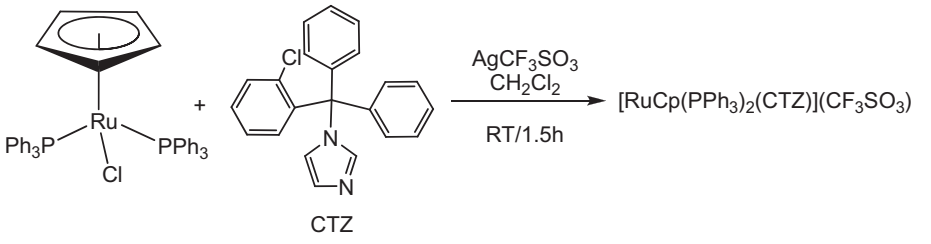

Figure 1. Scheme for the synthesis of $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$.

pathogenic fungi, and therefore, CTZ also displays moderate activity on *T. cruzi* [45, 46]. Five classical Ru-CTZ coordination compounds and four organometallic Ru-*p*-cymene CTZ compounds were developed by this group. Most of them displayed higher antiproliferative activity than free CTZ on *T. cruzi* suggesting a synergistic effect [35, 37, 44, 45]. The biological profile clearly changed when modifying the coordination sphere of the Ru(II), making further modification of the Ru(II)-CTZ environment interesting [10, 37].

As part of our efforts to rationally develop organometallic ruthenium compounds bearing activity on different trypanosomatid parasites but also on tumor cells, a new Ru(II)–cyclopentadienyl CTZ complex, $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$ (abbreviated as RuCpCTZ), was synthesized and characterized in this work. The compound was evaluated *in vitro* on the epimastigote form of *T. cruzi* (Y strain), the infective form of *T. brucei brucei* strain 427 (cell line 449) and the tumor cell lines A2780 (ovary), MCF7 (breast) and HeLa (cervix). To test selectivity, the cytotoxicity on J774 murine macrophages as mammalian cell model was evaluated. In order to get insight into the potential "dual" mechanism of antiparasitic action, emerging from the presence of Ru(II) and CTZ in a single molecule, the inhibitory effect of the complex on the biosynthesis of *T. cruzi* membrane sterols and the interaction with DNA were studied.

2. Results and discussion

Clotrimazole (CTZ, figure 1) is a classic imidazole ligand with a single nitrogen coordination site (N1) capable of binding to a metal ion. It usually reacts readily under mild conditions [37, 44, 45, 47–50]. A Ru-cyclopentadienyl complex (figure 2) with this bioactive

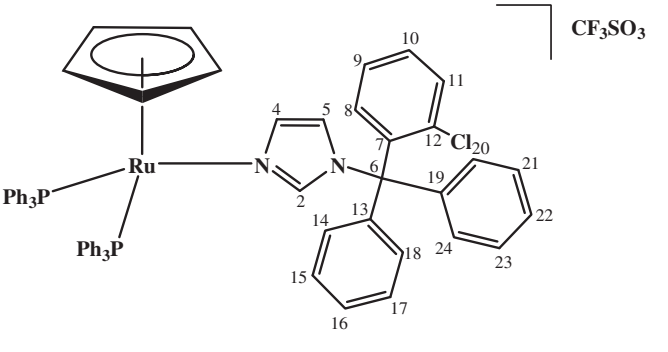

Figure 2. Proposed chemical structure for $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$.

ligand was synthesized with high purity and good yield by the reaction of the precursor [$RuCp(PPh_3)_2Cl$] and CTZ (figure 1). Unfortunately, it was impossible to obtain adequate single crystals of the complex for performing structural characterization by X-ray analysis. Nevertheless, the compound was fully characterized in the solid state and in solution using elemental analyses, conductometric measurements, and FTIR, and ^{1}H-, ^{13}C-, and ^{31}P-NMR spectroscopies.

Elemental analyses agree with the proposed formula. The molar conductivity value obtained for the complex in DMSO solution demonstrates that it is a 1 : 1 electrolyte [51]. No conductivity changes were observed during at least 5 days at 25 °C, hence suggesting that the complex is stable in air in DMSO.

Relevant solid FT-IR absorption bands were tentatively assigned to molecular vibration modes of the coordinated CTZ ligand [37], the triflate counterion [34] and the {RuCp} moiety [52]. Three new medium to strong bands were identified in the low-wavenumber region (600–400 cm^{-1}) at 572, 533, and 522 cm^{-1} that could correspond to metal-ligand vibrations.

A detailed characterization of the new complex in solution was performed by NMR spectroscopy. The resonances were assigned on the basis of 1-D ^{1}H-NMR, and 2-D homonuclear correlated spectroscopy (COSY) and heteronuclear correlation (HSQC) experiments as well as on the comparison with those for CTZ and other previously reported Ru(II)–CTZ complexes [37, 44, 45]. Table 1 shows the ^{1}H-NMR chemical shifts (δ) of the complex. Figure 2 shows the corresponding numbering scheme. ^{1}H-NMR integrations and signal multiplicities were in agreement with the proposed formula and structure.

^{1}H-NMR resonance of the η^5-cyclopentadienyl ring is in the characteristic range of cationic ruthenium(II) complexes [30]. The heteroaromatic ring protons of CTZ ligand are shielded upon coordination to Ru(II) center, with exception of the protons adjacent to the coordinated N; these two protons, H2 and H4, are deshielded by 0.31 and 0.16 ppm, respectively. This effect, perhaps due to the influence of the organometallic moiety on the ring current of the heteroaromatic ligand, was also observed in other piano stool Ru(II) η^5-cyclopentadienyl [34] and Ru(II) η^6-arene compounds with pyridylpyrazole, pyridylimidazole [53], and phenoxazine ligands [54], coordinated by N of the heteroaromatic rings.

^{13}C-NMR spectra revealed the same general effect observed for the protons, although it was not possible to unequivocally identify all the carbons, showing high complexity in the 127–138 ppm region with signals corresponding to both PPh_3 and CTZ ligands. However, analysis of the HSQC spectra allowed clear identification of C2 (138.2 ppm) and C4

Table 1. ^{1}H-NMR chemical shift values (δ) in ppm for [$RuCp(PPh_3)_2(CTZ)$](CF_3SO_3) and CTZ in DMSO-d_6 at 30 °C.

^{1}H-NMR/δ_H (integration) (multiplicity)		
Proton number	[$RuCp(PPh_3)_2(CTZ)$](CF_3SO_3)	CTZ
H2	7.75 (1, s)	7.44 (1, t)
H4	7.07 (1, dd)	6.91 (1, t)
H5	6.72 (1, dd)	6.73 (1, t)
H8	6.79 (1, m)	6.80 (1, d)
H11	7.38[a] (1, m)	7.40 (1, t)
H9, H10, H14, H16, H18, H20, H22, H24	6.79 (8, m)	7.32 (8, m)
H15, H17, H21, H23	6.66 (4, m)	7.01 (4, m)
Cp	4.50 (5, s)	–
PPh_3	7.38 (12, m), 7.27 (6, m), 7.21 (12, m)	–

[a]Overlapped with PPh_3.

(130.0 ppm) of the CTZ ligand. As previously reported, these are the most shifted CTZ carbon signals as a consequence of complexation [44]. The signal at 83.2 ppm corresponds to equivalent carbons of the Cp ligand [52]. Furthermore, a signal at 121.9 ppm corresponding to $CF_3SO_3^-$ counterion was also observed [55].

^{31}P-NMR spectra of the complex show a single sharp signal for the triphenylphosphane coligand at ~41 ppm, and the expected deshielding upon coordination is in accord with the σ-donor character of this ligand.

2.1. *Biological results*

2.1.1. Antiparasitic activity. The effect of RuCpCTZ on the epimastigote form of *T. cruzi*, Y strain, was evaluated and compared with that of CTZ. The results are presented in table 2. RuCpCTZ showed high cytotoxic activity on this parasite with an IC_{50} value in the submicromolar range. A sixfold increase of activity was observed with respect to free CTZ. In addition, the complex showed an activity about 30 times higher than the reference drug Nifurtimox (IC_{50} = 8.0 μM) [56].

For *T. brucei brucei* (strain 427), the new ruthenium compound showed growth inhibitory activity, inducing a dose-dependent antiproliferative effect on parasites treated for 24 h (table 2). CTZ displayed a lower cytotoxicity toward *T. brucei* (IC_{50} > 25 μM) than against *T. cruzi* (IC_{50} 1.8 μM). This selectivity is in good agreement with the sterol content in each species and life cycle stage of the parasites; *T. cruzi* epimastigotes contain 40% ergosterol whereas bloodstream *T. brucei* contains predominantly cholesterol incorporated from the medium by a receptor-mediated endocytic mechanism [57]. As a consequence of complex formation, the potency of RuCpCTZ against the infective form of African trypanosomes is 40-fold higher (IC_{50} = 0.6 μM) compared to the activity of the parental compound (IC_{50} > 25 μM). This suggests that the RuCp moiety of the CTZ complex is a major determinant for the biological activity of this compound against *T. brucei*.

In addition, RuCpCTZ showed a lower IC_{50} value on *T. brucei brucei* (strain 427) than previously reported $[Ru_2(p\text{-cymene})_2(L)_2]X_2$ complexes, where L = 5-nitrofuryl containing thiosemicarbazones and X = Cl^- or PF_6^- [39].

2.1.2. Cytotoxicity on J774 murine macrophages. The specificity of the antitrypanosomal activity of RuCpCTZ and CTZ was evaluated by analyzing their cytotoxicity against a murine macrophage-like cell line (J774). The results are depicted in table 2. These results enable the comparison of selectivity indexes (SI) of RuCpCTZ and CTZ calculated as SI = IC_{50} macrophages/IC_{50} parasites.

Although RuCpCTZ showed higher cytotoxicity than CTZ, RuCpCTZ showed fairly good selectivities toward both parasites (*T. cruzi* and *T. brucei*) with respect to mammalian

Table 2. *In vitro* activity (measured as the IC_{50} value, the half-inhibitory concentration) against *T. cruzi*, *T. brucei brucei*, cytotoxicity on murine macrophages, and selectivity index (SI) values of RuCpCTZ and CTZ.

Compound	*T. cruzi* IC_{50}/μM	*T. brucei* IC_{50}/μM	Macrophages IC_{50}/μM	SI (fold)[a]	SI (fold)[b]
RuCpCTZ	0.25 ± 0.08	0.6 ± 0.1	1.9 ± 0.1	8	3
CTZ	1.8 ± 0.4	> 25	55.1 ± 0.5	31	< 2

[a]SI: IC_{50} macrophages/IC_{50} *T. cruzi*.
[b]SI: IC_{50} macrophages/IC_{50} *T. brucei brucei*. Results are the mean value of three different independent experiments.

cells (J774 murine macrophages). In the case of *T. brucei*, the comparison of the selectivity index values reveals that RuCpCTZ (SI = 3) is not only more potent but also more selective than CTZ (SI < 2).

2.1.3. Cytotoxic activity in human tumor cells. The cytotoxic activities of RuCpCTZ and CTZ were evaluated on three human cancer cells, ovarian A2780, breast MCF7, and cervical HeLa, representing common cancer diseases and also presenting different degrees of resistance to metallodrugs. The cells were treated with the compounds in the concentration range 100 nM–100 μM during a 72-h incubation period. A colorimetric MTT assay was used to evaluate the cytotoxic activity measured as the half-inhibitory concentration (IC_{50}). IC_{50} values found for RuCpCTZ ranged between 0.5 and 5 μM while those obtained for CTZ spanned between 6 and 20 μM (table 3). The A2780 cells were particularly much more sensitive to both compounds, in particular to RuCpCTZ, than the MCF7 and HeLa cells. Complex formation seems to be responsible for the observed higher sensitivity of the cells to the Ru complex relative to the free ligand. Cisplatin was presented for comparison, since it is the metallodrug in clinical application. In the cell lines studied, the cytotoxic activity of RuCpCTZ is three to five times higher than that of the reference drug cisplatin. In addition, RuCpCTZ is 4- to 63-fold more active on A2780 tumor cells and 2- to 12-fold more active on MCF7 cells than a series of organometallic Ru compounds previously developed by our group [41] and shows a significantly lower IC_{50} value on HeLa and MCF7 cells than recently reported Ru(II) cytotoxic compounds [59, 60].

Table 3. IC_{50} values found for RuCpCTZ and CTZ in three different human cancer cells lines after 72-h incubation at 37 °C. Cisplatin is included for comparison.

	IC_{50} (μM)		
Compound	A2780	MCF7	HeLa
CTZ	5.7 ± 0.9	20.5 ± 8.1	18.4 ± 7.9
RuCpCTZ	0.54 ± 0.1	5.4 ± 2.0	3.6 ± 1.1
Cisplatin	1.9 ± 0.1[a]	28 ± 6.0[a]	7.00 ± 2.70[b]

[a]From Ref. [33].
[b]From Ref. [58].

2.2. *Mechanism of antiparasitic action*

RuCpCTZ was designed to include a bioactive ligand and the pharmacologically relevant Ru-cyclopentadienyl center, searching for a dual mechanism of action by affecting different targets or biologically significant processes in the parasite. Accordingly, studies were performed in order to validate proposed targets and to get insight into the probable mechanism of antiparasitic action of RuCpCTZ.

2.2.1. Inhibition of sterol membrane biosynthesis. CTZ mechanism of action involving α-14 C demethylase inhibition from the sterol membrane biosynthesis pathway is well known [45, 46]. Therefore, the effect of RuCpCTZ on this biochemical pathway was studied [61]. The obtained results (figure 3) showed that, while ergosterol is present in all the studied treatments, its concentration seemed to be lower for those incubations treated with

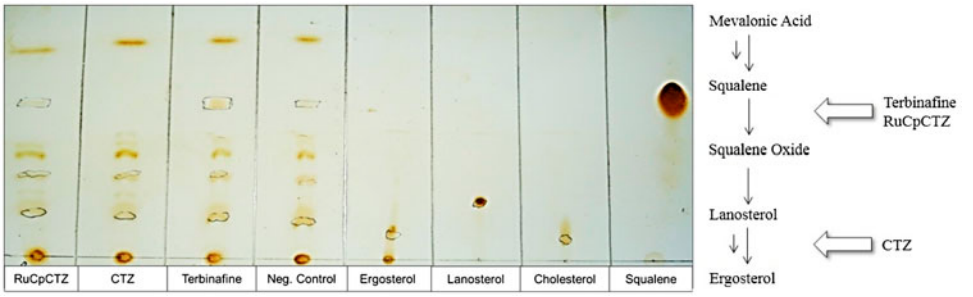

Figure 3. TLC of the lipidic extract of *T. cruzi* after treatment of the parasite with the compounds under study and terbinafine. The sterols used as standards are included. The TLC was revealed with vapors of iodine. Lane 1: RuCpCTZ, Lane 2: CTZ, Lane 3: terbinafine, Lane 4: negative control, Lane 5: ergosterol, Lane 6: lanosterol, Lane 7: cholesterol, Lane 8: squalene. Relevant pathways of the sterol biosynthesis are shown in the attached scheme (right).

RuCpCTZ, CTZ, or terbinafine than for the negative control. As expected, treatment with CTZ produced accumulation of lanosterol due to inhibition of this enzyme that catalyzes the conversion of lanosterol to ergosterol. On the other hand, the treatment with RuCpCTZ led to squalene accumulation, effect observed for terbinafine but not for free CTZ. The results indicate that RuCpCTZ affects sterol membrane biosynthesis at the same step as terbinafine, inhibiting squalene-2,3-epoxidase enzyme [62].

2.2.2. Interaction with DNA. Metal compounds and particularly ruthenium ones are usually able to interact with DNA. In addition, DNA has been proposed as a parasitic target for other Ru-CTZ complexes [45]. Therefore, this biomolecule was investigated as a potential target of RuCpCTZ. The well-established fluorescent DNA probe ethidium bromide (EB) was used to detect RuCpCTZ interaction with DNA. When EB is specifically intercalated into double-stranded DNA, the {DNA-EB} adduct shows a strong fluorescence emission (λ_{exc} = 510 nm) with a maximum at 594 nm in our experimental conditions [63, 64]. Results obtained for the titration of this {DNA-EB} adduct with RuCpCTZ are summarized in figure 4. No quenching in the emission of {DNA-EB} was observed upon increasing Ru

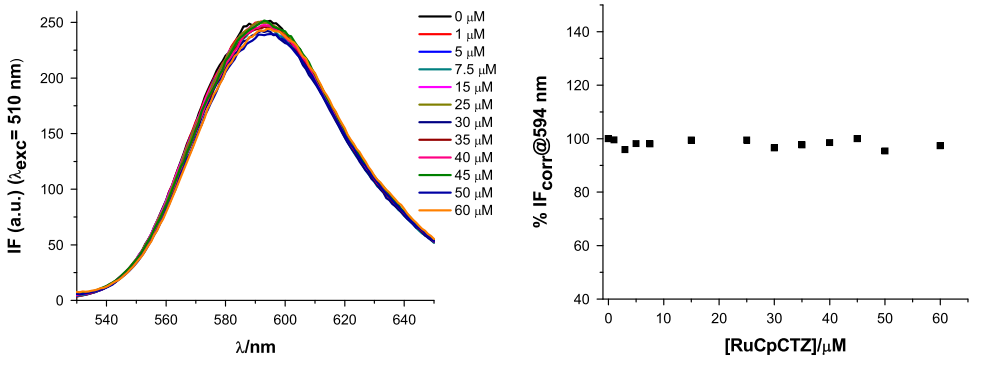

Figure 4. Left: Fluorescence data obtained for the competitive binding study of RuCpCTZ to {EB-DNA}. Right: Relative fluorescence intensity (%) at λ_{em} = 594 nm with increasing RuCpCTZ concentration. (C_{DNA} = 20 μM, C_{EB} = 10 μM, samples prepared in 2% DMSO/Tris-HCl medium, 30 min incubation at 37 °C).

complex concentration. This result indicates no strong interaction (through intercalation or covalent binding) between the Ru complex and the DNA, in agreement with what is observed in the direct quantification test for binding of RuCpCTZ to DNA described in section 4.5.2. The DNA binding level obtained by this method (nmol Ru mg^{-1} DNA ≈ 1) is negligible when compared to the values ranging from 40 to 300 nmol Ru mg^{-1} DNA reported for other Ru compounds that efficiently interact with DNA and for antitumor metal complexes having DNA as molecular target [39, 42, 65, 66].

3. Conclusion

A new organometallic ruthenium clotrimazole compound, $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$, was synthesized and fully characterized. This compound showed high cytotoxic activity on trypanosomatid parasites (*T. cruzi* and *T. brucei*), validating our proposal of the development of broad spectrum metal-based drugs active against multiple trypanosomatid protozoa as a potentially innovative approach for antiparasitic drug discovery. In addition, the compound shows high cytotoxicity on three tumor cell lines with different sensitivity to that of cisplatin. The compound is more cytotoxic on the parasites and on the tumor cell lines than the reference antitrypanosomal and antitumoral drugs. In all cases, $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$ is more active than free CTZ. The experiments performed gave insight into the probable mechanism of antitrypanosomatid action of the ruthenium compound. It affects the *T. cruzi* biosynthetic pathway responsible for the conversion of squalene to squalene oxide. According to the results obtained, $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$ could be considered a prospective antiparasitic and/or antitumor agent that deserves further development.

4. Materials and methods

4.1. *Materials*

All common laboratory chemicals were purchased from commercial sources and used without purification. All syntheses were carried out under dinitrogen using Schlenk techniques and the solvents used were dried by standard methods [67]. The precursor $[Ru(\eta^5\text{-}C_5H_5)(PPh_3)_2Cl]$ was synthesized from $RuCl_3$, triphenylphosphane, and cyclopentadienyl in ethanol according to a previously reported procedure [68].

4.2. *Synthesis of $[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$*

$[RuCp(PPh_3)_2Cl]$ (0.5 mmol, 0.320 g) and $AgCF_3SO_3$ (0.45 mmol, 0.116 g) were dissolved in dried distilled dichloromethane. The reaction mixture was kept at room temperature for 1.5 h. CTZ (0.5 mmol, 0.172 g) was added and allowed to react at room temperature for about 2 h. The reaction was followed by TLC (stationary phase silica gel GF254; mobile phase petroleum ether/acetone 1:1v/v) until no reactants were detected. AgCl was filtered off on line under dinitrogen pressure and the solvent was evaporated *in vacuo*. The yellow complex was obtained by separation through silica column under N_2 using petroleum ether/acetone (1:1v/v) as mobile phase.

$[RuCp(PPh_3)_2(CTZ)](CF_3SO_3)$. Yield: 190 mg, 32%. Yellow solid. Anal (%) Calcd for $C_{64}H_{52}ClF_3N_2O_3P_2RuS$ (%): C, 64.89; H, 4.42; N, 2.36; S, 2.71. Found: C, 64.75; H, 4.44; N, 2.38; S, 2.67. FT-IR (KBr, cm^{-1}, m = medium, w = weak, s = strong): 3055 (mw), ν(C–H from arene), $\nu(C_5H_5)$; 1479 (mw), 1446 (ms); 1430 (m), ν(C=N and/or C=C from arene); 696 (s), 520(s); 1273 (s), $\nu(CF_3SO_3)$. λ (DMSO) 380 nm ($\varepsilon = 2.8 \times 10^3\ M^{-1}\ cm^{-1}$). Λ_M(DMSO) = 34.6 $Scm^2\ mol^{-1}$.

4.3. *Physicochemical characterization*

C, H, and N analyses were carried out with a Thermo Scientific Flash 2000 elemental analyzer. Conductimetric measurements were done over time (5 days) at 25 °C in 10^{-3} M DMSO solutions using a Conductivity Meter 4310 Jenway to determine the type of electrolyte and to assess the stability of the complex in such medium [51]. The FTIR absorption spectra (4000–300 cm^{-1}) of the complex, the precursor, and CTZ were measured as KBr pellets with a Shimadzu IRPrestige-21 instrument. UV-vis spectrum was measured in DMSO from 300–800 nm with a spectrophotometer Shimadzu UV 1603 instrument. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 at 30 °C on a Bruker DPX-400 instrument (at 400 and 100 MHz, respectively). Homonuclear (COSY) and heteronuclear correlation experiments (2D-HETCOR), HSQC (heteronuclear single quantum correlation), were carried out with the same instrument. Tetramethylsilane was used as the internal standard. $^{31}P\{^1H\}$-NMR spectrum was recorded in DMSO-d_6 on a Bruker AVANCE III 400 instrument (at 161.98 MHz). Chemical shifts are reported in ppm.

4.4. *Biological activity*

4.4.1. Anti-*T. cruzi* activity. *T. cruzi* epimastigotes (Y strain) were grown at 28 °C in an axenic milieu (BHI-Tryptose) supplemented with 5% fetal bovine serum (FBS) as previously described [69, 70]. Cells from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture milieu to give an initial concentration of 10^6 cells mL^{-1}. Cell growth was followed every day by measuring the absorbance of the culture at 600 nm. Before inoculation, the milieu was supplemented with the indicated amount of the studied compound from a freshly prepared stock solution in DMSO. Nifurtimox (Nfx) was used as the reference trypanosomicidal drug. The final concentration of DMSO in the culture milieu never exceeded 0.4%, and the control was run in the presence of 0.4% DMSO and in the absence of the studied compounds. No effect on epimastigote growth was observed due to the presence of up to 1% DMSO in the culture milieu. The percentage of inhibition (PGI) was calculated as follows: PGI (%) = $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, where $Ap = A_{600nm}$ of the culture containing the studied compound at day 5; $A0p = A_{600nm}$ of the culture containing the studied compound just after addition of the inocula (day 0); $Ac = A_{600nm}$ of the culture in the absence of the studied compound (control) at day 5; $A0c = A_{600nm}$ in the absence of the studied compound at day 0. To determine IC_{50} values (50% inhibitory concentrations), parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. At day 5, the absorbance of the culture was measured and related to the control. The IC_{50} value was taken as the concentration of compound under study necessary to reduce the absorbance ratio to 50%.

4.4.2. Activity on *T. brucei brucei* strain 427. The infective form of *T. brucei brucei* strain 427, cell line 449 (encoding one copy of the tet-represor protein: Pleo^R [71]), was aerobically cultivated in a humidified incubator at 37 °C with 5% CO_2 in HMI-9 medium [72] supplemented with 10% (v/v) fetal calf serum (FCS), 10 U mL^{-1} penicillin, 10 μg mL^{-1} streptomycin and 0.2 μg mL^{-1} phleomycin. The assay was performed as previously described [73]. Ten mM stock solutions of the compounds were prepared using DMSO as solvent and then diluted in the culture medium to obtain seven experimental concentrations (from 25 to 0.3 μM). The maximum DMSO concentration used in the cytotoxicity assays did not exceed 1%, which is known to be nontoxic to *T. brucei* parasites. Compounds, DMSO (up to 1%) or medium, were immediately added at the concentrations described above, and the culture plate was incubated at 37 °C with 5% CO_2. After 24 h, living parasites were counted with a Neubauer chamber under the light microscope. Each condition was tested in triplicate.

For each compound concentration, cytotoxicity was calculated according to the following equation: Cytotoxicity (%) = {(experimental value - DMSO control)/(growth control - DMSO control)} × 100. The data were plotted as percentage cytotoxicity versus drug concentration. IC_{50} values were obtained from dose-response curves fitted to a sigmoidal equation (Boltzmann model) or extrapolated from nonlinear fitting plots.

4.4.3. Cellular viability assays in human tumor cells. Three human tumor cell lines: A2780 ovarian, MCF7 breast, and HeLa cervical (ATCC) were cultured in medium (Gibco, Invitrogen) RPMI 1640 (A2780) or DMEM containing GlutaMax I (MCF7, HeLa) supplemented with 10% FBS and 1% antibiotics at 37 °C, 5% CO_2 in a humidified atmosphere (Heraeus, Germany). The cells were adherent in monolayers and upon confluency were harvested by digestion with trypsin–EDTA (Gibco). Cell viability was evaluated using a colorimetric assay based on the tetrazolium salt MTT [74]. For this purpose, cells were seeded in 200-μL aliquots in complete media RPMI or DMEM into 96-well plates. Adequate cellular densities were chosen to ensure exponential growth of untreated control samples throughout the experiments (2–5 × 10^4 cells/well). For 24 h, cells were allowed to adhere followed by the addition of dilution series of the test compounds in fresh medium (200 μL/well). Ligand and complex were first solubilized in DMSO and then in medium, and added to final concentrations in the range 100 nM–100 μM. DMSO in cell culture medium did not exceed 1% (final concentration). DMSO does not show cytotoxic effect by itself at this concentration. After continuous treatment of the cells with the compounds for 72 h at 37 °C with 5% CO_2, the medium was replaced by 200 μL of MTT solution in PBS (0.5 mg mL^{-1}). After 3–4 h incubation, MTT solutions were removed and the formazan product formed was dissolved in DMSO (200 μL/well). The cellular viability was evaluated by measuring the absorbance at 570 nm using a plate spectrophotometer (PowerWave Xs, Bio-tek Instruments, VT, USA). The cytotoxic effects of compounds were quantified by calculating the IC_{50} value based on nonlinear regression analysis of dose-response curves (GraphPad Prism version 4.0). Evaluation was based on at least two independent experiments, each comprising six replicates per concentration.

4.4.4. Cytotoxicity on murine macrophages. The J774 mouse macrophage cell line was cultivated in a humidified 5% CO_2/95% air atmosphere at 37 °C in DMEM medium supplemented with 10% (v/v) FCS, 10 U mL^{-1} penicillin, and 10 μg mL^{-1} streptomycin. Stock

solutions of the compounds to be tested were prepared as described for anti-*T. brucei* activity tests and diluted in culture medium to obtain seven experimental concentrations (from 100 to 0.5 μM). The maximum DMSO concentration used did not exceed 1%, which is known to be tolerated by murine macrophages from J774 cell line. Each condition was tested in triplicate. The cytotoxic effect of the compounds toward macrophages was evaluated by colorimetric assay of cell viability with a tetrazolium salt (WST-1 reagent). The absorbance of the formazan dye produced by metabolically active cells was measured at 450 nm (reference wavelength at 630 nm) with an EL 800 microplate reader (Biotek). The methodological procedure is essentially as described previously [39]. Cytotoxicity was calculated and IC_{50} values were obtained as described for cytotoxicity on *T. brucei brucei*.

4.5. *Mechanism of action*

4.5.1. Inhibition of the biosynthesis of membrane sterols. Epimastigote form of *T. cruzi* (Tulahuen 2 strain) was maintained in an axenic medium (BHI-Tryptose). The experiments were carried out in cultures at 28 °C and with strong aeration. The studied compound was added to the culture (10×10^6 cells mL^{-1}) as a DMSO solution in a concentration equivalent to IC_{50}. Compound and parasites were incubated 72 h at 28 °C. Final concentration of DMSO in the culture never exceeded 0.4%. A control including 0.4% DMSO was prepared. Positive controls with terbinafine were also incubated. After incubation, the control and drug-treated parasites were centrifugated at 3000 rpm during 10 min and then the supernatants were discarded and the pellets were collected and suspended in 1-mL phosphate buffer (0.05 M, pH 7.4) and centrifugated at 3000 rpm during 10 min. The pellets were treated with 1 mL chloroform/methanol (2:1) during 12 h at 4 °C. After addition of saturated NaCl aqueous solution, a new extraction with 600 μL chloroform and a extraction with 600 μL hexane were performed. The total volume of extraction was seeded on a silica TLC plate. Squalene was identified by two chromatographic runs with hexane as mobile phase and ergosterol by one run with *n*-hexane:AcOEt (8:2). The TLC was developed by UV light and with iodine vapors. The compound was seeded together with a negative control, a positive control (terbinafine), and standards of squalene, lanosterol, cholesterol, and ergosterol [75].

4.5.2. Interaction with DNA.

4.5.2.1. *Fluorescence studies.* Experiments for competitive binding to calf-thymus DNA (ct-DNA, SIGMA, Type I, No. D-1501) with ethidium bromide (EB, SIGMA) were carried out in 10 mM Tris-HCl buffer at pH 7.4. Millipore® water was used for the preparation of all aqueous solutions. Fluorescence measurements were carried out on individually prepared samples to ensure the same pre-incubation time for all samples in each assay. Due to the low solubility of the complex in aqueous media, DMSO was used to prepare concentrated stock solutions followed by appropriate dilution to obtain the targeted concentration and the same content of DMSO (5% v/v) in the final samples. DNA stock solutions were prepared by hydrating ct-DNA in Tris–HCl buffer (1 mg mL^{-1}, ~2 $mM.nuc^{-1}$) during 3–4 days at 4 °C, swirling the solution about 4–5 times a day until full dissolution was attained, and a clear solution was obtained. This solution was kept at 4 °C (in the refrigerator) between measurements and discarded after 4 days. The concentration of each stock solution was determined by UV spectrophotometry using the molar extinction coefficient

$\varepsilon_{(260\ nm)} = 6600\ M^{-1}\ cm^{-1}$ [76]. A 5 mM EB solution was prepared in Tris–HCl buffer. ct-DNA was pre-incubated with EB at 4 °C for 24 h. Samples were prepared with a total concentration of DNA and of EB of 20 and 10 μM, respectively, varying the total complex concentration from 1 to 110 μM. They were incubated at 37 °C for 30 min. Samples with complex alone and samples with complex and EB but no DNA were used as blanks.

Fluorescence spectra were recorded from 520 to 650 nm at an excitation wavelength of 510 nm on a Shimadzu RF-5301PC spectrofluorimeter. Fluorescence emission intensity was corrected for the absorption and emission inner filter effect at the maximum emission wavelength (594 nm) using the UV–visible absorption data recorded for each sample according to the following equation [77].

$$IF_{corr} = IF \times 10^{\left(\frac{Abs_{\lambda exc} + Abs_{\lambda em}}{2}\right)}$$

4.5.2.2. *ct-DNA interaction experiments.* The complex was tested for their global DNA interaction ability using native ct-DNA by a modification of a previously reported procedure [78, 79]. ct-DNA (50 mg) was dissolved in water (30 mL) overnight. Solutions of the complex in DMSO (spectroscopy grade) (1 mL, 10^{-3} M) were incubated at 37 °C with solution of ct-DNA (1 mL) during 96 h. DNA/complex mixtures were exhaustively washed to eliminate the unreacted complex. Quantification of bound metal was done by atomic absorption spectroscopy on a Perkin Elmer 5000 spectrometer. Standards were prepared by diluting a metal standard solution for atomic absorption spectroscopy. Final DNA concentration per nucleotide was determined by UV absorption spectroscopy using molar absorption coefficient of 6600 $M^{-1}\ cm^{-1}$ at 260 nm [76].

Acknowledgements

E.R.A. and J.V. wish to thank ANII for a postgraduate grant. Authors thank CSIC-Universidad de la República (project 800), PEDECIBA, and ANII-SNI, Uruguay. M.A.C. acknowledges the support of FOCEM (MERCOSUR Structural Convergence Fund, COF 03/11). A.I.T. and T.M. thank the Portuguese Foundation for Science and Technology (FCT) for financial support (the IF Initiative IF/01179/2013 and post-doctoral grant SFRH/BPD/93513/2013). Portuguese coauthors also thank FCT for projects PTDC/QUI-QUI/118077/2010 and PEst-OE/QUI/UI0536.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

Supplemental data for this article can be accessed here [http://dx.doi.org/10.1080/00958972.2015.1062480].

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