



Cytogenotoxic effects of two potential anticancer Ruthenium(III) Schiff Bases complexes

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ABSTRACT

Introduction: Treatment of cancer has been subject of great interest. Researchers are continuously searching for new medicines. In this sense, ruthenium complexes have big potential. Some evidences suggest that ruthenium compounds possess anticancer activities. We synthesized two recently published ruthenium(III) complexes with bidentate O,N and tridentate O,O,N Schiff bases derived from 5-substituted salicylaldehyde and 2-aminophenol or aniline. These compounds showed affinity for binding to the DNA molecule, however, insufficient data are available regarding their possible toxic effects on biological systems.

Methods: In the present study we evaluated genotoxic, cytotoxic, and cytostatic effects of $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ and $\text{Na}[\text{Ru}(\text{L}^2)_2]$, using the *Allium cepa* assay.

Results: Different toxic effects were observed depending on the substance, tested concentration, and endpoint measured. In general, the tested compounds significantly lowered the root growth and mitotic index values as compared to the control group. Additionally, a wide range of abnormal mitotic stages, both clastogenic and non-clastogenic were observed in the treated cells. $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ significantly increased the frequency of sticky metaphases, chromosome bridges, micronuclei, impaired chromosome segregation, as well as number of apoptotic and necrotic cells over the controls. In contrast, $\text{Na}[\text{Ru}(\text{L}^2)_2]$ did not show significant evidence of genotoxicity with regard to chromosome aberrations and micronuclei, however, significant differences were detected in the number of apoptotic and necrotic cells when the highest concentration was applied.

Conclusions: In this study we demonstrated antiproliferative effects of $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ and $\text{Na}[\text{Ru}(\text{L}^2)_2]$. At clinical level, these results could be interesting for further studies on anticancer potential of the ruthenium(III) complexes using animal models.

Keywords: Ruthenium; Schiff bases; anticancer agents; apoptosis; chromosome aberrations; cytogenotoxic effects

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INTRODUCTION

In the past few decades, a large number of studies published data on a wide range of synthesized ruthenium-containing agents which have been tested for potential antitumor activity (1). Ruthenium preferentially accumulates in neoplastic rather than normal tissues possibly by binding to transferrin receptors, after which transferrin-ruthenium complexes are transported into tumor cells (2,3). Different ruthenium oxidation states (II, III, and IV) are all accessible under physiological conditions (4). Ruthenium(III) (Ru[III]) complexes appear to be more biologically inert than related Ru(II) and (IV) complexes (5). Moreover, ruthenium remains in relatively inactive Ru(III) oxidation state until it reaches the neoplastic cell. In such tissues where there is higher acidity and lower oxygen content when compared to the healthy tissues, reduction of the Ru(III) and (IV) complexes to the more reactive Ru(II) complex occurs (5,6). Some ruthenium agents show significant efficacy against tumor metastases by the inhibition of cancer cell detachment (7,8). NAMI-A and KP1019 are the only two Ru(III) complexes which have been described in the literature and are currently involved in human clinical trials (9). While NAMI-A has a specific anti-metastatic activity and low cytotoxicity, KP1019 demonstrates significant cytotoxicity by inducing apoptosis in human tumors (6,10). Furthermore, ruthenium possesses the ability to mimic iron in binding to serum transferrin and albumin (11). Since it has been previously reported that ruthenium is transferred into cells by transferrin, it appears that this transport is more efficient when transferrin is saturated with iron (12). Additionally, it is believed that the ability of ruthenium to mimic iron in binding to biological molecules leads to the general low toxicity of ruthenium drugs (13). Several *in vitro* studies confirmed the ability of Ru compounds to bind to the DNA molecule (14).

Ruthenium compounds showed prominent catalytic and biological activity (15,16). Well-known antimetastatic drug NAMI-A, Imidazolium *trans*-[tetrachloro(dimethylsulfoxide- κ S)(imidazole- κ N)ruthenate(III)] is currently in third phase of human clinical trials, while compounds like ICR (NAMI-A indazole analog) and KP1019, Indazolium *trans*-[tetrachlorobis(indazole- κ N)

ruthenate(III)] are also prominent candidates for biological application (3,16,17). Ruthenium complexes have several advantages over other platinum metals: (i) several oxidation states (+2, +3, +4) are physiologically accessible, (ii) possibility of iron mimicking, (iii) binding to plasma proteins, especially transferrin, (iv) possibility of preparation of pro-drugs which are activated by reduction or by hydrolysis in cancer cells, (v) lower cytotoxicity towards normal cells, (vi) higher selectivity to cancer cells, (vii) activity of its compounds is not limited to DNA binding, signaling pathways and enzyme inhibition are also included. Schiff bases derived from salicylaldehyde and aromatic amines are stable stereochemically flexible mono- or dibasic polydentate *O,N*-donor ligands, which showed antibacterial and antiviral activity making them good candidates for coordination on metals (18,19). Here we report *in vitro* cytotoxicity and genotoxicity of two ruthenium(III) complexes with Schiff base, sodium dichlorobis(*N*-phenyl-5-chlorosalicylideneiminato- κ^3 ON)ruthenate(III) and sodium bis[*N*-2-oxyphenyl-5-nitrosalicylideneiminato- κ^3 ONO]ruthenate(III) hereinafter Na[RuCl₂(L¹)₂] and Na[Ru(L²)₂] respectively, towards *Allium cepa* meristematic root cells. Additionally, we included ethidium bromide, a known DNA intercalator.

Ethidium bromide [(3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide)] is widely used to probe DNA structure. It binds to DNA and slips between adjacent base pairs causing stretch of double DNA helix (20). The intercalator ethidium bromide (EtBr) represents a four-ringed aromatic molecule with three of the rings being conjugated. It has been reported that the strong mode of binding of EtBr to DNA results in the intercalation of the planar phenanthridinium ring between adjacent base pairs on the DNA duplex (21). When intercalated into DNA, ethidium bromide increases the distance between the base pairs by 0.3 nm, unwinds the double DNA helix by 26° until the DNA duplex completely unfolds at a critical dye concentration. When EtBr concentration is further increased, the DNA double helix begins to wrap again in the opposite direction (21,22). Furthermore, EtBr modifies its light-absorbing properties and therefore its fluorescence (23).

Plants appear to be very sensitive to heavy metals (24). *Allium cepa* assay is standardized test

for studying cyto- and genotoxic effects of substances on chromosomes and cell division (25,26). Furthermore, *A. cepa* is widely used as an assay for biomonitoring of environmental pollutants (27). In addition to its sensitivity, cost-effectiveness and good correlation with mammalian test systems, the *A. cepa* assay gives some additional advantages which enable both macroscopic and microscopic endpoints to be measured reliably (25,28).

Therefore, the main objective of the present study is to evaluate, by means of the *A. cepa* assay, the cyto- and genotoxic effects of $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$, $\text{Na}[\text{Ru}(\text{L}^2)_2]$, and EtBr.

METHODS

Chemicals

$\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ and $\text{Na}[\text{Ru}(\text{L}^2)_2]$ were prepared according to previously published procedures. The synthesized compounds were characterized based on elemental analysis, ^1H nuclear magnetic resonance (NMR), MALDI-TOF (matrix-assisted laser desorption/ionization/time-of-flight) mass spectra, cyclic voltammograms, and electronic spectra (29,30). Ethidium bromide (CAS No. 1239-45-8) was purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of the test substances were prepared in dimethyl sulphoxide (DMSO) and kept at room temperature in the dark. All other chemicals used were of the highest grade commercially available.

Experimental procedures and root growth

A. cepa bulbs were grown in the dark at a temperature of $25 \pm 0.5^\circ\text{C}$ in incubator Nahita 636/2 (Berain, Navarra - Spain) for 48 hours. The onion bulbs (*Allium cepa* L.) were placed in glass vessels filled with tap water, which was renewed every 24 hours. The experimental procedures started after an incubation period of 48 hours by use of a series of three bulbs for each concentration and control group. The test concentrations of $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$, $\text{Na}[\text{Ru}(\text{L}^2)_2]$, EtBr, and DMSO (6.25; 12.50; 25; 50 $\mu\text{g}/\text{ml}$) were selected and prepared with tap water. At those concentrations, we incubated the onion bulbs for the next 48 hours. Fresh tap water was used as control group for all test compounds.

Macroscopic parameters

After an incubation period of 48 hours, before the treatment of the *A. cepa* bulbs with the test substances, root lengths were measured as previously described by Fiskesjö (31), after which the bulbs were exposed for 48 hours to the test solutions. At the end of the exposure period, the length of the roots from the experimental sets and control was also measured.

Microscopic parameters

A. cepa bulbs were exposed to the test solutions for 48 hours under the laboratory conditions as described above. At the end of the exposure period, root tips from each bulb were excised and fixed in ethanol/glacial acetic acid (3:1, v/v) and kept at 4°C within 24 hours. After hydrolysis for 15 minutes in 5 M HCl at room temperature, the root tips were placed into distilled water. Thereafter, apical 2 mm of the root were cut and placed on clean glass slide in a drop of 2% acetocein, and then squashed. For each tested concentration of all substances and control one slide per bulb was prepared. Cytogenetic analysis included determination of the mitotic index (MI), scoring of chromosome aberrations (CAs) and micronuclei (MNi) as biomarkers of genotoxicity, as well as counting apoptotic and necrotic cells as cytotoxicity endpoints. The MI was calculated as the ratio between the number of cells in cell division and the total number of cells analyzed, counting 1000 cells per slide. By observing 1000 interphase cells per slide for each concentration, the frequencies of micronuclei were determined. Chromosome aberrations were scored in 100 mitotic cells for each bulb. Apoptosis and necrosis were analyzed separately scoring 1000 cells per slide and were considered as cytotoxicity endpoints.

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA with the post-hoc multiple comparison test (Bonferroni) in terms of determining differences between test compounds and control group for all analyzed parameters. Differences were considered statistically significant at $p < 0.05$ level. All statistical analyses were conducted by use of Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism 6.0 for Windows (GraphPad Software Inc., USA).

RESULTS

Inhibition of *A. cepa* root growth

All compounds showed statistically significant reduction of the root growth compared to the control group (Table 1). At the same time, DMSO caused significant decrease in root length compared to the controls.

Genotoxicity and cytotoxicity in *A. cepa* root meristems

Exposure to the different concentrations (6.25; 12.5; 25; 50 µg/ml) of all the test compounds significantly inhibited the MI (Table 2) in the root tip cells of *A. cepa*. There were significant differences in the MI values compared to the control bulbs for all treatments, including DMSO.

As for the measured genotoxic endpoints, Na[RuCl₂(L¹)₂] showed statistically significant differences in the frequency of micronuclei (≥12.5 µg/ml), stickiness and chromosome missegregation (6.25 µg/ml), as well as for chromosome bridges (6.25; 50 µg/ml) compared to the control group (Table 3). On the other hand, Na[Ru(L²)₂] did not significantly induce any of the genotoxic parameters analyzed. EtBr increased the frequencies of sticky metaphases and chromosome bridges at the 6.25 and 25 µg/ml concentrations over the control values. At the lowest concentrations (≤12.5 µg/ml) DMSO showed an increase in the frequency of chromosome missegregation compared to the control group. Furthermore, DMSO (25 µg/ml) induced frequent appearance of micronuclei in mitotic *A. cepa* root cells. When assessing cytotoxic parameters, it was found that Na[RuCl₂(L¹)₂] (≥25 µg/ml) and Na[Ru(L²)₂] (50 µg/ml) significantly increased the frequency of both apoptotic and necrotic cells over the control values (Figure 1). On the other hand, EtBr and DMSO at the same concentrations (≥ 25 µg/ml) showed statistically significant differences only for the frequency of necrotic cells when compared to the control group.

DISCUSSION

Ruthenium complexes with Schiff bases are extensively studied mainly in the light of their catalytic and biological activity. Schiff bases are organic

TABLE 1. Root growth of *Allium cepa* bulbs before and after exposure to different concentrations of the test compounds (mean ± SD)

Concentration (µg/ml)	Na[Ru(L ²) ₂]		Na[RuCl ₂ (L ¹) ₂]		EtBr		DMSO		Control group	
	Root length ^a	Root length ^b	Root length ^a	Root length ^b	Root length ^a	Root length ^b	Root length ^a	Root length ^b	Root length ^c	Root length ^d
6.25	1.20±0.57***	1.55±0.77***	2.13±0.38	2.38±0.46****	2.31±0.18	2.66±0.52***	1.61±0.32**	2.20±0.45****		
12.5	1.89±0.77	2.01±0.70***	1.50±0.34****	1.69±0.34****	1.27±0.31****	1.46±0.21****	1.35±0.32****	1.55±0.34****	2.21±0.54	3.66±0.96
25	1.37±0.14****	1.42±0.15****	1.78±0.30*	1.88±0.38****	1.78±0.33*	1.81±0.31****	1.84±0.52	1.90±0.47****		
50	2.10±0.32	2.11±0.38**	2.69±0.32	2.70±0.08**	1.59±0.19**	1.62±0.21****	1.60±0.47**	1.69±0.46****		

Data are expressed in centimetres, SD: Standard deviation. ^aRoot length of the bulbs in the first 48 hours prior to exposure to different concentrations of the test compounds, ^bRoot length of the bulbs in the next 48 hours after exposure to different concentrations of the test compounds, ^cRoot length of the control bulbs in the first 48 hours, ^dRoot length of the control bulbs in the next 48 hours. Significant difference compared to the control group: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, EtBr: Ethidium bromide; DMSO: Dimethyl sulphoxide

TABLE 2. Mitotic index in meristematic cells of *Allium cepa* exposed to different concentrations of the test compounds for 48 hours (mean ± SD)

Concentration (µg/ml)	Na[Ru(L ²) ₂] MI	Na[RuCl ₂ (L ¹) ₂] MI	EtBr MI	DMSO MI	Control group MI
6.25	1.67±1.05*	4.43±0.98*	2.00±0.98*	2.00±0.86*	22.70±1.81
12.5	1.33±0.20*	3.43±0.40*	1.03±0.23*	1.50±0.78*	
25	0.87±0.40*	1.77±0.35*	0.87±0.15*	0.27±0.25*	
50	0.43±0.11*	0.70±0.17*	0.47±0.05*	0.20±0.26*	

Data are expressed in terms of percentages. SD: Standard deviation; MI: Mitotic index; Significant difference compared to the control group: **p*<0.0001, EtBr: Ethidium bromide; DMSO: Dimethyl sulphoxide

TABLE 3. The results of the genotoxicity and cytotoxicity in *Allium cepa* root tip cells exposed to different concentrations of the test compounds (mean ± SD)

Test compounds (µg/ml)	Micronuclei	Stickiness	Abnormal ana-telophases				Apoptotic cells	Necrotic cells
			Bridges	Vagrants	Chromosome missegregation	Multipolar		
Na[Ru(L₂)₂]								
6.25	0.33±0.57	1.67±1.15	0.67±0.57	n.o.	n.o.	n.o.	n.o.	n.o.
12.5	0.33±0.57	1.67±0.57	0.33±0.57	1.00±1.00	n.o.	n.o.	n.o.	n.o.
25	n.o.	1.33±1.52	n.o.	0.33±0.57	0.67±0.57	n.o.	n.o.	n.o.
50	0.67±1.15	1.00±1.73	0.33±0.57	n.o.	1.00±1.15	n.o.	5.67±1.52**	12.00±1.73****
Na[RuCl₂(L¹)₂]								
6.25	n.o.	7.00±1.73***	2.33±0.57**	2.67±1.52	4.33±2.51**	1.67±2.08	n.o.	n.o.
12.5	3.00±1.00*	3.33±1.52	n.o.	1.00±1.00	1.33±0.57	1.00±1.00	3.00±2.00	n.o.
25	4.33±0.57**	n.o.	1.00±1.00	n.o.	n.o.	n.o.	6.33±2.51**	5.00±2.00*
50	2.67±1.52*	1.33±0.57	3.33±0.57***	n.o.	n.o.	n.o.	8.67±2.51***	10.67±3.51****
EtBr								
6.25	n.o.	6.33±1.15****	3.67±1.52**	0.67±0.57	n.o.	n.o.	n.o.	1.67±0.57
12.5	n.o.	1.67±1.57	1.33±0.57	n.o.	n.o.	n.o.	n.o.	2.67±1.52
25	n.o.	3.33±1.15*	4.00±1.73**	n.o.	n.o.	n.o.	n.o.	4.33±0.57**
50	n.o.	2.33±0.57	0.67±0.57	n.o.	n.o.	n.o.	n.o.	9.00±1.73****
DMSO								
6.25	n.o.	0.33±0.57	0.67±1.15	n.o.	9.33±2.51***	n.o.	n.o.	n.o.
12.5	n.o.	1.00±1.73	0.67±0.57	n.o.	6.00±3.60*	n.o.	n.o.	n.o.
25	2.67±1.15**	n.o.	1.33±1.15	n.o.	n.o.	n.o.	n.o.	2.67±0.57***
50	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	6.33±1.15****
Control group	0.33±0.57	0.67±0.57	n.o.	0.67±1.15	n.o.	n.o.	n.o.	n.o.

n.o.: Not observed; SD: Standard deviation; Significant difference compared to the control group: **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001, EtBr: Ethidium bromide; DMSO: Dimethyl sulphoxide

molecules containing an azomethine group (-HC=N-) and having excellent chelating properties to bind many metals in stable complexes. Ru(II) and Ru(III) generally have preferential affinity toward N-donor ligands, including the azomethine group. The biological activity of metal complexes is determined by the transport mode, activation, and redox potential which must be taken into account

in designing the process of new compounds (32). The complex denoted as Na[RuCl₂(L¹)₂] contains Ru atom coordinated by two chlorides as easily leaving group and two O,N-Schiff bases derived from 5-chlorosalicylaldehyde and aniline. The compound has been described as antibacterial agent, especially against *Staphylococcus aureus* (33). It has been also described as a complex with weak to moderate

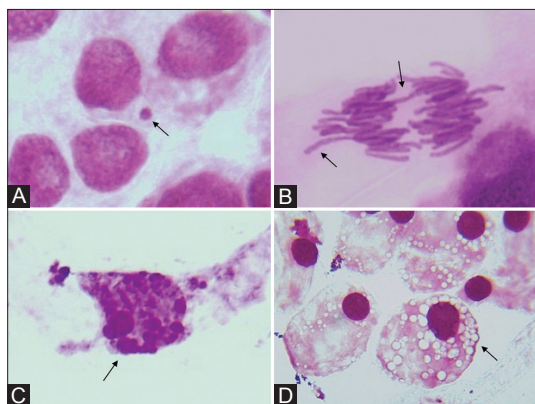


FIGURE 1. Representative images of various chromosome aberrations observed in *Allium cepa* meristematic cells. (A) Micronucleus caused by 50 µg/ml Na[RuCl₂(L¹)₂] in an interphase cell. (B) Chromosome bridge and vagrant chromosomes in anaphase induced by 12.5 µg/ml Na[Ru(L²)₂]. (C) Apoptotic cell after treatment with 50 µg/ml Na[RuCl₂(L¹)₂]. (D) Necrosis following exposure to 50 µg/ml Na[RuCl₂(L¹)₂].

antiproliferative effect *in vitro* for several primary tumor cell lines (34). In Na[Ru(L²)₂], the Ru atom is coordinated by two tridentate Schiff bases through two phenolic oxygen and azomethine nitrogen atoms. Both complexes are resistant toward hydrolysis and are able to moderately bind calf thymus DNA (CT DNA) by non-covalent mode. However, no data are available for genotoxic and cytotoxic properties of both compounds. Since, EtBr is a well-known intercalator, we comparatively used it in the present study along with our ruthenium complexes. Although, EtBr was more extensively investigated in terms of its mutagenic potentials and anti-mitotic activities, there are no data available in the literature about those effects in plants. This study has provided background data that contribute to the assessment and better understanding of the genotoxic and cytotoxic properties of these substances.

Since the different effects were observed depending on the substance, tested concentration, and endpoint measured, these three chemicals may, in a certain way, be considered as genotoxic, cytotoxic, and cytostatic substances. The results of the analysis of macroscopic parameters showed that root growth of the treated tips was reduced after all treatments (Na[RuCl₂(L¹)₂], Na[Ru(L²)₂], EtBr, and DMSO) and tested concentrations as compared to the controls (Table 1). Furthermore, all concentrations of

each tested substance in the present study significantly lowered the MI values (Table 2) when compared to the control set. It is demonstrated that root growth is regulated by the processes of cell division in actively dividing root meristematic zone. Subsequently root tip elongation occurs, where root growth can be disturbed by chemical inhibition and/or disruption (28). Additionally, the reduction in the MI values could be due to the inhibition of DNA synthesis, or blocking the G2 phase of the cell cycle, which prevents the cell to start mitosis (35). Clearly, the results of the present study confirm that all tested compounds disrupted these molecular events during cell division leading to the reduction of the MI values, which finally resulted in the inhibition of root growth in the treated root tips.

The reduced MI and root growth of *A. cepa* were accompanied by several chromosomal aberrations observed in the present study. Chromosomal alterations observed in the treatments were: sticky chromosomes, micronuclei, chromosomal bridges, vagrant chromosomes, chromosome missegregation, and multipolarity.

Chromosome stickiness is the major and most frequent alteration observed in this study. Stickiness could have been caused by adhesion or dissolution of chromosomal proteins (36,37). During the cell division, chromosome breakage leads to the micronuclei formation, which is not incorporated in the main nuclei. Therefore, the frequency of micronuclei reflects toxic effects of tested chemicals (38). Another commonly observed abnormality in the treated cells was chromosomal bridge. Chromosomal bridges arise from chromatid and/or chromosome breaks and fusion, and are considered as endpoints of clastogenic effects (39,40). According to Rank (41), vagrant chromosomes are indicators of spindle poisoning and disturbance. Similarly, impaired chromosome segregation may also indicate mitotic spindle disturbance (28). Cells with more than two centrosomes can undergo anaphase with multipolar spindles and segregate chromosomes irregularly to more than two daughter cells. This lethal event selectively targets the cancer cells with extra centrosomes, avoiding normal cells with two centrosomes (42).

The Na[RuCl₂(L¹)₂] treatments showed statistically significant incidence of spindle disturbances

like chromosome missegregation. In addition, many clastogenic abnormalities such as stickiness, chromosomal bridges as well as micronuclei were observed suggesting its destructive impact on chromosomes. The same chromosome aberrations (micronuclei and chromosome missegregation) were significantly induced by $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ and DMSO. Therefore, we cannot exclude cumulative effects of DMSO and $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ on these chromosomal abnormalities. The chromosomal aberrations induced by $\text{Na}[\text{Ru}(\text{L}^2)_2]$ are similar to those induced by $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$, but the differences in the frequency of genotoxic alterations induced by $\text{Na}[\text{Ru}(\text{L}^2)_2]$ were not significant as compared to the control group. These non-significant results for $\text{Na}[\text{Ru}(\text{L}^2)_2]$ do not exclude other relevant toxicological effects of this compound. The inhibition of mitotic activity and root growth in this study suggests that this substance may also cause toxic effects in plants. Based on these results, we could assume that observed differences between the two ruthenium complexes might be due to their different structures.

On the other hand, EtBr significantly induced sticky metaphases and chromosomal bridges, which also confirms its disruptive impact on chromosome structure. Interestingly, these findings indicate that there are no cumulative effects observed between EtBr and DMSO (Table 3).

While necrosis is usually seen as a cell rupture caused by exogenous damage, apoptosis is a process consisting of coordinated molecular events leading to morphological changes, removing unnecessary or damaged cells (43,44). DNA damage can induce programmed cell death. Hence, the frequencies of apoptosis and necrosis in the treated *A. cepa* cells were markers of cytotoxicity of the tested compounds in the present study.

As for the cytotoxicity endpoints analyzed, the two ruthenium complexes showed significant dose-dependent increase in the frequencies of apoptotic and necrotic cells. EtBr demonstrated similar cytotoxic effects but only with regard to the number of necrotic cells. This indicated that a significant number of EtBr and DMSO treated root tip cells of *A. cepa* were eliminated by necrosis. We also observed a significant number of necrotic cells after

the treatment with both ruthenium complexes, but we are not sure whether necrosis was caused by the tested ruthenium complexes or by DMSO, which was used as solvent. In addition, we had a significant number of apoptotic cells after the treatment with the tested ruthenium complexes. On the other hand, no apoptosis was observed in the EtBr and DMSO treated meristematic cells of *A. cepa*. This represents very interesting evidence, which corresponds with previously described antiproliferative effect of $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ on primary colon tumor cells (34).

It is generally accepted that the cytotoxicity of ruthenium complexes is associated with their ability to bind to DNA (45). Considering DNA as a target molecule, several *in vitro* studies have confirmed DNA-binding properties of Ru compounds and strongly suggested direct correlation between cytotoxic activity of such complexes and binding to DNA (14). The ruthenium complex cis-(dichloro)tetramineruthenium(III)chloride or cis- $[\text{RuCl}_2(\text{NH}_3)_4]\text{Cl}$ demonstrated cytostatic activity in studies conducted using human and mice tumor cells, by inhibiting the progression of the tumor cell cycle and inducing apoptosis (46), which corresponded with our results. Cytotoxic effects of the cis- $[\text{RuCl}_2(\text{NH}_3)_4]\text{Cl}$ were reported in BALB/c mice transplanted with Sarcoma 180 cells (14). Based on our findings, we cannot say whether the cytotoxic effects of the two tested ruthenium complexes are a consequence of their ability to react only with DNA, or with some cell proteins included in the cell cycle, or with both. It has been clearly demonstrated that ruthenium complexes form hydrogen bonds with proteins (47,48). The induction of apoptosis by $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ at 25 and 50 $\mu\text{g}/\text{ml}$ concentrations may be associated with the structure of this complex in which the Ru(III) atom is coordinated with two chlorides, which hydrolyze in aqueous solution making two active positions for possible binding to proteins. The complex $\text{Na}[\text{Ru}(\text{L}^2)_2]$ is not coordinated with easily leaving ligands. Therefore, the difference in activity between the two compounds can be attributed to the different ability to bind proteins, especially considering that the constants of non-covalent binding to CT DNA for both compounds are close (about 10^4M^{-1}) (29,30).

CONCLUSION

The results of the present study point out that $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$, $\text{Na}[\text{Ru}(\text{L}^2)_2]$, DMSO as well as EtBr possess highly genotoxic and cytotoxic potential. All tested compounds showed clear cytostatic effects in onion root tip cells. Observed alterations in this study such as micronuclei, sticky chromosomes, chromosomal bridges, vagrant chromosomes, chromosome missegregation, and multipolarity show that $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$, $\text{Na}[\text{Ru}(\text{L}^2)_2]$, DMSO and EtBr can be considered as clastogenic agents with significant potential for disruption of mitotic spindle.

Based on our results and described antiproliferative effects, $\text{Na}[\text{RuCl}_2(\text{L}^1)_2]$ and $\text{Na}[\text{Ru}(\text{L}^2)_2]$ could be interesting as potential anticancer substances, which suggests that it is necessary to conduct further examinations on tumor cell lines and animal models to confirm our findings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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