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New ruthenium(II)-arene complexes bearing hydrazides and the corresponding (thio)semicarbazones of 3- and 4-acetylpyridine: Synthesis, characterization, crystal structure determination and antiproliferative activity

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ABSTRACT

Metal semicarbazone and thiosemicarbazone complexes have attracted much attention due to their diverse biological activities. Because of the ability of ruthenium(II)-arene species to coordinate to different classes of ligands, they are suitable for fine-tuning chemical and pharmaceutical properties. Ruthenium(II) arene-complexes containing different types of ligands: namely caprylic hydrazide (a hydrazide with a long hydrocarbon chain), isonicotinic acid hydrazide (a hydrazide with an aromatic pyridine ring), thiosemicarbazones and semicarbazones (derived from the reaction of 3- and 4-acetylpyridine with either thiosemicarbazide or caprylic hydrazide), were obtained in the reaction of $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ with the corresponding ligands in a 1:2 or 1:2.2 molar ratio in methanol, ethanol or isopropanol with mild heating. The complexes were characterized by elemental analysis, mass spectrometry, IR and NMR spectroscopies. The structure of complex **1** was determined by X-ray crystallography. Antiproliferative activity of the investigated complexes, determined for three human cancer cell lines (HeLa, A549 and LS-174) revealed moderate activity without significant influence on the matrix metalloproteinases (MMP-2 and MMP-9) activity.

1. Introduction

Organometallic compounds are an extensively studied class of anticancer chemotherapy drug candidates. For two decades, there have been many publications on the subject of ruthenium complexes and some of them have resulted in very active anticancer drugs, like NAMI-A [1], KP1019 [2] and Ru(II)-arene complexes [3,4]. Ruthenium(II)-arene compounds are able to coordinate different classes of ligands and therefore are suitable for modulating properties such as chemical reactivity, solubility and pharmacokinetic behavior. One of the first attempts included linkage of metal-arene moieties with bioactive ligands. A typical example of that approach is seen with $[Ru(\eta^6\text{-}C_6H_6)Cl_2(\text{metronidazole})]$ [5].

Hydrazides themselves or mixed in more complex drugs have been used in medicine and veterinary medicine as antibacterial agents. Also, hydrazides produce oxygen radicals and consequently may induce irreversible damage to the prosthetic group of proteins and degrade polypeptide chains of cytochrome *c* [6]. The most frequently applied and studied antitubercular agent is isoniazid, isonicotinic acid hydrazide [7]. Determination of the coordination centers was the primary goal preceding a study of the biological activity. The nitrogen atom in the pyridine ring has a pK_a value of 3.56 [8] and is more basic than the N^1 atom of the hydrazide group, with a pK_a value of 1.91 [9]. Based on X-ray diffraction data, $CuCl_2$ and $Sm(NO_3)_3$ coordinate with the $CONHNH_2$ (hydrazinocarbonyl) group, while $MnCl_2$ can form bonds with both the pyridine and hydrazinocarbonyl groups [10]. The complex 5,10,15,20-tetraphenylporphyrinatozinc(II) is formed via coordination of the N^1 atom of the hydrazide group [8]. Long-chain isonicotinic ester li-

Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinases; RPMI, Roswell Park Memorial Institute; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay.

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gands bind to the ruthenium metal ion via the pyridine nitrogen and form complexes of the type [(arene)Ru(L)Cl_2]. Some of these complexes exhibit very high cytotoxicity in the investigated cell lines, with IC_{50} values equivalent to cisplatin [11].

Metal ions like Ce(III), Nd(III), Sm(III), Eu(III), Gd(III), Dy(III), Er(III) and Lu(III), on reaction with caprylic hydrazide give coordination compounds of the general formula $[ML_3]AsO_4{\cdot}nH_2O$ where $n = 0$, 1 or 2. Data obtained from IR spectroscopy showed that the hydrazide molecules act as bidentate chelating ligands, while the AsO_4^{3-} groups are in the outer coordination sphere of the complexes [12,13]. The outer sphere can also be occupied by three Cl^-, NO_3^- or NCS^- ions [14]. In the reaction of $Ln(NO_3)_3$ with caprylic hydrazide and Na_3AsS_4, complexes, $LnL_4(AsS_4){\cdot}nH_2O$ complexes are formed. The four hydrazide ligands act as bidentate chelators and AsS_4^{3-} is in the outer sphere [15,16].

Cd^{2+}, Co^{2+} and Ni^{2+} ions with the hydrazide of caprylic acid give compounds with an octahedral geometry of the general formulae $ML_3X_2{\cdot}nH_2O$ (where $X = Cl^-$, NO_3^- and $n = 1$, 2) and $ML_2(NCS)_2$, where the thiocyanate groups are N-bonded. The hydrazides act as bidentate-cyclic ligands bonded to the metals through the N atom of the primary amino group and the O atom of the carbonyl group [17,18].

Thiosemicarbazones represent an important class of nitrogen-sulfur donor ligands with diverse biological activities: antitumor, antiviral, antimalarial, antibacterial, anti-inflammatory and anti-HIV [19,20]. They exert a variety of coordination modes. In the monodentate fashion, they bind through the sulfur atom [21], while in the bidentate manner they bind through the sulfur atom and one of the nitrogen atoms of the hydrazine moiety, and therefore form four- or five-membered metallocycles [22,23]. If the aldehyde or ketone used for the preparation of the thiosemicarbazone has an additional functional group in a position that favors chelation, even tridentate coordination is possible [24]. Therefore, 2-heterocyclic thiosemicarbazones were the subject of extensive investigations [19]. If the attachment of the thiosemicarbazone moiety moves to the 3- or 4-position on the heteroaromatic ring, the ability for coordination and activity often decreases [25].

The possible use of thiosemicarbazones in cancer treatment has been a subject of research over the past few decades [26]. The compound 3-aminopyridine-2-carboxaldehyde, known as triapine, entered phase II clinical trials as a chemotherapeutic agent [27–29]. Thiosemicarbazones on reaction with platinum and palladium salts gave polynuclear and mononuclear complexes which can be considered as potential antitumor drugs [30–32]. Ruthenium(II) complexes with thiosemicarbazones showed cytotoxic activity [24,33–35].

Although DNA has been validated as an important target of the drug and adduct formation, protein targets have moved into the center of attention for ruthenium-based complexes [2,36]. It was shown that certain ruthenium complexes manifest higher efficiency against metastases than primary tumors [1,36–38]. As one of the important parts of metastatic progression includes interactions with the extracellular matrix and proteases, it is imperative for novel ruthenium complexes to investigate that aspect of action.

Over the past couple of years we have been working on ruthenium coordination chemistry. So far, we have reported the synthesis, characterization, kinetics and mechanism of reactions, hydrolysis, antiproliferative and antimetastatic properties of a series of Ru(II)-arene complexes with functionalized pyridines [39–42]. Here we report the synthesis, characterization and cytotoxic activity of ruthenium(II) arene-complexes containing different types of ligands: caprylic hydrazide (a hydrazide with a long hydrocarbon chain), isonicotinic acid hydrazide (a hydrazide with an aromatic pyridine ring), thiosemicarbazones and semicarbazones (derived from the reaction of 3- and 4-acetylpyridine with thiosemicarbazide or caprylic hydrazide). The aim was to study the influence of structural modifications of the ligands on the cytotoxic activity.

2. Experimental

2.1. Materials and measurements

All solvents and reagents, including ligands L^1 and L^6, were obtained from commercial suppliers and used without further purification. The thiosemicarbazones (L^2 and L^3) were prepared by refluxing ethanol solutions of 3- and 4-acetylpyridine with thiosemicarbazide, with the addition of 0.1 mol/dm^3 HCl [[43,44] and references there in]. Since references for the synthesis 3- and 4-acetylpyridine semicarbazones (L^4 and L^5) are lacking, they were prepared analogously to the synthesis of the previous thiosemicarbazones. The starting compound $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ was prepared following a published procedure [45]. Elemental analyses were carried out with an Elemental Vario EL III microanalyzer. Infrared spectra were recorded on a Nicolet 6700 FT-IR spectrometer using the ATR or KBr pill technique. The NMR spectra were recorded on a Varian Gemini 200 instrument or a Bruker Avance III 500 spectrometer (500.26 MHz for ^{1}H). Chemical shifts for ^{1}H and ^{13}C spectra were referenced to residual ^{1}H and ^{13}C presented in deuterated dimethylsulfoxide. Mass spectra measurements were carried out on an MS system consisting of a 6210 TOF LC/MS (G1969 A, Agilent Technologies) in methanol solutions.

2.2. Synthesis of the complexes

2.2.1. $[(\eta^6\text{-}p\text{-cymene})RuCl(L^1)]Cl{\cdot}1.5H_2O$ (**1**)

Ethanolic solutions of $[(\eta^6\text{-p-cymene})RuCl_2]_2$ (100 mg, 0.16 mmol) and caprylic hydrazide, L^1, (57 mg, 0.36 mmol) were mixed and the colour of the solution changed from red to orange. The obtained mixture was stirred for 2 h at 50 °C and then evaporated almost to dryness. The residue was dissolved in water and left in a refrigerator overnight to precipitate. The orange product was filtered off and dried in air (0.1 g, 64.77%). A crystal suitable for X-ray analysis was obtained by slow evaporation of the mother liquor. *Anal.* Calc. for $C_{18}H_{32}Cl_2N_2ORu{\cdot}1.5H_2O$ (M_r 491.45): C, 43.99; H, 7.18; N, 5.70. Found: C, 44.40; H, 6.86; N, 5.79%. ν_{max}(KBr)/cm^{-1}: 2957 (s), 2924 (s), 1641 (m), 1569 (m); ^{1}H NMR (500.26 MHz, DMSO-d_6, *J*/Hz) δ_H: 10.20 (1H, d, $J = 8.5$, N*H*), 7.40 (1H, d, $J = 8.5$, N*H*), 5.98 (1H, d, $J = 6$, C*H*(arene)), 5.87 (1H, d, $J = 6$, C*H*(arene)), 5.71 (1H, d, $J = 6$, C*H*(arene)), 5.62 (1H, d, $J = 6$, C*H*(arene)), 2.72 (1H, m, $J = 7$, C*H*$(CH_3)_2$), 2.25 (2H, m, $J = 7$, C(2)H_2(caprylic hydrazide)), 2.08 (3H, s, CH_3), 1.44 (2H, m, $J = 7$, C(3)H_2(caprylic hydrazide)), 1.19 (8H + 6H, m, C(4–7)H_2(caprylic hydrazide)) + CH(CH_3)$_2$), 0.83 (3H, t, $J = 7$, C(8)H_3(caprylic hydrazide)); ^{13}C NMR (125.79 MHz, DMSO-d_6) δ_C: 13.92 (*C*(8)H_3), 17.81 (*C*H_3), 21.67 and 21.99 (CH(*C*H_3)$_2$), 22.36 (*C*(7)H_2), 24.92 (*C*(3)H_2), 27.87 (*C*(4)H_2), 28.21 (*C*(5)H_2), 29.76 (*C*(6)H_2), 30.31 (*C*H$(CH_3)_2$), 31.04 (*C*(2)H_2), 78.76, 80.09, 81.11, 83.62, 96.31 and 99.86 (*C*H(arene)), 179.56 (*C*(1)=O); ESI-MS (MeOH) *m*/*z*: 451.19 [M+Na]$^+$, 429.12 [M+H]$^+$, 393.15 [M−Cl]$^+$.

2.2.2. $[(\eta^6\text{-}p\text{-cymene})RuCl_2(L^2)]$ (**2**)

To a solution of caprylic hydrazide (40 mg, 0.25 mmol) in ethanol (5 ml) at 50 °C was added 3-acetylpyridine (28 μl, 0,25 mmol). The reaction mixture was refluxed at 70 °C for 4 h. $[(\eta^6\text{-p-cymene})RuCl_2]_2$ (80 mg, 0.13 mmol) was added to the solution and stirring was continued for 2 h. The formed precipitate was filtered off and dried in air (0.06 g, 21.42%). *Anal.* Calc. for $C_{25}H_{37}Cl_2N_3ORu$ (M_r 567.56): C, 52.91; H, 6.57; N, 7.40. Found: C, 53.27; H, 6.74; N, 7.34%. ν_{max}(KBr)/cm^{-1} 2962 (m), 2928 (m), 2856 (m), 1689 (s), 1621 (m); ^{1}H NMR (199.97 MHz, DMSO-d_6, *J*/Hz) δ_H:

10.52 (1H, t, J = 21.8, N*H*), 8.94 (1H, s, C(2)pyr*H*), 8.56 (1H, s, C(6)pyr*H*), 8.10 (1H, d, J = 5.6, C(4)pyr*H*), 7.43 (1H, t, J = 5.6, C(5)pyr*H*), 5.79 (4H, d, J = 3.4, C*H*(arene)), 2.83 (1H, m, J = 6.2, C*H*$(CH_3)_2$), 2.50 (3H, s, –C=NCH_3), 2.26 (2H, s, C(2)H_2(caprylic hydrazide)), 2.09 (3H, s, CH_3), 1.58 (2H, s, C(3)H_2(caprylic hydrazide)), 1.21 (8H + 6H, m, C(4–7)H_2(caprylic hydrazide)) + CH(CH_3)$_2$), 0.85 (3H, s, C(8)H_3(caprylic hydrazide)); ^{13}C NMR (50.28 MHz, DMSO-d_6) δ_C: 14.15 (*C*(8)H_3), 18.07 (*C*H_3), 21.71 (CH(*C*H_3)$_2$), 22.28 (*C*(7)H_2), 24.50 (*C*(3)H_2), 25.35 (–C=NCH_3), 28.70 (*C*(4)H_2), 28.96 (*C*(5)H_2), 30.18 (*C*(6)H_2), 31.40 (*C*H(CH_3)$_2$), 32.58 (*C*(2)H_2), 85.75, 86.60, 100.33 and 106.60 (*C*H(arene)), 123.69 (*C*(5)pyrH), 133.54 (*C*(3)pyrH), 134.14 (*C*(4)pyrH), 147.34 (*C*(2)pyrH), 149.77 (*C*(6)pyrH), 169.67 (*C*(1)=O(caprylic hydrazide)), 175.66 (C=N); ESI-MS (MeOH) m/z: 532.17 $[M-Cl]^+$.

2.2.3. [(η^6-p-cymene)$RuCl_2(L^3)$] (**3**)

N′-(1-(pyridin-4-yl)ethyliden)octanehydrazide, L^3, (70 mg, 0,265 mmol) was dissolved in isopropanol (5 ml) at room temperature. To a warm solution of [(η^6-p-cymene)$RuCl_2$]$_2$ (70 mg, 0.12 mmol) in isopropanol (10 ml), a solution of the ligand was added in portions. The colour of mixture changed from red to orange. The obtained mixture was stirred for 22 h at 50 °C and then left to stand in a refrigerator for 2 days. The yellow-orange product was filtered off and dried in air (0.06 g, 44.05%). *Anal.* Calc. for $C_{25}H_{37}Cl_2N_3ORu$ (M_r 567.56): C, 52.91; H, 6.57; N, 7.40. Found: C, 52.67; H, 6.53; N, 7.29%. ν_{max}(KBr)/cm^{-1}: 2961 (s), 2929 (s), 2857 (m), 1686 (s), 1596 (m); ^{1}H NMR (500.26 MHz, DMSO-d_6, J/Hz) δ_H: 10.64 (1H, s, N*H*), 8.81 (1H, s, C(2)pyr*H*), 8.62 (1H, d, J = 10, C(6)pyr*H*), 7.81 (1H, s, C(3)pyr*H*), 7.75 (1H, m, C(5)pyr*H*), 5.82 (4H, dd, J = 5 and J = 15, C*H*(arene)), 2.83 (1H, m, J = 5 and J = 10, C*H*(CH_3)$_2$), 2.63 (3H, s, –C=NCH_3), 2.25 (2H, s, C(2)H_2(caprylic hydrazide)), 2.09 (3H, s, CH_3), 1.58 (2H, m, C(3)H_2(caprylic hydrazide)), 1.24 (8H, m, C(4–7)H_2(caprylic hydrazide)), 1.19 (6H, d, J = 10, CH(CH_3)$_2$), 0.85 (3H, d, J = 5, C(8)H_3(caprylic hydrazide)); ^{13}C NMR (125.79 MHz, DMSO-d_6) δ_C: 13.91 (*C*(8)H_3), 17.84 (*C*H_3), 21.47 (CH(*C*H_3)$_2$), 22.03 (*C*(7)H_2), 24.22 (*C*(3)H_2), 26.87 (–C=NCH_3), 28.46 (*C*(4)H_2), 28.70 (*C*(5)H_2), 29.94 (*C*(6)H_2), 31.14 (*C*H(CH_3)$_2$), 32.34 (*C*(2)H_2), 85.48, 86.33, 100.06 and 106.35 (*C*H(arene)), 121.27 (*C*(5)pyrH), 120.25 (*C*(3)pyrH), 149.30 (*C*(4)pyrH), 150.77 (*C*(2)pyrH) and (*C*(6)pyrH), 154.66 (*C*(1)=O(caprylic hydrazide)), 198.28 (C=N); ESI-MS (MeOH) m/z: 532.16 $[M-Cl]^+$.

2.2.4. [(η^6-p-cymene)$RuCl_2(L^4)$]·2H_2O (**4**)

2-(1-(Pyridin-3-yl)ethylidene)hydrazinecarbothioamide, L^4, (30 mg, 0.16 mmol) was added to [(η^6-p-cymene)$RuCl_2$]$_2$ (50 mg, 0.08 mmol) dissolved in methanol (10 ml). The mixture was stirred for 4 h and then left in a refrigerator to crystallize. The obtained product was filtered off and dried in air (0.06 g, 72.29%). *Anal.* Calc. for $C_{18}H_{24}Cl_2N_4RuS{\cdot}2H_2O$ (M_r 536.45): C, 40.29; H, 5.26; N, 10.44; S, 5.97. Found: C, 40.87; H, 5.37; N, 10.28; S, 6.28%. ν_{max}(KBr)/cm^{-1}: 3358 (s), 3209 (s), 2963 (m), 1605 (s), 1525 (m), 1501 (s), 1469 (m), 1431 (m), 1096 (m); ^{1}H NMR (199.97 MHz, DMSO-d_6, J/Hz) δ_H: 10.45 (1H, s, C=NN*H*), 9.24 (1H, d, J = 1.6, C(6)pyr*H*), 8.64 (2H, 2d, J = 5 and 7.4, C=SNH_2), 8.41 (1H, s, C(2)pyr*H*), 8.20 (1H, s, C(4)pyr*H*), 7.65 (1H, dd, J = 2.8 and 5.2, C(5)pyr*H*), 5.79 (4H, d, J = 3.2, C*H*(arene)), 2.83 (1H, m, J = 6.8, C*H*(CH_3)$_2$), 2.35 (3H, s, C=NCH_3), 2.08 (3H, s, CH_3), 1.21 (6H, d, J = 6.6, CH(CH_3)$_2$); ^{13}C NMR (50.28 MHz, DMSO-d_6) δ_C: 13.92 (–C=NCH_3), 18.07 (*C*H_3), 21.69 (CH(*C*H_3)$_2$), 30.16 (*C*H(CH_3)$_2$), 85.73, 86.59, 100.31 and 106.58 (*C*H(arene)), 124.86 (*C*(5)pyrH), 126.33 (*C*(3)pyrH), 129.06 (*C*(4)pyrH), 146.10 (*C*=N), 160.98 (*C*(6)pyrH), 179.46 (*C*(2)pyrH), 206.21 (*C*=S);

2.2.5. [(η^6-p-cymene)$RuCl_2(L^5)$]·2H_2O (**5**)

2-(1-(Pyridin-4-yl)ethylidene)hydrazinecarbothioamide, L^5, (30 mg, 0.16 mmol) was added to [(η^6-p-cymene)$RuCl_2$]$_2$ (50 mg, 0.08 mmol) dissolved in methanol (10 ml). The mixture was stirred for 4 h and then left in a refrigerator to crystallize. The obtained product was filtered off and dried in air (0.06 g, 72.29%). *Anal.* Calc. for $C_{18}H_{24}Cl_2N_4RuS{\cdot}2H_2O$ (M_r 536.45): C, 40.29; H, 5.26; N, 10.44; S, 5.97. Found: C, 39.82; H, 5.08; N, 10.50; S, 5.24%. ν_{max}(KBr)/cm^{-1}: 3140 (s), 3037 (s), 2962 (s), 1608 (s), 1526 (s), 1494 (s), 1467 (m), 1097 (m); ^{1}H NMR (500.26 MHz, DMSO-d_6, J/Hz) δ_H: 10.59 (1H, s, C=NN*H*), 8.71 (2H, d, J = 5.1, C(2)pyr*H* and C(6)pyr*H*), 8.21 (2H, d, J = 6.4, C=SNH_2), 7.10 (2H, d, J = 8.1, C(3)pyr*H* and C(5)pyr*H*), 5.79 (4H, 2d, J = 6.3, C*H*(arene)), 2.83 (1H, m, C*H*(CH_3)$_2$), 2.35 (3H, s, C=NCH_3), 2.08 (3H, s, CH_3), 1.18 (6H, 2d, J = 6.95, CH(CH_3)$_2$); ^{13}C NMR (50.28 MHz, DMSO-d_6) δ_C: 13.34 (–C=NCH_3), 17.87 (*C*H_3), 20.57 and 21.50 (CH(*C*H_3)$_2$), 29.98 (*C*H(CH_3)$_2$), 79.18, 85.51, 86.36, 100.11 and 106.41 (*C*H(arene)), 122.01 (*C*(4)pyrH), 126.09 (*C*(5)pyrH), 128.82 (*C*(3)pyrH), 134.57 (*C*=N), 145.32 (*C*(6)pyrH), 145.85 (*C*(2)pyrH), 179.50 (*C*=S); ESI-MS (MeOH) m/z: 216.05 $[M-2Cl+2H]^{2+}$, 430.07 $[M-2Cl+H]^+$.

2.2.6. [(η^6-p-cymene)$RuCl_2(L^6)$]·1.5H_2O (**6**)

To a warm solution of [(η^6-p-cymene)$RuCl_2$]$_2$ (100 mg, 0.16 mmol) in isopropanol (11 ml), isonicotinic hydrazide, L^6, (48 mg, 0,36 mmol) in isopropanol (5 ml) was added. The mixture was stirred for 3 h at 50 °C and then left in a refrigerator for 10 days to precipitate. The product was filtered off and dried in air (0.05 g, 33.22%). *Anal.* Calc. for $C_{16}H_{21}Cl_2N_3ORu{\cdot}1.5H_2O$ (M_r 470.33): C, 40.85; H, 5.14; N, 8.93. Found: C, 40.89; H, 5.12; N, 8.62%. ν_{max}(KBr)/cm^{-1}: 3054 (s), 1653 (w), 1616(m); ^{1}H NMR (500.6 MHz, DMSO-d_6, J/Hz) δ_H: 9.95 (1H, d, C=ON*H*), 8.77 (1H, td, C(2)*H*), 8.73 (1H, dd, C(6)*H*), 7.80 (1H, m, C(3)*H*), 7.76 (1H, dd, C(5)*H*), 5.79 (4H, 2d, C*H*(arene)), 2.80 (1H, m, J = 4, C*H*(CH_3)$_2$), 2.15 (2H, s, NH_2), 2.08 (3H, s, CH_3), 1.19 (6H, d, J = 7, CH(CH_3)$_2$); ^{13}C NMR (50.28 MHz, DMSO-d_6) δ_C: 18.11 (*C*H_3), 21.73 (CH(*C*H_3)$_2$), 30.21 (*C*H(CH_3)$_2$), 85.77 and 86.62 (*C*H(arene)), 123.07 (*C*3 and *C*5), 131.1 (*C*4), 138.93 (*C*2 and *C*6), 150.86 (*C*=O).

2.3. Crystallographic structure determination

X-ray diffraction measurements were performed on a Bruker X8 APEXII CCD diffractometer. The single crystal was positioned 40 mm from the detector and 1089 frames were measured, each for 2 s over a 1° scan width. The data were processed using SAINT software [46]. Crystal data, data collection parameters and structure refinement details are given in Table 1. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Non-H atoms were refined with anisotropic displacement parameters. H atoms were inserted in calculated positions and refined with a riding model (except for those attached to O4). The disorder in the aliphatic chain was resolved by using SADI, EADP and in a few cases ISOR instructions implemented in SHELXL. The following computer programs and hardware were used: structure solution, SHELXS-97; refinement, SHELXL-97 [47]; molecular diagrams, ORTEP [48] computer, Intel CoreDuo.

2.4. Analysis of cell growth inhibition by Ru(II)-complexes

2.4.1. Cell culture

The three tumor cell lines HeLa (human cervix adenocarcinoma cells), A549 (adenocarcinomic human alveolar basal epithelial cells) and LS-174 (human colon cancer cells) were maintained as monolayer cultures in nutrient medium, RPMI 1640. The powdered RPMI 1640 medium was purchased from Sigma–Aldrich Co. Nutrient medium RPMI 1640 was prepared in sterile ionized water, supplemented with penicillin (192 IU/mL), streptomycin (200 μg/mL), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM) and 10% of heat-inactivated fetal calf

Table 1
Crystal data and details of data collection for **1**.

	1
Empirical formula	$C_{18}H_{35}Cl_2N_2O_{2.5}Ru$
Fw	491.45
Space group	*Pccn*
a (Å)	21.0526(8)
b (Å)	24.3334(9)
c (Å)	8.7680(31)
V (Å^3)	4491.7(3)
Z	8
λ (Å)	0.71073
ρ_{calc} (g cm^{-3})	1453
Crystal size (mm^3)	0.20 × 0.20 × 0.17
T (K)	100(2)
μ (mm^{-1})	0.952
R_1[a]	0.0693
wR_2[b]	0.1492
Goodness-of-fit[c]	1.084

[a] $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$.
[b] $wR_2 = \{\sum[w(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]\}^{1/2}$.
[c] GOF = $\{\sum[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

serum (FCS) (pH 7.2). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

2.4.2. MTT assay

The antiproliferative activity of the investigated ruthenium complexes **1**–**6** was determined using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Co.) assay [49]. Cells were seeded into 96-well cell culture plates (NUNC), HeLa (2000c/w), A549 (2000c/w) and LS-174 (7000c/w) in culture medium and grown for 24 h. The compounds were dissolved in DMSO and further diluted with nutrient medium to the desired concentrations (up to 100 μM for **1**–**4** complexes and 300 μM for complexes **5** and **6**). The final concentration of DMSO never exceeded 1%. Solutions of various concentrations of the examined compound were added to the wells, except the control wells where only nutrient medium was added. All tests were carried out in triplicate. After incubation periods of 48 and 72 h, 20 μl of MTT solution, 5 mg/ml in phosphate buffer solution, pH 7.2 were added to each well. Samples were incubated for 4 h at 37 °C, with 5% CO_2 in a humidified atmosphere. Formazan crystals were dissolved in 100 μl of 10% sodium dodecyl sulfate (SDS). Absorbances were recorded on an enzyme-linked immunosorbent assay (ELISA) reader (ThermoLabsystems Multiskan EX 200-240 V) after 24 h at a wavelength of 570 nm. The IC_{50} (μM) values were determined from the cell survival diagrams.

2.4.3. Gelatin zymography

The potential of secreted MMP-2 and MMP-9, of drug-treated HeLa cells, to degrade gelatin was analyzed by zymography in 10% SDS-polyacrylamide gels impregnated with 0.1% gelatin. Cells were seeded in a 6-well plate (NUNC) in duplicate (6×10^5 c/w) in nutrition medium. At 70% of confluence, the cells were exposed to the investigated complexes at a sub-cytotoxic concentration of 50 μM in serum-free medium, for 24 h. Supernatants were collected and analyzed the same day. Equal amounts of supernatant proteins for each sample were mixed with the loading buffer (loading buffer: 0.125 M Tris–HCl, pH 6.8; 20% glycerol; 10% SDS and 0.25% bromophenol blue) before loading the SDS-polyacrylamide gels. Electrophoresis was performed at 125 V, gels were washed 3 times for 20 min in renaturing solution (2.5% Triton X-100) followed by a 48 h incubation at 37 °C in the enzyme assay buffer (50 mM Tris–HCl, pH 7.5; 0.2 M NaCl; 5 mM $CaCl_2$; 0.05% NaN_3) for the development of enzyme activity bands. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of MeOH:AcOH:H_2O (2.5:1:6.5) and destained in 4% MeOH with 8% AcOH. In order to increase the sensitivity, the gels were further destained in a solution of 1% Triton X-100 (1-2 h) [50,51]. The gelatinolytic activities were visualized as clear transparent bands against the blue background of Coomassie brilliant blue-stained gelatin.

3. Results and discussion

3.1. Synthesis of the ligands and metal complexes

Ligands $\mathbf{L^2}$–$\mathbf{L^5}$ were obtained as the products of the condensation of 3- or 4-acetylpyridine, either with caprylic hydrazide or thiosemicarbazide in ethanolic solution. The products precipitated directly from the reaction mixutre or after evaporating the solvent under reduced pressure. Complexes **1**–**6** were obtained from the reaction of $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ with the corresponding ligands in a 1:2 or 1:2.2 molar ratio in methanol, ethanol or isopropanol with mild heating.

3.2. Spectroscopy

The infrared spectra of the free ligands and the corresponding Ru(II) complexes were compared in order to gain preliminary information about the coordination of the ligands to the metal center. N^1H_2 stretching (asymetric and symetric) vibrations in the spectra of $\mathbf{L^4}$–$\mathbf{L^6}$ are in the range 3301–3383 cm^{-1}, and in the spectra of complexes **4**–**6** they are in the range 3358–3408 cm^{-1}, confirming coordination of the ligands in a neutral bidentate manner [52]. The thioamide IV band in the spectra of ligands $\mathbf{L^4}$ and $\mathbf{L^5}$ is at 806.8 and 820.8 cm^{-1} respectively, while in the spectra of the complexes it is at 815.9 and 802.3 cm^{-1}. The lower shifts (10–13 cm^{-1}) indicate that coordination occurs through pyridine the N atom [43].

The structures of the ligands and their atomic labellings are given in Fig. 1. Chemical shifts from ^{1}H and ^{13}C NMR spectra are represented in Tables 2 and 3 respectively. The ^{1}H NMR spectra of all the complexes have characteristic patterns originating from the *p*-cymene moiety. In the spectra of complexes **2**–**5**, the H^6 doublets shift considerably compared to the ligands, upon coordination through the pyridine nitrogen. The signals due to the N^2H protons in the free ligands (8.91–10.82 ppm) also appear in the spectra of complexes **1**-**6**, in the range 9.95–10.64 ppm, suggesting coordination of t he ligands as neutral species [51]. For complexes **4** and **5**, with acetylpyridine thiosemicarbazones as ligands, the N^1H_2 signal is found in the 8.21–8.64 range, similar to the case of the ligands (8.31–8.67 ppm), indicating that coordination is not through the S atom. The N^1H doublet for complex **1** is at $\delta = 7.40$, which is moved to upper field compared to the signal of the free ligand ($\delta = 4.14$), which indicates coordination of the ligand to the ruthenium center. For complexes **4** and **5**, the acetyl protons are not shifted to higher frequency (2.34 ppm) compared to the free ligands (2.32 ppm), which would be the case if the coordination was through N^1, as observed in the literature [53]. The ^{13}C NMR spectra for complexes **1**-**6** allowed assignment of all the signals. Resonances in the δ 17.87–18.11 range originate from methyl groups from *p*-cymene, signals at δ 21.50–22.57 and δ 29.98–31.40 come from the isopropyl group from *p*-cymene, while resonances from aromatic carbons are in the δ 78.90–106.60 range.

The ESI mass spectra of methanolic solutions of complexes **1**–**3**, recorded in the positive mode, showed peaks attributed to the $[M-Cl]^+$ ions. For complex **1**, the peak is at *m*/*z* 393.15, and for complexes **2** and **3** it is at *m*/*z* 532.17. A signal at *m*/*z* 429.12 in the spectrum of complex **1** is due to the $[M+H]^+$ ion. The ESI mass

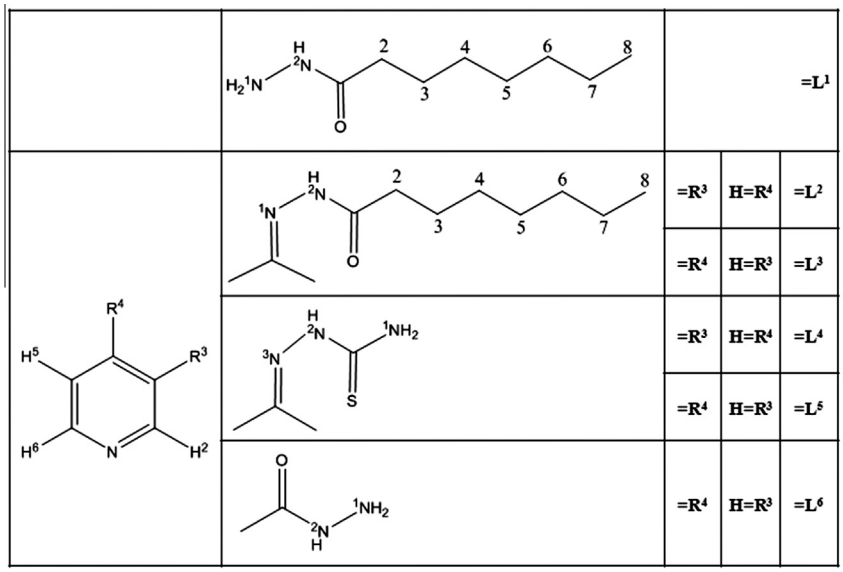

Fig. 1. Structures of the ligands used for synthesis of complexes **1**–**6**.

Table 2
Chemical shifts (ppm) from ^{1}H NMR spectra of complexes **1**–**6**.

H atom	Complex					
	1	**2**	**3**	**4**	**5**	**6**
CH(CH_3)$_2$	1.19	1.21	1.19	1.21	1.18	1.19
CH_3	2.08	2.09	2.09	2.08	2.08	2.08
C*H*(CH$_3$)$_2$	2.72	2.83	2.83	2.83	2.83	2.80
C*H*(arene)	5.98, 5.87, 5.71, 5.62	5.79	5.82	5.79	5.79	5.79
C(2)H_2(caprylic hydrazide)	2.25	2.26	2.25			
C(3)H_2(caprylic hydrazide)	1.44	1.58	1.58			
C(4–7)H_2(caprylic hydrazide)	1.19	1.21	1.24			
C(8)H_3(caprylic hydrazide)	0.83	0.85	0.85			
N^1*H*	7.40			7.65	8.21	2.15
N^2*H*	10.20	10.52	10.64	10.45	10.59	9.95
C(2)pyr*H*		8.94	8.81	8.41	8.71	8.77
C(3)pyr*H*			7.81		7.10	7.80
C(4)pyr*H*		8.10		8.20		
C(5)pyr*H*		7.43	7.75	7.65	7.10	7.76
C(6)pyr*H*		8.56	8.62	9.24	8.71	8.73
–C=NCH_3		2.50	2.63	2.35	2.35	

Table 3
Chemical shifts (ppm) from ^{13}C NMR spectra of complexes **1**–**6**.

C atom	Complex					
	1	**2**	**3**	**4**	**5**	**6**
C(2)H$_2$	31.04	32.58	32.34			
C(3)H$_2$	24.92	24.50	24.22			
C(4)H$_2$	27.87	28.70	28.46			
C(5)H$_2$	28.21	28.96	28.70			
C(6)H$_2$	29.76	30.18	29.94			
C(7)H$_2$	22.36	22.28	22.03			
C(8)H$_3$	13.92	14.15	13.91			
C(1)=O	179.56	169.67	154.66			150.86
–C=N*C*H$_3$		25.35	26.87	13.92	13.34	
C(2)pyrH		147.34	150.77	179.46	145.85	138.93
C(3)pyrH		133.54	120.25	126.33	128.82	123.07
C(4)pyrH		134.14	149.30	129.06	122.01	131.10
C(5)pyrH		123.69	121.27	124.86	126.09	123.07
C(6)pyrH		149.77	150.77	160.98	145.32	138.93
C=N		175.66	198.28	146.10	134.57	
C=S				206.21	179.50	

spectrum of complex **5** contains peaks at *m*/*z* 216.05 and 430.07 which are assigned to $[M-2Cl+2H]^{2+}$ and $[M-2Cl+H]^{+}$, respectively.

The caprylic hydrazide in complex **1** coordinates to ruthenium(II) in a bidentate fashion through the N atom of the primary amino group and the O atom of the carbonyl group in the neutral form. The available literature data [12,13,17,18,52] also showed that hydrazide molecules act as bidentate chelating ligands. Complexes **2**–**6** have ligands coordinated in a monodentate mode via the pyridine nitrogen. All these complexes have a typical "piano-stool" geometry, which is common for organoruthenium complexes with a *p*-cymene moiety.

3.3. X-ray crystallography

The structure of complex **1** was confirmed by X-ray diffraction. The compound crystallized in the orthorhombic space group Pccn. Complex **1** has the characteristic, three leg piano-stool" geometry, which is common for a large number of ruthenium(II) arene species, with an η^6 π-bound *p*-cymene ring forming the seat and caprylic hydrazide coordinated in a bidentate fashion along with one chlorido ligand acting as the legs of the piano-stool. Selected bond distances and angles are given in the legend of Fig. 2.

3.4. Results of the MTT assay

The antiproliferative activity of the investigated Ru(II)-*p*-cymene complexes was determined by an MTT assay. The results are shown in Table 4, in terms of IC_{50} values determined from 2 to 3 independent experiments. The activity of the investigated complexes on tumor cells was low, indicated by high IC_{50} values (>100 μM/>300 μM) for 48 h incubation. Prolongation of the incubation time from 48 h to 72 h, contributed to reaching IC_{50} in the investigated range of concentrations for complexes **2** and **3**. It is observed that the change of position of the acetyl group on the pyridine part of the ligand, from position 3 to position 4 (for

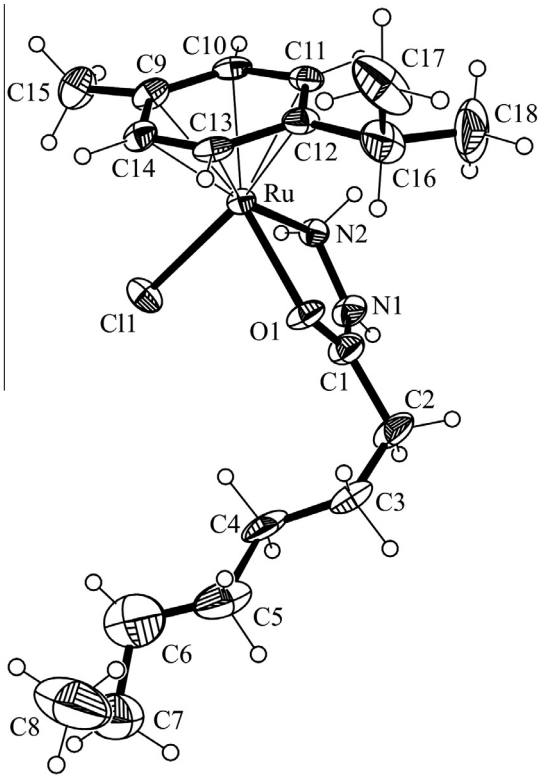

Fig. 2. ORTEP view of the molecule **1** with the atom-labelling scheme and thermal ellipsoids drawn at 50% probability level. Selected bond distances (Å) and angles (deg): Ru–O(1) 2.106(5), Ru–N(2) 2.110(5), Ru–Cl(1) 2.3901(17), Ru–C(9) 2.184(7), Ru–C(10) 2.144(6), Ru–C(11) 2.165(6), Ru–C(12) 2.176(7), Ru–C(13) 2.180(6), Ru–C(14) 2.173(6), O(1)–Ru–N(2) 77.4(2), O(1)–Ru–Cl(1) 85.20(15), N(2)–Ru–Cl(1) 82.83(16).

Table 4
Results of the MTT assay shown in terms of IC50 values, determined from 2 to 3 independent experiments.

Complex	IC_{50} (μM)		
	HeLa (cervix)	A549 (lung)	LS-174 (colon)
1	>100	>100	>100
2	85.38 ± 4.32	90.67 ± 8.07	>100
3	87.43 ± 8.60	87.82 ± 5.05	>100
4	>100	n.d.	>100
5	241.33 ± 12.13	243.06 ± 18.15	>300
6	>300	>300	>300

complexes **2** and **3**, respectively) does not changed the cytotoxicity of the complexes. Prolongation of the incubation time for complexes **4**–**6** does not contributed to their antiproliferative activity (IC_{50} > 100 μM).

3.5. Results of gelatin zymography

MMPs are a family of enzymes that play a central role in the turnover and remodeling of the extracellular matrix [54]. The role of MMPs in cancer was highlighted by Liotta et al. [54] in the early 1980s, when he identified proteolysis as one of the three essential steps of tumor invasion and a type IV collagenase being involved in melanoma invasion and metastasis [55,56]. Among the MMPs, MMP-9 and MMP-2 (capable of degrading gelatin, collagen type IV, I and V) are thought to be particularly important in the invasion of cancer cells [50]. As some ruthenium complexes have the potential of interfering with invasive and metastatic potential of tumor cells [41,57,58], it was desirable to determine the potential influence of the investigated ruthenium complexes on the activity of MMP-9 and MMP-2. The activities of secreted MMP-2 and MMP-9 after 24 h treatment with HeLa cells were analyzed by gelatin zymography. The results show that the investigated complexes do not show any important inhibitory activity on secreted MMP-2 and MMP-9 (Fig. 3).

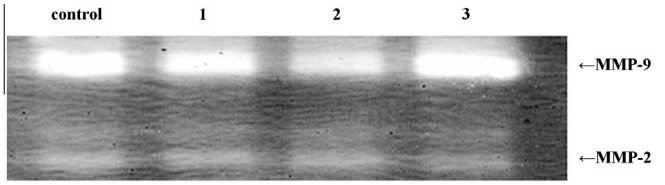

Fig. 3. Gelatin zymography of HeLa cells treated for 24 h with 50 μM of the investigated complexes.

4. Conclusion

We have prepared a series of new ruthenium-arene piano-stool complexes with different types of ligands: caprylic hydrazide (a hydrazide with a long hydrocarbon chain), isonicotinic acid hydrazide (a hydrazide with an aromatic pyridine ring), thiosemicarbazones and semicarbazones (derived from the reaction of 3- and 4-acetylpyridine either with thiosemicarbazide or caprylic hydrazide), with the aim of studying the impact of structural modifications of the coordinated ligands on their *in vitro* anticancer activity. The X-ray structure analysis confirms that caprylic hydrazide is coordinated in a bidentate manner through the hydrazide moiety, whereas spectroscopic data indicates that the semicarbazone and thiosemicarbazone lignads coordinate via the pyridine nitrogen. Of the 6 compounds tested, only compounds **2** and **3** exhibited mild cytotoxic activity againts HeLa and A549 cells, with IC_{50} values ranging from 85 to 91 μM. A change of the position of the acetyl group on the pyridine part of the ligand from position 3 to position 4 (in complexes **2** and **3**, respectively) does not changed the cytotoxicity of complex. Variations of the nature of the ligand, by introducing thiosemicarbazone and semicarbazone parts, significantly lowered the antiproliferative potential of the complexes (IC_{50} > 300 μM). Matrix metalloproteinases, especially MMP-2 and MMP-9, play an important role in the invasion, metastasis and angiogenesis of cancer cells. We investigated the potential of complexes **2** and **3** to interfere with the MMP activity and compared them with complex **1**, which showed no antiproliferative action. The results revealed no significant influence on the MMP-2/MMP-9 activity for any of our complexes. Further studies on their mechanism of anticancer activity and the structure and activity relationship are planned.

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Appendix A. Supplementary data

CCDC 923399 contains the supplementary crystallographic data for complex **1**. These data can be obtained free of charge via http://www.ccdc.cam.as.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

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