ORIGINAL ARTICLE
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UDC: 669.217::616-006-085
DOI: https://doi.org/10.2298/VSP190507002Z

New gold pincer-type complexes induce caspase-dependent apoptosis in human cancer cells *in vitro*

Novi kompleksi zlata pincer-tipa indukuju kaspaza-zavisnu apoptozu u humanim ćelijama raka *in vitro*

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Abstract

Background/Aim. The use of cisplatin as a chemotherapeutic opened the door to the new metal-based drug research. New complexes containing metals such as platinum, palladium, ruthenium and gold have recently been analyzed as potential antitumor agents. The aim of the study was to investigate the cytotoxicity of Au(III) complexes with pincer-type ligands against cervical carcinoma cells (HeLa), breast cancer cells (MDA-MB-231 and 4T1) and colon carcinoma cells (HCT116 and CT26), as well as to examine the type and mechanism of cell death that these complexes induced in cancer cells. **Methods.** The cytotoxicity of Au(III) complexes was investigated by MTT assay. The apoptosis of the treated cancer cells was measured by flow cytometry and applying Annexin V/7AAD staining. The expressions of active proapoptotic protein Bax, antiapoptotic protein Bcl-2 and the percentage of cells containing cleaved caspase-3 in the treated cancer cells were determined by flow cytometry. **Results.** Complex 1 showed the most potent antitumor effect on HeLa cells, both compared to other two examined gold complexes and compared to cisplatin. The IC_{50} values on HeLa cells after 72 hours were 1.3 ± 0.4 μM, 3.4 ± 1.3 μM, 5.7 ± 0.6 μM, 26.7 ± 6.5 μM for complexes 1, 2, 3 and cisplatin, respectively. Complex 1 also exhibited the highest cytotoxicity against MDA-MB-231 and HCT116 cells compared to other tested compounds. The results of Annexin V/7AAD staining showed that all three gold complexes induced apoptosis in the treated cells. Our Au(III) complexes induced apoptosis by caspase-dependent mechanism, but we did not observe that the activation of an internal pathway of apoptosis occurred in the treated cancer cells. **Conclusion.** According to the results of our *in vitro* study, all three gold compounds, and especially complex 1, are promising candidates for a new generation of anticancer drugs.

Key words:
antineoplastic agents; apoptosis; caspases; enzyme assays; gold, compounds; neoplasms; toxicity, tests.

Apstrakt

Uvod/Cilj. Upotreba cisplatine kao citostatika je otvorila vrata za istraživanje novih lekova koji u svojoj strukturi sadrže neki od metala. Novi kompleksi koji sadrže metale poput platine, paladijuma, rutenijuma i zlata se od nedavno ispituju kao potencijalni antitumorski lekovi. Cilj rada bio je da se ispita citotoksičnost Au(III) kompleksa sa ligandima tipa pincer protiv ćelija karcinoma grlića materice (HeLa), ćelija raka dojke (MDA-MB-231 i 4T1) i ćelija karcinoma kolona (HCT116 i CT26), kao i tip i mehanizam ćelijske smrti koji ti kompleksi indukuju u ćelijama raka. **Metode.** Citotoksičnost Au(III) kompleksa je ispitivana pomoću MTT testa. Apoptoza tretiranih ćelija raka je merena protočnom citometrijom i primenom bojenja Aneksin V/7AAD. Ekspresija aktivnog proapoptotičnog proteina Bax, antiapoptotskog proteina Bcl-2 i procenat ćelija koje sadrže aktivnu kaspazu-3 u tretiranim ćelijama raka određena je protočnom citometrijom. **Rezultati.** Kompleks 1 je pokazao najsnažniji antitumorski efekat na HeLa ćelije, kako u poređenju sa drugim ispitivanim kompleksima zlata, tako i u poređenju sa cisplatinom. Vrednosti IC_{50} kompleksa zlata na HeLa ćelije nakon 72 sata bile su 1,3 ± 0,4 μM, 3,4 ± 1,3 μM, 5,7 ± 0,6 μM, 26,7 ± 6,5 μM za komplekse 1, 2, 3 i cisplatin, redom. Kompleks 1 je takođe

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pokazao najvišu citotoksičnost prema MDA-MB-231 i HCT116 ćelijama u poređenju sa drugim testiranim jedinjenjima. Rezultati bojenja aneksinomV/7AAD pokazali su da sva tri kompleksa zlata indukuju apoptozu u tretiranim ćelijama. Naši kompleksi Au(III) indukovali su apoptozu mehanizmom koji je zavisio od kaspaze, ali nismo pokazali da je u tretiranim ćelijama raka došlo do aktivacije unutrašnjeg puta apoptoze. **Zaključak.** Prema rezultatima naše *in vitro* studije, sva tri jedinjenja zlata, a posebno kompleks 1, obećavajući su kandidati za novu generaciju antikancerogenih lekova.

Ključne reči:
antineoplastici; apoptoza; kaspaze; testovi, enzimski; zlato, jedinjenja; neoplazme; toksičnost, testovi.

Introduction

In the United States, it is assumed that about 40% of adults will be diagnosed with cancer at a certain age [1]. This is a worrying fact, but finding a cure for cancer is not easy. There are major differences between tumors of various tissues and organs in cellular morphology, tumor aggressiveness and treatment of tumors [1–3]. Such differences exist even among the tumors of the same organ, such as breast tumors [3]. Even in the course of a treatment, tumor cells are modified and may become resistant to the therapy, which even further complicates the treatment [4]. Therefore, one universal cure for cancer will most likely never be found and testing of a substance that has chemotherapeutic potential against two or three types of tumors *in vitro* cannot provide sufficiently precise results.

Deregulation in apoptotic cell death machinery is the main characteristic of cancer [5]. Apoptosis is the process of cell degeneration that is not associated with inflammation and damage to surrounding healthy cells, and therefore it is a more favorable mechanism for reducing the number of tumor cells compared to necrosis [5, 6]. Apoptosis happens spontaneously in malignant tumors, often noticeably delaying their growth, and it is increased in tumors responding to irradiation, chemotherapy, high temperature and hormone ablation [6]. Apoptosis alteration is responsible not only for tumor development and progression but also for tumor resistance to therapies [5]. On the other hand, much of the present attention in the process stems from the finding that it can be regulated by certain protooncogenes and the p53 tumor suppressor gene [5–7]. Two proteins located in the cytoplasm of the cells, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) and Bcl-2, are the activator and the inhibitor of apoptosis, respectively. It has been described, that Bax and Bcl-2, and their ratio, are predictive markers in different cancers [8]. Also, the significance of other molecules included in the apoptosis, such as caspases, has also been previously reported [9].

The use of cisplatin as a "standard" chemotherapeutic opened the door to the new metal-based drug research [10]. New complexes containing metals such as platinum, palladium, ruthenium and gold have recently been analyzed as potential antitumor agents [10–12]. Also, organometallic gold compounds occupied an important place in various anticancer researches due to their exceptional chemical characteristics with respect to gold coordination complexes [13]. Actually, many researchers have discovered that they could be utilized to create exceptionally capable metal-based drugs with potential relevance in the treatment of cancer [14].

In order to evaluate whether Au(III) compounds with pincer-type ligands might be used as possible anticancer agents, three new monofunctional Au(III) pincer complexes with three bispyrazolate ligands such as 2,6-bis(5-*tert*-butyl-1*H*-pyrazol-3-yl)pyridine (H_2L^{tBu}), 2,6-bis(5-*tert*-butyl-1-methyl-1*H*-pyrazol-3-yl)pyridine (Me_2L^{tBu}), and 2,6-bis((4*S*,7*R*)-1,7,8,8-tetramethyl-4,5,6,7-tetrahydro-1*H*-4,7-methanoindazol-3-yl)pyridine (Me_2*L) had been synthesized. The newly synthesized complexes, namely $[Au(H_2LtBu)Cl]Cl_2$ (1), $[Au(Me_2LtBu)Cl]Cl_2$ (2) and $[Au(Me_2*L)Cl]Cl_2$ (3) were characterized by elemental analysis, spectroscopic techniques (IR, UV-Vis, 1D and 2D NMR) and mass spectrometry (MS) methods: MALDI TOF and ESI Q-TOF. These three gold(III) complexes were also tested against three types of cancer cells (A549, A375, LS-174) *in vitro* [15].

However, these Au(III) complexes were not tested against other types of cancer cells and therefore further analyses were necessary. The aim of our research was to examine whether these Au(III) compounds with pincer-type ligands might be utilized as potential antitumor agents. Therefore, we examined the antitumor potential of all three Au(III) complexes against cell lines of cervical carcinoma (HeLa), breast cancer (MDA-MB-231 and 4T1) and colon carcinoma (HCT116 and CT26). We also included mouse carcinoma cells in our research (4T1 and CT26) in order to prove future research potential of these complexes for *in vivo* studies. We investigated their cytotoxic effect against five types of cancer cells, the type and mechanism of cell death that these complexes induce in cancer cells.

Methods

Synthesis of Au(III) pincer complexes with three bispyrazolate ligands

The synthesis and characterization of 2,6-bis(5-*tert*-butyl-1*H*-pyrazol-3-yl)pyridine (H_2L^{tBu}), 2,6-bis(5-*tert*-butyl-1-methyl-1*H*-pyrazol-3-yl)pyridine (Me_2L^{tBu}), and 2,6-bis((4*S*,7*R*)-1,7,8,8-tetramethyl-4,5,6,7-tetrahydro-1*H*-4,7-methanoindazol-3-yl)pyridine (Me_2*L) were discussed in detail in the previously published article [15]. The newly synthesized complexes, namely $[Au(H_2LtBu)Cl]Cl_2$ (1), $[Au(Me_2LtBu)Cl]Cl_2$ (2) and $[Au(Me_2*L)Cl]Cl_2$ (3) were characterized by elemental analysis, spectroscopic techniques (IR, UV-Vis, 1D and 2D NMR) and mass spectrometry (MS) methods: MALDI TOF and ESI Q-TOF.

Preparation of drug solutions

Complexes 1, 2, 3 and cisplatin were measured, dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific,

UK) at the concentration of 40 mM and then filtered through filters with a pore diameter of 0.22 mm. Afterward, the compounds were diluted in the complete medium so that the final concentration of DMSO was never greater than 0.5% (v/v). Dilution series of all compounds were prepared at the concentrations of 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM and 100 μM. All solutions used in the experiments were prepared exclusively on the day of the experiment, in order to avoid any potential modification in the chemical structure of the compounds tested. The control population consisted of cells that were not treated with the test substances and to which the same amount of DMSO, in comparison to the treated cells, was added. Blanks were microtiter plate wells that did not contain any cells or media.

Cell cultures

The study examined the effects of the Au(III) pincer complexes 1, 2 and 3, as well as cisplatin against five types of tumor cells: HeLa (human cervical cancer cells), MDA-MB-231 (human breast cancer cells), 4T1 (mouse breast cancer cells), HCT116 (human colon carcinoma cells) and CT26 (mouse colon carcinoma cells). All cells used in this study were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were cultured in a complete medium that was prepared as follows: DMEM (Dulbecco's Modified Eagle's Medium-High Glucose, D5796, Sigma Aldrich, Germany) and was supplemented with 10% FBS (Fetal Bovine Serum, Sigma Aldrich, Germany), 1% Penicillin-Streptomycin (Penicillin-Streptomycin, P4333, Sigma Aldrich, Germany), and 1% non-essential amino acids (MEM Non-essential Amino Acid Solution, M7145, Sigma Aldrich, Germany). The cells were maintained in 25 cm^2 flasks (Thermo Fischer Scientific, US) that were stored in CO_2 incubator at absolute humidity and 5% CO_2 at the temperature of 37ºC. Every 2–3 days, the cells were passaged in order to be used in all experiments in their exponential (Log) growth phase.

MTT assay

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (MTT) assay is the colorimetric test used to examine the viability of cells. The basic principle of this assay is based on the ability of viable or living cells to reduce yellow tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the purple formazan [16]. Namely, living cells contain NAD(P)H-dependent oxidoreductase enzymes that have this ability, so the purple color of the cellular suspension after the incubation period reflects the amount of viable cells. To be precise, in each well of 96-wells microtiter plate, 100 μL of a suspension containing 3×10^3 cells in the exponential growth stage was added. The cells were incubated in the atmosphere containing 5% CO_2 and at 37ºC for 24 hours, and then different concentrations (0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM) of the examined complexes or cisplatin were added. As we have previously mentioned, we only added complete medium containing the appropriate amount of DMSO in control wells. Each concentration of the investigated substances was tested in triplicate and in three independent experiments. After 72 hours of the incubation of tumor cells in the atmosphere containing 5% CO_2 and at 37ºC, supernatant was extracted from each well and 200 μL of the tetrazolium bromide solution in the medium (0.05 mg/mL) was added. After another 2–3 hours of the incubation under the previously mentioned conditions, the supernatant was again removed from each well, and 150 μL of DMSO was added to dissolve the crystals of violet formazan. The plates were then shaken in the dark for 15 minutes at room temperature and the intensity of the purple color in each well was measured using the plate reader (Zenyth 3100, Anthos Labtec Instruments, Austria) by analyzing the absorbance at 595 nm. The percentage of viable cells was calculated by dividing the value of the readout absorbance in the wells that contained the treated cells with the average absorbance value measured in the wells of the untreated cells, and the ratio thus obtained was multiplied by 100.

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

% of the cytotoxicity of the compound = 100% – % of the viable cells

All cytotoxicity results were presented graphically as the arithmetic mean of the cytotoxicity of the tested substances from all three repeated experiments and triplicates ± standard deviation (SD). The values that reduce the treated cell viability by 50% relative to the control (IC_{50} values) were calculated using Microsoft Office Excel 2010, via logarithm-transformed dose-response data, previously obtained by MTT assay.

Annexin V/7AAD assay

The type of cell death induced by Au(III) pincer complexes was tested by flow cytometry and using Annexin V/7AAD staining according to the manufacturer's recommendation [Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) Apoptosis Kit, BD Biosciences]. To be precise, two colors were used, Anneksin V, which binds to the residues of phosphatidyl serine and a viable color, 7-AAD. In viable cells that did not start apoptosis, the residues of phosphatidyl serine are turned to the inside of the cell membrane, so that there is no binding of Anneksin V to the residues of phosphatidyl serine. When initiating the apoptosis process, the parts of the cell membrane are drawn to the outside, so the cells that entered apoptosis bind Anneksin V. Such cells are grouped as Annexin V positive on the results of flow cytometry assay. On the other hand, 7-AAD is a color that binds the nucleus and color that does not pass through the cell membrane, so the cells that are 7-AAD positive are in fact the cells whose integrity of the cell membrane has been compromised. Such cells are either in the late stage of apoptosis or are necrotic. Therefore, it is assumed that the Annexin V(-)/7-AAD(-) cells are viable, Annexin

V(+)/7-AAD(-) are the cells at an early stage of apoptosis, Annexin V(+)/7-AAD(+) are the cells in the late stage of apoptosis and the necrotic cells are Annexin V(-)/7-AAD(+).

Therefore, we added 1 mL of cell suspension containing 10^6 cells/mL of complete medium in each well of 24-well plates. After 24 hours of incubation in the atmosphere containing 5% CO_2 and at 37 ºC, the medium was removed and 1 ml of the medium containing the half maximal inhibitory concentration (IC_{50}) of the tested substances was added. Each substance was tested in triplicate and in three independent experiments. The control population consisted of cells supplemented with complete medium with the same concentration of DMSO as in the treated cells. The cells were then incubated for 24 hours in order to detect substance-induced cellular changes [17]. After the incubation in the atmosphere containing 5% CO_2 and at 37 ºC, the cells were trypsinized, washed three times in ice-cold PBS (Phosphate Buffer Saline) and 1×10^5 cells were dissolved in 100 μL of binding buffer (BD Biosciences, USA). Then, 10 μL of Annexin-V-FITC and 20 μL of 7-AAD were added to the cells, followed by a 15-minute incubation at room temperature in the dark and the addition of 400 μL of binding buffer. The cells were then stirred and evaluated on the flow cytometer (The Cytomics FC500 Series, Beckman Coulter). At least 15000 events per sample were analyzed. The results were analyzed using FlowJo vX.0.7 and presented as the arithmetic means of the results obtained in three independent experiments and triplicates ± SD. We analyzed statistical significance between the percentages of cells found in early and late apoptosis against the percentage of necrotic cells for each test substance and for each type of tumor cells [18, 19].

Analysis of key proteins involved in the apoptosis process

The next step in our research was to examine the mechanism by which the process of apoptosis in the treated cells was activated. We wanted to determine if there was a change in the amount or activity of the key proteins involved in the apoptosis process. Therefore, we examined the cellular levels of the proapoptotic protein active-Bax, the antiapoptotic protein Bcl-2, and the percentage of the cells in which caspase-3 was active [18, 19]. We compared the populations of the cells treated with the half maximal inhibitory concentration (IC_{50}) of the tested substances (experimental group) to the population of the untreated cells (control group). Every type of examined cells was seeded in five 25 cm^2 flasks, i.e. four for the investigated compounds and one for the control group of cells. After 24 hours of incubation in the atmosphere containing 5% CO_2 and at 37 ºC and absolute humidity, the media was replaced in all cell culture flasks. In four flasks, we added a complete growth medium supplemented with the previously determined half maximal inhibitory concentration (IC_{50}) of complexes 1, 2, 3 and cisplatin. In one flask the complete growth medium was added and the cells from this flask were the control population of the cells. The cells were then incubated for another 24 hours in the atmosphere containing 5% CO_2 and at 37 ºC and absolute humidity in order to detect substance-induced cellular changes [17]. Afterward, the cells from all the flasks were trypsinized and from every cell culture flask were divided into nine tubes, i.e. three for active-Bax staining, three for Bcl-2 staining and three for active caspase-3 staining. All cells were washed three times in PBS, fixed and permeabilized according to manufacturer's instructions (Fixation and Permeabilization Kit, eBioscience). The cells we isolated for Bcl-2 staining were supplemented with primary antibody for Bcl-2 (mhbcl01, Life technologies) in the ratio of 1:1,000 for 15 min. Permeabilized HeLa cells were also incubated with primary antibody for active-Bax (N20, sc-493; Santa Cruz Biotech Inc.) and for activated caspase-3 (#9661, Cell Signing Technology) for 30 minutes and at the concentration of 1:1,000. Subsequently, these two groups of cells (for active-Bax and caspase-3) were incubated with the secondary, goat FITC-labeled antibody (goat anti-rabbit IgG FITC Ab6717-1, Abcam) for 30 minutes and at the concentration of 1:2,000. Afterward, all cells were washed in PBS and analyzed by flow cytometer FC500 (Beckman Coulter, US). At least 15,000 events per sample were analyzed. The mean fluorescence intensities (MFI) for Bcl-2 and Bax were calculated as the ratio of the measured fluorescences for Bcl-2 and Bax against the fluorescence of the isotype control and represent the concentrations of Bcl-2 and active-Bax in stained cells. Values for active caspase-3 are presented as the percentages of cells that emit fluorescence to active caspase-3. The experiment was repeated three times.

Statistical analysis

The distributions of the obtained data were evaluated for normality using the Shapiro-Wilk test. The values of MTT and Annexin assays were presented as mean ± SD. The values of apoptotic proteins were presented as medians due to large standard deviations and distribution of data that was not normal. All experiments were performed in triplicates and in 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

The cytotoxicity of complex 1 and its ligand was partially tested on the cell lines of colon carcinoma (LS-174), lung carcinoma (A549) and melanoma (A375) cell lines. The results of this study have shown that complex 1 exhibited stronger antitumor effect on all three types of tumor cells tested compared to cisplatin [15]. Since some compounds may show delayed toxicity, it is concluded that the analysis of cytotoxicity should be performed after an interval of at least 48 hours [20]. Therefore, we chose to analyze cytotoxicity after 72 hours, similarly to the previous experiments [21–23].

The results of MTT assay showed dose-dependent cytotoxic effects against all tested cancer cells (HeLa, MDA-MB-231, 4T1, HCT116 and CT26) *in vitro* (Figure 1).

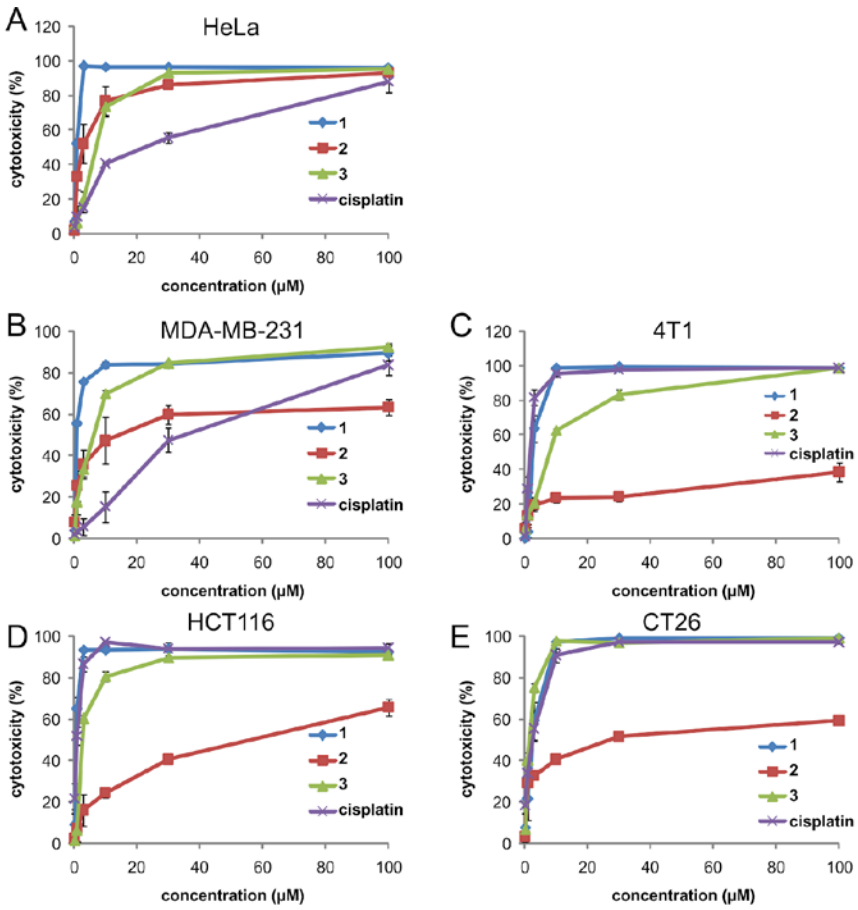

Fig. 1 – Gold (III) complex cytotoxic activities against cancer cells. MTT assay results after 72 hours of the incubation of complexes 1, 2, 3 and cisplatin against HeLa cells (A), MDA-MB-231 cells (B), 4T1 cells (C), HCT116 cells (D) and CT26 cells (E). The results are presented as mean ± standard deviation.

Table 1
IC_{50} values (μM) for complexes 1, 2, 3 and cisplatin after 72 h drug exposure

Cancer cells	1	2	3	cisplatin
HeLa	1.3 ± 0.4[a]	3.4 ± 1.3	5.7 ± 0.6	26.7 ± 6.5
MDA-MB-231	1.6 ± 0.3	15.1 ± 2.3	5.4 ± 0.5	30.8 ± 6.1
4T1	1.7 ± 0.2	> 100	6.2 ± 0.4	1.8 ± 0.2
HCT116	0.7 ± 0.2	53.9 ± 4.7	4.1 ± 0.4	1.6 ± 0.2
CT26	4.3 ± 0.3	26.04 ± 2.1	6.2 ± 0.3	2.6 ± 0.3

The results are presented as mean ± standard deviation determined from the results of MTT assay in three independent experiments.
IC – inhibitory concentration.

By further analysis of the results obtained by the MTT test, as described in material and methods section, we calculated the IC_{50} values that more accurately showed the cytotoxicity of the compounds tested. Complex 1 showed the most potent antitumor effect on HeLa cells, both compared to the other two examined Au(III) complexes and compared to cisplatin ($p < 0.05$). The IC_{50} values on HeLa cells after 72 hours were 1.3 ± 0.4 μM, 3.4 ± 1.3 μM, 5.7 ± 0.6 μM, 26.7 ± 6.5 μM for complexes 1, 2, 3 and cisplatin, respectively (Table 1).

The antitumor effects of our gold(III) complexes were also tested against human (MDA-MB-231) and mouse (4T1) breast cancer cells. All three gold(III) complexes showed stronger antitumor effects on both breast cancer cell lines compared to cisplatin ($p < 0.05$, Table 1). Complex 1 exhibited the highest cytotoxicity against MDA-MB-231 cells compared to other tested compounds ($p < 0.05$). The IC_{50} value of complex 1 against MDA-MB-231 cells was 1.6 ± 0.3 μM, which was significantly lower compared to complexes 2 and 3 (15.1 ± 2.3 μM and 5.4 ± 0.5 μM for complexes 2 and 3, respectively; $p < 0.05$). All three complexes displayed significantly increased cytotoxicity of MDA-MB-231 cells compared to cisplatin whose IC_{50} value against MDA-MB-231 cells was 30.8 ± 6.1 μM ($p < 0.05$).

Complex 1 exhibited a similar cytotoxic effect against the mouse breast cancer cells 4T1 as cisplatin ($p > 0.05$). The IC_{50} values for complex 1 and cisplatin were 1.7 ± 0.2 μM and 1.8 ± 0.2 μM, respectively (Table 1). Complexes 2 and 3 displayed significantly decreased cytotoxicity against 4T1 cells compared to cisplatin and the IC_{50} values for complexes 2 and 3 were > 100 μM and 6.2 ± 0.4 μM, respectively ($p < 0.05$, Table 1).

The most cytotoxic agent against human colon cancer cells HCT116, was complex 1 displaying twice as high cytotoxicity as cisplatin ($p < 0.05$; 1 vs. 2, 1 vs. 3 and 1 vs. cisplatin). The IC_{50} value for complex 1 and cisplatin against HCT116 cells was 0.7 ± 0.2 μM and 1.6 ± 0.2 μM, respectively. Complexes 2 and 3 exhibited less potent cytotoxic effects against HCT116 cells than cisplatin ($p < 0.05$, Table 1). It is worth noticing that our gold(III) complexes showed stronger cytotoxicity against the human MDA-MB-231 cells compared to the mouse 4T1 breast cancer cells ($p < 0.05$). Also, when we analysed MTT assay results of our gold(III) complexes against colon carcinoma cells, an identical phenomenon occurred ($p < 0.05$; 1(CT26) vs. 1(HCT116), 2(CT26) vs. 2(HCT116), 3(CT26) vs. 3(HCT116)). In this case, complexes 1, 2, and 3 showed weaker cytotoxicity against CT26 mouse colon carcinoma cells than against HCT116 human colon cancer cells. All three complexes exhibited lower cytotoxicity against CT26 cells compared to cisplatin ($p < 0.05$, Table 1).

We showed that our gold(III) complexes, and especially complex 1, displayed strong antitumor effects *in vitro* against all three types of tested human cancers cells, i.e. cervical cancer cells, breast cancer cells and colon cancer cells. Therefore, the next step of our research was to investigate the mechanism, or the type of cell death resulting in a decreased viability of the treated cells.

The results of Annexin V/7AAD staining showed that the apoptosis was induced by our gold complexes and cisplatin in all five types of the cancer cells tested (Figure 2, $p < 0.05$). In all cases, less than 4% of the total population of the cells were necrotic, while the rest of the non-viable cell population was in different stages of apoptosis. In general, a higher percentage of cells entered early apoptosis phase, and a slightly lower percentage of cells had already entered the late stage of apoptosis (Figure 2).

Consequently, the next step of our research was to investigate whether our gold complexes influenced the cytoplasmic concentration of antiapoptotic protein Bcl-2, the ac-

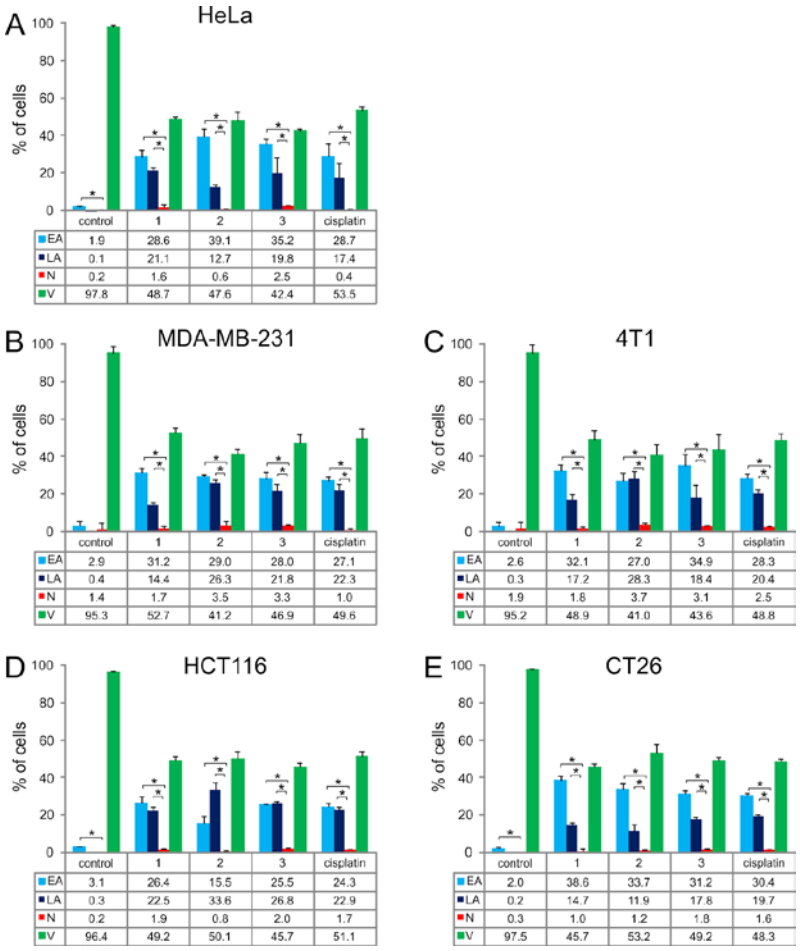

Fig. 2 – Annexin V/7AAD assay results after the treatment with inhibitory concentration $(IC)_{50}$ values of complexes 1, 2, 3 and cisplatin against HeLa cells (A), MDA-MB-231 cells (B), 4T1 cells (C), HCT116 cells (D) and CT26 cells (E). The results are presented as mean ± standard deviation. Control cells are untreated cells. EA – early apoptosis; LA- late apoptosis; N – necrosis; V – viability.

tivation of the proapoptotic protein Bax and the activation of the caspase cascade in HeLa cancer cells. We decided to investigate these events in HeLa cells considering that all three complexes exhibited the strongest cytotoxicity against this type of cancer cells, and these results were presented in Figure 3.

The results of our research have shown that all three gold complexes and cisplatin insignificantly reduced the amount of antiapoptotic protein Bcl-2 (Figure 3A, $p > 0.05$). Although this decrease was not statistically significant, it appeared as a noticeable trend. In addition, we showed that there was no statistically significant change in the activation of proapoptotic protein Bax in the groups of cancer cells cultivated in the presence of Au(III) complexes (Figure 3B, $p > 0.05$). However, in cisplatin-treated cells, a statistically significant increase of active-Bax was detected in comparison to the untreated cells (Fig. 3B, $p < 0.05$). If we observe Bcl-2/Bax ratio, there was also no significant change in the values following the effects of complexes 1–3 in comparison to control (Figure 3C, $p > 0.05$). Quite the opposite, cisplatin-treated cells exhibited a statistically significant increase of Bcl-2/Bax ratio in comparison to the untreated cells (Figure 3C, $p < 0.05$). On the other hand, a statistically significant increase in the amount of active caspase-3 in the cells treated with gold(III) complexes 1–3 and cisplatin in relation to the control population of the untreated cells was noted (Figure 3D, $p < 0.05$).

Discussion

Seeking new solutions to increase selectivity and specificity of chemotherapy in cancer cells has attracted much attention in science, recently. It was a widespread opinion that the cytotoxic effects of metal complexes are the result of direct damage to nuclear DNA [24]. However, gold(III) complexes exert their cytotoxic activities through mechanisms that are considerably diverse from those of platinum drugs [24] and the molecular mechanisms and targets of gold(III) complexes are still not precisely defined.

Hence in this work, three new gold(III) complexes with different hydro/lipophilic properties were evaluated for their anticancer activity *in vitro*. When we observed cytotoxic activities of our gold(III) complexes and cisplatin against all five types of the tested cancer cells, we discovered that complex 1 showed the strongest cytotoxicity in comparison to other complexes and compared to cisplatin in HeLa, MDA-MB-231, 4T1 and HCT116 cells. It is important to point out that complex 1 exhibited stronger cytotoxic effects than cisplatin against all three types of the human cancer cells: HeLa, MDA-MB-231 and HCT116 cells. The results of our research are in agreement with the results of other authors [25–27] who also synthesized gold(III) complexes that are significantly more effective against cancer cells in comparison to cisplatin. However, cisplatin showed statistically higher cytotoxicity against CT26 cells compared to complex 1.

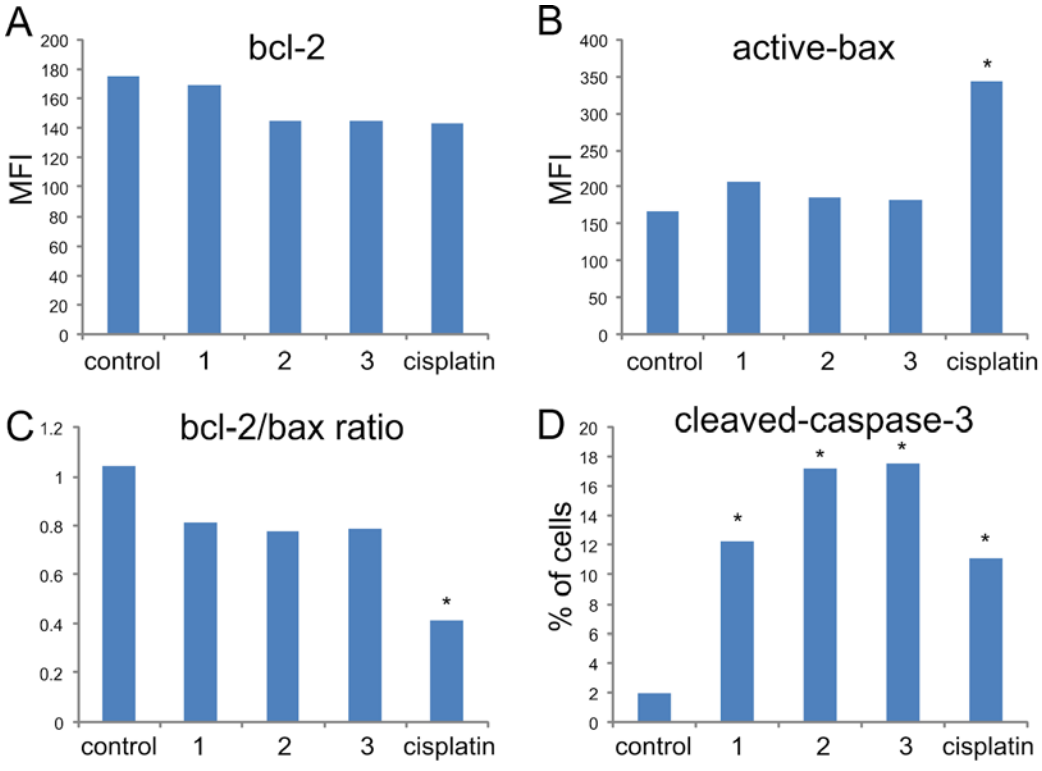

Fig. 3 – Gold (III) complexes induce apoptosis of HeLa cells via caspase-dependent pathway. (A) MFI (mean fluorescence intensity) values for antiapoptotic protein Bcl-2 of the untreated cells (control) or the cells treated with inhibitory concentration $(IC)_{50}$ values of complexes 1, 2, 3 or cisplatin; (B) MFI values for active proapoptotic protein Bax of the untreated cells (control) or the cells treated with IC_{50} values of complexes 1, 2, 3 or cisplatin; (C) Bcl-2/Bax ratio for both untreated and treated HeLa cells; (D) The percentages of cells displaying fluorescence for cleaved caspase-3. The results are presented as medians (*$p < 0.05$ compared to the untreated cells).

The results of our study showed that gold(III) complexes exhibit strong anticancer activity *in vitro*, which is in agreement with the results of some previous studies [25–27]. However, our gold complexes demonstrated stronger cytotoxicity against human cancer cells *in vitro* compared to gold complexes of some other authors. Williams et al. [25] synthesized seven new cyclometalated Au(III) complexes with five of them bearing an acridine moiety attached via (N^O) or (N^N) chelates, acyclic amino carbenes (AAC) and N-heterocyclic carbenes (NHC). However, the Williams et al. [25] complexes showed significantly lower cytotoxicity compared to our complexes, which was at least two-fold less in comparison to IC_{50} value of our complex 1 against MDA-MB-231 cells. Only complex 11 synthesized by Williams et al. [25] demonstrated an IC50 value against MCF-7 breast cancer cells comparable to our results. The complexes of Bertrand et al. [26] were biotin, 17α-ethynylestradiol and benzimidazole-based (NHC)AuCl conjugates connected by linkers of variable length. Bertrand et al. [26] investigated the cytotoxicity of their complexes against MCF-7, MDA-MB-231, A549, HCT116 and HEK-293 cancer cells. However, our complexes exhibited 3.5-fold stronger cytotoxicity *in vitro* compared to gold complexes of Bertrand and associates [26]. The series of lipophilic [AuIII(C^N^C)(NHC)]+ complexes synthesized by conjugation of an N-heterocyclic carbene (NHC) ligand to the [AuIII(C^N^C)]+ moiety, showed promising in vitro cytotoxicity towards a panel of cancer cell lines with IC50 values spanning between 0.17 and 1.2 mM [27]. These results are in line with the results of our study and show promising *in vitro* results of Au(III) complexes against cancer cells, in general. Li et al. [22] synthesized a series of cyclometalated gold(III) compounds [Aum-(C^N^C)mL]n+ (m = 1–3; n = 0–3; HC^N^CH=2,6-diphenylpyridine) by ligand substitution reaction of L with N-donor or phosphineligands. The cytotoxicity of their. Au(III) complexes was examined against HeLa cells under the same conditions in comparison to our study. Their IC_{50} values were similar or even significantly lower in comparison to the results that we obtained, and the proposed mechanism that decreased the viability of the treated cells was apoptosis.

The results of recent studies have pointed toward the induction of apoptosis as a major cytotoxic mechanism of gold(III) complexes against cancer cells [22, 28]. Apoptosis is mediated by two main pathways, an extrinsic pathway, which involves cell surface receptors, and an intrinsic pathway via mitochondria and the endoplasmic reticulum. The results of our research were completely in agreement with the results of other studies, where it was also shown that complexes of gold displayed cytotoxicity against the tested cancer cells by the induction of apoptosis [26]. This is very important because substances acting cytotoxically by the induction of apoptosis do not induce changes in surrounding healthy tissue; there is no process of inflammation or other adverse effects [6].

Therefore, the next step in our research was to examine the mechanism of apoptosis in the group of treated cancer cells. It has already been shown that the substances acting through the mitochondrial, internal pathway of apoptosis changed the activity or concentration of proapoptotic and antiapoptotic proteins [18, 19]. In addition, it has already been shown that gold complexes could induce apoptosis by activating the mitochondrial pathway of apoptosis [26, 29]. The activation of caspase-8 is typical for the external (receptor) pathway of apoptosis, while the increased active Bax and/or decreased Bcl-2 is rather connected with the internal (mitochondrial) pathway. In both cases, downstream activation of caspase-3 occurs. Afterward, caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating the DFF45/ICAD protein complex [30].

The results of our research indicated that most probably there had been no induction of apoptosis due to the effect of complexes 1–3 through the internal pathway of apoptosis. Further researches are necessary in order to confirm the following assumption, but we may suggest that the activation of the external pathway of apoptosis is the most probable cause of Au(III) complex-induced apoptosis [6, 31]. On the other hand, we undoubtedly concluded that all three gold(III) complexes induced apoptosis by activating the caspase cascade, as there was a statistically significant increase in the amount of active caspase-3 in the cells treated with IC_{50} values of gold(III) complexes 1–3 compared to the untreated cells ($p < 0.05$).

Although it has been shown that gold(III) complexes have a different mechanism of action in comparison to cisplatin, the precise mechanism of cytotoxic activity of gold(III) complexes has not yet been fully clarified.

Conclusion

It has been shown that gold(III) complexes could induce apoptosis by activating the internal pathway of apoptosis and activating the caspase cascade. The results of our research showed that gold(III) complexes tested induced apoptosis by the caspase-dependent mechanism, but we did not observe that an activation of the internal pathway of apoptosis occurred in treated cancer cells. The most probable cytotoxic mechanism of the investigated gold(III) complexes was the activation of the external pathway of apoptosis, but this assumption must be proven. However, all three investigated gold(III) complexes, especially complex 1, showed strong cytotoxicity against human cancer cells *in vitro* and induced apoptosis by caspase-dependent mechanism. It is therefore necessary to further investigate the mechanism of cytotoxicity of these complexes. According to the results of our *in vitro* study, and if some further *in vivo* investigations show promising results, complex 1 may be a good candidate for a new generation of anticancer drugs.

Acknowledgement

This work has been financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, under Grant No. 172011.

Conflict of interest

The authors declare no conflict of interest.

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Received on May 7, 2019
Revised on December 29, 2019
Accepted December 30, 2019
Online First January, 2020