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Synthesis, Characterization of Ruthenium Compounds and Studies of Biological Effects in MCF-7 Tumors Cell Lines

Andressa Teixeira de Barros Nunes Ribeiro[1*], Claudia Cristina Sousa[2], Adenilda Cristina Honório França[2], Eduardo Luzia França[2], Fabricio Tarso Moraes[1], Wagner Batista Santos[1*]

[1]Institute of Exact Sciences and Earth, Federal University of Mato Grosso, Barra do Garças/MT, Brazil
[2]Institute of Biological and Health Sciences, Federal University of Mato Grosso, Barra do Garças/MT, Brazil
Email: *atbnunes@hotmail.com, *wagner.santos@ufmt.br

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Abstract

This work presents the synthesis and characterization of compounds derived from the ruthenium transition metal with the nitrogenous ligand 4-aminopyridine (4-ampy). The synthesized compounds were characterized by FTIRmed spectroscopy and TG-DTA thermal analysis. For the cytotoxic evaluation of ruthenium compounds, a 66.0 μM aqueous solution containing the complex and the study of data observed in the biological assessment was performed using variance (ANOVA) analysis, followed by Tukey's multiple comparisons test. Differences between treatments were considered significant when the p-value was less than 0.05 ($p < 0.05$). TG/DSC thermal analysis for the first complex suggests a stoichiometry of $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$, which, due to the low solubility in an aqueous medium, was modified to increase its solubility for biological tests. The analysis of the spectra in the medium infrared region (FTIR) for the complex $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$, shows displacements of the bands observed at 1625 - 1566 cm^{-1} ν(C=C) e (C=N), indicating that coordination to the metallic center occurred by this group. Band displacements were observed in the modified Ru (III) complex, which suggests the presence of the 4-ampy ligand and the coordination by the groups ν(C=C) and (C=N) after the modification. In recent years, researchers worldwide have concentrated on obtaining, developing, and modifying drugs used as chemotherapeutic agents. The evaluation of the cell viability of the modified Ru (III) compound demonstrated cytotoxic effects in the MCF-7 cell line (15.33% ± DP 2.7) but did not affect normal cells (PBMC), which reflects the potential for possible applications.

Keywords

Ruthenium, MCF-7 Cells, Cytotoxic Evaluation, N-Heterocyclic Ligands

1. Introduction

In 2016, the World Health Organization (WHO) identified cancer as the second leading cause of death globally, responsible for the death of 8.8 million people, that is, one in every six deaths on the globe [1]. Among the most common causes of death were cancers of the lung, liver, colon, stomach, and breast [1]. Also, according to the WHO, there will be about 22.2 million new cases of cancer diagnosed each year worldwide by 2030 [2]. Moreover, in 2017, about 70% of deaths in low and middle-income countries will be due to cancer, placing even greater pressure on already vulnerable health systems [1]. Recent research shows that transition metals [3] [4] have interesting medicinal properties and can be used in combating several types of disease, including cancer [5]. In 1969, Rosenberg and his colleagues [6] casually discovered the anticancer properties of cisplatin, and today the derivatives of this study are among the most used drugs in the treatment of cancer [7]. Cisplatin-based drugs are currently the most widely used anticancer drugs worldwide; however, they have a variety of side effects such as gastrointestinal, nephrotoxicity, and neurotoxicity, in addition to drug resistance by some types of cancer after 4 to 6 treatment cycles [8] [9]. These negative characteristics have encouraged researchers around the world to look for new metal complexes which act similarly to cisplatin but have lesser side effects on the individual being treated [10] [11]. From this discovery, some compounds derived from Ru^{+3} and Ru^{+2} with amine [12] coordinated to N-heterocyclic ligands [13] [14] and alkyl sulfoxide ligand ligands [15] have also demonstrated a potential antimetastatic activity. In addition, new studies based on ruthenium [16] [17] [18] [19] have become the target of numerous therapeutic studies being applied in the fight against cancer. These properties cited above prompted us to make a new ruthenium-derived compound and test its antitumor potential. Thus, the synthesis and characterization of a new ruthenium compound are reported, the unique combination was biologically tested for a preliminary evaluation of its antitumor potential.

2. Experimental Section

2.1. Synthesis

Synthesis of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$: In a round-bottom flask, 1.0039 g of 4-aminopyridine (4-ampy) was added in 10.0 ml of distilled water, the resulting solution was subjected to stirring and gentle heating for 15 minutes. After this period, 1.0030 g of $RuCl_3\cdot 3H_2O$ metal was added, in the proportion of 1:2 (Starting compound/ligand). Then the system was contained under agitation, and the temperature adjusted to 85˚C, under reflux for 24 hours. Then, the solu-

tion was filtered, and the dark-colored precipitate formed was dried in an oven at 70˚C for 02 hours Purification: 0.5000 g of the complex was added in 50.0 ml of cold ethyl alcohol and subjected to stirring for 10 minutes. Finally, the compound was separated from the solvent by gravity filtration and left to dry in a desiccator—yield: 80%.

Synthesis of modification of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ complex (Incentive): In a 1000.0 ml beaker, 900.0 ml of an HCl solution (37, 5%), 1:1 water/HCl ratio, and then 0.5000 g of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ complex is added. The solution was subjected to stirring and heating until complete solubility of the complex, for later addition of 0.1076 g of KCl, thus guaranteeing the excess of the counter ion. After this step, the solution was filtered, still hot. The orange-colored supernatant was reduced to a volume of 20.0 ml in a bath with a controlled temperature at 80˚C. At the end of this stage, a precipitate formed. The brown-colored precipitate was separated by vacuum filtration, dried with ether, and taken to an oven at 80˚C for drying and later storage in a desiccator. Yield: 60%.

2.2. Cytotoxic Evaluation of Ruthenium Compounds

Preparation of Ruthenium Compound Solutions: For the cytotoxic evaluation of ruthenium compounds a 66.0 μM aqueous solution containing the complex.

Preparation of cell lines used

Tumor Cells: To evaluate the cytotoxicity of ruthenium compounds, we used breast adenocarcinoma-type (MCF-7) ATCC (American Type Culture Collection, USA) cell lines. The strain was grown and frozen in liquid nitrogen for storage and subsequent use. To perform the biological assays, tumor cells were cultured in RPMI medium, plus HEPES, penicillin, streptomycin, sodium bicarbonate, sodium pyruvate, and fetal bovine serum. Cells were cultured in cell culture flasks and kept in an oven at 37˚C at 5% CO_2 until cell monolayer formation. Subsequently, the culture flasks were washed with 5 mL RPMI and subjected to 1.0 mL trypsin-EDTA, until the cells detach from the bottom of it. For trypsin neutralization, cells were homogenized with an undefined volume of culture medium plus 10% fetal serum. The suspension containing MCF-7 cells was then adjusted to 2.0×10^4 cells/mL.

Healthy Cell Lines: As healthy cells, the PBMC strain was used. To obtain these cells, blood samples were collected in tubes containing the EDTA anticoagulant. Cell populations were separated by a ficoll-paque density gradient. In a falcon tube, 3.0 mL of ficoll-paque was added followed by the slow addition of ~ 5.0 mL of blood, thus forming two phases. The system was centrifuged at 1500 rpm for 40 minutes at room temperature. After this time, the mononuclear cell ring was removed and transferred to a new tube. To the tube containing the mononuclear cell, the ring was added 3 mL of PBS and stirred for homogenization of the system. After stirring the system was centrifuged for 10 minutes at 15,000 rpm at room temperature, the supernatant was then discarded and this step repeated, then 1 mL PBS was added. Cells were counted in a Neubauer cam-

era and cell suspension adjusted to 2.0×10^6 cells/mL.

Cell incubation: For the test, the cells were seeded in Falcon tubes, with or without ruthenium-derived compounds. The cells were incubated for 24 h in an oven at 37˚C and atmosphere containing 5% CO_2 (Table 1).

Cell viability assay by acridine orange staining method: At the end of the incubation period, the falcon tubes were centrifuged at 1500 rpm for 10 minutes and had their supernatant discarded. The pellet formed was stained with 200.0 µl of freshly prepared acridine orange solution (concentration 14.4 mg/mL) and allowed to stand for 1 minute for dye action. The resulting solution was resuspended in medium 199, after which the tubes were centrifuged and washed with PBS a further 2 times. Stained cells were placed on microscope slides (26 × 76 mm) and, after mounting with coverslips (24 × 24 mm), were analyzed by blind fluorescence microscopy (Nikkon Eclipse E200). The cell viability index was obtained by counting. For each treatment at least 100 cells were analyzed. Green cells were considered alive and orange cells considered dead.

Statistical analysis: For the proper statistical treatment, the analysis of variance test (ANOVA) was performed, followed by the multiple comparisons test, Tukey test. Differences between treatments were considered significant when the p-value was less than 0.05 ($p < 0.05$).

2.3. Characterization Methods

The synthesized compounds were characterized FTIRmed spectroscopy and TG-DSC thermal analysis.

The measurements in the FTIRmed region were obtained in a Perkin Elmer Fourier transform spectrophotometer, model Perkin Elmer Spectrometer 100. Resolution of 4 cm^{-1}, in the region between 4000 - 600 cm^{-1}, using an accessory for the technique of ATR with germanium crystal.

TG-DSC thermal analyzes of the synthesized compound were performed in a **TGA/DSC 1 da Mettler Toledo**, consisting of a horizontal mass comparator with a maximum capacity of 20.00 mg and a sensitivity of 1.00 µg. The analyzes were performed in the temperature range of 30˚C to 1000˚C, with a heating rate of 10˚C·min^{-1} and a dry air atmosphere with a flow rate of 100.0 mL·min^{-1}. The sample mass used was of the order of 7.00 mg in α-alumina crucible.

Table 1. Volume used in µl.

Group	Vol. (µl)
PBMC (1.0×10^6 cells)	500
PBMC (1.0×10^6 cells) + Incentive	500 + 50
MCF-7 (1.0×10^4 cells)	500
MCF-7 (1.0×10^4 cells) + Incentive	500 + 50
PBMC (1.0×10^6 cells)	500

3. Results and Discussion

Thermal Analysis—TG-DSC Curves: ***The*** TG/DSC curve (**Figure 1**), shown below, shows the thermal decomposition of the compound $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ up to 950˚C and formation of the RuClO residue with an error of 0.36% (TG = 55.99%; Calculated = 55.63%). In addition, studies carried out by Chagas [3] demonstrate the formation of the same type of waste.

In the TG/DSC curve, it still shows evidence of thermal decomposition of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$. **Table 2** shows this decomposition, which occurs in 3 consecutive steps.

The first loss step indicates a mass loss (135˚C), consistent with H_2O hydration output up to 100˚C and coordination water up to 135˚C. The second stage with maximum loss at 272˚C is attributed to the beginning of the oxidative decomposition of the 4-ampy ligand [20]. Literature shows that the free 4-ampy ligand decomposition step occurs at 210˚C, with 99% mass loss. Copper complex synthesized with the 4-ampy ligand shows that water (H_2O) as a ligand "leaves out" between 80˚C and 200˚C and that the 4-ampy ligand starts its thermal decomposition at 210˚C and 272˚C, with loss maximum at 240˚C and output [21]. The curves observed in this work proved to be similar for the Ru complex synthesized here. The third and last stage is attributed to the end of the ligand decomposition with the exit of the chloride ions and the formation of the RuClO residue, in a process similar to that observed in the literature [3] [16]. Qualitative tests with silver nitrate demonstrated the absence of the AgCl precipitate, indicating that the chloride ions were coordinated to the metallic center. The determination of stoichiometry, Δm% in stages, was not possible due to the loss of mass occurring consecutively. It was also not possible to propose a thermal decomposition mechanism for the material. Thus, for the calculations, with minimum formula, Δm% total was used.

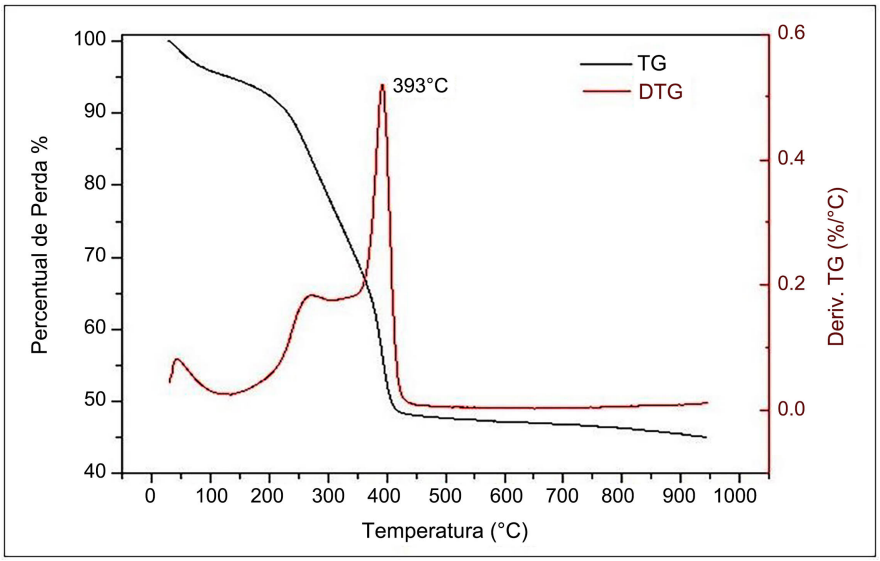

Figure 1. TG/DSC of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ complex. Source: The author.

Table 2. Thermal decomposition steps for $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$.

$[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$	Stages		
T °C	35 - 135	135 - 310	345 - 435
Δm%	4.9110	19.0000	27.9700

FTIR$_{med}$ Spectroscopy: Figure 2 shows the prominent characteristic bands of the 4-ampy ligand, observed at 3434 cm^{-1} for $_{\nu a}(NH_2)$, 3301 cm^{-1} $_{\nu s}(NH_2)$, 3093 - 3094 cm^{-1} $_{\nu}$(C-H) and 1645 cm^{-1} $_{\delta}(NH_2)$, the bands at 1594 - 1332 cm^{-1} refer to the $_{\nu}$(C=C) of the pyridinic ring, 1268 cm^{-1} $_{\nu}$(C-NH_2), 1215 cm^{-1} $_{\delta}$(CH) and 987 cm^{-1} (ϕ resp the ring).

In Figure 3, we present the overlap between the synthesized complexes $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ and modified Ru (III), which shows the presence of the characteristic bands of the 4-ampy ligand, with some displacements, suggesting the coordination of the ligand to the metallic center.

Such bands are similar to those observed by Buyukmurat [22] in his study by IR of the 4-ampy coordinated metals Fe and Zn. In addition, the spectra analysis can observe a displacement of the bands referring to the symmetrical and asymmetric vibrations of the group (NH_2) for regions of lower energy. For example, at 2971 cm^{-1}, the band corresponding to $_{\nu}$(C-H) decreases the vibration intensity and shifts to lower energy regions, an effect similar to that observed by Chandra [21]. The vibrations observed in 1630 - 1617 cm^{-1} of the complex $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ attributed to the groups $_{\delta}(NH_2)$ with $_{\nu}$(C=C), generated a band wide with two characteristic peaks, which can be attributed to the N-heterocyclic rings. Furthermore, comparing the free and complex ligand, a displacement of the vibration bands is observed, indicating that this is the site of the binding metal ligand. The "ring respiration" observed at 987 cm^{-1} in the free ligand and 1026 cm^{-1} in the complex indicates ligand-metal coordination. This band, commonly observed in benzene spectra, in pyridine and its derivatives, is attributed to the expansion and contractions of the ring [23]. Furthermore, this model is susceptible to coordination to the metallic center from the isolated pairs of nitrogen in the ring, causing an increase in the wavenumber caused by the coordination force [22], thus corroborating the proposed evidence. As for the modified Ru (III) complex, it is possible to observe significant differences when compared to the spectrum of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ complex. The bands in the 3200 - 3330 cm^{-1} region coincide with their precursor but are more intense. The displacement of the bands for regions with a higher wavenumber: 1652 cm^{-1} $_{\delta}(NH_2)$, 1625 - 1566 cm^{-1} $_{\nu}$(C=C) and (C=N); for a smaller number of waves: 1197 cm^{-1} $_{\delta}$(CH) and the band at 998 cm^{-1} (Φ resp. of the ring), give us indications of the presence of the 4-ampy ligand. The appearance of the bands at 1400 cm^{-1} and 786 cm^{-1} suggests changes in the stoichiometry of the $[Ru(Cl)_3(4\text{-ampy})(H_2O)_2]\cdot 1/2H_2O$ complex.

Cytotoxic Evaluation: The acridine orange staining assay allowed for the distinction of living and dead cells in each experimental group. It was aimed at

evaluating whether the tested compound would present toxicity to PBMC and MCF-7 cells. The data obtained by analysis of variance (ANOVA) followed by the multiple comparison test (Tukey) give us results with 95% accuracy (**Figure 4**).

The data analysis points out no significant differences in cell death between the PBMC, MCF-7, PBMC + modified Ru (III) complex (Incentive) groups and that the rate is similar and low. Furthermore, studies carried out by Tarso [16] indicated that ruthenium compounds as identical in the structure had similar effects to those observed here, that is, that statistically, the stimuli tested do not have toxic effects on cells used. However, when the analysis is restricted to MCF-7 + Incentive, the data showed significant differences, showing considerable toxicity to this strain. Compared to the group containing only MCF-7 cells,

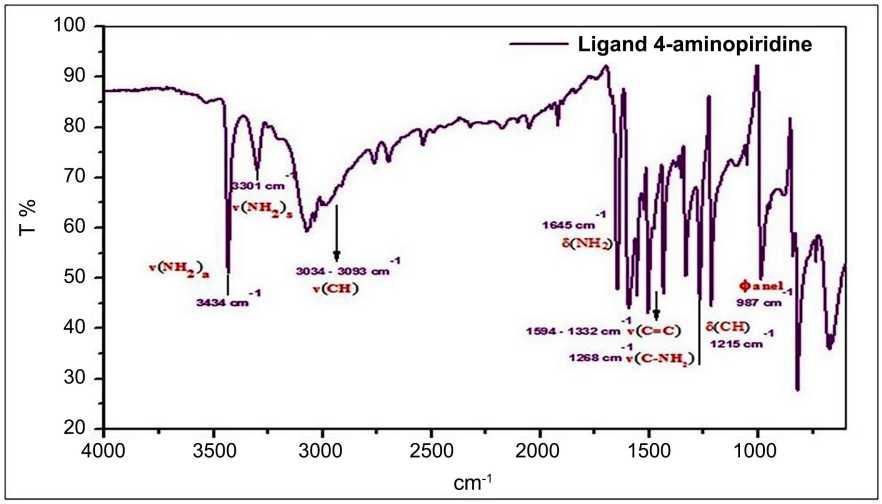

Figure 2. FTIRméd spectrum of the 4-ampy ligand. Source: The author.

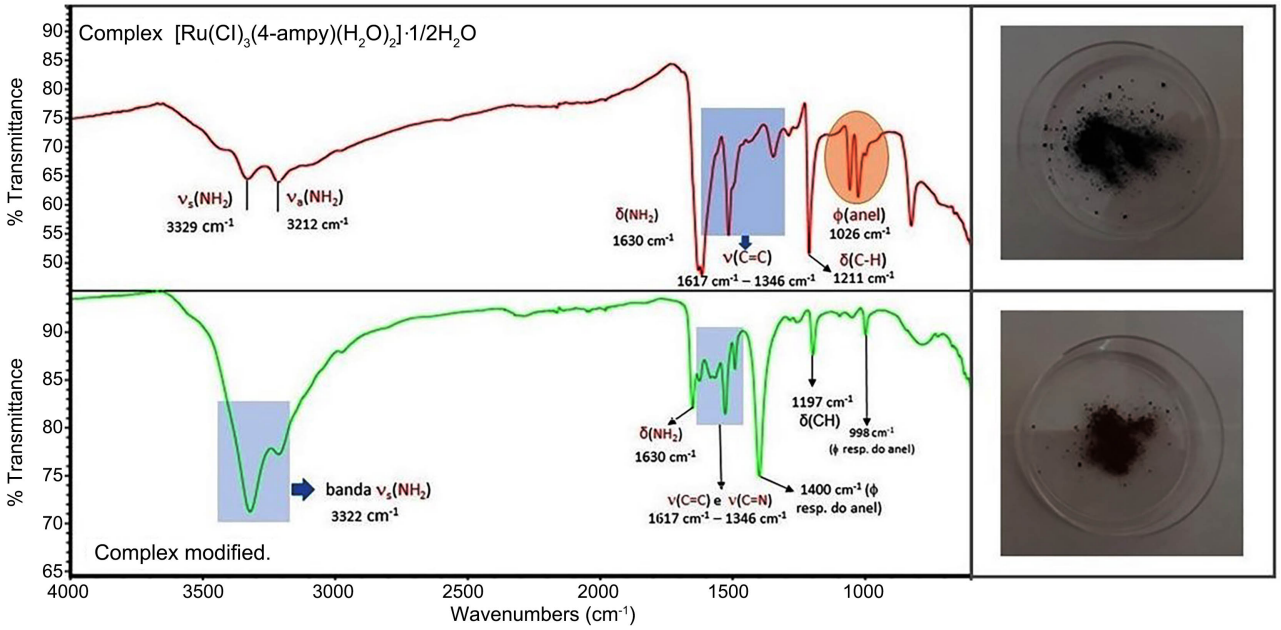

Figure 3. FTIRmed spectra of the [Ru(Cl)3(4-ampy)(H_2O)$_2$]·1/2H_2O and modified Ru (III) complexes. Source: The author.

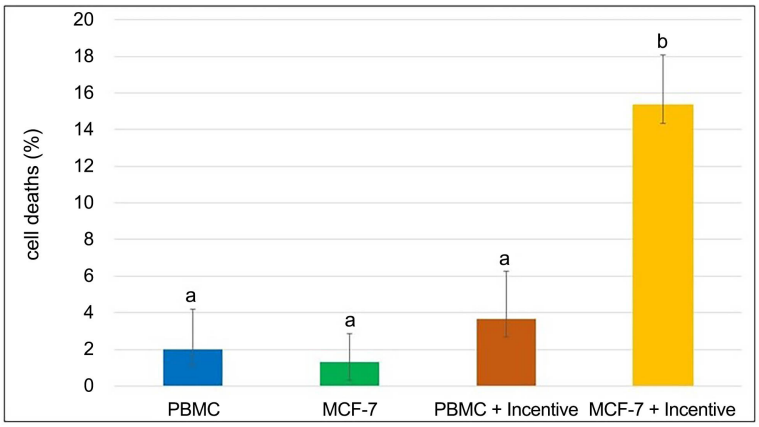

Figure 4. Comparative graph of cell deaths, using the Tukey test. Source: The author. Note: Different letters in the columns denote significant differences ($p < 0.05$).

an increase of 15.33% (±DP 2.7) is observed in cell death. The increase in selectivity for tumor cells goes against what is expected for compounds derived from ruthenium [5]; it is possible to state that the compound showed cytotoxicity against MCF-7 cells. Based on statistical analysis, the feasibility test reported here is possible to state that the compound was selective, has considerable cytotoxicity against the MCF-7 cell line, and could have antitumor activity in other cell lines. This antitumor activity deserves a more detailed analysis, aiming at a future application.

4. Conclusion

The work shows a simple and reproducible synthesis route for both $[Ru(4\text{-ampy})_1(H_2O)_2(Cl)_3]\cdot 1/2H_2O$ and modified Ru (III) complexes. Furthermore, the analysis of the TG/DTG curve, obtained for the ruthenium complex, showed the presence of the 4-ampy ligand, as well as the presence of water, allowing us to suggest the following stoichiometry $[Ru(4\text{-ampy})_1(H_2O)_2(Cl)_3]\cdot 1/2H_2O$, with a relative error of 0.36%. The low solubility of the synthesized compounds makes it challenging to investigate cell viability. Spectroscopy in the middle infrared region, performed for the compounds Ru ($[Ru(4\text{-ampy})_1(H_2O)_2(Cl)_3]\cdot 1/2H_2O$ and modified Ru (III) complex), showed the coordination of the metal center to the ligand in which the donor atom and the nitrogen of the pyridine ring. The analysis of the cell viability assessment of the modified Ru (III) compound demonstrated its cytotoxic effects with an increase in cell death for MCF-7 cells (15.33% ± DP 2.7). Data analysis shows no significant differences in cell death between the PBMC, MCF-7, PBMC + modified Ru (III) complex (Incentive) groups and a similar and low rate.

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Authors' Contributions

Conceptualization, Wagner Santos; Data curation, Andressa Fabricio Moraes and Andressa Ribeiro; Formal analysis, Fabricio Moraes and Andressa Ribeiro; Funding acquisition, Wagner Santos; Investigation, Claudia Sousa; Methodology, Fabricio Moraes, Andressa Ribeiro, and Claudia Sousa; Project administration, Wagner Santos; Resources, Adenilda Honorio-França, Eduardo França, and Wagner Santos; Software, Fabricio Moraes and Andressa Ribeiro; Supervision, Wagner Santos; Validation, Fabricio Moraes and Andressa Ribeiro; Visualization, Fabricio Moraes and Andressa Ribeiro; Writing-original draft, Andressa Ribeiro; Writing-review & editing, Wagner Santos.

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Conflicts of Interest

The authors declare no conflicts of interest and that the ruthenium complex $[Ru(4\text{-ampy})_1(H_2O)_2(Cl)_3]\cdot 1/2H_2O$ and modified Ru (III) complex synthesized in this work is unpublished. We further declare that no other studies involving MCF-7 tumor cells have been carried out with the ruthenium compounds reported here.

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