European Journal of Medicinal Chemistry 45 (2010) 4180–4187

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Antileishmanial activity of ruthenium(II)tetraammine nitrosyl complexes☆

José Clayston Melo Pereira [a], Vanessa Carregaro [b], Diego Luís Costa [b], João Santana da Silva [b], Fernando Q. Cunha [c], Douglas Wagner Franco [a,*]

[a] Departamento de Química e Física Molecular, Instituto de Química de São Carlos – Universidade de São Paulo (USP), São Carlos, SP, Brazil
[b] Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brazil
[c] Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 19 March 2010
Received in revised form
2 June 2010
Accepted 6 June 2010
Available online 12 June 2010

Keywords:
Nitric oxide
Ruthenium complexes
Nitroxyl
Leishmania major

ABSTRACT

The complexes *trans*-$[Ru(NO)(NH_3)_4L](X)_3$ (X = BF_4^-, PF_6^- or Cl^- and L = N-heterocyclic ligands, P$(OEt)_3$, SO_3^{-2}), and [Ru(NO)Hedta)] were shown to exhibit IC_{50pro} in the range of 36 (L = imN) to 5000 μM (L = imC). The inhibitory effects of *trans*-$[Ru(NO)(NH_3)_4imN](BF_4)_3$ and of the Angeli's salt on the growth of the intramacrophage amastigote form studied were found to be similar while the *trans*-$[Ru(NH_3)_4imN(H_2O)]^{2+}$ complex was found not to exhibit any substantial antiamastigote effect. The *trans*-$[Ru(NO)(NH_3)_4imN](BF_4)_3$ compound, administered (500 nmol kg^{-1} day^{-1}) in BALB/c mice infected with *Leishmania major*, was found to exhibit a 98% inhibition on the parasite growth. Furthermore, this complex proved to be at least 66 times more efficient than glucantime in *in vivo* experiments.

1. Introduction

Leishmaniasis is a neglected disease caused by haemoflagellates of the genus *Leishmania* [1] and is known to affect more than 12 million people around the world with an incidence of about 2 million new cases annually [2]. A common treatment of leishmaniasis consists of the use of pentavalent antimonials such as sodium stibogluconate (Pentostam) and N-methylglucamine antimoniate (Glucantime) [3]. This treatment is considered not to be efficient due to the resistance acquired by the parasite and the high toxicity of these drugs [4]. Amphotericin B, pentamidine or paromomycin are common alternative drugs for leishmaniosis. However, these compounds are also unsatisfactory to be considered as effective and rapid therapies [2]. Another well known drug for the treatment of leishmaniosis is Miltefosine. Miltefosine has been used as an oral drug for visceral leishmaniosis with cure rates of about 98%. Not withstanding, reports on its efficiency show that *Leishmania* is resistant to it under *in vitro* conditions [5]. In conclusion thereof, there is an urgent need for new efficient drugs against leishmaniasis [6–8].

Nitric oxide is very well known to have a crucial function in the defense mechanism of the host cells against *Leishmania* sp. [9]. Hence, the treatment of *Leishmania* promastigotes or amastigotes forms using nitric oxide gas or NO donors has been shown to lead to the inhibition of the parasite mitochondrial respiration [10]. In addition, nitrosothiols (RSNO) have been shown that inhibit *Leishmania* cysteine proteinase activity through transnitrosation reactions in which the nitrosyl group is transferred directly from the RSNO to the thiol group of the protein [9,11]. In this respect, there is currently an effort to find efficient pharmacologic sources of nitric oxide using NO-donor compounds – nitrosothiols [11], diazeniumdiolates [12], and metallic nitrosyls [13,14] as antileishmaniasis drugs.

In the present work, the *in vitro* antileishmanial activity of the ruthenium nitrosyl complexes against the intramacrophage amastigotes and the extracellular promastigote form of *Leishmania major* are examined. Experiments carried out on Leishmania-infected BALB/c mice are also described.

2. Chemistry

The *trans*-$[Ru(NO)(NH_3)_4L]X_3$ compounds (X = BF_4^-, PF_6^- or Cl^- and L = imN (**1**), 4-pic (**2**), pz (**3**), py (**4**), P$(OEt)_3$ (**5**), L-hist (**6**), isn (**7**), SO_3^{-2} (**8**), nic (**9**), imC (**11**)) and [Ru(NO)Hedta] (**10**) are capable of controlled release of NO when activated by one-electron reduction in the coordinated nitrosonium (NO^+) (Eqs. (1) and (2))

☆ This work is part of the Ph.D. Thesis of Pereira, JCM and is present in the Brazilian patent of invention PI 0705849-7.

* Corresponding author. Av. Trabalhador Sãocarlense, 400 Cx. Postal 780, CEP 13560-970, São Carlos, SP, Brazil. Tel./fax: +55 16 3373 9976.
E-mail address: douglas@iqsc.usp.br (D.W. Franco).

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doi:10.1016/j.ejmech.2010.06.010

[15–17] or by irradiation with light of a selected wavelength (Eq. (3)) [18,19].

$$trans-\left[Ru^{II}\left(NO^{+}\right)(NH_3)_4L\right]^{3+}+1e^{-}\xrightarrow{\text{fast}} trans-\left[Ru^{II}(NH_3)_4L\left(NO^{0}\right)\right]^{2+} \quad (1)$$

$$trans-\left[Ru^{II}(NH_3)_4L\left(NO^{0}\right)\right]^{2+}+H_2O\xrightarrow[\text{slow}]{k_{-NO}} trans-\left[Ru^{II}(NH_3)_4L(H_2O)\right]^{2+}+NO^{0} \quad (2)$$

$$trans-\left[Ru^{II}\left(NO^{+}\right)(NH_3)_4L\right]^{3+}+H_2O\xrightarrow{h\nu} trans-\left[Ru^{III}(NH_3)_4L(H_2O)\right]^{3+}+NO^{0} \quad (3)$$

The dissociation of NO can be controlled by selecting L resulting in specific rate constants (k_{-NO}) in the range of 0.02 (**9**) to 5.1 s^{-1} (**11**) and a $Ru^{II}NO^{+}/Ru^{II}NO^{0}$ couple reduction potential in the range of −0.320 V (**11**) to 0.132 (**5**) V *vs* NHE [20].

According to literature [21,22], it can be affirmed that nitrosyl ruthenium complexes react with excess cysteine to yield HNO (nitroxyl) not NO^{0}. In fact, a similar reaction occurs when **1** or **5** react with excess cysteine. That this is true is concluded from observed selective NO electrode measurements which were found to indicate a very low production of NO^{0} in the reaction between **1** or **5** and cysteine at a pH of 7.4. In addition, HNO was detected using the metmyoglobin test (Fig. 1A). Also, an increase in the concentration of NO was observed in the reaction between the complex and the thiol in chronoamperometric experiments with **5** when carried out in the presence of the ferrocyanide ion (Fig. 1B). This behavior is explained by the reaction (Eq. (4)) [23]:

$$\left[Fe^{III}(CN)_6\right]^{-3}+HNO\rightarrow\left[Fe^{II}(CN)_6\right]^{-4}+NO^{0}+H^{+} \quad (4)$$

Conversely, at pH = 4.0, the complex **5** yields more than 95% of the expected NO concentration according to data obtained using NO selective electrode (data not shown). Therefore, it is likely that the reaction of *trans*-$[Ru(NO)(NH_3)_4L]^{+3}$ with cysteine and others thiols occurs yielding NO and HNO (Eqs. (5) and (6)) with their respective concentrations being a function of the local experimental conditions. Hence the antiparasite effect of both compounds must be considered.

$$trans-\left[Ru^{II}\left(NO^{+}\right)(NH_3)_4L\right]^{+3}+2RS^{-}+H_2O+H^{+}\rightarrow trans-\left[Ru\,(NH_3)_4L(H_2O)\right]^{+2}+HNO+RSSR \quad (5)$$

$$trans-\left[Ru^{II}\left(NO^{+}\right)(NH_3)_4L\right]^{3+}+2RS^{-}+H_2O\rightarrow trans-\left[Ru(NH_3)_4L(H_2O)\right]^{+2}+NO^{0}+RS^{\bullet} \quad (6)$$

3. Pharmacology

NO synthase (NOS) is known not to be active in the absence of oxygen and thus, in any reducing environment typical of hypoxia resulting from infections [24], *L. major* exhibits defense mechanisms [25] which may interfere with the biological production of NO thus increasing the resistance of *L. major* to the macrophages [26].

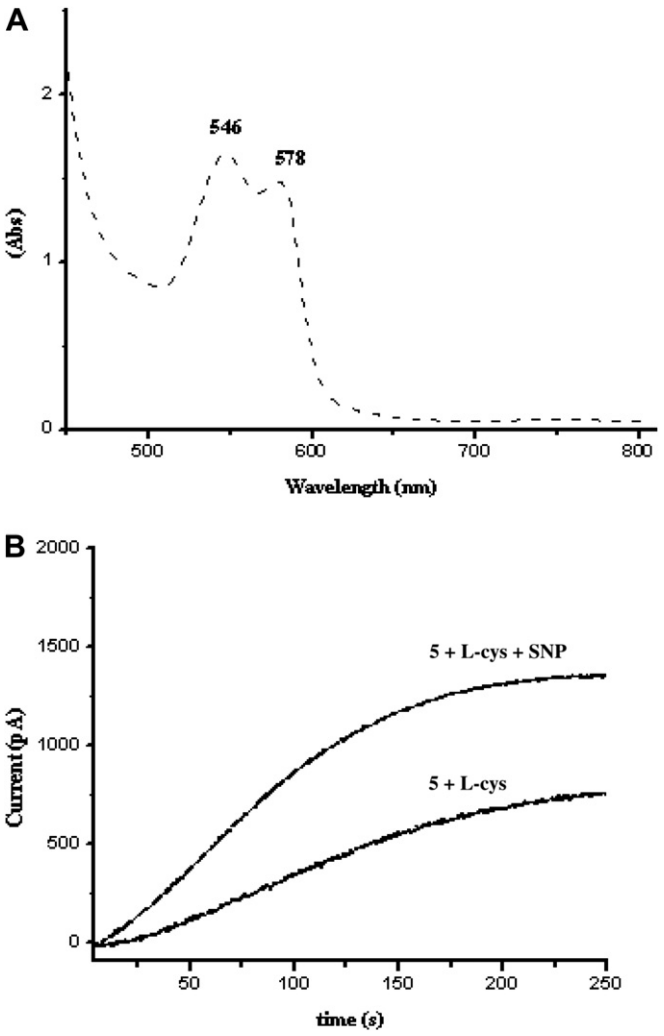

Fig. 1. A) Electronic spectrum from reaction between compound **5**, L-cysteine, and metmyoglobin. B) Chronoamperogram: reaction between **5** and L-cysteine, and reaction between **5**, L-cysteine, and $Na_3[Fe(CN)_6]$ (SNP). C_{Ru} = 500 μM, $C_{L\text{-}cys}$ = 500 μM, and C_{Fe} = 500 μM pH = 7.4. $T = 25 \pm 0.5$ °C.

Under hypoxia conditions, ruthenium nitrosyls are able to release nitric oxide (NO) after one-electron reduction hence providing a NO output and thus acting as an inorganic substitute of NOS [14–16,20,27–30]. Efforts aiming at the use of ruthenium(II) tetrammines nitrosyl complexes as NO carriers and thus exploring their ability as therapeutic agents against intracellular parasites [13,31] are currently being developed. These compounds have been shown to be quite promising metallopharmaceuticals since they are soluble and stable in water in the presence of oxygen [20] and also exhibit low toxicity *in vitro* and *in vivo* conditions [31–33].

Current observation from *in vitro* and *in vivo* experiments using *trans*-$[Ru(NO)(NH_3)_4L]X_3$, (X = BF_4^-, PF_6^- or Cl^- and L = imN, 4-pic, pz, py, $P(OEt)_3$, L-hist, isn, imC, nic, SO_3^{-2}), and [Ru(NO)Hedta] against *Trypanosoma cruzi* [13,31] showed the death of the protozoon parasite without any host cell damage. Encouraged by these results, studies are now being extended to others trypanosomatids.

4. Biological results and discussion

4.1. Antipromastigote activity

The antipromastigote screening of the compounds **1**–**11** was estimated by the IC_{50pro} (concentration that inhibits growth by