Cellular Sensitivity to β-Diketonato Complexes of Ruthenium(III), Chromium(III) and Rhodium(III)

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Abstract: The aim of this study was to investigate cellular response to several ruthenium(III), chromium(III) and rhodium(III) compounds carrying bidentate β-diketonato ligands: [(acac)-acetylacetonate ligand, (tfac)-trifluoroacetylacetonate ligand]. Cell sensitivity studies were performed on several cell lines (A2780, cisplatin-sensitive and –resistant U2-OS and U2-OS/Pt, HeLa, B16) using growth-inhibition assay**.** Effect of intracellular GSH depletion on cell sensitivity to the agents was analyzed in A2780 cells. Flow cytometry was used to assess apoptosis by Annexin-V-FITC/PI staining, and to analyze induction of caspase-3 activity. Possible DNA binding/damaging affinity was investigated, by inductively coupled mass spectrometry, and by ^{14}C-thymidine / ^{3}H-uridine incorporation assay. Cell sensitivity studies showed that the pattern of sensitivity to $Ru(tfac)_3$ complex of the two cisplatin-sensitive/-resistant osteosarcoma cell lines, U2-OS and U2-OS/Pt, was similar to that of A2780 cells (72 h exposure), with the IC_{50} being around 40 μM. The growth-inhibitory effect of $Ru(acac)_3$ ranged over 100 μM, while Cr(III) and Rh(III) complexes were completely devoid of antitumor action *in vitro*. $Ru(tfac)_3$ exhibited strong potential for apoptosis induction on A2780 cells (up to 40%) and caused cell cycle arrest in the S phase as well as decrease of the percent of G1 and G2 cells. $Ru(acac)_3$-induced apoptosis was slightly higher than 10%, whereas activation of caspase-3 in HeLa cells was moderate. DNA binding study revealed that only $Cr(acac)_3$ was capable of binding DNA, while Cr(III) and Ru(III) compounds possess potential to inhibit DNA/RNA synthesis. In conclusion, only Ru(III) complexes showed potential for antitumor action.

Key Words: Ruthenium(III), chromium(III) , β-diketonato, apoptosis, caspase-3.

INTRODUCTION

During the last two decades, the design of new antitumor drugs widely relied on the development of complexes of transition elements and in particular of platinum. Cisplatin (CDDP) and carboplatin, the first antitumor drugs of this type, have shown success in clinical use. The antitumor effect of platinum drugs is mainly due to reactions with nucleophilic sites on the DNA, which lead to interference with DNA synthesis and replication [1, 2]. As a result of a non tumor specific action of platinum drugs, toxicity to health tissue has emerged [3]. A further limitation to the use of platinum drugs has been the lack of activity against a spectrum of human malignancies, and /or the development of resistance to treatment [4].

In this context, the idea of new metal-based drugs that could act with more selectivity and less toxicity has emerged. Many complexes of metals such as ruthenium, chromium and rhodium have been synthesized and examined as potential antitumor agents [5-8]. Ruthenium drugs appear as promising compounds due to their low toxicity and the ability to accumulate into tumor cells almost specifically using nontoxic iron-transport system [9, 10]. Many Ru(II), Ru(III) and Ru(IV) complexes with ammine (11), dimethylsulfoxide [12**,** 13], and N-heterocyclic ligands [14-16] have been found to interact with DNA by forming DNA cross-links [17] or DNA-strand-breaks [18]. Ru(III) complexes are relatively stable so that ligands are retained in the complex for a long time, but under the hypoxic conditions found in tumor tissue, it is likely that Ru(III) becomes reduced to Ru(II) [19], which is far more reactive toward DNA [20, 21]. However, DNA-damage does not appear to be the basis of anticancer action of Ru(III) complexes [22]. The new generation of Ru(III)-based drugs such as Na[*trans*-$RuCl_4Me_2$ SO(imidazole)] (i.e.NAMI) and imidazolium *trans*-imidazoledimethylsulfoxide tetrachlororuthenate (ImH[*trans*-RuCl (4)(DMSO)Im]) (NAMI-A) can bind DNA, but in contrast to platinum-based drugs, are completely devoid of direct cytotoxicity against tumor cells *in vitro* [23**,** 24]. The antitumor effect of Ru(III) complexes is thought to occur through *in vivo*-selective antimetastatic action as well as through apoptosis induction [25]. It has been postulated that Ru(III) complexes can alter protein expression in cancer cells, either by binding to proteins or to RNA, causing thickening of the protein layer surrounding tumors and metastases, thereby inhibiting tumor cell dissemination [26].

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Among other transition elements, Cr(III) and Rh(III) have been regarded as a basis for development of novel complexes [27]. In particular, Cr(III) is normally present in the cells, and is non-toxic for the cell as it is implicated in insulin mediated glucose intracellular uptake [28]. At high concentrations, Cr(III) binds DNA in a dose-dependent manner, thereby forming DNA-DNA cross-links which prevent DNA replication and alter the kinetics and fidelity of DNA replication [29, 30]. Rhodium(III) complexes can also interact with DNA [31] exhibiting mutagenic characteristics and inducing SOS repair system in the microbial cells [32].

Based on the potential interest of novel metal complexes, the aim of this study was to investigate the cellular effects of complexes with different transition elements possibly characterized by new modes of binding to intracellular targets and capable of overcoming CDDP resistance. For this purpose, we have synthesized complexes of Ru(III), Cr(III) and Rh(III) carrying bidentate β-diketonato ligands [33, 34]. Diketonato ligands are arranged in octahedral, six-coordinated geometry, as opposed to the square planar structure of the platinum-based complexes, providing two additional coordination sites for interaction with the central metal ion. In particular, cellular response to novel β - diketonato complexes of Ru(III), Cr(III) and Rh(III), has been investigated in ovarian carcinoma A2780 cells, mouse melanoma B16, HeLa, and in osteosarcoma CDDP-sensitive (U2-OS) and -resistant (U2-OS/Pt) cells.

MATERIAL AND METHODS

Drugs and Chemicals

Complexes of Ru(III), Cr(III) and Rh(III) containing bidentate β-diketonato ligands were synthesized and kindly supplied by Faculty of Chemistry, Belgrade, Serbia & Montenegro [35, 36]. The chemical structures of compounds are presented in Fig. (**1**). The purity of complexes was analyzed by microanalysis and NMR analysis. Chemicals were purchased from Sigma-Aldrich Chemie Gmbh (Steincheim, Germany) unless otherwise noted. All complexes were dissolved in DMSO and dilutions were made in saline, immediately prior use.

Cell Cultures and Drug Sensitivity Studies

The A2780 human ovarian carcinoma cell line was cultured in RPMI-1640 medium (Invitrogen Italia, San Giuliano Milanese, Italy), supplemented with 10% of fetal bovine serum. The U2-OS osteosarcoma cell line and the U2-OS/Pt CDDP-resistant variant [37] were grown as monolayer in McCoy's 5A medium supplemented with 10% fetal bovine serum. HeLa cells, B16, were cultured as monolayer in RPMI 1640 medium pH 7.2, supplemented with 10% of heat inactivated fetal bovine serum.

The HeLa human cervix carcinoma and B16 murine melanoma cell lines were cultured as monolayer in RPMI 1640 medium, supplemented with 10% of fetal bovine serum.

Cell sensitivity to Ru(III), Cr(III) and Rh(II) complexes was examined on a panel of 3 cell lines, including A2780, U2-OS, and U2-OS/Pt, using growth inhibition assay [38]. Cells were seeded in duplicates into six-well plates ($4x10^4$ cells/ml for U2-OS and U2-OS/Pt; $2x10^4$ cells/ml for A2780 cell line). Twenty-four h later, cells were exposed to different drug concentrations (range 3-100 μM). After 24 h of exposure, medium was replaced with drug-free medium, and cells were incubated for 72 h. Cells were counted using a cell counter (ZBI, Coulter Electronics, Luton, UK). IC_{50} is defined as the drug concentration inhibiting cell growth by 50%.

MTT Assay

Cell sensitivity to $Ru(acac)_3$ and CDDP was analyzed using 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) assay [39, 40], in HeLa, B16, U2-OS, and U2-OS/Pt cells. Three hundred cells/well were seeded into 96-wells microtiter flat bottom plates. $Ru(acac)_3$ was added at various concentrations, with each concentration of compound tested in triplicate. After 48 or 72 h of drug incubation, 0.02 ml of MTT solution was added to each well for 4 h and formazan crystals were then solubilized by adding 0.1 ml of SDS-HCl solution /well (10% SDS in 0,01M HCl) . Absorbance was measured at 570 nm, using an ELISA- microplate reader .

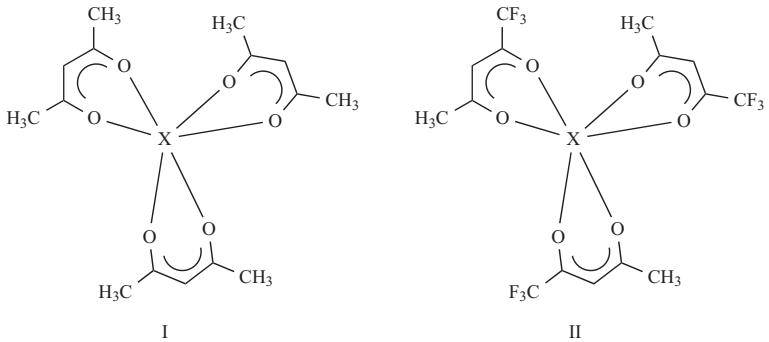

Fig. (1). Structural formulas of investigated complexes containing bidentate β–diketonate ligands.
I) Complex containing bis-acetylacetonate ligands (acac), coordinated to central metal ion X=Ru(III), Cr(III), Rh(III);
II) Complex containing bis-trifluoroacetylacetonate ligands (tfac), coordinated to central metal ion X=Ru(III).

Effects of GSH Depletion on Cellular Sensitivity to the Complexes

We investigated whether depletion of intracellular GSH *in vitro* may influence cell sensitivity to investigated agents. A2780 cells were incubated with 1 μg/ml buthionine sulfoximine (BSO), a synthetic amino acid that depletes glutathione by irreversibly inhibiting gamma-glutamylcysteine synthetase for 24 h, in co-incubation with investigated agent. After 24 h, the medium was replaced with a drug-free medium, and incubation was carried out for 72 h. Cell sensitivity was determined using the growth-inhibition assay (see above) [41].

Cellular DNA Binding

Three and a half million A2780 cells were seeded in 10 ml RPMI-1640 medium, into 75 cm^2 flasks. When growing to adequate confluence, cells were exposed to the metal complexes, at equimolar concentrations (100 μM), for 2 and 24 h. Both adherent and floating cells were collected and DNA was isolated according to Maniatis [42, 37]. DNA content was determined spectrophotometrically and the quantity of Ru(III), Cr(III) and Rh(III) metals bound to DNA was determined using inductively coupled plasma mass spectrometry.

***In Vitro* DNA Binding**

The affinity of the investigated complexes for the 123 bp DNA ladder [43] was analyzed by incubating 5 μg with two different concentrations of drug (0.3 or 3 μM). Incubation with CDDP was used as positive control. After 20 h of continual incubation at 37°C, fragments were precipitated by CH_3COONa/EtOH buffer pH=5. Plasmid-DNA content was determined spectrophotometrically, and metal binding to DNA was determined using inductively coupled plasma mass spectrometry [44].

Inhibition of DNA and RNA Synthesis

Ability of Ru(III), Cr(III) and Rh(III) complexes to inhibit incorporation of radioactive labeled nucleotides ^{14}C-thymidine / ^{3}H-uridine into DNA / RNA was analyzed on A2780 cell line [45]. For that purpose $7x10^4$ cells/ml were seeded into 96-well plates, in 100 μl of RPMI-1640 medium/ well. After 24 h of growing, the investigated complexes were added at various concentrations (10 μM-100 μM). Immediately after agents addition, 10 μl of ^{14}C-thymidine solution 50 μCi/ml, or 10 μl ^{3}H-uridine solution 1 μCi/ml were added at each well. After incubation at 37°C, for 6 h or 24 h, cells in the individual wells were detached in 200 μl of trypsin. Plates were placed into Harvester 96-Tomptec, and cells are collected from the plates to a paper sheet, in the 5 cycles of washing with deionized water. Paper sheets were incubated at 30°C, for 2 h. After incubation, radioactivity was measured from the paper sheet, using Liquid Scintillation Counter (1205 Betaplate, Perkin-Elmer). Analysis of cell cycle and apoptosis on A2780 cells and HeLa cells.

Analysis of Cell Cycle and Apoptosis in A2780 Cells

Analysis of drug-induced cell cycle perturbations and apoptosis, was performed using flow cytometry. Six-thousand A2780 cells were seeded in 10 ml of RPMI-1640 medium, into 75 cm^2 tissue culture flasks. Twenty-four h later, cells were exposed to equimolar concentrations of the agents (100 μM) for different times (24 and 48 h). Upon drug treatment, both detached and adherent cells were collected, washed in cold PBS by centrifugation (2000 rpm, 10 min) and resuspended in 1 ml PBS. For cell cycle analysis, $1x\ 10^6$ cells were fixed in the 70% ethanol, and then resuspended in PBS buffer containing RNAse (1 mg/ml) and PI (10 μg/ml) [46]. Cell cycle phase distribution was analyzed by using a flow cytometer equipped with an argon laser (Becton Dickinson, Mountain View, CA, USA). For analysis of apoptosis, $7x10^5$ cells were resuspended in 1 ml of buffer containing: RNase (1 mg/ml), propidium iodide (PI) (10 μg/ml), sodium citrate (0.1%), Triton-x-100 (0.1%), and incubated overnight, at 4°C. Apoptotic cells were detected as pre-G1 peaks with subdiploid DNA content by flow cytometric analysis.

Analysis of Cell Cycle in HeLa Cells

HeLa cells (4×10^5) were seeded in 2 ml RPMI-1640 medium into six-well plates. After 2 h of growth, medium was changed with fresh medium containing $Ru(acac)_3$, at concentrations of 1.5 x IC_{50}, and incubation continued for 48 h, and 72 h (IC_{50} were determined from cell growth inhibition diagram of MTT assay on HeLa cells). At the end of treatment, cells were collected by trypsinization, washed twice with ice-cold PBS, and fixed for 30 min in 70% Et-OH. Fixed cells were washed again with PBS, and incubated with RNAse A (1mg/ml) for 30 min, at 37°C. Cells were stained with PI at concentration of 50 μl/ml, just before flow-cytometric analysis. Subdiploid DNA content and cell cycle phases distribution were analyzed using FACS Calibur Becton Dickinson flow cytometer and Cell Quest computer software [47].

Assessment of Apoptosis by Annexin V-FITC and PI Double-Staining Flow Cytometry

HeLa cells ($2x\ 10^5$ /ml) were seeded in 2ml of RPMI-1640 medium, into six-well plates. After 24 h of growth, medium was replaced with fresh medium containing $Ru(acac)_3$ or CDDP, at concentration of 1.5 x IC_{50}. After short term (24h) and long term (48h) exposure, adherent cells were harvested by trypsinization (0.25%) and both floating and adherent cells were collected as a single cell suspension, and pelleted by centrifugation. Phosphatidylserine (PS) exposure on the surface of apoptotic cells was detected by Annexin V- FITC [Fluorescein isothiocyanate (FITC)] and PI double staining kit (BD Pharmingen, San Diego, CA, USA [48, 49]. Data were analyzed by flow cytometry (Becton Dickinson) using Cell Quest software. Early apoptotic cells were localized in the lower right quadrant of a dot-plot graph using AnnexinV-FITC *vs* PI staining.

Detection of Active Caspase-3 Protein

HeLa cells were used for determination of activation of caspase-3 by $Ru(acac)_3$. HeLa cells (2×10^5 /ml) were seeded in 2 ml RPMI 1640 medium in six-well plates. After 24 h of growth, cells were exposed to $Ru(acac)_3$ and CDDP at two

concentrations, corresponding to the calculated 5 x IC_{50} and 10 x IC_{50} for 5 h. At the end of the treatment, adherent cells were harvested by trypsinization (0.25%) and both floating and adherent cells were collected as a single cell suspension, and pelleted by centrifugation. Active caspase-3 protein was detected by flow cytometry (Becton Dickenson), using apoptosis staining kit (BD Pharmingen, San Diego, CA, USA.) that contains phycoerythrin (PE)-conjugated polyclonal antibody against human active caspase-3 [50, 51]. Collected data were analyzed using Cell Quest software (47).

Fluorescence Imaging

HeLa cells were seeded into six-well plates over sterile cover slip, at density of 2 x 10^5cells/ml RPMI medium. In the exponential phase of growing, cells were exposed to $Ru(acac)_3$ and CDDP, at concentration corresponding to their IC_{50} and 1.5 x IC_{50} values. After short term 24 h, and long term 48h and 72h agent action, cover slips were loaded on microscope glass, over the 10 μl of PBS solution containing acridine orange (AO, 0.01mg/ml) and ethidium bromide (EtBr, 0.2 mg/ml) [52]. Floating cells were collected separately and apoptosis was assessed also using AO-EtBr staining protocol. After 10 minutes of dye incubation, cells were examined by fluorescent microscopy using G 274 emitting filter, and photographed using digital imaging (Olimpus, Camedia 4040).

RESULTS

Cellular Sensitivity to Metal Complexes

Cell sensitivity of the ovarian carcinoma A2780 cell line and of CDDP-sensitive and –resistant osteosarcoma U2-OS and U2-OS/Pt cells was analyzed using the growth-inhibition assay. Among the tested compounds only $Ru(tfac)_3$ displayed an antiproliferative effect against A2780 cell line, as observed after short-term (24 h) IC_{50} 40.63 ± 15.48 (μM), as well as long-term (72 h) IC_{50} 25.2 ± 11.5 (μM) drug exposure, Table **1.** The pattern of sensitivity to $Ru(tfac)_3$ complex of the two osteosarcoma cell lines was similar to that of A2780 cells, the IC_{50} being 39.25 ± 10.45 (μM) in U2-OS and 37.75 ± 19.04 (μM) in U2-OS/Pt cells. $Ru(acac)_3$ was inactive up to a concentration of 100 μM. Cr(III) and Rh(III) did not possess growth inhibitory effect against A2780 cells. Cytotoxic action of $Ru(acac)_3$ up to a concentration of 300 μM was studied by MTT assay, on four different cell lines (U2-OS and U2-OS/Pt, B16 and HeLa). Cell growth inhibition diagrams are presented in Fig. (**2**), IC_{50} values determined for 48 h (U2-OS and U2-OS/Pt) and 72 h exposures (B16 and HeLa) ranged over 100 μM, Table **2**. U2-OS showed slightly higher sensitivity to $Ru(acac)_3$ in comparison to U2-OS/Pt cells.

In an attempt to define the determinants of cellular sensitivity to the Ru(III), Cr(III), Rh(III) complexes, we examined whether depletion of intracellular GSH level by buthionine sulfoximine (BSO) influenced cell sensitivity to tested compounds. $Ru(tfac)_3$ exhibited slightly higher but not significant cytotoxicity in A2780 cells [IC_{50} 38.73 ± 0.42 (μM)], in co-incubation with BSO (1 μg/ml, 24 h) when compared to activity without BSO [IC_{50} 40.63 ± 15.48 (μM)], Table **3**. Intracellular GSH depletion had no effect on cell sensitivity to Cr(III) and Rh(III) complexes, up to complex concentration of 200 μM.

Analysis of Drug-Induced Apoptosis and Cell Cycle Perturbations

Preliminary analyses of apoptosis and cell cycle perturbations induced by the investigated complexes were performed on A2780 cells, using flow cytometry. $Ru(tfac)_3$ exhibited strong potential for apoptosis induction (up to 40%) for 48 h of exposure, Table **4**, whereas among the other compounds, only $Ru(acac)_3$-induced apoptosis was slightly higher than 10% (48 h exposure). $Ru(tfac)_3$ caused a unique alteration of the profile of cell cycle progression as compared with the other tested compounds, Fig. (**3**), because it caused a time-dependent decrease of the percent of G1 and G2 cells, and delay in the S phase of cell cycle. Both Cr(III) and Rh(III) compounds did not induce cell cycle perturbations, whereas the perturbations generated by $Ru(acac)_3$ (slight arrest of cell cycle in the S phase, and decrease of the percent of the G1 and G2 cells,) were reversible,

Table 1. Cellular Sensitivity of Ovarian Carcinoma (A2780) and Osteosarcoma CDDP-Sensitive (U2-OS) and –Resistant (U2-OS/Pt) Cells to the Studied Metal Complexes [a]

Complex	Exposure time (h)	IC_{50} (μM)		
		A2780	U20S	U2OS/Pt
$Ru(tfac)_3$	24 72	40.63 ± 15.48 25.2 ± 11.5	39.25 ± 10.45	37.75 ± 19.04
$Ru(acac)_3$	24	> 100	> 100	> 100
$Cr(acac)_3$	24	> 200		
$Rh(acac)_3$	24	> 200		

[a] Cellular sensitivity was measured by growth-inhibition assay after 24 h or 72 h exposure. Cells were treated 24 h after seeding and were counted 72 h after the end of the exposure (for the 24 h exposure) or at the end of the exposure. The reported values are the mean of 2 to 3 independent experiments.. IC_{50} represents the concentration causing a 50% decrease of cell growth as compared to control cells.

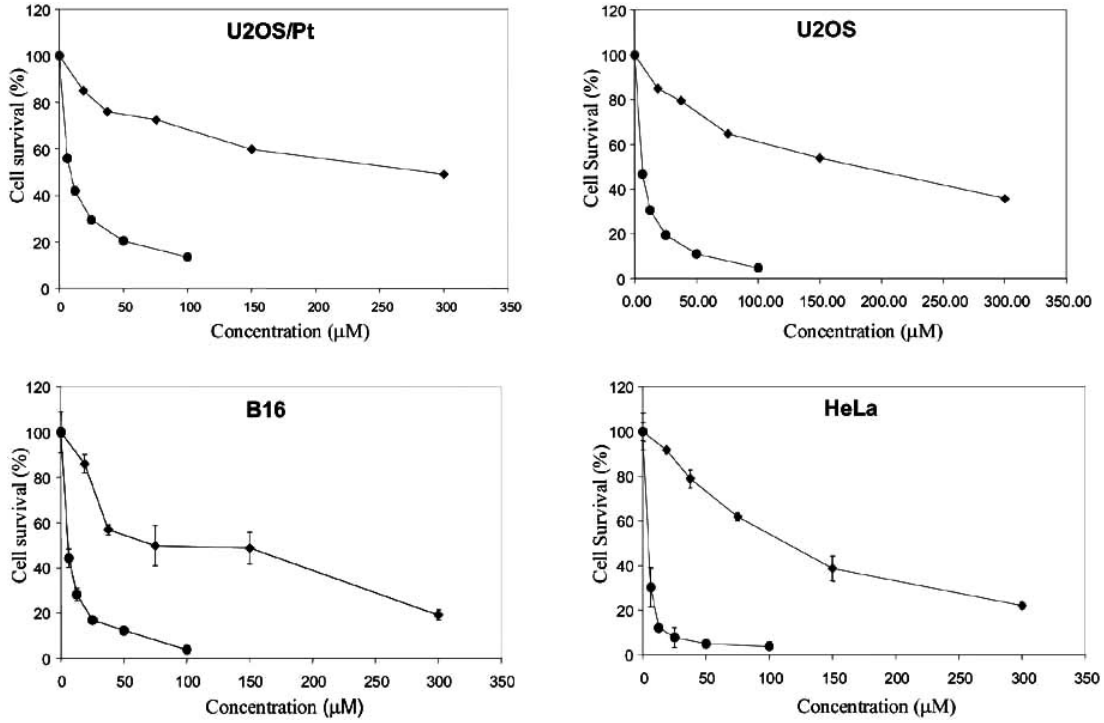

Fig. (2). Cell growth-inhibition diagrams for $Ru(acac)_3$ (◆) in comparison to CDDP (●), on panel of four cell lines: osteosarcoma CDDP-sensitive (U2-OS) and-resistant (U2-OS/Pt) cells, B16 and HeLa; measured by MTT assay for 48 h or 72 h of continual agent action. IC_{50} values determined from cell growth-inhibition diagrams represent the mean of three to four independent experiments.

Table 2. Results of MTT Assay After 72 h or 48 h of Continual Agent Action, Presented in the Terms of IC_{50} Values (Concentration of Drug Required to Cause 50% Inhibition of Cell Growth). Values of IC_{50} were Determined from the Cell Growth Diagrams and Represent the Average of the Three to Four Experiments

Complex	IC_{50} (μM)			
	[a] *HeLa*	[a] *B16*	[b] *U2OS*	[b] *U2OS/Pt*
$Ru(acac)_3$	114.25 ± 1	148.13 ± 1	173.16 ± 5.50	>300
CDDP	4.73 ± 0.7	5.68 ± 0.33	5.22 ± 0.59	12.25 ± 2.48

[a] Values of IC_{50} were determined after 48 h of agent action.

[b] Values of IC_{50} were determined after 72 h of agent action.

Table 3. Effect of Intracellular GSH Depletion on the Action of Investigated Complexes in A2780 Cells

Complex	IC_{50} (μM)	
	A2780	A2780 (BSO)
$Ru(tfac)_3$	40.63 ± 15.48	38.73 ± 0.42
$Ru(acac)_3$	> 100	>100
$Cr(acac)_3$	> 200	> 200
$Rh(acac)_3$	> 200	> 200

[a] Cellular sensitivity was measured by growth-inhibition assay after 24 h exposure. Cells were co-incubated with buthionine sulfoximine (BSO) 1 μg/ml and investigated agents for 24 h, and were counted 72 h after the end of the exposure. BSO did not affected cell viability at concentrations used in this study. IC_{50} represents the concentration causing a 50% decrease of cell growth as compared to control cells.

Table 4. Apoptosis Induction by Different Metal Complexes in A2780 Cells[a]

Complex	[a] Sub-G1 DNA content (%)	
	24 h	48 h
$Ru(tfac)_3$	3.36	46.71
$Ru(acac)_3$	9.93	14.70
$Cr(acac)_3$	8.88	4.75
$Rh(acac)_3$	9.47	1.61
Control	1.26	1.14

[a] Percent of apoptosis induced after 24 h or 48 h of continuous exposure (100 μM), measured as sub-G1 DNA content, using flow cytometry.

Fig. (**3**). Analysis of cell cycle on HeLa cells confirmed slight decrease in G1 and G2 cell cycle phases, induced by $Ru(acac)_3$, that was time dependent (48 h and 72 h incubation) and concentration dependent (IC_{50} and 1.5 x IC_{50}), Fig. (**4**). Accordingly, percent of apoptotic cell population (sub-G1 content) increased, and reached values around 10% (determined for 72 h incubation, 1.5 x IC_{50}), that was in correlation with the results obtained on A2780 cells.

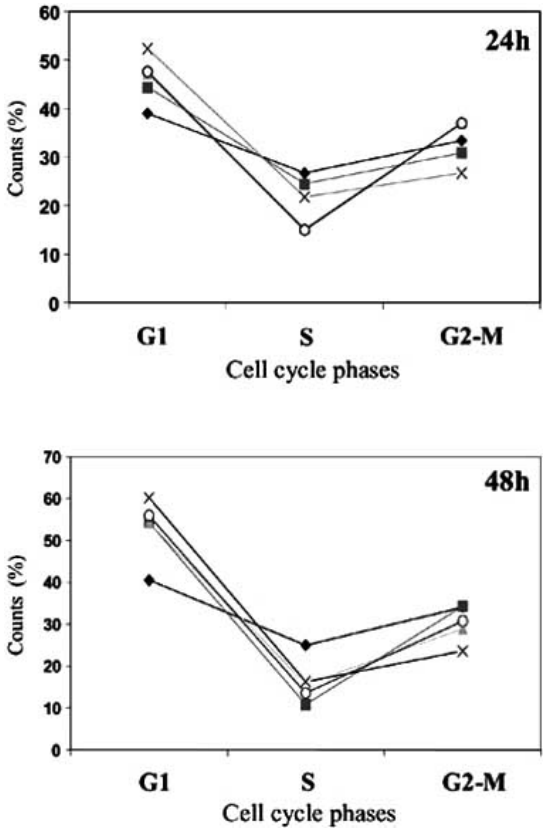

Fig.(3). Diagrams present the data of flow cytometry analysis of cell cycle phases perturbations in treated A2780 cells in comparison to control (❍) (untreated A2780 cells), after exposure to investigated complexes [$Ru(tfac)_3$ (◆), $Ru(acac)_3$ (■), $Cr(acac)_3$ (▲), and $Rh(acac)_3$ (✕)], in equimolar concentrations (100 μM), for 24 h and 48 h.

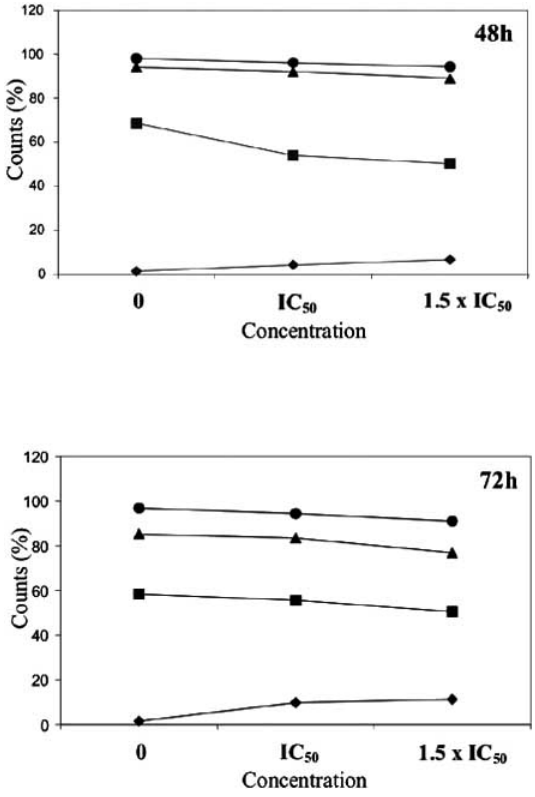

Fig. (4). Diagrams present the data of flow cytometry analysis of cell cycle phases [sub-G1(◆), G1(■), S(▲) and G2(●)] perturbations in HeLa cells, after exposure to $Ru(acac)_3$, in concentrations corresponding to IC_{50} or 1.5 x IC_{50}, for 48 h and 72 h.

DNA Binding

To assess the capability of the compounds to bind DNA, we used ICP-MS for analysis of *in vitro* binding to the 123 bp DNA ladder and of binding to cellular DNA. In the former case, CDDP was used as positive control for DNA binding. However, under our experimental conditions, among the studied novel compounds, only Cr(acac)3 exhibited binding to DNA (Table **5**). Again, the analysis of binding to cellular DNA of the A2780 cell line indicated that only Cr(acac)3 was capable of binding DNA.

Table 5. ***In Vitro*** **and Cellular Binding to DNA of the Studied Metal Complexes**[a]

	In vitro **binding (pg/DNA μg)**		**Cellular DNA binding (pg/DNA μg)**	
	0.3 μM	**3 μM**	**1 h**	**24 h**
$Ru(tfac)_3$	0	0	0	0
$Ru(acac)_3$	0	0	0	0
$Cr(acac)_3$	26.7	57.9	45.13	N.D.
$Rh(acac)_3$	0	0	0	0
CDDP	6613	N.D.	N.D.	N.D.

[a] DNA binding was assessed *in vitro* after exposure of the 123 bp DNA ladder to 0.3 and 3 μM of the studied compounds or after exposure of the A2780 cells to the compounds (100 μM) for 1 h or 24 h. Metal binding was measured by inductively coupled plasma mass spectrometry.

N.D. = not determined.

Inhibition of DNA and RNA Synthesis

The ability of the investigated complexes to interfere with DNA and RNA synthesis were studied by measuring inhibition of incorporation of ^{14}C-thimidine and ^{3}H-uridine in A2780 cells. All tested complexes showed ability to inhibit ^{14}C-thimidine-DNA incorporation in time and concentration dependent manner. $Ru(tfac)_3$ was the most potent compound in inhibiting both RNA and DNA synthesis at 100 μM, Fig. (**5**). The effect on RNA synthesis appeared reversible for 30 μM, as it was present after 6 h but disappeared after 24 h. Also $Ru(acac)_3$ and Cr(III) complexes caused a reversible decrease of RNA synthesis at 30 μM and 100 μM.

Assessment of Apoptosis by Annexin V-FITC and PI Double-Staining Flow Cytometry

Annexin-V and PI double staining procedure for detection of early apoptosis indicated apoptotic changes for both 24 h and 48 h exposure of $Ru(acac)_3$. Dot-plot graphs are presented in, Fig. (**6a**). Apoptosis induction was concentration and time-dependent, at the level similar to that obtained by CDDP, Fig. (**6b**). Apoptotic cell population induced by $Ru(acac)_3$ was 7.48% for 24 h action and 29.09% for 48h action, although percent of PI stained cells at 48 h was 31.78% indicating involvement of necrotic processes in cell death.

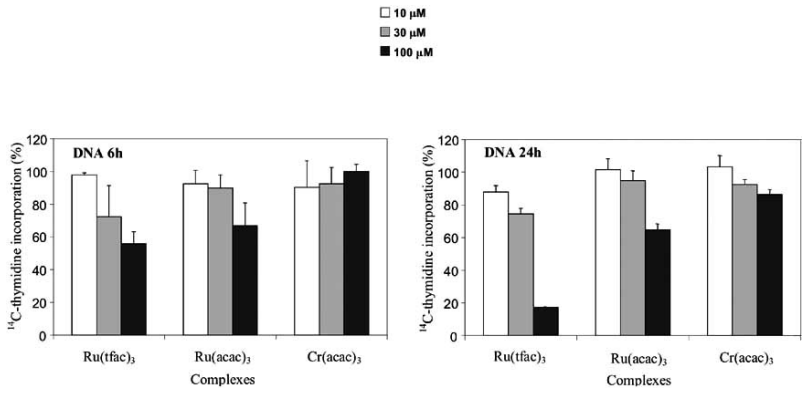

Fig. (5a). DNA synthesis inhibition caused by complexes of Ru(III) or Cr(III) for 6 h or 24 h of action on A2780 cells.

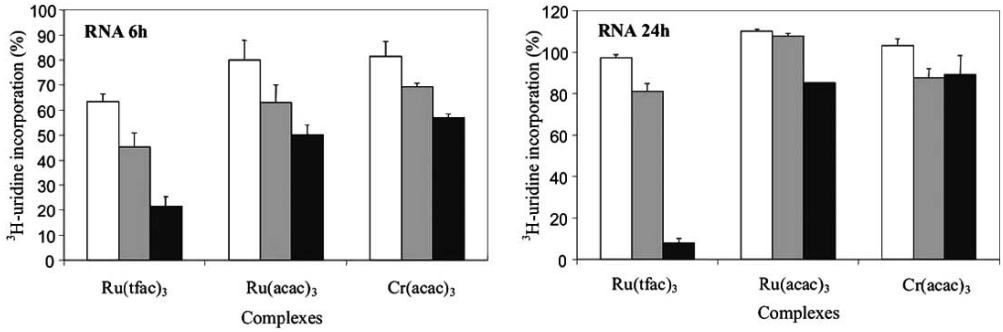

Fig. (5b). RNA synthesis inhibition caused by complexes of Ru(III) or Cr(III) for 6h or 24 h of action on A2780 cells.

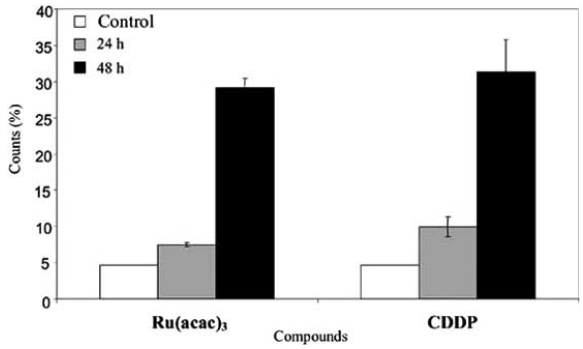

Fig. (6a). Percentage of apoptosis of HeLa cells induced by $Ru(acac)_3$ and CDDP, after 24 h (■) and 48 h (■) action in concentration of 1.5 x IC_{50}. Apoptosis was analyzed by flow cytometry using Annexin V-FITC/PI staining.

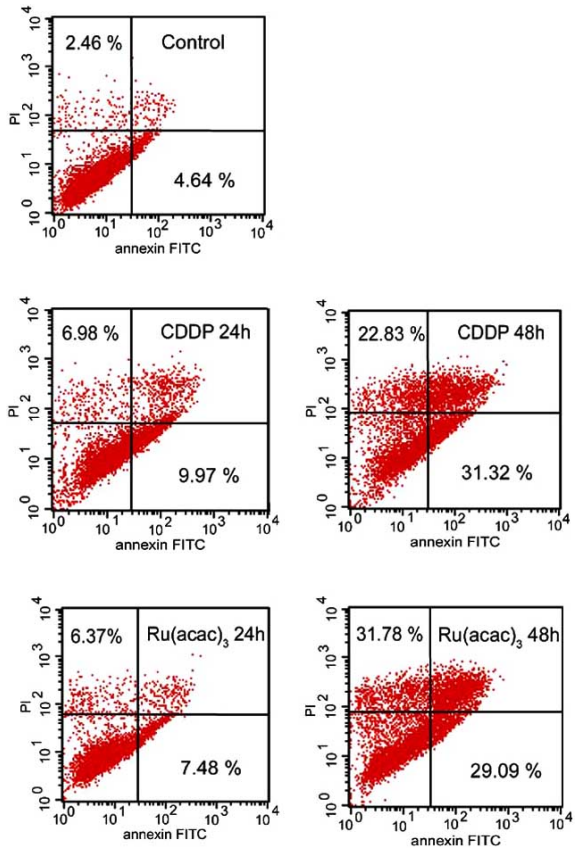

Fig. (6b). Dot plots graphs represent percentage of apoptotic HeLa cells, after exposure to $Ru(acac)_3$ in concentration corresponding to 1.5 x IC_{50}, for 24h and 48h; detected by flow cytometry using Annexin V-FITC/PI staining.

Detection of Active Caspase-3 Protein

Potential of $Ru(acac)_3$ to activate caspase-3 as marker of early apoptotic changes has been analyzed on HeLa cells upon 5 h treatment with two different concentrations: 5 x IC_{50} and 10 x IC_{50}, and was compared with CDDP, Fig. (**7**). CDDP has shown dose-dependent induction of caspase-3 activity being 5% for 5 x IC_{50} and 16.24% for 10 x IC_{50}. $Ru(acac)_3$ showed lower potential for caspase-3 induction in comparison to CDDP. There was low content of active caspase-3 at 5 IC_{50}, 0.9% and greater at 10 IC_{50}, 4.16%.

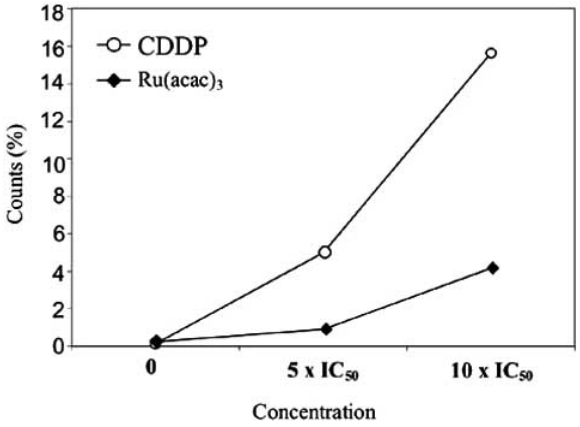

Fig. (7). Diagram representing percent of active-caspase-3 in HeLa cells after exposure to of $Ru(acac)_3$ (◆) or CDDP (○) for 5 h, in concentration corresponding to 5 x IC_{50} or 10 x IC_{50}; detected by Flow cytometry after staining by anti-active caspase-3 PE-conjugated monoclonal antibodies.

Morphological Study

Morphological study of cell death, using AO and EtBr staining revealed $Ru(acac)_3$-induced apoptotic morphological changes on HeLa cells, Fig. (**8**). After 48 h drug treatment at concentration of 1.5 x IC_{50}, cells were shrunk, less confluent in comparison to the control, and cell nuclei were condensed. After 72 h, drug action cells underwent terminal stage of apoptosis characterized by loss of cell membrane integrity, thus far incorporating AO.

DISCUSSION

In this study we evaluated and compared the action of several unconventional ruthenium(III), chromium(III) and rhodium(III) compounds carrying bidentate β-diketonato ligands. Synthesis and chemical structure of these compounds had been already determined [34-36]. Since it was shown that some bis-chelate Ru(II)/Ru(III) complexes [53] may be endowed with greater antitumor properties than the monochelates, we examined whether the chemical nature of the bidentate ligand or nature of central metal ion could determine the mode of the antitumor effects.

According to cell sensitivity studies performed on panel of cell lines (the ovarian carcinoma A2780 , the CDDP-sensitive and –resistant osteosarcoma U2-OS and U2-OS/Pt cells), only $Ru(tfac)_3$ displayed reasonable antiproliferative effect as tested on A2780 cell line, after short term (24 h, IC_{50} = 40.63 ± 15.48 μM) as well as long term (72 h, IC_{50} = 25.2 ± 11.5 μM) drug exposure. $Ru(tfac)_3$ exhibited similar activity on CDDP-sensitive and –resistant osteosarcoma variants the IC_{50} being 39.25 ± 10.45 μM in U2-OS and 37.75 ± 19.04 μM in U2-OS/Pt cells, that may indicate

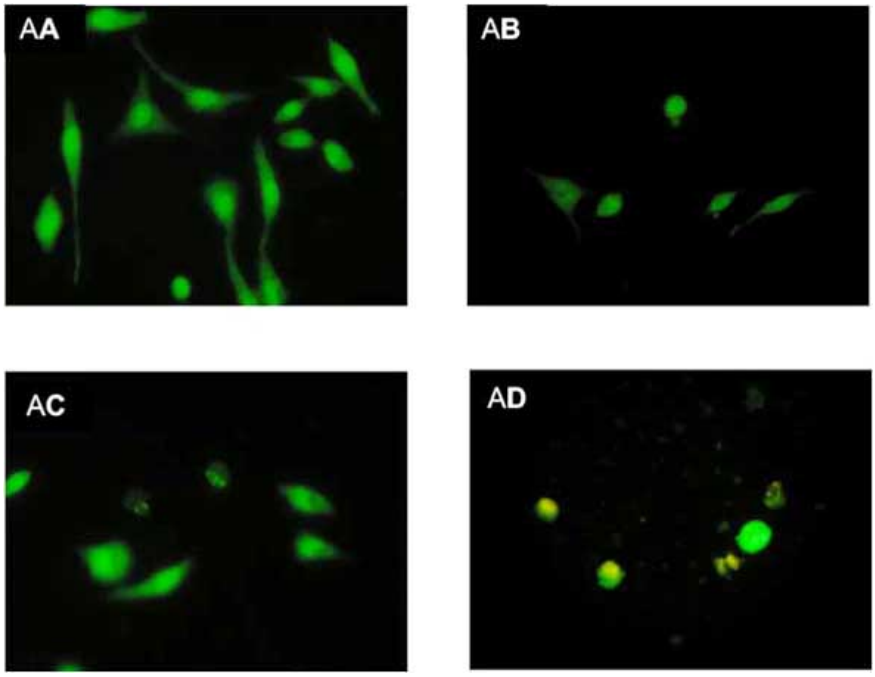

Fig. (8). Fluorescence photomicrographs of HeLa cells treated with $Ru(acac)_3$ at concentration 1.5 x IC_{50} for 24 h, 48 h or 72 h. A) Control; B) For 24 h drug exposure, there were less cells in comparison to control; C) for 48 h drug exposure, apoptotic morphological changes appeared: cells were rounded and cell nuclei were condensed; D) after 72 h drug exposure, there was a significant number of detached cells that incorporated EtBr, (thereafter nucleus fluoresces orange), indicating that cell membrane was disturbed at the late stage of cell death.

different patterns of cytotoxic action in comparison to CDDP.

Depletion of intracellular GSH level in A2780 cells affected cytotoxicity of $Ru(tfac)_3$ only marginally, showing that GSH may have a mild role, if any, in binding of $(tfac)_3$ containing Ru(III) complex, due to its greater intracellular reactivity in comparison to the other investigated complexes.

The growth inhibitory action of $Ru(acac)_3$, investigated up to 300 μM on HeLa and B16 cells, ranged over 100 μM [HeLa, IC_{50} = 114.25 ± 1 (μM); B16, IC_{50} = 148.13 ± 1 (μM)]. Lack of obvious growth inhibitory effect *in vitro* was expected for the Ru(III) compounds according to the data of the previous investigations [54].

While B16 and HeLa cell lines responded only after 72 h agent exposure, an effect in U2-OS and U2-OS/Pt cells was detected after 48 h exposure. U2-OS cells showed a slightly better sensitivity profile to $Ru(acac)_3$ in comparison to the U2-OS/Pt subline.

Cr(III) and Rh(III) complexes carrying bidentate β-diketonato ligands were completely devoid of any antiproliferative effect. Greater activity of Ru(III) compounds possessing (tfac) ligand in comparison to $Ru(acac)_3$ may be explained by possible intracellular formation of reactive Ru(II) species [19] that usually exhibit a cytotoxic effect on tumor cell lines [55-59].

To evaluate if possible DNA binding mode may be responsible for the presence/absence of cytotoxic activity of this different class of metal compounds, we have investigated binding of Ru(III), Cr(III) and Rh(III) β-diketonato complexes to cellular DNA on the A2780 cell line as well as *in vitro* DNA- binding after incubation of the 123 bp DNA ladder to 0.3 and 3 μM of complexes. Both studies showed that only $Cr(acac)_3$ was capable of binding DNA, indicating that there was no correlation between DNA binding and antiproliferative/cytotoxic effect, when Cr(III) and Ru(III) β-diketonate were considered.

Study of ability of investigated complexes to interfere with DNA- and RNA-synthesis by measuring inhibition of incorporation of ^{14}C-thymidine and ^{3}H-uridine in A2780 cells, revealed that Cr(III) and as well Ru(III) compounds, especially $Ru(tfac)_3$ possess potential to inhibit RNA and DNA synthesis at 100 μM. Inhibitory effect of Cr(III) complex may be explained as the result of direct DNA-interaction, according to our DNA- binding study.

Analyses of apoptosis and cell cycle perturbations performed on A2780 cells, using flow cytometry, showed that $Ru(tfac)_3$ exhibited strong potential for apoptosis induction (up to 40%) for 48 h of exposure. It also caused time dependent decrease of the percent of G1 and G2 cells, and cell cycle arrest in the S phase. This data together with the result of the DNA/RNA synthesis inhibition study indicated that $Ru(tfac)_3$ obviously interfered with process of DNA synthesis, although we did not confirmed direct Ru(III)-DNA binding.

Among the other tested compounds, $Ru(acac)_3$ induced apoptosis higher than 10% on two cell lines A2780 (48 h action) and HeLa (72 h action). $Ru(acac)_3$ induced cell

accumulation in the S phase (A2780 cells) was reversible. Except than for just slight decrease of the cell percent in G1 and G2 phases in HeLa cells, there were no great cell cycle alterations. This data indicated that both β-diketonato containing Ru(III) complexes may not belong to the same group of compounds as NAMI-A type complexes, characterized by G2-M cell cycle arrest [60, 61].

Cr(III) and Rh(III) compounds neither had any effect on cell cycle progression nor did they induce apoptosis.

Since there are lot of evidences underlying irrelevance of the direct cytotoxicity of Ru(III) complexes to its antitumor activity [24, 62] with NAMI-A, imidazolium *trans*-imidazoledimethylsulfoxide tetrachlororuthenate (ImH[*trans*-RuCl(4)(DMSO)Im]) as a representative of Ru(III) antimetastatic complexes that are devoid of direct cytotoxic activity *in vitro*, we have found $Ru(acac)_3$ β-diketonato complexes challenging for further analysis of antitumor action.

Apoptosis study on HeLa cells using Annexin V-FITC and PI double staining revealed that $Ru(acac)_3$ induced apoptosis in concentration and time-dependent manner, at the level similar to that obtained by CDDP. However, great PI staining at 48 h (31.78%) indicated involvement of necrotic processes during cell death.

Morphological analysis of cell death using AO/EtBr staining confirmed apoptotic morphological changes, during 48 h drug action at 1.5 x IC_{50}. At the late stage of cell death (72 h action), some cells incorporated EtBr that indicated secondary necrotic changes.

Potential involvement of caspase-3 in apoptotic changes induced by $Ru(acac)_3$ has been analyzed on HeLa cells upon 5 h treatment with two different concentrations: 5 x IC_{50} and 10 x IC_{50}. Data obtained showed that $Ru(acac)_3$ possesses low potential to induce caspase-3 activity in comparison to CDDP, being 0.9% at 5 x IC_{50} and progressing to 4.16% at 10 x IC_{50}. According to our results of Annexin V-FITC / PI double staining, $Ru(acac)_3$ acts as inducer of apoptosis in level similar to CDDP, but it seems that $Ru(acac)_3$-mediated apoptosis does not follow the same pattern.

CONCLUSION

This structural-activity study of several metal complexes differing according to the nature of the metal ion, and the nature of bidentate β-diketonato ligands, reveal that β-achetylacetonato ligands do not contribute to the cytotoxic action of tested complexes, since Cr(III) and Rh(III) complexes are completely devoid of antitumor action *in vitro*, while $Ru(acac)_3$ exhibits cytotoxicity over 100 μM, as investigated on several different cell lines. $Ru(tfac)_3$ is the only complex to show reasonable cytotoxicity and high capability to induce apoptosis and cell cycle alterations, on A2780 cells, possibly due to its greater intracellular reactivity in comparison to the other investigated agents. The pattern of sensitivity to $Ru(tfac)_3$ complex for the two CDDP-sensitive and -resistant osteosarcoma cell lines was similar, indicating that Ru(III) complexes may trigger some different cell downstream effects in comparison to CDDP. Thus, non-platinum metal complexes exhibiting cytotoxicity in tumor cells may have potential to overcome CDDP-resistance as was shown for some unconventional metal complexes based on platinum [63, 64]. The design of novel compounds of this class, especially Ru(II) derivatives [55, 65], is expected to generate agents endowed with a greater cytotoxic potency.

$Ru(acac)_3$ has no obvious cytotoxicity *in vitro,* as evaluated on several cell lines, although it shows potential for apoptosis induction. However, capability of $Ru(acac)_3$ to induce apoptosis, provides rationale to investigate ruthenium based compounds as sensitizer of tumor cells in combination therapy. These findings support the interest of Ru(III) complexes among the novel tested metal-complexes as promising compounds endowed with potential antitumor action.

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REFERENCES

[1] Eastman, A. *Pharmacol. Ther*., **1987**, *34*, 155.
[2] Douple, E.B. *Pharmacol. Ther*., **1984**, *25*, 297.
[3] Kelland, L.R. *Drugs*, **2000**, *59*, 1.
[4] Manic, S.; Gatti, L.; Carenini, N.; Fumagalli, G.; Zunino, F.; Perego, P. *Curr. Canc. Drug Targ.*, **2003**, *3*, 21.
[5] Sava, G.; Bergamo, A. *Anticancer Res.,* **1999**, 19, 1117.
[6] Kopf-Maier, P. *Eur. J. Clin. Pharmacol.*, **1994**, *47*, 1.
[7] Galanski, M.; Arion, V.B.; Jakupec, M.A.; Keppler, B.K. *Curr. Pharm. Des*., **2003**, *9*, 2078.
[8] Fruhauf, S.; Zeller, W.J. *Chemother. Pharmacol*., **1991**, *27*, 301.
[9] Allardyce, C.S.; Dyson, P.J. *Platinum Met. Rev.*, **2001**, *45*, 62.
[10] Kratz, F.; Hartmann, M.; Keppler, B.; Messori, L. *J. Biol. Chem*., **1994**, *269*, 2581.
[11] Rubin, J.R.; Sabat, M.; Sundaralingam, M. *Nucleic Acid Res.*, **1983**, *11*, 6571.
[12] Alessio, E.; Attia, W.; Calligaris, M.; Cauci, S.; Dolzani, L.; Mastroni, G.; Monti-Bragadin, C.; Nardin, G.; Quadrifoglio, F.; Sava, G.; Tamaro, M.; Zorzet, S. In *Proceedings of the Fifth International Symposium on Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*, Nicolini, M. Ed.; Martinus Nijhoff: Boston, **1988**, pp. 617-633.
[13] Dale, L.D.; Tocher, J.H.; Dyson, T.M.; Edwards, D.I.; Tocher, D.A. *Anti-Cancer Drug Design*, **1992**, *7*, 3.
[14] Keppler, B.K.; Rupp, W. *J. Cancer. Res. Clin. Oncol*., **1986**, *111*, 166.
[15] Novakova, O.; Kasparkova, J.; Vrana, O.; Van Vilet, P.M.; Reedijk, J.; Brabec, V. *Biochemistry*, **1995**, *34*, 12369.
[16] Malina, J.; Novakova, O.; Keppler, B.K.; Alessio, E.; Brabec, V. *J. Biol. Inorg. Chem*., **2001**, *6*, 435.
[17] Van Houten, B.; Illenye, S.; Qu, Y.; Farrell, N. *Biochemistry*, **1993**, *32*, 11794.
[18] Chan, P.K.; Skov, K.A.; James, B.R.; Farrell, N. *Chem. Biol. Interact*., **1986**, *59*, 247.
[19] Grguric-Sipka, S.R.; Vilaplana, R.A.; Perez, J.M.; Fuertes, M.A.; Alonso, C.; Alvarez, Y.; Sabo, T.H.; Gonzalez-Vilxhez, F. *J. Inorgan. Biochem*., **2003**, *97*, 215.
[20] Hotze, A.C.; Bacac, M.; Velders, A.H.; Jansen, B.A.; Kooijman, H.; Spek, A.L.; Haasnoot, J.G.; Reedijk, J. *J. Med. Chem.,* **2003**, *46*, 1743.
[21] Chan, H.L.; Liu, H.Q, Tzeng. B,C.; You, Y.S.; Peng, S.M.; Yang, M.; Che, C.M. *Inorg. Chem*., **2002**, *41*, 3161.
[22] Mura, P.; Camalli, M.; Messori, L.; Piccioli, F.; Zanello, P.; Corsini, M. *Inorg. Chem*., **2004**, *43*, 3863.

[23] Sava, G.; Gagliardi, R.; Bergamo. A.; Alessio, E.; Mestroni, G.; *Anticancer Res*., **1999**, *19*, 969.
[24] Sava, G.; Pacor, S.; Mestroni, G.; Alessio, E. *Clin. Exp. Metastasis*., **1992**, *10*, 273-80.
[25] Alessio, E.; Mestroni, G.; Bergamo, A.; Sava, G. *Curr. Top. Med. Chem*., **2004**, *4*, 1525.
[26] Zorzet, S.; Bergamo, A.; Cocchietto, M.; Sorc, A.; Gava, B.; Alessio, E.; Iengo, E.; Sava, G. *J. Pharmacol. Exp. Ther*., **2000**, *295*, 927.
[27] Hirohumi, A. H.; Rohana, A. R.; Naoui, M.; Tajmir-Riahi, H.A. *J. Biol. Chem*., **2000**, *275*, 10150.
[28] Wang H.; Kruszewski A.; Brautigan D. L.; *Biochemistry*, **2005**, *44*, 8167.
[29] Shrivastava, H.Y.; Ravikumar, T.; Shanmugasundaram, N.; Babu, M.; Unni, N.B. *Free Radic. Biol. Med*., **2005**, *38*, 58-69.
[30] Balamurugan. K.; Rajaram, R.; Ramasami, T.; Narayanan, S. *Free Radic. Biol. Med*., **2002**, *33*, 1622.
[31] Katsaros, N.; Anagnostopolou, A. *Crit. Rev. Oncol. Hematol*., **2002**, *42*, 297.
[32] Dale, L.D.; Edwards, D.J. *Lett. App. Microb*., **1989**, *9*, 53.
[33] Keller, H.J.; Keppler, B.K.; Schmähl, D. *Drug. Res*., **1982**, *8*, 806-807.
[34] Bischoff, H.; Berger, M.R.; Keppler, B.K.; Schmahl, D. *J Cancer Res. Clin. Oncol*., **1987**, *113,* 446.
[35] Wolf, L.; Butter, E.; Weinelt, H. *J. Annorg. Allg. Chem*., **1960**, *306*, 87.
[36] Juranic, N. *Inorganica Acta*, **1984**, *87*, 37.
[37] Perego, P.; Caserini, C.; Gatti, L.; Carenini, N.; Romanelli, S.; Supino, R.; Colangelo, D.; Viano, I.; Leone, R.; Spinelli, S.; Pezzoni, G.; Manzotti, C.; Farrell, N.; Zunino, F. *Molecul. Pharmacol*., **1999**, *55*, 528.
[38] Perego, P.; Beretta, G.L.; Gatti, L. In *Handbook of Proteomic Methods*; Conn, P.M. Ed.; Humana Press: New Jersey**, 2003**.
[39] Supino, R. In *Methods in Molecular Biology, In vitro toxicity testing protocols*. O'hare, S.; Atterwill, C.K. Ed.; Humana Press: New Jersey, **1995**, pp. 137-149.
[40] Arandjelovic, S.; Tesic, Z.; Juranic, Z.; Radulovic, S.; Vrvic, M.; Potkonjak, B.; Ilic, Z. *J. Exp. Clin. Canc. Res.*, **2002**, *4*, 519.
[41] Perego, P.; Paolicchi, A.; Tongiani, R.; Pompella, A.; Tonarelli, P.; Carenini, N.; Romanelli, S.; Zunino, F. *Int. J. Cancer,* **1997**, *71*, 246.
[42] Maniatis, T.; Fritsh, E.F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: New York, **1982**.
[43] Fink, D.; Nebel, S.; Aebi, S. *Cancer Res*., **1996**, *56*, 4881.
[44] Bonetti, A.; Apostoli, P.; Zannineli, M.; Pavanel, F.; Colombatti, M.; Cetto, G.L.; Franceshi, T.; Spperetto, L.; Leone, R. *Clin. Cancer. Res*., **1996**, *2*, 1829.
[45] Ciusani, E.; Perego, P.; Carenini, N.; Corna, E.; Facchinetti, F.; Boiardi, A.; Salmaggi, A.; Zunino, F. *Biochem. Pharmacol*., **2002**, *63*, 881.
[46] Spinozzi, F.; Agea, E. *Scand. J. Immunol*., **1995**, *41*, 504.
[47] Ormerod, M.G. In *Flow cytometry, a practical approach*. Ormerod, M.G. Ed.; Oxford University Press: New York, **1994,** pp. 119-125.
[48] Raynal, P.; Pollard, H.B. *Biochem. Biophys. Acta*, **1994**, *1197*, 63.
[49] Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. *J Immunol. Meth*., **1995**, *184*, 39.
[50] Fujita, N.; Tsuruo, T. *Biochem. Biophys. Res. Comm.*, **1998**, *246*, 484.
[51] Patel, T.; Gores, G.J.; Kaufmann, S.H. *FASEB J*., **1996**, *10*, 5877.
[52] Banada, N.K.; Satterfield W.C.; Dunlap, A.; Steimer, K.S.; Kurrle, R.; Finkel, T.H. *Apoptosis*, **1996**, *1*, 49.
[53] Bregant, F.; Pacor, S.; Ghosh, S.; Chattopadhyay, S.K.; Sava, G. *Anticancer Res*., **1993**, *13*, 1011.
[54] Sava, G.; Pacor, S.; Bergamo, A.; Cocchietto, M.; Mestroni, G.; Alessio, E. *Chem. Biol. Interact*., **1995**, *95*, 109.
[55] Hayward, R.L.; Schornagel, Q.C.; Tente, R.; Macpherson, J.S.; Aird, R.E.; Guichard, S.; Habtemariam A, Sadler, P.; Jodrell, D.I. *Cancer. Chemother. Pharmacol*., **2005**, 55, 577.
[56] Strasberg, R. M.; Anzellotti, A.; Sanchez-Delgado, R.A.; Rieber, M. *Int. J. Cancer*, **2004**, *112*, 376.
[57] Hotze, A.C.; Bacac, M.; Velders, A.H.; Jansen, B.A.; Kooijman, H.; Spek, A.L.; Haasnoot, J.G.; Reedijk, J. *J. Med. Chem*., **2003**, *46*, 1743.
[58] Marx, K.A.; Kruger, R.; Clarke, M.J. *Mol. Cell. Biochem*., **1989**,*86*:155.
[59] Bergamo, A.; Zorzet, S.; Gava, B.; Sorc, A.; Alessio, E.; Iengo, E.; Sava, G. *Anticancer Drugs*, **2000**, *11*, 665.
[60] Bergamo, A.; Zorzet, S.; Cocchietto, M.; Carotenuto, M.E.; Magnarin, M.; Sava, G. *Anticancer Res*., **2001**, *21*, 1893.
[61] Bergamo, A.; Gava, .;Alessio, E.; Mestroni, G.; Serli, B.; Cocchietto, M.; Zorzet, S.; Sava, G. *Int. J. Oncol*., **2002**, *21*, 1331.
[62] Sava; G., Pacor.; S., Bergamo, A.; Cocchietto, M.; Mestroni, G.; Alessio, E. *Chem. Biol. Interact.*, **1995**, *95*, 109.
[63] Arandjelovic, S.; Tesic, Z.; Radulovic, S. *Med. Chem. Rev.-Online***, 2005**, *2*, 415.
[64] Radulovic, S.; Tesic, Z.; Manic, S. *Curr. Med. Chem.*, **2002**, *9*, 1611.
[65] Aird, R.E.; Cummings, J.; Ritchie, A. A.; Muir, M.; Morris, R. E.; Chen, H.; Sadler, P. J.; Jodrell, D. I. *B. J. Cancer*, **2002,** *86*, 1652.

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