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Toward a novel metal-based chemotherapy against tropical diseases. Part 5. Synthesis and characterization of new Ru(II) and Ru(III) clotrimazole and ketoconazole complexes and evaluation of their activity against *Trypanosoma cruzi*

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Abstract

The complexes $RuCl_3(CTZ)_3{\cdot}2CH_3OH$ (**1**) and $RuCl_3(KTZ)_2(H_2O){\cdot}2H_2O$ (**2**) were prepared by reaction of $RuCl_3{\cdot}3H_2O$ with CTZ and KTZ, respectively, while $RuCl_2(KTZ)_2$ (**4**) was prepared by reaction of $RuCl_2(CH_3CN)_4$ with KTZ (CTZ = 1-[(2-chlorophenyl)diphenylmethyl-1*H*-imidazole, and KTZ = *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine. All the complexes were characterized by NMR spectroscopy and for the paramagnetic species EPR spectroscopy was also employed. The new compounds were tested for in vitro activity against cultures of epimastigotes of *Trypanosoma cruzi*, the causative agent of Chagas disease, and compared with $RuCl_2(CTZ)_2$ (**3**) (reported previously) in order to establish some structure–activity correlations. At concentrations of 10^{-6} M (DMSO), all the complexes showed higher activity than the parental organic drug against the epimastigote form of the parasite, and Ru(II) complexes seem to be more active than their Ru(III) counterparts for a given nitrogen-donor ligand.

Keywords: Ruthenium; Clotrimazole; Ketoconazole; Paramagnetic; Metal complexes; *Trypanosoma cruzi*; Chagas

1. Introduction

Ruthenium complexes have shown promise in the development of several types of pharmaceutical, particularly as anticancer agents [1]. As part of our research program on novel chemotherapies against tropical diseases, we have been searching for new metal-based drugs with a low level of toxicity and high biological activity against parasites responsible for ailments like malaria [2] and Chagas disease [3]. The latter is caused by *Trypanosoma cruzi*, an hemoflagelate protozoon, which is transmitted to humans by a cone-nosed bug known as Reduviid or Triatomid. This endemic and essentially incurable illness afflicts close to 20 million people in the Latin American subcontinent, ranking as the third largest parasitic disease worldwide, after malaria and schistosomiasis [4]. In view of the urgent need for an effective treatment against Chagas disease, the most varied approaches have to be considered.

Some very encouraging results have been disclosed recently by Urbina et al. [5] and by others [6] using azole-type molecules which have been found to exert their therapeutic effect through a mechanism involving the inhibition of the biosynthesis of sterols essential for

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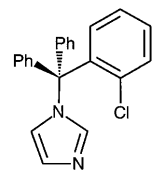
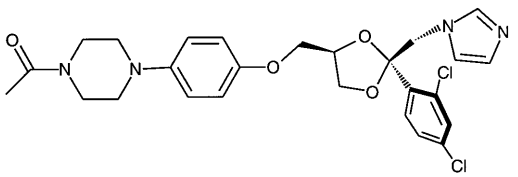
the parasite. Another alternative which has been emerging from our laboratories as an interesting possibility makes use of such azoles (collectively known as SBIs, sterol biosynthesis inhibitors) as N-donor ligands in complexes of various metals; the general strategy we have been employing is the synthesis of metal derivatives of particular organic ligands which themselves have shown activity against *T. cruzi*, *e.g.* clotrimazole, CTZ = 1 - [(2 - chlorophenyl)diphenylmethyl-1*H* - imidazole) and ketoconazole, KTZ = *cis*-1-acetyl-4 - [4 - [[2 - (2,4 - dichlorophenyl) - 2 - (1*H* - imidazol - 1 - ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl] piperazine [5,6].



CTZ



KTZ

Recently, we described the synthesis and characterization of a series of metal–CTZ complexes (Ru, Rh, Pt, Au and Cu) which showed promising biological activity for the case of Chagas disease. The most efficacious compound against *T. cruzi* was the Ru(II) derivative $RuCl_2(CTZ)_2$ which prompted us to study it in greater detail; this led us to a preliminary proposal for a mechanism of therapeutic action according to which the metal complex first acts as a transport agent across the parasite's membrane, and it is subsequently hydrolyzed in order to liberate CTZ which goes on to produce its known SBI action. Since a synergistic effect due to the presence of the metal fragment was observed, together with intracellular disorganization at the level of the nucleus, it was thought that Ru–DNA binding provides an additional means of damaging the parasite, and some preliminary studies indicated that some Ru–DNA interaction was in fact taking place [3]. Nevertheless, other factors such as partition coefficients at the cellular level may be influencing the selective transport and toxicity effects required for antiparasitic activity; in order to gain further insight into the mode of biological action of our complexes, we have engaged on a systematic study aimed at establishing possible structure–activity correlations.

Both CTZ and KTZ in their free forms have been evaluated against *T. cruzi*, and the latter has been found to be more active than the former [5,6]. It is also known that both Ru(II) and Ru(III) covalently bind to DNA, the binding generally being faster for the divalent state [7,8]. In order to shed some additional light on the mechanism of anti *T. cruzi* action of metal–azole derivatives, in this paper we present the synthesis, characterization and some preliminary data on the in vitro evaluation of the biological activity of a new series of Ru complexes in both the (II) and (III) oxidation states, containing either CTZ or KTZ. In this way we hope to reach a better understanding of the relative importance of the two main components of our mechanistic proposal.

2. Experimental

2.1. General procedure

Solvents of analytical grade were distilled from appropriate drying agents immediately prior to use. CTZ, KTZ, and $RuCl_3 \cdot H_2O$ were used as received. $RuCl_2(CH_3CN)_4$ and $RuCl_2(CTZ)_2$ were prepared according to the literature and characterized by comparison of their IR and NMR spectra with reported values [4]. Elemental analyses were performed at the Chemistry Center (IVIC) on a Fisons analyzer EA 1108; IR spectra were recorded on a Nicolet 5DCX FT spectrometer while UV–Vis spectra were obtained by use of a diode array Hewlett Packard 8453 instrument fitted with deuterium and mercury lamps, using chloroform solutions (10^{-4} M) of the complexes. EPR spectra were measured at room temperature (r.t.) in a Varian E-line spectrometer working in the X-band ($v = 9.3$ Hz) with a cylindrical cavity and a homemade coaxial microwave coupler for the X-band; experimental conditions (microwave power and modulation field) were adjusted to avoid saturation of signal intensity. NMR spectra were obtained in DMSO-d_6 solutions at 500 MHz with 8000 data points on a Bruker AVANCE500 spectrometer, ^{1}H NMR shifts being recorded relative to residual resonances in the deuterated solvent. For the paramagnetic complexes the NMR spectra were obtained using a 90° pulse of 10 ms. An inversion-recovery pulse sequence (180°–τ–90°–Aq) was used to obtain nonselective proton longitudinal relaxation times (T_1) with the carrier frequency set at different positions to ensure the validity of the measurements. Signal:noise ratios were improved by applying a line-broadening factor of 30 Hz to the FID prior to Fourier transformation.

2.2. $RuCl_3(CTZ)_3 \cdot 2CH_3OH$ (1)

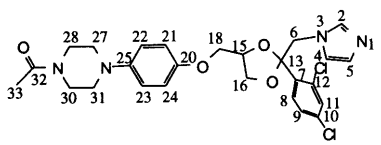
$RuCl_3 \cdot 3H_2O$ (154 mg, 0.59 mmol) was dissolved in freshly distilled methanol (15 ml), CTZ (876 mg, 2.54 mmol) in methanol (20 ml) was added to the solution which was stirred under reflux in a nitrogen atmosphere for 24 h. The volume of the solvent was reduced until precipitation began and the mixture was allowed to stand overnight at −10°C, after which the green solid obtained was filtered, washed with diethyl ether and dried under vacuum (yield, 480 mg, 62%). *Anal.* Calc. for $C_{68}H_{59}Cl_6N_6O_2Ru$: C, 62.5; H, 4.55; N, 6.4. Found: C, 62.1; H, 4.54; N, 6.9%. IR (cm^{-1}): ν(C=C) 1500, ν(C=N) 1440. λ_{max} ($CHCl_3$, 10^{-4} M) = 241, 325, 432 nm.

2.3. $RuCl_3(KTZ)_2(H_2O) \cdot 2H_2O$ (2)

$RuCl_3 \cdot H_2O$ (150 mg, 0.57 mmol) was dissolved in freshly distilled methanol (10 ml), KTZ (609 mg, 1.15 mmol) in methanol (20 ml) was added to the solution which was stirred under reflux in a nitrogen atmosphere for 24 h. The volume of the solvent was reduced until precipitation began and the mixture was allowed to stand overnight at −10°C, after which the brown solid obtained was filtered, washed with diethyl ether and dried under vacuum (yield, 70%). *Anal.* Calc. for $C_{52}H_{62}Cl_7N_8O_{11}Ru$: C, 47.1; H, 4.7; N, 8.5. Found: C, 46.8; H, 4.3; N, 8.3%. IR (cm^{-1}): ν(C=C) 1500, ν(C=N) 1440. λ_{max} ($CHCl_3$, 10^{-4} M) = 247, 296 nm.

2.4. $RuCl_2(KTZ)_2$ (4)

A 2:1 mixture of KTZ and $RuCl_2(CH_3CN)_4$ (60 mg, 0.18 mmol) was dissolved in methanol and allowed to react for 14 h at r.t., after which a white solid precipitated, which was filtered off, washed with diethyl ether and dried under vacuum (yield 60%). *Anal.* Calc. for $C_{52}H_{56}Cl_6N_8O_8Ru$): C, 50.6; H, 4.38; N, 9.09. Found: C, 51.3; H, 4.79; N, 9.28. IR (cm^{-1}): ν(C=C) 1510, ν(C=N) 1450. ^{1}H NMR ($CDCl_3$) δ, ppm: 7.92 (s, 1H) *H*(2); 7.69 (d, $J = 2.1$ Hz, 1H) *H*(11); 7.58 (d, $J = 8.7$ Hz, 1H) *H*(8); 7.46 (dd, $J^1 = 3.0$, $J^2 = 2.1$ Hz, 1H) *H*(9); 7.20 (s, 1H) *H*(4); 6.91 (s, 1H) *H*(5); 6.82 (AA′XX′, 4H) *H*(21), *H*(22), *H*(23), *H*(24); 4.62 (AB, 2H) *H*(6); 4.31 (m, 1H) *H*(15); 3.84 (m, 1H) *H*(18); 3.54 (m, 6H) *H*(27), *H*(31), *H*(16), *H*(18′); 3.43 (m, 1H) *H*(16′); 2.95 (dt, $J^1 = 3.3$, $J^2 = 4.9$ Hz, 4H) *H*(28), *H*(30); 2.02 (s, 3H) *H*(33). λ_{max} ($CHCl_3$, 10^{-4} M) = 247, 296 nm (Scheme 1).



Scheme 1. NMR numbering scheme for KTZ.

2.5. Biological tests against epimastigotes of *T. cruzi*

Tests were conducted following a method reported previously [5b]. An EP stock of the epimastigote form of *T. cruzi* was used throughout this study. The epimastigotes were cultured in liver infusion–tryptose medium supplemented with 10% calf serum at 28°C with strong stirring (120 rpm) as previously described; the cultures were initiated with a cell density of 2×10^6 epimastigotes per ml; CTZ, KTZ and compounds **1**–**4** were added as DMSO solutions (10^{-6} M) when the culture reached a cellular density of 10^7 epimastigotes per ml. Parasite proliferation was followed daily by the use of an electronic particle counter (model ZBI; counter Electronics, Inc, Hialeah, FL.) and by direct counting with an hemacytometer.

3. Results and discussion

3.1. Synthesis and characterization of the new ruthenium(III) CTZ and KTZ complexes

The Ru(III) complexes $RuCl_3(CTZ)_3$ (**1**) and $RuCl_3(KTZ)_2(H_2O) \cdot 2H_2O$ (**2**) were prepared by reaction of $RuCl_3 \cdot 3H_2O$ with CTZ (1:4) and KTZ (1:2), respectively, in methanol, yielding dark green (**1**) and brown (**2**) paramagnetic compounds which unfortunately did not provide crystals suitable for an X-ray structure determinations. The oxidation state of the ruthenium was confirmed by their characteristic rhombic EPR spectra, as shown by the data collected in Table 1. For both complexes the room temperature spectra displayed three absorption bands typical of low spin d^5 ions in an octahedral field; the theory of the EPR spectra of distorted octahedral low spin d^5 complexes (idealized t_{2g}^5, ground term $^2T_{2g}$) is well documented in the literature [9–11]. The spectra of **1** and **2** are rather compressed, g values being very close to one another in both complexes; this is attributable to a rhombic distortion with a very small anisotropy which can be ascribed to a considerable delocalization of the unpaired electron. Interestingly, an unusually high intensity of the low field line in both spectra indicates

Table 1
EPR spectroscopic data for the new Ru(III) complexes

Complex	g_1	g_2	g_3
$RuCl_3(CTZ)_3$ (**1**)	2.11	2.04	1.94
$RuCl_3(KTZ)_2(H_2O)_2 \cdot 2H_2O$ (**2**) [a]	2.22	2.21	2.07

[a] An unidentified paramagnetic impurity ($g = 2.00$) was observed in the spectrum of this compound.

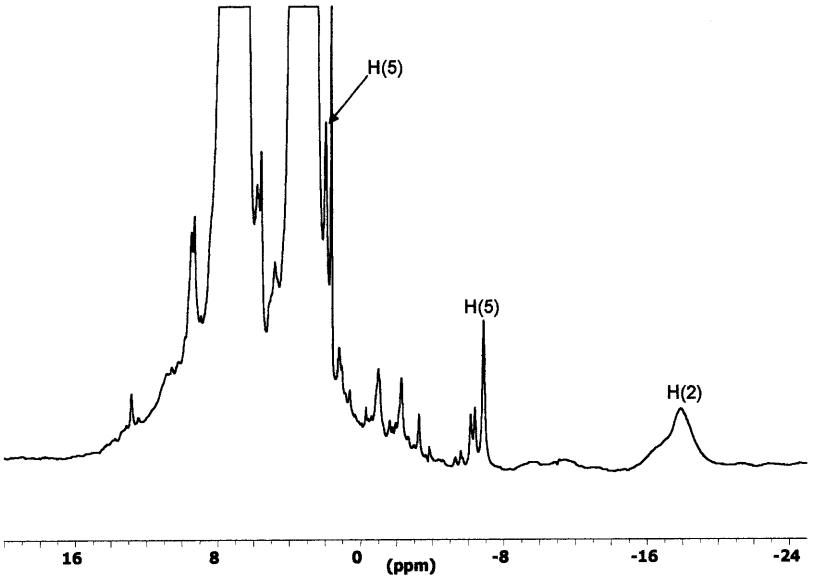


Fig. 1. ^{1}H NMR spectrum of $RuCl_3(CTZ)_3$ (**1**) in DMSO-d_6.

that the projection of the magnetic moment along the g_1 direction is large, as would be expected for systems with this type of symmetry. This spectral features are indicative also of a meridional rather than a facial structure for complex **1**; a facial Cl_3N_3 coordination would have a C_3 symmetry and therefore result in only two EPR signals [11]. A meridional arrangement of lower symmetry, which is in fact sterically more favorable for bulky ligands like CTZ, is also consistent with the NMR data presented below. In the case of **2** only two KTZ ligands can be accommodated around the ruthenium atom, presumably because of its larger size, so that the normal octahedral coordination sphere is probably attained by coordination of a water molecule; a rhombic EPR spectrum would be expected also in this case. By analogy with **1** and on the basis of NMR data reported below, a *mer* arrangement of the chlorides with two mutually *cis* KTZ ligands is the most likely stereochemistry for **2**.

3.2. NMR spectra of Ru(III) complexes

Several Ru(III) complexes containing imidazoles and related *N*-heterocycles as ligands have attracted attention, mainly because of use as radiosensitizers and of their promising antitumor properties; those compounds have been well characterized in the solid state but their solution properties are less well understood [1]; some rather extensive reports on the spectroscopic properties of imidazole complexes of Ru(III) have come out of the groups of Clarke [10] and Beauchamp [12]. We have carried out a ^{1}H NMR study of the new paramagnetic Ru(III) complexes **1** and **2**; the relaxation times (T_1) of the protons, which in the free ligand are in the range 10–0.5 s, decrease upon complexation to 3.0–0.5 s for **1** and 2.3–0.5 s for **2** (for the signals in the diamagnetic region), as a result of the contribution of the unpaired electron to the fluctuating magnetic fields that affect the relaxation of the nuclei in these systems.

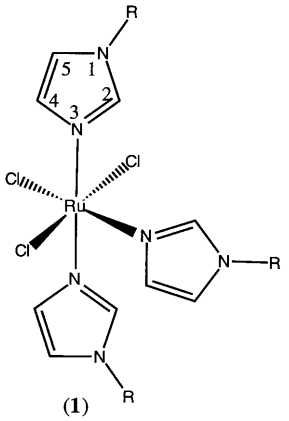
The spectrum of complex **1** in DMSO-d_6 (see Fig. 1) displays typical isotropically shifted signals for coordinated imidazoles at about -7 ppm (sharp, $T_1 = 10.1$ ms) and 2 ppm (sharp, $T_1 = 14$ ms), as well as a broad signal between -18 and -20 ppm; this high field signal is in fact composed of two peaks centered at about -17.5 and -16.5 ppm, with relative integrals of approximately 2:1 and T_1 values of 0.36 and 0.42 s, respectively (see Fig. 1). The sharp peak at 2 ppm is very close to strong signals in the diamagnetic region and therefore its integration is not reliable, but its relation to the peak at -7 ppm was established mainly on the basis of their very similar T_1 values, also close to those reported in the literature for related systems [10]. The paramagnetic region of this spectrum is further complicated by the appearance of other smaller signals between 1 and -14 ppm, possibly due to the formation of closely related solvation derivatives, as has been reported for *trans*-$[RuCl_4Im_2]^-$ in aqueous solution [12]. The spectrum of complex **2** in DMSO-d_6, shown in Fig. 2, is more straightforward as it exhibits three clearly distinct isotropically shifted signals located at -1.8 ppm (sharp), -8.7 ppm (broad) and -16.0 ppm (broad) with relative integrals 2:1:1 and T_1's of 11, 0.6, and 0.4 s. In CD_3OD two well separated signals are observed for H(5) at about -5.5 and -5.7 ppm.

On the basis of reported previously NMR spectra of other imidazoles coordinated to ruthenium(III) [10,12], the sharp signals at -5 and -2 ppm (in **1**) and at -1.8 ppm (in **2**) can be confidently assigned to the H5 protons of the CTZ and KTZ ligands, respectively. This imidazole proton is the one located furthest away from Ru and therefore it is the least affected by the paramagnetic influence of the metal center; as a result the H5 reso-

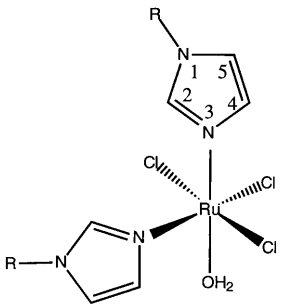
nances are the least broadened, but they experience significant upfield shifts.

As for the broad high-field signals, the situation is less clear cut, since previously reported data appear to be contradictory. Clarke's work on Ru(III)-imidazole complexes, which included extensive substitution studies in order to assign the proton resonances, concludes that the NMR signals for H(2) are broad and always appear at high fields, whereas the H(4) peak, also very broad, is generally shifted downfield although it may also be shifted upfield [10]. In contrast, Anderson and Beauchamp report spectra for Ru(III)–imidazole complexes in which two high field broad peaks are attributed to H(4) and H(2), respectively, on the basis of deuteration experiments [12]. In our case, deuteration or methylation of the ligands is not straightforward; however, the values of T_1 observed for the high field broad signals, taken together with their relative integrals and the EPR data discussed above, suggest that they correspond to two separate H(2) signals of nonequivalent CTZ (2:1) or KTZ (1:1) ligands, while the peaks corresponding to H(4) in both complexes were not observed probably because of their extreme broadness and possible location in the rather crowded diamagnetic region of the spectrum. The two separate signals observed for H(5) of **2** in CD_3OD are also in good agreement with two nonequivalent KTZ ligands.

From a perusal of the data available to us and the extensive known chemistry of Ru(III) [13], compound **1** is most likely octahedrally coordinated with the three *N*-donor atoms and the three chlorides in a *mer* stereochemistry so as to minimize the steric repulsion between the three bulky CTZ ligands; this implies that there are two (equivalent) mutually *trans* CTZ ligands while the third one is located *trans* to Cl, in accord with the 2:1 integral ratio observed for the H(2) protons in the NMR spectrum. Complex **2** is proposed to consist of an octahedral arrangement in which the three chlorines are in a meridional disposition and the two nonequivalent KTZ ligands are located mutually *cis*, thus requiring one to be *trans* to Cl, while the other is *trans* to the water molecule that completes hexacoordination.



(**1**)



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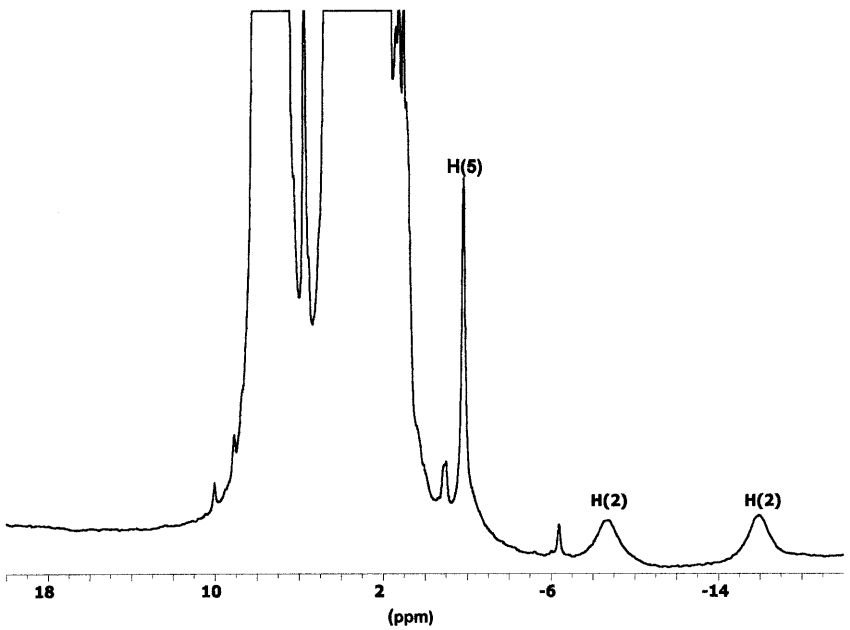


Fig. 2. ^{1}H NMR spectrum of $RuCl_3(KTZ)_2(H_2O)$ (**2**) in DMSO-d_6.

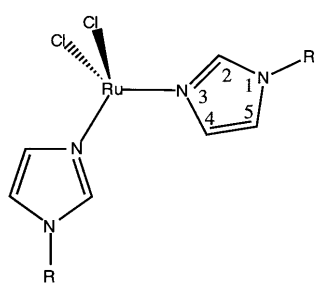
Table 2
Effect of CTZ, KTZ and their ruthenium complexes and on the proliferation of the epimastigotes of *T. cruzi* [a]

Compound	% Inhibition
CTZ	60.8
KTZ	20.3
$RuCl_2(CTZ)_2$	82.4
$RuCl_3(CTZ)_3$	65.9
$RuCl_2(KTZ)_2$	73.0
$RuCl_3(KTZ)_2$	70.3

[a] Complexes used as 10^{-6} M DMSO solutions. For details, see Section 2.

3.3. Ruthenium(II) complexes

The Ru(II) complexes **3** and **4** were prepared by displacement of the labile acetonitriles in $RuCl_2(CH_3CN)_4$ with 2 equiv. of the required ligand (CTZ or KTZ) in MeOH. We have described complex **3** in detail, as well as the X-ray structure of a closely related BTZ derivative (BTZ is the bromo analogue of the CTZ ligand) in a previous publication [3b]. These compounds are unusual diamagnetic 14-electron species with a distorted tetrahedral structure in an approximately C_{2v} arrangement of the donor atoms around the metal. Coordination through the unsubstituted N(3)-atom of the imidazole in **4** was deduced, as previously reported for **3**, from the displacement of the ^{1}H NMR signal of the proton located on the carbon atom α to that nitrogen (labeled H2 in Section 2), with respect to the free ligand ($\Delta\delta$ 0.48 ppm); this variation in chemical shift is by far larger than those observed for any other proton in the molecule. According to the analytical and NMR data and by analogy with **3**, complex **4** most likely possesses a tetracoordinated 14-electron Ru(II) configuration, which seems to be particularly adequate when the nitrogen-donor ligands are voluminous within a $RuCl_2N_2$ core. Although we have been unable to obtain crystals of **4** of X-ray quality, we presume that the structure of this complex is analogous to that of the $RuCl_2(BTZ)_2$ analogue.



(**3**, **4**)

3.4. In vitro activity of complexes **1**–**4** against epimastigotes of T. cruzi

The effect of DMSO solutions (10^{-6} M) of complexes **1**–**4** on the proliferation of in vitro cultures of the epimastigote form of *T. cruzi* (equivalent to the form present in the Reduviid vector [2]) was evaluated as described in the experimental section. Table 2 summarizes the results of such tests. The activities of the metal derivatives were all higher than the free parental compounds CTZ or KTZ, emphasizing our previous finding that binding to metal centers enhances the anti *T. cruzi* activity of azoles. Some interesting additional observations can be extracted from these preliminary biological data:

1. For CTZ, the Ru(II) complex displayed a noticeably higher activity (82.4% inhibition) than the Ru(III) derivative (73% inhibition). For KTZ the difference in activity observed was much less pronounced (73% for Ru(II) vs. 70% for Ru(III)).
2. The increase in efficacy of KTZ when attached to ruthenium is greater than that for CTZ; the trend in the activity ratio (Ru complex/free ligand) is Ru(II)–KTZ (3.60) > Ru(III)–KTZ (3.46) > Ru(II)–CTZ (1.35) > Ru(III)–CTZ (1.08).
3. At the concentrations used in this study, the most active compound was the Ru(II)–CTZ complex **3**.

In terms of our mechanistic proposal [3b], it is of interest to note that rapid metal–DNA binding is thought to be a key feature of the therapeutic action. It has been suggested that in order for DNA binding to take place, a chloro ligand must be replaced by water [14,15] but since Ru(III) is normally considered substitutionally inert in physiological conditions [16], activation by in situ reduction to a more labile Ru(II) species may actually be taking place [17]. The fact that Ru(II) appears to more active against *T. cruzi* than Ru(III) would then be in agreement with either a lower rate of hydrolysis for the Ru(III) compounds or with the need to reduce them to Ru(II) before they can exert their action. The hydrolysis of Ru(III) chloro–imidazole complexes has been studied in some detail by Anderson and Beauchamp [12] and we note that the NMR spectrum of complex **1** seems to indicate that solvolysis processes are indeed taking place in DMSO solution. Also interesting, the observed difference in activity between Ru(II) and Ru(III) was very small for KTZ, but if one takes into account that the Ru(III)–KTZ complex already contains a labile water in its coordination sphere, the need for a hydrolysis step is less pronounced. The larger increase in activity observed for KTZ with respect to CTZ as a result of coordination to Ru may be interpreted in terms of transport effects across the parasite's membrane, and/or, again, to the relative rates of hydrolysis for the different Ru complexes. Clearly more work is needed in order to further

clarify the mechanistic aspects of the observed biological activity, and a series of experiments aimed at addressing some of these issues are currently in progress.

Acknowledgements

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