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Cytotoxic, clastogenic and genotoxic effects of *cis*-tetraammine(oxalato)ruthenium(III) dithionate on human peripheral blood lymphocytes

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ABSTRACT. There is a concern about stablishing the clinical risk of drugs used for cancer treatment. In this study, the cytotoxic, clastogenic and genotoxic properties of *cis*-tetraammine(oxalato)ruthenium(III) dithionite - *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$, were evaluated *in vitro* in human lymphocytes. The mitotic index (MI), chromosomal aberrations (CA) and DNA damage by comet assay were also analyzed. The MTT test revealed that the ruthenium compound showed a slight cytotoxic effect at the highest concentration tested. The IC_{50} value for the compound after 24 hours of exposure was 185.4 µM. The MI values of human peripheral blood lymphocytes treated with 0.015, 0.15, 1.5 and 150 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ were 6.1, 3.9, 3.2 and 0.2%, respectively. The lowest concentration, 0.015 µM, did not show any cytotoxic activity. The CA values for the 0.015, 0.15 and 1.5 µM concentrations presented low frequency (1.5, 1.6 and 2.3%, respectively), and did not express clastogenic activity when compared to the negative control, although it was observed clastogenic activity in the highest concentration tested (150 µM). The results obtained by the comet assay suggest that this compound does not present genotoxic activity at lower concentrations. The results show that *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ has no cytotoxic, clastogenic or genotoxic *in vitro* effects at concentrations less than or equal to 0.015 µM. This information proves as promising in the treatment of cancer and is crucial for future trials.

Keywords: ruthenium complexes; mitotic index; chromosomal aberrations; comet assay.

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Introduction

One of the major challenges in anti-cancer therapy is to overcome the high toxicity of the drugs applied to healthy cells and the multi-drug resistance of cells to chemotherapeutics (Matejczyk, Świderski, Świsłocka, Rosochacki, & Lewandowski, 2018). Doxorubicin (DOX) is an anthracycline drug widely used as a first chemotherapeutic drug treatment of a variety of solid tumors (breast, lung and ovary) (Adjei & Rowinsky, 2003) and hematological diseases, such as leukemia and Hodgkin's disease (Pilco-Ferreto & Calaf, 2016). However, the use of doxorubicin has been limited due to the significant toxicity and debility that causes unwanted side effects, including cardiac, renal, pulmonary, hematological and testicular toxicity (Matejczyk et al., 2018; Pilco-Ferreto & Calaf, 2016; Turner, Biganzoli, & Di Leo, 2015; Mitry & Edwards, 2016).

These unwanted side effects can occur during or some years after treatment, especially because DOX cannot discriminate healthy cells from malignant ones in a rapidly and non-selectively way, accumulating in healthy tissues (Mitry & Edwards, 2016).

Therefore, it has become essential to search for more selective, efficient and safer anti-cancer agents, which eliminate malignant cells and also maintain the integrity or minimally affect the normal cells. In particular, the ruthenium complexes have emerged against many types of cancers with great potential as antitumor agents with the aim of overcoming the unwanted effects (Bergamo & Sava, 2011; Sánchez-Suárez et al., 2008; Kamatchi, Chitrapriya, Kim, Fronczek, & Natarajan, 2013).

The antitumor activity pathway of ruthenium complexes works by the kinetic ligand exchange, favored by the octahedral molecular form; more specifically, such activity is related to the capacity to mimic iron in

binding to biological molecules and to the reduction of the oxidative state of ruthenium III (RuIII) to ruthenium II (RuII), which is favored in hypoxic environments with low pH and high levels of glutathione (Allardyce & Dyson, 2001; Dougan, Habtemariam, McHale, Parsons, & Sadler, 2008; Clarke, 2003). The physiologic state in which cancer cells are found, gives to ruthenium the ability to become activated and to effectively bind to DNA (Keene, Smith, & Collins, 2009). Otherwise, the mechanism of action of doxorubicin includes an intercalation in DNA, the inhibition of topoisomerase II activity and induction of DNA breaks in cancer cells, resulting in DNA damage and cell death (Matejczyk et al., 2018).

Cancer treatments with alkylating agents result in an increased frequency of chromosomal aberrations and they can elevate the chance of the patients develop a second neoplasia (Sánchez-Suárez et al., 2008). To detect clastogenic agents in peripheral human blood lymphocytes, chromosomal aberrations must be analyzed in detail (Albertini et al., 2000).

This study has the aim of analyzing the *in vitro* cytotoxic, clastogenic and genotoxic effects of the *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ complex on normal human blood lymphocytes.

Material and methods

Chemicals

The *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ was synthesized following a standard protocol (Pavanin, Giesbrecht, & Tfouni, 1985). Doxorubicin (Oncodoxdoxorubicin chloridate 50 mg Meizler®) was used as the positive control for lymphocyte cultures at a concentration of 0.2 μg mL^{-1} (IC_{50} of reference = 0.002 μM) (Ribeiro et al., 2009).

Cell Culture

Human peripheral blood lymphocytes (PBL) were collected using heparinized vials from six healthy 20-30-year-old donors with no history of smoking, drinking, or chronic drug use. Lymphocytes were isolated and independently cultured in RPMI-1640 medium (Gibco®, Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Gibco®), penicillin (5 μg mL^{-1}), streptomycin (10 μg mL^{-1}), and 2% phytohemagglutinin (Cultilab Campinas-SP, Brazil), according to standard procedures (Moorhead, Nowell, Mellman, Battips, & Hungerford, 1960).

The protocol (N. 043/2007) for these experiments was approved by the Research Ethics Committee at the *Universidade Federal de Goiás*, and prior to joining the study, all blood donors have signed an informed consent.

Cell viability assay (MTT assay)

Cytotoxic activity of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ in peripheral human blood lymphocytes was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (Moorhead et al., 1960). In brief, 1×10^5 cells were harvested in 96-well tissue culture plates and exposed to different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ (0.015, 0.15, 1.5 and 150 μM) for 48 hours. After treatment, 10 μL of MTT (5 mg mL^{-1}) were added to each well, and the plates were incubated at 37°C for an additional 3 hours. The purple formazan crystals were dissolved in 50 μL of SDS, and absorbance was determined at 545 nm using a Stat Fax 2100 microplate reader (Awareness Technology, Palm City, FL, USA). Cell viability was calculated as follows: viability (%) = (absorbance of the treated wells) / (absorbance of the control wells) × 100. The IC_{50} (micromolar concentration of complex that results in 50% of reduction in cellular viability) was obtained from dose-response curves using GraphPad Prism 5.0 for Windows (San Diego, California, USA, www.graphpad.com).

Cytogenetic Studies

Metaphase preparations were made to analyze chromosomal aberrations (CA). In brief, 5 mL of venous blood were collected from each donor in a heparinized vial (5.000 IU mL^{-1}; Liquemine, Roche, São Paulo-SP, Brazil), and the cells were incubated (37 °C, 5% CO_2) for 24 hours before *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ exposure. Then, the cells were exposed to *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ (0.015, 0.15, 1.5 and 150 μM) and to positive control doxorubicin (0.002 μM) and then incubated for 24 hours. Untreated cells were used as negative control. Colchicine was added at a final concentration of 0.0016% 1h prior to harvesting. Cells were

harvested by centrifugation (800 rpm), treated with 0.075 M KCl at 37 °C for 20 min., centrifuged (800 rpm) again, and fixed in 1:3 (v v^{-1}) acetic acid–methanol. Finally, the slides were air-dried, and stained with 3% Giemsa solution (pH 6.8) for 5 min. They were then analyzed using a light microscope, structural (chromosome/chromatid gaps and breaks) and numerical CA were examined in both the ruthenium complex-treated cultures and negative/positive controls. For assessment of chromosomal aberrations, one hundred cells were analyzed per treatment. The frequencies of chromosomal aberrations in 100 metaphases per culture and MI were determined. The MI was calculated for each treatment as the number of metaphases per 5,000 lymphocytes.

Comet Assay

The genotoxic effect of ruthenium complex *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ was evaluated using the alkaline version of the comet assay. For this assay, 1×10^5 lymphocyte cells were treated separately in the absence or presence of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ compound at 0.015, 0.15, 1.5 and 150 µM and as positive control it was used the doxorubicin (0.002 µM) for 24 hours.

After exposure, 300 µL of the cell suspension were centrifuged for 5 min. (500 rpm). The resulting pellet was homogenized with 80 µL of a low melting-point agarose (0.5%), spread onto microscope slides pre-coated with normal melting-point agarose (1.5%), and covered with a coverslip. After 5 min. at 4 °C, the coverslip was removed, and the slides were immersed in cold lysis solution (2.4 M NaCl, 100 mM ethylenediamine tetraacetic acid (EDTA), 10 mM Tris, 10% DMSO, and 1% Triton-X, pH 10) for 24 hours. After lysis, the slides were placed in an electrophoresis chamber and covered with electrophoresis buffer (300 mM NaOH per 1 mM EDTA, pH>13) for further 20 min. to allow DNA unwinding. The electrophoresis proceeded for 20 min. at 25 V (0.7 V cm^{-1}) and approximately 300 mA. Afterwards, the slides were submerged for 15 min. in a neutralization buffer (0.4 M Tris-HCl, pH 7.5), dried at room temperature, and fixed in 100% ethanol for 5 min. All steps were conducted in the dark to prevent additional DNA damage. Slide staining was performed immediately before analysis using ethidium bromide (20µg mL^{-1}). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) in a fluorescent microscope (Zeiss, Germany) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm using a × 40 objective. The nucleus were classified according to the migration of the fragments using the software CometScore 15 as: class 0 (no damage); class 1 (little damage with a short tail length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length once or twice the diameter of the nucleus); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus); class 4 (significant damage with a tail longer than three times the diameter of the nucleus) (Burlinson et al., 2007).

Statistical Analysis

Statistical analyses of the results were performed using One-way analysis of variance followed by Tukey's post hoc test for multiple comparisons with a control. All statistical analyses were undertaken using the statistical software GraphPad 5.0 for windows (San Diego California USA, www.graphpad.com). A probability of 0.05 or less was deemed as statistically significant. The following notation is used throughout the manuscript: *, $p < 0.05$ and **, $p < 0.01$ relative to the control.

Results and discussion

Cell viability assay

The cytotoxic effects of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ on peripheral human blood lymphocytes were investigated using MTT assay. As shown in Table 1, after exposure to 0.015, 0.15, 1.5 and 150 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ for 24 hours, cell viability represented 94.06, 87.55, 82.29 and 75.99%, respectively. The IC_{50} value was approximately 185.4 µM after 24 hours of exposure. Cell viability has shown significant difference compared to the untreated control ($p < 0.05$) at concentration of 150 µM (data not shown).

Table 1. Cytotoxic activity of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ in peripheral human blood lymphocytes by MTT assay

Concentration	% Viability	SD
Ru 0.015 µM	94.06	±9.40659
Ru 0.15 µM	87.55	±10.89899
Ru 1.5 µM	82.29	±9.447006
Ru 150µM	75.99	±5.536723

Mitotic Index

The results from the analysis of mitotic index (MI), (which estimates the frequency of cellular division), revealed a concentration-dependent decrease of MI in the treated peripheral human blood lymphocytes (PBL). The cells treated with 0.015, 0.15, 1.5 and 150 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ compound presented a mitotic index of 6.1, 3.9, 3.2 and 0.2%, respectively (Figure 1). The mitotic index of PBL exposed to the positive control drug (doxorubicin) was 2.9% and the negative control was 5.6%.

A concentration of 0.015 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ did not show cytotoxic effect, did not inhibit the mitotic index of the cells, and resulted in increased proliferation, when compared to the negative control ($p > 0.01$). It was observed a decrease in MI for 0.15 and 1.5 µM concentrations, and a highly significant reduction in the MI in the 150 µM concentration, of 0.2 % (**$p < 0.01$). The positive control, doxorubicin (0.002 µM), presented a MI similar to that found to 1.5 µM of the ruthenium complex, which represents a concentration approximately 750 x higher than that used for doxorubicin (Figure 1).

(A)

Concentration	*MI(%)*
Negative Control	5.6
Ru 0.015 µM	6.1
Ru 0.15 µM	3.9
Ru 1.5 µM	3.2
Ru 150 µM	0.2
DXR (0.002 µM)	2.9

(B)

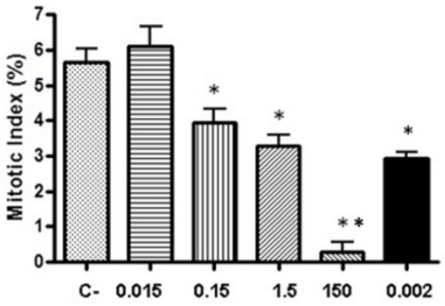

Figure 1. A) Mitotic Index (MI) was obtained from the analysis of the number of metaphases per 5,000 lymphocytes exposed to each of the four different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$, negative control and to positive control (doxorubicin). B) Graphical representation of Mitotic Index. Significant differences from the negative control are indicated by *$p < 0.05$, **$p < 0.01$.

Chromosomal Aberration

Table 2 contains the results of the chromosomal aberrations assay in the PBL that were exposed to different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$, negative control (cells with no exposition) and positive control (cells exposed to doxorubicin). The chromosomal aberrations of cells treated with ruthenium complex at concentrations of 0.015, 0.15 and 1.5 µM were similar to values found in the negative control (2.0%), with frequencies of 1.5, 1.6 and 2.3%, respectively. These values compared to the negative control were not statistically significant ($p > 0.05$).

The ruthenium complex concentration of 150 µM presented approximately one-third of chromosomal aberrations observed for doxorubicin ($p < 0.01$).

Table 2. Chromosomal aberrations of peripheral human blood lymphocytes exposed to different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$.

Concentration	Ctg	Ctb	Chg	Chb	Total	F(%)
Negative Control	6	4	2	0	12	2.0±0.30
Ru 0.015 µM	8	0	1	0	9	1.5±0.32
Ru 0.15 µM	5	4	1	0	10	1.6±0.26
Ru 1.5 µM	4	10	0	0	14	2.3±0.43
Ru 150 µM ***	37	179	24	18	258**	43±0.92
DXR (0.002 µM)	353	176	149	16	694**	115±17.20

For each treatment 100 cells were analyzed; Ctg=chromatid gaps, Ctb = chromatid breaks, Chg = chromosome gaps, Chb = chromosome breaks, DXR = doxorubicin. ** Indicates significant difference in comparison to negative control ($p < 0.01$). Data are mean ± S.D. *** Frequency estimates a total of 195 metaphases, different from the other concentrations, which were calculated on a total of 600 metaphases per treatment.

Figure 2 shows chromosomal aberrations observed on metaphase of peripheral human blood lymphocytes exposed to 150 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ (Figure 2C), compared to negative control (Figure 2A) and positive control (Figure 2B).

The concentration of 150 µM induced DNA damages and breaks (red arrows 2 and 3, demonstrating significant clastogenic activity when compared to the negative control ($p < 0.01$), predominantly through chromatidic failure, although a greater amount of chromosomal aberrations was observed for positive control (red arrow 1).

All the chromosomal aberrations analyzed for doxorubicin were higher than that found for the ruthenium complex tested, with exception to chromatid breaks and chromosome breaks, for which the highest concentration of the complex (150 µM) presented similar results of CA related to doxorubicin (0.002 µM). The total number of CA verified for doxorubicin were approximately the triple of the number for the highest concentration of ruthenium complex and 49 times higher than found for the concentration of 1.5 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$.

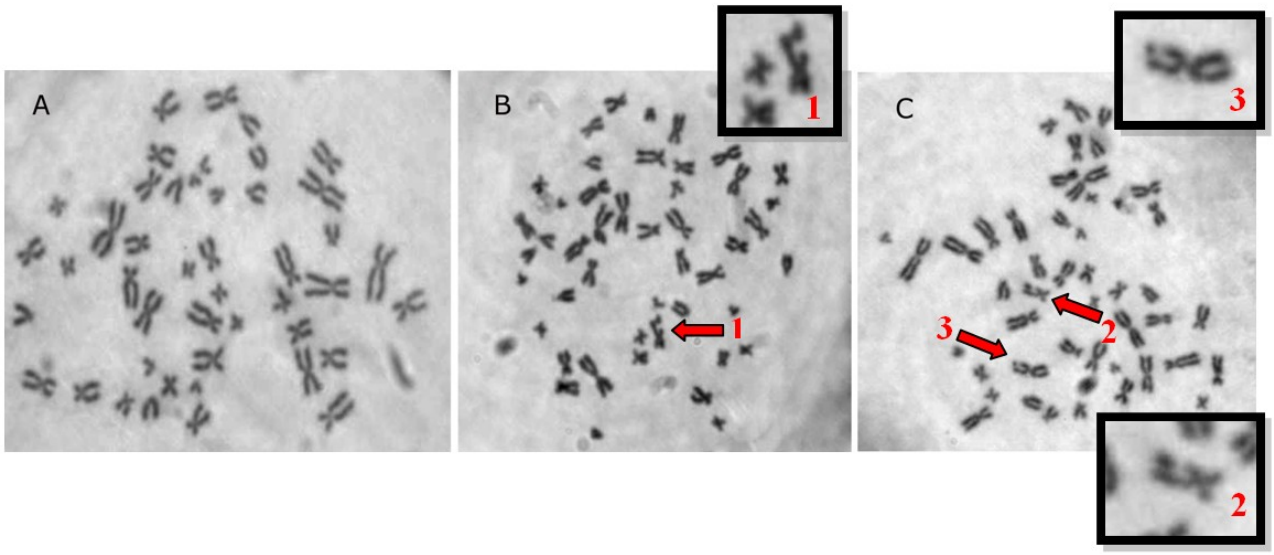

Figure 2. Chromosomal aberrations observed on Metaphase of peripheral human blood lymphocytes. A. Negative Control - Normal metaphase; B. Cells exposed to doxorubicin (1- Chromatidic break); C. Cells exposed to *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ complex at a concentration of 150 µM (2 - Chromatidic gap; 3 - Chromatidic break).

Genotoxicity assay

Comet assay was performed to analyze the genotoxicity potential in peripheral human blood lymphocytes treated with different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$. The concentration of 0.015 µM did not present DNA damage and was similar to that observed in the negative control ($p > 0.01$). The DNA damages indexes were concentration dependent, as the highest concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ caused more DNA damages when compared to the negative control (Figure 3).

Although the DNA damage indexes were considered statistically significant when compared to the negative control at concentrations of 0.15, 1.5 and 150 µM, just the two highest concentrations presented significant percentage of DNA damage. Otherwise, doxorubicin, despite widely used in the treatment of many types of cancer, when used in this study in a very low concentration, presented significant DNA damage in human blood lymphocytes.

The use of current antineoplastic drugs requires the knowledge about their cytotoxicity, clastogenicity and genotoxicity, in order to obtain treatments with greater selectivity and less side effects.

Ruthenium complexes have demonstrated selective cytotoxic activity to tumor cells with low toxicity to normal cells. Ribeiro et al. (2009) has demonstrated that *cis*-(dichloro)tetraammineruthenium(III) complex

did not cause DNA damage in human lymphocytes. Furthermore, this ruthenium(III) complex also presented immune stimulatory activity, and a high concentration of this complex had induced damage to the normal peripheral blood mononuclear cells (Silveira-Lacerda et al., 2010a).

(A)

Frequency of Cell Nuclei (%)

Treatment	*Class of comet assay*					*DNA damage Index*
	0	1	2	3	4	
Control	78	09	10	03	00	38
Ru (0.015 µM)	70	24	05	01	00	37
Ru (0.15 µM)	38	33	21	08	00	99
Ru (1.5 µM)	11	10	32	46	01	216
Ru (150 µM)	09	12	30	25	24	243
Doxo (0.002 µM)	26	24	25	22	03	152

(B)

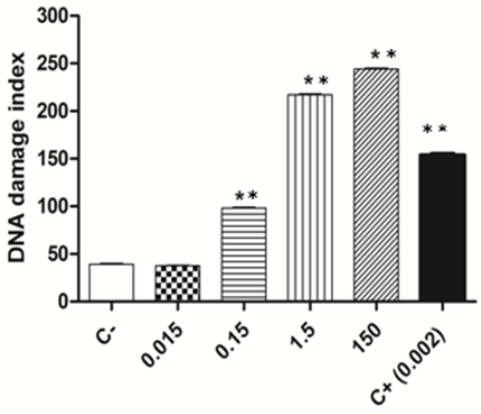

Figure 3. A) Concentration-dependent effects of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ on the DNA damage index, assessed by the comet assay. B) Graphical representation of DNA damage Index of Peripheral human blood lymphocytes treated with different concentrations of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ (0.015-150 µM), negative control (C-) and positive control doxorubicin (C+). ** Indicates significant difference in comparison to negative control ($p < 0.01$).

Studies with *cis*-tetraammine(oxalato)ruthenium(III) dithionate complex tested on the root meristem cells of *Allium cepa* showed no significant cytotoxic and genotoxic activity (Pereira et al., 2009). The *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ compound has presented *in vitro* cytotoxicity activity in sarcoma 180 cells (Pereira et al., 2015) and in chronic myeloid leukemia K562 cells (Pereira et al., 2014).

Cytogenetic methods are used in clinical screening to evaluate primary DNA damage caused by chemical and physical agents. The most frequently used methods include CA and the comet assay, which have the potential to detect cytotoxic and genotoxic activities (Bender, Preston, Leonard, Pyatt, & Gooch, 1989; Wojewódzka, Kruszewski, Iwaneñko, Collins, & Szumiel, 1998; Fenech, 2000; Hartmann, Plappert, Poetter, & Suter, 2003; Collins, 2004, Singh, 2005) and all the methods used in the primary evaluation of DNA damage include the comet assay (Tice et al., 2000; Trzeciak, Barnes, & Evans, 2008).

Peripheral human blood lymphocyte cultures are an excellent method for testing the capacity of chemical agents to cause DNA damage (Lima et al., 2007).

The ruthenium (III) complex has been studied because it has favorable antitumor properties, so there is a need to test it for cytotoxic and genotoxic effects in human normal cells, using the test established by ANVISA (National Health Surveillance Agency) and FDA (Food and Drug Administration) for the release of new drugs.

Because of the promising results from previous *cis*-tetraammine(oxalato)ruthenium(III) studies (Pereira et al., 2009; Pereira et al., 2014), the need has risen for research into the possible cytotoxic, clastogenic and genotoxic effects of this complex in peripheral human blood lymphocytes *in vitro*.

In this study, using the MTT assay, we found that *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ reduced the viability of peripheral human blood lymphocyte cells only at the highest concentration tested (150 µM) relative to untreated cells ($p < 0.05$); on the other concentrations tested (0.015, 0.15 and 1.5 µM) there were no significant reduction in cellular viability ($p > 0.05$).

Otherwise, Pereira et al. (2014) and Silveira-Lacerda et al. (2010a; 2010b) demonstrated that the *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ and *cis*-$[RuCl_2(NH_3)_4]Cl$ complexes, respectively, showed expressive selectivity for tumor cells such as Jurkat, A549, S-180 and K562 with IC_{50} of 18.6, 19.1, 24.5 and 29.2 µM, respectively. In contrast to the verified cytotoxicity in tumor cells, this study using human primary normal cells demonstrated that cytotoxic, clastogenic and genotoxic effects were observed at a much higher concentration than that required to cause cytotoxicity in tumor cells. On the other hand, the positive control doxorubicin has caused cytotoxic effects in these normal cells in a very low concentration (0.002 µM). The cytotoxic properties of other ruthenium complexes have been investigated comparing tumor and

normal cells; and these complexes are selective for tumor cells when compared to normal cells (Djinovic, Todorovic, Zizak, Sabo, & Juranic, 2009).

Based on the IC_{50} value of earlier studies with tumor cells, we suggest that the cytotoxicity of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ for human cancer cells is much stronger than its cytotoxicity for normal peripheral human blood lymphocyte cells (IC_{50} 184.5 µM). This value was ten times higher than the IC_{50} for K562 tumor cell found by Pereira et al (2014), which was 18.28 µM, revealing a great selectivity associated to this ruthenium complex, with values of SI ⩾ 2.0 considered significant. The same study noticed that K562 cells suffered apoptosis and presented genotoxic effects after treatment with this complex, with a significant increase in DNA damage index in all of the concentrations tested in tumour cells, in a dose-dependent manner (10 - 150 µM).

In order to evaluate the effect of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ on the mitotic index in human lymphocytes, we used four concentrations of the complex (0.015 -150 µM), as well as a positive control, doxorubicin, at 0.002 µM. The MI at a 0.015 µM concentration of ruthenium complex did not reveal cytotoxicity for human lymphocytes (Figure 1). Similarly, Pereira et al. (2009) evaluated the cytotoxic and genotoxic activities in *Allium cepa* roots treated with *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$, showing that a higher concentration of the complex was necessary for a significant reduction in MI.

Previous studies using the *cis*-$[RuCl_2(NH_3)_4]Cl$ ruthenium complex showed that this compound inhibited cellular division (MI=0%) (Ribeiro et al., 2009) and presented cytotoxicity, reduction of tumor growth and increased survival time of animals on murine sarcoma S180 using *in vitro* and *in vivo* assays. Additionally, the cytotoxic activity of the *cis*-$[RuCl_2(NH_3)_4]Cl$ ruthenium complex in peripheral blood mononuclear cells was lesser than that in tumor cells, presenting a high IC_{50} of 8157 µg ml^{-1} (De Paula Silveira-Lacerda et al., 2010a). This expresses the higher selectivity of this ruthenium complex towards tumor cells in relation to normal cells. These results corroborate with the findings of the present study, once the increased concentrations of the *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ complex were necessary to induce cytotoxicity in peripheral human blood lymphocytes.

The low toxicity related to ruthenium compounds can be explained by the ability of ruthenium to mimic iron in respect of binding to several biomolecules, such as transferrin and albumin. Since cancer cells divide rapidly, the demand for iron is greater, so they increase the number of transferrin receptor on their surface. Consequently, ruthenium-loaded transferrin is sequestrated in higher quantity than in normal cells. Additionally, regarding to Ru(III) complexes, it is believed that their anticancer activity occurs with initial reduction to Ru(II) at the cancer tissue, due to the altered and more acidic physicochemical environment shown in tumour cells (Allardyce & Dyson, 2001).

The CA analysis of metaphases after treatments showed that 0.015, 0.15 and 1.5 µM of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ did not induce CA, but instead presented similar values to those of the negative control. The positive control doxorubicin presented a higher frequency of chromosomal abnormalities (Table 2), predominantly through chromatin failures and breaks.

The *cis*-$[RuCl_2(NH_3)_4]Cl$ complex did not show any metaphase at the highest concentration of 380 µM, probably because the cell cycle was blocked at the interphase, and thus interfered with the repair mechanism and blocked mitosis (Ribeiro et al., 2009).

In the present study, the CA assay did not reveal cytotoxic or clastogenic effects in human lymphocytes exposed to *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$. The CA frequency in the negative control of human lymphocytes was 2.0%, which is within the range for healthy individuals (around 2%) (Julian Preston, San Sebastian, & McFee, 1987). The 0.015, 0.15 and 1.5 µM concentrations of the complex did not demonstrate clastogenic effects when compared to the negative control either. However, there was an increase in the clastogenic activity of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ in human lymphocytes when the 150 µM was used, predominantly manifested as chromatic breaks and failures.

The present study also used the comet assay to evaluate DNA damage. This test is a sensitive method for detecting simple breaks, double breaks and crosslinking of DNA (Tice et al., 2000). In this study, the lowest concentration (0.015 µM) of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ complex did not demonstrate statistically significant difference of DNA damage when compared to the negative control ($p > 0.01$). On the other hand, DOX has caused approximately four times more damage to DNA in a concentration seven times lower than that of the minor concentration of ruthenium complex (0.015 µM).

Conclusion

This study indicates that *cis*-$[Ru(C_2O_4)(NH_3)_4]_2(S_2O_6)$ *in vitro* has no potential cytotoxic, clastogenic or genotoxic effects on lymphocytes at concentrations lesser than or equal to 0.015 µM. The compound deserves further evaluation as a chemotherapeutic agent for human cancers with a view to finding reliable alternatives to anthracycline drugs, especially for the treatment of leukemia.

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