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ORIGINAL ARTICLE

Antitumor activity of Ru(III) complexes carrying β-diketonato ligands in vitro and in vivo

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Summary

Purpose: *To investigate the antitumor activity of two newly synthesized ruthenium(III) [Ru(III)] compounds carrying bidentate ligands: (acac)-acetylacetonate, [Ru(acac)$_3$], and (tfac)-trifluoroacetylacetonate [Ru(tfac)$_3$].*

Materials and methods: *The activity of ruthenium(III) analogues was evaluated on HeLa, B16, and Femx cell lines for cytotoxicity in vitro using MTT assay, and inhibition on tumor invading ability in vitro using cell migration and invasion assays, whereas inhibition of tumor growth in vivo was estimated on advanced B16 murine melanoma model. Both compounds were also investigated in combinations with cisplatin, oxaliplatin, or poly ADP-ribose polymerase-1 (PARP-1) inhibitor, in order to determine the pattern of mutual interactions.*

Results: *Applied as single drugs, Ru(tfac)$_3$ showed high cytotoxic activity against HeLa and Femx cell lines, while Ru(acac)$_3$ did not reach the IC$_{50}$ on any of the cell lines tested. In combinations, Ru(acac)$_3$ with cisplatin gained synergistic interaction, antagonistic with oxaliplatin, and of different kind with (PARP-1) inhibitor in concentration-and cell line-dependent manner.*

Ru(acac)$_3$ exhibited inhibition of HeLa cell migration and gelatinolytic activity of MMP-2 and MMP-9. Ru(tfac)$_3$ complexes did not induce significant reduction of melanoma growth in vivo, whereas Ru(acac)$_3$ did, but the latter failed to contribute in lifespan improvement.

Conclusion: *The investigated ruthenium complexes showed different levels of antitumor activity in vitro and in vivo, implicating on different mechanisms of their action as well as diverse perspectives in cancer treatment.*

Key words: antitumor activity *in vivo/in vitro,* MMP, ruthenium(III) - β-diketonato

Introduction

NAMI-A (imidazolium-*trans*-imidazoledimethylsulfoxidetetrachlororuthenate) is the first ruthenium drug to be endowed with selective antimetastatic properties, that entered clinical studies [1]. Indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14A) [2] is just the second ruthenium-based anticancer agent after NAMI-A which was developed to the stage of clinical trials. Both of them have shown to enter the cell via the transferrin receptor pathway [3]. These Ru(III)-based drugs induce apoptosis at non-toxic levels via the mitochondrial pathway, the feature that distinguish them from the established platinum anticancer drugs and suggest that different types of cancer might be treatable with such compounds. Indeed, promising activity against certain types of tumors, which are not successfully treatable with cisplatin, and only a very low incidence of acquired resistance has been observed in *in vitro* and *in vivo* studies. However, the nature of the target(s) responsible for the antimetastatic activity of Ru(III) drugs remains unclear. Common to any type of NAMI-A type compound, both monomeric and dimeric, cytotoxicity (which is generally very low) is not sufficient to explain their potent and peculiar antitumor activity. All active NAMI-A type compounds share the capacity to modify important parameters of metastasis such as tumor invasion, matrix metalloproteinases activity and cell cycle progression [4].

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In the present study, we investigated the antiproliferative and antimetastatic action of Ru(III) compounds, $Ru(acac)_3$ and $Ru(tfac)_3$, and compared it to cisplatin and oxaliplatin. These Ru(III) complexes differ significantly in comparison to NAMI-A type compounds according to stereochemical features, which may cause their different mode of action [5,6]. We previously reported the potential of Ru(III) complexes to induce apoptotic response of cancer cells *in vitro*. The combined activity of $Ru(acac)_3$ with cisplatin or oxaliplatin was investigated. Disruption of poly(ADP-ribose) polymerase (PARP) pathways by inhibitors of PARP catalytic domain has been shown to increase the antitumor activity of some cytotoxic agents [7].

Herein we tested whether 5-aminoisoquinoline hydrochloride (5-AIQ), an inhibitor of PARP [8], might enhance Ru(III)-based drug efficacy. The influence of two Ru(III) compounds on cell cycle distribution and cancer cell migration *in vitro*, as well as tumor growth reduction *in vivo* was tested.

Materials and methods

Compounds

Ru(III) complexes used in the experiment were synthesized as described previously [9,10] and kindly supplied by Dr. Tesic, Faculty of Chemistry, University of Belgrade, Serbia. The chemical structures of the complexes are presented in Figure 1. All chemicals, except indicated, were purchased from Sigma Chemical Co. Nutrient medium RPMI 1640 was prepared in sterile ionized water, and supplemented with penicillin (192 U/ml), streptomycin (200 µg/ml), HEPES (25 mM), L-glutamine (3 mM), and 10% of heat-inactivated fetal calf serum (FCS), pH=7.2. Complexes were dissolved in DMSO/0.9% NaCl mixture, prior to use. 5-AIQ was kindly provided by Prof. M. Prostran, University of Belgrade, Serbia.

Cell growth inhibition assay

Human cervix carcinoma cells (HeLa), murine melanoma cells (B16) and human melanoma (Femx) were cultured as monolayer in RPMI 1640 medium pH 7.2. Cells were incubated at 37° C, in highly humidified air atmosphere, with 5% CO_2, and subcultured twice weekly.

Drug-induced cytotoxicity was analyzed using MTT assay [11]. Cells (3000 cells /well) were seeded into wells of 96-well microtiter flat bottom plate (Linbro). After 24 h of cell growth, the tested compounds were added at various concentrations, with each concentration of compound tested in triplicate. After 48 h of continual agent action, 0.02 ml MTT solution was added to each well, and 4 h later, 0.1 ml of SDS-HCl solution (10% SDS in 0.01M HCl) was added to each well. After 20 h, absorbance was measured at 570 nm, using ELISA reader.

Median-effect principle for dose-effect analysis

The activity of 5-AIQ [8] in combination with cisplatin or $Ru(acac)_3$ was investigated on 3 different cell lines (HeLa, B16 and Femx) in order to explore the mode of mutual interaction of these drugs. The multiple drug effect analysis of Chou and Talalay [12] was used to calculate the combined drug effects. Dose-effect curves for each agent and for combinations, in multiple diluted concentrations (being 3; 10; 30 µM and 0.1; 0.3 µM for the investigated compounds and 5-AIQ, respectively) were plotted using the median-effect equation $fa/fu = (D/Dm)^m$, where D is the dose, Dm is the dose required for 50% effect, fa is the fraction affected by dose D, fu is the unaffected fraction and m is a coef-

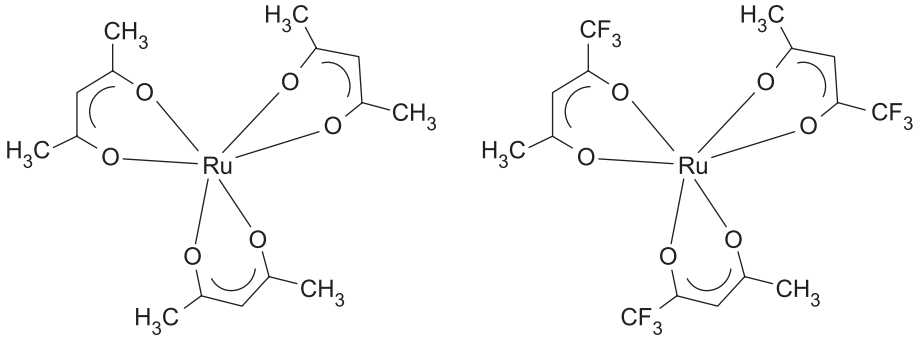

Figure 1. Graphical presentation of formulas of the tested Ru(III) complexes.

ficient of the sigmoidicity of the dose-effect curve. The conformity of the data to the median-effect principle was determined by the linear correlation coefficient *r*. The combination index (CI) equation for mutually non-exclusive drugs CI = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/(D_x)_1(D_x)_2$, where drug 1, $(D)_1$, and drug 2, $(D)_2$, in combination inhibit x%, and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 alone, respectively, inhibiting x%, was employed for measuring synergism and antagonism. CI < 1, = 1, and > 1 indicates synergism, additive effect and antagonism, respectively. Quantitations by computerized analysis were done using the Calcusyn software (Biosoft). Combinational drug studies were also performed for $Ru(acac)_3$ with cisplatin or oxaliplatin on HeLa cells for 72 h of drug action.

Analysis of cell cycle

B16 cells were seeded in 3 ml of RPMI-1640 medium into 6-well tissue culture plates and exposed to different concentrations of the agents for 72 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, containing RNAse (1 mg/ml) and PI (10 μg/ml). Cell cycle phase distribution was analyzed by using a flow cytometer equipped with an argon laser (Becton Dickinson, Mountain View, CA, USA).

Migration assay

4×10^5 HeLa cells were seeded into 6-well plates. After 24 h of treatment with $Ru(acac)_3$ in concentration of 200 and 400 μM, treated cells were deprived in FCS-free RPMI medium containing 0.2% bovine serum albumin (BSA), for 24 h. For quantification of the migration capacity, cells were collected by centrifugation, and 2×10^5 cells were transferred into cell culture inserts (BD bioscience). The test includes compartment system where cells may be induced to migrate from an upper chamber (with 0.2% BSA) into a lower compartment (with 10% FCS), thus following the gradient of chemoattractant. Cells were stained by Calcein-AM solution (BD bioscience), according to the application protocol for quantification of migratory cells [13] and fluorescence was measured at emission wavelength of 520 nm, after excitation at wavelength of 485 nm.

MMP-2 and MMP-9 gelatin-zymography and activity

U2-OS cells (10^6) were cultured overnight in 6-well plates. Cells were treated with $Ru(acac)_3$, 100 μM and 200 μM for 3 h. After drug treatment cells were rinsed twice with Ca^{2+} and Mg^{2+}-free PBS and incubated in serum-free RPMI 1640 medium. After 24h incubation, the supernatant was collected, and spun at 3000 rpm for 10 min at 4° C in order to eliminate remaining cells, and such cell-free medium was further used for detection of gelatinase activity. Gelatin zymographs were performed according to the method reported in [14] with some modifications. Briefly, samples were applied to SDS-polyacrylamide gels (10%, w/v) containing 0.1% (w/v) gelatin. After electrophoresis the gels were rinsed twice (30 min and 1 h each) in 2.5% Triton X-100 to remove SDS, and incubated for 18h at 37° C in incubation buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 200 mM NaCl).

In vivo effects on mouse melanoma B16 tumors

Antitumor activity of ruthenium compounds was evaluated on melanoma-bearing mice. Animals were housed in cages, under constant temperature and humidity conditions, exposed to light and dark for 12 h respectively, with food and water available *ad libitum*. All procedures involving animals were approved by the Ethical Committee of the National Cancer Research Center of Serbia, in compliance with the Guidelines for the Use and Care of Laboratory Animals (NIH Publication 85-23).

Ruthenium compounds were tested in separate experiments, but under the same dosing schedules and experimental conditions. Both $Ru(acac)_3$ and $Ru(tfac)_3$ were initially dissolved with DMSO as a stock solution (300×10^{-6} mol/L). Further dilutions were made with saline (0.9% NaCl) up to a concentration calculated for individual animal in correspondence to the tested dose and body weight, and applied as 0.1 ml volume as intraperitoneal injection. With this procedure DMSO never exceeded the amount of 10% in a daily dose. Control mice received the equivalent concentration of DMSO in saline only.

C57 black female mice, 9 weeks old, were subcutaneously injected in the right flank with B16 murine melanoma cell suspension. Tumors were allowed to grow up to a volume of 250-300 mg, when animals were randomly divided into 3 groups and treated as follows: group I – control animals received vehicle only; group II – animals received 25 mg/kg of $Ru(acac)_3$ or $Ru(tfac)_3$ daily; group III – animals received 50 mg/kg of $Ru(acac)_3$ or $Ru(tfac)_3$ daily. The first day of treatment was considered as the experimental day 1. Treatments lasted for 10 days, with tumor size and body weight of all animals measured on a daily basis. In each experiment control and experimental groups consisted of 12 animals.

Antitumor activity and toxicity of ruthenium compounds on melanoma-bearing mice were estimated using conventional statistical methods and several parameters adjusted for small sample size analysis:

1. Relative tumor volume (RTV) represents the rate of tumor growth, calculated for each animal as RTV = TVn/TV1, where TVn is tumor volume on the day n of treatment, and TV1 is tumor volume on the experimental day 1;

2. Percent inhibition rate in tumor volume (IRTV%) represents the inhibition of tumor growth in the treated animals regarding the non-treated group, calculated for each day of treatment as IRTV% = [1-(RTVt/RTVc)] × 100, where RTVt is the mean of the relative tumor volume in the treated group, and RTVc is the mean of the relative tumor volume in the control group, for the same day of treatment;

3. Percent of body weight reduction (BWR%) represents the body weight change during treatment, calculated as BWR% = [1-(RBWn/RBW1)] × 100, where RBWn is the mean of the relative body weight on the day n, RBW1 is the mean of the relative body weight on the experimental day 1, and RBW calculated as the measured body weight minus the measured tumor weight (tumor weight equals to tumor volume since its density is assumed to be 1);

4. Percent increase in lifespan (ILS%) represents the effect of the investigated drug on the life length in the treated animals regarding the non-treated group, calculated as ILS% = [(msT/msC)-1] × 100, where msT is median survival in the treated group, and msC is median survival in the control group.

Statistical analysis

Statistical analysis was performed using RTV values on days 3, 7, and 11 with Kruskal-Wallis test and Mann-Whitney post test [15].

Results

Drug cytotoxicity study

Drug cytotoxicity studies performed for 48 or 72 h of continual action using MTT assay indicated that $Ru(acac)_3$ possessed no cytotoxicity up to 100 μM on all tested cell lines. After prolonged incubation (72 h and 96 h) $Ru(acac)_3$ reached IC_{50} values but in concentrations higher than 100 μM and not in all cell lines (excepting IC_{50} of 11 μM after 96 h action on Femx cells). $Ru(tfac)_3$ showed much higher cytotoxicity compared to $Ru(acac)_3$ on HeLa and Femx cells but B16 cells remained resistant up to concentration of 100 μM. IC_{50} values determined from cell survival graphs are presented on Table 1.

Combination drug studies in vitro

In order to establish whether the apoptosis-related effects of the different treatments could be relevant to HeLa, B16 and Femx cell response to Ru(III)-based agents, combination drug studies were performed. The effects of the agents, used as single agents and in combinations, were evaluated after 72 h of treatment.

The CI equation was employed for determining synergistic, antagonistic, or additive effects. The combination drug effect varied, depending on the drug concentrations used, but in general most of the interactions of 5-AIQ with cytotoxic drugs, at higher 5-AIQ concentration, proved antagonistic, as tested on HeLa, B16 and Femx cell lines (Table 2). However, the combination of 5-AIQ with the non-toxic ruthenium-agent, happened to be fully synergistic at low 5-AIQ concentration. 5-AIQ stimulated cisplatin cytotoxicity almost 90 times as tested on mouse melanoma B16.

Interaction between the $Ru(acac)_3$ and cisplatin or oxaliplatin (additive, synergistic and antagonistic) was also evaluated after HeLa cells exposure to two-drug combinations, with various sequences of administration. In the combination study on HeLa cells, all sequences of $Ru(acac)_3$ and cisplatin demonstrated synergistic interaction (CI values < 1) (Figure 2a). However, the combination of oxaliplatin with $Ru(acac)_3$ exhibited antagonistic effect (Figure 2b).

Table 1. Cellular sensitivity of cervix carcinoma (HeLa), human melanoma (Femx) and mouse melanoma (B16) cells to the studied metal complexes

Treatment	*Exposure time (hours)*	*IC_{50} (μM)* *HeLa*	*Femx*	*B16*
$Ru(tfac)_3$	48	11	28	>100
	72	6	12	>100
$Ru(acac)_3$	48	>100	>100	>100
	72	>100	>100	>100
	96	>100	11	>100
Cisplatin	48	4	2	9
	72	4	3	12
	96	2	7	13
Oxaliplatin	72	6	ND*	ND*

Cellular sensitivity was measured by growth-inhibition assay. The reported values are the mean of 2 to 3 independent experiments, whereas standard deviations were less than 15%. IC_{50} represents the concentration causing a 50% decrease of cell growth as compared to control cells.
* not determined

Table 2. The results of mutual interactions between cisplatin or $Ru(acac)_3$ with 5-AIQ expressed as combination index (CI) values, where CI>1 indicates antagonistic, CI=1 additive, and CI<1 synergistic interaction between drugs in combination

Combined treatment		*CI values*		
5-AIQ (μM) + Cisplatin (μM)		*HeLa*	*Femx*	*B16*
	0.1	0.86	0.64	0.041
3	0.3	1.04	0.58	0.073
	1.0	0.81	0.81	0.18
	0.1	1.97	5.69	0.056
10	0.3	1.75	1.97	0.082
	1.0	0.85	1.25	0.17
5-AIQ (μM) + $Ru(acac)_3$ (μM)				
	3	0.42	0.91	23.35
3	10	0.28	0.80	0.44
	30	0.27	1.27	1.25
	3	0.66	1.60	2.07
10	10	2.35	1.03	1.18
	30	0.43	3.15	1.62

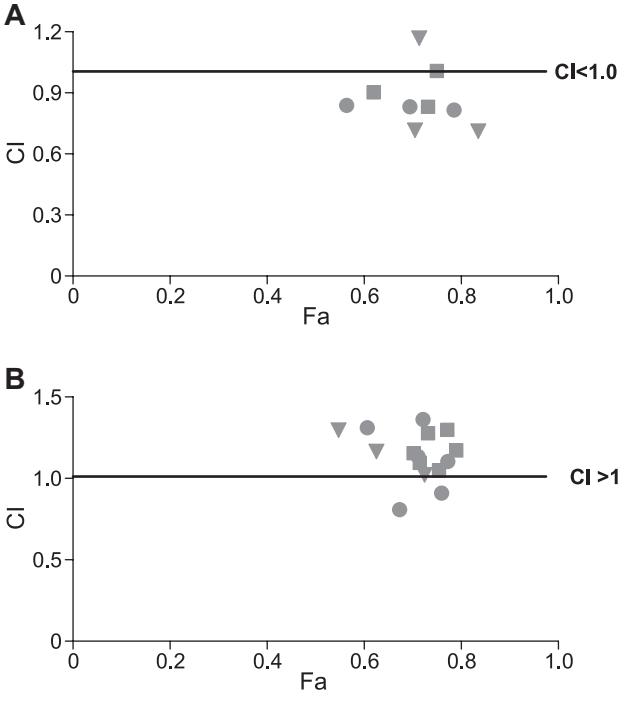

Figure 2. Graphical presentation of combination index (CI), for simultaneous action of $Ru(acac)_3$ as test agent, with cisplatin (**A**) or oxaliplatin (**B**) as standard agents , on HeLa cells, using Calcusyn software. The combination drug study was performed after 72 h of drugs action. Every CI value represents mean of 3-5 independent experiments. Drugs were tested in the following combination of doses: ▲75:25% test agent / standard agent; ■ 50:50% test agent / standard agent; ● 25:75% test agent / standard agent. 100% of single drug dose corresponds to concentration of $2 \times IC_{50}$, evaluated after 72 h of drug action.

Cell cycle perturbation

The effect of Ru(III) complexes on cell cycle progression on B16 cells was analyzed for 3 concentrations corresponding to IC_{25}, IC_{50} and IC_{75} values for $Ru(acac)_3$ (and 10; 30; 100 μM for $Ru(tfac)_3$), after 72 h of agent action (Figures 3a, 3b). Both Ru(III) complexes induced cell cycle arrest in S phase, this effect being greatest at IC_{75} (highest concentration). Accordingly, the percent of cells in G1 and G2/M phases decreased.

Effects on migration of HeLa cervix carcinoma cells

HeLa cells, treated *in vitro* with 200 and 400 μM $Ru(acac)_3$ for 24 h, showed significantly reduced capacity to invade and migrate through a barrier in the transwell chamber. The evaluation of cell invasion, performed at 72h is presented on Figure 4.

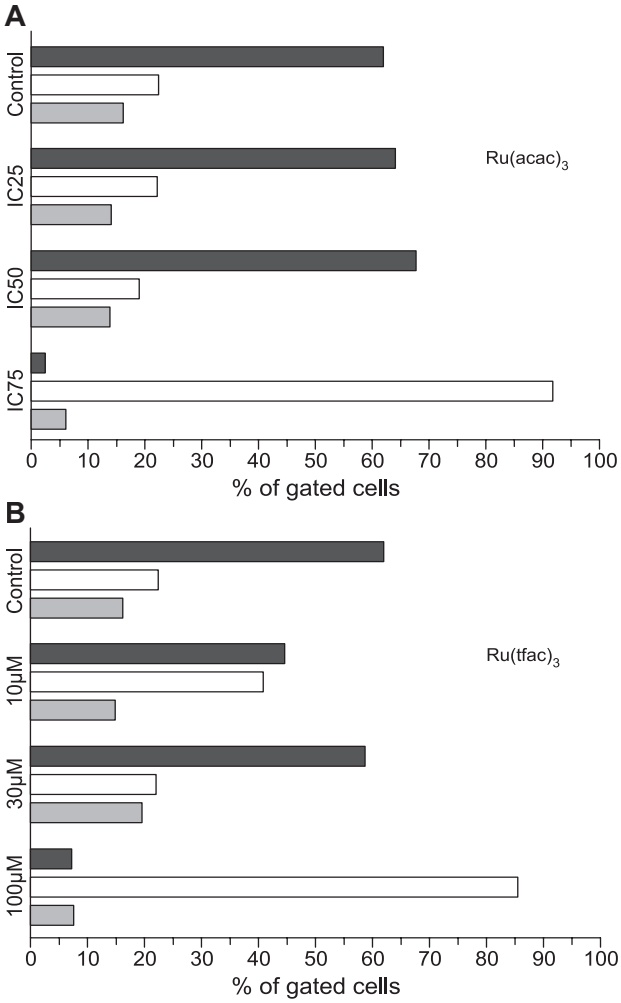

Figure 3. Distribution of B16 cells in G0/G1 (black bars), S (white bars), and G2/M (gray bars) phases of the cell cycle after 72 hours of incubation with $Ru(acac)_3$ (**A**), or $Ru(tfac)_3$ (**B**). The applied concentrations of $Ru(acac)_3$ were chosen according to the level of its cytotoxic activity, whereas $Ru(tfac)_3$, which is lacking cytotoxic activity, was applied in 3 concentrations chosen from the range of tested (1-100 μM).

Effect on MMP-2 and MMP-9 activity

The study of the direct effects of $Ru(acac)_3$ on MMP-2 and MMP-9 was performed using gelatin-zymography test. Both gelatinases, obtained from human osteosarcoma (U2-OS) culture medium supernatant after cell starvation in serum-free RPMI medium for 24 h (Figure 5) showed that osteosarcoma cells secreted overlapping amounts of MMP-2 and MMP-9, whose activity was inhibited in the same extend by both concentrations of $Ru(acac)_3$.

Effects on tumor growth in vivo

Treatment with $Ru(acac)_3$ at higher dose (50 mg/kg) for 10 days induced substantial inhibition of tumor bulk growth in treated mice (Figures 6a and 6b). At the end of therapy, the mean tumor size in the treated group increased < 5 times compared to an increase in relative tumor volume of > 15 times in the non-treated mice (Figure 6a). Statistical comparison showed significant reduction on days 3 ($p<0.001$), 7 ($p<0.001$), and 11 ($p<0.05$) when compared to the control group. As shown in Figure 6b, treatment with $Ru(acac)_3$ had sustained antitumor activity during treatment. After initial weight loss during the first 4 days, the animals gained body mass thereafter and a mean increase of 6% in body weight was recorded on day 10 (Figure 6c). However, despite significant tumor size reduction, $Ru(acac)_3$ in a dose of 50 mg/kg did not prolong the lifespan of mice. Animals started dying 2 days after therapy had been completed (Figure 6d). Thus, the lifespan of treated mice was just 7% longer than in those without treatment. $Ru(acac)_3$ at a dose of 25 mg/kg gave inferior antitumor effect compared with 50 mg/kg dose. In this group tumors increased 6 times in size, but when compared with the control group the difference was not statistically significant (Figure 6a). During the treatment time no considerable loss in body weight was recorded (Figure 6c). However, the lifespan in animals treated with lower dose of $Ru(acac)_3$ was reduced compared with the non-treated controls.

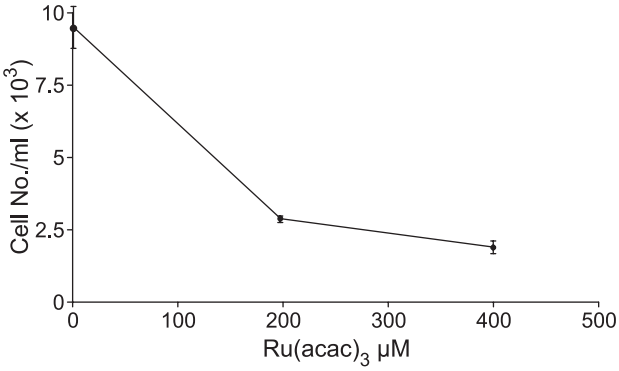

Figure 4. *In vitro* migration by HeLa cells through polycarbonate filters of 24-well trans well chamber. HeLa cells, exposed previously to $Ru(acac)_3$, were sown on the top compartment of a trans well chamber. Data represent cells that had completely passed through the Matrigel-coated barrier after 72 h and are located in the bottom compartment of the trans wells. Data are expressed as percentage ± SE of invading cells relative to the corresponding controls.

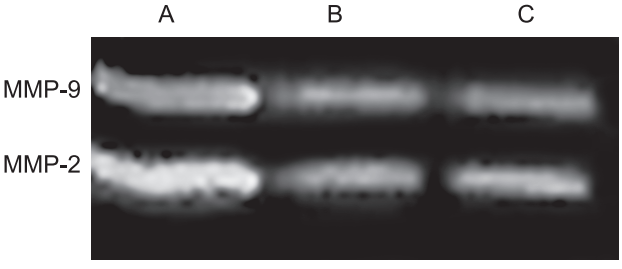

Figure 5. Direct effect of $Ru(acac)_3$ on MMP-2 (M_r 72,000) and MMP-9 (M_r 92,000) gelatinases. MMP-2 activity was evaluated from a densitometric scan of bands appearing on a SDS/polyacrylamide/gelatin gel loaded with culture medium of human osteosarcoma cells (U2-OS) increasing concentrations of $Ru(acac)_3$ ranging from 200 – 400 μM. Lane identification: **A:** control (supernatant of untreated cells); **B:** $Ru(acac)_3$ 200 μM; **C:** $Ru(acac)_3$ 400 μM. Lanes B, and C are darker due to $Ru(acac)_3$ inhibited gelatinolytic activity of MMPs.

Similar to $Ru(acac)_3$, therapy with $Ru(tfac)_3$ significantly inhibited melanoma growth only when applied at higher dose. On the first day after treatment termination (day 11) in mice receiving 50 mg/kg of $Ru(tfac)_3$, tumors increased 7 times on average compared to 10 and 13 times in the group receiving 25 mg/kg and the non-treated controls, respectively (Figure 7a). The higher dose reached its pick antitumor activity on day 5 with a modest 40% inhibition rate (Figure 7b), but statistical significance was observed only on day 7 ($p<0.05$, compared to control group). In both treatment groups, the lifespan was shorter than in non-treated mice, again with greater reduction in the group under 25 mg/kg (-38%) than in the group under 50 mg/kg (-8%) dosing schedule (Figure 7d). In all treated animals body weight gain was recorded (Figure 7c). Treatment of animals with intraperitoneal administration of $Ru(tfac)_3$ proved highly toxic, inducing tachypnea, stiff hair, hair loss during the experiment and signs of tardive dyskinesia.

Discussion

In our previous study [5], both tested Ru(III) compounds induced apoptotic cell response (approximately > 10% of the treated cell were apoptotic, as analyzed by PI and Annexing V-FITC staining using flow cytometry on A2780 and HeLa cells), but the mechanisms underlying such actions were not clear. In that study, we showed

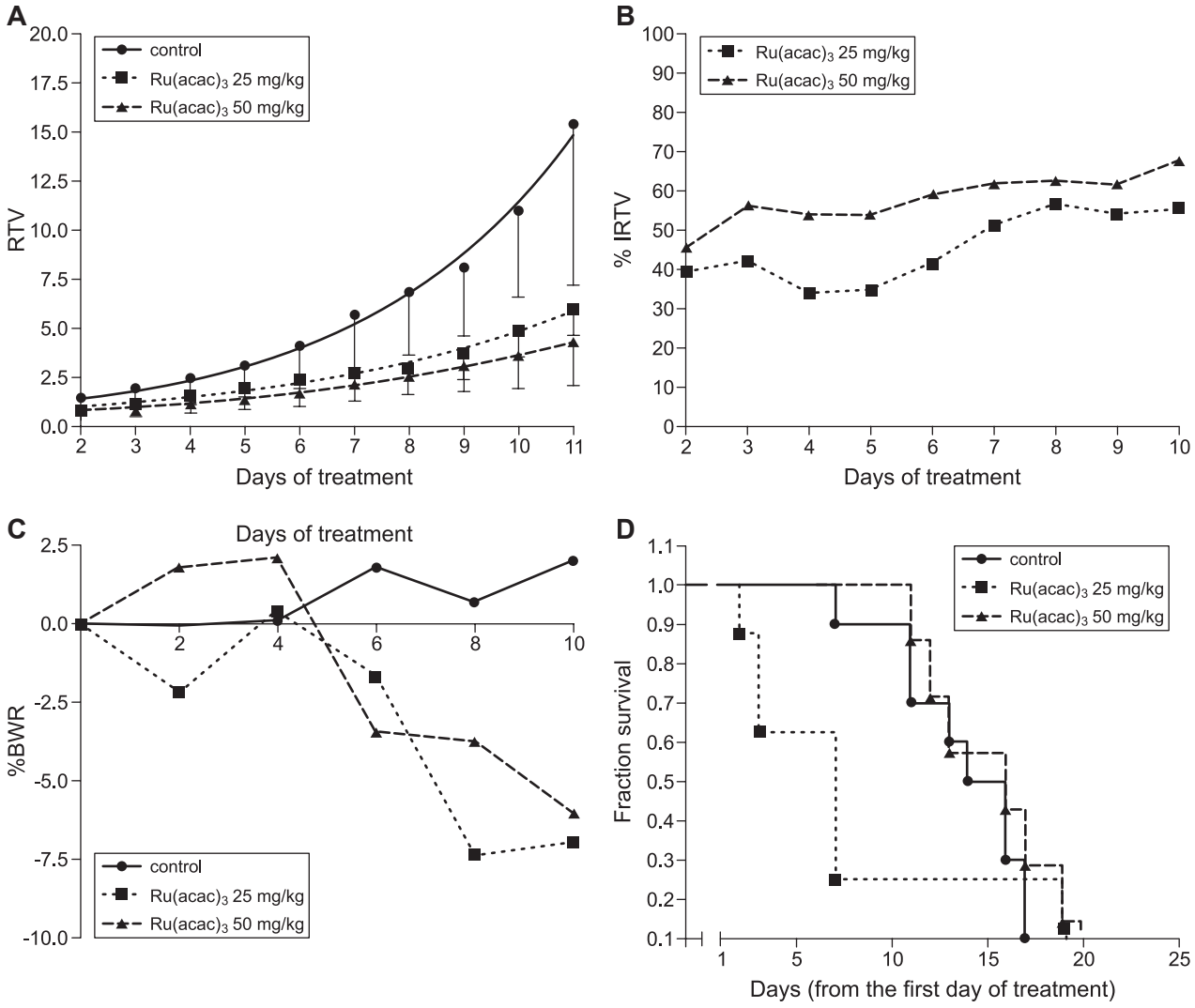

Figure 6. Effect of $Ru(acac)_3$ treatment on tumor growth rate (**A**), inhibition rate in tumor volume (**B**), body weight change (**C**), and lifespan (**D**) in melanoma-bearing mice. Ruthenium compound was given for 10 days. There were 12 animals in each experimental group.

that both Ru(III) complexes induced significant arrest in the S phase of the cell cycle, indicating possible interaction with DNA or DNA replication/repair machinery. It is likely that delay of the cell cycle in the S phase may not be due to direct DNA binding [5] and may be secondary effect of drug cytotoxic effect. 5-AIQ at lower doses exhibited synergistic effect in combination with Ru(III) complexes, thus potentiating the apoptosis-inducing ability of ruthenium agents. Similar result was obtained for 5-AIQ in combination with cisplatin which acts as a sole adductor on DNA. We investigated also the cytotoxic action between two metal bases complexes which share no structural similarities: Ru(III)-complex with bidentate ligands and Pt(II) based drugs. $Ru(acac)_3$, although devoid of reasonable cytotoxicity *in vitro*, demonstrated synergistic effect in combination with cisplatin (IC < 1), acting as sensitizer of tumor cells in combination therapy. Our data showed that the combination of oxaliplatin with $Ru(acac)_3$ resulted in marked antagonism irrespective of the application schedule. That result may account for differences between two platinum(II)-based agents [16], particularly in the mechanisms involved in processing DNA lesions. Although oxaliplatin is at least as potent as cisplatin in inhibiting the growth of cancer cells, the biological role of DACH-Pt(II) adducts differs compared to cisplatin-DNA adducts, and they are differently processed in the cell [17]. Since the activity of the DNA nucleotide excision repair (NER) pathways is activated by oxaliplatin adducts [18], attenuation of oxaliplatin cytotoxicity in combination with non-toxic Ru(III) drug may be due to inhibition of the activity of NER pathways, mediated by $Ru(acac)_3$. Also, it may be possible that $Ru(acac)_3$ interfere with oxaliplatin intracellular transport, thus reducing oxaliplatin intracellular uptake, as well as its cytotoxic activity.

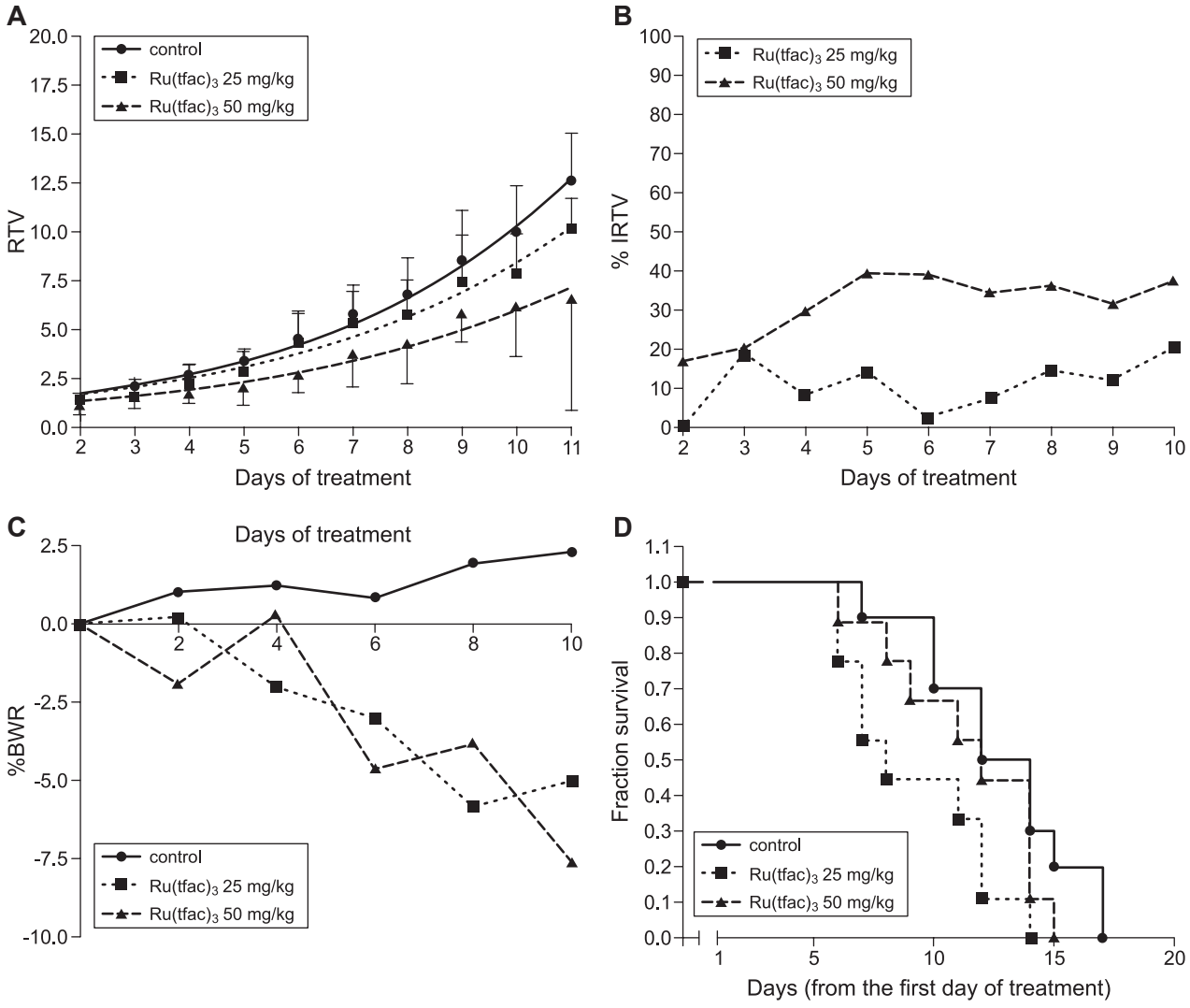

Figure 7. Effect of $Ru(tfac)_3$ treatment on tumor growth rate (**A**), inhibition rate in tumor volume (**B**), body weight change (**C**), and lifespan (**D**) in melanoma-bearing mice. Ruthenium compound was given for 10 days. There were 12 animals in each experimental group.

Ruthenium complexes also inhibited the invasive potential of human cervix carcinoma (HeLa) and mouse melanoma (B16) cell line, as evidenced by *in vitro* tests. The ability of HeLa cells to migrate through the reconstituted basement membrane in the transwell culture system was decreased by $Ru(acac)_3$ in a dose-dependent manner. $Ru(acac)_3$ exhibited slight inhibition of MMP-9 and MMP-2 action, thus showing antimetastatic potential [19]. $Ru(acac)_3$ as well as $Ru(tfac)_3$ induced significant inhibition of tumor growth *in vivo* at both administered doses *(*25 or 50 mg/kg), comparable to the action of cisplatin at 7.5 mg/kg. Despite the ability to decrease tumor volume both $Ru(acac)_3$ and $Ru(tfac)_3$ were not capable to prolong median survival of animals bearing B16 tumors. On the contrary, the lifespan of treated animals was significantly shortened. It seems that doses (25 or 50 mg/kg) and/or schedule (days 1-10), administered intraperitoneally to mice bearing B16 melanoma, were too toxic and should be redrawn. The doses we used were chosen from similar experiments on mice with MCa mammary carcinoma treated with NAMI-A (35 mg/kg/day for 6 consecutive days) [20]. There are not too many data on *in vivo* toxicity of Ru(III) complexes. NAMI-A induces glomerular and tubular changes on mice kidney as well as significant liver toxicity [21]. In our experiments, $Ru(tfac)_3$ appeared much more toxic to the C57 black mice compared to $Ru(acac)_3$, inducing signs of neurotoxicity and early deaths.

Thus the apoptotic and antimetastatic potential of Ru(III) agents, even when they are devoid of reasonable cytotoxicity *in vitro*, provides rationale to investigating ruthenium-based compounds as promising agents in combination therapy. 5-AIQ may represent an alternative strategy to enhance the efficacy of Ru(III)-based drugs against invasive melanoma. The *in vivo* antitumor

activity of $Ru(acac)_3$ and $Ru(tfac)_3$, rather peculiar as it appears, deserves further attention and investigation.

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