REDOX PROPERTIES OF RUTHENIUM COMPLEX WITH CATECHOL ARE INVOLVED IN TOXICITY TO GLIAL CELLS

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ABSTRACT

Since the biological activity of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ has never been tested, its cytotoxicity to glial cells was assayed and correlated with its redox properties. Coordinated catechol oxidizes faster than catechol in the presence of oxygen, but controlled potential electrolysis showed that its oxidation involves only one-electron. However, the oxidation of the free ligand by oxygen involves two electrons, which could generate more reactive oxygen species. Indeed, catechol was more cytotoxic than $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex to human glioblastoma GL-15 cells and also to rat astrocytes. $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ -induced cytotoxicity was related to the generation of reactive oxygen species and $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$. However, other mechanisms should be involved since antioxidant enzymes and deferoxamine only partially protected GL-15 cells.

INTRODUCTION

Ruthenium is an element that has industrial, pharmaceutical and medical applications. Ruthenium can be used in the reduction of noxious oxides from industrial emissions¹. Ruthenium dioxide and tetroxide are useful in oxidation reactions for the preparation of therapeutic compounds such as HIV protease inhibitors^{2, 3}. Ruthenium can also be utilized to build medical devices, such as endoprostheses⁴. $[\text{RuC}(\text{NH}_3)_5]^+$ has some interesting chemical and biological properties. $[\text{RuC}(\text{NH}_3)_5]^+$ can carry out electrocatalytic water oxidation⁵, it can bind to transferrin⁶, and has also been used in the preparation of ruthenium (II) complexes since chloride is rapidly displaced^{7, 8}.

Catechol is a compound that has also industrial and pharmaceutical applications. Catechol has been used as a protecting group in the synthesis of new drugs⁹, and it is also useful in synthetic chemistry because this compound has nucleophilic oxygen⁹. A number of novel estrane skeletons have been synthesized using catechol estrogens¹⁰. Catecholates are good siderophores, chelating various metals¹¹.

In this article, $[\text{RuCl}(\text{NH}_3)_5]\text{Cl}_2$ and catechol were used to synthesize $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ ^{12,13}. As the biological activity of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex was never tested, its cytotoxicity to glial cells was assayed. Glial cells were chosen because biomolecules bearing catechol groups are present in the central nervous system. Moreover, glial cells play important roles in physiological and pathological conditions such as neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, and brain cancers like glioblastoma. The redox properties and the role of reactive oxygen species (ROS) in $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ -induced cytotoxicity were also studied.

EXPERIMENTAL

Chemistry

In this study catechol was obtained from Riedel- de Haën (Buchs, Switzerland). $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ was synthesized by dropping a solution containing 0.53 mmol catechol in 7 M NH_3 into a solution containing 0.34 mmol $[\text{RuCl}(\text{NH}_3)_5]\text{Cl}_2$ in 7 M NH_3 at 55 °C for 30 min under argon atmosphere. NaBF_4 was added after 6 h, and the mixture volume was reduced to dryness to give a blue solid crystal. The crude solid was washed with deaerated acetone/ethanol solution and excess ligand was removed. The product was purified under argon by a chromatographic technique. The complex was dissolved in a minimum water/ethanol (1:2) solution and placed on a column with dimensions of 10 cm x 2 cm packed with neutral alumina. The complex was efficiently recovered by evaporation of the solvent under vacuum leading to the formation of a blue solid crystal, which was washed with deaerated ethanol (average yield: 62 %).

Analysis for $\text{C}_6\text{H}_4\text{O}_2\text{RuBF}_4$ (364.09), calculated C, 19.79; H, 4.43; N, 15.39; found C, 19.10; H, 4.5; N, 14.90. Since elemental analysis of the compound was consistent with the formulation, conductometry measurements

were not necessary. Complexes were also characterized by UV-visible and infrared spectra. Redox processes were studied using controlled potential electrolysis, cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Electrochemical measurements were carried out in a PARC model 273 potentiostat – galvanostat, using a conventional three-electrode cell. Glassy carbon and platinum gauze were respectively used as working electrodes for voltammetry and coulometry, whereas Ag/AgCl electrode was used as reference, and a platinum wire as auxiliary electrode. Spectroelectrochemical measurements were carried out using a gold mini-grid working electrode, Ag/AgCl as reference electrode and platinum wire as auxiliary electrode in a quartz cell with 0.3 mm optical path. Successive spectra were recorded during the redox process at 25 °C. $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$ complex was synthesized from $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})](\text{BF}_4)$ in 1 M HCl by oxidation using controlled potential electrolysis at + 400 mV versus Ag/AgCl electrode, with a concomitant recording of sequential UV-Vis spectra. Following electrolysis, complex solution was evaporated to dryness leading to the formation of crystals. Methods used in this article verified the purity of the complexes and complemented the characterization by EPR and FT ¹H NMR published previously elsewhere¹². This methodology is also in accordance to the characterization of analogous complexes¹³.

Oxygen consumption

Oxygen consumption rates during oxidation of 1 mM catechol (n = 3) or $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ (n = 5) in 50 mM phosphate buffer (pH 7.4) at 37 °C were measured using a Clark-type oxygen electrode connected to an oxygen monitoring system (YSY model 53 oxygen monitor, USA). Reaction rates were obtained from linear regression plots during the first 12 minutes. Results were compared using the Student's *t*-test. *P* < 0.05 was considered as a significant value.

Glial cell cultures

Isolated cortical astrocytes were prepared as described previously¹⁴. Briefly, astrocytes were prepared from the neocortex of 1-day-old Wistar rat brains, maintained in Dulbecco's modified Eagle's medium (DMEM, Cultilab, Campinas, Brazil) supplemented with 10% fetal calf serum (Cultilab), 2 mM L-glutamine (Sigma, S. Paulo, Brazil), 100 IU/mL penicillin (Cultilab), and 100 µg/mL streptomycin (Cultilab). One-day-old Wistar rats were obtained from the Department of Physiology of the Health Sciences Institute, Federal University of Bahia (Salvador, BA, Brazil). Only four newborn rats were needed to obtain the necessary amount of cells to prepare primary astrocyte cultures. This minimum quantity of animals used in experimental protocols is according to the regulations established by the Ethical Committee for Animal Experimentation of the Federal University of Bahia. Further experiments were carried out using human glioblastoma GL-15 cell line of clonal origin, which has been previously established¹⁵. GL-15 cell cultures were prepared as described previously¹⁶. Cells were grown in a humidified 5% CO₂ and 95% air atmosphere at 37 °C, and the culture medium was replaced three times a week. At the time of the experiment, confluent cells were trypsinized and plated in

a 96-well plate. Astrocytes were plated at a density of 7.8×10^4 cells/cm² and GL-15 cells were plated at a final density of 3×10^4 cells/cm². Experiments were initiated 72 hours after plating.

In vitro evaluation of cytotoxicity¹⁷

Logarithmic dilutions in the range between 0.01 mM and 6 mM were used to examine catechol, $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ or $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$ cytotoxic effects. Eight replicates were used for each dose per 96-well plate. Cells were exposed to catechol or complexes for 72 hours. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as described previously¹⁷. In brief, this colorimetric assay measures the MTT reduction by mitochondrial succinate dehydrogenase in intact cells. Because MTT reduction can only occur in metabolically active cells, the activity level has been used as a measure of cell viability. Cell viability was normalized to data measured under control conditions. Catechol and complex oxidation in the medium was monitored spectrophotometrically at 420 nm, based on the formation of quinones¹⁸⁻²¹. Data were expressed as medians, 25%, and 75% percentiles. A nonlinear regression was performed to fit concentration-response curves using GraphPad Prism Software (version 3.02, San Diego, USA). Data were analyzed using the Kruskal-Wallis non-parametric ANOVA method followed by the Student-Newman-Keuls post hoc test.

Involvement of reactive oxygen species

In order to study the involvement of ROS in the $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex-induced GL-15 cell death, superoxide dismutase (SOD; 200 U; EC 1.15.1.1; Sigma; n = 8), catalase (CAT; 500 IU; EC 1.11.1.6; Sigma; n = 8), and the iron chelator deferoxamine (DFO; 0.6 mM; Sigma; n = 7) were incubated with 2.6 mM $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ for 72 h at 37 °C. Similarly, the effect of boiled inactivated catalase (iCAT; n = 5) and SOD (iSOD, n = 4) on complex-induced cell death was also studied. The protective effect of both SOD (200 U) and catalase (500 IU) was also investigated (Both; n = 8). Each group was compared to the control group or the group treated with the complex alone using the Student's *t*-test or the Mann-Whitney rank sum test depending on the normality test and the equal variance test.

RESULTS AND DISCUSSION

In neutral aqueous solution, catechol is unstable because it undergoes spontaneous oxidation constituted by a multi-step reaction process resulting in ROS, semiquinone and quinone formation. 1,2-Dioxolene ligands are redox-active molecules and their properties are modified when coordinated to transition metal ions. Since they are able to shuttle reversibly through the sequence quinone/semiquinone/catechol, their metal complexes can display both the ligand-centered and the metal-centered electron transfers, which in some cases can overlap or trigger internal charge reorganizations²². Hence, the redox properties of the complex were studied to better understand its biological effects.

The electrochemical behaviors of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex and catechol were examined. DPV of catechol showed a large anodic peak (E_{pa} +525 mV) associated with a cathodic peak (E_{pc} +670). These peaks were attributed to the redox reaction catechol/semiquinone. Another observed cathodic peak (E_{pc} -15 mV) was attributed to the reduction of quinone to semiquinone. The formation of quinone is coupled to catechol/semiquinone electrode process. DPV and CV of ruthenium complex showed two pairs of peaks, one at a positive and another at a negative potential. The redox reaction catechol/semiquinone in the complex occurred at the positive potential (E_{pa} +230 mV, E_{pc} +190 mV, E_{r} +210 mV, $i_{\text{pa}}/i_{\text{pc}} \approx 1$). These data demonstrate that this electrode process is reversible and involves one electron. This monoelectronic process was further supported by controlled potential electrolysis, which showed a consumption of 1 F/mol. Peaks at more negative potentials (E_{pa} -630 mV, E_{pc} -720 mV, E_{r} -653 mV, $i_{\text{pa}}/i_{\text{pc}} \approx 0.8$) occurred at the metal ($\text{Ru}^{\text{III}}/\text{Ru}^{\text{II}}$) and this process also involves one-electron. However, $i_{\text{pc}}/i_{\text{pa}}$ ratio suggests a reversible process with a coupled reaction. According to data found for the free ligand and analogous $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catecholamine})]^+$ complexes^{23,24}, all these data, show that Ru^{III} is coordinated to catecholate.

The spectrum of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ in phosphate buffer (pH 7.4) (Merck, Darmstadt, Germany) showed a band at 655 nm ($\log \epsilon$ 2.97), which was attributed to a ligand to metal charge transfer (LMCT) and another at 285 nm ($\log \epsilon$ 3.23) considered as internal ligand (IL). The electrochemical oxidation of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex at 400 mV was monitored spectrophotometrically in the UV-Vis region. Spectra showed a progressive

decrease of LMCT and IL bands, and the appearance of a new band of light absorption at 520 nm concomitant with ligand oxidation and a well-determined isosbestic point at 610 nm (Figure 1). These energy bands were also seen during $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catecholamine})]^+$ complex oxidation²³. The band at 520 nm was assigned to a metal to ligand charge transfer (MLCT), which probably arises from $d_{\text{Ru}^{\text{II}}} \rightarrow \pi^*(\text{quinone})$ electronic transition during the oxidation of coordinated catechol to form $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$.

Figure 1: Absorption spectrum during the oxidation of 10^{-3} M $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex at 400 mV. Scans were repeated every three minutes.

Oxygen consumption rates during catechol and the complex oxidation were compared. Oxygen consumption increased approximately four times during 1 mM $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ oxidation ($2.47 \pm 0.18 \mu\text{M} \cdot \text{min}^{-1}$) compared to the rate obtained for the free ligand under the same conditions ($0.58 \pm 0.01 \mu\text{M} \cdot \text{min}^{-1}$; $P < 0.0001$). These data agree with oxidation potentials measured by CV and DPV, which showed that the reaction of the complex with oxygen is more thermodynamically favored. However, the oxidation of catechol to quinone in the presence of oxygen involves two electrons meanwhile during the oxidation of the complex one electron reduces oxygen and the other is transferred to the metal.

Since the oxidation leads to the formation of ROS and reactive quinones, we investigated the cytotoxicity of catechol, $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$, and $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$ to glial cells.

Catechol- and $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ -induced cytotoxicity to rat astrocytes was studied to determine the concentration that kills effectively 50% of cells (EC_{50}). Catechol- and also complex-induced cytotoxicity depended on the concentration of these compounds (Figure 2).

Figure 2: Dependence of the astrocyte viability (% V) on the logarithmic concentration ($\log [c]$, mM) of catechol (▼---▼) or $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ (■—■).

Catechol-induced cytotoxicity was fitted to equation [1]:

$$V = 27.45 + \{77.35 / [1 + 10^{(7.99 + 16.30 \log [c])}]\}; (R^2 = 0.9838) \quad [1]$$

in which V corresponds to cell viability normalized to data measured under control conditions, and [c] is the catechol concentration. The calculated EC_{50} for catechol on astrocytes after 72 hours was 0.342 mM. Complex-induced cytotoxicity was fitted to equation [2]:

$$V = 19.57 + \{81.03 / [1 + 10^{(-0.31 + 3.75 \log [c])}]\}; (R^2 = 0.9862) \quad [2]$$

The calculated EC_{50} for the complex was 1.380 mM. It means that the complex was 4-fold less toxic than catechol to rat astrocytes, probably because less ROS is formed during its oxidation.

Catechol-induced cytotoxicity to human GL-15 glioblastoma cells was also studied (Figure 3). Catechol-induced cytotoxicity depended on its concentration and was fitted to equation [3]:

$$V = 30.75 + \{70.25 / [1 + 10^{(1.27 - 3.45 \log [c])}]\}; (R^2 = 0.9945) \quad [3]$$

The EC_{50} for catechol on GL-15 cells, after 72 hours, was 0.568 mM. This means that catechol is 1.7-fold less toxic to human GL-15 cells than to rat astrocytes. Furthermore, the formation of quinone increased with catechol concentration and was fitted to equation [4]:

$$O.D._{420\text{ nm}} = -0.01 + \{0.63 / [1 + 10^{(-0.84 - 1.59 \log [c])}]\}; (R^2 = 0.9987) \quad [4]$$

in which $O.D._{420\text{ nm}}$ corresponds to optical densities measured at 420 nm that correlates to the formation of quinone in the medium, and [c] is the catechol concentration. In this study it was found that there is a trend in cell viability to decrease depending on the formation of quinone according to equation [5]:

$$V = -154.6 O.D._{420\text{ nm}} + 123.1; (R^2 = 0.9353; P < 0.002) \quad [5]$$

Another study also showed that catechol-induced cytotoxicity to GL-15 cells is associated with the formation of quinones²⁵.

Figure 3: Dependence of GL-15 cell viability (% V; ■—■) and the formation of quinone ($O.D._{420\text{ nm}}$; ▼—▼) on logarithmic concentration ($\log [c]$, mM) of catechol.

$[Ru^{III}(NH_3)_4(catechol)]^+$ -induced cytotoxicity to human GL-15 glioblastoma cells also depended on its concentration (Figure 4) and was fitted to equation [6]:

$$V = 13.08 + \{84.95 / [1 + 10^{(-1.27 + 3.36 \log [c])}]\}; (R^2 = 0.9889) \quad [6]$$

The EC_{50} for the complex on GL-15 cells, after 72 hours, was 2.600 mM. It means that human glioblastoma cells were 1.9-fold more resistant to the complex than rat astrocytes, and that the complex was 4.6-fold less toxic than catechol to GL-15 cells. Once again, the formation of quinone increased with the concentration of the complex and was fitted to equation [7]:

$$O.D._{420\text{ nm}} = 41 / [1 + 10^{(2.33 - 0.83 \log [c])}]; (R^2 = 0.9939) \quad [7]$$

There was also a trend in cell viability to decrease depending on the formation of quinone according to equation [8]:

$$V = -110.6 O.D._{420\text{ nm}} + 105.7; (R^2 = 0.9620; P < 0.0001) \quad [8]$$

Figure 4: Dependence of GL-15 cell viability (% V; ■—■) and the formation of quinone ($O.D._{420\text{ nm}}$; ▼—▼) on logarithmic concentration ($\log [c]$, mM) of $[Ru^{III}(NH_3)_4(catechol)]^+$.

Since there was an inverse linear correlation between $[Ru^{III}(NH_3)_4(catechol)]^+$ -induced cytotoxicity and the formation of quinone, $[Ru^{II}(NH_3)_4(quinone)]^{2+}$ -induced toxicity to GL-15 cells was tested (Figure 5). Quinone-induced cytotoxicity also depended on its concentration and was fitted to equation [9]:

$$V = 8.28 + \{90.44 / [1 + 10^{(-0.04 + 3.59 \log [c])}]\}; (R^2 = 1.00) \quad [9]$$

The EC_{50} for $[Ru^{II}(NH_3)_4(quinone)]^{2+}$ on GL-15 cells, after 72 hours, was 1.073 mM. This means that the oxidized complex was 2.4-fold more toxic than $[Ru^{III}(NH_3)_4(catechol)]^+$.

To examine the role of ROS on $[Ru^{III}(NH_3)_4(catechol)]^+$ complex-induced cytotoxicity, we evaluated the effects of antioxidant enzymes, such as SOD and catalase, and also the iron chelator DFO. A complete protection was not achieved by any treatment. However, SOD and catalase, alone or associated, and also DFO protected cells partially but significantly against complex-induced cytotoxicity (Figure 6). These results showed that complex cytotoxicity is at least in part mediated by ROS and iron. We showed previously that catechol-induced toxicity to GL-15 cells is also due to the formation of superoxide and reactive quinones²⁵.

Figure 5: Dependence of GL-15 cell viability (% V) on logarithmic concentration ($\log [c]$, mM) of $[Ru^{II}(NH_3)_4(quinone)]^{2+}$.

Figure 6: Dependence of $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ complex-induced cytotoxicity to GL-15 cells on ROS generation. *, $P < 0.0005$ vs. control group (Ctrl). ●, $P < 0.01$ vs. cells treated with the complex alone (Cplx).

CONCLUSIONS

This study investigated for the first time $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ -induced cytotoxicity. Catechol was more cytotoxic than $[\text{Ru}^{\text{III}}(\text{NH}_3)_4(\text{catechol})]^+$ -complex to human glioblastoma GL-15 cells and also rat astrocytes. Although the coordinated catechol oxidizes faster than catechol in the presence of oxygen, controlled potential electrolysis showed that oxidation to quinone involves only one-electron. However, the oxidation of the free ligand to quinone by oxygen involves two electrons, which could lead to more ROS generation. Experiments involving SOD, catalase and deferoxamine showed that oxidative stress is an important, but not unique, cytotoxic pathway. The formation of $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{quinone})]^{2+}$ is also responsible for the complex-induced cytotoxicity. To better understand the cytotoxicity of the complex, other mechanisms of cell death must be studied.

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