ORIGINALNI RAD / ORIGINAL ARTICLE

UDC: 616-006-085
546.96-386
DOI: 10.5937/erhs2201006Z

ANTITUMORSKA AKTIVNOST RUTENIJUM (II) KOMPLEKSA NA HCT 116 ĆELIJE *IN VITRO*

Ivanka Zelen[1], Milan Zarić[1], Petar Čanović[1], Danica Igrutinović[2], Ana Rilak Simović[3]
[1]Univerzitet u Kragujevcu, Srbija, Fakultet medicinskih nauka, Katedra za biohemiju
[2]Univerzitet u Kragujevcu, Srbija, Fakultet medicinskih nauka
[3]Univerzitet u Kragujevcu, Srbija, Prirodno-matematički fakultet, Katedra za hemiju

ANTITUMOR ACTIVITY OF RUTHENIUM(II) COMPLEXES ON *HCT 116* CELL LINE *IN VITRO*

Ivanka Zelen[1], Milan Zaric[1], Petar Canovic[1], Danica Igrutinovic[2], Ana Rilak Simovic[3]
[1]University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Biochemistry
[2]University of Kragujevac, Serbia, Faculty of Medical Sciences
[3]University of Kragujevac, Serbia, Faculty of Science and Mathematics, Department of Chemistry

Sažetak

Istraživanja su pokazala da kompleksi koji u svojoj strukturi sadrže rutenijum imaju snažan citotoksični efekat na različite cisplatin rezistentne malignitete. Takođe, Ru(II) I Ru(III) kompleksi su pokazali visok stepen selektivnosti prema maligno izmenjenim ćelijama, veoma slab efekat prema maligno neizmenjenim ćelijama, kao i antimetastatski efekat. Značajno je da rutenijum kompleksi imaju mogućnost vezivanja za DNK molekul tumorskih ćelija i na taj način smanjuju vijabilnost kancerskih ćelija. Pokazano je da se kompleksi rutenijuma mogu vezati za albumin i transferin čime se olakšava dopremanje ovih kompleksa do maligno izmenjenih ćelija. Stoga, cilj našeg istraživanja je ispitivanje biološkog efekta dva sintetisana Ru (II) kompleksa [Ru(Cl-Ph-tpy)(phen)Cl]Cl (K1) i [Ru(Cl-Ph-tpy)(*o*-bqdi)Cl]Cl (K2) na ćelije karcinoma kolona HCT 116, kao i definisanje mehanizma antitumorske aktivnosti kompleksa. Rezultati naše studije su nedvosmisleno pokazali da kompleksi rutenijuma obeleženi kao K1 i K2 imaju snažan citotoksični efekat na HCT116 tumorske ćelijske linije. Kompleks obeležen kao K1 je pokazao snažniji antitumorski efekat od K2 kompleksa i cisplatina. Takođe, kompleksi K1 I K2 su ispoljili citotoksični efekat na tumorske ćelijske linije indukcijom unutrašnjeg mitohondrijalnog puta.

Ključne reči: kompleksi rutenijuma, apoptoza, karcinom kolona

Abstract

In the field of non-platinum complexes, ruthenium complexes have shown very strong antitumor activity on various types of cisplatin-resistant tumors. In addition, Ru(II) and Ru(III) complexes have shown a high degree of selectivity towards cancer cells as well as antimetastatic effects. Importantly, ruthenium compounds can bind to the DNA molecule of a tumor cell and thus reduce the viability of cancer cells. Moreover, ruthenium complexes can bind to human serum albumin and transferrin, which makes their transfer to tumor cells more efficient than platinum compounds. Consequently, the research aim was to investigate the antitumor effect of two synthesized Ru(II) complexes [Ru(Cl-Ph-tpy)(phen)Cl]Cl (K1) and [Ru(Cl-Ph-tpy)(*o*-bqdi)Cl]Cl (K2) on the *HCT 116* cell line, and to define the mechanism of cell death that these compounds induce in *HCT 116* cancer cells. Results of our research clearly showed that the two investigated ruthenium complexes K1 and K2 showed very strong antitumor activity against the *HCT 116* tumor cell line. Additionally, ruthenium complex K1 showed higher antitumor activity than ruthenium K2 complex and cisplatin after 72 hours of treatment. Our findings demonstrated that both K1 and K2 ruthenium compounds exhibited strong antitumor activity against *HCT 116* cell line by induction of early apoptosis.

Keywords: ruthenium complexes, apoptosis, colon cancer

Introduction

One of the most used drugs in the therapy of malignant diseases is cisplatin (1). The therapeutic use of cisplatin has proven very successful in the treatment of various types of cancer (1, 2). Despite its successful application in the treatment of various tumors, cisplatin therapy can lead to a series of side effects such as ototoxicity, neurotoxicity, nephrotoxicity, etc (2, 3). Also, it is very significant that cancer cells can develop drug resistance after long-term therapy with cisplatin (1, 3). In the field of non-platinum complexes, ruthenium complexes have shown very strong antitumor activity on various types of cisplatin-resistant tumors (4, 5). In addition, Ru(II) and Ru(III) complexes have shown a high degree of selectivity towards cancer cells as well as antimetastatic effects (4, 6). Importantly, ruthenium compounds can bind to the DNA molecule of a tumor cell and thus reduce the viability of cancer cells (7, 8). Moreover, ruthenium complexes can bind to human serum albumin and transferrin, which makes their transfer to tumor cells more efficient than platinum compounds (6, 9).

Apoptosis is the process of programmed cell death in which antiapoptotic *Bcl-2* and proapoptotic *Bax* protein play key roles (10). An innovative and effective therapeutic method in the treatment of cancer is based on the initiation of the process of apoptosis (7, 11). In addition, changes in the concentration of *Bax* and *Bcl-2* molecules can lead to the initiation or inhibition of programmed cell death (11, 12). Studies have shown that Ru(II) complexes demonstrated a strong antitumor effect by inducing early apoptosis (7, 13, 14).

Consequently, the aim of the research was to investigate the antitumor effect of two synthesized Ru(II) complexes [Ru(Cl-Ph-tpy)(phen)Cl]Cl (K1) and [Ru(Cl-Ph-tpy)(*o*-bqdi)Cl]Cl (K2) on the *HCT 116* cell line, and to define the mechanism of cell death that these compounds induce in *HCT 1116* cancer cells. The previously mentioned ruthenium complexes K1 and K2 showed selectivity and strong cytotoxic effect on various tumor cell lines (7), but the effect on colorectal cancer cells was not investigated.

Materials and Methods

MTT Assay

The primary step in our research was to define the cytotoxic effect of two Ru(II) complexes on the *HCT 116* cell line by MTT test (7). The cancer cells were harvested from the culture flasks during the exponential growth phase, counted and 5×10^3 cells/well were seeded into 96-well culture plates. Furthermore, tumor cells were incubated in an atmosphere containing 5% CO_2 and at 37ºC for 24 hours and then treated with various concentrations (0.3, 1, 3, 10, 30, and 100 μM) of Ru(II) complexes, and with the fresh complete medium as a control. *HCT 116* cells were incubated at 37°C in an atmosphere containing 5% CO_2 and at absolute humidity for 24, 48, and 72 hours. After incubation, the medium was separated and MTT solution was added to each well. Next, the solution was gently removed and formazan crystals were dissolved in DMSO. Microtiter plates were shaken in the dark for 10 minutes and absorbance was measured at 595 nm with a multiplate reader (Zenyth 3100, Anthos Labtec Instruments, Austria). Experiments were performed in triplicates and repeated in three independent series. The percentage of viable cells was calculated by dividing the value of the readout absorbance in the wells that contained treated cells with the average absorbance value measured in the wells of untreated cells, and the ratio thus obtained was multiplied by 100.

% of the viable cells = ((absorbance of treated cell-absorbance of blank) / (absorbance of untreated cell-absorbance of blank)) * 100

The IC_{50} values (values that reduce the treated cells' viability by 50% relative to the control) were determined using Microsoft Office Excel 2010 via logarithm-transformed dose-response data, previously obtained by MTT assay.

Annexin V/7AAD Assay

Apoptosis of *HCT 116* tumor cell line was estimated by annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (BD Biosciences). *HCT 116* cancer cells were incubated with previously calculated IC_{50} values of Ru(II) complexes or with media alone (control) for 24h at 37°C in an atmosphere of 5% CO_2 and absolute humidity. Additionally, *HCT 116* cells were trypsinized, washed in phosphate buffer saline (PBS), centrifuged, and resuspended in 100 μL of ice-cold binding buffer. Then, we stained cells with both 10 μL of Annexin V-FITC and 20 μL of PI, incubated for 15 minutes in the dark at room temperature, and to each tube, 400 μL of binding buffer was added. Samples were measured using the flow cytometer Cytomics FC500 (Beckman Coulter). Also, obtained data were analyzed using FlowJo V10 Software. Measurements were presented as density plots of Annexin V-FITC and PI stainings.

Assessment of Apoptosis

One of the key objectives of our study was to analyze the expression of the proapoptotic protein

Bax, antiapoptotic protein *Bcl-2*, and the percentage of cells containing active caspase-3. *HCT 116* cells were incubated for 24 h with IC_{50} concentration of K1 and K2 complexes or in a complete cell culture medium (control). Additionally, *HCT 116* cells were washed three times with ice-cold PBS, resuspended, fixed, and permeabilized (Fixation and Permeabilization Kit, eBioscience). For *Bcl-2* staining, the cells were incubated with 1:1000 *Bcl-2* fluorescein isothiocyanate (FITC) primary antibody (mhbcl01, Life technologies) for 15 minutes at room temperature. Additional staining included incubation of permeabilized *HCT 116* cells for 30 minutes with 1:1000 of primary antibodies for active-Bax (N20, sc-493; Santa Cruz Biotech Inc.) and cleaved caspase-3 (#9661, Cell signaling Technology). Also, cells had been washed with PBS and incubated with the 1:2000 secondary goat anti-rabbit IgG-FITC antibody (Ab6717-1, Abcam) for 30 minutes. Afterwards, cells were washed in PBS and analyzed by flow cytometry. Fluorescence of at least 15000 events/sample had been measured using FC500 (Beckman Coulter). Fluorescence intensity was standardized using isotype-matched negative control antibodies. The mean fluorescence intensities for *Bax* and *Bcl-2* (MFIs) were calculated as the ratio of raw mean channel fluorescence to isotype control levels, respectively, and represented the level of expression of these proteins. The cleaved caspase-3 concentrations were evaluated as the percentages of cells displaying the fluorescence.

Statistical Analysis

The distributions of the obtained data were evaluated for normality using the Shapiro-Wilk test. The values of MTT and apoptotic protein assays were presented as mean ± standard deviation (SD). The values of annexin and cell cycle assays were presented as medians due to large standard deviations and the distribution of these data that was not normal. All experiments were performed in triplicates and three separate repetitions. Commercial SPSS version 20.0 for Windows was used for statistical analysis. Statistical evaluation was performed by Student's T-test for paired observations, or one-way ANOVA depending on data distribution. P values less than 0.05 were considered to indicate a statistically significant difference.

Results

Cytotoxicity of complexes

The antineoplastic effect of two Ru(II) complexes K1 and K2 was evaluated on a human colon cancer cell line *HCT 116* by MTT test following 24, 48, and 72h of treatment. In addition, due to its clinical application, the antitumor effect of cisplatin has been examined. The results demonstrated that two Ru(II) complexes and cisplatin show very strong and dose-dependent cytotoxic effects against the *HCT 116* cell line *in vitro* (Figure 1).

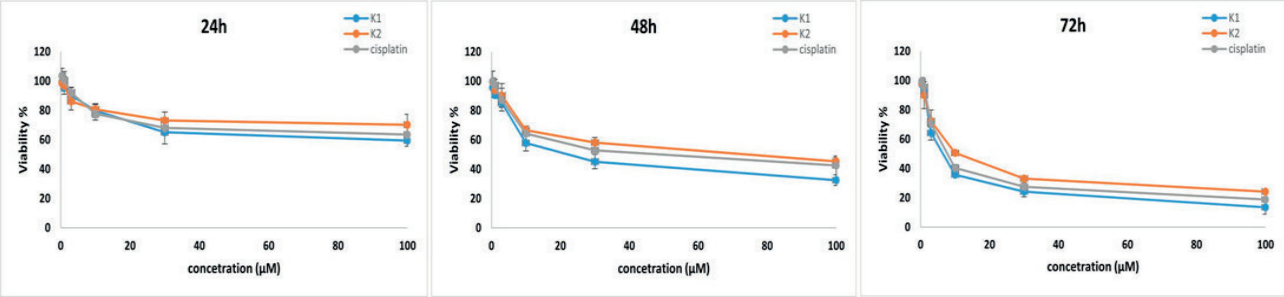


Figure 1. The effects of Ru(II) complexes (K1-K2) and cisplatin on the viability of human colorectal cancer cells *HCT 116*.

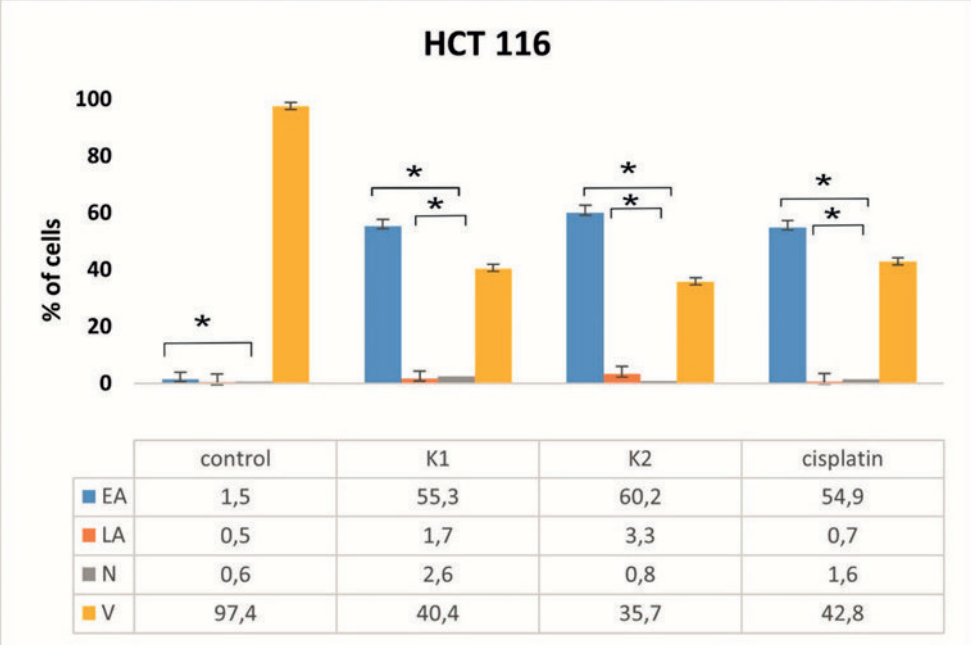
The calculated IC_{50} values for K1 and K2 complexes are represented in Table 1. Moreover, cisplatin also showed strong cytotoxicity against the *HCT 116* cell line, showing an IC_{50} value of 8.2 μM after 72h of treatment. Additionally, we can conclude that both Ru(II) complexes and cisplatin demonstrated high cytotoxicity against *HCT 116* cells after 72h of treatment (Table 1).

HCT 116			
IC_{50} (μM)	K1	K2	cisplatin
24h	107,2	118,7	110,4
48h	19,1	38,2	27,5
72h	7,8	10,3	8,2

Table 1. IC_{50} values for Ruthenium(II) complexes K1, K2, and cisplatin after 24, 48, and 72h drug exposure.

Effects of Ru(II) complexes on apoptosis

The previously defined findings of our study undoubtedly demonstrated that two Ru(II) complexes revealed a strong and dose-dependent antineoplastic effect against the *HCT 116* cell line. Hence, the next point of our research was to explore the type of *HCT 116* cell death caused by K1 and K2 ruthenium complexes. The results revealed that early apoptosis was induced by two Ru(II) complexes (Figure 2). Additionally, an insignificant percentage of the *HCT 116* cells were necrotic and in late apoptosis.



	control	K1	K2	cisplatin
EA	1,5	55,3	60,2	54,9
LA	0,5	1,7	3,3	0,7
N	0,6	2,6	0,8	1,6
V	97,4	40,4	35,7	42,8

Figure 2. Ru(II) complexes K1 and K2 reduce the viability of treated *HCT 116* cells predominantly by induction of early apoptosis.

Ru(II) complexes induce apoptosis via the mitochondrial pathway

In the final phase of our study, we wanted to examine whether Ru(II) complexes changed the concentration of antiapoptotic protein *Bcl-2* and pro-apoptotic protein *Bax*. Moreover, we estimated the activation of caspase-3 in malignant tumor *HCT 116* cells treated with both K1 and K2 ruthenium complexes. The findings revealed that two ruthenium complexes K1 and K2 significantly decreased the amount of antiapoptotic protein *Bcl-2* (Figure 3, $p<0,05$). Furthermore, there was a significant increase in the concentration of proapoptotic *Bax* protein in *HCT 116* cells treated with tested ruthenium complexes (Figure 3, $p<0,05$). Crucially, the percentage of tumor *HCT116* cells containing active caspase-3 was also increased (Figure 3). Therefore, both tested ruthenium complexes K1 and K2 decreased the *Bcl-2*/*Bax* ratio which managed the activation of caspase-3 and initiation of early apoptosis through the mitochondrial pathway.

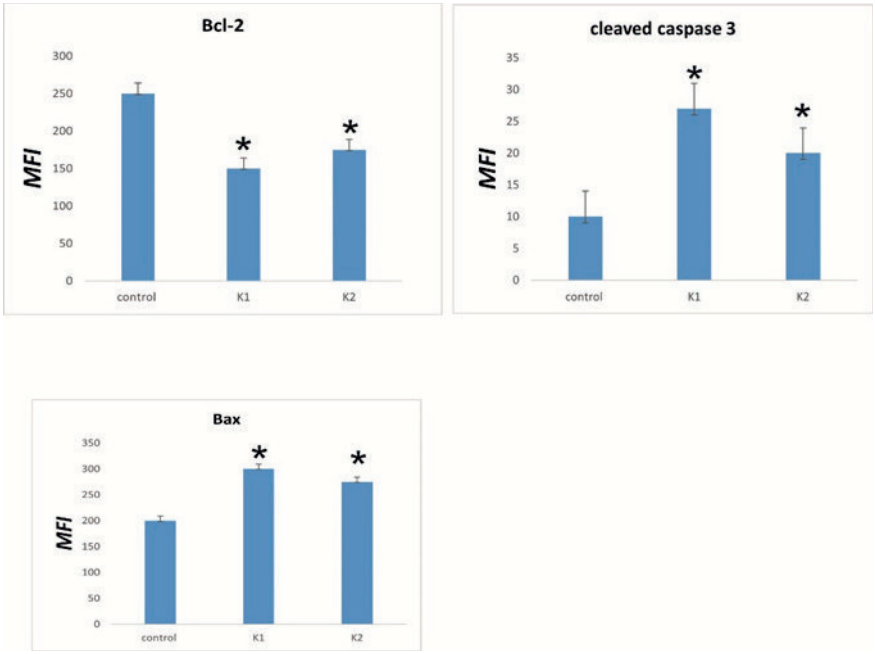


Figure 3. Ru(II) complexes K1 and K2 induce apoptosis of human colorectal cancer cells *HCT 116* via the mitochondrial pathway.

Discussion

Ruthenium complexes have become vital in medical studies due to their physicochemical properties, multiple oxidation states, and stereochemistry (15, 16). Our research aimed to investigate the biological activity of ruthenium compounds K1 and K2. Furthermore, we analyzed the mechanism of action against human colorectal cancer cells *HCT 116*. The previously presented results of our research clearly showed that the two investigated ruthenium complexes K1 and K2 showed very strong antitumor activity against the *HCT 116* tumor cell line. Additionally, ruthenium complex K1 showed higher antitumor activity than ruthenium K2 complex and cisplatin after 72 hours of treatment.

Similarly to our findings, Gatti et al. study demonstrated that ruthenium (II) complexes exhibited strong antitumor activity against human colorectal cancer cells *HCT 116* (17). Also, Baliza et al. research showed that ruthenium complexes displayed strong cytotoxic activity against the *HCT 116* cell line (14). Moreover, Savic et al. report undoubtedly demonstrated that ruthenium (II) complexes showed significant cytotoxic activity against human and mouse colon carcinoma cells *in vitro* (18). We can undoubtedly conclude that the findings of our research are in agreement with the findings of the previously mentioned sources.

Programmed cell death is initiated by two essential signaling pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (19-21). Each apoptotic pathway involves certain triggering signals to start an energy-dependent cascade of molecular actions (22, 23). Both extrinsic and intrinsic pathways activate their initiator caspase which in turn will activate the executioner caspase-3 (24, 25).

Our research findings demonstrated that both K1 and K2 ruthenium compounds exhibited strong antitumor activity against *HCT 116* cell line by induction of apoptosis. In addition, Faria et al. study showed that ruthenium complexes induce programmed cell death in cancer cell lines *in vitro* (26). Moreover, Jiang et al. research demonstrated that Ru(II) polypyridyl complexes induce apoptosis by regulating the expression of Bcl-2 family proteins (27). The findings of our research were in agreement with the conclusions of the previously cited research papers.

ACKNOWLEDGMENTS

This study was financially supported by the Faculty of Medical Sciences, University of Kragujevac (JP 19/16).

References

1. Kryczka J, Kryczka J, Czarnecka-Chrebelska KH, Brzeziańska-Lasota E. Molecular Mechanisms of Chemoresistance Induced by Cisplatin in NSCLC Cancer Therapy. Int J Mol Sci. 2021;22(16):8885.

2. Makovec T. Cisplatin and beyond: molecular mechanisms of action and drug resistance development in cancer chemotherapy. Radiol Oncol. 2019;53(2):148-58.

3. Ghosh S. Cisplatin: The first metal based anticancer drug. Bioorg Chem. 2019;88:102925.

4. Sun Q, Li Y, Shi H, Wang Y, Zhang J, Zhang Q. Ruthenium Complexes as Promising Candidates against Lung Cancer. Molecules. 2021;26(15):4389.

5. Fabijańska M, Kasprzak MM, Ochocki J. Ruthenium(II) and Platinum(II) Complexes with Biologically Active Aminoflavone Ligands Exhibit In Vitro Anticancer Activity. Int J Mol Sci. 2021;22(14):7568.

6. Mohamed Subarkhan MK, Ren L, Xie B, Chen C, Wang Y, Wang H. Novel tetranuclear ruthenium(II) arene complexes showing potent cytotoxic and antimetastatic activity as well as low toxicity in vivo. Eur J Med Chem. 2019;179:246-56.

7. Čanović P, Simović AR, Radisavljević S, Bratsos I, Demitri N, Mitrović M, Zelen I, Bugarčić ŽD. Impact of aromaticity on anticancer activity of polypyridyl ruthenium(II) complexes: synthesis, structure, DNA/protein binding, lipophilicity and anticancer activity. J Biol Inorg Chem. 2017;22(7):1007-28.

8. Devi CS, Thulasiram B, Satyanarayana S, Nagababu P. Analytical Techniques Used to Detect DNA Binding Modes of Ruthenium(II) Complexes with Extended Phenanthroline Ring. J Fluoresc. 2017 Nov;27(6):2119-130.

9. de Melo ACC, Santana JMSVP, Nunes KJRC, Rodrigues BL, Castilho N, Gabriel P, Moraes AH, Marques MA, de Oliveira GAP, de Souza ÍP, Terenzi H, Pereira-Maia EC. New Heteroleptic Ruthenium(II) Complexes with Sulfamethoxypyridazine and Diimines as Potential Antitumor Agents. Molecules. 2019;24(11):2154.

10. Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: disease message and therapeutic target potentials. Biosci Rep. 2019;39(1):BSR20180992.

11. Park SH, Shin I, Kim ND, Shin I. An Inhibitor of the Interaction of Survivin with Smac in Mitochondria Promotes Apoptosis. Chem Asian J. 2019 Nov 18;14(22):4035-41.

12. Rahmani M, Nkwocha J, Hawkins E, Pei X, Parker RE, Kmieciak M, Leverson JD, Sampath D, Ferreira-Gonzalez A, Grant S. Cotargeting BCL-2 and PI3K Induces BAX-Dependent Mitochondrial Apoptosis in AML Cells. Cancer Res. 2018;78(11):3075-86.

13. Chen T, Mei WJ, Wong YS, Liu J, Liu Y, Xie HS, Zheng WJ. Correction: Chiral ruthenium polypyridyl complexes as mitochondria-targeted apoptosis inducers. Medchemcomm. 2018;9(4):745.

14. Baliza IRS, Silva SLR, Santos LS, Neto JHA, Dias RB, Sales CBS, Rocha CAG, Soares MBP, Batista AA, Bezerra DP. Ruthenium Complexes With Piplartine Cause Apoptosis Through MAPK Signaling by a p53-Dependent Pathway in Human Colon Carcinoma Cells and Inhibit Tumor Development in a Xenograft Model. Front Oncol. 2019;9:582.

15. Lin K, Zhao ZZ, Bo HB, Hao XJ, Wang JQ. Applications of Ruthenium Complex in Tumor Diagnosis and Therapy. Front Pharmacol. 2018;9:1323.

16. Gałczyńska K, Drulis-Kawa Z, Arabski M. Antitumor Activity of Pt(II), Ru(III) and Cu(II) Complexes. Molecules. 2020;25(15):3492.

17. Gatti A, Habtemariam A, Romero-Canelón I, Song JI, Heer B, Clarkson GJ, Rogolino D, Sadler PJ, Carcelli M. Half-Sandwich Arene Ruthenium(II) and Osmium(II) Thiosemicarbazone Complexes: Solution Behavior and Antiproliferative Activity. Organometallics. 2018;37(6):891-99.

18. Savic M, Arsenijevic A, Milovanovic J, Stojanovic B, Stankovic V, Rilak Simovic A, Lazic D, Arsenijevic N, Milovanovic M. Antitumor Activity of Ruthenium(II) Terpyridine Complexes towards Colon Cancer Cells In Vitro and In Vivo. Molecules. 2020;25(20):4699.

19. Giacomello M, Pyakurel A, Glytsou C, Scorrano L. The cell biology of mitochondrial membrane dynamics. Nat Rev Mol Cell Biol. 2020;21(4):204-24.

20. Popov LD. Mitochondrial biogenesis: An update. J Cell Mol Med. 2020;24(9):4892-99.

21. Singh A, Kukreti R, Saso L, Kukreti S. Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. Molecules. 2019;24(8):1583.

22. Riley JS, Tait SW. Mitochondrial DNA in inflammation and immunity. EMBO Rep. 2020;21(4):e49799.

23. Priesnitz C, Becker T. Pathways to balance mitochondrial translation and protein import. Genes Dev. 2018;32(19-20):1285-96.

24. Jiang M, Qi L, Li L, Li Y. The caspase-3/GSDME signal pathway as a switch between apoptosis and pyroptosis in cancer. Cell Death Discov. 2020;6:112.

25. Nagata S. Apoptosis and Clearance of Apoptotic Cells. Annu Rev Immunol. 2018 Apr 26;36:489-517.

26. Faria RS, Silva HD, Mello-Andrade F, Pires WC, de Castro Pereira F, de Lima AP, de Fátima Oliveira Santos S, Teixeira TM, da Silva PFF, Naves PLF, Batista AA, da Silva Oliveira RJ, Reis RM, de Paula Silveira-Lacerda E. Ruthenium(II)/ Benzonitrile Complex Induces Cytotoxic Effect in Sarcoma-180 Cells by Caspase-Mediated and Tp53/p21-Mediated Apoptosis, with Moderate Brine Shrimp Toxicity. Biol Trace Elem Res. 2020;198(2):669-80.

27. Jiang GB, Xie YY, Lin GJ, Huang HL, Liang ZH, Liu YJ. Synthesis, characterization, DNA interaction, antioxidant and anticancer activity studies of ruthenium(II) polypyridyl complexes. J Photochem Photobiol B. 2013;129:48-56.

Korespondent: Petar Čanović, e-mail: petar.c89@gmail.com
Corresponding author: Petar Čanović, e-mail: petar.c89@gmail.com