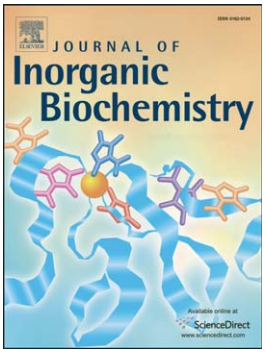

Ru(III) anticancer agents with aromatic and non-aromatic dithiocarbamates as ligands: Loading into PF127 micelles and preliminary biological studies

S. Scintilla, L. Brustolin, A. Gambalunga, F. Chiara, A. Trevisan, C. Nardon, D. Fregona

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Ru(III) anticancer agents with aromatic and non-aromatic dithiocarbamates as ligands: loading into PF127 micelles and preliminary biological studies.

Scintilla S. [a,#], Brustolin L. [a,#], Gambalunga A.[b], Chiara F. [b], Trevisan A. [b], Nardon C. [a,*], Fregona D. [a,*]

[a] Department of Chemical Sciences, University of Padova, Via F. Marzolo 1, 35131 Padova, Italy

[b] Department of Cardiac, Thoracic and Vascular Sciences, University of Padova, Via Giustiniani, 2, 35128 Padova, Italy

[*] corresponding authors

[#] these authors contributed equally to this work

In memory of Professor Alessandro Bagno (Dept. of Chemical Sciences, University of Padova), for his contribution to NMR spectroscopy of species containing paramagnetic centers.

Abstract

Since the discovery of cisplatin in the 1960s, other metal complexes have been investigated as potential antitumor agents to overcome the side-effects associated with the administration of the Pt-based drug. In line with our previous research, in this work we report the synthesis and characterization of mono- and dinuclear Ru(III) complexes with the pyrrolidinedithiocarbamate (PDT) ligand and the more sterically-hindered carbazole-dithiocarbamato ligand (CDT), to compare their properties (both at the chemical and antiproliferative level), in an attempt to assess a structure-activity rationale. Moreover, to overcome the scarce solubility under physiological conditions of the Ru(III)-dithiocarbamato compounds, the biocompatible copolymer Pluronic® F127 has been used, to encapsulate the metal derivatives in water-soluble micellar carriers. Finally, preliminary biological evaluations on CDT and PDT compounds along with their nanoformulations, open intriguing perspectives in anticancer chemotherapy. In particular, comparing the structure of the Ru(III) derivatives, the ionic dinuclear PDT complex shows an important cytotoxic action in comparison to its neutral counterparts. Moreover, the micellar carrier improves the overall activity of the encapsulated Ru(III)-PDT chemotherapeutics. On the other hand, the nanoformulation of the CDT derivatives allows us to solubilize both the 1:3 and the 2:5 complexes and to state their inactivity .

Keywords: Ru(III) complexes, dithiocarbamate, Pluronic F127, cancer, micelle, nanoformulation.

1. Introduction

Cancer is now the leading cause of death worldwide [1] and intense efforts are still devoted to develop new and more effective therapies [2–4]. Since the successful application of cisplatin against a variety of solid tumors [5], a large number of metal-based complexes - including both platinum and non-platinum (Au, Cu, Os, Pd, Ru, Rh and Ir) centers, combined with an even larger variety of ligands- have been extensively investigated to increase the efficiency and overcome the drawbacks exhibited by their parent compound [6–9].

The side interactions of metallodrugs with biomolecules (mainly those involving sulfur aminoacids, such as glutathione or cysteine) are crucial in their pharmacology and clearance.[10]In the light of this consideration, in the early stages of clinical use, sulfur-containing nucleophiles were administered after treatment with Pt(II)-based drugs in the attempt to modulate the related patient-disabling side-effects. The results, obtained by using sodium diethyldithiocarbamate (DEDTNa) as a chemoprotectant for cisplatin [11], inspired us to develop compounds which combine - in a single molecule - the chemoprotective properties of this type of organic moiety with the cytotoxic activity of a chosen metal center [12]. Besides platinum, our research group has been studying also Ru(III) derivatives, inspired by the promising activity of the three complexes NAMI-A, KP1019 and NKP-1339 (which have reached human clinical trials [14–16]) and other ruthenium-based complexes exhibiting activity against cisplatin-resistant tumors with less severe side-effects compared to platinum drugs [13]. Starting from the works of Pignolet and Hendrickson [17–20], our research group has developed novel Ru(III) dithiocarbamato (dtc) complexes (Fig. 1, panel I) with the ligands pyrrolidyldithiocarbamate (PDT), *N*,*N*-dimethyldithiocarbamate (DMDT) and alkyl-sarcosyldithiocarbamate (RSDT, R = Me, Et, tBu) (Fig. 1, panel II) [21–24].

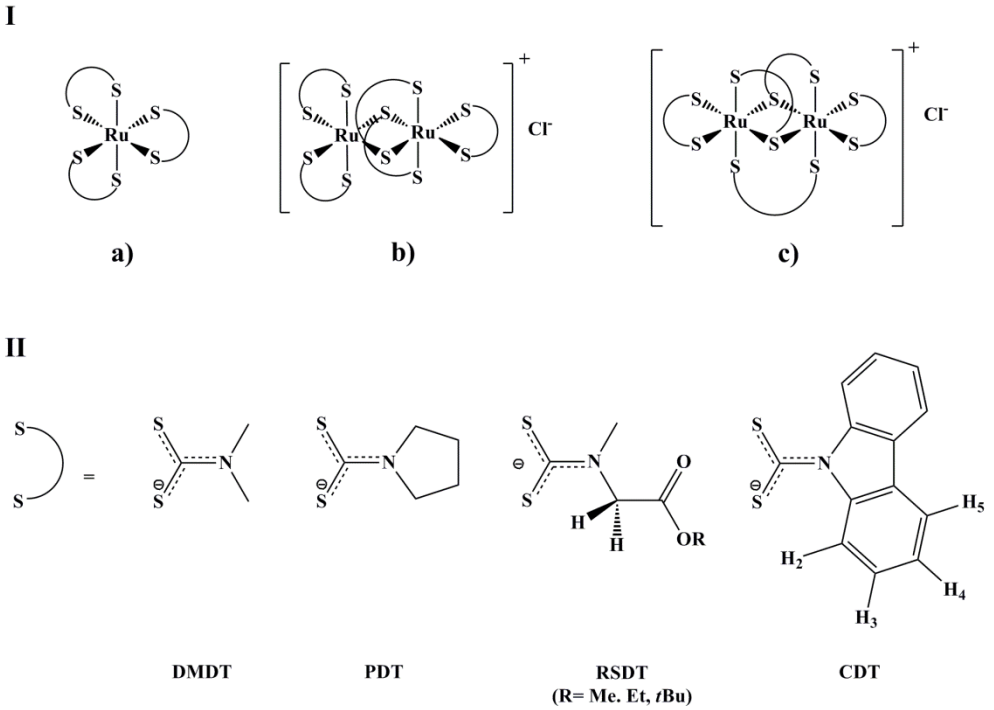


Fig. 1. I, Chemical structure of our Ru(III)-dithiocarbamato complexes [Ru(dtc)$_3$] (a), α-[Ru$_2$(dtc)$_5$]Cl (b) and β-[Ru$_2$(dtc)$_5$]Cl (c). II, Chemical structure of the investigated dithiocarbamato ligands. *N,N*-Dimethyl dithiocarbamate (DMDT), Pyrrolidyl dithiocarbamate (PDT), Sarcosyl-alkyl-ester dithiocarbamate (RSDT) and Carbazolyl dithiocarbamate (CDT).

In vitro cytotoxicity assays performed on different human tumor cell lines indicated that the ionic dinuclear complexes (in particular in the case of α-[Ru$_2$(DMDT)$_5$]Cl and α-[Ru$_2$(TSDT)$_5$]Cl (TSDT = *tert*-butyl sarcosine dithiocarbamate), Fig. 1, panel II) had superior anticancer activity than cisplatin against solid tumors (IC_{50} < 1 μM). On the contrary, the monomeric species were more selective towards leukemic cells. Recently, it has been reported that [Ru$_2$(PDT)$_5$]Cl is ten-fold more active than cisplatin against NCI-H1975 line (non-small cell lung cancer). Very attractively, the antiproliferative activity is strongly affected by the chemical nature of the coordinated dithiocarbamato-ligand. Ru(III) complexes containing PDT ligands are much more active than the DMDT counterparts, underlining the crucial role of the ligand moiety for the efficacy of the drug [25].

In this work we report the synthesis and physico-chemical characterization (elemental analysis, ESI-MS, ^{1}H-NMR spectroscopy, UV-Vis and FT-IR spectrophotometries) of novel Ru(III)-carbazolyl-dithiocarbamato-derivatives (CDT, Fig. 1, panel II). Compared to pyrrolidine, carbazole possesses two aromatic rings, condensed on the central heterocycle, thus conferring a pronounced hydrophobic character on the complex, which may favor interactions with the cell membrane and specific regions of proteins and organelles, possibly enhancing its cytotoxicity. Both Ru(III)-PDT and -CDT derivatives have been tested as anticancer chemotherapeutics towards two human tumor

epithelial cell lines, HeLa (cervix adenocarcinoma) and HCT 116 (colon carcinoma), with the aim to get structure/activity relationships. For the *in vitro* tests, Pluronic® F127, a non-ionic surfactant, has been used to increase the water solubility and, hence, the bioavailability of the investigated Ru(III) complexes. The choice of Pluronic® F127 was not accidental. The cytotoxicity exhibited by Pluronic® micelles towards non-cancerous cells was significantly lower than that observed for cancerous cells [26], pointing out intriguing selectivity properties. Its combination with Pluronic® L61 has been used to obtain doxorubicin-loaded mixed micelles, which have already reached Phase III stage in human clinical trials on patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction [27,28]. These interesting properties have recently led our research group to investigate PF127 as a carrier for Au(III)-dithiocarbamato complexes, thus providing a smart solution for the implementation of *in vivo* tests of metallodrugs [29,30]. In addition, literature reports of attempts to incorporate Ru(III)-based drugs into other nanosystems [31,32].

The results collected with PF127 micelles loaded with Ru(III) compounds are discussed in comparison with those obtained *via* the common methodology, involving DMSO as a solubilizing agent [24].

2. Experimental

2.1. Materials

All reagents and solvents were used as supplied, unless otherwise stated, from Sigma Aldrich without any further purification: ruthenium(III) chloride hydrate ($RuCl_3 \cdot 3H_2O$), carbazole, sodium hydride (NaH) 60% - dispersion in mineral oil -, carbon disulfide (CS_2), sodium pyrrolidine-dithiocarbamate (NaPDT), cisplatin, *n*-octanol, saline solution, sodium sulfide (Na_2S), sulfuric acid (H_2SO_4) 98%, dichloromethane (CH_2Cl_2), diethyl ether (Et_2O), ethanol (EtOH), methanol (MeOH), acetone, acetonitrile, dimethylsulfoxide (DMSO), tetrahydrofuran (THF), chloroform ($CHCl_3$), pentane, hexane, deuterated chloroform ($CDCl_3$), dimethylsulfoxide-d_6 (DMSO-d_6), deuterium oxide (D_2O). Tetrahydrofuran (THF) was dried using standard distillation procedures. The synthetic procedures to obtain the carbazolyl dithiocarbamate and Ru(III) derivatives were performed under controlled nitrogen atmosphere with a Schlenk line and Schlenk glassware. All the aqueous reactions were conducted in distilled water, purified by means of ionic exchange membrane filters.

A dialysis tubing cellulose membrane (avg. flat width 25 mm, MW cut-off 11,181 Da, Sigma-Aldrich) was used for release assays and prepared as reported in section 2.12.

For *in vitro* cytotoxicity studies, the following products were purchased and used as provided by suppliers: HeLa cells, HCT 116 cells (American Type Culture Collection, ATCC); Dulbecco Modified Eagle's Medium (D-MEM), L-glutamine, penicillin, streptomycin, fetal bovine serum

(FBS), trypsin (0.05%, EDTA 0.02% in PBS) (Euro Clone); Pluronic® F127, DMSO (>99.9%, for biological treatments), *in vitro* toxicology assay kit (resazurin based) (Aldrich).

2.2. FT-IR spectroscopy

Near-FT-IR spectra (4000-400 cm^{-1}) were recordered at room temperature (32 scans, resolution 2 cm^{-1}) by Nicolet Nexus 5SXC spectrophotometer. KBr pellets of samples were prepared according to standard procedures. Far-FT-IR spectra (600-50 cm^{-1}) were registered at room temperature with a Nicolet Nexus 870 spectrophotometer. For the analysis, films of sample dispersed in nujol were loaded on polyethylene discs (250 scans, resolution 4 cm^{-1}). Spectra were processed with OMNIC 5.2 (Nicolet Instrument Corporation).

2.3. NMR spectroscopy

NMR spectra were recorded from samples with a typical concentration of 5mM at 298 K on a Bruker Avance DRX300 spectrometer equipped with a BBI [1H, X] probe-head and on a Bruker Avance DRX400 equipped with a BBI-5 mm z-field gradient probe-head. Typical one-dimensional ^{1}H-NMR spectra were acquired with 128 scans, recycle delay of 1-4 s, spectral window 0÷14 ppm. ^{1}H spectra of paramagnetic samples were acquired with 256 scans, recycle delay of 40 ms, with -50÷50 ppm spectral window. The ^{1}H-NMR chemical shifts (δ) of the signals are given in ppm and referenced to residual protons in the deuterated solvents: chloroform-*d* ($CDCl_3$, 7.26 ppm), dimethylsulfoxide-d_6 (DMSO-d_6, 2.50 ppm), deuterium oxide (D_2O, 4.80 ppm). Data processing was carried out by means of MestReNova version 6.2.0 (Mestrelab Research S.L.).

2.4. UV-Vis spectroscopy

Absorption spectra of freshly prepared solutions of the investigated complexes were acquired at 298 K or 310 K in the range 200-800 nm by an Agilent Cary 100 UV-Vis double beam spectrophotometer. Samples were dissolved in the appropriate solvents and the resulting solutions were placed in QS quartz cuvette (path length 1 cm). According to the Lambert-Beer law, molar extinction coefficients for the investigated complexes were extrapolated by calibration curves, assessed by recording spectra at different concentrations (7, 5, 4, 3 and 2 $\times 10^{-5}$ M).

2.5. ESI-MS spectrometry

ESI-MS spectra were recorded on a Mariner Perspective Biosystem instrument, setting a 5 kV ionization potential and a 20 μL/min flow rate. A mixture of coumarin and 6-methyl-triptophan was used as a standard. Samples were dissolved in methanol or acetonitrile, whereas methanol with 1% formic acid was used as eluent. ESI-MS spectra have been processed by the software Data Explorer.

2.6. Silica gel and thin layer chromatography

Analytical TLC and preparative TLC were performed on Kiesegel F254 and Kiesegel 60 (thickness 2 mm) (Fluka), respectively. UV light (λ= 254 nm) allowed spot after TLC runs. Gravity column chromatography was carried out on silica gel Kiesegel 60 (40-63 μm) (Fluka); the elution of the loaded compound was obtained by using the proper eluent mixture.

2.7. Elemental analysis

Elemental analyses were carried out at the Microanalysis Laboratory of the Department of Chemical Sciences, University of Padua by using a microanalyzer Fisons EA-1108 CHNS-O and a microanalyzer Carlo Erba 1108 CHNS-O.

2.8. Synthesis of Ru(III) precursors

2.8.1. Synthesis of [(DMSO)$_2$H][trans-Ru(DMSO)$_2$Cl$_4$]. The synthetic procedure was carried out as described elsewhere [33]. After precipitation of red-orange crystals the supernatant was removed and the crystals washed with acetone, Et_2O and dried under vacuum in presence of P_2O_5.

Yield: 35%

^{1}H-NMR (D_2O, 300.13 MHz, 298 K, δ/ppm): 2.7 (br, 6H, S-C*H*$_3$ $[DMSO_2H]^+$, cationic moiety), -17.0 (br, 6H, S-C*H*$_3$, coordinated DMSO).

2.8.2. Synthesis of Na[trans-Ru(DMSO)$_2$Cl$_4$]. The synthetic procedure was carried out as described elsewhere [33]. The product rapidly precipitated from the mixture as light orange microcrystals, which were filtered off, washed with cold EtOH, Et_2O and dried under vacuum dried in presence of P_2O_5.

Yield: 75%

^{1}H-NMR (D_2O, 300.13 MHz, 298 K, δ/ppm): -16.8 (br, 6H, S-C*H*$_3$).

Anal. Calc. for $C_4H_{12}Cl_4O_2S_2RuNa$ (M.W.= 422.14) H 2.87; C 11.38; S 15.19. Found H 2.89; C 11.25; S 15.32.

2.9. Ru-based PDT complexes

2.9.1. Synthesis of [Ru(PDT)$_3$]. The synthetic procedure was carried out as described previously [21]. To a solution of Na[*trans*-Ru(DMSO)$_2$Cl$_4$] (42.2 mg, 0.10 mmol) in EtOH (4 mL), a solution of NaPDT (64.6 mg, 0.38) in EtOH (4 mL) was added. The mixture was stirred for 20 minutes at room temperature, leading to the formation of a dark brown solid which was filtered off and washed with cold EtOH (2 x 2.0 mL). The solid was re-dissolved in CH_2Cl_2 and purified by silica gel chromatography, using CH_2Cl_2 to elute the mononuclear complex first. Successively, the mixture

was switched to the more polar eluent CH_2Cl_2/MeOH 95:5 to obtain dinuclear complexes (as byproducts).

Yield: 14%, dark green solid

R.f. (on silica gel, CH_2Cl_2): 0.85

^{1}H-NMR ($CDCl_3$, 300.13 MHz, 298 K, δ/ppm): 44.4 (br, 2H, (N)CH_2), 36.0 (br, 4H, CH_2-N-CH_2), 0.3 (br, 4H,CH_2-CH_2).

ESI-MS $[M]^+$ 539.4 m/z (calc. = 539.8).

Anal. Calc. for $C_{15}H_{24}N_3S_6Ru$ (M.W.= 539.84) H 4.48; C 33.37; N 7.78; S 35.64. Found H 4.58; C 33.45; N 7.56; S 36.02.

2.9.2. Synthesis of [Ru$_2$(PDT)$_5$]Cl (α and β mixture). According to the literature [21], to a solution of $RuCl_3 \cdot 3H_2O$ (0.40 g, 1.50 mmol) in water (4 mL), 10 mL of a NaPDT aqueous solution (0.78 g, 4.60 mmol in 10 mL) were added dropwise. A dark brown solid precipitated instantaneously. The mixture was stirred for 1 h, then the solid was filtrated and washed with cold water (3 x 3.0 mL) and cold Et_2O (2 x 2.5 mL). The isolated product was re-dissolved in CH_2Cl_2 and purified by silica gel chromatography, by using CH_2Cl_2 to elute the mononuclear byproduct and, afterwards, a mixture CH_2Cl_2/MeOH 95:5 to elute the desired isomer mixture complexes.

Yield: 19%, dark red solid

R.f. (on silica gel, CH_2Cl_2/MeOH 92:8): 0.23

^{1}H-NMR ($CDCl_3$, 300.13 MHz, 298 K, δ/ppm): 4.25-3.15 (m, 20H, CH_2-N-CH_2), 2.15-1.04 (m, 20H, CH_2-CH_2).

ESI-MS $[Ru_2(PDT)_5]^+$ 933.1 m/z (calc. = 933.5).

Anal. Calc. for $C_{25}H_{40}N_5S_{10}Ru_2Cl$ (M.W.= 968.87) H 4.16; C 30.99; N 7.23; S 33.10. Found H 4.01; C 31.12; N 7.42; S 33.02.

2.9.3. Isomerisation of the α/β mixture to β-[Ru$_2$(PDT)$_5$]Cl. The isomerization from α-$Ru_2(PDT)]_5Cl$ to β-$[Ru_2(PDT)]_5Cl$ was carried out according to a method described in literature [20], in order to achieve the most thermodynamically stable isomer. 0.14 g (0.14 mmol) of α,β-$[Ru_2(PDT)]_5Cl$ mixture was heated under reflux into a minimum volume of MeOH (3 mL) for 8 h. Successively, the mixture was allowed to cool down and a dark red solid was then separated and dried under vacuum.

^{1}H-NMR ($CDCl_3$, 300.13 MHz, 298 K, δ/ppm): 3.98-3.40 (m, 20H, CH_2-N-CH_2), 2.18-1.80 (m, 20H, CH_2-CH_2).

ESI-MS $[Ru_2(PDT)_5]^+$ 933.1 m/z (calc. = 933.5).

Anal. Calc. for $C_{25}H_{40}ClN_5S_{10}Ru_2$ (M.W.= 968.87) H 4.16; C 30.99; N 7.23; S 33.10. Found H 4.20; C 31.15; N 7.22; S 32.97.

2.10. Synthesis of Ru-based CDT complexes

2.10.1. Synthesis of Na(CDT). The sodium salt of carbazole-dithiocarbamate (NaCDT) was synthetized by following a synthetic procedure reported in literature properly modified [34]. According to this procedure, to 0.24 g (1.44 mmol) of carbazole previously dissolved in freshly distilled THF under inert N_2 atmosphere, 0.12 g (2.88 mmol) of NaH 60% suspension were added at 0 °C. After the reaction was kept under vigorous stirring for 10 minutes, the mixture was filtered under nitrogen to remove NaH residues. Then, 173 μL (2.88 mmol) of dry CS_2 were added to the resulting solution under stirring at 0 °C. After 2 h the solvent was removed under reduced pressure and a bright yellow-orange solid was obtained. The product is conserved under inert atmosphere, as it resulted highly hygroscopic and air sensitive, decomposing within 30 s into a black tar. The product is used without any further purification (Fig. 1, panel I).

Yield: 80%

^{1}H-NMR ($CDCl_3$, 300.13 MHz, 298 K, δ/ppm): 8.82-8.75 (m, 1H, H_5), 8.05-8.12 (m, 1H, H_2), 7.93-7.87 (m, 1H, H_3), 7.46-7.41 (m, 1H, H_4).

2.10.2. Synthesis of [Ru(CDT)$_3$] and α-[Ru$_2$(CDT)$_5$]Cl. By using a Schlenk-line apparatus under N_2 atmosphere, 0.31 g (1.15 mmol) of synthesized Na(CDT) and 0.24 g (0.92 mmol) of $RuCl_3 \cdot 3H_2O$ were dissolved in 18 mL of freshly distilled THF. The mixture was left under stirring for 15 h at room temperature. The solvent was successively removed under reduced pressure, leaving a black solid that was washed with cold pentane (5 x 5.0 mL). The dinuclear complex was isolated by several precipitation cycles in THF/pentane mixture (1:1 v/v). The THF/pentane solution is dried leaving a dark green solid that was dried under vacuum and purified by silica gel chromatography using an eluting mixture CH_2Cl_2/hexane 4:6 to isolate the mononuclear complex.

[Ru(CDT)$_3$]: Yield: 11%, aspect: dark green

R.f. (on silica gel, CH_2Cl_2/hexane 1:1): 0.88

^{1}H-NMR ($CDCl_3$, 400 MHz, 298 K, δ/ppm): 9.03-9.01 (d, 1H, H_3), 8.91-8.88 (t, 1H, H_4), 7.73-7.71 (d, 1H, H_5).

ESI-MS $[M]^+$ 828.0 m/z (calc. = 827.9).

Anal. Calc. for $C_{39}H_{24}N_3S_6Ru$ (M.W.= 828.09) H 2.92; C 56.57; N 5.07; S 23.23. Found H 3.28; C 56.52; N 4.86; S 23.57.

α,β-[Ru$_2$(CDT)$_5$]Cl: Yield: 17%, aspect: dark orange

R.f. (on silica gel, CH_2Cl_2/hexane 1:1): 0.80

^{1}H-NMR ($CDCl_3$, 400 MHz, 298 K, δ/ppm): 9.19-9.17 (d, 1H, H_2), 8.00-7.98 (d, 1H, H_5), 7.53-7.46 (m, 1H, H_3+H_4).

ESI-MS $[M+CH_3CN]^+$ 1490.5 m/z (calc. = 1490.9).

Anal. Calc. for $C_{65}H_{40}ClN_5S_{10}Ru_2$ (M.W.= 1449.30) H 2.87; C 53.87; N 4.83; S 22.12. Found H 2.70; C 53.79; N 4.67; S 22.18.

2.10.3. Isomerisation of α/β mixture to β-[Ru$_2$(CDT)$_5$]Cl. The reaction of isomerization was carried out by following the methodology described in Section 2.9.3.

Aspect: dark orange

^{1}H-NMR ($CDCl_3$, 400 MHz, 298 K, δ/ppm): 9.19-9.17 (d, 1H, H_2), 8.00-7.98 (d, 1H, H_5), 7.53-7.46 (m, 1H, H_3+H_4).

ESI-MS $[M+CH_3CN]^+$ at 1490.5 m/z (calc. = 1490.9)

Anal. Calc. for $C_{65}H_{40}ClN_5S_{10}Ru_2$ (M.W.= 1449.30) H 2.87; C 53.87; N 4.83; S 22.12. Found H 2.91; C 53.50; N 4.51 ; S 22.49.

2.11. In vitro cytotoxicity studies

2.11.1. Preparation of samples for cytotoxicity assays. Tests were carried out using Ru(III)-pyrrolidinedithiocarbamato and Ru(III)-carbazoledithiocarbamato complexes. Due to their low solubility in water, Pluronic® F127 surfactant was used to facilitate solubilization of the compounds in physiological media. The samples were prepared by dissolving both the polymer (a weighted amount to yield a final concentration of 40 mg/mL in saline solution – see below) and the complex in CH_2Cl_2 and successively evaporating the solvent so to obtain a homogeneous thin layer. The subsequent addition of saline solution (0.9% NaCl w/v) and sonication for 2 minutes led to the formation of a mixture 140 μM in Ru complex ready for cell treatment, upon proper dilution with cell culture medium (D-MEM). DMSO (sterile) solutions of carbazole, [Ru(PDT)$_3$] and β-[Ru$_2$(PDT)$_5$]Cl were also prepared and, after *ad hoc* dilution with D-MEM medium, tested on the cell cultures to compare the polymer-mediated release of the investigated compounds with the activity found in DMSO vehicle. The notation PL{compound} stands for the use of micellar systems.

2.11.2. Cell lines and culture conditions. Cell cultures have been incubated at 37 °C in a 5% carbon dioxide controlled atmosphere of a Hera Cell 150i CO_2 incubator (Thermo Scientific). Cellular vitality was determined by absorbance measurements at 595 nm, using a plate reader ELISA

Microplate Reader Model 550 (Bio-Rad). Cell counting was performed with a Bruker camera emocytometer. Data were obtained and processed by Microplate Manager 4.0 and Origin 8.0 software.

For this work, HeLa and HCT 116 cells were grown in a culture flask (surface 75 cm^2) in Dulbecco's modified Eagle's medium (D-MEM), with addition of FBS (10%), L-glutamine (5 mM) and antibiotics (streptomycin, penicillin) and incubated at 37 °C in a 5% carbon dioxide controlled atmosphere. After the removal of the medium, cells were washed with 6 mL of PBS (phosphate buffered saline) solution and 1 mL of trypsin was added. The culture flask was incubated for 3 min and then shaken to remove cells from the flask surface. 3 mL of D-MEM were successively added to the flask, to block the action of trypsin. After that, cellswere counted, transferred to the test plates (polystyrene, 96-well) and incubated for 24 h before treating with complexes.

2.11.3. Cell growth inhibition assay. The treatments were carried out for either 24 or 72 hours starting from the following cell densities: 20,000 and 25,000 cells/mL in 24 hours-experiments for HeLa and HCT 116 lines, respectively; 10,000 and 15,000 cells/mL in 72 hours-experiments for HeLa and HCT 116 line, respectively. Briefly, when confluence was about 70-80%, D-MEM was removed from the cell cultures. The previously described saline solutions (NaCl 0.9% w/v) of the micellar samples were diluted with D-MEM (saline < 29% v/v) to yield the final tested concentrations (typically in the range 0.1 - 20 μM) and hence added to the corresponding wells (200 μL/well). Similarly, DMSO solutions were properly diluted with medium at a safe DMSO concentration of 0.1% v/v. At least three independent experiments were carried out under quadruplicate conditions for every tested μM concentration. Cisplatin (in saline solution, NaCl 0.9% w/v) has been used as a reference drug.

Growth inhibition was evaluated by Resazurin-test [35,36] , by removing the cell treatment medium and adding 100 μL of a 10% Resazurin solution in D-MEM with an incubation time of 2 hours at 37 °C. Cellular viability was determined by absorbance measurements at 595 nm. Cytotoxicity data were expressed as IC_{50} values, *i.e.* the concentration of the test complex inducing 50% reduction in cell number compared with control cultures.

2.12. Release tests on PL{β-[Ru_2(CDT)$_5$]Cl} and PL{β-[Ru_2(PDT)$_5$]Cl}

2.12.1. Preparation of the dialysis tubing cellulose membrane. The dialysis tubing cellulose membrane (Sigma Aldrich, avg. flat width 25 mm, MW cut-off 11,181 Da) was washed and activated as follows: i) glycerol included as humectant was removed by washing with running water for 3-4 hours; ii) removal of sulfur compounds was obtained by a treatment with a 0.3% (w/v)

solution of sodium sulfide at 80 °C for 1 minute; iii) the tubing was washed with water at 60 °C for 2 minutes; iv) 0.2% (v/v) solution of sulfuric acid was added and a final rinse with hot water allowed the removal of the acid.

2.12.2. Release test on PL{β-[Ru_2(CDT)$_5$]Cl} and PL{β-[Ru_2(PDT)$_5$]Cl}. A saline solution (NaCl 0.9% w/v) of each complex ($1.4 \cdot 10^{-4}$ M) was obtained by re-suspending PF127/complex thin layer (see section 2.11.1). 2 mL of each solution was injected in the previously described dialysis membrane, with a final PF127 concentration of 40 mg/mL (the ideal dissolving concentration for [Ru_2(dtc)$_5$]Cl complexes). Then, the membrane was opportunely sealed with clips and put in a container with 80 mL of saline solution and incubated at 37 °C under stirring. UV-Vis measurements were recorded overtime up to 96 hours. For each measure, an aliquot of 1 mL was withdrawn from the bulk, immediately replenished with 1 mL of saline solution to keep the total volume constant.

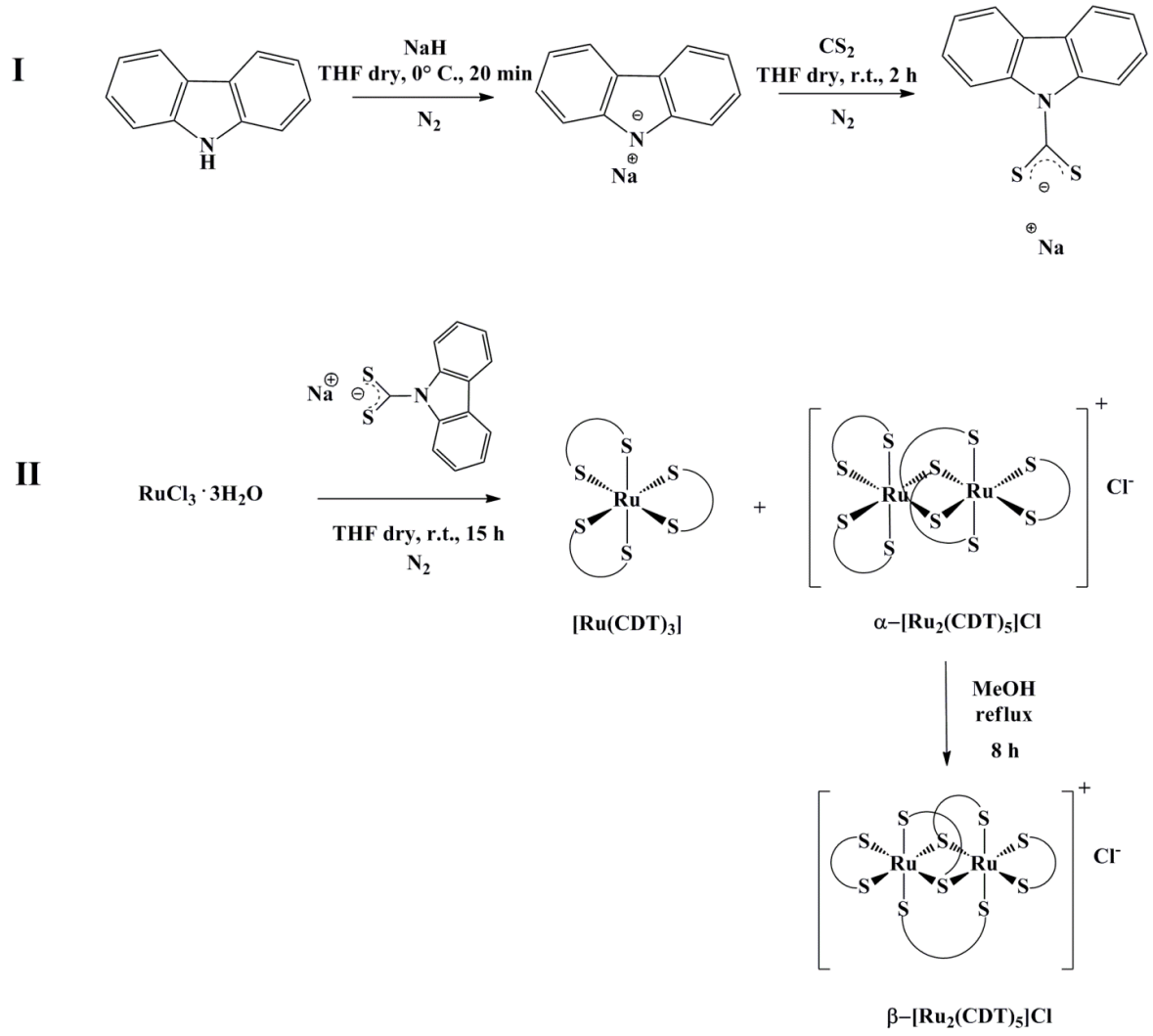
2.13. Log P evaluation

For the evaluation the of partition coefficient *P*, *n*-octanol was pre-saturated with deionized water for 24 hours under vigorous stirring, then let to equilibrate for 6 hours at 25 °C. After that, weighted amounts of all Ru-dtc complexes were dissolved in a defined aliquot of the organic phase and shaken for 2 hours in the presence of deionized water at 25 °C. Subsequently, the mixture was left to equilibrate for 30 minutes. The concentration of every Ru-dtc derivative in the organic phase before (C_0) and after partitioning (C_1) was measured by UV-Vis spectrophotometry, followed by the evaluation of the corresponding *n*-octanol/water partition coefficient (*P*) as log P = log (C_1)/(C_0-C_1) [37].

3. Results and discussion

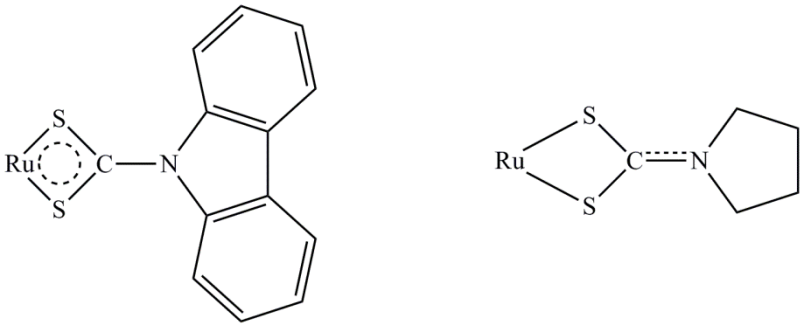
3.1. The Ru(CDT) complexes

Scheme 1 reports the synthetic pathway to the mono- and dinuclear Ru(III) carbazole-dithiocarbamato (CDT) complexes. Bereman and Nalewajek reported in 1978 the synthesis of Cu, Ni, and Zn-CDT derivatives but, at the best of our knowledge, the synthesis reported here represents the first attempt to obtain Ru(III)-CDT complexes [34]. The dithiocarbamic salt of carbazole (NaCDT, Scheme 1, I) is very hygroscopic and extremely air sensitive and was used as above indicated (Scheme 1, II) to obtain the Ru(III)-CDT derivatives. In the following sections, FT-IR and NMR characterizations are reported. ESI-MS spectra confirmed the nature of the compounds and are reported in the SI (Fig. S1 and S2).



Scheme 1. Reaction scheme for the synthesis of Ru(CDT) compounds. (I); synthesis of NaCDT (II): synthesis of the mononuclear complex $[Ru(CDT)_3]$ and the α-dinuclear complex $[Ru_2(CDT)_5]Cl$; the α-dinuclear complex is then converted to its β-isomer by refluxing the mixture in MeOH.

3.1.1. FT-IR characterization of Ru(CDT) complexes. Dithiocarbamato salts possess the ability to be, at the same time, strong- and weak-field ligands. The dithiocarbamate can be considered as a strong-field ligand when the dithiocarbamic mononegative form (hereinafter I) is dominating (Fig. 2, left). On the other hand, a significant contribution of the thioureide resonance hybrid (hereinafter II, Fig. 2, right) is associated with a weak-field ligand behavior [38–40]. The contribution of the latter (presenting a positive charge on the nitrogen with each sulfur atom negatively charged) makes these ligands capable to stabilize metal ions in a wide range of oxidation states [38]. Carbazole-dithiocarbamato (CDT) ligand exhibits only the resonance form (I), as the resonance structure (II) previously described would hamper the aromaticity of the molecule (Fig. 2) [34].



Ru-CDT **Ru-PDT**

Fig. 2. Binding of the ligands CDT and PDT to Ru(III) ion. In the case of CDT (left), the dithiocarbamic resonance form (I) mainly contributes to the final structure. For ligands such as PDT (right), the thioureide form (II) is the most influent resonance, thus endowing the ligand with the ability to stabilize metal ions in a wide range of oxidation states.

These important features can be proved by comparing FT-IR analysis of both the Ru(III)-CDT complexes and their PDT [24] analogues (Table 1, Fig. S3-S6).

Table 1. FT-IR data of Ru(III) complexes with CDT and PDT ligands in the region 4000-400 cm^{-1}.

Assignment	$[Ru(CDT)_3]$	$[Ru(PDT)_3]$	$[Ru_2(CDT)_5]Cl$	$[Ru_2(PDT)_5]Cl$
ν(SSC-N)	1363s; 1324s; 1294s cm^{-1}	1487s; 1469s; 1444s cm^{-1}	1364s; 1326s; 1299s cm^{-1}	1498s; 1472s; 1443s cm^{-1}
ν_{asym}(S-C-S)	1036m cm^{-1}	941m cm^{-1}	1038 m cm^{-1}	947m cm^{-1}
ν_{sym}(S-C-S)	585 cm^{-1}	571 cm^{-1}	585 cm^{-1}	569 cm^{-1}
ν_{asym}(Ru-S)	459 cm^{-1}	438 cm^{-1}	467, 440 cm^{-1}	438, 429 cm^{-1}

ν_{sym}(Ru-S)	413 cm^{-1}	342 cm^{-1}	416 cm^{-1}	340 cm^{-1}

(asym= asymmetric, sim= symmetric; s= strong, m= medium, w= weak)

The FT-IR spectra present, for both mono- and dinuclear complexes, three strong and well defined ν(SSC-N) bands (Fig. S3 and S5). In the case of mononuclear complexes, such a pattern is due to their distorted octahedral geometry, as similarly reported for [Fe(PDT)$_3$][41], resulting in three not equivalent dtc ligands [22]. This is visible in the X-ray crystal structures obtained also for dinuclar derivatives [24]. A shift to lower energies of the ν(SSC-N) bands is detected for Ru(III)-CDT complexes with respect to their Ru(III)-PDT analogues. This shift is ascribable to the partial double character of SSC-N bond, caused by the predominant contribution of the resonance form (II) for coordinated PDT, contrarily to Ru(III)-CDT complexes, for which the limiting form (I) is dominating (single SSC-N bond). At the same time the ν(S-C-S) as well as the ν(Ru-S) bands, are shifted to higher energies in CDT-complexes than in PDT derivatives. This energy increase suggests the presence of a metal-to-ligand back-donation, leading to a multiple bond character for the RuSCS four-membered ring of Ru(III)-CDT complexes (Table 1, Fig. S4 and S6). In addition, the detection of a single band at *ca.* 1000 cm^{-1} in all the complexes clearly indicates a symmetrical bidentate coordination of the -NCSS moiety to the metal ion, as confirmed by the Bonati-Ugo criterion (Fig. S3 and S5) [42].

Interestingly, the dinuclear species show a splitting of the band attributable to the asymmetric ν(Ru-S) (467 and 440 cm^{-1}, 438 and 429 cm^{-1}, respectively, Fig. S4 and S6, Table 1), owing to the simultaneous presence of differently coordinated (bidentate or bridged) dithiocarbamato ligands. Similarly to mononuclear complexes, for [Ru$_2$(CDT)$_5$]Cl the ν(S-C-S) and ν(Ru-S) occur at higher wavenumbers with respect to [Ru$_2$(PDT)$_5$]Cl, because of the presence of metal-to-ligand back-bonding, and hence of a multiple bond character for the RuSCS ring (Fig. 2).

Table S1 shows the bands related to the aromatic moiety of Ru(III)-CDT complexes (Fig. S3 and S5) [43].

3.1.2 ^{1}H-NMR characterization of Ru(III)-CDT complexes. ^{1}H-NMR data for the Ru-CDT complexes, together with the sodium carbazoledithiocarbamate and the carbazole precursor is shown in Table 2.

Table 2. ^{1}H-NMR data in $CDCl_3$ found for Ru(III)-CDT complexes, Na(CDT) and carbazole (400 MHz, 298 K).

Compound	**NH δ/ppm**	**H_5 δ/ppm**	**H_4 δ/ppm**	**H_3 δ/ppm**	**H_2 δ/ppm**
Carbazole	8.03 (s)	8.08 (d)	7.24 (t)	7.42 (m)	7.42 (m)
Na(CDT)	-	8.82-8.75 (m)	7.46-7.41 (m)	7.93-7.87 (m)	8.12-8.05 (m)

[Ru(CDT)$_3$]	-	7.73-7.71 (d)	8.91-8.88 (t)	9.03-9.01 (d)	-
α-[Ru$_2$(CDT)$_5$]Cl	-	8.00-7.98 (d)	7.53-7.46 (m)	7.53-7.46 (m)	9.19-9.17 (d)
β-[Ru$_2$(CDT)$_5$]Cl	-	8.00-7.98 (d)	7.53-7.46 (m)	7.53-7.46 (m)	9.19-9.17 (d)

m= multiplet, s= singlet, d= doublet, t= triplet, br= broad.

The signals related to H_2 nuclei of [Ru(CDT)$_3$] (see Fig. 1, Fig. S7) are broadened under the limit of detection. Generally, both the coordination to a paramagnetic metal ion and the presence of an aromatic ring causes a broadening of the proton signals [44]. Nevertheless, no difference was found in the recorded spectra by increasing the relaxation time up to 10 seconds, suggesting that the paramagnetic influence of Ru(III) ion is the main cause of such behavior.

In the case of [Ru(PDT)$_3$] the paramagnetic effect of the metal center not only affects the broadening of the signals but also determines a large downfield shift of the resonances (up to 40 ppm) [24]. Remarkably, in the spectra of the mixture of the two Ru(III)-CDT dinuclear isomers, the signals related to H_2 (Fig. 1) are detected. In fact, the two Ru(III) nuclei are antiferromagnetically coupled [21], thus quenching the paramagnetic effect of both metal centers (Fig. S8). The spectra recorded for the two Ru(III)-CDT dinuclear isomers are basically identical, making it impossible to assess the degree of the α/β conversion (Scheme 1, Fig. S8), contrarily to what found for α/β-[Ru$_2$(PDT)$_5$]Cl [24]. In the light of this evidence, ^{1}H-NMR spectroscopy on Ru(dtc) complexes does not allow a deep structural characterization although being useful to assess the purity of the isolated species.

3.1.3. Ru(III)-CDT and Ru(III)-PDT: UV-Visible characterization and stability studies in solution. UV-Vis spectra (spectral range 240-800 nm) of the ruthenium(III) complexes with PDT and CDT ligands (hereinafter L) were recorded in CH_2Cl_2 over time to assess their stability in solution (neutral monomers Fig. S9 and S10; dinuclear analogues Fig. S11 and S12). The most significant spectroscopic features found for the compounds [RuL$_3$] and [Ru$_2$L$_5$]Cl are summarized in Table 3.

Table 3. UV-Vis spectral data recorded in CH_2Cl_2 at 25 °C for the monomeric species [RuL$_3$] and the dinuclear complexes [Ru$_2$L$_5$]Cl (L = PDT, CDT).

Compound	**λ_{max}/nm (ε / M^{-1} cm^{-1})**					
[Ru(PDT)$_3$]	241 (21400)	255^{sh} (19762)	280^{sh} (13092)	362 (5334)	467 (1468)	565 (963)
[Ru(CDT)$_3$]	- [a]	- [a]	279 (18552)	341 (15559)	445 (9687)	626 (1441)
[Ru$_2$(PDT)$_5$]Cl	247	268	288	333^{sh}	468	

	(23419)	(24516)	(24223)	(9401)	(1031)
$[Ru_2(CDT)_5]Cl$	- [a]	- [a]	278[sh] (36794)	361 (28260)	421[sh] (16943)

sh= shoulder; a= band occurring at wavelength lower than solvent cut-off (240 nm)

All the analyzed complexes have shown high stability in CH_2Cl_2 solution, as no significant spectral change was observed even after days. The first band at *ca.* 240 nm for PDT complexes (Fig. S9 and S11) is not detectable for CDT derivatives (Fig. S10 and S12) in dichloromethane but is found at about 210 nm for the latter in saline solution (Fig. S14 and S16). The corresponding transition cannot be attributable unambiguously as it is still subject of debate in literature and in most cases it has been not even ascribed. Anyway, it could be due either to an intraligand $\pi^* \leftarrow \pi$ transition located in the –NCSS moiety or to an intraligand $p \leftarrow d$ transition between levels originated by sulfur atoms [45]. The bands at around 260 nm and 280-290 nm have been attributed to the intraligand $\pi^* \leftarrow \pi$ transition located on the N-C-S and S-C-S moieties of the dithiocarbamato ligands, respectively [46,47]. In the range 300-700 nm, the spectra of the two mononuclear species (Fig. S9 and S10) consist of three absorption bands, significantly differing from each other in terms of intensity and spectral shape. In fact, based on the high value of the molar extinction coefficient ($\varepsilon > 10^3$ M^{-1} cm^{-1}), the absorption band at about 330-360 nm can be assigned to a charge-transfer (CT) transition rather than to *d-d* transitions. As the dithiocarbamato ligand is characterized by both filled π (bonding) and empty π^* (antibonding) orbitals localized on the sulfur atoms, an interaction with the orbitals t_{2g} of the metal ion is conceivable. Although both the transitions of the type ligand-to-metal ($d \leftarrow \pi$, LMCT) and metal-to-ligand ($\pi \leftarrow d$, MLCT) charge transfer are theoretically allowed, the first one is the most favored in this case, as it can easily occur when the metal is in a high oxidation state. On the other hand, the bands at 440-460 nm and at *ca.* 600 nm may be assigned to *d-d* transitions. According to the Tanabe-Sugano diagrams, the crystal field ground state of low-spin Ru(III) centers is $^2T_{2g}$ arising from the $t_{2g}^5 e_g^0$ configuration in an O_h environment. All *d-d* transitions are spin-allowed and the absorption spectrum should show four bands, as the excited states are ^2E_g, $^2T_{2g}$, $^2A_{1g}$ and $^2A_{2g}$,$^2T_{1g}$ (with the last two being degenerate) [40]. It is known that the *d-d* transitions have small molar extinction coefficients (*ca.* 1-100 M^{-1} cm^{-1}) since they are Laporte-forbidden. Nevertheless, the X-ray structure of $[Ru(PDT)_3]$ shows a slightly distorted octahedral geometry of the complex, therefore, upon loss of symmetry, it shows more intense *d-d* transitions (ε *ca.* 1000 M^{-1} cm^{-1}) [22]. The high molar extinction coefficients observed for the bands at 440-460 nm can be explained by considering also the contribution of charge-transfer transitions. Interestingly, in the case of $[Ru(CDT)_3]$, the aromatic nature of the ligand emphasizes this aspect, giving rise to a ε value of *ca.* 9000 M^{-1} cm^{-1}. Finally, for these Ru(III) complexes, it is worth noting

that the absorption bands observed in the visible region are very broad, supporting the hypothesis that some transitions are overlapped.

The absorption spectra of the $[Ru_2L_5]Cl$ derivatives show an intense band at 330-360 nm and a broad weak band at 420-470 nm (Fig. S10 and S12). Due to their quite dissimilar structures, any correlation between the spectra found for mono- and di-nuclear complexes cannot be established. Dinuclear complexes involve two ruthenium(III) ions antiferromagnetically coupled, overall annihilating the paramagnetic effect of each metal center [21]. Therefore, since a strong metal-metal interaction may take place, the absorption band at 330-360 nm can be ascribed to a metal-metal to ligand charge transfer transition (MMLCT), whereas the very broad band at 420-470 nm may be due to a metal-to-metal charge transfer (MMCT) (Fig. S10 and S12). In fact, their molar extinction coefficient values are higher than those expected for *d-d* transitions. In particular, the former (MMLCT) involves a metal–metal bonding orbital as the donor, similarly to other ruthenium dinuclear complexes with diverse bridging ligands[48,49]. On the other hand, the latter (MMCT) is unlikely to be a *d–d* transition in light of the strong interaction between the two metal centers [21].

On passing from Ru(III)-PDT dinuclear derivatives to CDT counterparts, about a 3-fold and 17-fold increase of the molar extinction coefficient is observed for MMLCT and MCT transitions, respectively. In addition, a bathochromic and a hypsochromic shift is detected for the same bands. To elucidate these phenomena, DFT calculations are planned. However, the stronger the sulfur-metal bond, the shorter the Ru-Ru distance (see Section 3.1.1). This may account for a higher ε value for the aromatic derivatives.

3.2. Pluronic® F127 block copolymer as a micellar nanocarrier

3.2.1. Pluronic® F127. Generally, metal compounds with scarce solubility in aqueous solutions such as cell culture media (*i.e.*, D-MEM) or other aqueous media suitable for cell culture conditions (*e.g.*, saline solution or PBS), are first dissolved in an organic solvent (*e.g.*, DMSO, EtOH) to facilitate the dissolution. These organic solvents, if added in cell culture medium at low concentrations (< 0.5% v/v), are well tolerated by cells; thus, their presence under this limit does not affect the IC_{50} values found for the investigated cytotoxic species. Ru(III)-PDT derivatives are soluble in DMSO. A previous work reported the stability of $[Ru(PDT)_3]$ and of the two $[Ru_2(PDT)_5]Cl$ isomers in such organic solvent for several days. Moreover, these compounds proved stable also in D-MEM cell-culture medium by means of UV-Vis spectrophotometry [22].

Due to the low solubility of Ru(III)-CDT species in the aforementioned organic solvents, new strategies are required to treat cells with these compounds in aqueous media.

The copolymer Pluronic® F127, through a process of micelle formation (micellization), allows for the solubilization of our Ru-complexes in aqueous media. Such block copolymer is a non-ionic surfactant consisting of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO), arranged in A-B-A tri-block structure (PEO_{106}-PPO_{70}-PEO_{106}), with an average molecular weight of 12,600 and a critical micelle concentration (CMC) of $2.8 \cdot 10^{-6}$ M [50,51]. The micelle structure will comprise a hydrophobic PPO region covered by a hydrophilic shell, made up of PEO chains. Internalization of low-MW compounds into Pluronic® micelles can increase their solubility and stability in aqueous media. In case of biologically active species, this would significantly enhance their bioavailability [52].

Pluronic® F127 is one of the least toxic copolymers among all those commercially available [53]. Remarkably, the cytotoxicity exhibited by Pluronic® micelles towards non-cancerous cells was significantly lower than that observed in case of cancerous cells [26], pointing out inherent selectivity properties. In fact, drug encapsulation in micelles reduces extravasation into normal tissues. The diffusion is indeed increased in tumor tissues by means of the enhanced permeability and retention (EPR) effect, which is related to the abnormal high permeability of tumor blood vessels, thus resulting in a passive drug targeting to tumors. Because of these interesting properties, Pluronic® F127 has been used in combination with Pluronic® L61 to obtain doxorubicin-loaded mixed micelles, which have already reached Phase III stage in human clinical trials [53,28]. Our research group has recently studied a Pluronic® F127-based supramolecular system encapsulating Au(III)-based compounds to yield a new strategy potentially exploitable in anticancer chemotherapy [29,30]. Successively, there have been attempts to load Ru(III)-based complexes into similar systems [31,32].

In this work, the loaded compound/copolymer ratio (w/w) has been optimized to obtain full solubilization of the Ru(III) complex and to avoid gelification processes in aqueous media. In order to promote the micellization process, each sample was sonicated for 5 min to favor hydrophobic interactions of the compounds with the poly(propylene oxide) domains (Fig. 3).

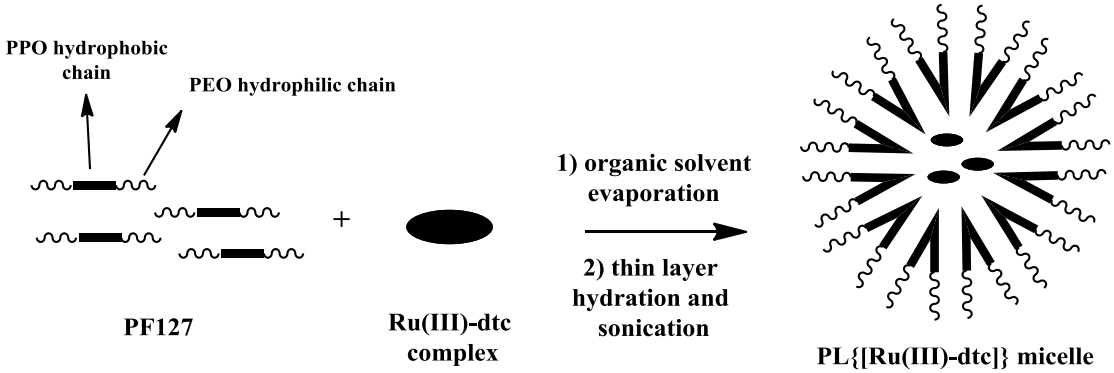


Fig. 3. Scheme of the micelle formation and Ru(III) complex internalization process by Pluronic® F127 block copolymer.

3.2.2. PF127 micelle-loaded Ru(III)-CDT and Ru(III)-PDT complexes: UV-Vis stability studies. Generally DMSO has been used so far as a vehicle for *in vitro* testing of our poor water soluble complexes. In this work, supramolecular carriers have been taken into account to enhance the bioavailabilty of our hydrophobic Ru(III)-dtc complexes. In particular, Pluronic® F127 micelles were loaded with mono and dinuclear Ru complexes of CDT and PDT and tested for their stability in saline solution (NaCl 0.9% w/v) over 72 hours at 37°C by UV-Vis spectrophotometry.

No significant change was observed over time. The absence of any modification of Ru(III) complexes after encapsulation (Fig. S13-S16) points out Pluronic® F127 as a suitable solubilizing agent for *in vitro* cytotoxicity studies on the investigated complexes.

3.3. In vitro cytotoxicity test on HeLa and HCT 116 cell lines

The *in vitro* antiproliferative activity of Ru(III)-CDT and Ru(III)-PDT, both mononuclear and dinuclear species, has been evaluated against HeLa and HCT 116 human tumor cell lines, over 24- and 72-h treatment. HeLa (cervix adenocarcinoma) cells represent a starting point for preliminary testing in light of their widespread use in the last sixty-five years [55]. HCT 116 colon carcinoma cells were chosen as this tumor represents the third most commonly diagnosed cancer in males and the second in females [1,55].

All the complexes were tested in saline solution upon micellization with PF127 or, when possible (Ru(III)-PDT complexes), upon previous dissolution in DMSO. Cisplatin was instead dissolved in saline solution, to avoid the formation of the less cytotoxic species *cis*-$[PtCl(NH_3)_2(DMSO)]^+$ [57]. Remarkably, the dilution of the cell culture medium with the saline solution (up to 29% v/v) did not affect the growth conditions. In fact, for each test, the amount of grown cells was comparable in the presence of either pure or diluted medium (both over 24 h and 72 h). Over 24 hours, none of the compounds reached the IC_{50} value, including the reference drug. This is in agreement with the slow ligand-substitution kinetics of both Ru(III) and Pt(II) complexes [58]. Therefore, the attention was addressed to the 72-h treatment, which is a commonly used incubation time in preliminary screening tests, involving also cisplatin for comparison purposes. The calculated IC_{50} values after 72-h treatment are summarized in Table 4.

Table 4. IC_{50} values (μM) evaluated after 72-h treatment with carbazole and Ru(III)-dtc (CDT and PDT) complexes in Pluronic® F127 micelles and DMSO (reference drug: cisplatin).

Compound	HeLa	HCT 116

PL{[Ru(CDT)$_3$]}	>20	>20
PL{[Ru(PDT)$_3$]}	5.9 ± 0.9	4.9 ± 0.2
PL{α-[Ru$_2$(CDT)$_5$]Cl}	>20	>20
PL{β-[Ru$_2$(CDT)$_5$]Cl}	>20	>20
PL{β-[Ru$_2$(PDT)$_5$]Cl}	0.28 ± 0.04	0.56 ± 0.08
PL{carbazole}	>20	>20
[Ru(PDT)$_3$][a]	>20	>20
β-[Ru$_2$(PDT)$_5$]Cl[a]	0.63 ± 0.06	0.90 ± 0.03
Carbazole[a]	>20	>20
Cisplatin[b]	6.6 ± 0.3	15.96 ± 0.08

PL{compound} = micellar derivatives; a = solubilized in DMSO; b = solubilized in saline solution.
(The error was evaluated as standard deviation of the average IC_{50} value deriving from three or more independent experiments)

Among the investigated formulations, three showed promising antiproliferative activity against both the human tumor cell lines with IC_{50} values comparable or lower than cisplatin, which was found more active towards HeLa cells than HCT ones. As an example, Fig. 4 reports a plot related to the results obtained by treating HeLa and HCT116 cells with the micelle formulation PL{β-[Ru$_2$(PDT)$_5$]Cl}.

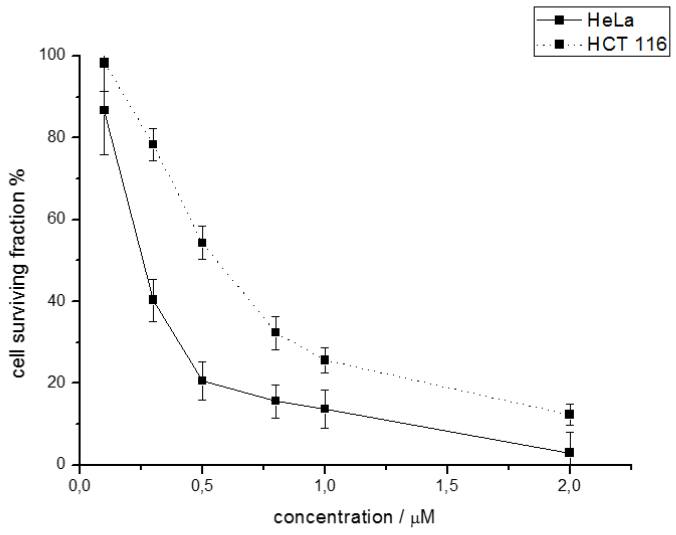


Fig. 4. Sensitivity profile of HeLa (continuous line) and HCT166 (dotted line) cell lines to PL{β-[Ru$_2$(PDT)$_5$]Cl} as a function of compound concentration. The plots come from the average of three independent experiments, each one being carried out under quadruplicate conditions.

Interestingly, the Ru(III)-PDT dinuclear complex displayed a significantly higher activity when loaded into Pluronic® F-127 micelles if compared to the standard administration *via* DMSO. This phenomenon occurs also for the mononuclear PDT complex, associated with at least 3-fold increase of anticancer activity.. The intrinsic nature of Pluronic® F-127 may account for such a behavior. In fact, Batrakova and Kabanov already proposed that this class of copolymers may act as a biological response modifier [52,59]. Pluronic® surfactants sensitize multi-drug resistant (MDR) cancer cells since they can (i) be incorporated into membranes, changing their microviscosity; (ii) induce a dramatic reduction in ATP levels in cancer and barrier cells; (iii) inhibit drug efflux transporters; (iv) induce release of cytochrome c and increase reactive oxygen species (ROS) levels in the cytoplasm, thus favoring cell death; (v) enhance pro-apoptotic signaling and decrease anti-apoptotic defense; (vi) inhibit the glutathione/glutathione *S*-transferasedetoxification system and (vii) hamper drug sequestration within cytoplasmic vesicles. Therefore, in order to assess the PF127 vehicle cytotoxic effects we have also treated cells with empty Pluronic® F-127 micelles at different concentrations of copolymer (comparable to those used for solubilizing Ru(III) complexes), without observing any relevant effect.

In the light of the obtained results, the presence of the rigid PDT ligand has been associated with a great anticancer activity *in vitro*. This result has been observed not only with ruthenium, but also with gold and copper complexes [24,60,61].

Ru(III)-CDT complexes encapsulated in Pluronic® F127 micelles did not result active and, because of their poor solubility, they could not be tested with DMSO or EtOH as vehicles.

3.4. Release tests and log P comparison

The ability of a molecule to pass through the biological barriers (*e.g.*, cell membrane, gastrointestinal barrier) is often described by the *n*-octanol-water partition coefficient (log *P*) [62]. Table 5 summarizes the log *P* values obtained for the investigated mono- and dinuclear Ru(dtc) complexes.

Table 5. Log *P* values of the evaluated Ru(III) derivatives as partition coefficient *n*-octanol/water.

Compound	**log *P* (pH 7; 25 °C)**
$Ru(PDT)_3$	>4
$Ru(CDT)_3$	>4

β-[$Ru_2(PDT)_5$]Cl	0.92 ± 0.07
β-[$Ru_2(CDT)_5$]Cl	1.56 ± 0.09

All the Ru(III) derivatives showed a log *P* value > 0, pointing out a great affinity to lipophilic systems, including the phospholipidic layer of cell membrane and, on the other hand, a great affinity for the hydrophobic core of supramolecular carriers, both features being essential for biological applications [63]. The results well correlate with the neutral and hydrophobic nature of mononuclear complexes (log *P* > 4) as well as with the ionic character of their dinuclear counterparts, the latter being associated with a lower log *P* value than the former. Moreover, concerning the dinuclear derivatives, it should be underlined the presence of the aromatic moiety in the Ru-CDT derivative, bearing five hydrophobic ligands, results in a higher log *P* value than the PDT counterpart.

Only the ionic derivatives were chosen for dialysis studies as (i) they are endowed with a pharmacologically-relevant partition coefficient [64] and (ii) upon release, they possess more chances to be spectrophotometrically detectable, due to the higher solubility in saline solution and higher molar extinction coefficient. UV-Vis measurements were carried out to monitor the micelle-unloading of the encapsulated Ru-PDT and Ru-CDT dinuclear complexes, through a dialysis membrane in saline solution (as described in section 2.13). The samples collected for both the complexes displayed a similar spectral trend, with a band at 204 nm increasing in intensity over time and progressively undergoing a weak red-shift (Fig. S17, panel a and b). By considering both the membrane cut-off and the equilibrium of micellar aggregates with their monomeric components at concentrations higher than the CMC ($2.8 \cdot 10^{-6}$ M), this behavior is ascribable to the presence of free PF127 monomer (MW 12,600) in the saline solution (Fig. S17, panel c) [50,51]. On the other hand, the total lack of spectral features attributable to the Ru(III) complexes (see Fig. S10 and S12) confirms the high stability of the investigated supramolecular formulations (previously discussed in section 3.2.2), thus hampering compound release over 96 hours. On the contrary, even if a defined amount of compound was released, this was not detected because of a very poor solubility of the tested complexes in saline solution. Precipitation phenomena were indeed observed inside the dialysis bag after eight days as a brown mud for both complexes.

These findings could correlate with the higher *in vitro* activity of PF127-encapsulated Ru-PDT dinuclear complex, compared to its DMSO counterpart. In fact, a clathrin-mediated internalization process could be involved in the uptake and hence activity of the studied compounds [65]. In other words, we hypothesize the interaction between the carrier and the cell membrane plays a key role in

a passive and huge delivery of ruthenium-containing molecules with PDT ligand. On the contrary, the aromatic nanoformulation does not trigger cancer cell death, likely because of a non-proper release of its Ru-CDT cargo. In fact, the strong hydrophobic character of the CDT moiety, as well as its planarity, may prevent the complex release as a consequence of a too long-lasting supramolecular interaction (longer than 72 hours) either in the cytoplasm upon cell-uptake or in the cell culture medium.

4. Concluding remarks

In this work, we have reported the synthesis and characterization of mono- and dinuclear Ru(III) complexes in which the metal ion coordinates to dithiocarbamato derivatives of cyclic aromatic or aliphatic amines, forming homoleptic compounds. The structural features of the novel derivatives $[Ru(CDT)_3]$ and $[Ru_2(CDT)_5]Cl$ have been compared with their Ru-pyrrolidine dithiocarbamato (PDT) analogues, with the main goal of obtaining structure-activity relationships. In contrast with PDT complexes, the aromatic heterocyclic moiety of CDT leads to a resonance limiting form in which the C-N bond possesses mainly a single bond character due to the hampered nitrogen lone pair donation. Consequently, sulfur atoms have empty low-energy orbitals which can accept π-back-donation from metal *d* orbitals. These features have been fully characterized by FT-IR spectrophotometry. The thorough characterization has pointed out a stronger Ru-S bond for the aromatic derivatives compared to the PDT ones, thus explaining the lower reactivity, under biological conditions, observed for the former. Moreover, Pluronic® copolymer was used to encapsulate the Ru(III) derivatives, ultimately improving their water solubility, stability and bioavailability. The use of supramolecular architectures (*e.g.*, micelles, liposomes) for poor water-soluble metal derivatives is a new horizon in cancer treatment [29,32] and some platinum-based formulations have already reached clinical trials [28]. Our results on HeLa and HCT 116 cancer cell lines highlight the activity of the $[Ru_2(PDT)_5]Cl$ derivative, which increases when delivered *via* the PF127 carrier. These promising findings are the base for future *in vivo* investigations. On the other hand, the formulations with Ru(III)-CDT have shown no significant activity against the tested human cancer cell lines. Log *P* evaluations have underlined the pronounced hydrophobic nature of the complexes, entailing a strong interaction with the PPO units of the Pluronic® F127 micelles, and hence preventing the release of the loaded compounds. A number of studies are planned to elucidate the mechanism of action of micelles loaded with the Ru(III)-PDT complexes. Concluding, this work paves the way to the use of micellar formulations for the solubilization of our hydrophobic complexes overcoming the use of DMSO in future *in vivo* experiments.

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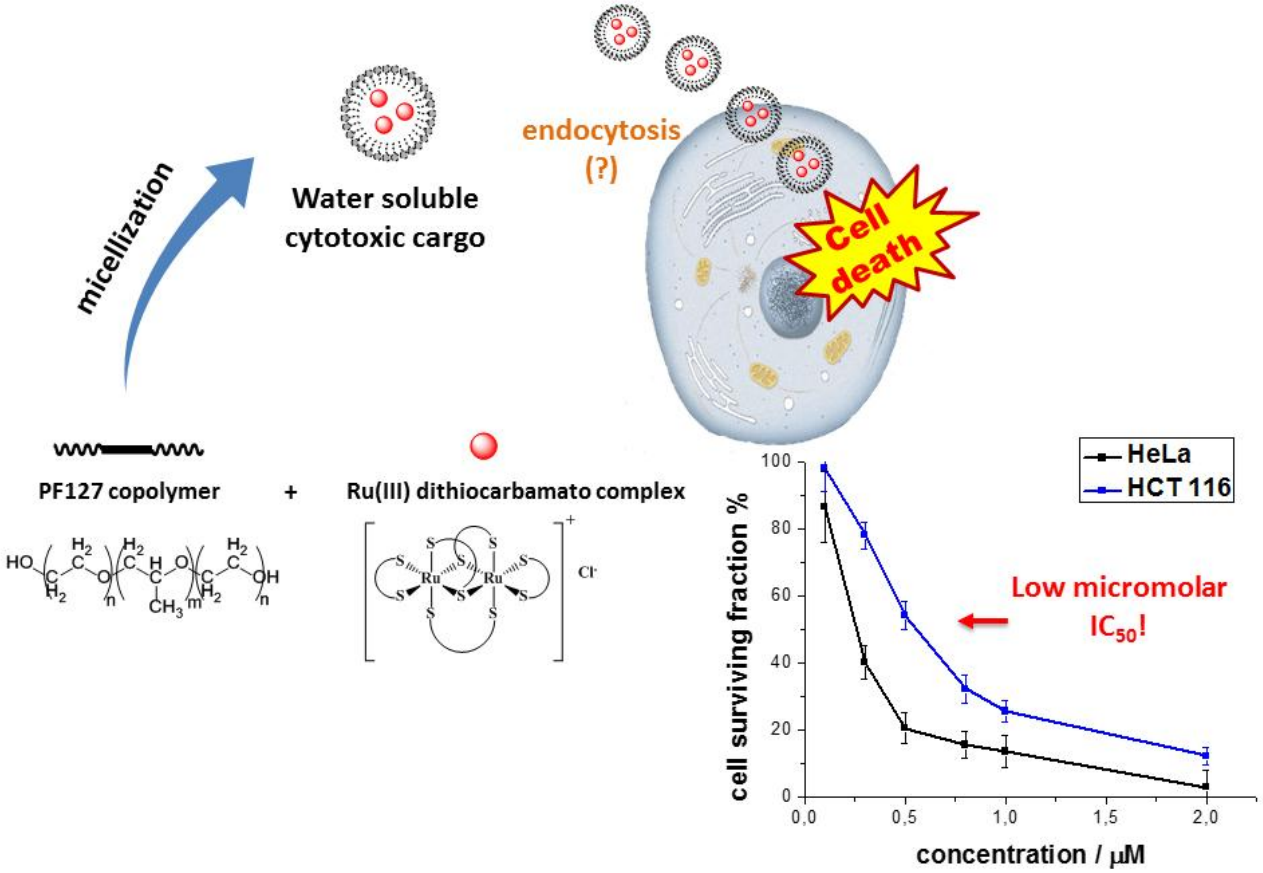
Synopsis

This work presents the synthesis and characterization of Ru(III) complexes with the aromatic carbazole-dithiocarbamato ligand, to compare their anti-proliferative properties with those of the aliphatic pyrrolidine-dithiocarbamato analogues (structure-activity relationships). The nonionic surfactant PF127 was used in *in vitro* cytotoxicity experiments, thus overcoming the poor water solubility of the compounds.



Graphical abstract

Highlights

- Investigated four Ru(III) complexes with aromatic and aliphatic dithiocarbamato ligands
- Their peculiar electronic structure and hydrophobicity result in different cytotoxicity
- Their encapsulation in micellar nanocarriers improves water solubility
- Dinuclear ionic compounds show higher activity than their mononuclear counterparts