Semisandwich cobalt(III) complexes as antitumor agents: high intracellular ROS activity and low *in vivo* toxicity

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ABSTRACT

Cp*Co(III) complexes based on the ligand 1-amidino-2-thiourea (guanylylthiourea) have been synthesized and characterized. The antitumor potential of the synthesized complexes was evaluated in vitro using HeLa and HepG2 cell lines. The cytotoxicity of the complexes was assessed, along with their effect on the production of reactive oxygen species (ROS), activation of apoptotic pathways, and cell cycle progression. Finally, the acute toxicity of the complexes was evaluated using the model organism *Caenorhabditis elegans*.

Keywords:
Semisandwich cobalt(III)
Guanidine ligands
Cytotoxicity
Reactive oxygen species
In vivo toxicity

INTRODUCTION

Research into innovative cancer therapies is vital to public health, as cancer is one of the leading causes of death worldwide. A complex aspect of this disease is the relationship between cancer and the generation of reactive oxygen species (ROS).[1],[2] One promising approach to cancer treatment is to significantly increase ROS generation in tumor cells.[3] This triggers a cascade of events that activates intracellular signaling pathways and promotes apoptosis in tumor cells. These processes not only compromise cell viability but also disrupt tumor survival mechanisms, highlighting their importance as an antitumor strategy and a promising alternative to current oncological treatments.[4],[5], [6] Selective modulation of reactive oxygen species (ROS) in cancer cells, without affecting normal cells, is a critical challenge for the development of effective and safe cancer therapies.

In 2017, Liu *et al.* reported on a ruthenium(II) semisandwich complex (Figure 1, complex **I**) with a chelated imino-pyridyl ligand as a selective anticancer agent. This complex increases intracellular ROS compared to the control and eliminates A549 cancer cells by disrupting mitochondrial membrane potential.[7] Subsequently, they developed another ruthenium(II) semisandwich complex with enhanced ROS production, which acted as an anticancer and antimetastatic agent.[8] Chen *et al.* synthesized a ruthenium polypyridyl complex with mixed ligands containing an orthophenolic group. This complex can pass into cancer cells via endocytosis and then translocate from lysosomes to mitochondria, where it increases intracellular ROS levels to selectively kill cancer cells.[9]. Later, they discovered that the solvolysis of the ruthenium complex (Figure 1, complex **II**) can effectively increase its hydrophilicity and cellular uptake, significantly enhancing its anticancer efficacy by inducing mitochondrial dysfunction and consequently increasing intracellular ROS levels.[10]

Other authors have discussed cyclometalated iridium complexes as another type of anticancer agent that increases ROS levels.[11],[12] Iridium complexes (Figure 1, complex **III**) were described as inducers of necroptosis in cisplatin-resistant cancer cells, which can selectively accumulate in mitochondria, disrupt mitochondrial membrane potential, and increase ROS levels.[13] Platinum(II) and palladium(II) complexes (Figure 1, complex **IV**) that caused damage to DNA and mitochondria, increasing intracellular ROS levels and leading to apoptosis.[14],[15] Copper(II) complexes acting through the same mechanism have also been described (Figure 1, complex **V**).[16] Recently, same other metal complexes have been developed to eliminate cancer cells by increasing intracellular ROS levels, using copper as the metal (Figure 1, complexes **VI** and **VII**),[17],[18] rhodium, [19] gold, [20] rhenium, [21] and iridium.[22]

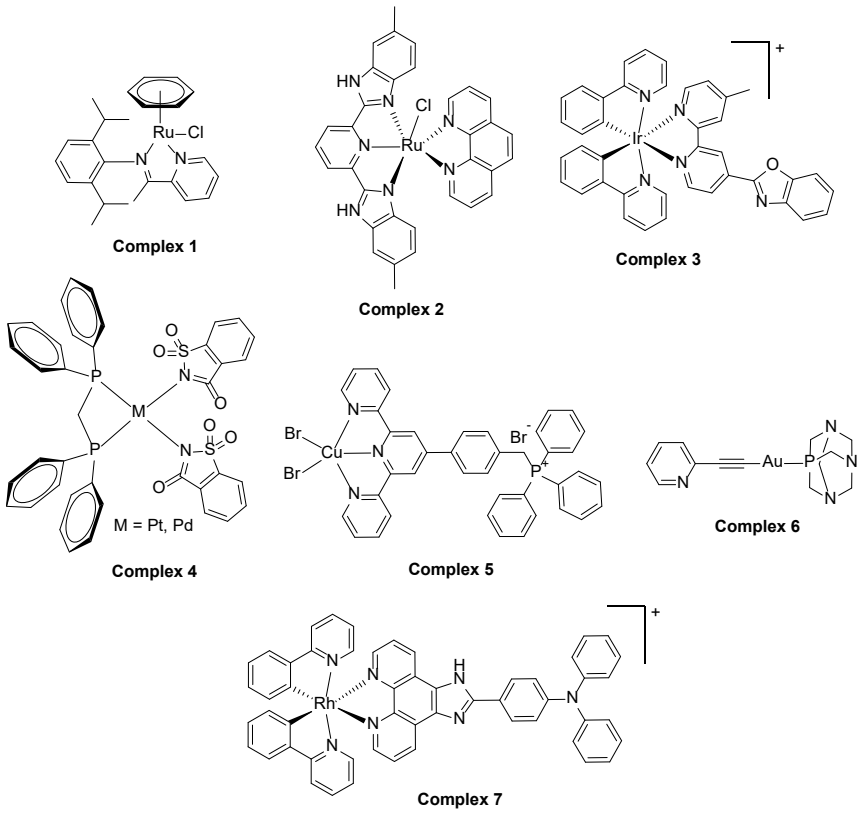


Figure 1. *Metal complexes with anticancer properties described in the literature.*

The use of first transition series metals (3d) as metallodrugs offers several advantages over those from the second and third transition series: lower metal-associated toxicity (many of them are essential trace elements), abundance in the Earth's crust, and socioeconomic accessibility [23], [24]. Specifically, cobalt exhibits high affinity as it is present in various biological systems, such as the metal center in vitamin B12 and other cobalamins [25].

On the other hand, pentamethylcyclopentadienyl (Cp*) and guanidine-type ligands are ligands that help reduce drug toxicity, improve selectivity, and decrease side effects associated with certain medical treatments. For instance, the pentamethylcyclopentadienyl ligand coordinated to a metal center offers the advantage of controlling hydrophilicity and hydrophobicity, thereby modulating cellular uptake and targeting of the metal complex. In addition, they are robust ligands and kinetically inert to substitution, occupying three or two coordination positions. This will allow us to have control over ligand substitution in the complex.

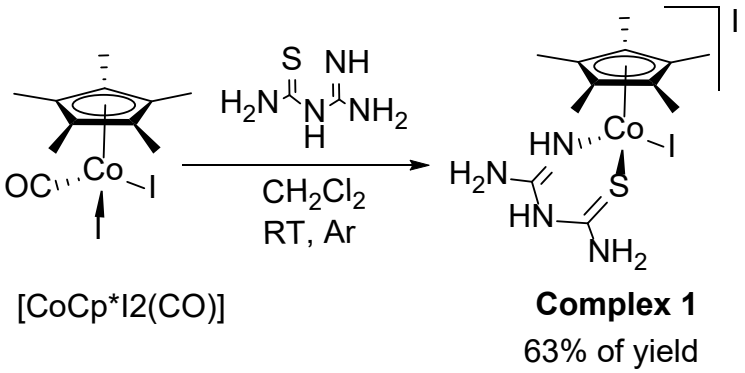
In this study, we present the synthesis, characterization, and biological evaluation of new semisandwich cobalt(III) complexes with 1-amidino-2-thiourea (guaniltiourea) ligand. These complexes exhibit high cytotoxicity in HeLa and Hep G2 cell lines, inducing apoptotic cell death. The complexes cause an extraordinary increase in ROS levels, reaching levels up to six times higher than those observed in control cells. However, despite the elevated ROS levels, all complexes exhibit very low *in vivo* toxicity in C. elegans.

RESULTS AND DISCUSSION

Synthesis and characterisation

Scheme 1 depicts the synthesis of the Co(III) metal complex from the precursor [$CoCpI_2(CO)$].[26] The complex [CpCoI(guanidinothiourea)][I] (**1**) is obtained by

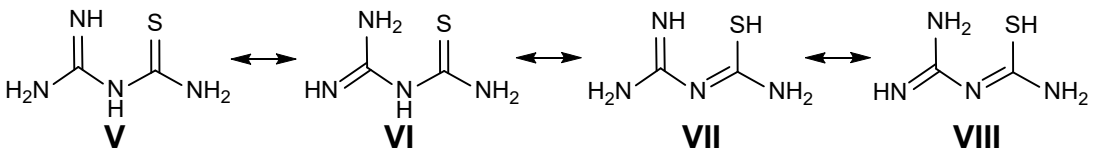
reacting one equivalent of [$CoCp^*I_2(CO)$] with one equivalent of the guanidinothiourea ligand in dry and deoxygenated CH_2Cl_2. The guanidine ligand coordinates to the metal in a bidentate manner, yielding the product with a 63% yield.



*Scheme 1. Synthesis of complex **1**.*

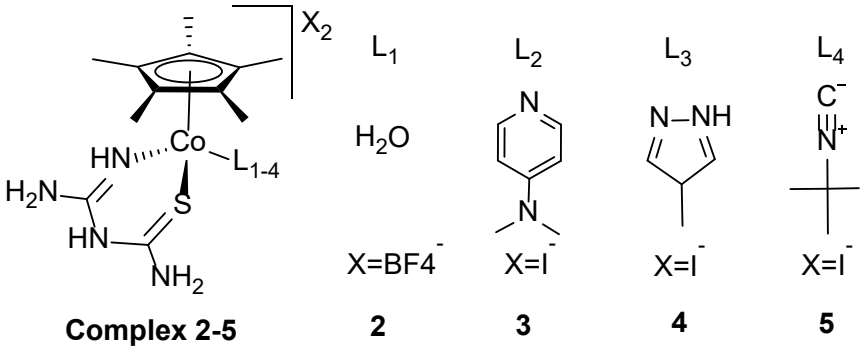
In the NMR spectrum of complex **1**, we observe a single set of signals for the 15 hydrogen atoms of the Cp* group, appearing as a singlet centered at 1.69 ppm, which is shifted 0.55 ppm upfield compared to the Cp* in the metal precursor. This chemical shift to lower frequencies occurs because of the substitution of two ligands—one π-acceptor σ-donor (CO) and another π-donor σ-donor (I^-)—by a fundamentally σ-donor bidentate ligand. The ligand substitution allows the electron density of the metal center to be more efficiently back donated to the Cp* group, causing the Cp* hydrogen atoms to shift upfield. The mass spectrum (MS) of complex **1** shows the molecular ion peak $[M\text{-}I^-\text{-}I^-\text{-}H^+]^+$ at 311.0730 m/z (calcd., 311.3075 m/z), which includes the mass of the guanidinothiourea ligands, Cp*, and the cobalt atom minus one H^+. This data confirms the presence of the {Cp*Co(guanidinothiourea)} fragment in complex **1**. The IR spectrum of complex **1** does not show any signals corresponding to a CO ligand. Therefore, during the formation of complex **1**, the CO group has become uncoordinated.

Additionally, two signals at 1678 cm^{-1} (ν(N=C)) and 1573 cm^{-1} (ν(S=C)) corresponding to the coordinated ligand are observed. These frequencies are much higher than those found for the uncoordinated guanidinothiourea ligand (ν(N=C) = 1615 cm^{-1}; ν(S=C) = 1511 cm^{-1}), indicating a strengthening of the two double bonds N=C and S=C in complexes **1**. This reinforcement of the double bonds in **1** can be understood according to the stabilization of the resonant form **V** (Escheme 2) due to coordination to the metal center.



Scheme 2. Resonant forms of complex guanidinothiourea.

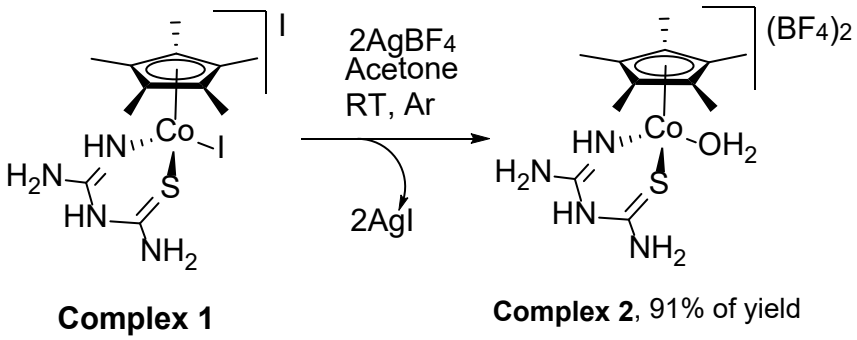
Once the complex [Cp*CoI(guanidinothiourea)][I] (**1**) was synthesized, a family of complexes **2-5** with the stoichiometry [Cp*Co(guanidinothiourea)$\mathbf{L}_{1\text{-}4}$][X]$_2$ (X = I^- or BF_4^-) was synthesized, where **L** represents the monodentate ligands H_2O ($\mathbf{L}_1$), 4-dimethylaminopyridine ($\mathbf{L}_2$), 4-methylpyrazole ($\mathbf{L}_3$), and t-butyl isocyanide ($\mathbf{L}_4$) (Scheme 3).



*Scheme 3. Synthesis of the **complex 2-5**.*

The exchange of I^- for a harmless molecule of H_2O (**L1**) in the coordination sphere can help determine the impact of the presence of the two I^- ions on biological activity. Regarding 4-dimethylaminopyridine (**L2**), it acts as an inhibitor of K^+ channels, which are involved in the growth and proliferation of cancer cell lines. Inhibiting these channels blocks proliferation and triggers cell apoptosis.[27] Pyrazole derivatives, such as 4-methylpyrazole (**L3**), are widely used as antimicrobial agents, anticancer drugs, enzyme inhibitors, antivirals, and anti-inflammatory agents. Finally, although t-butyl isocyanide (**L4**) is an unusual pharmacophore, several isocyanide-type compounds have been reported with antiviral, antimalarial, antibacterial, and antitumor activity. Regarding the latter, cytotoxic activity has been discovered in leukemia cells with isocyanide-derived compounds, as well as inhibition of the enzyme β-glucuronidase, which is overexpressed in various types of cancer.[28]

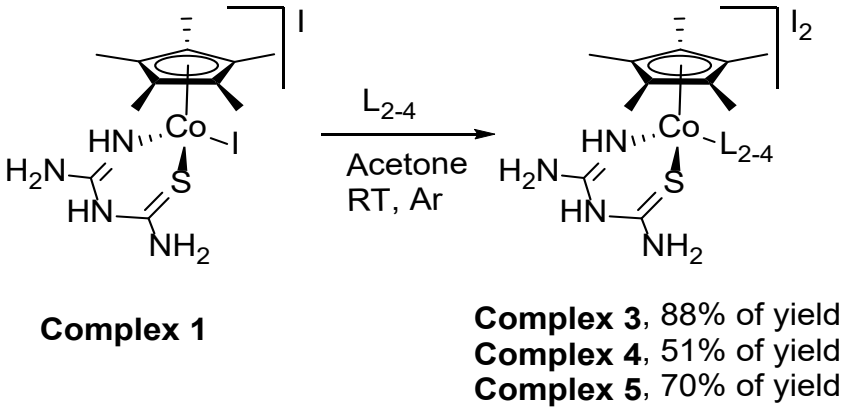
The synthesis of the complex [Cp*Co(guaniltiourea)**L1**][BF_4]$_2$ (**2**) was carried out by reacting one equivalent of complex **1** with two equivalents of $AgBF_4$ in acetone, as shown in Scheme 3. A purple solid was obtained with a yield of 91%.



Complex 1 **Complex 2**, 91% of yield

*Scheme 2. Synthesis of **complex 2**.*

The synthesis of compounds **3-5** follows a similar procedure. A solution of complex **1** in acetone is reacted with the corresponding ligand, 4-dimethylaminopyridine (**L2**) for complex **3**, 4-methylpyrazole (**L3**) for **4**, and t-butyl isocyanide (**L4**) for **5** (Scheme 4).



Scheme 3. Synthesis of ***complex 3-5****.*

Complexes **2-5** were characterized using standard analytical methods and various spectroscopic techniques, including HRMS, IR, UV-Visible, and NMR spectroscopy, utilizing one-dimensional techniques (^{1}H and ^{13}C) as well as two-dimensional techniques (COSY ^{1}H-^{1}H, NOESY ^{1}H-^{1}H, HSQC ^{1}H-^{13}C, and HMBC ^{1}H-^{13}C). The molecular structures of complexes **3** and **5** were determined by X-ray diffraction. The IR spectroscopy for complexes **1-5** (Table 1) shows that, in all cases, similar to complex **1**, the N=C and S=C double bonds of the guanilthiourea ligand are strengthened upon coordination to the metal, indicating that the guanilthiourea ligand is coordinated in a κ^2-*S*,*N* manner.

Table 1. IR frequencies for N=C y S=C bonds of complexes **1-5**.

Compound	ν(N=C) (cm^{-1})	ν(S=C) (cm^{-1})
Free ligand	1615	1511
1	1678	1573
2	1683	1581
3	1678	1558

4	$1670_{solapado}$	1591
5	1679	1566

In the ^{1}H-NMR and HMBC ^{1}H-^{13}C spectra of complex **2**, the Cp* group appears as a single signal in the proton spectrum (1.45 ppm), which correlates with the aromatic carbons of the Cp* group resonating at 95.70 ppm. The Cp* group in solvated complex **2** resonates at approximately 0.17 ppm lower than in complex **1**. Given the presence of a single Cp* group, it is concluded that the complex [Cp*Co(guanilthiourea)H_2O][BF_4]$_2$ (**2**) forms with complete diastereoselectivity.

In the ^{1}H-NMR spectrum of complex [Cp*Co(guanilthiourea)(**L**$_2$)][I]$_2$ (**3**) (**L**$_2$ = 4-dimethylaminopyridine), a singlet is observed at 1.43 ppm corresponding to the 15 hydrogens of the Cp* group, which is 0.26 ppm lower than in compound **1**. A broad singlet (brs) integrating for 6 protons appears at 3.22 ppm, assigned to the two methyl groups of ligand **L**$_2$. The four protons attached to the sp^2 carbons of ligand **L**$_2$ resonate at 6.76 ppm (br, 2H in the meta position) and 8.33 ppm (br, 2H in the ortho position). In the infrared spectroscopy, complex **3** shows an increase in the number of signals in the region of 1683-1533 cm^{-1} due to the addition of the signals from the imine group of the guanilthiourea ligand and the signals corresponding to the 4-dimethylaminopyridine (**L2**) ligand.

Complexes [Cp*Co(guanilthiourea)(**L3**)][I]$_2$ (**4**) (**L3** = 4-methylpyrazole) and [Cp*Co(guanilthiourea)(**L4**)][I]$_2$ (**5**) (**L4** = t-butyl isocyanide) present a single signal for the Cp* group in their respective ^{1}H-NMR spectra, at 1.20 ppm for **4** and 2.02 ppm for **5**. The NMR signals for the 4-methylpyrazole ligand (**L3**) in **4** appear at 2.39 ppm (methyl group) and 7.11 ppm, assigned to the two H of the pyrazole ring. Meanwhile, the t-butyl group of the t-butyl isocyanide ligand (**L4**) in **5** resonates at 1.53 ppm (br).

The IR spectroscopy for **4** shows a band centered at 3430 cm^{-1}, corresponding to the N-H bond of the 4-methylpyrazole ligand. In the IR spectrum of compound **5**, a signal centered at 2194 cm^{-1} is observed, characteristic of the C≡N bond of the isocyanide ligand.

Single crystals of **3** and **5** were obtained from solutions in acetone/ethyl ether and in dichloromethane/ethyl ether, respectively. Compound **3** tends to crystallize as twinned samples, and the poor quality of the obtained X-ray data prevents us from discussing any specific metrical parameter, although, the atomic connectivity has been unambiguously established. The main features of the coordination sphere of complex **5** are reported in Table 2.

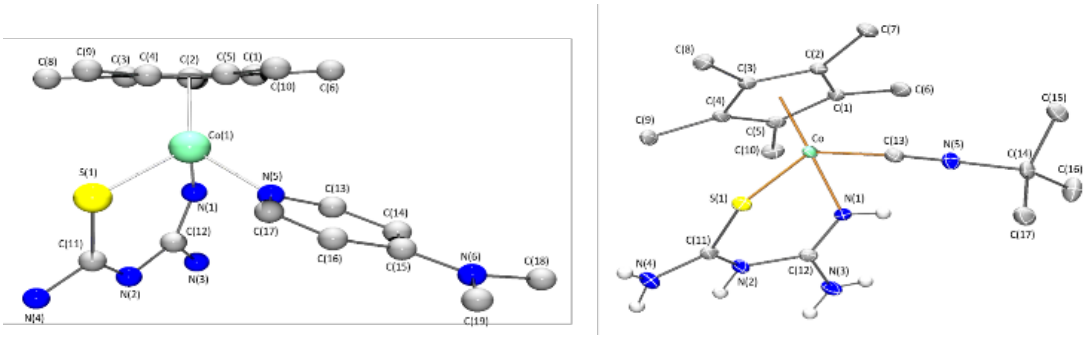


*Figure 2. a) Connectivity of cationic **3** complex. b) Structure of cationic **5** complex. For clarity hydrogen atoms (except those coordinated to N atoms) have been omitted.*

As displayed in Figure 2, in both complexes the cation exhibits a pseudo-octahedral geometry, also known as "three-legged piano stool", with the Cp* ligand coordinated pentahapto to the metal center, occupying three coordination positions. The other three coordination positions are occupied by the guaniltiourea ligand coordinated chelately through the sulfur and nitrogen atoms of the N=C group, and by the respective monodentate ligand, 4-dimethylaminopyridine in **3** and t-butyl isocyanide in **5**. The solid state structure of **3** and **5** are therefore fully consistent with the characterization by

NMR in solution and confirms the anticipated bidentate coordination mode of the guaniltiourea ligand. Notably, both bond lengths of this coordination in **5** (Co(1)-S(1): 2.2380(8) Å and Co(1)-N(1): 1.926(2) Å) are notably shorter than those reported in [Co(III) tris(3-methyl-1-(2-pyridyl)imidazolyl-2-thione)] cation (Co-S: 2.470(8) Å, Co-N: 2.198(2) Å).[30] Crystal packing of **5** shows an extensive hydrogen bonding network, involving-H fragments of thiourea, iodine counterions, and crystallization water (See Supplementary Material).

*Table 2. Bond lengths (Å) and angles (°) of the metal coordination sphere of complex **5**.*

Co-Ct(1)[a]	1.6893(4)	Ct(1)[a]-Co-N(1)	124.22(8)
Co-S(1)	2.2380(8)	Ct(1)[a]-Co-C(13)	125.41(9)
Co-N(1)	1.926(2)	S(1)-Co-N(1)	90.51(8)
Co-C(13)	1.872(3)	S(1)-Co-C(13)	94.19(9)
Ct(1)[a]-Co-S(1)	123.98(3)	N(1)-Co-C(13)	88.21(12)

[a] Ct(1) is the centroid of the Cp* ring.

Cytotoxic Activity

In this study, HeLa and $HepG_2$ tumor cell lines were used as a cellular model to determine the ability of cobalt complexes to cause their cytotoxicity. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [29]. Compounds are soluble in the cell culture medium.

Based on the dose-response curves of cell death, we calculated the IC_{50} values (IC_{50} is the concentration of a compound that is necessary to reduce the cell population by half) (**2**) for each cobalt compound and both cell lines.

Table 3. IC_{50} by MTT assay calculated by non-linear regression. Results are cobalt complexes concentration values expressed in µM (mean ± SEM, n=3).

Compound	HeLa	HepG2
[CoCp*I$_2$(CO)]	NA*	NA*
1	0.25 ± 0.052	0.80 ± 0.045

2	0.31 ± 0.020	0.71 ± 0.063
3	0.23 ± 0.038	0.52 ± 0.074
4	0.25 ± 0.037	0.27 ± 0.034
5	0.28 ± 0.045	0.75 ± 0.090

*NA: no activity detected

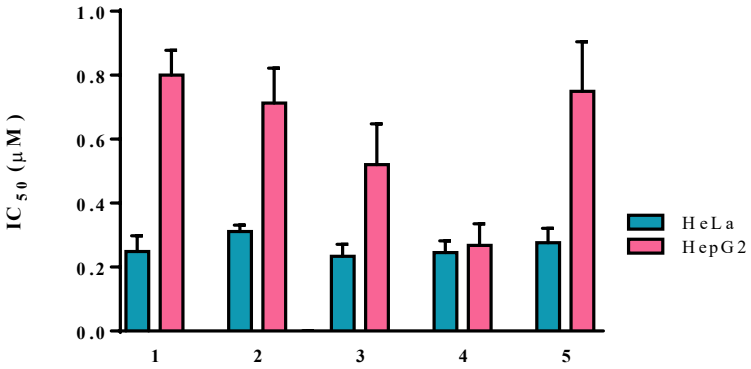


Figure 3. Representation of the IC_{50} values of Cobalt complexes in tumoral cell line HeLa and $HepG_2$.

All complexes, except the precursor compound [CoCp*I_2(CO)], exhibit excellent cytotoxic activity against the HeLa tumor cell line (Table 3). This value increases in all cases for the $HepG_2$ tumor cell line, so it can be concluded that there is greater selectivity of the complexes towards the HeLa cell line except in complex **4**, which is similar (Figure 3). All complexes are active, and their difference may depend mainly on the auxiliary ligand used in each case. The most active complexes are **1** and **3**.

Production of Reactive Oxygen Species in HeLa

To elucidate the potential mechanism of action of the synthesized cobalt complexes, a study was conducted to evaluate the effect of complexes on ROS production capacity of HeLa cells. To evaluate the ROS production capacity of the

synthesized complexes, the protocol used by G. Cásedas et al. applied to the HeLa cell line.[31]

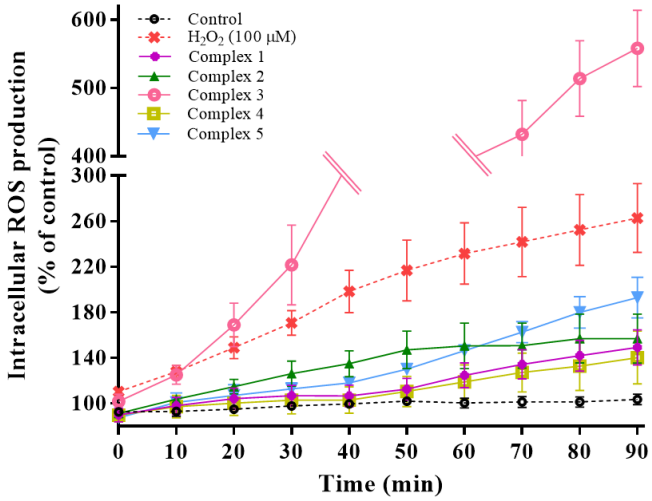


Figure 4. Representation of the generation of intracellular reactive oxygen species from cobalt(III) complexes in HeLa cell line. The concentration tested is 70 percent of the IC_{50} (mean ± SEM µM, n=3).

As shown in Figure **4**, the cobalt complexes synthesized in this study exhibit a significant increase in intracellular ROS in tumor cells compared to control cells. It should be highlighted that complex **3**, as it exhibits a dramatic increase in these species surpassing the positive control (H_2O_2 100µM). This promising result suggests their potential to selectively induce cell death in cells. In the case of complex **3**, an increase in ROS levels approximately six times compared to control cells is observed. To the best of our knowledge, these values of intracellular ROS production are the highest reported in the scientific literature for metal complexes. The semisandwich iridium(III) complex [(η^5:κ^1-$C_5Me_4CH_2py$)Ir(κ^2-phenylpyridine)][PF_6], $C_5Me_4CH_2py$ = 2-((2,3,4,5-tetramethylcyclopentadienyl)methyl)pyridine, synthesized by Pizarro *et al.*[32] and the photosensitizer complexes of the type [Cp*Ir(CΛN)L]$^+$ with expanded π ligands, synthesized by Gonzalo-Navarro *et al.*,[33] show the highest increases in ROS reported in the literature, approximately 5 times higher than the control. Other representative

examples include Elesclomol (N-malonyl-bis(N-methyl-N-thiobenzoylhydrazide)) with a ROS activity 2.4 times greater,[34] and the Ir(III) complex $[Ir(C^N)_2(XY)]$ (C^N = bidentate anionic ligand; XY = Schiff base) with a 2.7 times increase in ROS activity.[35] The complex $[(\eta^5\text{-CpX})Ir(N\Lambda N)Cl][PF_6]$ shows a 2.3 times increase,[36] and the Co(III) complexes derived from curcumin [Co(curcumin)(tetradentate phenolate ligand)]Cl_2 exhibit a 2.1 times increase.[37] These bibliographic results highlight the uniqueness and potential of our compounds, which show increases of 6 times compared to control cells.

Cell Apoptosis Study

Tumor cells are characterized by an imbalance between cell division and cell death, with disease progression resulting from dysregulation in cell death regulatory pathways. Therefore, understanding the mechanism by which the complexes induce cell death is of great importance. Cell death can occur through two distinct pathways, namely, apoptosis and necrosis. Apoptosis is a process of programmed cell dethat and it is governed by genetic programs and requires the prior synthesis of certain proteins, such as caspases. These caspases are key proteins in the transduction and execution of the apoptotic signal, existing within the cell as inactive precursors that need to be activated to initiate their activity. Necrosis results from severe physical, chemical, or osmotic damage, leading to a pronounced inflammatory response associated with the dispersal of cellular components due to membrane rupture. [38] Thus, it is evident that an apoptotic cell death mechanism in the synthesized complexes is the anticipated outcome as seen in Figure 5, the images obtained after 24 hours of cell incubation with the metal complex suggest the occurrence of cell death by apoptosis. This interpretation is based on the observation that the cell membrane of the treated cells appears clearly

defined with the morphology of the cell changed compared to the control cells. These alterations are typical of the apoptotic process.

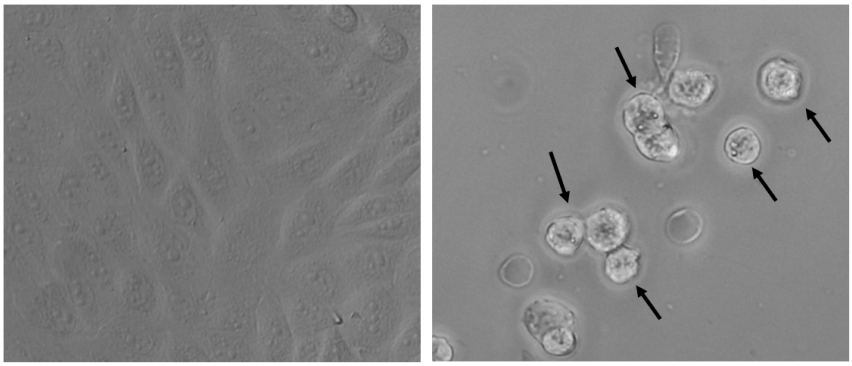


*Figure 5. HeLa cells. Control (right and cells treated with complex **2** after 24 hours (left) at the IC_{50} concentration in which the cell membrane can be observed without alterations (the unaffected membranes are marked with arrows).*

The mechanism of cell death was analyzed through Western Blot in order to elucidate whether these compounds activate caspases 3 and 9, related to the intrinsic pathways of cellular apoptosis. For this purpose, the HeLa cell line was used. The selected compounds were **1** and **3**. These compounds were chosen because both are highly active in the cytotoxicity assay.

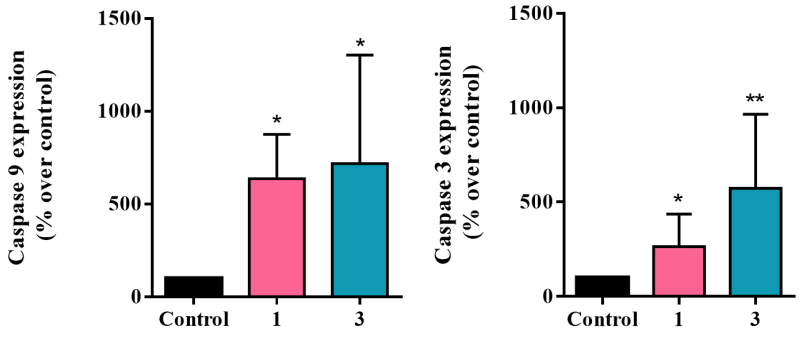


Figure 6. Representation of caspase 3 and 9 activation versus normalized control cells (n=3). Differences compared to the control group were considered significant at $p < 0.05$ (*).

In Figure 6, both complexes significantly activated both caspases regarding the control value, indicating that they act via the intrinsic pathway of cellular apoptosis. The activation of both caspases is a key indicator of the initiation of the apoptotic cascade.

Cell cycle

The effect of **complexes 1** and **3** on the cell cycle of the HeLa cells line was evaluated using flow cytometry for this 20.000 cells per condition and duplicated.

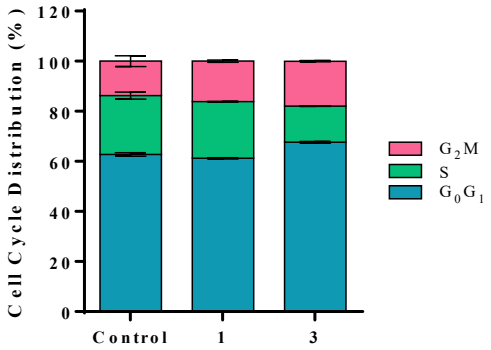


Figure 7. Representation of the arrested cells in each phase of the cell cycle to the control cells in percentage.

As seen in Figure 7, complex **1** shows a behavior like control cells in all phases, not being specific to any phase of the cell cycle. In contrast, complex **3** significantly increases the cell arrest in G0/G1 at 7%, thus acting selectively in this part of the cell cycle.

***In vivo* studies with *Caenorhabditis elegans* (*C. elegans*)**

C. elegans model was used to evaluate the acute toxicity of the complexes in a liquid medium.[39] This model was used because it is a multicellular organism that shares basic physiological processes and stress responses with higher organisms, such

as humans. Additionally, *C. elegans* retains between 60% and 80% of human genes and 12 out of 17 signaling transduction pathways. Moreover, it is a powerful research model due to its easy maintenance and low cost, short life cycle, and ability to produce a large number of offspring.[40] Currently, *C. elegans* has been employed to investigate the characteristics of drug-induced neuropathies used in anticancer therapies, such as cisplatin, due to its resemblance to chemotherapy-induced peripheral neuropathy in humans[41] as a toxicity model.[42].

The synchronized L_4 wild-type *C. elegans*[43] were exposed to compounds **1** and **3** at a concentration ranging from 0 to 3.5 µM for 24h incubation. Toxicity us easily screened by survival of the worms (existence of movement). The higher concentrations tested (3.5 µM to compound **1** and 2.9 µM to compound **3**) had a negative impact on the viability of the nematodes ($p < 0.0001$). The mortality to these concentrations increased by approximately in 2.8% to compound **1** and 3.3% to compound **3** with respect to the control, without reaching in both cases the lethal concentration 50 (LC_{50} is the necessary concentration of drug to reduce the population by half), this indicates that our results are promising due to the difference in the effective dose required *in vitro* model and the low toxicity observed in the *in vivo* model. We are not aware of any bibliographic examples where the toxicity of cobalt(III) organometallic complexes has been studied. We have selected the work of Boubaker *et al.* in 2024,[44] who studied the toxicity of semi-sandwich Ru(II) complexes [Ru_2(SDX)] (where SDX is a bridging trithiolato ligand modified with sulfadoxine) in the biomodel *Toxoplasma gondii*, an alternative model organism to *C. elegans*. This Ru(II) dimer has an LC_{50} of 0.112 µM for *in vivo* exposure in *T. gondii*, a concentration which, although not directly comparable, is at least 30 times lower than the maximum concentration used in our *in vivo* assays in *C. elegans*.

CONCLUSIONS

Semisandwich Co(III) complexes based on the guanylthiourea ligand have been synthesized and characterized. These complexes exhibit high cytotoxicity in HeLa and HepG2 tumor cell lines, inducing apoptosis via a significant increase in intracellular reactive oxygen species (ROS). Among the complexes studied, compounds **1** and **3** are particularly effective, achieving up to sixfold increase in ROS levels compared to control cells, the highest value in the scientific literature. Despite their potent *in vitro* cytotoxic activity, these complexes exhibit low *in vivo* toxicity in the *C. elegans* model. These findings suggest that the studied Co(III) complexes hold potential for the safe and effective development of oncological therapies.

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