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ORIGINAL ARTICLE

Anticancer activity of two ruthenium(II)-DMSO-chalcone complexes: Comparison of cytotoxic, pro-apoptotic and antimetastatic potential

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Summary

Purpose: *Recently, we reported the synthesis and characterization of two complexes of general formula cis-[Ru(S-DMSO)3(R-CO-CH=CH-R')Cl] (R=2-hydroxyphenyl for both, R'=thiophene (1), 3-methyl thiophene (2)) that showed remarkable topoisomerase II inhibition and strong binding with DNA. The aim of this study was the investigation of cytotoxic properties of these complexes against a panel of human tumor cell lines, with elucidation of their anticancer mechanisms in HeLa cells.*

Methods: *Characterization of anticancer activity of the investigated ruthenium complexes 1 and 2 included analysis of cytotoxicity by MTT assay. Cell cycle phase disruption of HeLa cells treated with complexes 1 and 2 was analyzed by flow cytometry after propidium iodide (PI) staining. Annexin V-FITC/PI double staining and further flow cytometry analysis and acridine orange (AO)/ethidium bromide (EB) double staining and fluorescent microscopy were used to determine the apoptotic potential of the investigated ruthenium complexes. The inhibitory effect on gelatinases (MMP-2 and MMP-9) as an indication of possible antimetastatic potential was also analyzed using gelatine zymography.*

Results: *The 50% cell growth inhibition (IC50) values of the investigated complexes ranged between 22.9 and 76.8 μM, with complex 2 being more cytotoxic. Both complexes induced G2 phase cell cycle arrest and apoptosis in HeLa cells. Inhibitory effect of complex 2 on MMP-2 activity was detected.*

Conclusions: *This work revealed the potential of the investigated Ru(II)-DMSO-chalcone complexes as anticancer agents with cytotoxic and pro-apoptotic activity and indicated complex 2 as leading compound for further chemical modifications and anticancer research.*

Key words: *apoptosis, chalcones, cytotoxicity, matrix metalloproteinase, ruthenium(II) complexes*

Introduction

The discovery of cisplatin by Rosenberg was a major breakthrough in the treatment of malignant diseases [1]. Nowadays, when a great number of non-platinum metal complexes has been synthesized and examined for their anticancer activity, ruthenium complexes are recognized as the most perspective ones because of their low general toxicity and a variety of modes of action, different from those of platinum compounds currently in use [2,3].

To date, two ruthenium complexes such as NAMI-A [(ImH)(trans-Ru(DMSO)(Im)Cl4), Im=imidazole] and KP1019 [(IndH)(*trans*-Ru(Ind)$_2$Cl$_4$), Ind=indazole] have entered clinical trials. Despite their structural and chemical similarities, these two Ru(III) complexes show distinct antitumor

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behaviors [4-7].

Beside Ru(III) complexes, a large number of Ru(II) compounds have been prepared and tested for antitumor activity [5]. The ruthenium(II) species shows a higher propensity for ligand exchange reactions and might therefore interact with target molecules more rapidly. Their cytotoxicity often correlates with the DNA binding ability [8,9]. Entirely new classes of Ru(II) compounds were developed with many different ligands like arene, tetramine and polypyridine ligands [10-12].

In recent years, chalcones emerged as important class of ligands. Chalcones are structurally simple and large class of plant secondary metabolites that can be considered as important component in construction of different, potentially bioactive molecules due to their great diversity of structures and an impressive broad spectrum of biologic activities, including antifungal, antiviral, antibacterial, antiinflammatory, anticancer and antiinvasive [13].

In our previous work, 5 new ruthenium(II)-DMSO-chalcone complexes of type cis-[Ru(DMSO)3L-Cl] containing substituted chalcone derivatives as ligands were synthesized and characterized. Two complexes containing heterocyclic core in chalcone ligand (L=R-CO-CH—CH-R'; R=2-hydroxyphenyl; R´=thiophene (1), 3-methyl thiophene (2)) showed remarkable topoisomerase II inhibition and strong binding with the minor groove of DNA [14]. However, cytotoxicity and the biological activities of these complexes have not been studied. Therefore, it was interesting to examine and compare potential anticancer activities underlying the chemical structures of these complexes (Figure 1).

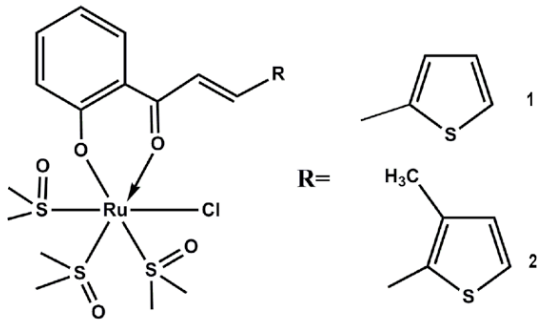

Figure 1. Chemical structures of the two investigated Ru(II)-DMSO-chalcone complexes.

Methods

Synthesis

The complexes of structural formula *cis*-[Ru(DMSO)$_3$LCl], where L=R-CO-CH—CH-R'; R=2-hydroxyphenyl; R´=thiophene (*1*), 3-methyl thiophene (2) were synthesized and characterized as previously described [14].

Cell culture

Human cervix carcinoma cells (HeLa), human melanoma cells (FemX), human breast cancer cells (MDA-MB-231), human lung adenocarcinoma epithelial cells (A549), and human foetal lung fibroblast cells (MRC-5) were maintained as monolayer culture in Roswell Park Memorial Institute nutrient medium (RPMI 1640). Human myelogenous leukaemia cells (K562) were maintained in suspension culture. Powdered RPMI 1640 medium was purchased from Sigma Aldrich Co. Nutrient medium RPMI 1640 was prepared in sterile ionized water, supplemented with penicillin (192 IU/mL), streptomycin (200 µg/mL), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM) and 10% of heat-inactivated foetal calf serum (FCS) (pH 7.2). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

Cytotoxicity assay

Cytotoxicity of the investigated ruthenium complexes was determined using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay [15]. Cells were seeded in 96-well cell culture plates (NUNC) in the following densities (cells/well, c/w): HeLa (3000 c/w), FemX (5000 c/w), MDA-MB-231 (5000 c/w), A549 (5000 c/w), and MRC-5 (5000 c/w) in culture medium and grown for 24 hrs. K562 (5000 c/w) cells were seeded 2 hrs before treatment. Stock solutions of the investigated agents were made in DMSO at a concentration of 10 mM, and afterwards diluted with nutrient medium to the desired final concentrations (ranging up to 100 µM). Cisplatin (CDDP) stock solution was made in 0.9% NaCl at a concentration of 1.66 mM and afterwards diluted with nutrient medium to desired final concentrations (ranging up to 100 µM). Solutions of various concentrations of the examined compounds were added to the wells, except the control wells where only nutrient medium was added. All samples were done in triplicate. Nutrient medium with the corresponding agent concentrations but without target cells was used as a blank, also in triplicate.

Cells were incubated for 48 hrs with the test compounds at 37°C, with 5% CO_2 in humidified atmosphere. After incubation, 20 µL of MTT solution, 5 mg/mL in phosphate buffer solution (PBS), pH 7.2, were added to each well. Samples were incubated for 4 hrs at 37 °C with 5% CO_2 in humidified atmosphere. Formazan crystals were dissolved in 100 µL 10% sodium dodecyl sulphate (SDS). Absorbance was recorded on the ThermoLabsystems 408 Multiscan EX 200–240 V after 24 hrs at a wavelength of 570 nm. Concentration IC_{50}

Table 1. Results of MTT assay presented as IC_{50} (μM) values obtained after 48-h treatment

	IC_{50} [μM] (mean ± SD)					
Complex	*HeLa*	*FemX*	*MDA-MB-231*	*K562*	*A549*	*MRC-5*
1	42.36 ± 3.15	76.87 ± 0.40	50.43 ± 4.71	41.49 ± 1.07	> 100[a]	45.70 ± 3.27
2	27.79 ± 1.47	37.56 ± 8.43	38.33 ± 2.17	30.64 ± 2.24	41.80 ± 6.14	22.86 ± 2.51
Cisplatin	7.79 ± 2.32	10.77 ± 0.88	29.79 ± 4.65	19.77 ± 0.89	17.20 ± 0.70	30.26 ± 2.98

IC_{50} values were calculated as mean values obtained from three independent experiments and quoted with their standard deviations.
[a] The sign > (in front of the maximum value of the concentration) indicates that IC_{50} value is not reached in the examined range of concentrations.

(μM) was defined as the concentration of drug producing 50% inhibition of cell survival and was determined from the cell survival diagrams.

Cell cycle analysis

HeLa cells were seeded at density of $2x10^5$ cells /well at 6-well plate (NUNC) and grown in nutrition medium. After 24 hrs, cells were continually exposed to the tested compounds with concentrations that corresponded to IC_{50} (determined for 48 hrs treatment). After 24 and 48 hrs of continual treatment, cells were fixed with 70% ethanol according to standard procedure [16] and after staining with PI were analysed using fluorescence activated sorting cells (FACS) Calibur Becton Dickinson flow cytometer and Cell Quest computer software.

Quantification of apoptotic cells by flow cytometry/Annexin V/PI staining

HeLa cells were seeded at density of $2x10^5$/well at 6-well plate and grown in nutrition medium. After 24 hrs of growth, cells were continually exposed with IC_{50} concentrations of 1 and 2 complexes and CDDP for 24 and 48 hrs. After treatment, cells were prepared and evaluated by Annexin V–FITC apoptosis detection kit, according to the manufacturer's instructions (BD Biosciences Cat. No. 65874x, Pharmingen, San Diego, CA, USA) and then analysed within an hour using a FACS Calibur Becton Dickinson flow cytometer and Cell Quest Pro computer software.

Morphological analysis of HeLa cell death using fluorescent microscopy

Briefly, $1×10^5$ HeLa cells were seeded on glass slide in Petri dishes, and treated after 24 hrs with IC_{50} concentration of the investigated compounds for 24 and 48 hrs. The cells were then stained with 10 μL of a mixture of working concentration of AO/EB (3 μg/ml AO and 10 μg/ml EB in PBS) [17] and observed in the next 30 min under a fluorescence microscope using a fluorescein isothiocyanate (FITC) filter set.

In vitro antimetastatic potential - Gelatine zymography

The collagenolytic activity in drug-treated and untreated HeLa cells was analysed and identified by gelatine zymography in 10% SDS-polyacrylamide gels impregnated with 0.1% gelatine. Cells/sample ($2x10^6$) were treated with subcytotoxic concentrations of the investigated complexes to achieve final concentrations of 15, 7.5 and 3.5 μM. Cells were treated with the investigated compounds for 24 hrs in serum-free medium. The supernatants of the treated cells were prepared and analyzed according to procedure previously described [18,19].

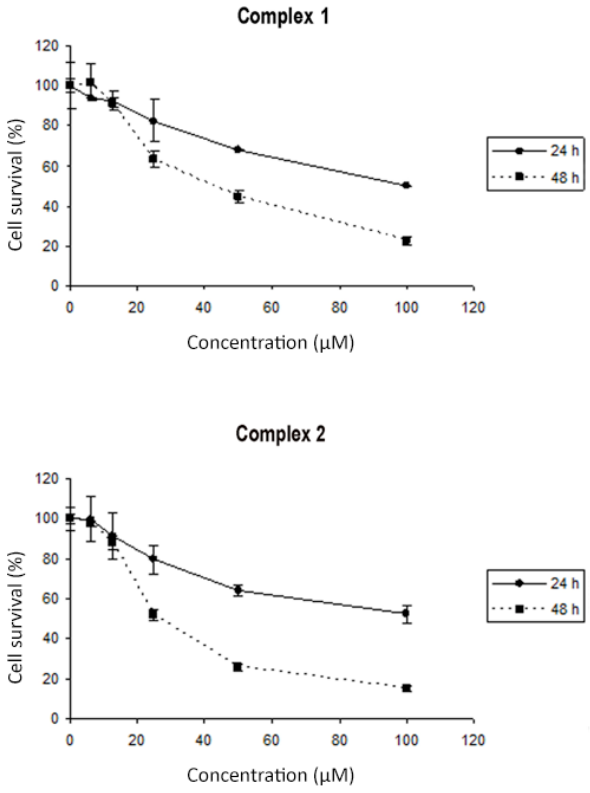

Figure 2. Time and dose dependent drug efficacy curves for Complex *1* and *2* on HeLa cells.

Results

The cytotoxic activity of the investigated ruthenium complexes *1* and *2* was determined on five tumour cell lines (HeLa, FemX, MDA-MB-231, K562, and A549) and one normal cell line (MRC-

5) using MTT assay. Both investigated complexes induced similar, moderate cytotoxic activity to all investigated cell lines with IC_{50} values ranging between 22.9 and 76.8 μM. A549 cells were least sensitive with IC50 values not reaching 100 μM for complex *1* and being 41.8 μM for complex 2 (Table 1). It is notable that complex *2* induced higher cytotoxic effect on all the investigated cell lines in comparison to complex *1*. Antiproliferative activity of both complexes on HeLa cells was not only concentration-dependent but time-dependent as well (Figure 2).

Cell cycle changes induced by the investigated ruthenium complexes on HeLa cells were apparent only after 48 h treatment (Figure 3 and Figure 4, lower image). In comparison to the control population, cell cycle data clearly showed that the investigated ruthenium complexes altered the normal phase distribution, causing arrest in G2 phase (from 17.1% in the control population to 27.6% for complex *1* and 27.9% for complex *2*) and increased the percentage of cells in sub-G1 phase (from 7.3% in the control population to 15.8% for complex *1* and 16.4% for complex *2*).

The potential of ruthenium complexes to induce apoptotic cell death was initially investigated using Anexin-V-FITC/PI staining for the determination of early marker of apoptosis (translocation of phosphatidylserine). After 24 hrs of HeLa cells treatment with the investigated agents with IC_{50}^{48h} concentration, the number of early apoptotic cells (A+P-) increased from 1.91% in the control to 16.06% for complex *1*, and 9.45% for complex *2*, similar to cisplatin treatment (16.71%) (Figure 5A). The percent of cells in late apoptosis and necrosis (A+P+) increased also, from 5.47 % in the control to 12.55, 10.50 and 12.15% for complex *1*, *2* and CDDP treated cells, respectively.

The type of cell death induced by the investigated complexes was further investigated by fluorescent microscopy to confirm apoptotic potential. Microphotographs of AO/EB double stained HeLa cells, pretreated for 24 and 48 hrs with cisplatin and ruthenium complexes (IC_{50} concentration) are presented in Figure 5B. The control cells (untreated) were dense, light green color, elongated and spindle-shaped. After treatment with cisplatin and the investigated complexes, the number of cells was reduced compared to control, cells lost their normal morphology and became rounded. This was followed by cytoplasmic shrinkage and nuclear condensation, which are characteristics for apoptosis. These early apoptotic markers were obvious already after 24 hrs treatment with CDDP and complex 2, while complex 1, even after prolonged treatment for 48 hrs, induced milder effects.

The potential effect of the investigated complexes on the activity of MMP-9 and MMP-2 (gelatinases) was determined by gelatine zymography using supernatants of treated HeLa cells for 24 hrs with subcytotoxic concentrations of the complexes. The results revealed inhibitory effect of complex *2* on MMP-2 activity at its highest concentration applied (Figure 6).

Discussion

In our previous study, synthesis and characterisation of five Ru(II)-DMSO-chalcone complexes was reported along with their DNA binding, nuclease and topoisomerase II in vitro inhibitory activity. Two complexes bearing thiophene derivatives in their structural framework (complexes *1* and *2*) bound to DNA with almost the same affinity and Topo II inhibitory activity, indicating a similar mechanism of molecular action [14]. Having in mind that studies performed in cell-free system provided limited information regarding many aspects of the mechanism of action, examination of in vitro anticancer activity was of major interest.

In the present study we investigated the potential anticancer effect *in vitro*, and gained preliminary insights of the mechanism of action of these two complexes.

The cytotoxic activity of ruthenium complexes with thiophene (*1*) and 3-methyl thiophene (*2*) in their ligand skeleton determined on five tumor cell lines of different origin (cervix, breast, blood, lung, skin) revealed that both investigated complexes induced moderate cytotoxic activity to all investigated cell lines. Comparison of cytotoxic activity of the investigated complexes showed that complex *2* is 2-fold more potent than complex *1*, which could be related to the presence of methyl group as substituent in the thiophene part of chalcone ligand. Higher cytotoxicity of complex *2* is in concordance with results obtained from DNA cleavage experiments as DNA cleavage by complex *2* is significantly higher than that observed with complex *1* [14]. Based on these data we can assume that the cytotoxicity of the investigated complexes is partially due to its DNA damaging effects, as DNA damage can be a potent stimulus for apoptotic cell death.

Cell cycle changes examined by flow cytometry on HeLa cells were apparent only after 48 hrs treatment, and, in comparison to the control population, data showed that the investigated ruthenium complexes caused arrest in G2 phase, in

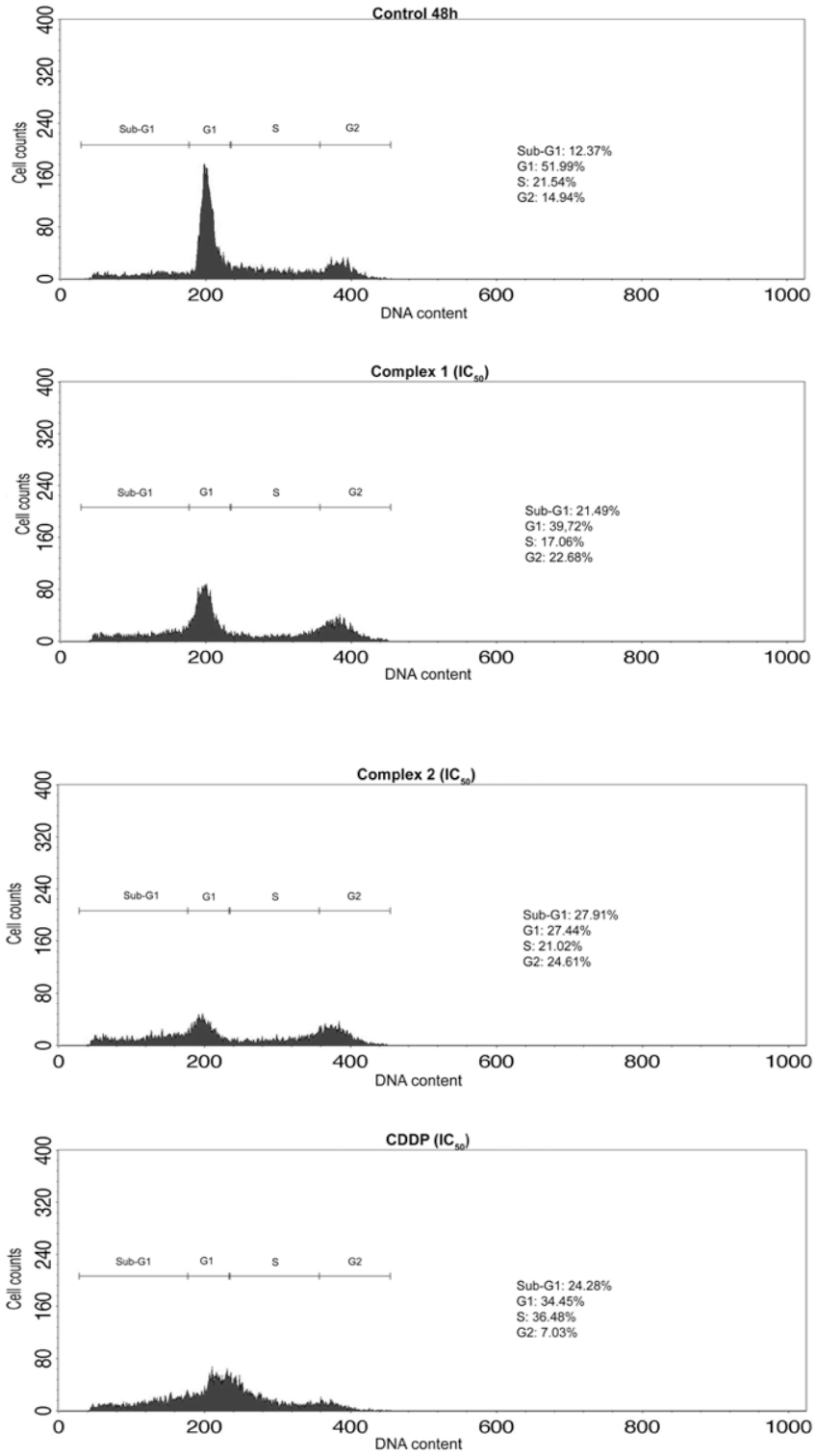

Figure 3. Representative experiment histograms for cell cycle analysis of HeLa cells after 48 h continual incubation with medium (control) and investigated ruthenium complexes and cisplatin ($IC_{50}^{48\text{-h}}$).

contrast to cisplatin treatment which caused arrest in the S phase. The investigated complexes, like cisplatin, induced increase of percent of cells in sub-G1 phase (cells with fragmented DNA) which may be the evidence of apoptotic potential of the investigated complexes.

The mechanism of action of some drugs used for cancer chemotherapy is based on inducing

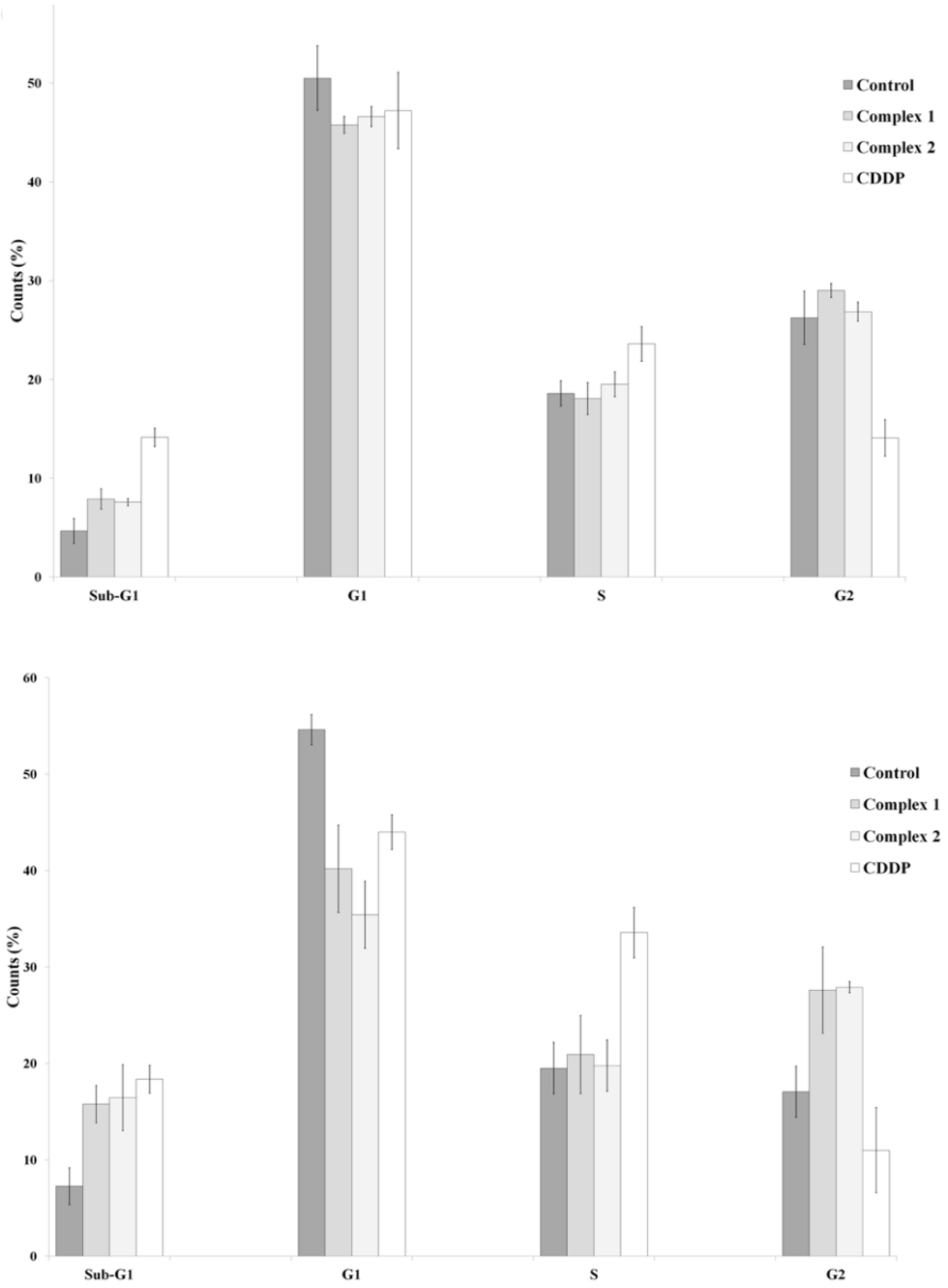

Figure 4. Diagrams representing cell cycle phase distribution of treated HeLa cells, obtained by flow-cytometric analysis of the DNA content in fixed cells, after staining with PI. HeLa cells were collected following 24-h (upper image) and 48-h (lower image) treatment with the tested complexes or cisplatin at a concentration corresponding to IC_{50}^{48h}. Bar graphs represent mean ± SD in three independent experiments.

DNA damage in cells, which result in apoptotic death [20]. Detecting the proapoptotic potential of novel, developing drugs has become an essential step in basic investigations. The apoptotic potential of the investigated ruthenium complexes *1* and *2* was determined on HeLa cells after 24 hrs treatment with IC_{50}^{48h} concentration and Annexin V-FITC/PI flow cytometric analysis and further confirmed with AO/EB staining and fluorescent microscopy. Annexin V-FITC/PI flow cytometric analysis results revealed increased percentage of early and late apoptotic populations of HeLa cells after 24 hrs incubation period with complex *1* and *2*, compared to the control population (Figure 5A). This clearly indicates that the investigated Ru(II)-DMSO-chalcone complexes induced apoptosis in cancer cells, which is important prerequisite for cytotoxic agents. Moreover, apoptosis-in-

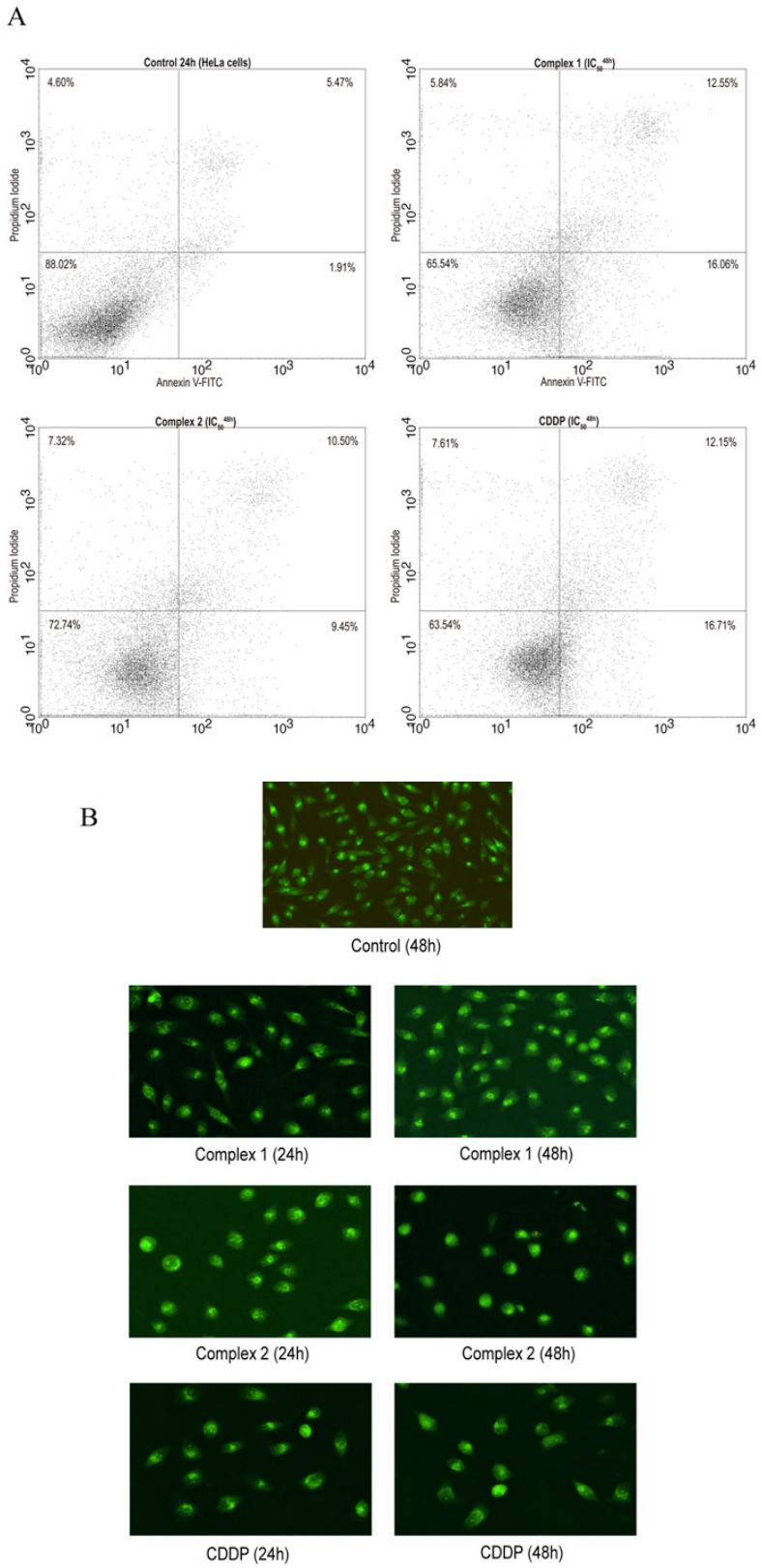

Figure 5. (**A**) The potential of Ru complexes to induce apoptotic cell death, comparison with cisplatin (24 h). FITC(-)/PI(-) (lower-left quadrant) are intact cells, FITC(+)/PI(-) (lower-right quadrant) are early apoptotic cells, FITC(+)/PI(+) (upper-right quadrant) are late apoptotic or necrotic cells and FITC(-)/PI(+) (upper-left quadrant) are necrotic cells. (**B**) Double stained HeLa cells with AO and EB: untreated (control) and treated with cisplatin and the investigated ruthenium complexes ($1 \times IC_{50}$ concentration).

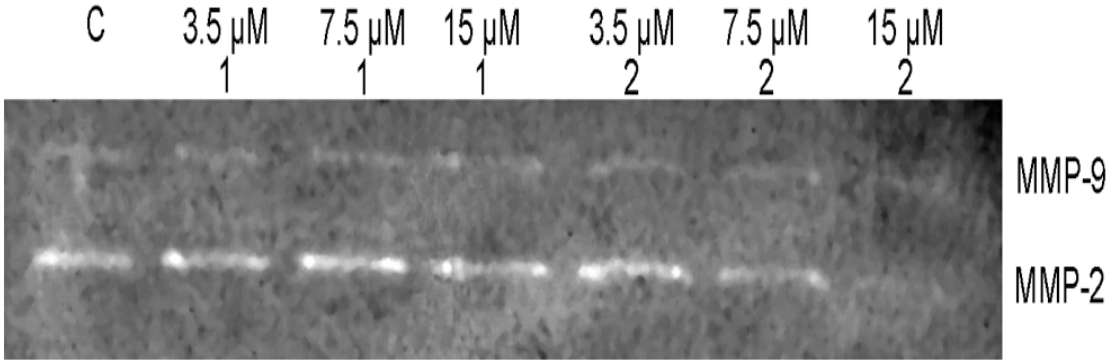

Figure 6. Effect of the investigated complexes on the activity of MMP-9 and MMP-2 (gelatinases) determined by gelatine zymography using supernatants of treated HeLa cells for 24 hrs with sub-cytotoxic concentrations of the investigated complexes.

ducing potential was further confirmed using AO/EB staining and fluorescent microscopy. After 24 and 48 hrs of incubation with both investigated complexes HeLa cells changed their morphology from spindle-shaped to round, nuclear material became more condensated, and cell density decreased, which was especially prominent in cells treated with complex *2* (Figure 5B).

Tumor metastasis is a complex multistep process that involves invasion, adhesion, direct migration, and proteolytic activity to degrade extracellular matrix (ECM) barriers [21,22]. Matrix metalloproteinases (MMPs) are a large family of proteolytic enzymes involved in tissue remodelling [22]. As proteolysis is one of three essential steps of tumor invasion, MMPs (especially MMP-9 and MMP-2) are thought to be particularly important in the invasion of cancer cells [21]. Thus, they become important target for potential antitumor drugs [23]. For ruthenium-based complexes it is known that they can exhibit unique antimetastatic properties regardless of their cytotoxic effect [24,25]. In the present study complex *2* inhibited MMP-2 activity of HeLa cells at its highest concentration applied.

The results of this research indicate that two structurally similar Ru(II)-DMSO-chalcone complexes (*1* and *2*) had important cytotoxic effect on all investigated tumor cell lines. The presence of methyl group in the 3- position on the thiophene part of chalcone ligand in complex *2* resulted in enhanced cytotoxicity. Both complexes exerted their cytotoxic effect through G2 phase cell cycle arrest. Complex *2*, in addition to greater cytotoxicity, had greater potential of apoptosis induction (more apparent after fluorescent microscopy analysis).

In conclusion, the results provided in this study suggest that the investigated Ru(II)-DMSO-chalcone complex *2* holds antitumor potential and represents a leading compound for further chemical modifications and anticancer research.

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