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Study of dinuclear Rh(II) complexes of phenylalanine derivatives as potential anticancer agents by using X-ray fluorescence and X-ray absorption

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In vitro antitumor efficacy of several dinuclear bridgings and one chelate structure dirhodium(II) complex of *N*-protected phenylalanine derivatives were tested on HT-29 cells. The following synthesized and previously characterized complexes were applied in the present work: $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ (n = 1–4, $^-$O-Phe-*Z* = *N*-benzyloxycarbonyl-L-phenylalaninate), $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ (n = 1–4, $^-$O-Phe-Ac = *N*-acetyl-L-phenylalaninate), $Rh_2(OAc)_2$(*N*-Me-D-Phe-O)$_2$ corresponding to *N*-methyl-D-phenylalaninate as well as $Rh_2(OAc)_4$ ($^-$OAc = acetate). Depending on the complex ligand type and its coordination number, the intracellular rhodium (Rh) content determined by total reflection X-ray fluorescence (TXRF) spectrometry in the HT-29 cells varied between 25 and 2500 ng/10^6 cells. *In vitro* cytotoxicity and cytostatic evaluations of the compounds on HT-29 human cell culture were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Compared to $Rh_2(OAc)_4$, the Rh compounds containing one or two $^-$O-Phe-*Z* moieties proved to be the most effective on the HT-29 cells. Moreover, synchrotron radiation TXRF–X-ray absorption near edge structure measurements suggested a change of the molecular symmetry of the dirhodium(II) center for the moderately *in vitro* cytotoxic, lipophilic L-phenylalanine derivative complexes, characterized also by low ligand exchange rate when they were studied on HT-29 cells.

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1. Introduction

The successful application of *cis*-$Pt(NH_3)_2Cl_2$ as metal-based antitumor drug has paved the way to the development of other chemotherapeutic anticancer agents with improved specificity, reduced toxicity and cell resistance. In recent years, interactions of dinuclear, frequently acetato-bridged metal species of molybdenum, rhenium, ruthenium (Ru) and rhodium (Rh) with nucleobases have been intensively studied [1] following early reports of considerable antitumor effects of dirhodium(II) carboxylates [2,3]. The main mode of action of many antitumor drugs consists of the disruption of transcription and related processes. Generally, the inhibition of transcription by these drugs is achieved through modification or damage of the DNA template. Metal species can interact with DNA in one of the two following ways: i) directly via coordination to phosphate oxygen atoms, sugar oxygen atoms, atoms of the heterocyclic bases (N, C, O) or combination of thereof; ii) indirectly via its other ligands. This latter possibility includes H-bond formation (between aqua or amine ligands and suitable acceptors of the nucleic acids) or π–π interactions such as intercalation and groove binding (between nucleic acid and metal entity containing heteroaromatic auxiliary ligands) [1]. The platinum-based drugs existing so far in clinical use are DNA-targeted metal containing compounds that are believed to bind DNA coordinatively [4,5]. The main concerns with cisplatin, such as high systemic toxicity, limited

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range of activity [6], and development of tumor resistance [7] have led to the need of designing alternative anticancer drugs, *i.e.*, transition metal complexes. Ruthenium and Rh complexes (with their different oxidation states and also di- or trinuclear compounds) may be expected to exhibit different reactivity towards cellular targets as compared to cisplatin.

Dirhodium(II, II) compounds play an important role in the field of metal–metal chemistry [8]. Formation of a dinuclear Rh–Rh single bond is a key factor in stabilizing the Rh_2^{4+} units. According to the molecular orbital theory, eight of the 14 electrons are distributed in the σ-, π- and δ-orbitals and the remaining six electrons occupy π*- and δ*- antibonding orbitals, resulting in a net Rh–Rh bond order of one and no unpaired electrons. Complexes with Rh_2^{4+} cores have a stable paddlewheel (~ lantern) structure and, generally, they possess one or two Lewis base-type axial ligands [9] usually weakly binding one or two nucleophilic solvent molecules (*i.e.*, dimethyl sulfoxide (DMSO), acetonitrile, water) in axial position, as well (Fig. 1a). Dirhodium(II) μ-carboxylate complexes are most commonly obtained by reduction of Rh(III) compounds in alcohols but the most efficient synthesis method involves refluxing of Rh(III) salt in a carboxylic acid solution or an alcohol and carboxylic acid mixture [10]. By rapid ligand exchange reactions, the acetate ions of $Rh_2(OAc)_4$ (Fig. 1a) can be replaced by mono- and other bidentate ligands. Since axial ligands are quite labile, bridging or chelating complexes may be formed by a slow rearrangement [8,11]. Adduct formation starting with $Rh_2(OAc)_4$ is a stepwise process where the initial interaction is axial, which is followed by a shift of the newly incorporated axial ligand to an equatorial position [12]. Several papers have been published on synthesis and characterization of dinuclear Rh complexes with amino acids as ligands [13–17]. Lately, the more specific syntheses with unprotected [18,19] and protected amino acids [20,21] have been also reported. Other articles focused on dirhodium(II) complexes serving as reagents for cross-linking of proteins [22] or inhibition of protein synthesis [23].

The studies on the *in vitro* cytotoxicity of Rh complexes have been focused on dirhodium(II) compounds, mainly on carboxylates, which are among the most promising non-platinum antitumor complexes [9]. Early studies in the 1970's showed that $Rh_2(O_2CR)_4$ (R = Me, Et, Pr) exhibit *in vivo* antitumor activity against L1210 tumors [24], and Ehrlich ascites [25]. In some cases, the antitumor activity *in vitro* was reported to be of the same order of magnitude as that of the anticancer drug cisplatin [26,27]. The antitumor activity increased with the lipophilicity of the alkyl group but further lengthening of the carboxylate moiety reduces the therapeutic efficacy of the compound [9]. It was also shown that the partially oxidized species in the solution, $[Rh_2(OOR_4)]^+$, are also active against tumors [28]. The availability of the axial coordination site is presumably responsible for their biological activity [29,30].

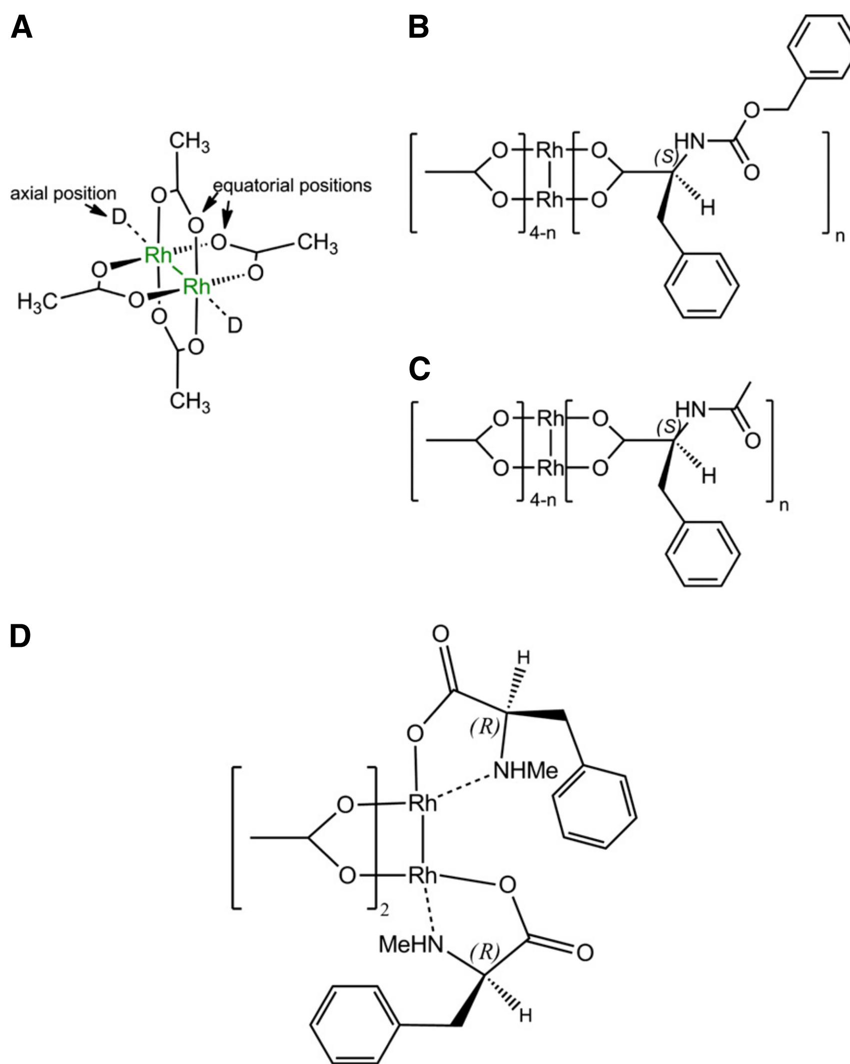


Fig. 1. Structural formulae of the investigated dirhodium(II) complexes: A) $Rh_2(OAc)_4D_2$, where D = nucleophilic ligands in axial position; B) $Rh_2(OAc)_{4-n}$(*N*-benzyloxycarbonyl-L-phenylalaninate)$_n$ (n = 1–4); C) $Rh_2(OAc)_{4-n}$(*N*-acetyl-L-phenylalaninate)$_n$ (n = 1–4); D) $Rh_2(OAc)_2$(*N*-methyl-D-phenylalaninate)$_2$. Complexes illustrated in figures A–C are bridging type, while complex indicated at D has a chelate type of structure.

Steric hindrance is a key factor for lower biological activity, since it precludes the access of biological targets to the axial and equatorial sites of the dirhodium(II) core [9]. Fine tuning of other features such as charge, solubility, lability of bridging groups, *etc.* has led to the design of additional dirhodium(II) complexes to reach a compromise between *in vitro* and/or *in vivo* antitumor activity [9]. For example, Rh_2(L-Phe)$_2$(OAc)$_2$ had a strong activity towards human colon adenocarcinoma cells and its effect was not accompanied by generation of reactive oxygen species neither by activation of caspase-3 [18]. Dirhodium(II) compounds interact with DNA [30], bind to DNA [31] and potent inhibitors of transcription [29,32,33] although their precise mechanism of action has not yet been elucidated. In 1976, by studying the amino acid coordination to dirhodium(II) tetraacetate complex, it was suggested that binding was achieved through the amino acid side chains with hetero atoms [34].

Previously, several chiral complexes with amino acid ligands having different hydrophobic properties were synthesized and chemically characterized [21]. The aims of the present study were: i) investigation of the cellular Rh content originating from dirhodium(II) complexes of D- and L- isomers of phenylalanine derivatives and chelate and bridging type of geometry, respectively, as potential anticancer drugs; ii) determination of *in vitro* cytotoxicity and cytostatic activities of these compounds; iii) characterization of the chemical environment of these Rh containing complexes in HT-29 cells. In order to achieve these goals, total-reflection X-ray fluorescence (TXRF) spectrometry [35] complemented with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and X-ray absorption near edge structure (XANES) analysis were performed for quantitative determination and elemental speciation of cellular samples [36,37], respectively.

2. Materials and methods

2.1. Chemicals

Throughout the experiments, deionized Milli-Q (Millipore, Billerica, MA, US) water with a relative conductivity of 18.2 MΩ cm was used. All chemicals were of analytical grade, if not stated otherwise. The stock solution of 1000 mg L^{-1} Ga was purchased from Merck (Darmstadt, Germany). Concentrated nitric acid (65%) and hydrogen peroxide (30%) of Suprapur quality needed for sample preparation of cells were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS), prepared from Sigma-Aldrich reagents, had the following composition: 0.2 g L^{-1} KCl, 8 g L^{-1} NaCl, 2.2 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$ and 0.2 g L^{-1} KH_2PO_4. Rhodium(III) chloride ($RhCl_3$) hydrate and dirhodium(II) μ-tetracarboxylate were purchased from Sigma-Aldrich (Budapest, Hungary). Fetal calf serum (FCS), RPMI-1640 (Roswell Park Memorial Institute), L-glutamine, gentamicin and trypsin were supplied by Sigma-Aldrich (Budapest, Hungary).

2.2. Synthesis of the dirhodium(II) complexes

The $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ complexes (n = 1–4 and $^{-}$O-Phe-*Z* corresponding to *N*-benzyloxycarbonyl-L-phenylalaninate) were prepared in different solvents and identified according to our previous work [21]. The same procedure was applied for the synthesis of $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ complexes (n = 1–4 and $^{-}$O-Phe-Ac corresponding to *N*-acetyl-L-phenylalaninate [38] and $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$ corresponding to *N*-methyl-D-phenylalaninate) [20]. All representatives of these complexes are illustrated in Fig. 1b–d.

2.3. Cell culture and sample preparation for TXRF and TXRF–XANES

The adherent, epithelial-like human HT-29 colorectal adenocarcinoma cells (HT-29, ATCC® number: HTB-38) were cultured in a complete medium prepared from RPMI-1640 supplemented with 10% v/v FCS, 2 mM L-glutamine and 160 μgmL^{-1} gentamicin; at 37 °C in 5% v/v CO_2 and water saturated atmosphere [39,40]. Twenty-four hours prior to treatment, the cells were plated into a 6-well culture plate (with initial 10^6 cell/1000 μL complete RPMI-1640 medium/well). Cells were washed with serum-free RPMI-1640 medium before the treatment and then they were treated for 3 h with 100 μM of each of the following compounds in serum-free medium: $RhCl_3$ hydrate, $Rh_2(OAc)_4$, $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$, $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ and $Rh_2(OAc)_2$(*N*-Me-D-Phe-O) for TXRF and TXRF–XANES measurements, respectively. Simultaneously, control cells were also cultured in serum-free RPMI-1640 medium. For TXRF measurements, cells were harvested with a trypsin solution after incubation. Trypsinization was stopped by complete RPMI-1640 medium and then, the cells were washed twice with 1 mL of PBS. The cells were subjected to an acidic microdigestion method as described before [41]. For the TXRF–XANES measurements, HT-29 cells were treated for 3 h separately with 20 μM $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ and Rh_2(O-Phe-*Z*)$_4$ each. Then, treated cells were further processed according to our previously published method [37]. Briefly, cells were harvested by trypsin, washed twice with an isotonic NaCl solution and centrifuged at 7000 rpm. After the second centrifugation, the cells were resuspended in 100 μL of an isotonic NaCl solution and 5 μL of cell suspension was pipetted onto quartz carrier plates for XANES analysis. The final suspension volume corresponded to 5000–10,000 cells/TXRF carrier plate.

All standards for TXRF–XANES were dissolved in water and hexane, except for $RhCl_3$ hydrate and Rh_2(O-Phe-*Z*)$_4$, which were dissolved only in water and hexane, respectively. All aqueous/organic phase standard solutions and cellular samples were evaporated to dryness prior to XANES analysis.

2.4. Cell culture and evaluation of in vitro cytoxicity and cytostatic effects of the compounds using MTT assay

HT-29 cells were cultured as described in Section 2.3. Twenty-four hours before the treatment, cells were plated into a 96-well flat bottom culture plate (with initial 5000 cells/100 μL complete RPMI-1640 medium/well and 10,000 cells/100 μL complete RPMI-1640 medium/well for cytostasis and cytotoxicity, respectively) [39,40]. After 24 h incubation at 37 °C, cells were treated with the compounds in 100 μL of serum-free medium having a final concentration of 1.0% v/v DMSO. Cells were incubated with the compounds from 5×10^{-2} to 2.5×10^2 μM concentration range for overnight. Control cells were treated with serum-free medium only or with 1.0% v/v DMSO. After washing the cells twice with serum-free medium, cell viability was tested using MTT test [42–44]. Briefly, 45 μL of MTT solutions were added to each well (2 mgmL^{-1}, dissolved in serum-free medium). Following a 4 h of incubation, plates were centrifuged at 2000 rpm for 5 min, and the supernatant was carefully removed. The precipitated purple crystals were dissolved in 100 μL DMSO, and after agitation for 10 min, the absorbance was determined at $\lambda = 540$ nm and 620 nm using ELISA plate reader (iEMS Reader, Labsystems, Finland).

In the case of cytostatic effect determination, cells were cultivated for further 72 h in serum containing RPMI-1640 medium at 37 °C after the washing steps. After 72 h, viability was determined by the MTT assay. The 50% inhibitory concentration (IC_{50}) value is defined as the concentration of a compound required to achieve half maximal activity, a parameter that is indicative of cytotoxic or cytostatic potency. The higher the IC_{50} value means lower *in vitro* cytotoxic or cytostatic effect. The IC_{50} values were determined from the dose–response curves using Microcal™ Origin 8.6 software and they were expressed as the mean of three independent experiments (each one performed with four replicates).

The percent of cytotoxicity or cytostasis was calculated using the following equation:

$$\text{cytoxicity/cytostasis}\ (\%) = \left[1 - \frac{OD_{\text{treated}}}{OD_{\text{control}}}\right] \times 100,$$

where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of the treated and the control cells, respectively. In each case, three independent experiments were carried out with four replicates.

2.5. Determination of intracellular Rh content by TXRF

Samples were analyzed by TXRF spectrometry according to our previous method [41]. Briefly, a TXRF 8030C spectrometer (Atomika Instruments GmbH, Oberschleissheim, Germany), equipped with a 3 kW fine focus X-ray tube containing a Mo/W alloy anode, a W/C multi-layer monochromator, adjusted to obtain an excitation energy of 33 keV selected out from the Bremsstrahlung was used. A Si(Li) detector with an active area of 80 mm^2 was in operation with a resolution of 150 eV at 5.9 keV. Ten microliter of 100 mg L^{-1} Ga was added to the samples prior to the TXRF analysis as an internal standard for the quantification procedure. Due to the imprecision of cell counting, the Rh results were normalized to the Zn or S content of the samples. Applicability of TXRF for the elemental analysis of human cells has been demonstrated earlier [35].

2.6. Rh speciation by synchrotron radiation TXRF–XANES

The K-edge XANES measurements in fluorescence mode and grazing incidence geometry were carried out using the setup with an 8-stage sample changer at the beamline L at the Hamburger Synchrotronstrahlungslabor (HASYLAB) at DESY. All measurements were performed in vacuum. A Si(311) double crystal monochromator was used for selecting the energy of the exciting beam from the continuous X-ray spectrum emitted by the 1.2 T bending magnet at beamline L. The primary beam was collimated horizontally and vertically by a cross-slit system. The incident X-ray intensity was monitored with an ionization chamber. During the measurements, the excitation energy of the incident beam was tuned in varying steps (10–0.5 eV) across the K-edge of Rh at 23,220 eV. At each energy increment, the fluorescence spectra were recorded by a 50 mm^2 silicon drift detector. For each specimen ($n = 8$), not less than three consecutive scans were performed. Spectra of highly substituted Rh complexes were recorded in hexane due to their low water solubility. Due to the time demand of the synchrotron radiation–TXRF–XANES measurements, only the cells of mono- and tetrasubstituted Rh complexes of $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ were measured.

The evaluation of the XANES spectra was done using the Athena program of the IFEFFIT software suite [45]. The energy calibration of each spectrum was performed by aligning the simultaneously measured XANES edge of a reference foil. Normalized spectra of the samples were compared to those of prepared standards, which were measured during the same beam time.

3. Results and discussion

3.1. Intracellular Rh content after treatment with dirhodium(II) complexes determined by TXRF

Depending on the type of Rh compounds used for the incubation of HT-29 colorectal adenocarcinoma cells, considerable differences were observable in the Rh content (Fig. 2). Thus, the intracellular Rh content in the HT-29 cancer cell line varied between approximately 25 and 2500 ng/10^6 cells. There were large differences in the Rh content of investigated samples. Therefore, due to its high dynamic range for quantitative determination, TXRF is an ideal tool to evaluate the Rh uptake by HT-29 cells, since alternative instrumental analytical techniques such as atomic absorption spectrometry and inductively coupled plasma mass spectrometry suffer from limited dynamic range and molecular ionic interferences, respectively. The lack of sensitivity of the Rh determination by TXRF was not a critical issue in the present study, since the Rh content of the cells was relatively high.

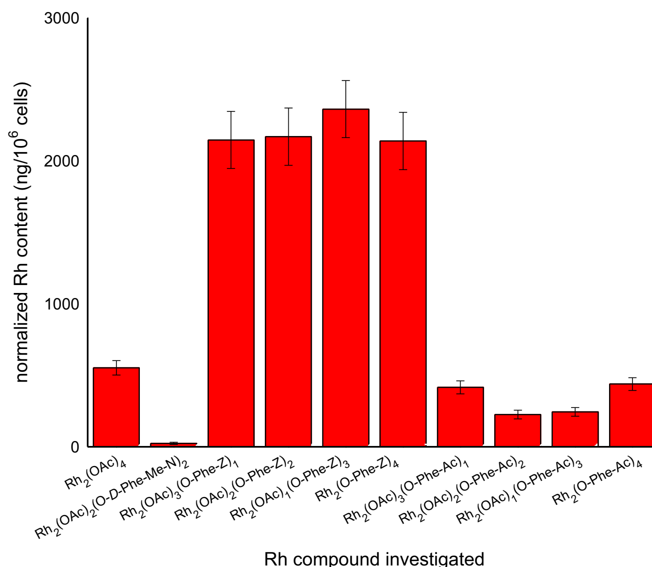


Fig. 2. Comparison of intracellular Rh content after treatment of HT-29 cell lines ($n = 6$) with different Rh compounds.

Complexes of $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ with different acetate ligand displacement showed the highest Rh content. Nevertheless, the Rh content of the cells from $Rh_2(OAc)_4$ was also considerable (Fig. 2). By relating the intracellular Rh content of the complexes to that of the $Rh_2(OAc)_4$, the increase in the Rh content ranged between 3.8 and 4.3. The intracellular Rh content was lower for the $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ than that of the treatments performed with $Rh_2(OAc)_4$ complexes by a factor of 0.4–0.8 (Fig. 2).

In order to understand the differences in the Rh content, several factors had to be considered: i) chemical nature of ligand; ii) lipophilicity of the ligands; iii) the presence of open coordination sites or the steric hindrance caused by the ligands found in axial position; iv) type of complex (bridging *vs.* chelate); v) lability of the ligands bound to the metal core or the leaving groups. It was expected that the increase of the ligand displacement of the complex would increase its non-polar character and, consequently, the Rh uptake by cells. However, an unequivocal relationship between the ligand displacement of the original $Rh_2(OAc)_4$ molecule with either Z-protected L-phenylalanine or *N*-acetyl-L-phenylalanine and the intracellular Rh content could not be established.

By comparing the Rh content of HT-29 cells treated with different $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ and $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ species, a considerable difference was observed. One possible explanation is that the polarity of the $^-$O-Phe-Ac moiety is higher than that of the$^-$O-Phe-*Z* one. Moreover, an additional π interaction between the benzyl groups of the $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ with cellular membrane compounds, compared to the series of compounds containing acetate groups, seems to corroborate the increased lipophilicity and subsequent Rh content.

Contrary to the above-mentioned bridging type of complexes, the treatment of HT-29 cells with $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$, a chelating complex, resulted in a very low Rh content. The low Rh content of the cells in the case of $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$ seems to be related to the fact that this complex forms a chelate ring with one of the Rh atoms of the original dinuclear complex after the ligand exchange reaction. L-phenylalanine [18] and *N*-methyl-D-phenylalanine [20] built up dirhodium complexes with two amino acid units where the binding generally occurs in equatorial positions in a chelating structure through the N atom of the amino and the O atom of the carboxyl groups, respectively. In the ligand exchange procedure, only one product was formed,

namely $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$ [20], due to the special directing influence of the first $^-$O-D-Phe-Me-*N* ligand on the subsequent substitution steps [46]. This chelate type of binding is quite different because of the formation of a bridging bidentate structure where the carboxylic groups are in equatorial positions like in the case of $Rh_2(OAc)_4$. Besides the steric hindrance, the chelate type of complex seems to be more stable than the equatorially bound ligand in the bridging type of structure. Furthermore, the ligand binding in chelating complexes is achieved through mixed hetero donor atoms, so the $^-$O-D-Phe-Me-*N* ligands are not labile enough. Similarly, the higher toxicity of $Rh_2(OAc)_4$ as compared to the toxicity of N–N diimine part containing complexes may be attributed to the greater lability of the carboxylate groups as compared to the chelating N–N diimine groups [47]. Thus, their properties are more complex with respect to their electronic and/or structural effects than those of the dirhodium(II) carboxylates. For example, this structure is more rigid than the bridging one decreasing the possibility to cross the flexible cell membranes. These phenomena can be the reason for the low Rh content of the investigated cells and our results are in good agreement with literature data [47].

3.2. In vitro cytotoxicity and cytostatic activities of the dirhodium(II) complexes

After the incubation of HT-29 cells with different Rh containing compounds, the viability was determined by the MTT assay (Table 1). An IC_{50} of approximately 50 μM after an overnight incubation of cells was an indicator of a moderate cytotoxicity. The higher the IC_{50} value means lower *in vitro* cytotoxic or cytostatic effect. The tetrasubstituted dirhodium(II) complexes (both ligands $^-$O-Phe-*Z* and $^-$O-Phe-Ac) have no *in vitro* cytotoxic activity on HT-29 cells at the concentration up to 500 μM (Table 1).

The *in vitro* cytostatic activity of the trisubstituted compounds is considerably lower than that of the monosubstituted complexes. Thus, the mono- and disubstituted compounds can be considered as the most effective in both series of the bridging complexes. These observations can be explained with several reasons: i) the ligand displacement rate of complex by exchanging the acetate ions with protected L-phenylalanine derivatives increases the steric hindrance in axial position and decreases the possibility of the binding of the newer ligand, ii) acetate ligand is a better leaving group than the $^-$O-Phe-*Z* and $^-$O-Phe-Ac, thus the number of the acetate groups and, consequently, the possibility of the ligand exchange decrease. The different *in vitro* cytotoxic activity in the case of $n = 1–4$ of the $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ complexes is related to the steric hindrance of the coordination site(s), especially in the axial positions. It was shown that the benzyl group of the urethane protecting group (Z−) is less fixed in the case of monosubstituted $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ and it can interact in axial position with nucleophilic reagents by ligand exchange reaction [38]. Furthermore a π–π interaction between the aromatic moieties is supposed to result in the formation of dimers that may decrease the efficacy.

According to our conformational calculations, a further explanation for the efficacy of the $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ and $Rh_2(OAc)_2$(O-Phe-*Z*)$_2$ complexes is that the phenyl ring of the Z protecting group interacts in the vicinity of the ligand entering in the axial coordination site of the dirhodium core through either σ–π or π–π interactions depending on the nature of this ligand [38].

The *in vitro* cytotoxicity and cytostatic activities for 4 h of the $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$ complex were low but slightly higher compared to the $^-$O-Phe-*Z* and $^-$O-Phe-Ac tetrasubstituted bridging complexes. The *in vitro* inactivity may be explained with the low Rh content of the treated HT-29 cells. However, Frade et al. found that a similar chelating complex showed antiproliferative activity on HT-29 cells [18]. According to our experience, the D and L isomers of amino acid derivatives did not show different biological activity. Thus, the cytostasis on HT-29 cells expressed as IC_{50} was higher than 100 μM for $Rh_2(OAc)_2$(O-L-Phe-Me-*N*)$_2$ as well as for D and L isomers of unprotected amino acids such as phenylalanine, valine, proline, homoproline (data not shown). This is in high contrast to the relative activities of the geometric isomers of platinum(II) and Ru(II) complexes. For example, it was found that the trans-[$RuCl_2(DMSO)_4$] complex was much more cytotoxic than its *cis*-counterpart [48].

3.3. Rh speciation in HT-29 cells treated with responsive and non-responsive dirhodium(II) complexes by TXRF–XANES

The evaluation of the XANES spectra was performed by comparing the measurements of the samples to those of the standards. The XANES of the $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ and $Rh_2(OAc)_4$ complexes were almost identical (Fig. 3). Prior to the TXRF–XANES spectra acquisition, the compounds of interest had to be dissolved. The shape of the spectra of these complexes did not depend on the solvent (water or *n*-hexane) used for the dissolution of the investigated compounds (Fig. 3). The XANES of the tetrasubstituted compound of the aforementioned complex series are missing from Fig. 3, since it is hardly soluble in water. However, the XANES of this tetrasubstituted complex recorded in *n*-hexane were identical to those shown in Fig. 3 (results not shown). Similar spectra were recorded for the $Rh_2(OAc)_{4-n}$(O-Phe-Ac)$_n$ and $Rh_2(OAc)_2$(O-D-Phe-Me-*N*)$_2$ complexes (results not shown). Besides the different types of dirhodium(II) complexes, the TXRF–XANES spectra of $RhCl_3$ hydrate standard were also recorded (Fig. 4). The TXRF–XANES spectra of the dirhodium(II) complexes were different to those of $RhCl_3$ ones. This difference can be related to the symmetry of these compounds: $Rh_2(OAc)_4$ molecule has a D_{4h} symmetry [21], while $Rh(H_2O)_6^{3+}$ has an octahedral geometry with a regular (O_h) symmetry in water, the latter having three chloride ions as counterpart after crystallization. Thus, the TXRF–XANES spectra of the $RhCl_3$ hydrate standard were used as a reference for further comparisons. Given the difference in the toxicity of the investigated $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ complexes, the cellular samples of the mono- and tetrasubstituted compounds were further investigated by TXRF–XANES. Clear differences could be observed between TXRF–XANES spectra of *in vitro* effective and ineffective dirhodium(II) complexes characterized by a high Rh content (Fig. 5). Interestingly, the TXRF–XANES spectra of the cells treated with the cytotoxic $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ complex and that of the $RhCl_3$ were found to be very similar (Fig. 5a). This indicated a modification of the chemical environment of the Rh(II) center which may be related to the change of the molecular symmetry or cleavage of the

Table 1
In vitro cytotoxicity and cytostatic activities of the dirhodium(II) complexes using MTT assay.

Compound	$IC_{50} \pm SD$ (μM)		Compound	$IC_{50} \pm SD$ (μM)	
	Cytotoxicity	Cytostasis		Cytotoxicity	Cytostasis
$Rh_2(OAc)_4$	56.7 ± 5.7	10.9 ± 1.9	$Rh_2(OAc)_2$(O-D-Phe-Me-N)$_2$	395 ± 50.6	>100[a]
$Rh_2(OAc)_3$(O-Phe-*Z*)$_1$	60.3 ± 6.0	18.3 ± 1.8	$Rh_2(OAc)_3$(O-Phe-Ac)$_1$	87.0 ± 11.7	32.6 ± 4.8
$Rh_2(OAc)_2$(O-Phe-*Z*)$_2$	70.2 ± 8.5	15.3 ± 1.3	$Rh_2(OAc)_2$(O-Phe-Ac)$_2$	82.0 ± 18.3	48.8 ± 3.7
$Rh_2(OAc)_1$(O-Phe-*Z*)$_3$	97.2 ± 12.4	45.8 ± 6.7	$Rh_2(OAc)_1$(O-Phe-Ac)$_3$	197 ± 20.7	78.3 ± 6.8
Rh_2(O-Phe-*Z*)$_4$	>500[a]	>100[a]	Rh_2(O-Phe-Ac)$_4$	>500[a]	>100[a]

IC_{50} = cytotoxicity and cytostasis values (μM) through half maximal inhibitory concentration.
[a] Not toxic (see explanation in text).

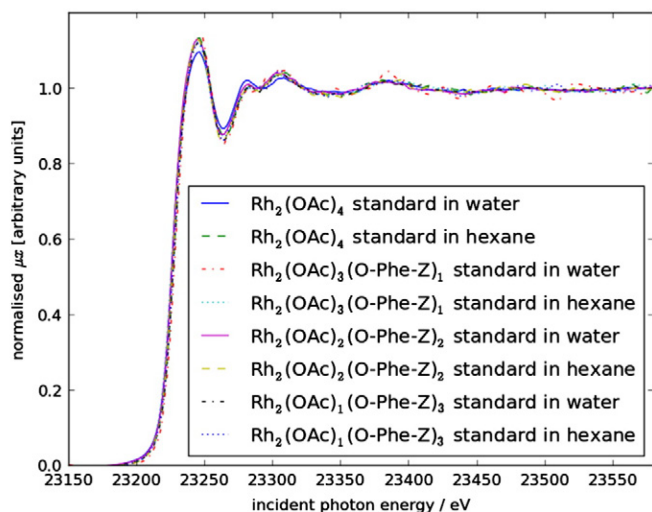


Fig. 3. XANES of $Rh_2(OAc)_4$ and $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$, $Rh_2(OAc)_2$(O-Phe-*Z*)$_2$, and $Rh_2(OAc)_1$(O-Phe-*Z*)$_3$ standards recorded in water and hexane. Adequate amounts from each compound were pipetted onto the carrier in order to investigate 10 ng of Rh.

dinuclear bond. The similarity in the shape of the spectra of the cells treated with $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ and $RhCl_3$ indicates that a change in the molecular symmetry occurred in the cytosol but it cannot be unequivocally related to the protein or DNA binding. On the contrary, the XANES spectra recorded for the cells treated with the non-cytotoxic tetrasubstituted complex did not resemble that of $RhCl_3$ (Fig. 5b). Apart from a small shift in the edge position, the features of the spectral shape for this sample was similar to all dirhodium(II) complex standards (Fig. 5b) suggesting that the dinuclear Rh(II) core remained intact.

Nevertheless, no difference was found in the edge energy of the $RhCl_3$ and any of the investigated dirhodium(II) complex could be observed. This implies that other oxidation states could not be demonstrated within resolution limits of the applied TXRF–XANES analysis.

4. Conclusions

The structure–activity relationship resulting from the comparative analysis of the intracellular Rh content and *in vitro* evaluation of dirhodium(II) compounds with antitumor actions on HT-29 human colon adenocarcinoma cells was performed. The Rh content of HT-29 cells treated with several dirhodium(II) complexes may be in a strong relationship with the cellular Rh uptake. Among the compounds reported in this contribution, the derivatives exerted a dose–dependent *in vitro* antitumor activity at micromolar concentrations. The treatment of HT-29 cells with $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ has resulted in the same cellular Rh content independently of the substitution rate and approximately four times higher than the cellular uptake rate of $Rh_2(OAc)_4$. Among the $Rh_2(OAc)_{4-n}$ (O-Phe-Ac)$_n$, the internalization rate was significantly lower. In contrast, there was a low internalization in the case of the *N*-methyl-D-phenylalanyl moiety containing compound. Among the protected amino acid derivatives, $Rh_2(OAc)_{4-n}$(O-Phe-*Z*)$_n$ compounds ($n = 1$ or 2) proved to be active on the HT-29 cells. In the case of $^{-}$O-Phe-Ac derivatives, the *in vitro* antitumor activity was gradually diminished with the increasing number of incorporated ligands in contrast with similar internalization rate.

The presence of the *N*-methyl-D-phenylalanyl moiety as ligand destroyed the *in vitro* internalization and biological activity. Our results suggest that a certain level of cellular Rh uptake is required. However, this was insufficient to ensure higher *in vitro* efficacy *per se*.

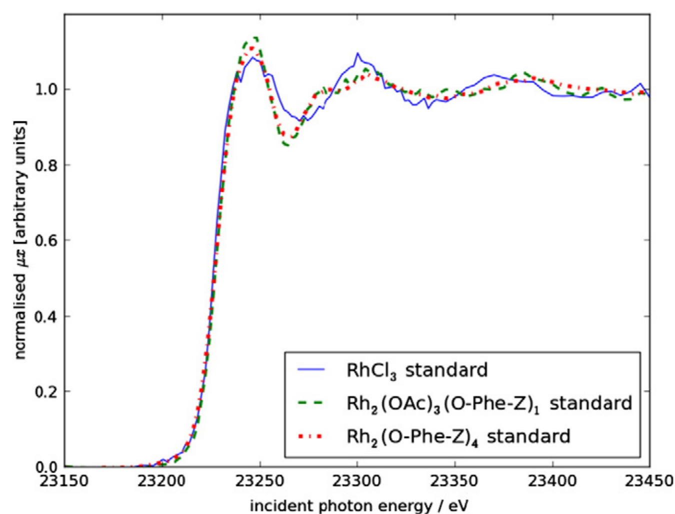
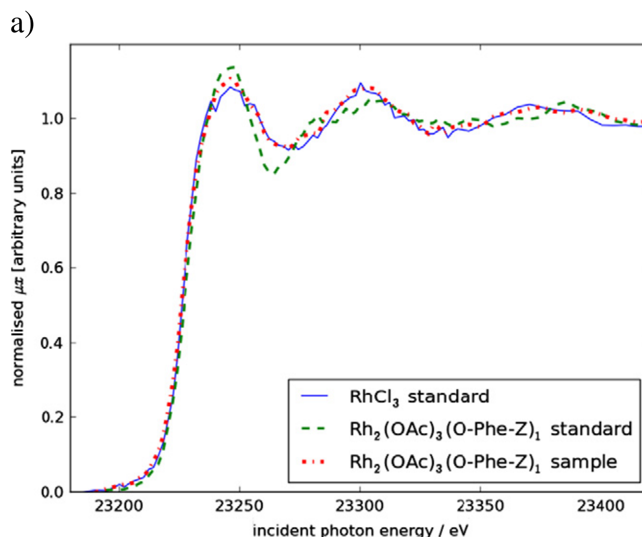


Fig. 4. XANES of $RhCl_3$ hydrate, $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ and Rh_2(O-Phe-*Z*)$_4$ standards. Adequate amounts from each compound were pipetted onto the carrier in order to investigate 10 ng of Rh.



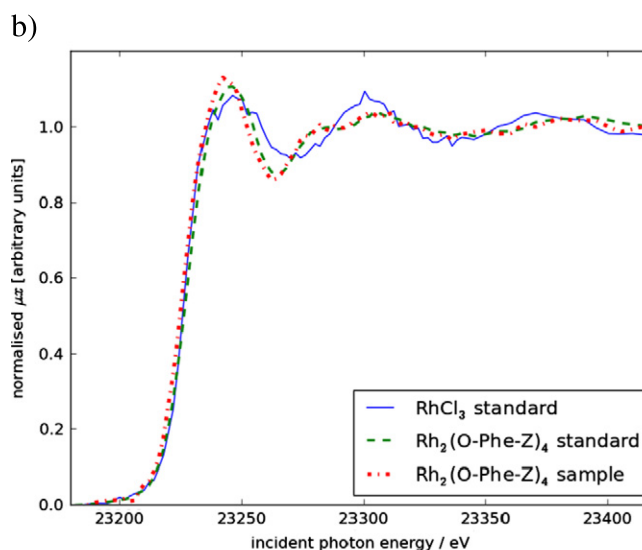


Fig. 5. a) XANES of $RhCl_3$ hydrate and $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ standards as well as HT-29 cells treated with $Rh_2(OAc)_3$(O-Phe-*Z*)$_1$ as sample; b) XANES of $RhCl_3$ and Rh_2(O-Phe-*Z*)$_4$ standards as well as HT-29 cells treated with Rh_2(O-Phe-*Z*)$_4$ as sample.

Speciation studies by TXRF–XANES on selected HT-29 cells incubated with dirhodium(II) complexes characterized by high intracellular Rh content and low acetate ligand displacement rate indicated a possible change of the molecular symmetry of the dirhodium(II) center or cleavage of the dinuclear bond. The results obtained by TXRF and TXRF–XANES could be useful for supporting biochemical, *in vitro* and complex chemistry knowledge on these dirhodium(II) complexes.

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