The ruthenium complex *cis*-(dichloro)tetramminеruthenium(III) chloride induces apoptosis and damages DNA in murine sarcoma 180 cells

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Ruthenium(III) complexes are increasingly attracting the interest of researchers due to their promising pharmacological properties. Recently, we reported that the *cis*-(dichloro)tetramminеruthenium(III) chloride compound has cytotoxic effects on murine sarcoma 180 (S-180) cells. In an effort to understand the mechanism responsible for their cytotoxicity, study we investigated the genotoxicity, cell cycle distribution and induction of apoptosis caused by *cis*-(dichloro)tetramminеruthenium(III) chloride in S-180 tumour cells. *cis*-(dichloro)tetramminеruthenium(III) chloride treatment induced significant DNA damage in S-180 cells, as detected by the alkaline comet assay. In the cell cycle analysis, *cis*-(dichloro)tetramminеruthenium(III) chloride caused an increase in the number of cells in G1 phase, accompanied by a decrease in the S and G2 phases after 24 h of treatment. In contrast, the cell cycle distribution of S-180 cells treated with *cis*-(dichloro)tetramminеruthenium(III) chloride for 48 h showed a concentration-dependent increase in the sub-G1 phase (indicating apoptosis), with a corresponding decrease in cells in the G1, S and G2 phases. In addition, *cis*-(dichloro)tetramminеruthenium(III) chloride treatment induced apoptosis in a time-dependent manner, as observed by the increased numbers of annexin V-positive cells. Taken together, these findings strongly demonstrate that DNA damage, cell cycle changes and apoptosis may correlate with the cytotoxic effects of *cis*-(dichloro)tetramminеruthenium(III) chloride on S-180 cells.

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1. Introduction

Apoptosis is an active physiological process that results in cellular self-destruction involving specific morphological and biochemical changes in the nucleus and cytoplasm (Kerr *et al.* 1994; Kaufmann and Hengartner 2001). Agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. While many anticancer agents have been developed, unfavourable side-effects and resistance to apoptosis are serious problems (Lowe and Lin 2000; Fesik 2005). Thus, there is growing interest in the design, synthesis and pharmacological evaluation of metal-based antitumour agents for the

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Abbreviations used: A-20, murine B-cell lymphoma; *cis*-[$RuCl_2(NH_3)_4$] Cl, *cis*-(dichloro)tetramminеruthenium(III) chloride; DI, damage index; FITC, fluorescein isothiocyanate; Jurkat, human T-cell leukaemia; KP1019, indazolium *trans*-[tetrachlorobis (1*H*-indazole) ruthenate(III)]; NAMI-A, ImH[*trans*-RuCl4(DMSO)Im]; PBL, peripheral blood lymphocytes; RDC, ruthenium-derived compound; PI, propidium iodide; SCGE, single-cell gel electrophoresis; S-180, murine sarcoma 180 cells

treatment of various cancers, and the development of safer and more effective therapeutic agents (Kostova 2006; Jakupec *et al.* 2008; Alama *et al.* 2009).

Ruthenium(III) complexes represent a new family of promising metal-based anticancer drugs that offer the potential of reduced toxicity compared with the antitumour platinum(II) complexes currently used in the clinic, a novel mechanism of action, the prospect of non-cross-resistance and a different spectrum of activity (Kostova 2006; Allardyce and Dyson 2001; Clarke 2003; Brabec and Nováková 2006). In addition, some chemical properties, such as the rate of ligand exchange, the range of accessible oxidation states and the ability of ruthenium to mimic iron in binding to certain biological molecules make these compounds well suited for medicinal applications as an alternative to platinum antitumour drugs for the treatment of cancer cells that are resistant to cisplatin and its analogues, thus justifying the further development of this novel and interesting group of metal complexes (Allardyce and Dyson 2001; Brabec and Nováková 2006).

The two ruthenium compounds: ImH[*trans*-$RuCl_4$(DMSO)Im] (NAMI-A) and indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate(III)] (KP1019) are the first ruthenium-based anticancer drugs to be entered into clinical trials (Lakahai *et al.* 2004; Hartinger *et al.* 2006), and many other compounds that include ruthenium centres are being developed and tested (Clarke 2003).

Among the ruthenium complexes studied for anticancer application, the *cis*-(dichloro)tetrammineruthenium(III) chloride (*cis*-$[RuCl_2(NH_3)_4]Cl$) complex has shown promising results on tumour cells in humans and mice (Silveira-Lacerda *et al.* 2009). Recently, it was demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ exerts antitumour activity against both *in vitro* and *in vivo* models of the sarcoma 180 (S-180) cell line (Menezes *et al.* 2007; Silveira-Lacerda *et al.* 2009). However, no studies on induction of apoptosis and DNA damage have been reported for this cell line. In an effort to understand the effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on these cells, we investigated the effect of the compound on cell cycle distribution, apoptosis and DNA damage.

2. Materials and methods

2.1 *Synthesis of* cis-*$[RuCl_2(NH_3)_4]Cl$*

The *cis*-$[RuCl_2(NH_3)_4]Cl$ complex (figure 1) was prepared as follows. Air-free concentrated ammonium hydroxide (25 ml) was added to $[RuCl(NH_3)_5]Cl_2$ (1.0 g, 3.4×10^{-3} mols), which was then refluxed under argon until a burgundy-coloured solution was produced. Sodium dithionate (0.7 g) was added to the hot solution, which was subsequently cooled in an ice bath. An off-yellow-cream solid, $[RuOH(NH_3)_5]S_2O_6$, precipitated, was collected by filtration and air-dried. A second crop of crystals was obtained by the addition of anhydrous ethanol (50 ml). This material was collected by filtration, washed with ethanol and air-dried. The $[RuOH(NH_3)_5]S_2O_6$ was dissolved in saturated oxalic acid (11 ml) and then refluxed for approximately 10 min under argon, forming a yellow solid. The mixture was then cooled to complete the precipitation. The crystals of *cis*-$[Ru(C_2O_4)(NH_3)_4]_2S_2O_6$ were isolated by filtration, washed with ethanol and air-dried. The tetraammineoxalatoruthenium(III) dithionate crystals were dissolved in 5 M HCl (12 ml), and the solution was refluxed for 10 min. The hot solution was filtered, and absolute ethanol (30 ml) was added to the yellow filtrate. A yellow solid, *cis*-$[RuCl_2(NH_3)_4]Cl$, was precipitated. After the mixture had cooled, the *cis*-product was collected by filtration, washed with ethanol and air-dried. The yield was 55%. The compound was characterized by electronic spectra at room temperature using quartz cells in an HP 8453 spectrophotometer with a diode arrangement that interfaced with a compatible PC (HP Vectra XM). The electronic spectrum of the *cis*-$[RuCl_2(NH_3)_4]Cl$ complex presented bands at 260, 308 and 350 nm. Briefly, the absorption spectra in aqueous solution were dominated by the ligand-to-metal charge transfer bands with λ_{max} at 350 nm ($\varepsilon = 1.5\times10^3$ M^{-1} cm^{-1}), 308 nm ($\varepsilon = 1.3\times10^3$ M^{-1} cm^{-1}) and 260 nm sh ($\varepsilon = 500^3$ M^{-1} cm^{-1}). Carbon, hydrogen and chloride microanalyses were performed by the staff of the Analytical Center of the Chemical Institute of Universidade de São Paulo. The calculated proportions were N, 20.3; H, 4.4; Cl, 38.6. The observed proportions were N, 20.2; H, 4.5; Cl, 38.5.

2.2 *Cell culture*

Murine (S-180) were cultured in suspension in RPMI 1640 medium (Sigma Chemical Co., MO) supplemented with

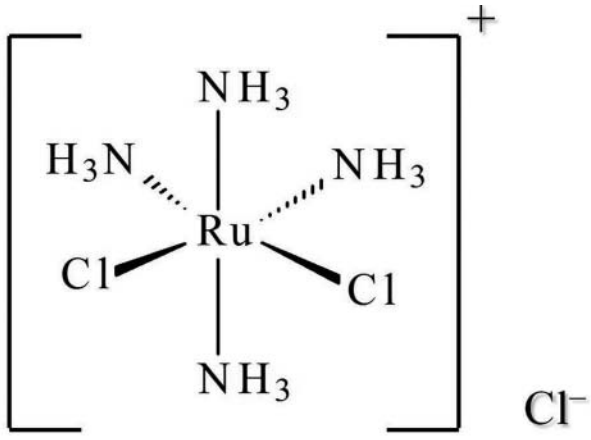

Figure 1. Chemical structure of *cis*-(dichloro)tetraammineruthenium(III) chloride {*cis*-$[RuCl_2(NH_3)_4]Cl$}.

10% foetal calf serum, 100 UI/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C in 5% CO_2.

2.3 *DNA analysis by the comet assay*

The presence of DNA damage was examined by single-cell gel electrophoresis (SCGE) (Comet assay), according to published methods (Singh 1988). Briefly, 2×10^4 S-180 cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38, 3.8, 19 and 95 μM) and methotrexate (25 μM) for 24 and 48 h. After incubation, the S-180 cell suspension was homogenized with 100 μl of a low melting point agarose (0.5%), spread onto microscope slides pre-coated with a normal melting point agarose (1.5%) and covered with a coverslip. After 10 min at 4°C, the coverslip was removed and the slides were immersed in cold lysis solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) for 24 h. 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 20 min to allow for unwinding of the DNA. The electrophoresis proceeded for 20 min (25 V and 300 mA). The slides were then 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. Slide staining was performed immediately before analysing using ethidium bromide (20 μg ml^{-1}). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. The nucleus was classified visually according to the migration of the fragments, as previously proposed (Kobayashi *et al.* 1995) as follows: 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 one or two times 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); and class 4 (significant damage with a long tail of damage more than three times the diameter of the nucleus). A value (damage index, DI) was assigned to each comet according to its class, according to the following formula:

$$DI = (0 \times n0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4),$$

where n = number of cells in each class analysed.

DI thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage; 100 cells × 4).

2.4 *Annexin V/PI staining*

Apoptosis-mediated cell death of S-180 tumour cells was examined using a FITC-labelled annexin V/propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Briefly, 1 x 10^6 cells were harvested and washed with PBS. Cells were re-suspended in 400 μl binding buffer. Next, 5 μl annexin V-FITC and 1 μl PI were added. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a flow cytometer (FACS Canto, BD Biosciences) using the Diva software. Cells in the early stages of apoptosis were annexin V positive and PI negative, whereas cells in the late stages of apoptosis were both annexin V and PI positive.

2.5 *Cell cycle analysis*

Cell cycle analysis of the S-180 cells was performed using flow cytometry. After treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 μM) for 24 and 48 h, cells were harvested by centrifugation, washed with PBS, fixed with 70% (v/v) cold aqueous ethanol and stored overnight at –20°C. The fixed cells were washed with PBS and incubated with PI (50 μg/ml) containing 0.05% RNase. Samples were incubated at 4°C in the dark and analysed by flow cytometry (FACS Canto, BD Biosciences). The percentage of cells in G1, S, G2 and sub-G1 phases was determined using the Diva software.

3. Results

3.1 *Induction of DNA damage by* cis-*$[RuCl_2(NH_3)_4]Cl$*

To directly determine if *cis*-$[RuCl_2(NH_3)_4]Cl$ induces DNA damage, S-180 cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$. Figure 2 shows the effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on DI in S-180 cells. After 24 and 48 h of exposure, *cis*-$[RuCl_2(NH_3)_4]Cl$ significantly increased the DI in relation to the negative control ($P<0.01$). In addition, the DNA damage occurred in a concentration- and time-dependent manner.

3.2 cis-*$[RuCl_2(NH_3)_4]Cl$ induces apoptosis in S-180 cells*

As shown in figure 3, after treatment with 19 and 95 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 24 and 48 h, the early apoptotic cells (annexin V positive and PI negative) represented 6.9% and 4.1% (24 h, figure 3a), and 3.7% and 1.7% (48 h, figure 3b) of the total cells, respectively. Meanwhile, the late apoptotic cells (annexin V positive and PI positive) represented 29.6% and 47.1% (24 h, figure 3a) and 82.6% and 59% (48 h, figure 3b) of the total cells, respectively. These results suggest that the induction of apoptosis by *cis*-$[RuCl_2(NH_3)_4]Cl$ accounts for part of its cytotoxic activity.

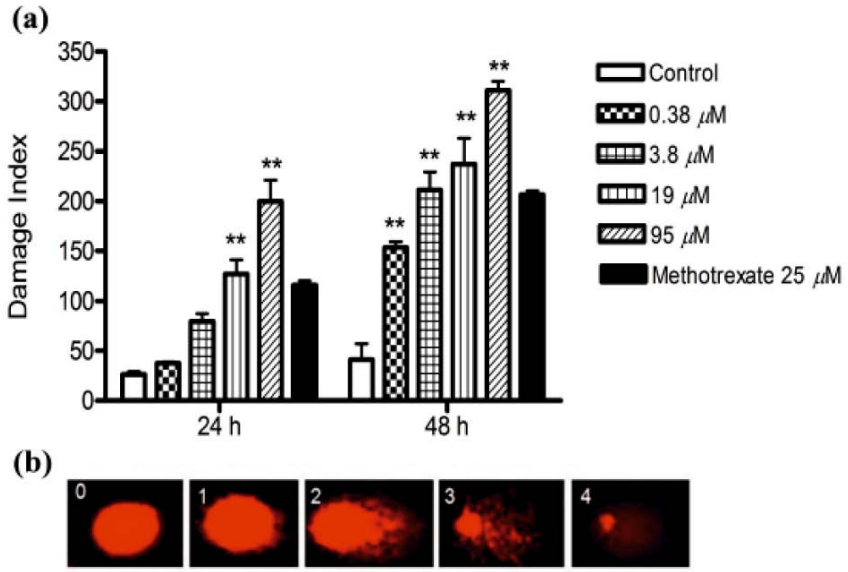

Figure 2. Effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the DNA damage index, assessed by the comet assay. S-180 cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (0.38–95 μM) for 24 and 48 h. Data represent the mean ± SD of two experiments. Significant differences compared to the untreated control are indicated by **P*<0.05 and ***P*<0.01 **(a)**. Comet images of S-180 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$. The comets illustrate the visual scoring classification: class 0 represents undamaged nucleoids, classes 1–4 represent increasing DNA damage reflected by the increasing length of the tail **(b)**.

3.3 cis-*[$RuCl_2(NH_3)_4$]Cl induces alteration in the cell cycle distribution of S-180 cells*

To test whether *cis*-$[RuCl_2(NH_3)_4]Cl$ affects the cell cycle of S-180 cells, cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ were subjected to flow cytometric analysis. After 24 h, cells treated with 95 μM of *cis*-$[RuCl_2(NH_3)_4]Cl$ showed a statistically significant increase in the G1 fraction (44.2%) accompanied by a decrease in the S (9.5%) and G2 (20.5%) fractions (figure 4a). In contrast, the cell cycle distribution of S-180 cells treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 μM) for 48 h showed a concentration-dependent increase in the sub-G1 peak (49.7% and 50.0%) (indicating apoptosis) with a corresponding decrease in cells in the G1 (15.6% and 27.35%), S (15.25% and 9.1%) and G2 (14% and 8.8%) phases, respectively (figure 4b).

4. Discussion

In the past few years, clinical trials using metal-based drugs for the treatment of tumours have become increasingly widespread in cancer therapy (Bakhtia and Ochiai 1999; Jakupec *et al.* 2008). There have been reports that *cis*-$[RuCl_2(NH_3)_4]Cl$ exhibits antitumour effects on S-180 cell lines. However, in an effort to understand the mechanism behind their cytotoxicity, we investigated whether *cis*-$[RuCl_2(NH_3)_4]Cl$ can induce DNA damage, change the cell cycle and induce apoptosis. Previous studies have demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces cytotoxicity in S-180 tumour cells (IC_{50} 63 μM) (Silveira-Lacerda *et al.* 2009). Besides being cytotoxic to tumour cells *in vitro*, it was recently reported that *cis*-$[RuCl_2(NH_3)_4]Cl$ exhibits antitumour effects on S-180 tumour cells *in vivo* and results in tumour volume reduction and an increased survival time in treated animals (Menezes *et al.* 2007)

To assess DNA damage caused by *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells, the comet assay was used. This assay has achieved the status of a standard test in the battery of tests used to assess the safety of novel pharmaceuticals or other chemicals, and is now well established as a sensitive assay for detecting strand breaks in the DNA of single cells (Fairbairn *et al.* 1995).

It is commonly believed that the main target for ruthenium compounds and other antitumour metal complexes is DNA; as shown for platinum drugs, the antitumour action of ruthenium(III) complexes would be the consequence of direct DNA binding and damage (Gallori *et al.* 2000; Brabec and Nováková 2006). Our study clearly showed an increase in DNA damage after treatment with *cis*-$[RuCl_2(NH_3)_4]Cl$, in relation to the negative control. The DI increased from 41 in controls to 237 and 311 in cells treated with 19 and 95 μM *cis*-$[RuCl_2(NH_3)_4]Cl$ for 48 h, respectively. These results demonstrated that DNA lesions induced by the ruthenium complex are difficult to eliminate with cell-repair mechanisms over 24 and 48 h. In addition, when compared

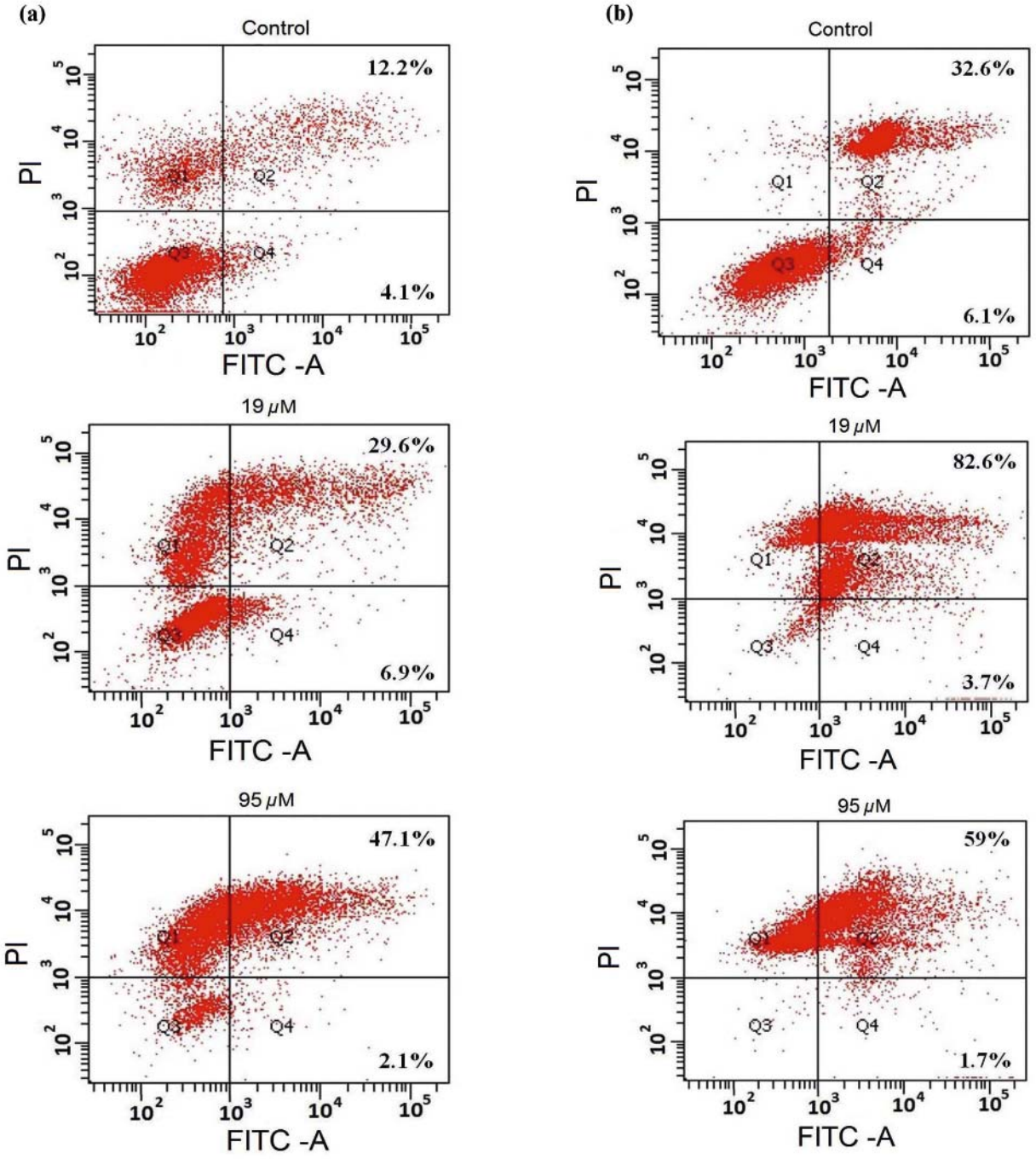

Figure 3. Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining) in S-180 cells undergoing apoptosis. The cells were treated with *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 μM) for 24 h **(a)** and 48 h **(b)**. The dual parametric dot plots combining annexin V-FITC and PI fluorescence show the viable cell population in the lower left quadrant annexin V(-)/PI(-), the early apoptotic cells in the lower right quadrant annexin V(+)/PI(-), and the late apoptotic cells in the upper right quadrant annexin V(+)/PI(+).

to a positive control (methotrexate), it is important to note that the ruthenium compound causes greater DNA damage when compared to another chemotherapeutic agent after 48 h of treatment (figure 2). Several studies have shown that ruthenium complexes induce DNA damage in the tumour cells, mainly by intercalant effects (Kapitza *et al.* 2005a; Vilaplana *et al.* 2006). A previous report indicated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces internucleosomal DNA

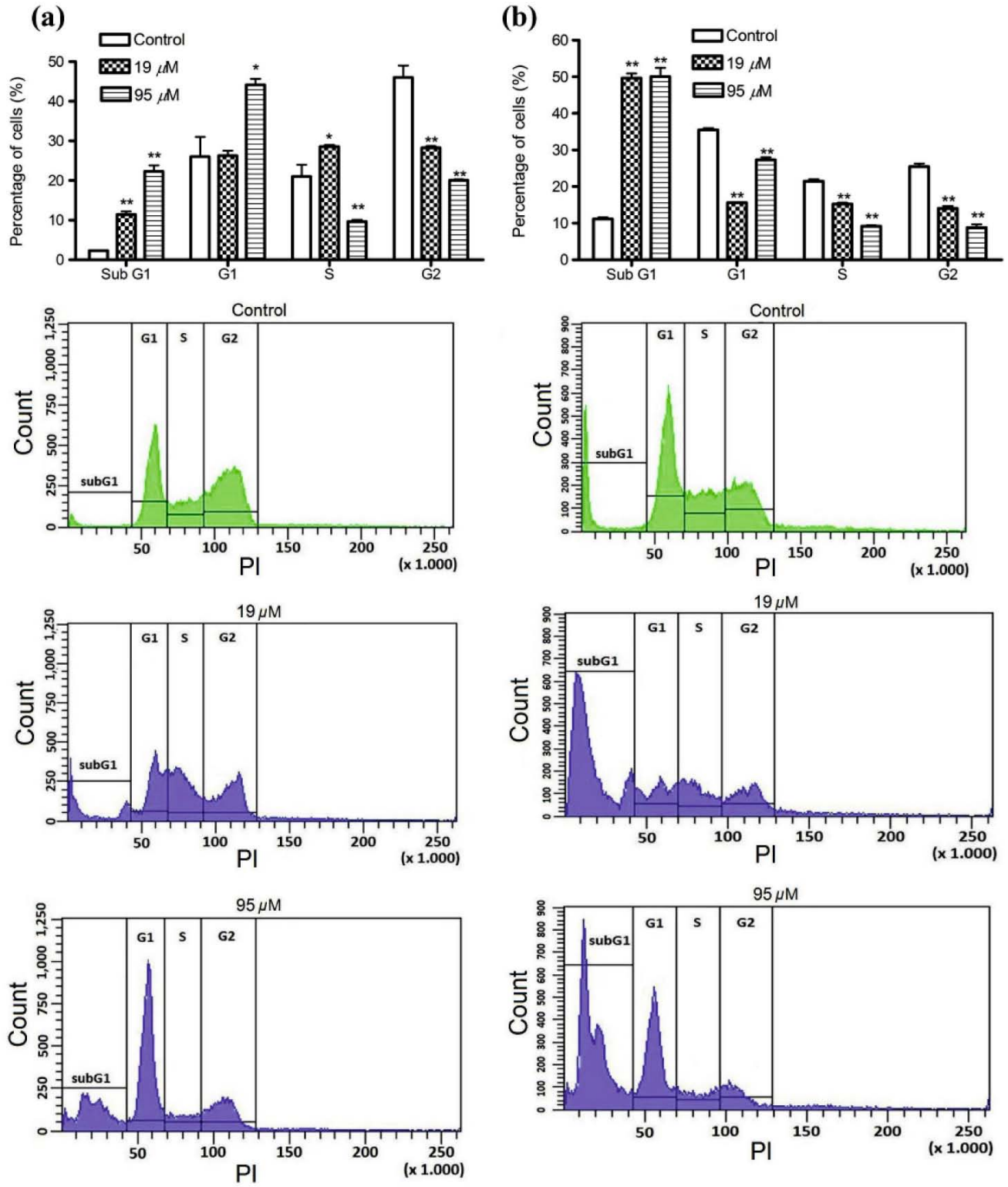

Figure 4. Effect of *cis*-$[RuCl_2(NH_3)_4]Cl$ on the cell cycle distribution of S-180 cells. Cells were treated with different concentrations of *cis*-$[RuCl_2(NH_3)_4]Cl$ (19 and 95 μM) for 24 h **(a)** and 48 h **(b)**. The percentages of cell cycle distribution were then evaluated by flow cytometric analysis. Data represent the mean ± SD of two experiments. Significant differences compared to the untreated control are indicated by **P*<0.05, ***P*<0.01.

fragmentation in Jurkat leukaemia cells (Silveira-Lacerda *et al.* 2009). Unlike the results of DNA damage presented in this study in S-180 cells, genotoxic studies using the alkaline version of the comet assay to evaluate the possible DNA-damaging effects of *cis*-$[RuCl_2(NH_3)_4]Cl$ in human peripheral blood lymphocytes (PBL) showed that this compound does not cause significantly increased DNA damage in normal cells (Ribeiro *et al.* 2009), suggesting that the genotoxic activity of this compound could be selective to tumour cells.

Thus, it is possible that the cytotoxicity of *cis*-$[RuCl_2(NH_3)_4]Cl$ to several cancer cell lines *in vitro* (Silveira-Lacerda *et al.* 2009) is partially due to its DNA damaging effects, as DNA damage is a potent stimulus for apoptotic cell death. This finding led us to evaluate the nature of cell death involved in the cytotoxicity of *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells.

Cell apoptosis is important for destroying undesirable cells during the development and homeostasis of multi-cellular organisms, and it is characterized by distinct morphological changes, such as plasma membrane blebbing, cell shrinkage, mitochondrial depolarization, chromatin condensation and DNA fragmentation (Hetts 1998; Danial and Korsmeyer 2004). Therefore, killing tumour cells through the induction of apoptosis has been recognized as a strategy for the identification of antitumour drugs and as a valuable tool for cancer treatment (Kaufmann and Earnshaw 2000; Lowe and Lin 2000). In this study, our results clearly showed that *cis*-$[RuCl_2(NH_3)_4]Cl$ induced apoptosis in S-180 cells in a time-dependent manner, as evidenced by a significant increase in annexin V-positive apoptotic cells. Consistent with our results, previous studies have demonstrated that *cis*-$[RuCl_2(NH_3)_4]Cl$ induces increased numbers of annexin V-positive Jurkat cells (Silveira-Lacerda *et al.* 2009). Thus, the mechanisms by which *cis*-$[RuCl_2(NH_3)_4]Cl$ induced apoptosis may be associated with the increase in DNA damage, as demonstrated by the comet assay.

Several reports have indicated that ruthenium complexes inhibit the proliferation of cells by inhibiting cell cycle progression and inducing apoptosis (Capozzi *et al.* 1998; Kapitza *et al.* 2005b). As shown in our cell cycle profile analysis, *cis*-$[RuCl_2(NH_3)_4]Cl$ induced G1 cycle arrest after 24 h of treatment. After 48 h of treatment, *cis*-$[RuCl_2(NH_3)_4]Cl$ caused a significant increase in the number of cells in the sub-G1 phase (indicating apoptotic DNA), with a corresponding decrease of cells in the G1, S and G2 phases. The results obtained herein suggest that the complex was capable of preventing cell cycle progression and inducing apoptosis, as the percentage of cells entering the G1, S and G2 phases was decreased, correlating with an increased proportion of cells in the sub-G1 peak, probably as a consequence of DNA damage generation.

Recently, it was shown that *cis*-$[RuCl_2(NH_3)_4]Cl$ reduced the number of murine B-cell lymphoma (A-20) cells in G0/G1 but not in S and G2/M. In contrast, in human T-cell leukaemia (Jurkat) cells, this compound slightly diminished the number of cells in G0/G1 and greatly reduced the number of cells in G2/M (Silveira-Lacerda *et al.* 2009).

It is well established that cell cycle progression is a tightly ordered and regulated process that involves multiple checkpoints. These checkpoints respond to a variety of growth signals, alterations in cell size and DNA integrity (Alberts *et al.* 2004). Thus, it is possible that the ruthenium complex may act on these checkpoints, thereby preventing further cell division and subsequently initiating death via apoptosis. However, this theory has not yet been proven experimentally. Studies with the ruthenium complex compound-derived ruthenium (RDC) showed that this compound induced the arrest of cells in G1 and induced high p21 protein levels, an inhibitor of the cell cycle which blocks CDK activity (Gaiddon *et al.* 2005).

The mechanisms by which ruthenium complexes induce apoptosis remain largely unknown. However, ruthenium(III) complexes might interact with DNA, causing cross-linking similar to that induced by cisplatin, or inducing strand break (Vilaplana *et al.* 2006; Pizarro and Sadler 2009). In contrast, ruthenium belongs to the same group of elements as iron (iron-triad), which is reflected by its strong affinity for transferrin and by the necessity of its reductive activation in cells. It can be assumed that ruthenium(III) can substitute for iron(III), which induces Fenton type redox processes and intracellular radicals (Frasca *et al.* 2001; Clarke 2003; Kapitza *et al.* 2005a). This may result in cellular damage that induces apoptosis.

In conclusion, the results presented herein demonstrate that DNA damage, apoptosis and changes in the cell cycle may correlate with the cytotoxic effects exhibited by *cis*-$[RuCl_2(NH_3)_4]Cl$ in S-180 cells.

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