Unexplored features of Ru(II) polypyridyl complexes - towards combined cytotoxic and antimetastatic activity

Ilona Gurgul, Olga Mazuryk, Michal Łomzik, Philippe Gros, Dorota Rutkowska-Zbik, Malgorzata Brindell

► To cite this version:

Ilona Gurgul, Olga Mazuryk, Michal Łomzik, Philippe Gros, Dorota Rutkowska-Zbik, et al.. Unexplored features of Ru(II) polypyridyl complexes - towards combined cytotoxic and antimetastatic activity. *Metallomics*, 2020, 12 (5), pp.784-793. 10.1039/D0MT00019A . hal-02514957

HAL Id: hal-02514957

<https://hal.science/hal-02514957>

Submitted on 19 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Metallomics

Integrated biometal science

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: I. Gurgul, O. Mazuryk, M. omzik, P. C. Gros, D. Rutkowska-Zbik and M. Brindell, *Metallomics*, 2020, DOI: 10.1039/D0MT00019A.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The impact of metal-based drugs on cellular processes is crucial to understand their mode of action and enables to determine the key points important for improvement of their therapeutic index.

View Article Online
DOI: 10.1039/D0MT00019A

The presented data underline that ruthenium polypyridyl complexes except well-documented cytotoxic activity can have a high potency as anti-metastatic drugs.

We show that by appropriate modification of ligands it is possible to obtain compounds, which strengthen cells' adherent properties reducing the chance of metastatic cells to escape to surrounding tissue.

For the first time, we correlate the observed influence on cell adhesion properties with inhibition of the released and membrane-bound metalloproteinases on in vitro level as well as using isolated enzymes.

We postulate that metalloproteinases might be important cellular targets for ruthenium polypyridyl complexes, which are responsible for their potency in inhibition of metastasis.

Unexplored features of Ru(II) polypyridyl complexes - towards combined cytotoxic and antimetastatic activity

Ilona Gurgul^a, Olga Mazuryk^{a,*}, Michał Łomzik^{a,b,c}, Philippe C. Gros^c, Dorota Rutkowska-Żbik^d, Małgorzata Brindell^{a,*}

^aDepartment of Inorganic Chemistry, Faculty of Chemistry, Jagiellonian University in Kraków, Gronostajowa 2, 30-387 Krakow, Poland

^bDepartment of Organic Chemistry, Faculty of Chemistry, University of Łódź, ul. Tamka 12, 91-403 Łódź, Poland

^cUniversité de Lorraine, CNRS, L2CM, F-54000 Nancy, France

^dJerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Niezapominajek 8, 30-239 Krakow, Poland

Abstract

The well-documented cytotoxic activity of coordinatively saturated and substitutionally inert polypyridyl Ru(II) complexes substantiates to their high potency as antiproliferative agents against primary tumors. However, the primary cause of cancer morbidity and mortality responsible for about 90% of cancer deaths is the occurrence of metastasis. Therefore, scientists have to concentrate their efforts in designing compounds affecting not only the primary tumor, but also inhibiting efficiently the metastasis. Herein, we report two families of Ru(II) polypyridyl complexes bearing 2,2'-bipyridine substituted by a semicarbazone 2-formylpyridine moiety as one of the ligands and, 4,4'-di-*tert*-butyl-2,2'-dipyridyl or 4,7-diphenyl-1,10-phenanthroline as auxiliary ligands. These complexes strengthen cells' adherent properties as well as inhibit the activity of metalloproteinases (MMPs) *in vitro*, which is relevant in anti-metastatic treatment. The *in vitro* studies were performed on human lung adenocarcinoma (A549) and human pancreatic cancer (PANC-1) cells that have a well-documented invasive potential. The induced alteration of tumor cells' adhesion properties correlated with the high cytotoxic effect exerted by the complexes and their excellent cellular uptake. It was also proved that both complexes directly inhibit MMP2 and MMP9 enzyme activities, which are essential for the development of tumor metastasis. The results of this study indicate that the biological properties of polypyridyl Ru(II) complexes extend beyond the standard cytotoxic activity and represent an important step towards designing new anti-metastatic agents.

Introduction

Most compounds tested for their anticancer properties act as cytotoxic agents and their activity is focused on influencing primary tumors. However, the biggest problem in the treatment of cancer is the development of metastases. It is estimated that over 90% of deaths due to cancer are associated with metastases.[1, 2] So far, no effective for inhibition of metastasis formation are available on the market. Clinical trials concentrated primarily on the extracellular matrix metalloproteinase inhibitors and inhibitors of angiogenesis, however none

of the tested compounds have exhibited the desired therapeutic effect.[3, 4] It is postulated that affecting a number of other targets related to the process of metastasis, might be beneficial. It is believed that an essential precondition for successful clinical development requires targeting the correct portions of the metastatic cascade.[4, 5]

The detachment of cancer cells from their parent tumors is an initial event in metastasis. It often requires losing cell-cell and cell-matrix contact, followed by migration through the basement membrane (involving remodeling of actin cytoskeleton). Cancer cells must also degrade the surrounding tissue to move outside the primary organ. This can be achieved by an increased production of matrix metalloproteinases (MMPs), which are actively involved in many other steps of the metastatic cascade like intravasation, angiogenesis or formation of an appropriate metastatic niche.[6] In particular, it was shown that overexpression of MMP-2 and MMP-9[2, 7, 8] was frequently associated with the invasive and metastatic potential of tumor cells[7] and is a bad prognostic factor in a tumor.[9] Affecting any of the mentioned processes or ideally influencing several of them can bring benefits in inhibition of metastasis at its initial phase.

For many years there has been an increased interest in developing new metal based drugs using coordinatively saturated polypyridyl Ru(II) compounds bearing inert substituents.[10-13] The recent research has been directed particularly towards designing site-specific or targeted drugs that can be obtained either through appropriate modification of ligands by conjugation of specific molecules for targeting cancer cells or by application of stimuli-responsive prodrugs.[12, 13] Most of the *in vitro* studies were focused on the determination of the cytotoxic activity of Ru polypyridyl complexes as well as on the elucidation of the mechanisms responsible for the observed biological effects (cell death mechanism, induction of reactive oxygen species formation, the uptake and localization in cell, etc.).[11] There are only a handful of studies, that considered their potential antimetastatic activity. It was shown that Ru polypyridyl complexes can influence the cell-matrix adhesion properties[14, 15], disrupt microtubule function by acting as microtubule stabilizing agents[16] as well as decrease cell migration capability.[14, 17-19] Inspired by these findings, we have decided to thoroughly investigate the *in vitro* behavior of a group of Ru(II) polypyridyl complexes containing 2,2'-bipyridine ligand substituted by a semicarbazone2-formylopyridine moiety (**L1**, Fig.1 and Scheme 1), focusing on their impact on the alteration of tumor cells' adhesion properties that is relevant in anti-metastatic treatment.

Complexes **Ru1** and **Ru4** were recently prepared by our group and the mechanism of their cytotoxicity was reported.[20] Herein, we also describe the synthesis and characterization of the three new Ru(II) complexes from this series **Ru2**, **Ru3** and **Ru5**, including their photophysical properties as well as their structure and characterization of frontier orbitals obtained by applying Density Functional Theory (DFT) approach. In this study we examine the effect of auxiliary ligands in Ru(II) complexes on the cytotoxicity, cell adhesion properties, activity of matrix proteinases (MMPs) and other cellular responses. For *in vitro* studies human lung adenocarcinoma (A549) and human pancreatic cancer (PANC-1) cell lines have been chosen due to their well-documented invasive potential[21, 22] and their expression of MMP-2 and MMP-9 largely involved in tumor invasion and metastasis. [2, 7, 8]

Our study is aimed to speculate that the ruthenium complexes impact on cancer cells can be tuned towards their combined cytotoxic and antimetastatic activity by a selection of appropriate substituents in the polypyridyl ligands.

Figure 1. The studied ruthenium complexes.

Results and discussion

Synthesis and photophysical properties

The target complexes **Ru1** to **Ru5** were prepared in acceptable yields by reacting semicarbazone (SC)-based ligand **L1**, prepared according to the published reference[23], with the appropriate Ru(NN)₂Cl₂ complex in absolute ethanol under reflux (Scheme 1). The Ru(NN)₂Cl₂ complexes were prepared from ruthenium(III) chloride and the proper ligand in DMF under microwave irradiation. Interestingly, the coordination of ruthenium occurred selectively on the bipyridine side of ligand **L1** and no trace of ruthenium coordination by the SC moiety was observed.

Scheme 1. Synthesis of complexes **Ru1** to **Ru5**.

Despite all our efforts, the growing suitable crystals for **Ru2**, **Ru3** and **Ru5** was unsuccessful and thus no X-Ray diffraction-based structure of the complexes could be obtained. Thus, we focused on quantum chemical calculations within DFT approach for the structural determination of the newly synthesized compounds as well as the characterization of their frontier orbitals. Structural data are given in Supplementary Information while plots of HOMO and LUMO of all studied compounds are given in Fig. S1.[23] In **Ru1**, **Ru2**, and **Ru3** compounds, the HOMO spanned over the **L1** ligand, whereas LUMO was located on the bpy ligands. Similarly, for **Ru4**, HOMO is located on the **L1** moieties, while LUMO on the bpy residue of the **L1** ligand. In **Ru5**, HOMO is located on one of the SO₃⁻ groups and LUMO on the bpy of the **L1** moiety.

All the studied Ru(II) complexes exhibited broad and intense absorption bands in the visible region of the spectrum (400-500 nm), which is predominated by metal-to-ligand charge transfer (¹MLCT) character. Additionally, the studied ruthenium complexes exhibited a very intense absorption band in the UV region of the spectrum (250-300 nm). These bands were assigned to the intraligand $\pi \rightarrow \pi^*$ transition. Electronic absorption spectra of the studied complexes are presented in Fig. S3 and the main absorption bands wavenumbers with molar absorption coefficients are collected in Table 1. The Time-Dependent DFT (TD-DFT) calculations confirmed that the most intensive bands in the visible part of the spectrum show the MLCT character, and that they can be attributed to the excitation from a mixture of Ru d orbitals (mixed to a low extent with π orbitals of -SC-py fragment of **L1** ligand) to the π^* of bpy fragments of all ligands (for the schematic representation of the most intense absorption bands and their assignment as computed with TD-DFT see Fig. S2)

The studied Ru(II) complexes showed luminescence after excitation into the MLCT band and the emission bands are observed in the 610 - 640 nm range (Fig. S3, Tab. 1). In comparison to the excitation wavelength, a significant red shift of the emission energies (ca. 200 nm) is observed along with a quite high quantum yield and relatively long emission lifetime (Table 1). This should make them perfectly detectable by fluorescent microscopy when uptaken by cells.

Table 1. Photophysical data for all studied Ru(II) complexes in air-equilibrated and deoxygenated aqueous solutions. Absorption band maxima (λ), molar extinction coefficients (ϵ) emission wavelengths (λ_{em}) and quantum yields (Φ_{em}) were measured at 25 °C while emission lifetimes (τ_{em}) at room temperature.

	$\lambda_{\pi \rightarrow \pi^*}$ [nm] ($\epsilon \times 10^3$ [M ⁻¹ cm ⁻¹])	λ_{MLCT} [nm] ($\epsilon \times 10^3$ [M ⁻¹ cm ⁻¹])	λ_{em} [nm]	Φ_{em} [%] Air (Anaerobic)	τ_{em} [μ s] Air (Anaerobic)
Ru1	286 (108.6 $\pm$ 0.4) ^{a,b}	427 (12.3 $\pm$ 1.8) ^{a,b} 455 (14.8 $\pm$ 2.1) ^{a,b}	617 ^a	0.92 $\pm$ 0.09 ^a (1.62 $\pm$ 0.02) ^a	0.27 $\pm$ 0.03 ^a (0.36 $\pm$ 0.06) ^a
Ru2	286 (107.9 $\pm$ 0.3)	431 (11.6 $\pm$ 1.1) 459 (13.5 $\pm$ 1.3)	626	0.68 $\pm$ 0.01 (1.16 $\pm$ 0.03)	0.26 $\pm$ 0.02 (0.36 $\pm$ 0.04)
Ru3	286 (91.2 $\pm$ 0.5)	431 (9.6 $\pm$ 0.8) 458 (11.0 $\pm$ 0.9)	624	0.74 $\pm$ 0.04 (1.03 $\pm$ 0.02)	0.29 $\pm$ 0.05 (0.39 $\pm$ 0.02)
Ru4	279 (204.8 $\pm$ 0.8) ^{a,b}	435 (21.8 $\pm$ 1.7) ^{a,b}	623 ^a	1.18 $\pm$ 0.03 ^a	0.65 $\pm$ 0.03 ^a

		457 (22.2 ± 1.6) ^{a,b}		(2.62 ± 0.04) ^a	(1.72 ± 0.07) ^a
Ru5	279 (159.0 ± 0.9)	440 (18.0 ± 0.2)	618	2.21 ± 0.13	0.86 ± 0.04
		464 (17.4 ± 0.2)		(8.10 ± 0.09)	(2.90 ± 0.12)

^a taken from ref. [23], ^b the absorption coefficient were corrected considering hygroscopic properties of compounds

Cytotoxicity and uptake

The viability of human lung adenocarcinoma cells (A549) was inhibited by the studied Ru(II) complexes in a dose-dependent manner. The measured IC₅₀ values are listed in Table 2. The parent compound with two 2,2'-bipyridyl (bpy) ligands (**Ru1**) exhibited a low cytotoxicity, while the introduction of *tert*-butyl groups on the pyridine rings (**Ru3**) dramatically increased complex's potency in inhibition of the cell growth. **Ru4**, which has two 4,7-diphenyl-1,10-phenanthroline (dip) ligands, was the most cytotoxic compound. Further modification of phenyl rings in dip with sulfonate groups (**Ru5**) led to a change in the overall charge of Ru(II) complex from positive to negative and resulted in a substantial decrease in cytotoxicity. Our previous studies showed that the cytotoxicity of **L1** was at the same level as **Ru1**. [20] Under the tested conditions all Ru(II) complexes were more cytotoxic than cisplatin (IC₅₀ 1630 ± 60 μM). Another cancer cell line (human pancreas carcinoma cells (PANC-1) was even more sensitive towards the studied Ru(II) complexes than A549 cells (see Table S1).

Table 2. Cytotoxicity (IC₅₀) and uptake ([Ru]_{cell}/[Ru]_{medium})^a of the studied Ru(II) complexes evaluated for A549 cells along with determined lipophilicity (logP_{o/w})^b.

	IC ₅₀ /μM	[Ru] _{cell} /[Ru] _{medium} ^a	logP _{o/w} ^b
Ru1	>240	~2×	-1.54 ± 0.08
Ru2	158 ± 15	~18×	-0.86 ± 0.02
Ru3	14.1 ± 0.3	~90×	-0.11 ± 0.02
Ru4	10.7 ± 0.7	~85×	0.36 ± 0.01
Ru5	>240	~15×	-0.90 ± 0.10

^a The average value was obtained after the calculation the ratio between [Ru] in cell (determined by ICP-MS) and [Ru] in cell medium used for cell treatment independently determined for three concentrations: 3.5, 7 and 14 μM.

^blogP_{o/w}, where P_{o/w} is 1-octanol/water partition coefficient

The cytotoxicity of the studied Ru(II) complexes was found to be strongly dependent on their propensity to accumulate in the cells. The cell uptake of all the studied complexes was dose-dependent (Fig. 2). The direct quantification of the ruthenium concentration inside live A549 cells using ICP-MS revealed an equally excellent uptake of **Ru3** and **Ru4**, which was manifested in an approximately 90-fold or 85-fold increase in the concentration of ruthenium accumulated in cells in relation to the concentration in the cell medium for **Ru3** and **Ru4**, respectively (Table 1 and Fig. 2A). Cytotoxicity and uptake of the Ru complexes

are positively correlated with their lipophilicity (Pearson's r are 0.971 and 0.936, respectively, $p < 0.05$, Fig. S5). Such correlation between lipophilicity and IC_{50} has been already reported for several Ru complexes.[24, 25] Ru(II) complexes with the highest values of $\log P_{o/w}$ (0.36 for **Ru4** and -0.11 for **Ru3**) showed the highest uptake and the lowest IC_{50} values. On the other hand, **Ru1** with the lowest $\log P_{o/w}$ value exhibited poor accumulation and cytotoxic parameters. Even though the lipophilicity of **Ru4** was much higher than that of **Ru3** its uptake and cytotoxicity were at a similar level pointing out that the structure is another critical factor affecting the accumulation. **Ru1**, **Ru2**, **Ru3** and **Ru4** are +2 charged, so they enter the cells most probably via a passive diffusion facilitated by the membrane potential difference.[14] **Ru5** has a similar lipophilicity and uptake values as **Ru2** despite the overall charge -2, indicating that its accumulation goes either via energy-dependent pathway (endocytosis) or that its charge is at least partially compensated during accumulation with a suitable counter ion. Despite similar accumulation levels, **Ru2** and **Ru5** exhibited different cytotoxicity values, further confirming the relevance of structure on the biological activity of Ru complexes.

All studied Ru(II) complexes were luminescent, so their cellular uptake was followed using flow cytometry assuming that the emitted light by the Ru treated cells was proportional to the amount of the accumulated Ru(II) complexes (Fig. 2B). Since the luminescence quantum yield for these compounds is different (see Table 1) and strongly influenced by the environment[24] their concentration cannot be compared between each other but only separately for each of them. It is a fast and simple method for checking the internalization of luminescent Ru(II) complexes.

View Article Online
DOI: 10.1039/D0MT00019A

Fig. 2. Ruthenium accumulation in A549 cells determined after 24 h incubation with **Ru1** (green), **Ru2** (red), **Ru3** (blue), **Ru4** (purple) and **Ru5** (orange) presented as A) a concentration in a single cell (obtained from ICP-MS measurements) or B) luminescence increase vs. control (determined by flow cytometry, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 700 \text{ nm}$).

Impact of Ru complexes on cell adhesion properties

To check the changes in adhesion properties of A549 and PANC-1 cells upon exposure to the Ru(II) complexes their sensitivity to trypsin was evaluated. Cells, after 24 h of incubation with the tested compounds, were exposed to a diluted trypsin solution for a short time to avoid cells damage, and the amount of remaining trypsin-resistant cells was quantified using a resazurin assay. As shown in Figs. 3 and S6 all studied Ru(II) complexes significantly decreased the cells susceptibility to detachment by trypsin, on both plastic and collagen surfaces, and the effect was more pronounced for plastic. The observed effect was not concentration dependent, however, it can be directly correlated to the uptake, cytotoxicity and lipophilicity of the studied compounds. **Ru4** had the strongest impact on cells adhesion. It is a result of a synergistic effect of the hydrophobic, bulky dip ligands and quite elongated **L1** ligand with its hydrophilic end. Neither **L1** ligand alone nor $[\text{Ru}(\text{dip})_2(\text{bpy})]^{2+}$ induced such a strong resistance against trypsin (Fig. 3). The *in vivo* studies proved that trypsin-sensitive subpopulation of cancer cells generated more metastases than the trypsin-resistant subpopulation.[26] Therefore, by decreasing the ability of cancer cell to detach using Ru(II) treatment we may block cancer cells ability to leave the primary site and in this way decrease their metastatic potential. Recently, it was shown that genistein (derivative of isoflavone), can inhibit cell detachment by increasing cell adhesion *in vitro* and *in vivo* as well as in human clinical trials. Together with its inhibitory effect on protease production and cellular invasion made this compound a potent anti-metastatic agent.[27]

Fig. 3. A549 cell adherence to A) plastic or B) collagen coated surface evaluated as the percentage of remained adherent cells upon controlled trypsin treatment. Cells were treated for 24 h with **Ru1** (green), **Ru2** (red), **Ru3** (blue), **Ru4** (purple), **Ru5** (orange), [Ru(dip)₂(bpy)]²⁺ (pink) and **L1** (grey). Untreated cells were used as control (100%).

Change in the activity of matrix proteinases

Inhibition of matrix metalloproteinases (MMPs) suppresses the invasive potential of tumors. MMPs take part in physical barriers breakdown, which is a crucial step in tumor migration. They are involved in among others the degradation and remodeling of basement membrane and extracellular matrix (ECM) as well as cell adhesion properties.[28, 29] To determine if the observed changes in cells adhesion might arise from Ru complexes effects on MMPs, their cellular enzymatic activity was monitored using a fluorogenic substrate FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂; Mca = methoxycoumarin-4-acetyl). Some of MMPs, are only partly released into a soluble form whereas, the majority remains

attached to the cell surface. Furthermore, the six-membrane types of MMPs (MMP-14, -15, -16, -17, -24, and -25) are not released at all.[6] To determine the activity of dissolved MMPs the hydrolysis of FS-6 was measured in a cell-free conditioned medium from the A549 cells (supernatant), while cell-associated MMPs activity was investigated by addition of FS-6 into the A549 cell culture immediately after removing the supernatant. Proteolysis of FS-6 was also measured in supernatants and on the cell surface in the presence of the broad-spectrum MMPs inhibitor GM6001 (I1) and the serine protease inhibitor AEBSF (I2) as positive controls. As shown in Fig. 4 **Ru3** and **Ru4** exhibited the strongest inhibition of both, cell associated and dissolved MMPs in A549 cells, regardless of the applied concentration. Both of them are as potent as GM6001, which inhibition constant for several MMPs (MMP-1, 2, 3, 8 and 9) was in the low nanomolar range.[4] **Ru1** also inhibited MMPs activity but only at higher concentrations (from 4 to 8 μ M). No significant influence was observed for **Ru2** and **Ru5**. Inhibition of MMPs *in vitro* by the studied Ru complexes correlates well with their impact on cells adhesion.

Fig. 4. Hydrolysis of FS-6 (substrate for MMPs) by A549 cells A) *in situ* and B) by cell free supernatant after 24 h incubation with various concentrations of Ru complexes (1, 2, 4 and 8 μ M) versus control. MMPs inhibitor GM6001 (I1, 10 μ M) and serine protease inhibitor AEBSF (I2, 1mM) were used as positive control. Bars represent mean and SD from triplicate experiments.

A549 cells, are known to produce MMP-2 and MMP-9 [2, 7] that are key ECM-degrading enzymes in lung cancer cells. It was shown that inhibition of MMP-2 led to a decrease in tumor growth and invasiveness, so MMP-2 is an interesting target for antimetastatic therapy.[30] In turn, MMP-9 was recognized as an important enzyme involved in the formation of the metastatic niche[31], related to among others its ability to liberate vascular endothelial growth factor and thereby supporting angiogenesis.[32] To closer examine the observed inhibitory effect of **Ru3** and **Ru4** on MMPs *in vitro*, their impact on enzyme activity was evaluated on commercially available MMP2 and MMP9 enzymes (see Table S2). A potent broad-spectrum hydroxamate metalloproteinase inhibitor, GM6001, was used for comparison.[33] **Ru4** was highly potent with IC_{50} as low as 0.4 and 1.8 μ M for MMP2 and MMP9, respectively (Fig. 5). **Ru3** was ca. 25 times more potent in inhibition of MMP2 over MMP9 while **Ru4** had a similar effect on both enzymes. The obtained data suggest that the inhibition of MMPs might be engaged in alteration of adhesion properties by preventing the degradation of surface adhesion receptors. The detailed mechanism by which the studied Ru complexes interact with MMPs is currently under investigation, there is also a lack of data in the literature concerning applying Ru complexes as MMPs inhibitors.

Fig. 5. IC_{50} values for inhibition of MMP-2 (A) and MMP-9 (B) by **Ru3** and **Ru4** as well as the reference metalloproteinase inhibitor GM6001. Experimental conditions: [FS-6] = 2.5 μ M, [enzyme] = 0.5 nM, 0.1M Tris, pH 7.4 at 37 $^{\circ}$ C (MMP-9 was activated using 1 mM APMA, 4 $^{\circ}$ C, overnight and buffer was supplemented with 0.1M NaCl, 10mM $CaCl_2$, 0.1mM $ZnCl_2$ and 0.05% Brij35). Bars represent mean and SD from triplicate experiments.

Cellular responses upon treatment with Ru(II) complexes

To get better insight into changes induced by the treatment with Ru(II) complexes, several parameters were evaluated for A549 cells. The level of oxidative stress induced by the

studied Ru(II) complexes was evaluated by using various fluorescent probes (Fig. S7). Treatment of A549 cells with **Ru4** caused a pronounced increase in general ROS production, in particular pointing to the formation of H₂O₂ (see the explanation in supplementary material) as well as singlet oxygen and superoxide anion radical formation in a dose-dependent manner (Fig. S7). The high level of oxidative stress induced by **Ru4** correlates with its high cytotoxicity. The cell death in this case might be related to the huge ROS formation efficiency.[23] For **Ru3**, which has its toxicity and uptake similar to **Ru4**, only a slight elevation in ¹O₂ formation was determined which pointed out a different mechanism in cell death induction. Treatment of cells with **Ru2** also caused a small increase in H₂O₂ and singlet oxygen production. **Ru1** did not cause any significant ROS production most probably due to its poor accumulation.

Changes in mitochondrial membrane potential caused by the treatment of A549 cells with Ru(II) complexes were evaluated using JC-1 probe (Fig. S8). A marked decrease in the mitochondrial membrane potential was determined only for cells incubated with **Ru3** while other compounds (**Ru2**, **Ru4**, **Ru5**) had a tendency to slightly increase it. Similar disruption in the mitochondrial transmembrane potential observed for **Ru3** (regardless of the applied concentration in the range of 2.2-9.0 μM) as for both highly toxic positive controls gramicidin[20] (10 μM) and valinomycin (20 μM) suggests that this feature of **Ru3** might be responsible for its high cytotoxicity.

Cytosolic calcium concentration evaluated using Fluo-8 AM probe was altered only in the presence of **Ru3** and **Ru4** complexes in an opposite way (Fig. S9). **Ru3** greatly increased it while **Ru4** decreased it. This finding further supports different mechanisms of the induced cytotoxicity by both complexes proposed based on previously discussed induced oxidative stress and changes in the mitochondrial membrane potential.

Cellular re-adhesion properties – preliminary studies

Tumor cells, which have survived the treatment with cytotoxic drugs, managed to detach from the primary tumor mass, invade ECM to migrate towards blood vessels and finally succeeded in entering into a blood vessel can circulate until they are able to attach to a vessel wall and start extravasation. The attachment of cancer cells to new places is crucial in starting proliferation, which is the beginning of a secondary growth. Thus, the effect of the treatment of A549 cells with non-toxic dose of the studied Ru(II) complexes on anti-adhesion properties of cancer cells was examined by evaluating the ability of the treated cells to re-adhere to plastic surface. The decrease in re-adhesion of A549 cells to a plastic surface, as shown in Fig.6, occurred for cells treated with **Ru2**, **Ru3**, **Ru4** and **Ru5**, usually at higher concentrations. The most significant inhibition of cell adhesion was observed for **Ru4** and this effect became gradually larger as the concentration increased. A similar effect was observed for PANC-1 cell line (Fig. S10). Similar results were obtained for parent compound [Ru(dip)₂(bpy)]²⁺ (Fig. 6) as well as for compound, in which bpy was modified with a nitroimidazole unit (4-[3-(2-nitro-1H-imidazol-1-yl)propyl]).[14] The results are very promising, however to evaluate the relevance of this property, further studies using the *in vitro* flow adhesion assay on endothelial cells are planned to be performed.

Fig 6. Influence of **Ru1** (green), **Ru2** (red), **Ru3** (blue), **Ru4** (purple), **Ru5** (orange) and $[Ru(dip)_2(bpy)]^{2+}$ (pink) on A549 cells' ability to re-adhere to plastic surface, measured after 24 h incubation of cells with Ru(II) complexes.

Conclusions

In conclusion, among the studied series of Ru(II) polypyridyl complexes two of them **Ru3** and **Ru4** demonstrated very interesting biological properties. They exhibited high cytotoxicity against studied cancer cells that correlated very well with their excellent uptake by cells. In addition, both of them strongly influenced the adhesion properties of cells. The strengthening of cells' adherent properties upon treatment with **Ru3** and **Ru4** along with inhibition of released and membrane-bound MMPs was observed. Prevention of the detachment of tumor cells from cancer tissue, reduces the chance of metastatic cells to escape to the surrounding tissue. Therefore, these types of compounds might be beneficial not only in the treatment of solid tumors in standard chemotherapy as cytotoxic agents but also in inhibition of metastasis development. However, due to their high toxicity the use of drug delivery systems to increase selectivity towards cancer cells for in vivo application is necessary. In addition, preliminary studies showed that cells pre-treated with non-toxic doses of the studied compounds had lower ability to re-adhere to new place after detachment. This effect may contribute to a decrease in their ability for extravasation (needed to complete metastatic colonization), which requires arresting of circulated cells that is supported by specific adhesive interactions. We anticipate that our findings will draw the attention of scientists to unrecognized, underrated properties of polypyridyl Ru(II) complexes towards their application as antimetastatic agents. Still, there is a huge knowledge gap not only in the experimental work demonstrating such properties but particularly in the understanding of the molecular foundations of the observed effects, that will provide a mechanistic rationale for designing new chemotherapeutics.

Experimental

Metallomics Accepted Manuscript

Synthesis and photophysical characterization

View Article Online
DOI: 10.1039/D0MT00019A

All solvents were of analytical grade and were used without further purification. Reagents were purchased from Sigma-Aldrich. **Ru1**, **Ru4**, and ligand **L1** were obtained according to the published procedures.[20]

Synthesis of studied complexes – general procedure

A solution of **L1** (0.06 mmol) in absolute ethanol (3 mL) was added to a solution of *cis*-Ru(NN)₂Cl₂ (0.05 mmol) in absolute ethanol (5 mL). The resulting mixture was refluxed under argon atmosphere for 16h. After that time, solvent was removed under reduced pressure, and red-orange residue was dissolved in water (10 mL) and filtrated. Filtrate was evaporated until dry, dissolved in a small amount of dichloromethane with a few drops of methanol and precipitated out with diethyl ether. Orange powder was washed with diethyl ether and dried to give the pure product.

Synthesis of **Ru2**

NN = 4,4'-dimethyl-2,2'-bipyridine, yield 79% (36 mg); ¹H NMR (600 MHz, DMSO-*d*₆) 2.46 (d, J=6Hz, ~15H, superimposed with solvent signal), 2.94 (t, J=7.2 Hz, 2H); 3.07 (t, J=7.2 Hz, 2H); 6.64 (brs, 2H); 7.19-7.24 (m, 1H); 7.27-7.30 (m, 1H); 7.32-7.34 (m, 4H); 7.43 (d, J=11.4 Hz, 1H); 7.46 (d, J=6.6 Hz, 1H); 7.50-7.52 (m, 3H); 7.65 (dd, J=8.4 and 1.8 Hz, 1H); 8.09 (d, J=8.4 Hz, 1H); 8.31 (s, 1H); 8.68-8.73 (m, 6H); 10.59 (s, 1H). HRMS calculated for C₄₆H₄₄N₁₀ORuCl₂ m/z = 427.1366 (M²⁺ -2Cl), found m/z = 427.1370 (M⁺ -2Cl).

Synthesis of **Ru3**

NN = 4,4'-di(tert-butyl)-2,2'-bipyridine, yield 76% (33 mg); ¹H NMR (600 MHz, DMSO-*d*₆) 1.32 - 1.37 (m, 36H); 2.51 (s, ~3H superimposed with solvent signal); 2.69 (t, J=6.0 Hz, 2H); 3.09 (t, J=6.0 Hz, 2H); 7.38 (dd, J=6.0 and 2.4 Hz, 2H); 7.46-7.58 (m, 10H); 7.63 (d, J=24 Hz, 1H); 7.90 (dd, J=8.4 and 1.8 Hz, 1H); 8.48 (s, 1H); 8.78-8.89 (m, 6H); 10.61 (s, 1H); 12.73 (s, 1H). HRMS calculated for C₅₈H₆₈N₁₀ORuCl₂ m/z = 1021.4537 (M²⁺-2Cl -H⁺), found m/z = 1021.4468 (M²⁺-2Cl -H⁺)

Synthesis of **Ru5**

NN = : 4,7-Diphenyl-1,10-phenanthroline-disulfonic acid disodium salt trihydrate, Product was purified by flash chromatography (neutral Al₂O₃, dichloromethane/methanol 5-10% (gradient). Yield 42% (22 mg); ¹H NMR (600 MHz, DMSO-*d*₆): (ppm) 2.52 (s, superimposed with solvent signal); 2.93-2.99 (m, 2H); 3.06-3.11 (m, 2H); 7.58-7.69 (m, 16H); 7.78-7.83 (m, 10H); 7.89 (s, 4H); 8.18 (s, 2H); 8.23-8.31 (m, 3H); 8.35 (s, 1H); 8.37 (s, 2H). HRMS: calculated for C₇₀H₄₈N₁₀O₁₃S₄RuNa₄ m/z = 779.0454(M²⁺), found m/z = 779.0450 (M²⁺)

Spectroscopic measurements were performed according to previously published procedures and its overview is given in the supplementary information along with the details for computational characterization and other photophysical measurements.[23]

Cell culturing and cytotoxicity assay

Human lung adenocarcinoma A549 and human pancreas carcinoma PANC-1 cell were routinely cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 1% penicillin-streptomycin solution (100 units/ml-100µg/ml) (v/v), at 37°C in humidified atmosphere with 5% CO₂ (v/v). Cell viability upon treatment with Ru(II) complexes was determined using the MTT assay. Cells were seeded into 96-well plate with the density of 3 × 10⁴ cells per cm² in complete medium and cultured for 24 h. Then the medium was removed

and various concentrations of Ru(II) complexes in basic medium (without FBS) were added to the wells. Stock solutions of the Ru(II) complexes were prepared in DMSO. The final DMSO concentration in a cell culture was fixed (0.1% (v/v) for Ru3 and Ru4 and 0.5% (v/v) for Ru1, Ru2 and Ru5). After 24 h of incubation cells were washed with PBS and MTT solution (0.5 mg/ml, 100 μ L) was added to each well. Cells were incubated for 3 h at 37°C. Next, MTT was removed and the formed violet formazan crystals were dissolved in a 100 μ L of DMSO:methanol (1:1) mixture. The absorbance was measured using Tecan Infinite 200 microplate reader at 565 nm with 700 nm as a reference wavelength. Experiments were performed in triplicates and repeated three times. Results are presented as mean values and standard error of the mean. IC₅₀ parameters were determined using the Hill equation (OriginPro 2018).

Cellular uptake of Ru compounds

Cellular uptake of Ru complexes was determined using A549 cell line. A549 cells were seeded in a 6-well plate with the density of 4×10^4 cells per cm² in complete medium and cultured for 24 h. Then the medium was removed and cells were treated with various concentrations of Ru(II) complexes in basic medium for 24 h. In order to correctly measure accumulation of Ru complexes only non-toxic concentrations were used. Afterwards the incubated cells were washed, detached by trypsin treatment and counted. Additional attention was paid to eliminate dead cells by washing cells' monolayer with PBS. The cells were isolated by centrifugation and digested in concentrated nitric acid overnight at room temperature. The solutions were diluted with Millipore water to a final nitric acid concentration of 10%. The Ru content in the samples was measured by inductively coupled plasma mass spectrometry (ICP-MS). Experiments were repeated three times. Results were calculated as ruthenium concentration per cell and presented as a mean values and standard deviation of mean. Additionally, after detachment by trypsin cells were suspended in PBS buffer and analysed by BD FACSVerse cytometer (λ_{ex} = 488 nm and λ_{em} = 700 ± 27 nm). Experiment was performed twice and the mean values and standard error of the mean are presented.

Trypsin resistance assay

The ability of A549 and PANC-1 cells to detach from the surface after their exposure to Ru(II) complexes was evaluated by checking their resistance to trypsin treatment. Cells were seeded into a 96-well plate on the uncoated bottom of plastic wells or on collagen coated surfaces with the density of 3×10^4 cells per cm² in a complete medium and cultured for 24h. Then the complete medium was removed and various concentrations of the studied complexes were added to the wells for a 24h incubation. Afterwards the cells were washed and 30 μ L of a trypsin solution (0.05%) was added to each well and incubated for 10 min at 37°C. Then the cells were washed with PBS and a resazurin assay was performed to quantify the adherent cells according to the procedure published by us.[23] The obtained results were normalized with respect to corresponding wells without the trypsin treatment to exclude the possible toxicity of the studied compounds and are presented as a percentage of untreated cells. Experiments were performed in triplicates and each experiment was repeated five times to obtain mean values and the standard error of the mean.

Activity of matrix proteinases assays

In vitro MMPs' inhibition assays

The MMP enzymatic activity was quantitated using FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂) fluorogenic substrate (Sigma-Aldrich). Cells were seeded into a 96-well plate with the density of 3×10^4 cells per cm² in a complete medium and cultured for 24 h. Then the medium was removed and various concentrations of the studied Ru complexes were added for 24 h incubation. To determine the soluble MMP activity, supernatant (80 μ L) was transferred to a black 96-well plate, FS-6 (final concentration 5 μ M) was added to each well and incubated for 30 min. The activity of cell membrane-associated MMP was measured using Ru(II) treated cells by adding FS-6 solution (final concentration 5 μ M) directly to the cells and incubating for 30 min. After that, 80 μ L of the obtained solution were transferred to a black 96-well plate. Next the enzymatic reactions were terminated by the addition of 90 μ L of 3% (w/v) sodium acetate. Fluorescence of the samples was detected at 400 nm using a 345 nm excitation wavelength (Tecan Infinite 200 microplate reader). MMP inhibitor GM6001 (10 μ M) and serine protease inhibitor AEBSF (1 mM) incubated with the cells for 4 h were used as a positive control. Experiments were performed in triplicates and results are presented as a mean values and standard error of the mean.

MMP-2 and MMP-9 inhibition

Inhibitor potency of Ru₃, Ru₄ and GM6001 (as a reference) against human MMP-2 and MMP-9 was studied using the fluorogenic substrate FS-6. MMP-2 and MMP-9 enzymes were obtained from Sigma-Aldrich. MMP-9 was activated using APMA (1 mM, 4 °C, overnight) prior to the experiments. MMP-2 assays were performed in 0.1 M Tris/HCl (pH 7.4, 37 °C), while for the MMP-9 evaluation Tris/HCl buffer was additionally enriched with 0.1 M NaCl, 10 mM CaCl₂, 0.1 mM ZnCl₂ and 0.05% Brij35. The final concentrations of enzyme and substrate were kept constant at 0.5 nM and 2.5 μ M, respectively. Kinetic fluorescence measurements were performed using a Perkin Elmer LS55 spectrofluorimeter ($\lambda_{\text{ex/em}}$ = 325/400 nm). The enzyme activity in the presence of inhibitors was expressed as a fraction of initial reaction rate (v_i/v_0 ratio, v_0 - initial reaction rate, v_i – initial reaction rate in the presence of inhibitor). Experiments were repeated three times and results are presented as mean values and standard deviation of the mean. IC₅₀ parameters were calculated from a dose-response plot of enzyme fractional activity as a function of inhibitor concentration, using the Hill equation (OriginPro2018).

Re-Adhesion assay

The effect of the studied complexes on adhesion properties of cancer cells was also examined by evaluating the ability of the treated cells to re-adhere. Cells were seeded into a 6-well plate with the density of 3×10^4 cells per cm² in complete medium and cultured for 24 h. Then, the medium was removed and various concentrations of the studied Ru(II) complexes were added and incubated with the cells for 24 h. Afterwards, the cells were washed, and incubated with a fresh portion of PBS without Mg and Ca ions for 20 min. Then, the cells were detached with a cell dissociation solution, counted and seeded into 96-well plates with the density of 6×10^4 cells per cm². Plates were incubated for 1 h in humidified atmosphere at 37 °C and then were

washed with PBS to remove non-adherent cells. Resazurin assay was performed to quantify the adherent cells according to the published procedure.[23] Detachment of cells treated with Ru4 was not possible by using the cell dissociation solution so instead a trypsin solution (0.05%) was used. Experiments were performed in triplicates and each experiment was repeated five times to calculate mean values and the standard error of the mean.

Influence of Ru(II) complexes on other cellular processes

Evaluation of oxidative stress, changes in mitochondrial membrane potential and cytosolic calcium concentration were performed using various fluorescent probes according to the manufacturers protocols and full experimental details are given in the supplementary information.

Statistical analysis

For in vitro experiments, all data were expressed as the mean $\pm$ standard error of the mean (SEM). Pearson correlation coefficients were calculated using Statistica 13 software. Probabilities of $p < 0.05$ were considered as statistically significant.

Acknowledgments

M.B. thanks for financial support from National Science Center (DEC-2016/21/B/NZ7/01081). I.G. acknowledges the support of InterDokMed project no. POWR.03.02.00-00-I013/16. The research was carried out with equipment purchased with financial support from the European Regional Development Fund within the framework of the Polish Innovation Economy Operational Program (contract no. POIG.0 2.01.00-12-0 23/08).

References

- [1] P.S. Steeg, Targeting metastasis, *Nat. Rev. Cancer*, 16 (2016) 201-218
- [2] J. Araya, M. Maruyama, K. Sassa, T. Fujita, R. Hayashi, i.S. Matsu, T. Kashii, N. Yamashita, E. Sugiyama, i.M. Kobayash, Ionizing radiation enhances matrix metalloproteinase-2 production in human lung epithelial cells, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 30-38 (2001).
- [3] D.-Y. Lu, T.-R. Lu, H.-Y. Wu, New insights into individualized antimetastatic therapy., *Adv. Tech. Biol. Med.*, 1 (2013) 1000106 (1000101-1000103).
- [4] R.E. Galaray, M.E. Cassabonne, C. Giese, J.H. Gilbert, F. Lapierre, H. Lopez, M.E. Schaefer, R. Stack, M. Sullivan, B. Summers, R. Tressler, D. Tyrrell, J. Wee, S.D. Allen, J.J. Castellot, J.P. Barletta, G.S. Schultz, L.A. Fernandez, S. Fisher, T.Y. Cui, H.G. Foellmer, D. Grobelyny, W.M. Holleran, Low Molecular Weight Inhibitors in Corneal Ulceration, *Ann. N. Y. Acad. Sci.*, 732 (1994) 315-323.
- [5] A.F. Chambers, I.C. MacDonald, E.E. Schmidt, V.L. Morris, A.C. Groom, Clinical targets for anti-metastasis therapy, *Adv. Cancer Res.*, 79 (2000) 91-121.
- [6] K. Kessenbrock, V. Plaks, Z. Werb, Matrix Metalloproteinases: Regulators of the Tumor Microenvironment, *Cell*, 142 (2010) 52-67.
- [7] R. Vinodhkumar, Y. Song, V. Ravikumar, G. Ramakrishnan, T. Devaki, Depsipeptide a histone deacetylase inhibitor down regulates levels of matrix metalloproteinases 2 and 9 mRNA and protein expressions in lung cancer cells (A549), *Chem.-Biol. Interact.*, 165 (2007) 220-229.

- [8] S.E. Ghlban, T. Kasai, T. Shigehiro, H.X. Yin, S. Sekhar, M. Ida, A. Sanchez, A. Mizutani, T. Kudoh, H. Murakami, M. Seno, Chlorotoxin-Fc Fusion Inhibits Release of MMP-2 from Pancreatic Cancer Cells, *BioMed Res. Intern.l*, 2014 (2014) 152659.
- [9] Z.H. Yao, T. Yuan, H.Y. Wang, S.N. Yao, Y. Zhao, Y. Liu, S.L. Jin, J.F. Chu, Y.L. Xu, W.P. Zhou, S.J. Yang, Y.Y. Liu, MMP-2 together with MMP-9 overexpression correlated with lymph node metastasis and poor prognosis in early gastric carcinoma, *Tumor Biol.*, 39 (2017) No. 700411
- [10] F.E. Poynton, S.A. Bright, S. Blasco, D.C. Williams, J.M. Kelly, T. Gunnlaugsson, The development of ruthenium(II) polypyridyl complexes and conjugates for in vitro cellular and in vivo applications, *Chem. Soc. Rev.*, 46 (2017) 7706-7756.
- [11] A. Notaro, G. Gasser, Monomeric and dimeric coordinatively saturated and substitutionally inert Ru(II) polypyridyl complexes as anticancer drug candidates, *Chem. Soc. Rev.*, 46 (2017) 7317-7337.
- [12] X.H. Wang, X.Y. Wang, S.X. Jin, N. Muhammad, Z.J. Guo, Stimuli-Responsive Therapeutic Metallodrugs, *Chem. Rev.*, 119 (2019) 1138-1192.
- [13] E. Villemin, Y.C. Ong, C.M. Thomas, G. Gasser, Polymer encapsulation of ruthenium complexes for biological and medicinal applications, *Nat. Rev. Chem.*, 3 (2019) 261-282.
- [14] O. Mazuryk, F. Suzenet, C. Kieda, M. Brindell, Biological effect of nitroimidazole derivative of polypyridyl ruthenium complex on cancer and endothelial cells, *Metallomics*, 7 (2015) 553-566.
- [15] U. Schatzschneider, J. Niesel, I. Ott, R. Gust, H. Alborzinia, S. Wolfl, Cellular Uptake, Cytotoxicity, and Metabolic Profiling of Human Cancer Cells Treated with Ruthenium(II) Polypyridyl Complexes [Ru(bpy)₂(N--N)]Cl₂ with N--N=bpy, phen, dpq, dppz, and dppn, *ChemMedChem*, 3 (2008) 1104-1109.
- [16] N. Alatrash, F.H. Issa, N.S. Bawazir, S.J. West, K.E. Van Manen-Brush, C.P. Shelor, A.S. Dayoub, K.A. Myers, C. Janetopoulos, E.A. Lewisc, F.M. MacDonnell, Disruption of microtubule function in cultured human cells by a cytotoxic ruthenium(II) polypyridyl complex, *Chem. Sci.*, (2019) 10.1039/C1039SC05671H.
- [17] C. Wang, Q.Q. Yu, L.C. Yang, Y.Y. Liu, D.D. Sun, Y.C. Huang, Y.H. Zhou, Q.L. Zhang, J. Liu, Ruthenium (II) polypyridyl complexes stabilize the bcl-2 promoter quadruplex and induce apoptosis of Hela tumor cells, *Biometals*, 26 (2013) 387-402.
- [18] W.Q. Cao, W.J. Zheng, T.F. Chen, Ruthenium Polypyridyl Complex Inhibits Growth and Metastasis of Breast Cancer Cells by Suppressing FAK signaling with Enhancement of TRAIL-induced Apoptosis, *Sci. Rep.*, 5 (2015) No. 9157.
- [19] A.F. Tikum, Y.J. Jeon, J.H. Lee, M.H. Park, I.Y. Baea, S.H. Kim, H.J. Lee, J. Kim, Cytotoxic and anticancer properties of new ruthenium polypyridyl complexes with different lipophilicities *J. Inorg. Biochem.*, 180 (2018) 204-210.
- [20] M. Łomzik, O. Mazuryk, D. Rutkowska-Zbik, G. Stochel, P.C. Gros, M. Brindell, New ruthenium compounds bearing semicarbazone 2-formylpyridine moiety: Playing with auxiliary ligands for tuning the mechanism of biological activity, *J. Inorg. Biochem.*, 175 (2017) 80-91.
- [21] M. Fujita, K. Imadome, T. Imai, Metabolic characterization of invaded cells of the pancreatic cancer cell line, PANC-1, *Cancer Sci.*, 108 (2017) 961-971.
- [22] M.-W. Lin, C.H. Law, H.C. Chou, Identifying the invasive nature of a distinct population from A549 adenocarcinomic lung cancer cell, *Biomark. Gen. Med.*, 6 (2014) 159-162.
- [23] M. Łomzik, O. Mazuryk, D. Rutkowska-Zbik, G. Stochel, P.c. Gros, M. Brindell, New ruthenium compounds bearing semicarbazone 2-formylpyridine moiety: Playing with auxiliary ligands for tuning the mechanism of biological activity, *J. Inorg. Biochem.*, 175 (2017) 80-91.

- [24] O. Mazuryk, K. Magiera, B. Rys, F. Suzenet, C. Kieda, M. Brindell, Multifaceted interplay between lipophilicity, protein interaction and luminescence parameters of non-intercalative ruthenium(II) polypyridyl complexes controlling cellular imaging and cytotoxic properties, *J. Biol. Inorg. Chem.*, 19 (2014) 1305-1316.
- [25] G.H. Ribeiro, L. Colina-Vegas, J.C.T. Clavijo, J. Ellena, M.R. Cominetti, A.A. Batista, Ru(II)/N-N/PPh₃ complexes as potential anticancer agents against MDAMB-231 cancer cells (N-N=diimine or diamine), *J. Inorg. Biochem.*, 193 (2019) 70-83.
- [26] C. Morata-Tarifa, G. Jiménez, M.A. García, J.M. Entrena, C. Griñán-Lisón, M. Aguilera, M. Picon-Ruiz, J.A. Marchal, Low adherent cancer cell subpopulations are enriched in tumorigenic and metastatic epithelial-to-mesenchymal transition-induced cancer stem-like cells, *Sci. Rep.*, 6 (2015) 18772.
- [27] M.J. Pavese, S.N. Krishna, R.C. Bergan, Genistein inhibits human prostate cancer cell detachment, invasion, and metastasis, *Am. J. Clin. Nutr.*, 100 (2014) suppl. 431S-436S.
- [28] A.T. Ho, E.B. Voura, P.D. Soloway, K.L.M. Watson, R. Khokha, MMP Inhibitors Augment Fibroblast Adhesion through Stabilization of Focal Adhesion Contacts and Up-regulation of Cadherin Function, *J. Biol. Chem.*, 276 (2001) 40215-40224.
- [29] A. Das, M. Monteiro, A. Barai, S. Kumar, S. Sen, MMP proteolytic activity regulates cancer invasiveness by modulating integrins, *Sci. Rep.*, 7 (2017) 14219.
- [30] O. Kargiotis, C. Chetty, C.S. Gondi, A.J. Tsung, D.H. Dinh, M. Gujrati, S.S. Lakka, A.P. Kyritsis, J.S. Rao, Adenovirus-mediated transfer of siRNA against MMP-2 mRNA results in impaired invasion and tumor-induced angiogenesis, induces apoptosis in vitro and inhibits tumor growth in vivo in glioblastoma, *Oncogene*, 27 (2008) 4830-4840.
- [31] R.N. Kaplan, R.D. Riba, S. Zacharoulis, A.H. Bramley, L. Vincent, C. Costa, D.D. MacDonald, D.K. Jin, K. Shido, S.A. Kerns, Z. Zhu, D. Hicklin, Y. Wu, J.L. Port, N. Altorki, E.R. Port, D. Ruggero, S.V. Shmelkov, K.K. Jensen, S. Rafii, D. Lyden, VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche, *Nature*, 438 (2005) 820-827.
- [32] G. Bergers, R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, D. Hanahan, Matrix metalloproteinase- triggers the angiogenic switch during carcinogenesis., *Nat. Cell Biol.*, 2 (2000) 737-744.
- [33] A.L. McNulty, Weinberg J. B., F. Guilak, Inhibition of matrix metalloproteinases enhances in vitro repair of the meniscus. , *Clin. Orthop. Relat. Res.*, 467 (2009) 1557-1567