Electronic Supplementary Material (ESI) for Dalton Transactions.

Supporting Information

"*In vitro* Cytotoxicity and *in vivo* Zebrafish Toxicity Evaluation of Ru(II)/2-Mercaptopyrimidine Complexes".

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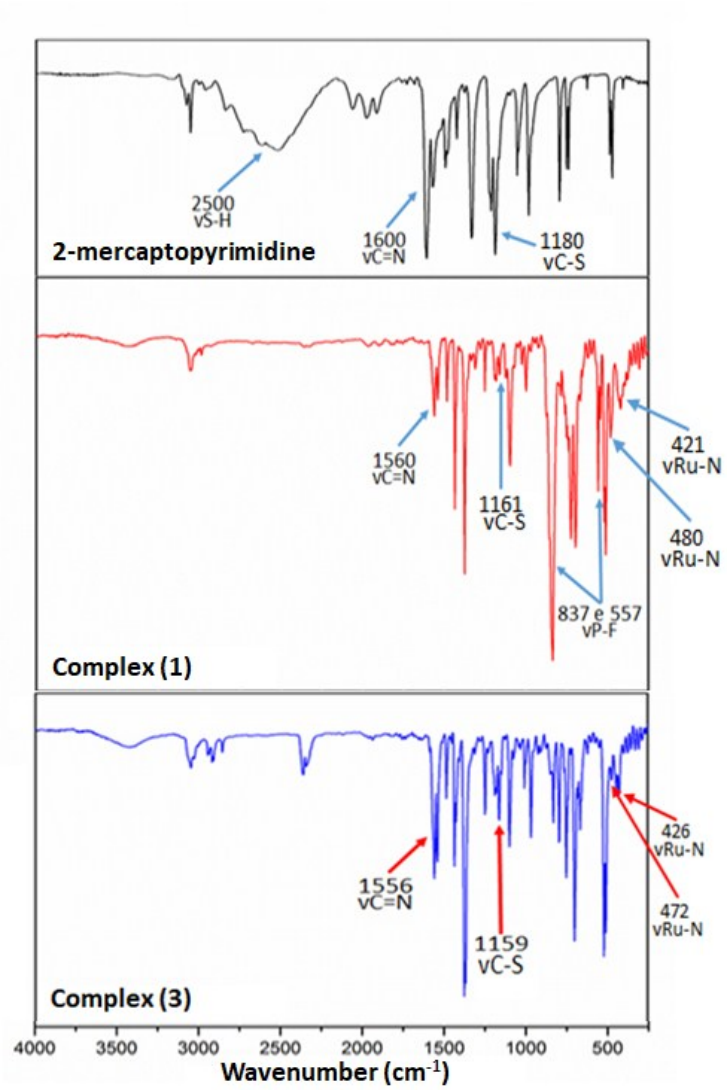

Fig. S1 - Infrared spectra of the 2-mercaptopyrimidine and the complexes 1 and 3.

Table S1 Vibrational frequency assignments (cm^{-1}) in the infrared spectra of ruthenium complexes with 2-mercaptopyrimidine.

Assignments	Wavenumber (cm^{-1})				
	(1)	**(2)**	**(3)**	**(4)**	**pymS**
υ(C-H) sp^2	3055	3056	3047	3049	3057
υ(C-H) sp^3	2987	2948 e 2933	2945 e 2918	2943 e 2915	-
υ(S-H)	-	-	-	-	2500
υ(C=N)	1560	1566	1556	1557	1600
υ(C=C)	1540 ,1483	1533 e 1434	1537 e 1433	1535 e 1434	-
υ(C-S)	1161	1161	1159	1158	1180
υ(C-P)	1097	1097	1097	1097	-
υ(P-F)	837 e 557	837 e 557	-	-	-
υ(Ru-S)	480	482	472	475	-
υ(Ru-N)	421	422	426	425	-

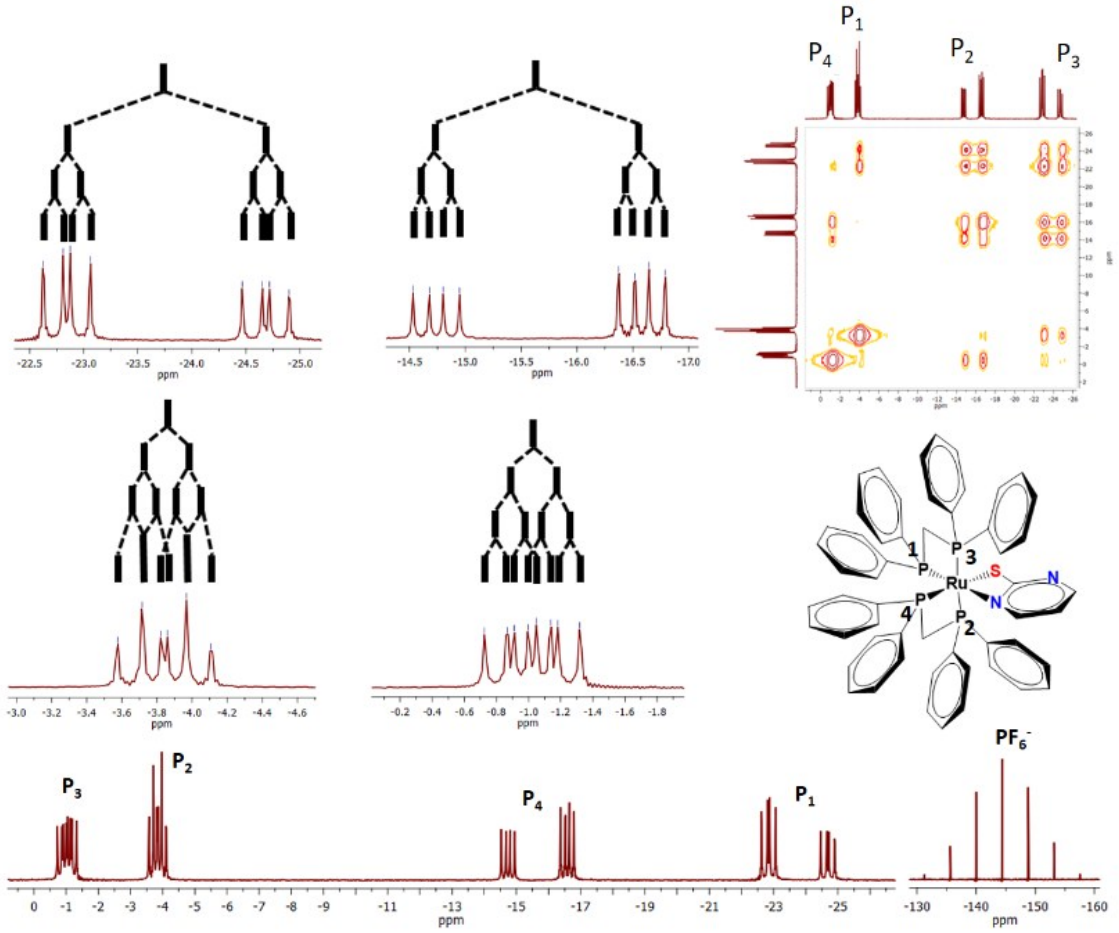

Fig. S2 - COSY (^{31}P - ^{31}P) spectruum of complex [Ru(pymS) $(dppm)_2$]PF_6 (1), in CD_2Cl_2.

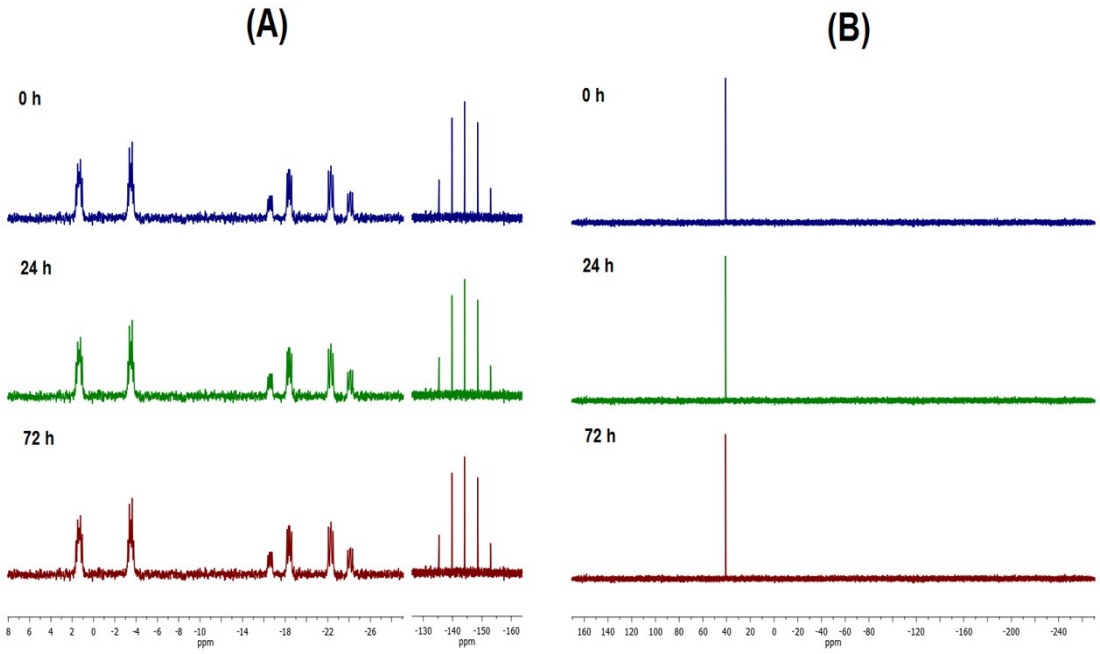

Fig. S3 - ^{31}P-{^{1}H} NMR monitoring of the stability of a 1 mM solution of the complexes (A) $[Ru(pymS)(dppm)_2]PF_6$ and (B) $[Ru(pymS)_2(dppp)]$ in tris buffer (pH 7.4) with 40% DMSO.

Table S2 - Main lengths (Å) of the structures of the synthesized complexes

Bonds	**Bond length**			
	(1)	(2)	(3)	(4)
Ru-P_1	2.32(17)	2.39(3)	2.27(7)	2.33(11)
Ru-P_2	2.38(16)	2.34(3)	2.26(7)	2.30(11)
Ru-P_3	2.33(16)	2.34(3)	-	-
Ru-P_4	2.33(16)	2.39(3)	-	-
Ru-S_1	2.45(17)	2.44(4)	2.42(8)	2.44(11)
Ru-S_2	-	-	2.43(8)	2.38(11)
Ru-N_1	2.14(5)	2.29(8)	2.13(2)	2.12(3)
Ru-N_2				1.17(3)
Ru-N_3	-	-	2.13(2)	
C_1-S_1	1.73(7)	1.71(7)	-	-
C_5-S_2	-	-	-	-
C_{31}-S_1	-	-	1.74(3)	-
C_{35}-S_2	-	-	1.72(3)	-
C_{11}-S_1	-	-	-	1.73(4)
C_{21}-S_2	-	-	-	1.72(4)

Table S3 Crystal and refinement data for (**1**), (**2**), (**3**) and (**4**). (supplementary material)

	[Ru(pymS)(dppm)$_2$]Cl	**[Ru(pymS)(dppe)$_2$]PF$_6$**	**[Ru(pymS)$_2$(dppp)]**	**[Ru(pymS)$_2$(PPh$_3$)$_2$]**
Empirical formula	[C54H47N2P4SRu]Cl	[C56H51N2P4SRu]PF6	C35 H32 N4P2 Ru S2	C44 H36 N4 P2 Ru S2
Formula weight	1016.40	1153.97	735.78	847.90
Temperature (K)	293(2)	293(2)	296(2)	296(2)
Wavelength (Å)	0.71073	0.71073	0.71073	0.71073
Crystal system	Monoclinic	Monoclinic	Triclinic	Triclinic
Space group	P21/c	P21/c	P-1	P-1
Unit cell dimensions				
a (Å); α (°)	21.538(1)	13.9790(4)	11.1616(4); 90.5870(10)	11.1851(2); 77.084(1)
b (Å); β (°)	11.555(1)	20.091(1)	11.3093(4); 104.7410(10)	13.2033(3); 71.312(1)
c (Å); γ (°)	22.094(1)	21.149(1)	14.4332(4); 111.1510(10)	14.8420(4); 66.439(2)
Volume (Å^3)	5166.7(6)	5932.1(4)	1632.7(9)	1891.7(7)
Z	4	4	2	2
Density (calculated) (Mg/m3)	1.307	1.292	1.497	1.489
Absorption coefficient (mm^{-1})	0.556	0.489	0.738	0.648
F(000)	2088	2360	752	868

Crystal size (mm^3)	0.20 x 0.15 x 0.02	0.22 x 0.10 x 0.06	0.17 x 0.13 x 0.08	0.187 x 0.116 x 0.057
Theta range for data collection	2.99 to 25.00°	3.07 to 25.00°	1.47 to 25.11°	2.81 to 26.00 º
Index ranges	-25 ≤ h ≤ 25 , -13 ≤ k ≤ 13, -24 ≤ l ≤ 26	-16 ≤ h ≤ 16 -23 ≤ k ≤ 23, -25 ≤ l ≤ 25	13 ≤ h ≤ 13, -13 ≤ k ≤ 13, -14 ≤ l ≤ 17	-13 ≤ h ≤ 13 -16 ≤ k ≤ 16, -18 ≤l ≤ 18
Reflections collected	22946	20049	28558	32642
Independent reflections	9079 [R(int) = 0.0967]	10392 [R(int) = 0.1127]	5671 [R(int) = 0.0211]	7393 [R(int) = 0.0720]
Completeness to theta = 25°	99.7 %	99.6 %	97.6 %	99.3%
Refinement method	Semi-empirical from equivalents	Semi-empirical from equivalents	Semi-empirical from equivalents	Full-matrix least-squares on F2
Data / restraints / parameters	9079 / 577	10392 / 1 / 569	5671 / 0 / 397	7393 / 0 / 406
Goodness-of-fit on F2	1.037	1.018	1.067	1.035
Final R indices [I>2sigma(I)]	R1 = 0.0696, wR2 = 0.1698	R1 = 0.1189, wR2 = 0.3187	R1 = 0.0303, wR2 = 0.0733	R1 = 0.0479, wR2 = 0.1047
R indices (all data)	R1 = 0.1182 , wR2 = 0.1939	R1 = 0.1495, wR2 = 0.3326	R1 = 0.0358, wR2 = 0.0770	R1 = 0.0941, wR2 = 0.1254
Largest diff. peak and hole ($e.Å^{-3}$)	0.955 and -0.884	2.360 and -1.045	1.026 and -0.447	0.854 and -0.749

Experimental Section of Biological Studies

Cell Culture

The cell lines used in this study were MDA-MB-231 (human triple negative metastatic breast adenocarcinoma), A549 (human lung carcinoma), and HaCat (normal human skin keratinocytes). All cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., MO) supplemented with 10% fetal calf serum, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were kept in an incubator with 5 % CO_2 and a humidified incubator (Thermo Scientific) at 37 °C.

Cell viability assay (MTT assay)

Cell lines viability after treatment for 48 h with the complexes were evaluated using the MTT colorimetric method in order to obtain the IC_{50} values (μM concentration which inhibits 50% of cell viability).[58] For conducting the assay, 1.5×10^4 cells/well were seeded in 96-well plates (Nunc, Nalge, Rochester, NY, USA) for 24 h. Then, the cell lines were treated with the ruthenium complexes dissolved in sterile DMSO 1.0% [59] and cisplatin dissolved in sterile DMF 1.0% [13], both solutions were used in concentrations ranging from 0.2 to 200 μM and incubated for 48 h at 37 °C and 5% CO_2. The complexes (3) and (4) were not soluble enough, thus their cytotoxicity could not be determined. After exposure to the tested complexes, cells were incubated with 50 μL of MTT solution (1 mg mL^{-1}) for 3 h. Then, it was added 50 μL of SDS and the absorbance was determined at 545 nm the next day, using a Stat Fax 2100 microplate reader (Awareness Technology, Palm City, FL, USA). The percentage of cell viability was determined using the formula: Viability % = Absorbance of Treatment/Absorbance of negative control * 100. The IC_{50} value was determined by concentration response curve using the GraphPad Prism 5.0 statistical program (GraphPad Software, San Diego, CA, USA). The IC_{50} values were used to calculate the selectivity indexes (SI) of different cell lines treated with the complexes. These indexes indicate the selectivity of the complexes against tumour cells and provide information about their potential for pre-clinical *in vivo* tests, and for clinical tests. The selectivity indexes (SI) were determined using the formula: SI = IC_{50} normal cell/IC_{50} tumour cell.

Clonogenic Assay

Clonogenic assay is commonly used for determining the effects of cytotoxic agents and other anti-cancer therapeutics on colony forming ability in different cell lines.[60-61] For this, MDA-MB-231 cells ($1x10^3$ cells/well) were seeded in 6-well plates. On the next day, after adherence, cells were treated with IC_{25} and IC_{50} concentrations of complex (1) and complex (2), for 48 h and in the negative controls no treatment was performed. The next step was to replace the supernatant containing the solution used in the treatment without the complex, and to monitor the clonogenic capacity of the treated and untreated cells by inverted microscopy for up to two weeks. On the fourteenth day, the medium was discarded and the cells were washed with PBS (1X). Cells were then fixed with a 1: 1: 8 methanol/acetic acid/distilled water solution for 30 min. After fixation, cells were stained with Giemsa/Buffer Phosphate at the ratio of 1:20 for 15 to 30 min. For densitometric analysis, colonies with 50 or more cells were analyzed in each well of 6-well plates using the plugin Colony Area for Image J software.[42]

Cell Cycle Analysis

MDA-MB-231 cells were treated with the IC_{25} and IC_{50} values of complexes (1) and (2) and incubated at 37 °C, 5% CO_2 for 48 h in 12-well plates. Briefly, 1.5×10^5 MDA-MB-231 cells were harvested by centrifugation, washed with PBS, fixed with 70% (v/v) cold ethanol and stored overnight at -20 °C. The fixed cells were washed with PBS (1X) and incubated with propidium iodide (PI; Sigma) containing 0.05% RNase. Samples were incubated at 4 °C in the dark and analyzed by flow cytometry (FACS Calibur, BD Biosciences). The percentages of cells in the G0/G1, S, G2/M and sub-G1 phases were analyzed using ModFit software. This percentage of cells in each phase of the cell cycle is possible to obtain due to the capacity of propidium iodide to bind to DNA.

Morphologic Detection of Apoptosis and Necrosis – HO/PI in MDA-MB-231 cells

For the morphologic detection, Hoechst 33342 (HO) and Propidium Iodide (PI) were used for fluorescence imaging analysis of the apoptosis or necrosis stage. Hoechst 33342 is membrane-permeant blue dye used for specifically staining the nuclei of living or fixed cells and tissues.[50] Propidium iodide is a molecule that intercalates in any DNA, as long as the cell membrane is permeable. After treatment with the complexes (1) and (2) for 48 h, cells previously trypsinized, were centrifuged at 1800 RPM for 5 min. After pouring the culture medium, the cells were resuspended in 50 μL of PBS, and simultaneously

incubated with Hoechst 33342 (1 mg mL^{-1}) and propidium iodide (1 mg mL^{-1}) at a 1: 1 ratio, for 10 min at 37 °C. After that, 50 µL of this cell solution was placed on a slide, which was covered with a cover slip for visualization under a fluorescence microscope (Carl Zeiss). In this test, 300 cells were analyzed per treatment, i.e. triplicate of 100 cells for each analysis, and normal cells, early apoptosis, late apoptosis and necrotic cells were discriminated, based on the differential staining and morphological aspects of the cells, which allowed them to be identified.

Annexin V-FITC/PI double-staining and analysis by flow cytometry

The apoptosis detection procedure by Annexin V-FITC/propidium iodide is possible by specific binding of the human placental protein annexin V to phosphatidylserine (PS), on the destabilized cell membrane of initiating apoptosis cells, as well as the binding of propidium iodide to DNA of cells in the final process of apoptosis. The binding of fluorochrome-conjugated annexin V to exposed PS can be detected by flow cytometry.[62] For apoptosis detection, the Annexin V/propidium iodide kit (PI) (Sigma-Aldrich, St. Louis, MO) was used according to the manufacturer's instructions. For this assay, 1.5 x 10^5 MDA-MB-231 cells were harvested in the absence (negative control) or presence of complexes (1) and (2) and washed with PBS (1X). The cells were re-suspended in 400 µL binding buffer. Next, 5 µL of Annexin V-FITC and 1 µL of PI were added. Cells were incubated at room temperature for 15 min. Then, data acquisition and analysis were performed on a flow cytometer (FACSCalibur, BD Biosciences). The results, such as the percentage of Annexin-V cells (An -) / (PI-) (viable cells), Annexin-V (An +) / (PI-) (early apoptosis), Annexin-V (An +) / (PI +) (late apoptosis) and Annexin-V (An-)/ (PI +) (necrosis) were performed on a flow cytometer using Cell Quest software.

***In vivo* Fish Embryo Toxicity (FET)**

For the Fish Embryo Toxicity Test (FET), the OECD 236 guideline was the tool that assessed the potential toxic effects of complexes (1) and (2) on the zebrafish model. To obtain embryos, groups of male and female fish, at a proportion of 2: 1, respectively, were placed in aquarium the day before the test, separated by an acrylic barrier. The barrier was removed the next day, allowing contact between males and females and, consequently, spawning. Zebrafish eggs were collected from the spawning cage within 30 min after natural mating, rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation) and the unfertilized or injured eggs (with irregularities during cleavage) were discarded. For this assay, a total of 20 newly fertilized eggs per treatment were distributed in 24-well microplates. The test was performed in triplicate. As soon as possible, they were exposed to the complexes (1) and (2) at seven different concentrations (0.5; 1.2; 2.9; 7.1; 17.1; 41.4 and 100 mg L^{-1}) and the vehicle control (dimethylsulfoxide [DMSO], 1.0 %) for periods of 24, 48, 72 and 96 h. As a negative control of the test, fertilized eggs were used with the fish farming system water. During the incubation period, the embryos were kept with strictly controlled water parameters, in a climatic chamber at a temperature of 26 ± 1 º, conductivity at 750 ± 50 µS/cm, pH at 7.0 ± 0.5 and dissolved oxygen ≥ 95 % saturation, and they were submitted to a photoperiod cycle 12:12h, light: dark.

Embryos were observed daily in a stereomicroscope (Stemi 2000-C, Zeiss, Germany), using a 70x magnification lens for the embryos and 40x for the larvae. In the embryo phase, the following parameters were evaluated every 24h from the beginning of the experiment: coagulation of fertilized eggs, absence of somite formation, absence of tail detachment, lack of heart beat, craniofacial malformation, microphthalmia, pigmentation, otolith, cardiac edema, yolk sac absorption, tail malformation, altered balance, larval hatching and other anomalies that may appear during the experiment. The hatch rate values, mortality rate and median lethal concentration (LC_{50}) of complexes (1) and (2) were calculated based on the results obtained after 96 h post fertilization (hpf).

Statistical analysis

Statistical results were expressed as mean ± standard deviation of the means from triplicates of each independent experiment. Statistical analysis of the results was performed using the unpaired t test or one-way ANOVA followed by Dunnett's or the Bonferroni post hoc test for multiple comparisons with a control. All statistical analyses were performed using the GraphPad Prism statistical software, version 5. A probability of 0.05 or less was considered statistically significant. The following notations were used throughout the manuscript: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, relative to the negative control.