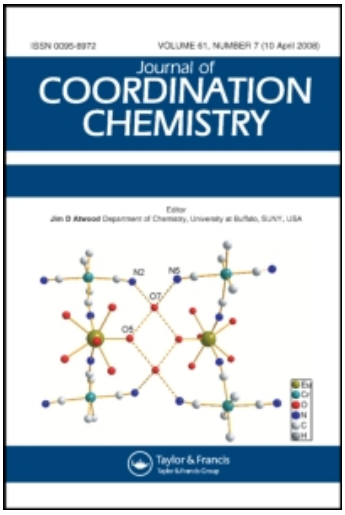

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Ru(III) complexes derived from *N*-methyl derivatives of glycine and 1,3-propylenediamine-*N*,*N*′-diacetato ligands and their activities against HeLa, K562 cell lines and human PBMC

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Ruthenium(III) complexes formulated as $K_2[Ru(sar)Cl_4] \cdot H_2O$ and $K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$ containing bidentate chelates *N*-methylglycine (sarcosine, sar) or *N*,*N*-dimethylglycine (dmgly) as well as $K[Ru(pdda)Cl_2] \cdot 2.5H_2O$ complex with tetradentate 1,3-propylenediamine-*N*,*N*′-diacetato were synthesized. The complexes were obtained by direct reaction of ruthenium(III) chloride with the corresponding bidentate or tetradentate ligand followed by addition of a base (KOH). These new complexes were characterized by elemental analysis, IR and electronic absorption spectroscopy. To assess selectivity in the antitumor action of these complexes, their antiproliferative activities against human adenocarcinoma HeLa cells, human myelogenous leukemia K562 cells, human breast carcinoma MDA-MB-361 and MDA-MB-453 cells and normal immunocompetent PBM cells were determined.

Keywords: Ruthenium(III); *N*,*N*-dimethylglycine; Sarcosine; 1,3-Propylenediamine-*N*,*N*′-diacetate

1. Introduction

Cisplatin is one of the most efficient chemotherapeutic agents displaying clinical activity against a wide variety of solid tumors. However, its application suffers from toxicity and drug resistance [1]. Thus, several strategies have been employed to improve cisplatin properties, including synthesis and testing of numerous cisplatin-based complexes [2–4]. However, in spite of a significant contribution to the field of anti-cancer chemotherapy, complete success has not been achieved. Increasing demand for effective new anticancer drugs able to circumvent the problem of tumor resistance to cisplatin and related platinum compounds, reducing side-effects and with higher selectivity towards tumor cells, continues to stimulate investigations in the field of non-platinum tumor-inhibiting metal complexes, particularly ruthenium compounds [5]

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exhibiting a number of interesting properties. Reduction of ruthenium(III) compounds in hypoxic tumor areas can afford more active species capable of rapid binding to cellular DNA [6]. Under these conditions, amine ruthenium complexes may be activated *in vivo* to coordinate a nucleobase in a similar way to platinum-based anticancer drugs [7–10].

Ruthenium compounds have been shown to be active against certain tumors, both *in vivo* and *in vitro*, with fewer side effects than cisplatin [11–13]. For example, imidazolium *trans*-imidazoledimethylsulfoxide-tetrachloro ruthenate (NAMI-A), a ruthenium complex that is a likely candidate for therapeutic application, expressed a selective action against lung metastases of solid experimental tumors in mice, irrespective of the lack of significant reduction of primary tumor growth [14, 15]. Similarly, this agent showed no direct cytotoxic activity against tumor cells *in vitro*, but markedly suppressed tumor cell invasion *in vivo* [16]. The lack of cytotoxic NAMI-A properties might explain a low level of host toxicity of this compound. Such favorable properties of NAMI-A led to phase I clinical trials of this agent as an anti-metastasis drug [13, 17].

In order to improve antitumor properties of ruthenium compounds, ruthenium complexes using polyaminocarboxylic molecules as chelating agents were prepared [18]. Ruthenium(III) complexes with polydentate chelating agents (''secondary ligands'') may be considered as prodrugs against different tumors. The secondary ligand plays a role in stabilizing the ruthenium(III) cation, thus preventing the metal delivery to the tissues during transport in the body and keeping the complex unchanged. In this way, a drastic lowering of the host toxicity produced by these potential drugs has been observed [19, 20]. Once the complex reaches the hypoxic areas of tumor tissues, the metal ion is reduced *in situ*, promoting the accumulation of ruthenium in tumor masses [19, 20]. As reported previously [19], a PDTA-Ru(III) complex (PDTA = 1,2-propylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid) accumulates in tumor tissues in higher proportion than other ruthenium compounds, e.g. $[Ru(en)_2Cl_2]$. At the same time, the ratio drug/g tissue of PDTA-Ru found in liver and kidneys was much lower than that of the $[Ru(en)_2Cl_2]$.

The ability of newly synthesized ruthenium(III) and the corresponding platinum(IV) complexes with bidentate *N*-methyl derivative of glycine to affect tumor cell viability has been tested on rat astrocytoma C6 cells [21]. This investigation has shown that substitution of *N*,*N*-dimethylglycine (dmgly) with two sarcosine (sar) ligands in the coordination sphere of the ruthenium (figure 1) significantly reduced antitumor properties of these agents. Among previously investigated ruthenium complexes, the compound containing one dmgly coordinated to ruthenium(III) [complex **4**, figure 1(b)] markedly reduced C6 cell viability, slightly influenced primary astrocyte viability and did not affect viability of primary rat macrophages, thus displaying tumor-specific action [21]. The influence of ruthenium(III) complex with two sarcosine ligands (sar) [complex **5**, figure 1(b)] on rat astrocytoma C6 cell viability was less than that observed for ruthenium(III) complex with dmgly [complex **4**, figure 1(b)] [21]. Taking into account the above mentioned reports, we found it of interest to examine whether the change in the number of *N*,*O*-bidentate ligands coordinated to ruthenium(III) could also lead to ruthenium complexes displaying promising cytotoxic properties (figure 1).

As part of our studies on this class of compounds, we present here the synthesis and characterization of new ruthenium(III) complexes with different numbers of

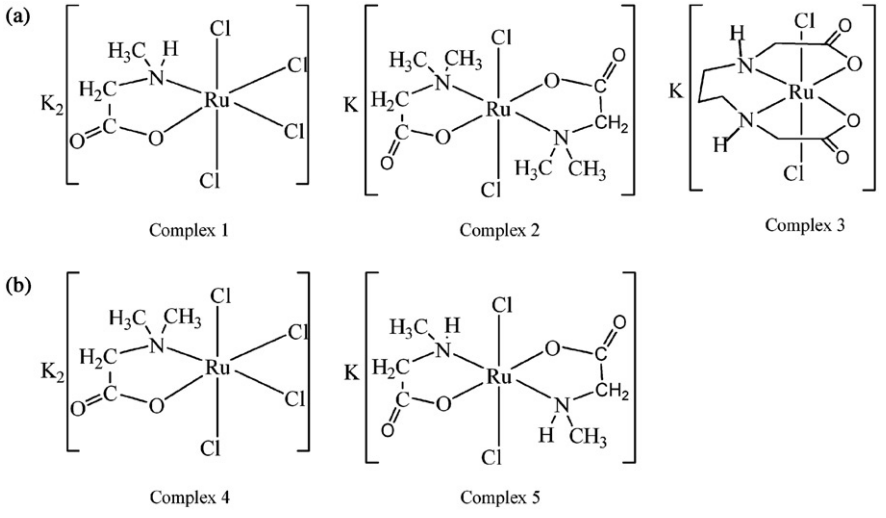

Figure 1. Structural formulas of the complexes used in the experiments (a) and those of previously described compounds (b).

coordinated bidentate chelates: *N*-methylglycine (sarcosine) or *N*,*N*-dimethylglycine, as well as ruthenium(III) complex with tetradentate 1,3-propylenediamine-*N*,*N*′-diacetato (pdda). Cytotoxic properties of these complexes against four human tumor cell lines (cervical carcinoma HeLa, chronic myelogenous leukemia K-562 and breast carcinoma MDA-MB-361 and MDA-MB-453 cells) were examined. Non-stimulated and phytohaemaglutinin (PHA) stimulated PBMCs from healthy donors served as controls.

2. Experimental

2.1. *Syntheses*

All chemicals used in the experiments were Sigma (St. Louis, MO, USA) products, unless otherwise stated. Commercial hydrated ruthenium(III) chloride was dissolved in concentrated HCl and refluxed for 30 min. After evaporation to dryness, the compound was stored in a desiccator over $CaCl_2$.

2.1.1. Synthesis of $K_2[Ru(sar)Cl_4]\cdot H_2O$ (1). $K_2[Ru(sar)Cl_4]\cdot 2H_2O$ (**1**) was synthesized by heating aqueous solution of 0.057 g sar (0.625 mmol) with 0.130 g $RuCl_3$ (0.625 mmol), molar ratio = 1 : 1, for 2 h on a steam bath. After that, the mixture was stirred and refluxed for 3 h. During this period, 0.1 mol dm^{-3} aqueous KOH solution was introduced to adjust pH to 3.0, the solution evaporated to a small volume and was left overnight. After acetone addition, a hydrosoluble, stable to air, black powder was obtained. Yield: 0.187 g (70.5%). Anal. Calcd for $K_2[Ru(sar)Cl_4]\cdot H_2O$ (%): C, 8.43; H, 1.87; N, 3.28. Found: C, 8.22; H, 1.49; N, 3.05.

2.1.2. Synthesis of *trans,trans*-K[Ru(dmgly)$_2$Cl$_2$] · 3H$_2$O (2). *Trans,trans*-K[Ru(dmgly)$_2$Cl$_2$] · 3H$_2$O (**2**) was prepared as for **1** using 0.130 g $RuCl_3$ (0.625 mmol) and 0.225 g dmgly (2.19 mmol) instead of sar. In this case, molar ratio of the corresponding reactants was 3.5 : 1. The compound was isolated as a black powder. Yield: 0.207 g (71.3%). Anal. Calcd for *trans,trans*-K[Ru(dmgly)$_2$Cl$_2$] · 3H$_2$O (%): C, 20.46; H, 4.69; N, 5.97. Found: C, 20.64; H, 4.28; N, 5.87.

2.1.3. Synthesis of *trans*-K[Ru(pdda)Cl$_2$] · 2.5H$_2$O (3). *Trans*-K[Ru(pdda)Cl$_2$] · 2.5H$_2$O (**3**) was prepared as described for **1** using 0.130 g $RuCl_3$ (0.625 mmol) and 0.200 g pdda · 2HCl (0.758 mmol) instead of sar. The isolated compound was a black powder. Yield: 0.191 g (69.3%). Anal. Calcd for *trans*-K[Ru(pdda)Cl$_2$] · 2.5H$_2$O (%): C, 18.91; H, 3.83; N, 6.30. Found: C, 19.25; H, 4.01; N, 6.35.

The presence and number of water molecules in all prepared complexes were confirmed by thermogravimetric experiments. Thermal degradation of the isolated complexes clearly demonstrated a gradual loss of crystal water molecule(s) at approximately 137, 120 and 131°C for **1**, **2** and **3**, respectively. Further thermal decomposition of the complexes was observed at 240°C for **1** and at 143°C for **2**. The anhydrous **3** remained unchanged from 131°C to 350°C, as judged by qualitative observations. All prepared complexes are soluble in water, but cannot be dissolved in acetone, DMF, DMSO and acetonitrile. All isolated complexes are highly hygroscopic.

2.2. *Characterization of the complexes*

Infrared spectra were recorded on a Perkin-Elmer FTIR 31725-X spectrophotometer using KBr pellets. Electronic absorption spectra were recorded on a Varian GBC UV-Visible Cintra 40 spectrophotometer on aqueous 1.0 mM solutions. The electronic absorption spectra of aqueous solutions of K$_2$[Ru(sar)Cl$_4$] · H$_2$O (**1**), K[Ru(dmgly)$_2$Cl$_2$] · 3H$_2$O (**2**) and K[Ru(pdda)Cl$_2$] · 2.5H$_2$O (**3**) show bands at 316 nm (4800 mol^{-1} dm^3 cm^{-1}) and 400 nm (2800 mol^{-1} dm^3 cm^{-1}), 315 nm (2553 mol^{-1} dm^3 cm^{-1}) and 403 nm (1366 mol^{-1} dm^3 cm^{-1}) and 310 nm (2500 mol^{-1} dm^3 cm^{-1}) and 475 nm (700 mol^{-1} dm^3 cm^{-1}), respectively. Elemental analyses were done on a Vario III CHNOS elemental analyzer, Elemental Analyses system GmbH. Thermal behavior was investigated using a Boetius PMHK instrument.

2.3. In vitro *cytotoxicity studies*

Before use, aqueous 1.0 mM stock solutions of the ruthenium(III) complexes were filtered (Millipore, 0.22 μm) and diluted with nutrient medium to various working concentrations. Nutrient RPMI 1640 medium without phenol red was supplemented with L-glutamine (3 mM), streptomycin (100 μg mL^{-1}) and penicillin (100 IU mL^{-1}), 10% fetal bovine serum (FBS) and 25 mM Hepes and adjusted to pH 7.2 by bicarbonate solution. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was dissolved in phosphate buffered saline (PBS), pH 7.2 (5 mg mL^{-1}) and filtered (Millipore 0.22 μm filter) before use.

2.3.1. Cell cultures. Human cervical carcinoma HeLa cells, human breast carcinoma MDA-MB-361 and MDA-MB-453 cells were cultured in a monolayer. Human myelogenous leukemia K562 cells were grown as a suspension in complete nutrient medium at 37°C in 5% CO_2 and humidified air. For the growth of MDA-MB-361 and MDA-MB-453 cells and all subsequent experiments, complete medium was enriched with 1.11 g L^{-1} glucose.

2.3.2. Treatment of cell lines. HeLa (2,000 cells per well), MDA-MB-361 (10,000 cells per well) and MDA-MB-453 cells (3,000 cells per well) were seeded into 96-well microtiter plates. Twenty hours later, after the cell adherence, the investigated complexes in five different concentrations ranging from 12.5–200 μM were added to the wells. Equivalent volume of nutrient medium was added to control cells. K562 cells were seeded at 3,000 per well, 2 h before addition of the examined compounds prepared as stock solutions and diluted to desired final concentrations within the above range.

2.3.3. Preparation of normal, peripheral blood mononuclear cells, PBMC. PBMC were separated from whole heparinized blood of healthy volunteers by Lymphoprep™ (Oslo, Norway) gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g L^{-1} gelatin polymers, pH 7.4), were counted and resuspended in nutrient medium (RPMI-1640 + 10% FCS).

2.3.4. Treatment of PBMC. PBMC were seeded at the density of 150,000 cells per well in a pure nutrient medium, or enriched with 5 μg mL^{-1} phytohaemaglutinin PHA (Welcome Diagnostics, England) in 96-well microtiter plates. Two hours later, the investigated complexes in five different concentrations were added to the wells (final concentrations ranging from 12.5–200 μM) containing non-stimulated and PHA-stimulated PBMC.

2.3.5. Determination of cell survival. Cell survival was determined by MTT test 14 72 h after addition of the complexes. Briefly, in the MTT test, 20 μL of MTT solution (5 mg mL^{-1} in PBS) was added to each well. The samples were incubated for a further 4 h at 37°C in 5% CO_2 and humidified air, then 100 μL of 10% sodium dodecyl sulfate (SDS) was added to the wells. The absorbance (A) at 570 nm was measured the next day. To get cell survival (S%), A of a sample with cells grown in the presence of various concentrations of the investigated agents was divided with control absorbance Ac (the A of control cells grown in nutrient medium only) and multiplied by 100. It is implied that A of blank was always subtracted from A of the corresponding sample with target cells. Concentration $IC50_{72h}$ was determined as the concentration of a drug inhibiting cell survival by 50%, as compared to the control.

3. Results and discussion

3.1. *Synthesis and characterization of the complexes*

The complexes were obtained by direct reaction of ruthenium(III) chloride with the corresponding bidentate or tetradentate ligand followed by the addition of KOH.

The preparation of *trans*,*trans*-K[Ru(dmgly)$_2$Cl$_2$] · 3H$_2$O (**2**, figure 1a) required a lower metal to bidentate ligand ratio (1 : 3.5) than for *trans*,*trans*-K[Ru(sar)$_2$Cl$_2$] · 1/2H$_2$O [complex **5**, figure 1(b)] (1 : 2) [21], likely from the presence of two methyl groups at the *N*,*N*-dimethylglycine nitrogen with higher steric demands. The complexes described here are water soluble.

Geometrical isomers of the prepared compounds containing tetradentate pdda or two bidentate sar ligands are possible (figure 2). The geometry of the isolated compounds has been proposed [figure 1(a)] by analyzing their infrared spectra and their similarity to the spectra of the corresponding Pt(IV) analogues whose geometry was confirmed by X-ray analysis. However, unambiguous evidence of the geometry by X-ray structural studies remains to be provided. Our attempts to prepare the complexes in a crystalline form have been unsuccessful.

3.1.1. Electronic absorption spectra. Electronic absorption spectra of ruthenium(III) complexes with *N*,*O*-coordinated ligands contain bands arising from one or more of the following types of transitions: intraligand, metal to ligand charge transfer, ligand to metal charge transfer and ligand field [22]. Spectra of ruthenium(III) complexes containing ONNO-type tetradentate ligand, as well as two *N*,*O*-bidentate ligands, show similarities to those of the complexes described here and can be used to assign the bands [21–23]. Spectra of the complexes described in the present study, together with those of some related ruthenium(III) complexes, are listed in table 1.

Electronic absorption spectra of the aqueous solutions of the prepared complexes show two bands, λ_1 and λ_2, attributed to charge transfer bands. Due to high absorptions in the relatively low energy region (charge transfer bands), d–d bands are obscured in the electronic spectra of all three complexes.

3.1.2. Infrared spectra. Asymmetric stretching frequencies of the carboxylate moieties distinguish between protonated (1700–1750 cm^{-1}) and coordinated (1600–1650 cm^{-1}) carboxylate groups.

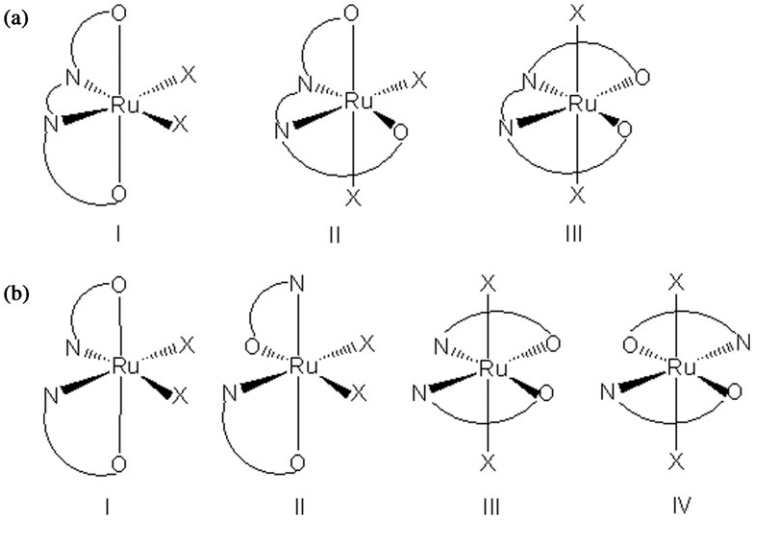

Figure 2. Possible geometrical isomers of [Ru(pdda)X$_2$] (a) and [Ru(dmgly)$_2$X$_2$] (b).

All synthesized complexes, $K_2[Ru(sar)Cl_4] \cdot H_2O$, *trans*,*trans*-$K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$ and *trans*-$K[Ru(pdda)Cl_2] \cdot 2.5H_2O$, show a very strong absorption band at 1630, 1632 and 1624 cm^{-1}, respectively, indicating coordination of carboxylate. Moderately strong bands in the 2830 cm^{-1} range of $K_2[Ru(sar)Cl_4] \cdot H_2O$ and *trans*,*trans*-$K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$ complexes can be related to the asymmetric CH_3 stretching vibrations, while those at 1371 cm^{-1} and 1369 cm^{-1} of the same complexes, respectively, are due to degenerate symmetric vibrations. The N–H stretching bands of $K_2[Ru(sar)Cl_4] \cdot H_2O$ and *trans*-$K[Ru(pdda)Cl_2] \cdot 2.5H_2O$ complexes are in the range of 3440–3410 cm^{-1}, overlapped with IR bands of H_2O also observed in the spectra of all prepared compounds.

The spectrum of the *trans* isomer is expected to be simpler than that of the *cis* isomer [24]. The IR spectra of the investigated *trans*,*trans*-$K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$ and *trans*-$K[Ru(pdda)Cl_2] \cdot 2.5H_2O$ complexes and the IR spectra of previously synthesized Pt(IV) analogous complexes (the complex with two sar, *trans*,*trans*-$Pt(sar)_2Br_2$) [25], as well as the complex with one pdda (*trans*-$[Pt(pdda)Cl_2]$) [26], where *trans* arrangement of the corresponding ligands was confirmed by X-ray crystallography, are almost identical, especially in the CO-stretching region. On the basis of these observations, *trans* arrangement of the corresponding ligands in *trans*,*trans*-$K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$ and *trans*-$K[Ru(pdda)Cl_2] \cdot 2.5H_2O$ are proposed.

3.2. In vitro *cytotoxicities of the Ru(III) complexes*

Antiproliferative activity of all examined compounds (**1**–**3**) tested against human HeLa, K562, MDA-MB-361 and MDA-MB-453 cell lines is presented in figure 3. Compound **3** was also screened for cytotoxic activity against nonstimulated and phytohemaglutinin (PHA)-stimulated normal human peripheral blood mononuclear cells (PBMCs) (figure 4) serving as a control. PBMC do not divide unless stimulated for proliferation by PHA and the IC_{50} values are related to direct toxic action of the investigated compounds. In the presence of PHA, PBMC are stimulated for proliferation and the IC_{50} values are related not only to the direct toxic action of the examined compounds, but also to reproductive cell death.

All investigated complexes, with the exception of **3**, had either no activity or very mild antiproliferative action toward target tumor cell lines and PBMC ($IC_{50} > 200\,\mu M$). Compound **3** exerted a moderate antiproliferative activity toward myelogenous leukemia K562 cells ($IC_{50} = 89.92 \pm 6.20\,\mu M$) and PBMC from one volunteer ($IC_{50} = 177.58\,\mu M$ and 161.96 μM for nonstimulated and PHA-stimulated PBMC, respectively), while being

Table 1. Electronic absorption spectra of Ru(III) complexes with aminocarboxylato ligands: wavelengths (λ, nm) and extinction coefficients (ε, $mol^{-1}\,dm^3\,cm^{-1}$).

Complex	λ_1	ε_1	λ_2	ε_2	Ref.
$K[Ru(eddp)Cl_2] \cdot 3H_2O$	470	600	300	2140	[23]
$K[Ru(sar)_2Cl_2] \cdot 0.5H_2O$	443	329	304	600	[21]
$K_2[Ru(dmgly)Cl_4] \cdot 2H_2O$	380	875	285	1750	[21]
$K[Ru(pdda)Cl_2] \cdot 2.5H_2O$	475	700	310	2500	*
$K_2[Ru(sar)Cl_4] \cdot H_2O$	400	2800	316	4800	*
$K[Ru(dmgly)_2Cl_2] \cdot 3H_2O$	403	1366	315	2553	*

*Complexes described in this article.

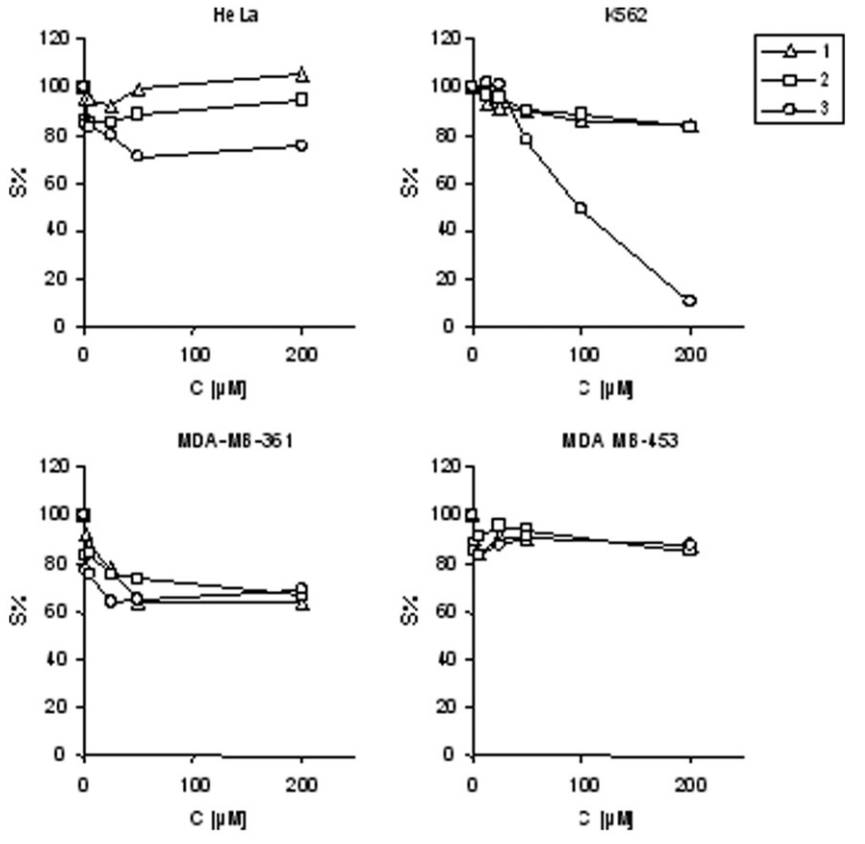

Figure 3. Representative graphs showing survival of tumor cells grown for 72 h in the presence of increasing concentrations of the investigated complexes determined by MTT test.

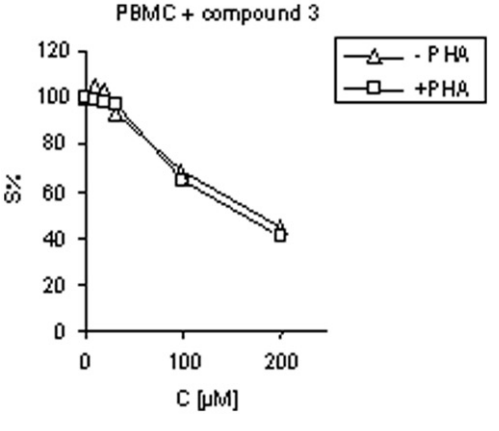

Figure 4. Representative graph showing survival of non-stimulated and PHA-stimulated PBMC for 72 h in the presence of increasing concentrations of **3** determined by MTT test.

inactive toward other tumor cell lines and PBMC from two other volunteers ($IC_{50} > 200\,\mu M$). These results indicate that tumor cell survival (HeLa, MDA-MB-361 and MDA-MB-453 cells) was not significantly decreased by increasing the dose of the investigated complexes (figure 3). Replacement of dmgly with sarcosine (sar) in previously prepared $K_2[Ru(dmgly)Cl_4] \cdot 2H_2O$ displaying tumor-specific cytotoxic action [complex **4**, figure 1(b)] [21], obviously led to $K_2[Ru(sar)Cl_4] \cdot H_2O$ [complex **1**, figure 1(a)] unable to reduce the survival of treated tumor cells (figure 3).

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