A ruthenium(II)-trithiacyclononane curcuminate complex: Synthesis, characterization, DNA-interaction, and cytotoxic activity

Magda Carvalho Henriquesa, Maria Amparo F. Faustinoa, Artur M. S. Silvaa, Juliana Felgueirasb, Margarida Fardilhab and Susana Santos Bragaa

aDepartment of Chemistry, QOPNA Research Unit, University of Aveiro, Aveiro, Portugal; bLaboratory of Signal Transduction, Institute for Research in Biomedicine, Medical Sciences Department, University of Aveiro, Aveiro, Portugal

ABSTRACT
The coordination of ruthenium(II) complexes to anionic oxygen-based donors are very rare. This study describes a simple, one-pot method for obtaining [ruthenium(II)(trithiacyclononane)(curcumin)(S-DMSO)]-Cl (1) in 37% yield. The structural characterization of complex 1 by elemental analysis, FT-IR, 1-D and 2-D NMR, ESI+-MS as well as UV–vis and fluorescence spectroscopies are presented. The DNA-melting temperature (Tm) assay shows that salmon sperm DNA (smdNA) in the presence of complex 1 has a higher melting temperature, with ΔTm = 7.4 °C, while in the presence of curcumin the melting temperature remains unaltered. The in vitro cytotoxic activities of curcumin and complex 1 were investigated using the tumor human prostate cell line, PC-3, and the healthy cell line, PNT-2. Complex 1 is innocuous toward normal prostate epithelial cells and, whereas curcumin is toxic, with inhibition rates of ca. 35 and 65% at 50 and 80 μM, respectively. On the tumor cell line PC-3, complex 1 did not cause viability changes, whereas curcumin exhibited dose-dependent inhibition, with ca. 73% inhibition at the highest concentration tested, i.e. 80 μM. This study suggests that coordination with the trithiacyclononane ruthenium(II) scaffold stabilizes the photochemical properties of curcumin and strongly changes its biologic activity.

© 2017 Informa UK Limited, trading as Taylor & Francis Group

KEYWORDS Curcumin; ruthenium complexes; spectroscopic characterization; DNA-intercalation; cytotoxicity

ARTICLE HISTORY
Received 14 March 2017
Accepted 17 May 2017

CONTACT Susana Santos Braga sbraga@ua.pt
Supplemental data for this article can be accessed at https://doi.org/10.1080/00958972.2017.1336232.

© 2017 Informa UK Limited, trading as Taylor & Francis Group
1. Introduction

Curcumin is a natural yellow pigment occurring in various plants, the most well-known and curcumin-rich being turmeric or *Curcuma longa* L. Curcumin is the main pigment in the rhizomes, giving them a strong yellow color and being thus used as a natural textile dye for centuries. The first attempt at its isolation and characterization dates back to 1815 but only in 1870 curcumin was first obtained pure as orthorhombic crystals [1]. Nowadays, it can be quickly and easily identified in foodstuff and other products by means of advanced analytical methods [2]. The interest in curcumin has grown strongly in the past decades due to the recognition of its therapeutic properties. Curcumin is a molecule capable to modulate many cell signaling pathways, exhibiting anti-inflammatory [3,4], antioxidant [5–8] and antitumor [9–12] activities. The value of curcumin as a new anticancer agent is reinforced by reports on the antiangiogenic ability and apoptotic action that it displays on certain kinds of tumors [13], which has led to the development of various cytotoxic curcumin derivatives [14,15]. Many of these new compounds, besides aiming at improved activity, are also designed to overcome curcumin’s poor intestinal absorption rate and extensive inactivation due to hepatic first-pass metabolism [16], the two main barriers for its successful administration by the oral route.

Presently, there are a number of curcumin-based dietary supplements in the market that present solutions for circumventing its low bioavailability. These include innovative formulations with nanoparticles and liposomes or the formation of prodrugs [17]. Another useful strategy is the coordination of curcumin to a suitable metallic precursor, bringing increased solubility and stability in physiological media [18,19]. Curcumin is known to form complexes with halogenates of zinc(II) [19–21], iron(III) [22], copper(II) [23, 24], palladium(II) [25] and vanadium, as vanadyl bis(acetoacetonate), VO(acac)₂ [26]. Curcumin zinc(II) complexes, with dinonyl-2,2′-bipyridine (bpy-9) and 4,4′-bis(hydroxymethyl)-2,2′-bipyridine as spectator ligands, presented antitumor activity on several prostate cancer and neuroblastoma human cell lines, with IC₅₀ values at 72 h of incubation in the micromolar range (12–37 μM) [20, 21].

Also, curcumin palladium complexes are reported with activity against several colon cancer cell lines (IC₅₀ values in the 10–34 μM range) [27]. Ruthenium complexes are particularly useful for fine-tuned anticancer activity, since they are not toxic and some are quite selective for cancer cells, likely due to the ability of ruthenium to mimic iron in binding to biomolecules [28]. Several organometallic ruthenium(II)-arene complexes of curcumin were reported [28–30]. The results of the cytotoxic studies on these complexes proved them effective against several human cancer cell lines with significantly lower toxicity on non-tumor cell lines. Of these, the most promising were [Ru(hexamethylbenzene)(curcumin)(PTA)] and [Ru(p-cymene)(curcumin)(PTA)] (PTA = 1,3,5-triaza-7-phospha-adamantane), which were able to inhibit both cisplatin-sensitive and cisplatin-resistant breast cancer cells of the A2780 line at sub-micromolar concentrations [31]. Their IC₅₀ values were around 0.4 μM, approximately 10 times lower than free curcumin (IC₅₀ = 4.3 μM). Their safety, evaluated on a healthy embryonic kidney cell line, HEK293, was relatively high, with IC₅₀ values of 4.3 and 9.1 μM, respectively. Ru(III) complexes with new curcumin condensates as the ligand are also reported [32]. The ligands were obtained by replacing the active hydrogen of curcumin with p-hydroxybenzaldehyde and 4-hydroxy-3,5-dimethoxybenzaldehyde and their resulting Ru complexes have displayed high cytotoxicities against HeLa cells, being also active against HepG2, MDA-MB-231 and HT-29 cells.
The present paper reports the simple, one-pot synthesis of a new Ru(II)-curcumin complex bearing trithia cyclononane ([9]aneS3) as the face-capping ligand in place of the aforementioned arene ligands. Curcumin acts as an O,O′-donor ligand, forming a five-membered heterocyclic ring containing the Ru(II) atom. The DNA-binding ability and in vitro cytotoxic activity against human prostate cell lines (PC-3 and PNT-2) of the new complex was studied, using curcumin as reference.

2. Experimental

2.1. Chemicals

Sodium methoxide (95%) and low molecular weight salmon sperm deoxyribonucleic acid (smDNA) were acquired from Sigma Aldrich. [Ru(II)([9]aneS3)(S-DMSO)Cl2] was prepared according to the procedures described in our previous work [33–37]. Curcumin was extracted and purified from dry turmeric powder using an adaptation of the method described by Revathy et al. [38] (for more details, refer to the Electronic Supplementary Information, section S1). Solvents were at least of analytical grade and used without purification.

2.2. Instrumentation

\(^1\)H and \(^{13}\)C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer at 300.13 and 75.47 MHz, respectively, at room temperature. Unequivocal \(^1\)H and \(^{13}\)C assignments were made using 2-D homonuclear correlation spectroscopy (cozy, \(^1\)H,\(^1\)H), while \(^{13}\)C assignments were made based on 2-D heteronuclear single quantum coherence spectroscopy (HSQC, \(^1\)H,\(^{13}\)C), and heteronuclear multiple bond correlation (HMBC, delay for long-range \(J_{\text{C/H}}\) couplings were optimized for 7 Hz) experiments. Deuterated chloroform (CDCl₃) was used as solvent (\(^1\)H \(\delta\) 7.26 ppm and \(^{13}\)C \(\delta\) 77.03 ppm) and tetramethylsilane (TMS) as internal reference. Chemical shifts are quoted in parts per million (ppm) and the coupling constants (\(J\)) in Hertz (Hz).

Fourier-transform infrared (FT-IR) spectra, in the range of 4000–380 cm\(^{-1}\), were collected as KBr pellets using a Unican Mattson Mod 7000 FTIR spectrophotometer by averaging 64 scans at a maximum resolution of 2 cm\(^{-1}\). In a typical preparation, 2 mg of sample were mixed in a mortar with 200 mg of KBr (Sigma-Aldrich, \(\geq 99\%)\).

Mass spectra were recorded by C. Barros on a Micromass® Q-TOF 2 mass spectrometer using methanol as solvent and electrospray ionization (ESI-MS). The \(m/z\) ratios presented in the characterization data of the sample are monoisotopic, calculated using the mass of the most abundant natural isotope of each element (\(^1\)H, \(^{12}\)C, \(^{14}\)N, \(^{16}\)O, \(^{32}\)S, \(^{35}\)Cl, and \(^{102}\)Ru).

Ultraviolet–Visible (UV–Vis) solution spectra, used to determine the maximum of absorption of each compound, were obtained at 25 °C using 1 × 1 cm quartz optical cells and recorded on a Shimadzu UV-2501 PC spectrophotometer using DMF as solvent. Molar absorptivity of each compound was determined in concentration range where Beer–Lambert law is valid. The fluorescence spectra were recorded using a Horiba Jobin-Yvon FluoroMax-3 spectrofluorimeter.

Elemental analysis for CHNS was performed by M. Marques in a TruSpec 630-200-200 CHNS Analyser. The absorbance at 260 nm of smDNA in the presence or absence of complex
was performed on a GBC Cintra 500 UV–Vis spectrophotometer equipped with a temperature controller (GBC Thermocell).

Cell optical density was measured at 540 and 630 nm using a Tecan Infinite® 200 PRO-series microplate reader.

2.3. Synthesis of [Ru(II)([9]aneS₃)(curcumin)(S-DMSO)]Cl (1)

A solution of curcumin (67.0 mg, 0.182 mmol) in methanol (20 mL) was treated with sodium methoxide (9.83 mg, 0.182 mmol). The solution was stirred and refluxed under nitrogen until the color changed from saffron yellow to dark red (ca. 30 min), indicating deprotonation of curcumin. After the change in color, a methanol (20 mL) solution of [Ru([9]aneS₃)(S-DMSO)Cl₂] (62.5 mg, 0.182 mmol) was added to the reaction vessel and the mixture was allowed to reflux for 24 h (Scheme 1). It was then cooled to room temperature under rest for ca. 30 min. Following, methanol was evaporated to near dryness and diethyl ether (~5 mL) was added. This mixture was kept at 4 °C for three days to obtain a microcrystalline dark red precipitate, which was isolated using a Willstätter filter, washed with diethyl ether (~15 mL), and vacuum-dried (49.1 mg, 37% yield).

Elemental analysis for [Ru(C₆H₁₂S₃)(C₂₁H₁₉O₆)(C₂H₆SO)]Cl·(NaCl)·2.5(H₂O) (Mr = 865.0): C, 40.23; H, 4.89; S, 14.78. Found: C, 40.10; H, 4.62; S, 14.35%.

Scheme 1. Reaction scheme for the two-step synthesis of complex 1.
FT-IR ν (tilde) = 3400 m, 2925 m, 2852 w, 1618 m, 1601 m, 1508 vs, 1505 vs, 1452 s, 1425 s, 1410 s, 1397 s, 1384 s, 1280 m, 1226 m, 1163 m, 1124 m, 1078 m, 1017 m, 986 m, 973 m, 906 w, 823 m, 681 w, 641 v, 610 v, 594 v, 565 v, 556 v, 544 v, 529 w, 482 w, 454 w, 425 w, 391 v, 386 v, 303 v, 291 v, 284 v.

ESI⁺-MS m/z (relative intensity %): 727 ([Ru([9]aneseS₃)(curc)(S-DMSO)]⁺, 100%); 621 ([Ru([9]-

1H NMR (300.13 MHz, CDCl₃): δ (ppm) 7.30 (2H, d, J 16.7 Hz, H-4,4'), 7.10 (2H, dd, J 1.8 and 8.3 Hz, H-10,10'), 7.01 (2H, d, J 1.8 Hz, H-6,6'), 6.94 (2H, d, J 8.3 Hz, H-9,9'), 6.49 (2H, d, J 16.7 Hz, H-3,3'), 5.64 (1H, s, H-1) 3.96 (6H, s, 7,7'-OCH₃), 2.96 (6H, s, S-CH₃), 3.66–3.58, 3.42–3.38, 2.93–2.80, 2.72–2.65 (12H, m, CH₂ of trithiacyclononane).

13C NMR (75.47 MHz, CDCl₃): δ (ppm) 178.3 (C-2,2'), 148.6 (C-8,8'), 147.5 (C-7,7'), 138.0 (C-4,4'), 129.2 (C-5,5'), 124.5 (C-3,3'), 121.9 (C-10,10'), 115.4 (C-9,9'), 110.4 (C-6,6'), 102.2 (C-1), 55.1 (7,7'-OCH₃), 42.2 (SO(CH₃)₂), 33.6, 32.1, 29.5 (CH₂ of trithiacyclononane).

UV–Vis (DMF): λ_max (log ε) = 412 nm (4.51).

2.4. Fluorescence quantum yield

The fluorescence spectra of complex 1 and curcumin in DMF were measured in 1 × 1 cm quartz optical cells under normal air conditions. The fluorescence quantum yields (Φ_F) were calculated by comparison of the area below the corrected emission spectrum (450–800 nm) with that of 5,10,15,20-tetraphenylporphyrin (TPP). TPP is used as fluorescence standard, having a Φ_F = 0.12 in DMF when operating at λ_exc = 430 nm with a 5 nm slit [39]. In all cases, the absorbance of the sample and standard solutions was kept at 0.02 at the 430 nm (the excitation wavelength). The fluorescence quantum yield is calculated according to Equation (1):

\[
Φ_F^{sample} = Φ_F^{ref} \frac{AUC^{sample}(1 - 10^{-Abs^{ref}})}{AUC^{ref}(1 - 10^{-Abs^{sample}})}
\]

where AUC is the integrated area under the fluorescence curves of each compound and the reference and Abs is the absorbance of the samples and the reference at the excitation wavelength.

2.5. Photooxidation of 9,10-dimethylanthracene

The ability of complex 1 and curcumin to generate singlet oxygen was evaluated by monitoring the photooxidation of 9,10-dimethylanthracene (DMA), a singlet oxygen quencher [40,41]. Solutions of complex 1 or curcumin in DMF (Abs430 ~ 0.10) were aerobic irradiated in quartz cuvettes with monochromatic light (λ = 430 nm) in the presence of DMA (30 μM). TPP was used as reference (Φ_A = 0.65) [42]. The kinetics of DMA photooxidation was studied by following the decrease in its absorbance at 378 nm and the result registered in a first-order plot for the photooxidation of DMA photosensitized by 1, curcumin and TPP in DMF. The kinetics of DMA photooxidation in the absence of any compound was also studied and no significant photodegradation was observed under irradiation at 430 nm in DMF. The results are expressed as mean and standard deviation obtained from three independent experiments.
2.6. *DNA-denaturation temperature assay*

The thermal denaturation temperature of *sm*DNA and of the mixture 1/*sm*DNA (1:10) was determined in a 10 mM phosphate buffer saline solution (PBS, composition Na₂HPO₄/NaH₂PO₄ in ultrapure water, pH 7.4) containing 1.5 × 10⁻⁴ M of *sm*DNA and 1.5 × 10⁻⁵ M of complex 1. Melting curves were recorded at 260 nm with samples being heated in steps of ca. 5 °C with 10-min intervals between each step. For each temperature point, three absorbance readings were collected and averaged before plotting. The melting temperature is defined as the temperature at which 50% of the DNA denaturates into a single strand, and it is obtained from the middle point of the melting curve. Experimental ΔTₘ values were estimated to be accurate within ± 1 °C.

The concentration of the stock solution of *sm*DNA (prepared by dissolution of 5.13 × 10⁻⁷ g in 5 mL of PBS) was determined by spectrophotometry using the molar extinction coefficient ε₂₆₀ nm = 6600 M⁻¹ cm⁻¹ [43] to afford a value of [DNA] = 356 μM. Furthermore, this solution gave a ratio of UV absorbance at A₂₆₀ nm/A₂₈₀ nm of ca. 1.90, indicating that *sm*DNA was sufficiently free of protein [44].

2.7. *Cytotoxicity assays*

In this study, two human prostate cell lines were used: PNT-2 (normal cell line), kindly given by Dr. Ricardo Perez-Tomás (University of Barcelona, Spain) and PC-3 (neoplastic androgen-independent cell line), kindly given by Dr Rui Medeiros (University of Porto, Portugal). Cells were cultured in Roswell Park Memorial Institute media (RPMI)-1640, supplemented with 10% FBS and 1% penicillin/streptomycin mixture, and maintained in a humidified atmosphere at 37 °C containing 5% CO₂. Cells having a narrow range of passage number were used for all experiments. Fresh stock solutions of curcumin and complex 1 were prepared in DMSO and protected from light. Further dilutions of these stock solutions were made in RPMI-1640, keeping only 1% of DMSO, to obtain the final concentrations required for the biological assays.

The cytotoxicity of curcumin and complex 1 was determined using the AlamarBlue (AB) assay. This assay is based on the ability of resazurin (blue, non-fluorescent) to change color in a reducing environment to form the pink-colored fluorescent resorufin. It is widely used to monitor cellular health, since resazurin reduction may represent an impairment of the cellular metabolism, an interruption of electron transport or a mitochondrial dysfunction [45]. When added to cell cultures, the oxidized form of the AB is converted to the reduced form by mitochondrial enzymes. This redox reaction is accompanied by a shift in color of the culture media from blue to pink, which can be easily measured by colorimetric reading at 570 and 630 nm.

Cells were seeded in 96-well plates (100 μL per well) at an average density of 5000 cells per well and incubated at 37 °C for 24 h to allow them to adhere. Curcumin and complex 1, at concentrations of 20, 50, and 80 μM were added. The treated cells were incubated at 37 °C for 48 h, and then the growth medium was removed and replaced with fresh medium and the cells were further incubated for another 24 h. Sixteen hours before the endpoint of the experiment, 10 μL of AB solution were added to each well. RPMI with 10% AB was used as blank, whereas cells treated with 1% DMSO were used as control. For each condition, including the control conditions, three replicates were prepared. The assay was performed in three independent experiments.

2.7.1. *Statistical analysis*

Statistical analysis of the cytotoxicity data were carried out using the GraphPad Prism 7.01 software for *Windows*. The results are expressed as a mean and standard deviation obtained
from three independent experiments, each comprising three replicate measurements performed for each concentration of each compound tested plus the untreated control. A p-value of < 0.05 was considered significant.

3. Results and discussion

3.1. Synthesis of complex 1

The reaction of [Ru([9]aneS₃)(DMSO)Cl₂] with equimolar amounts of sodium methoxide and curcumin in methanol yielded complex [Ru(II)([9]aneS₃)(curcumin)(S-DMSO)]Cl (1). The complex is microcrystalline and dark red in color. It was obtained in a fairly good yield (37%), higher than those reported for other ruthenium complexes with O-coordinated ligands [46]. It is soluble in polar solvents such as DMSO or methanol, but insoluble in water.

3.2. Spectroscopic properties

3.2.1. Fourier-Transform Infrared spectroscopy in the solid state

The most relevant FT–IR band frequencies of the powdered samples of curcumin and complex 1 are presented in Table 1. The most intense vibrations are associated with the diketonato group, which is directly coordinated to the ruthenium core atom in complex 1. It must be noted that the 1628 and 1604 cm⁻¹ bands have a strong mixed character, comprising ν(C=C) and ν(C=O) contributions [47]. Nevertheless, a red shift upon metal coordination was expected, since it was previously observed with curcumin copper complexes [23]. A combination of stretching and bending modes account for the most intense spectral band, observed at 1510 cm⁻¹ in pure curcumin [47] and at 1508 and 1505 cm⁻¹ in complex 1. Also noteworthy is the observation of a new band at 482 cm⁻¹ in the spectrum of complex 1, attributed to the Ru–O stretch [48, 49].

3.2.2. UV–Vis spectroscopy in solution

The UV–Vis absorption spectrum of complex 1 is dominated by an intense absorption band in the blue region, with a maximum at 413 nm (in DMF). It is worth mentioning that pure curcumin peaks at 432 nm, thus meaning that its chelation causes a hypsochromic displacement of 19 nm in regard to its free form (Figure 1(a)).

The emissive properties of curcumin and complex 1 were assessed in DMF at room temperature after excitation at 430 nm. Curcumin exhibits a fluorescence emission dominated by a band centered at 524 nm with a fluorescence quantum yield (Φₑ) of 0.18 (Figure 1(b)). In contrast, the emission of complex 1 after excitation at the same wavelength is practically

| Table 1. Selected FT-IR vibrational frequencies (in cm⁻¹) for curcumin and complex 1 in the solid-state, collected from KBr pellets. |
|---|---|---|
| Curcumin | Complex 1 | Approximate description |
| 1628 | 1618 | ν(C=C), ν(C=O) |
| 1604 | 1601 | ν(C=C), ν(C=O) |
| 1510 | 1508, 1505 | ν(C=O), δ(CC10C), δ(CC=O) |
| 482 | Ru–O | |

Curcumin group band frequencies are described according to the work of Kolev et al. [47]; the Ru–O band is attributed based on references [48] and [49].
non-existent. In pure curcumin, the emission of fluorescence is well-known to result from curcumin’s delocalized π-conjugated electronic system which is strongly influenced by solvents, tautomerism, and structural modifications. Indeed, coordination to ruthenium(II) and other metal transitions may quench the fluorescence through either energy or electron transfer processes [50]. In other Ru complexes, namely the organometallic compounds [Ru(p-cymene)(curcuminate)(PTA)] and [Ru(hexamethylbenzene)(curcuminate)(PTA)], the absence of fluorescence emission of coordinated curcumin was also observed [31].

Figure 1. Absorption and fluorescence spectra of curcumin and complex 1 in DMF at ambient temperature: (a) the absorption spectra in DMF are normalized and compared with those obtained in DMSO solution; (b) the emission spectra were collected following excitation at 430 nm, using a 5-nm slit.
3.3. NMR spectroscopy

Solution phase NMR determined the structure of complex 1 and geometry of coordination of the curcuminate ligand, showing the presence of DMSO in the first coordination sphere (1H and 13C spectra are depicted in Figures S1 and S2, respectively, of the Electronic Supplementary Information). Several curcumin proton signals present shifts upon coordination, as listed in Table 2 (refer to Scheme 2 for curcumin atom labeling). Indeed, when compared to those of free curcumin, the H-1 and H-4,4′ resonances appear shielded as a result of increased electronic density. This effect evidences O,O'-coordination. The resonances of the aromatic ring protons are not affected and the signal of the 2′-OH is, as expected, absent. Regarding the thioether macrocycle, [9]aneS₃, the resonances of the methylene protons are observed as four multiplets at δ = 3.66–3.58, 3.42–3.38, 2.93–2.80, and 2.72–2.65 ppm. The resonances of protons ascribed to the two equivalent methyl groups of coordinated dimethylsulfoxide give rise to a singlet at δ = 2.96 ppm, thus evidencing S'-coordination for DMSO.

The 2D NMR experiments (HSQC and HMBC spectra, presented in the Electronic Supplementary Information) helped in the attribution of the carbon resonances. 1JC-H HSQC correlations permitted identifying the signals of the methoxyl carbons (deemed 7,7'-OCH₃), the carbons of coordinated DMSO (deemed S-(CH₃)₂) and those of the trithiacyclononane ligand (deemed CH₂-[9]aneS₃), whereas the 2/3JC-H HMBC correlations allowed the identification of the other carbon signals. In the 13C NMR spectrum of complex 1, the resonance ascribed to the DMSO methyl groups appears at 42.2 ppm and the six methylene carbons of [9]aneS₃ are found in the 33.6–29.5 ppm region. The 13C NMR spectrum of complex 1 also showed the curcumin carbon resonances, some slightly shifted in regard to the values observed for pure curcumin (Table 3). As expected, the resonances ascribed to C-1 and C-2,2′ were the most affected, suffering shifts of ca. 8 and 5 ppm, respectively. Furthermore, also the C-4,4′, C-5,5′, and C-6,6′ appear shifted.

3.4. Mass spectrometry

The ESI⁺-MS spectrum of complex 1 (in methanol) showed the most intense peak at m/z 727, corresponding to the molecular cation, [Ru([9]aneS₃)(curcumin)(DMSO)]⁺. The second most intense peak occurred at m/z 621, corresponding to a fragment generated by loss of one ethylene unit of the macrocycle along with the coordinated DMSO, [Ru([9]aneS₃–CH₂CH₂)

Table 2. 1H NMR chemical shifts (in ppm) and proton coupling constants (in Hz) of complex 1 in CDCl₃ in comparison with those of free curcumin.

| Attribution | Curcumin 1H (ppm) | Complex 1 1H (ppm) | J (Hz) | J (Hz) |
|-------------|------------------|--------------------|--------|--------|
| 2′-OH       | 16.07 (s)        | 7.30 (d)           | 16.7   |
| H-4,4′      | 7.60 (d)         | 7.10 (d,d)         | 8.3/1.8|
| H-10,10′    | 7.13 (d,d)       | 7.01 (d)           | 1.8    |
| H-6,6′      | 7.06 (d)         | 6.94 (d)           | 8.3    |
| H-9,9′      | 6.94 (d)         | 6.49 (d)           | 16.7   |
| H-3,3′      | 6.48 (d)         | 5.86 (s)           | –      |
| 8,8′-OH     | 5.86 (s)         | 5.80 (s)           | 5.64 (s)|
| H-1         | 5.80 (s)         | 3.95 (s)           | 3.96 (s)|
| –OCH₃       | –                | –                  | 2.96   |
| –S(CH₃)₂    | –                | –                  | –      |
| CH₂-[9]aneS₃| –                | –                  | 3.66–2.65 | m (m) |

The TMS signal (0.0 ppm) was used as reference.
Scheme 2. Structure of complex 1 depicting the adopted numbering for the carbon atoms of the curcumin fragment.

Table 3. $^{13}$C NMR chemical shifts (in ppm) of complex 1 in CDCl$_3$, in comparison with those of free curcumin.

| Attribution | Curcumin | Complex 1 |
|-------------|----------|-----------|
| C-2,2'      | 183.5    | 178.3     |
| C-8,8'      | 148.0    | 148.6     |
| C-7,7'      | 146.8    | 147.5     |
| C-4,4'      | 140.5    | 138.0     |
| C-5,5'      | 127.8    | 129.2     |
| C-3,3'      | 123.1    | 124.5     |
| C-10,10'    | 121.9    | 121.9     |
| C-9,9'      | 115.8    | 115.4     |
| C-6,6'      | 114.8    | 110.4     |
| C-1         | 109.7    | 102.2     |
| 7,7'-OCH$_3$| 56.0     | 55.1      |
| S(CH$_3$)$_3$| –       | 42.2      |
| CH$_3$(9)aneS$_3$| –   | 33.6, 32.1, 29.5 |

The CDCl$_3$ signal (78.76, 78.70, and 78.49 ppm) was used as the internal reference.

This fragmentation pattern is typical of ruthenium(II)-trithiacyclononane complexes, as demonstrated by studies developed by the group of Santana-Marques [51, 52].
3.5. **Photooxidation of 9,10-dimethylanthracene**

The ability of complex 1 and curcumin to generate singlet oxygen was assessed by the 9,10-dimethylanthracene (DMA) photobleaching assay. The DMA is a well-known singlet oxygen ($^{1}\text{O}_2$) quencher that reacts selectively with $^{1}\text{O}_2$ to form a non-absorbing endoperoxide derivative [40, 41]. It was observed that DMA photooxidation caused by curcumin is higher than that caused by complex 1 and lower than the one caused by TPP, considered a good oxygen producer. In all cases the DMA photooxidation follows a first-order kinetic and the observed rate constants ($K_{\text{obs}}$) are given in Table 4. The DMA-photosensitization followed the order TPP $>>$ curcumin > complex 1. Considering that the reported $^{1}\text{O}_2$ quantum yield ($\Phi_{\Delta}$) of TPP in DMF is 0.65 [42], the $\Phi_{\Delta}$ of curcumin and complex 1 are 0.06 and 0.04, respectively. Note also that no significant photodecomposition of DMA was detected in the absence of a photosensitizer.

3.6. **Influence of 1 on the thermal denaturation of DNA**

This assay provides evidence for the intercalation of small molecules into the DNA double helix, measurable from the increase in the DNA-melting temperature ($T_m$), i.e. the mean

**Table 4.** Kinetic constants for the photooxidation of 9,10-dimethylanthracene (DMA) in DMF by complex 1, curcumin and TPP.

| Photosensitizer | $K_{\text{obs}}$ (s$^{-1}$) |
|----------------|-----------------------------|
| TPP (reference) | $1.21 \times 10^{-3} \pm (5.01 \times 10^{-5})$ |
| Curcumin       | $1.20 \times 10^{-4} \pm (5.29 \times 10^{-5})$ |
| Complex 1      | $6.71 \times 10^{-5} \pm (2.47 \times 10^{-5})$ |

**Figure 2.** Comparison of the thermal denaturation curves ($A_{260}$ as a function of temperature) for pure smDNA and the mixtures of smDNA/curcumin and smDNA/complex 1 in a 10:1 molar ratio. Curves were recorded under equilibrium conditions in PBS (pH 7.2).
temperature at which double-stranded DNA denatures into single-stranded DNA. The melting curves of salmon-sperm DNA (smDNA), either pure or in the presence of curcumin or complex 1, are shown in Figure 2. Under our experimental conditions, the $T_m$ value of smDNA (100 μM in PBS, see Experimental section for details) was calculated at 84.8 °C. It was not significantly altered by the presence of curcumin, but in the presence of complex 1, at a concentration ratio of [smDNA]/[1] = 10:1, the melting temperature was higher. The calculated $\Delta T_m$ is 7.4 °C (Table 5), which in other ruthenium complexes has been associated with a strong binding to DNA [53].

### 3.7. Cytotoxicity assays

The effect of complex 1, in comparison with that of curcumin, on the viability of cultured human prostate PC-3 cells was studied using the Alamar Blue assay. To establish the safety profile of curcumin and complex 1, they were also incubated with pre-neoplastic epithelial prostate cells of the PNT-2 cell line. Cells were incubated for a period of 48 h in RPMI-1640 medium in the presence of curcumin or complex 1 at concentrations of 20, 50, and 80 μM (Figure 3). Given that curcumin is a photosensitive compound, the manipulation and culture were carried out under reduced light conditions. After 48 h of incubation, the culture media was replaced by fresh media (without compounds).

At the tested concentrations, complex 1 was innocuous against the normal prostate epithelial cells (PNT-2 cell line). However, it also showed no effect on the viability of the cells of the prostate cancer line (PC-3). Such results are somewhat unexpected, given the ability of complex 1 to intercalate with the DNA (section 3.6) and the positive results reported for the [Ru(II)(arene)(curcumin)] analogs [29–31]. Furthermore, the cytotoxic activity of complexes of [Ru(II)(trithiacyclononane)] family was demonstrated on several tumor cell lines, both human (MG-63, PC-3 and MDA-MB-231) [35, 36] and murine (TS/A) [54]. In particular, [Ru([9]aneS3)(phpz)Cl2] (phpz = 5-(2-hydroxyphenyl)-3-[(4-methoxystyryl)pyrazole]) presented cytotoxic effect against the PC-3 cells ($IC_{50} = 32.3 \mu M$), though this activity is lower than that of cisplatin (6.6 μM) and free phpz (9.9 μM) against the same cell line [36]. Decrease in ligand cytotoxicity upon coordination to a Ru(II) complex is not infrequent, being also reported for [Ru(II)(p-cymene)(curcumin)Cl] [29] and [Ru(II)(hexamethylbenzene)(curcumin)Cl] [30]. Lipophilicity was also recently appointed as an important factor for a high cytotoxicity in other Ru(II) curcumin compounds [55]. In this way, using [9]aneS3 macrocycle as the face-capping ligand, instead of the arene, affords complex 1 with a much stronger polar character which may account for the lack of noticeable cytotoxic activity.

#### Table 5. Thermal denaturation values calculated for smDNA and its 10:1 mixed solutions with curcumin or complex 1.

| Sample                  | $T_m$ (°C) | $\Delta T_m$ (°C) |
|-------------------------|------------|-------------------|
| smDNA                   | 84.8       |                   |
| smDNA/curcumin          | 85.2       | 0.4               |
| smDNA/1                 | 92.2       | 7.4               |

Lipophilicity was also recently appointed as an important factor for a high cytotoxicity in other Ru(II) curcumin compounds [55]. In this way, using [9]aneS3 macrocycle as the face-capping ligand, instead of the arene, affords complex 1 with a much stronger polar character which may account for the lack of noticeable cytotoxic activity.
4. Conclusion

The complex \([\text{Ru}([9\text{aneS}_3](\text{curcumin})(\text{DMSO})])\text{Cl (1)}\) herein reported is obtained in a yield of 37% with a simple one-pot synthesis. Characterization using both solution and solid-state techniques, namely elemental analysis, FT–IR, 1-D and 2-D NMR, and ESI⁺-MS, has allowed to unequivocally determine its composition and geometry, with S-coordinated DMSO and curcumin binding as a bidentate ligand. Mononuclear Ru(II) complexes with O, O'-bound ligands are rare, and complex 1 herein reported is a worthy example of such species. Another fascinating example of these complexes is \([\text{Ru}(2,2'-\text{bipyridine})(2-\text{hydroxynicotinate})(\text{dppb})]\text{PF}_6 [\text{dppb} = 1,4-\text{bis(diphenylphosphino)}\text{butane}],\) recently reported by Barbosa et al. as a promising novel anti-tuberculosis agent [56].

Ruthenium complexes, as mentioned in the introductory section of this work, are often used to reduce toxicity of active ligands on healthy cells, thus increasing the selectivity of antitumor compounds. In the case of complex 1, \textit{in vitro} studies showed that it is indeed innocuous toward the normal epithelial prostate cells (PNT-2 line), whereas curcumin inhibits 65% of the cells at 80 μM, the highest concentration tested. However, the cytotoxic action...
of curcumin is so strongly decreased upon coordination to ruthenium(II)-trithiacyclononane that complex 1 was shown not to interfere with the viability of the tumor cell line PC-3 (with inhibition rates at 80 μM being similar to those of the control). Possible causes for this result are the high photochemical stability of complex 1 and its high polarity. A careful choice of the spectator and the face-capping ligands, increasing lipophilicity, is the most obvious path for future developments on this family of complexes.

Regarding complex 1 itself, its polarity and DNA-binding abilities make it suitable for interfering with the cell lifecycle, providing that it can be conveyed in more lipidic carrier to facilitate its cell uptake. A follow-up work will thus include the encapsulation of this complex into different carriers such as liposomes or solid-lipid nanoparticles, the evaluation of its uptake by different cell types and unicellular microorganisms, and the study of its potential application as a cytotoxic or biocide agent.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the University of Aveiro and FCT/MEC (Fundação para a Ciência e a Tecnologia, Ministério da Educação e da Ciência), through national founds and, where applicable, co-financed by the FEDER (European Fund for Regional Development) within the PT2020 Partnership Agreement, through the project FCT UID/QUI/00062/2013 supporting the QOPNA research unit, through support to the Portuguese NMR Network and through the project FCT UID/BIM/04501/2013 supporting iBiMED – Aveiro Institute for Research in Biomedicine.

References

[1] (a) J. Pelletier, A. Vogel. J. Pharm., 1, 289 (1815); (b) F.W. Daube. Ber. Dtsch. Chem. Ges., 3, 609 (1870).
[2] I. Ali, A. Haque, K. Saleem. Anal. Methods, 6, 2526 (2014).
[3] P.R. Holt, S. Katz, R. Kirshoff. Dig Dis Sci, 50, 2191 (2005).
[4] S. Shishodia, G. Sethi, B.B. Aggarwal. Ann N. Y. Acad. Sci., 1056, 206 (2005).
[5] M. Subramanian, Sreejayan, T.P.A. Devasagayam, B.B. Singh, B.B. Singh. Mutat. Res. Fund. Mol. Mech. Mut, 311, 249 (1994).
[6] M.N. Sreejayan. J. Pharm. Pharm., 46, 1013 (1994).
[7] M.L. Kuo, T.S. Huang, J.K. Lin. BB-Mol. Basis Dis., 1317, 95 (1996).
[8] M. Iqbal, S.D. Sharma, Y. Okazaki, M. Fujisawa, S. Okada. Pharm. Toxicol., 92, 33 (2003).
[9] S. Busquets, N. Carbó, V. Almendro, M.T. Quiles, F.J. López-Soriano, J.M. Argilés. Cancer Lett., 167, 33 (2001).
[10] T.-H. Leu, M.-C. Maa. Curr. Med. Chem. Anticancer Agents, 2, 357 (2002).
[11] A. Duvoix, R. Blasier, S. Delhalle, M. Schnekenburger, F. Morceau, E. Henry, M. Dicato, M. Diederich. Cancer Lett., 223, 181 (2005).
[12] G. Garcea, D.P. Berry, D.L.J. Jones, R. Singh, A.R. Dennison, P.B. Farmer, R.A. Sharma, W.P. Steward, A.J. Gescher. Cancer Epidemiol. Biomarkers Prev., 14, 120 (2005).
[13] N.G. Vallianou, A. Evangelopoulos, N. Schizas, C. Kazazis. Anticancer Res., 35, 645 (2015).
[14] I. Ali, A. Haque, K. Saleem, M.F. Hsieh. Bioorg. Med. Chem., 21, 3808 (2013).
[15] A.R. Reddy, P. Dinesh, A.S. Prabhakar, K. Umasankar, B. Shireesha, M.B. Raju. Mini-Rev. Med. Chem., 13, 1769 (2013).
[16] P. Anand, A.B. Kunnumakkara, R.A. Newman, B.B. Aggarwal. Mol. Pharm., 4, 807 (2007).
[17] M.C. Henriques, M.A.F. Faustino, S.S. Braga. Anti-Cancer Agents Med. Chem., 17 (2017).
[18] S. Banerjee, A.R. Chakravarty. Acc. Chem. Res., 48, 2075 (2015).
[19] R. Sareen, N. Jain, K.L. Dhar. Pharm. Develop. Technol., 17, 1 (2015).
[20] D. Pucci, A. Crispini, B.S. Mendiguchia, S. Pirillo, M. Ghedini, S. Morelli, L. De Bartolo. Dalton Trans., 42, 9679 (2013).
[21] D. Pucci, T. Bellini, A. Crispini, I. D’Agnano, P.F. Liguori, P. Garcia-Orduña, S. Pirillo, A. Valentini, G. Zanchetta, J. Reedijk, D. Pucci. Med. Chem. Comm., 3, 462 (2012).
[22] F. Kühlwein, K. Polborn, W. Beck. Z. anorg. allg. Chem., 623, 1211 (1997).
[23] X.-Z. Zhao, T. Jiang, L. Wang, H. Yang, S. Zhang, P. Zhou. J. Mol. Struct., 984, 316 (2010).
[24] D. Pucci, A. Crispini, A. De Martino, R. Condello, C. Stellitano, G. Rotilio, M. Ghedini, G. Federici, S. Bernardini, D. Pucci. J. Med. Chem., 52, 484 (2009).
[25] K.H. Thompson, K. Böhmerle, E. Polishchuk, C. Martins, P. Toleikis, J. Tse, V. Yuen, J.H. McNeill, C. Orvig. J. Inorg. Biochem., 98, 2063 (2004).
[26] N. Miklášová, E. Fischer-Fodor, R. Mikláš, L. Kucková, J. Kožíšek, T. Liptaj, O. Soritau, J. Valentová, F. Devínsky. Inorg. Chem. Commun., 46, 229 (2014).
[27] P.J. Dyson, G. Sava. Dalton Trans., 1929 (2006).
[28] F. Caruso, M. Rossi, A. Benson, C. Opazo, D. Freedman, E. Monti, M.B. Gariboldi, J. Shaulky, F. Marchetti, C. Pettinari, A.M. Eleuteri. Chem. Med. Chem., 7, 2010 (2012).
[29] J. Marques, T.M. Braga, F.A.A. Almeida Paz, T.M. Santos, M.F.S. de Fátima Silva Lopes. BioMetals, 22, 541 (2009).
[30] J. Marques, T.M. Santos, M.P.M. Marques, S.S. Braga. Dalton Trans., 9812 (2009).
[31] J. Marques, J.A. Fernandes, F.A.A. Almeida Paz, M.P.M. Marques, S.S. Braga. J. Coord. Chem., 65, 2489 (2012).
[32] J. Marques, V.L.M. Silva, A.M.S. Silva, M.P.M. Marques, S.S. Braga. Complex Metals, 1, 7 (2014).
[33] S.S. Braga, J. Marques, E. Heister, C.V. Diogo, P.J. Oliveira, F.A.A. Paz, T.M. Santos, M.P.M. Marques. BioMetals, 27, 507 (2014).
[34] S. Revathy, S. Elumalai, M. Benny, B. Antony. J. Experim. Sci., 2, 119 (2011).
[35] M.E. Elisa Milanesio, M.G. Gabriela Alvarez, J.J. Silber, V. Rivarola, E.N. Durantini. Photochem. Photobiol. Sci., 2, 926 (2003).
[36] B. Stevens, B.E. Algar. J. Phys. Chem., 72, 2582 (1968).
[37] R. Castro-Olivares, G. Günter, A.L. Zanocco, E. Lemp. J. Photochem. Photobiol. A: Chem., 207, 160 (2009).
[38] J.C.J.M.D.S. Menezes, M.A.F. Faustino, K.T. Oliveira, M.P. Uliana, V.F. Ferreira, S. Hackbarth, B. Röder, T.T. Tasso, T. Furuyama, N. Kobayashi, A.M.S. Silva, M.G.P.M.S. Neves, J.A.S. Cavaleiro. Chem. Eur. J., 20, 1 (2014).
[39] G.D. Fasman. Handbook of Biochemistry and Molecular Biology, vol. 2 - Nucleic Acids, CRC Press, Cleveland, Ohio, USA (1975).
[40] J. Marques. Ruthenium(II)-Trithiacyclononane Complexes as Potential Antitumourals. Dissertation thesis, Universidade de Aveiro (2013).
[41] T.M. Kolev, E.A. Velcheva, B.A. Stamboliyska, M. Spiteller. Int. J. Quantum Chem., 102, 1069 (2005).
[42] J.R. Durig, W.A. McAllister, E.E. Mercer. J. Inorg. Nucl. Chem., 29, 1441 (1967).
[43] M. Ashok, A.V.S.S. Prasad, V. Ravinder. J. Braz. Chem. Soc., 18, 1492 (2007).
[44] (a) R. Ghosh, J.A. Mondal, D.K. Palit. J. Phys. Chem. B, 114, 12129 (2010); (b) A.K. Renfrew, N.S. Bryce, T.W. Hambley. Chem. Sci., 4, 3731 (2013); (c) V.W.-W. Yam, K.M.-C. Wong. Chem. Commun., 47, 11579 (2011).
[45] M.G.O. Santana-Marques, F.M.L. Amado, A.J.F. Correia, M. Lucena, J. Madureira, B.J. Goodfellow, V. Félix, T.M. Santos. J. Mass Spectrom., 36, 529 (2001).
[52] R.A. Izquierdo, J. Madureira, C.I.V. Ramos, M.G.O. Santana-Marques, T.M. Santos. *Int. J. Mass Spectrom.*, **301**, 143 (2011).

[53] J. Aldrich-Wright, C. Brodie, E.C. Glazer, N.W. Luedtke, L. Elson-Schwab, Y. Tor. *Chem. Commun.*, **1018** (2004).

[54] B. Serli, E. Zangrando, P.J. Dyson, T. Gianferrara, A. Bergamo, C. Scolaro, E. Alessio. *Eur. J. Inorg. Chem.*, **2005**, 3423 (2005).

[55] F. Caruso, R. Pettinari, M. Rossi, E. Monti, M.B. Gariboldi, F. Marchetti, C. Pettinari, A. Caruso, M.V. Ramani, G.V. Subbaraju. *J. Inorg. Biochem.*, **162**, 44 (2016).

[56] M.I.F. Barbosa, R.S. Corrêa, L.V. Pozzi, Érica de O. Lopes, F.R. Pavan, C.Q.F. Leite, J. Ellena, S.P. Machado, G. Poelhsitz, A.A. Batista. *Polyhedron*, **85**, 376 (2015).