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Effects of tethered ligands and of metal oxidation state on the interactions of cobalt complexes with the 26S proteasome

Dajena Tomco^a, Sara Schmitt^b, Bashar Ksebatia^a, Mary Jane Heeg^a, Q. Ping Dou^b, and Cláudio N. Verani^{a,*}

^aDepartment of Chemistry, Wayne State University, 5101 Cass Ave. Detroit, MI 48202, USA

^bThe Prevention Program, Ann Barbara Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, MI 4820, USA

Abstract

In this paper we report on the synthesis and characterization of three cobalt complexes described as $[\text{Co}^{\text{II}}(\text{L}^1)_2]$ (**1**), $[\text{Co}^{\text{II}}(\text{L}^2)]$ (**2**), and $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{ClO}_4$ (**3**). These complexes contain the deprotonated forms of the $[\text{NN O}]$ tridentate ligand **HL**¹ and its newly synthesized $[\text{N}_2\text{N}_2\text{O}_2]$ hexadentate counterpart **H₂L**², namely, 2,4-diiodo-6-((pyridine-2-ylmethylamino)methyl)phenol and 6,6 -((ethane-1,2-diylbis((pyridin-2-ylmethyl) azanediyl))bis(methylene))bis(2,4-diiodophenol). Characterizations for **1–3** include electrospray ionization (ESI) spectrometry, infrared, and UV–visible spectroscopies, and elemental analyses. A detailed ¹H-NMR study was conducted for **3** and X-ray structural data was obtained for **2**. The viability of this series as potential agents for proteasome inhibition and cell apoptotic induction involving PC-3 cancer cells is presented comparing the behavior of the untethered $[\text{NN O}]_2$ six-coordinate **1** and **3** and the tethered counterpart **2** with a 1:1 metal-to-ligand ratio. It is observed that the tethering in **2** decreases inhibition activity. When **1** and **3** are compared, the most inert, but redox-active, cobalt(III) species shows the highest chymotrypsin-like activity inhibition on purified proteasome and PC-3 cancer cells. A hypothesis based on the role of oxidation states for proteasome inhibition is offered.

Keywords

Proteasome inhibition; Cobalt complexes; NMR spectroscopy; Cell apoptosis

1. Introduction

Our groups have been interested in the development of coordination complexes capable of inhibiting the activity of the 26S proteasome in tumorous prostate cells. The 26S proteasome is a large protein complex responsible for the destruction of faulty proteins and enzymes [1,2]. In tumor cells the activity of the 26S proteasome goes in overdrive fostering the proteolysis of inhibition factors such as I B releasing NF B nuclear factor that support the development of blood vessels and promote tumor cell growth [3,4]. Inhibition of this activity leads to cellular apoptosis, or programmed cell death.

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*Corresponding author at: Department of Chemistry, Wayne State University, 501 Cass Ave, Detroit, MI 48202, USA. Tel.: +1 313 577 1076; fax: +1 313 577 8022. cnverani@chem.wayne.edu (C.N. Verani).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.09.013.

We have demonstrated recently that a series of gallium complexes $[\text{Ga}^{\text{III}}(\text{L}^{\text{NN}}\text{O})_2]^+$ (with the ligand 2,4-di-X-6-((pyridine-2-ylmethylamino)methyl)phenol, where X = bromo or iodo) [5,6] were active against the proteasome, as measured by the inhibition of its chymotrypsin-like activity (CT) and resulting accumulation of ubiquitinated proteins. This activity triggered cell death both *in vitro* and *in vivo*. Thus, we proposed that in order to inhibit the proteasome activity, this species would likely bind to available amino acids along the channels of the proteasome, or to the terminal threonines of the active sites in a similar way as reported to the antineoplastic agent bortezomib (valcade) [7]. These $[\text{ML}_2]^+$ species would need to be converted into a $[\text{ML}]^{2+}$ species, in order to establish a metal/amino acid chemical bond. Evidence for such mechanism was gathered when related 2:1 and 1:1 copper(II) complexes showed comparable CT inhibition activities in purified 20S and 26S proteasomes, in C4-2B and PC-3 cell extracts, as well as in intact cells [8]. Consistently we have also observed that $[\text{ML}_2]$ complexes with labile zinc(II) ions show considerable activity, whereas more inert and redox-inactive metal ions such as nickel(II) show negligible results [9].

In this paper we further this investigation by comparing the inhibition activity of three cobalt complexes described as $[\text{Co}^{\text{II}}(\text{L}^1)_2]$ (**1**), $[\text{Co}^{\text{II}}(\text{L}^2)]$ (**2**), and $[\text{Co}^{\text{III}}(\text{L}^1)_2\text{ClO}_4]$ (**3**) (Scheme 1).

We are interested in the comparison of **1** and **2** that allows further analysis for the need of ligand dissociation, where the newly designed ligand used in **2** hinders such dissociation. Similarly we want to compare **1** and **3** to examine the role of the oxidation states and the potential of redox changes in proteasome inhibition. The antitumor activity of cobalt species is fairly understudied, [10] but activity against prostate cancer has been recently demonstrated by McNail et al. for bivalent cobalt. [11] On the other hand, Teicher, Ware, Hambley, [12] and more recently Donnelly [13] have taken advantage of bioreductive activation of its trivalent counterpart for the treatment of hypoxic tumors. In this case, intracellular redox conversion of Co(III) into Co(II) releases alkylating mustards.

2. Experimental

2.1. Materials and methods

All of the reagents were used as received from commercial sources. Methanol was dried using calcium hydride, and dichloromethane was doubly purified using alumina columns in an Innovative Technologies solvent purification system. Infrared spectra were measured from 4000 to 400 cm^{-1} as KBr pellets on a Bruker Tensor 27 FTIR spectrophotometer. Electrospray ionization mass spectra in the positive mode (ESI positive) were measured in a Micromass Quattro LC triple quadrupole mass spectrometer, and experimental assignments were simulated for peak position and isotopic distribution. Elemental analyses were performed by Midwest Microlab: Indianapolis, Indiana. Visible spectroscopy from 1.0×10^{-4} M N,N-dimethylformamide solutions were performed using a Cary 50 spectrometer in the range of 250 to 1100 nm. Trypan blue exclusion dye was purchased from Sigma Aldrich (St. Louis, MO). The peptide substrate SucLLVYAMC (for the proteasomal chymotrypsinlike activity) was purchased from Calbiochem, Inc. (San Diego, CA). RPMI 1640, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Aleken Biologicals (Nash, TX). Antibodies against ubiquitin, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against human PARP was purchased from BIOMOL International LP (Plymouth Meeting, PA).

2.2. X-ray structural determination of [Co^{III}L²]

Diffraction data were measured on a Bruker *X8 APEX-II* kappa geometry diffractometer with Mo radiation and a graphite monochromator. Frames were collected at 100 K with the detector at 40 mm and 0.3° between each frame and were recorded for 10 s. *APEX-II* [14] and *SHELX* [15] software were used in the collection and refinement of the model. Crystals of [Co^{III}L²] (**2**) appeared as amber plates. A total of 23770 reflections were measured, yielding 3750 unique data (*R*_{int} = 0.049). Hydrogen atoms were placed in calculated positions. The asymmetric unit contains one neutral complex without solvate. The complex crystallized in 1:1 ratio of dichloromethane and methanol solution (Table 1).

2.3. NMR spectroscopic measurements

NMR spectra were measured using a Varian Mercury-400 and VNMRS-500 MHz spectrometers. Proton, carbon-13, distortionless enhancement by polarization transfer (DEPT), homonuclear correlation spectroscopy (COSY), and heteronuclear multiple quantum coherence (HMQC), and nuclear Overhauser effect (NOE) spectroscopy experiments were acquired in CDCl₃, DMSO-*d*₆ at 298 K and DMF-*d*₇ between 233 and 273 K.

2.4. Biological assays

2.4.1. Proteasomal activity in purified 20S proteasome—Purified human 20S proteasome (35 ng) was incubated with 10 μM of CT substrate, SucLLVYAMC, in 100 μL of assay buffer [20 mM Tris–HCl (pH 7.5)] in the presence of complexes **1–3** at various concentrations, as well as the salt [Co(H₂O)₆](ClO₄)₂ and DMSO solvent as control experiments. After 2 h of incubation at 37 °C, the production of hydrolyzed AMC groups was measured using a Wallac Victor3 multilabel counter with an excitation filter of 365 nm and an emission filter of 460 nm [16].

2.4.2. Cell cultures and whole cell extract preparation—PC-3 human prostate cancer cells were grown in RPMI1640 supplemented with 10% fetal bovine serum and maintained at 37 °C and 5% CO₂. A whole cell lysate was prepared as previously described [17].

2.4.3. Cell proliferation assay—Cells were seeded in quadruplicate in a 96 well plate and grown to 70–80% confluence, followed by treatment with the indicated agents for 18 h followed by measurement of cell proliferation by the 3 (4,5dimethylthiazol-2-yl)2,5diphenyltetrazolium bromide (MTT) assay as described previously [18].

2.4.4. Proteasome CT-like activity in cells—Proteins extracted from cells after each treatment were incubated for 2 h at 37 °C in 100 μL of assay buffer (50 mM Tris–HCl, pH 7.5) with 10 μM of fluorogenic CT substrate SucLLVYAMC, as described previously [17].

2.4.5. Western blot analysis—Cell extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. A western blot analysis was performed using specific antibodies to PARP or ubiquitin, followed by visualization using the HyGLO reagent (Denville Scientific, Metuchen, NJ).

2.5. Syntheses

2.5.1. Ligand syntheses—The iodo-substituted ligand **HL**¹ was synthesized as previously described [19,20] by the treatment of 2-hydroxy-3,5-diiodobenzaldehyde with pyridin-2-ylmethanamine in methanol followed by reduction with sodium borohydride.

The organic precursor 2,4-di-iodo-6-(chloromethyl)phenol was synthesized as previously reported [21]. Yield: 90%. IR (KBr, cm^{-1} , s = strong) 1450(s) (C = C_{Ar}); 1265 (s) (C – O); ^1H – NMR [, ppm; s = singlet, 400 MHz, CDCl_3 , 300 K] = 4.60, [2x s 2x 1H (CH_2)]; 7.60 [s, 1 H (aryl)]; 7.93 [s, 1 H (aryl)].

The new tethered ligand 6,6 -((ethane-1,2-diylbis((pyridin-2-ylmethyl)azanediyl)) bis(methylene))bis(2,4-diiodophenol) **H₂L²** was synthesized by adapting available procedures for similar species [22]. The precursor *N*¹,*N*²-bis(pyridin-2-ylmethyl)ethane-1,2-diamine was obtained when a 40 mL methanolic solution (0.9 g, 15 mmol) of ethane-1,2-diamine, was treated with two equivalents of picolinaldehyde (3.21 g, 30 mmol) and refluxed for 2 hours. The resulting yellow Schiff base was reduced by addition of sodium borohydride (1.4 g, 37 mmol) at 0 °C. The solvent was rotoevaporated and the crude product was dissolved in 100 mL of brine. Extraction with 4×25 mL of dichloromethane followed, and the combined were dried over MgSO_4 . The solution was rotoevaporated and the product was obtained as a viscous oil.

The precursor *N*¹,*N*²-bis(pyridin-2-ylmethyl)ethane-1,2-diamine (0.31 g, 1.3 mmol) was dissolved in 40 mL dichloromethane, when triethylamine base (0.6 g, 6.4 mmol) was added dropwise and followed by the addition of 2-(chloromethyl)-4,6-diiodophenol (1.0 g, 2.5 mmol). The solution was refluxed for 48 h. The product was extracted with 3×25 mL of dichloromethane to remove the triethylammonium chloride salt. The organic layer was dried over MgSO_4 and rotoevaporated to give a yellow amorphous foamy solid which was then recrystallized in methanol to yield a microcrystalline solid. Yield: 75%. Mp 158–160 °C; IR (KBr, cm^{-1} , s = strong, m = medium) 2818(s) (C-H), 1596(s), 1540(s) (C = N_{Py}, C = C_{Ar}); 1363(m) (C-O); 1250 (s), (C-N); ^1H NMR [, ppm; s = singlet, d = doublet, dd = doublet of doublet, 400 MHz, CDCl_3 , 300 K] 2.67, [4x s, 4x 1H (2 CH_2) -N- CH_2 - CH_2 -N-]; 3.61, [4x s, 4x 1H (2 CH_2) -N- CH_2 -phenol]; 3.72, [4x s, 4x 1H (2 CH_2) -N- CH_2 -pyridyl]; 7.11-7.13, [4x 4x 1H (phenol)]; 7.20-7.23, [2x dd, 2x 1H (py-2)]; 7.66-7.70, [2x dd, 2x 1H (py-3)]; 7.91, [2x d, 2x 1H (py-4)]; 8.59, [2x d, 2x 1H (py-1)]; ESI⁺ in MeOH: *m/z* (100%) = 959.1 for [**H₂L²** + H⁺]⁺.

2.5.2. Complex syntheses—*Note: Perchlorate salts of the metal complexes are potentially explosive and should be handled with care.*

[Co^{II}(L¹)₂] (1): Synthesis of this complex followed the previously described procedure [21]. Yield: 85%; IR data: (KBr, cm^{-1} , s = strong, m = medium) 1603(m), 1560(s), 1446(s) (C = N_{Py}, C = C_{Ar}); 1328 (m) (C-O); ESI⁺ in MeOH: *m/z* (100%) = 990.1 for [Co^{II}(L¹)₂ + H⁺]⁺; Anal. Calc. (%) for **1·H₂O** (%), C₂₆H₂₄CoI₄N₄O₃, FW = 1007.04 g.mol⁻¹) C, 31.01; H, 2.40; N, 5.56. Found (%): C, 31.14; H, 2.16; N, 5.39.

[Co^{II}L²] (2): [Co(H₂O)₆](ClO₄)₂ (0.12 g, 0.31 mmol) was dissolved in 5 mL of methanol and was added dropwise under anaerobic conditions to a 10 mL dichloromethane solution containing (0.3 g, 0.31 mmol) of the **H₂L²** ligand and Et₃N (0.079 g; 79.2 mmol). The solution was stirred at room temperature for 3 h, when a color change was observed from yellow to brown. No precipitate was formed observed. The solution gave orange colored crystals after 48 h. Yield: 65%; IR data: (KBr, cm^{-1} , s = strong, m = medium) 2839 (m) (C-H), 1558(s), 1483(s) (C = N_{Py}, C = C_{Ar}); 1363(m) (C-O); ESI⁺ in MeOH: *m/z* (100%) = 1015.7 for [Co^{II}L²]⁺; Anal. Calc. for **2** (%), C₂₈H₂₄CoI₄N₄O₂, FW = 1014.74 g.mol⁻¹); C, 33.13; H, 2.38; N, 5.52. Found (%): C, 33.25; H, 2.44; N, 5.44.

[Co^{III}(L¹)₂]ClO₄ (3): The [Co(H₂O)₆](ClO₄)₂ salt (0.37 g, 1.0 mmol) was dissolved in 5 mL of methanol and added to a 30 mL dichloromethane solution containing the ligand **HL¹** (0.98 g, 2.0 mmol) and Et₃N (0.28 mL; 2.0 mmol) under aerobic condition. The solution

was refluxed for 4 h, when a brownish product was vacuum filtered and washed with cold methanol and ether. Yield: 70%; IR data (KBr, cm^{-1} , s = strong, m = medium): 1603(m), 1560(s), 1446(s) ($\text{C} = \text{N}_{\text{Py}}$, $\text{C} = \text{C}_{\text{Ar}}$); 1099(s) cm^{-1} (ClO_4) 1328(m) ($\text{C}-\text{O}$); ESI⁺ in MeOH: m/z (100%) = 989 for $[\text{Co}^{\text{III}}(\text{L}^1)_2]^+$; Anal. Calc. for **3** (%), $\text{C}_{26}\text{H}_{22}\text{CoI}_4\text{N}_4\text{O}_6\text{Cl}$, FW = 1088.48 $\text{g}\cdot\text{mol}^{-1}$) C, 28.69; H, 2.04; N, 5.15. Found (%): C, 28.72; H, 2.17; N, 5.05.

3. Results and discussion

3.1. Synthesis and characterization

Both ligands **HL**¹ and **H₂L**² were synthesized in moderate yields following procedures available in the literature [21,22]. The condensation reaction of picolinaldehyde with ethane-1,2-diamine produced the corresponding Schiff base ligand. This was followed by reduction with sodium borohydride to generate the intended *N*¹,*N*²-bis(pyridin-2-ylmethyl)ethane-1,2-diamine precursor. Formation of the new **H₂L**² ligand, as shown in Scheme 2, was obtained by reaction of this amino-pyridine precursor with 2-(chloromethyl)-4,6-diiodophenol in the presence of base.

The ¹H-NMR spectra of the ligands were measured and will be relevant for future comparisons. The spectrum for **HL**¹ was recorded in DMSO-*d*₆ in order to compare with the ¹H NMR of **3**. A complete characterization of **HL**¹ ligand including the ¹H-NMR spectra and ¹H-¹H COSY is given in the Supporting Materials (Figures S1, S2, S3). From the ¹H-NMR spectra, the two methylene protons from both the pyridine rings (H5, H5') and phenol (H6, H6') appear as two distinct singlet resonances centered at 3.83 ppm and 3.87 ppm, respectively, whereas the aromatic protons lie between the 7.28–8.60 ppm region. The exchangeable protons from the aliphatic nitrogen atom (H9) and the phenol group (H10) are observed as a broad resonance between 6.60 and 7.10 ppm. The (H1) proton from the pyridine ring resonates as a doublet at 8.54 ppm.

The spectrum for **H₂L**² recorded in CDCl₃, provides evidence for a symmetric ligand in the solution, as Orvig et al. [23] and Neves et al. [24] have similarly observed with other related H₂bbpen ligands. Therefore, three sharp singlet resonances are shown at 2.67 ppm, 3.61 ppm, and 3.72 ppm representing the four protons from ethane-1,2-diamine, phenolic-methyl, and pyridyl-methyl groups, respectively; whereas the aromatic protons are displayed between the region of 7.11–8.60 ppm.

Complexes **1–3** were synthesized by the reaction of the metal salt with the appropriate ligand in methanol or dichloromethane in the presence of triethylamine base. Characterization of all of the metal complexes includes spectroscopic and spectrometric techniques consisting of IR, NMR, ESI-MS, and elemental analyses. Infrared analysis confirms that both $\text{C} = \text{N}$ and $\text{C} = \text{C}_{\text{Ar}}$ stretching modes shift *ca.* 40–60 cm^{-1} to lower frequencies due to metal coordination. Characteristic for the infrared spectrum of the $[\text{Co}^{\text{III}}(\text{L}^1)_2]\text{ClO}_4$ (**3**) complex is the presence of a strong broad band at 1098 cm^{-1} , corresponding to the perchlorate counterion.

Spectrometric analysis for complexes **1–3** was performed by ESI-MS in the positive mode and in methanol solutions. The results showed excellent agreement between the experimental and the simulated data as presented in Fig. 1. As previously published, [21] complex **1** shows the presence of the molecular ion peak observed at $m/z^+ = 989.9$ (100%) which corresponds to the $\{[\text{Co}(\text{L}^1)_2] + \text{H}^+\}^+$ species. Alternatively, for complex **3** the main peak is detected at $m/z^+ = 988.9$ (100%) and assigned to the $[\text{Co}(\text{L}^1)_2]^+$ species providing strong evidence for the coordination of the cobalt(III) ion. ESI-MS for complex **2** shows the parent peak for $\{[\text{Co}(\text{L}^2)] + \text{H}^+\}^+$ species at $m/z^+ = 1015.7$ (100%). The isotopic distribution for both complexes **2** and **3** are shown in Fig. 1. All of the above data confirm the expected

configurations of 1:2 and 1:1 metal-to-ligand species containing six-coordinate cobalt ions bound to **HL**¹ or **H₂L**², respectively.

3.2. Molecular structural characterization of [Co^{II}L²] (**2**)

X-ray diffraction was used to determine the molecular structure of single crystals of compound **2** isolated from slow solvent evaporation of a 1:1 mixture of dichloromethane and methanol. Fig. 2 displays the ORTEP diagram for complex **2** with selected bond lengths and angles. Compound **2** consists of a neutral cobalt(II) species with coordination environment arranged about a fully deprotonated hexadentate ligand (L²)²⁻, containing two [NN O] donor sets. The pseudo-octahedral cobalt(II) center is oriented in a two-fold rotation axis with each half of the ligand in a facial arrangement. We have previously reported on similar coordination spheres for cobalt(II) complexes containing two independent [NN O] ligands, in which all equivalent donors are positioned *trans* to each other [21,25]. In contrast, complex **2** displays an arrangement of *cis* phenolates, *trans* pyridines, and *cis* nitrogen atoms, rather observed for iron(III) and manganese(III) complexes with similar hexadentate ligand [26,27,28]. The short cobalt-donor bonds, along with the absence of counterions support the bivalent nature of the metal ion. The observed Co-O_{phenolate} distance is 2.00 Å, whereas Co-N_{pyridine} is 2.14 Å, and Co-N_{amine} is 2.23 Å. These values are in good agreement with the literature [21].

3.3. ¹H NMR spectroscopic studies

The ¹H-NMR data for ligands **HL**¹ and **H₂L**² has been discussed in Section 3.1. The spectra for compound **3** were taken in DMSO-*d*₆ at 298 K, and are shown in Fig. 3. A comparison of the proton chemical shifts of **HL**¹ and **3** is given in Table 2 and (Table S1). The ¹H-NMR spectrum of **3** showed eleven distinctive resonances between 3.41 and 9.00 ppm, indicating that the compound has a diamagnetic 3d⁶_{low-spin} electronic configuration. The ¹³C-NMR spectrum of complex **3** confirmed the presence of thirteen different carbon chemical peaks corresponding with half the number of carbons present in the molecule, suggesting that this complex is symmetric in solution. A combination of DEPT, COSY (Figures S4 and S5), and HMQC experiments were used to establish proton and carbon connectivity in the isolated spin systems for **3**, whereas NOE was employed to confirm the proposed structure. DEPT analysis revealed two signals corresponding to two methylene groups, and six distinct CH peaks giving a total of ten protons, whereas ¹³C-NMR/DEPT studies confirmed the presence of five fully-substituted aromatic carbons. Characteristic for the ¹H NMR spectra for complex **3** is the observation of the chemical shifts and splittings of the proton resonances attributed to metal coordination for the two methylene protons (Fig. 3a). The original singlet peak observed at 3.83 ppm (H5) in the non-metallated ligand now appears as the two doublets observed at 4.39 and 5.58 ppm for the complex, possibly due to the coupling with the aliphatic amine proton (H9). The same phenomenon was observed for the initial singlet peak at 3.87 ppm (H6), which transforms into two distinct resonances appearing as doublets at 3.48 and 3.66 ppm for **3**. The connectivity of the protons to the carbons for complex **3** was supported by the HMQC experiment, as shown in Fig. 3b and Figure S6; where the identity of the exchangeable proton attached to the aliphatic nitrogen atom (H9) was revealed. In this spectrum, one resonance corresponding to the proton on the aliphatic amine (H9) appears as a broad singlet and is shifted downfield to 8.33 ppm. Also, in comparison to the ¹H NMR spectra of the ligand **HL**¹, where the exchangeable proton of the phenol group is centered between 6.60 and 7.10 ppm, complex **3** has no such peak present which indicates ligand deprotonation. The aromatic proton found on the 1-position of the pyridine ring (H1) shifts downfield to 8.99 ppm and appears as a doublet of a doublet due to metal coordination. Only slight changes of the proton chemical shifts were observed for all aromatic protons as shown in Table 2, and no broadening of the signal indicative of dynamic equilibria [29] were observed. To confirm the thermodynamic stability of **3** in solution, variable-temperature ¹H-

NMR experiments were performed in DMSO- d_6 (25 to 80 °C, not shown), as well as in DMF- d_7 (25 to –40 °C, Figure S7). No significant changes were observed, thus supporting the idea that the $3d^6_{\text{low spin}}$ assignment for **3** is the stable conformation in the timeframe of these experiments.

3.4. UV–visible spectroscopy

The UV–visible spectra of complexes **1–3** were taken in 1.0×10^{-4} M DMF solutions to assure consistency of solubility properties (Fig. 4). The noticeable absence [21] of a ligand-to-metal charge transfer band at about 450 nm is diagnostic of the $3d^7_{\text{high spin}}$ cobalt(II) center in complexes **1** and **2**. This very characteristic process is observed for complex **3** at 441 nm corresponding to a $p_{\text{phenolate}} - d^*_{\text{cobalt(III)}}$ charge transfer. Another band at 630 nm usually attributed to d-d transitions is also present, confirming the trivalent character of the $3d^6_{\text{low spin}}$ cobalt(III) species. Other observed features include the intense $\pi - \pi^*$ intraligand bands at about 320 nm for all species. These results are in good agreement with the previously published data on cobalt complexes containing [NN O] donor ligands [10,19].

3.5. Biological results

Biological data were gathered regarding the inhibition activity of species **1–3** toward cell proliferation, the purified 20S proteasome, intact PC-3 prostate cancer cells, and finally, toward tumor cell selectivity. Inhibition of cell proliferation in human PC3 cells was successful with species **3**. Cell proliferation was decreased by 40% upon treatment with 20 μM , and reached nearly 100% inhibition at a concentration of 30 μM . A less remarkable activity was observed for **1**, where noticeable inhibition required considerably higher concentrations, e.g. ~30% inhibition at 50 μM (Fig. 5). Initially, this observation seems to contradict the proposed ligand exchange mechanism observed for similar copper, nickel and zinc complexes with similar ligands [8,9].

To test the *in vitro* 20S proteasome inhibition ability of these species, a comparison of the inhibitory activity of **1**, **2** and **3** to the 20S proteasomal activity was performed under cell-free conditions. Human purified 20S proteasome was incubated with the $\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ salt as control, as well as with **1**, **2**, and **3** at different concentrations, followed by measurement of CT-like activity. This activity was marginally inhibited by the ligands and was inhibited by the highest concentration (50 μM) of cobalt salt at a 50% level. This result confirms that in 20S proteasome – where the regulatory 19S caps have been removed – metal ions will show some inhibitory effect, as previously observed for gallium salts [30]. In spite of modest activities, comparison between complexes **1** and **2**, displayed in Fig. 6 (top) shows the highest inhibition for **1** (40% at 10 μM), where two independent ligands are present. This result reinforces the notion that facilitated ligand exchange will foster inhibition. No activity is observed for **2** even at 50 μM .

The activities of **1** and **3** were also compared, as shown in Fig. 6 (bottom). These species show constant 1:2 metal-to-ligand ratio but allow for the respective comparison between a labile $3d^7$ and an inert $3d^6$ metal center. The latter is expected to display little or no inhibitory effects, as observed for the equally inert nickel(II) ion [9]. Unexpectedly, species **3** displays remarkably better CT-like activity inhibition of the 20S core than its labile counterpart.

Intrigued by the previous results, proteasome inhibition and apoptosis induction were tested in intact PC-3 human prostate cancer cells to confirm the potential activity of **3**. Cells were first treated with different concentrations (up to 50 μM) of **1** and **3** for 18 h, followed by measurement of proteasome inhibition. PC-3 cells treated with **3** showed a dose-dependent inhibition of the proteasomal activity by ~35% at 30 μM and ~95% at 50 μM (Fig. 7).

Consistently, levels of ubiquitinated proteins were increased in a dose-dependent manner in PC-3 cells for **3**, whereas **1** showed negligible inhibition.

Previous reports have indicated that inhibition of proteasomal chymotrypsin-like activity in tumor cells may result in the induction of apoptosis [31]. To investigate whether proteasome inhibition is associated with apoptotic cell death, apoptotic-specific caspase-3 induction (Fig. 8) and related PARP disappearance (Fig. 9) were measured spectrophotometrically and by Western blotting, respectively. Dramatic induction of caspase-3 was observed in cells treated with **3** at 40 μ M. As expected, abrogation of full length PARP only occurred in cells treated with 30–50 μ M of **3**, whereas cells treated with **1** at the highest concentration tested had little visible effect. These results show that induction of apoptosis by **3** in PC-3 cells is associated with inhibition of proteasomal chymotrypsin-like activity.

These results confirm the need for ligand dissociation, as shown by comparison between the activities of **1** and **2**, both with a labile 3 d⁷ configuration. However, it is puzzling that compound **1** has proven to be less active than the inert 3 d⁶ metal-containing complex **3**. Unlike the previously studied inert 3 d⁸ nickel(II) ion, cobalt(III) is a redox-active species capable of being reduced to cobalt(II) within the reducing cellular environment by available reductants. This reduction has been demonstrated individually by several groups [12,13] and utilized for release of alkylating agents such as nitrogen mustards. The redox potential for the cytosolic environment is reported to be around –0.3 V vs. NHE by Østegaard [32]. A preliminary cyclic voltammogram for **3** in DMSO/TBAPF₆ suggests the Co(III)/Co(II) couple at around –0.5 V vs. NHE. Although more negative, the result suggests bioreductive activation as a valid working hypothesis in need of further exploration.

4. Conclusions

In this paper we have investigated the interaction of cobalt complexes with the 26S proteasome. We have compared the behavior of 1:2 and 1:1 metal-to-ligand six-coordinate cobalt species toward cell proliferation, the purified 20S proteasome, and intact PC-3 prostate cancer cells. The 1:2 species described as **1** and **3** are formed respectively between cobalt(II) or cobalt(III) ions and two deprotonated [NN O] ligands (L¹)[–], whereas the 1:1 species **2** is based on a cobalt(II) ion and the new [N₂N₂O₂] ligand **H₂L²** in its deprotonated form. Detailed characterization along with a crystal structure allows for unquestionable identification of **2**, whereas meticulous NMR spectroscopic evaluation indicated configurational stability of **3** from 80 to –40 °C. The CT-like activity inhibition is severely hampered for the 1:1 species **2**, reinforcing the current hypothesis of ligand dissociation as a requirement for proteasome inhibition. Surprisingly, the kinetically inert **3** showed remarkable proteasome inhibition, far superior to that observed for the labile **1**. We hypothesize that this difference is due to the fact that cobalt is redox-active and species **3** is likely to be reduced intracellularly. In this process a labile cobalt(II) species would be generated, favoring ligand dissociation and interaction with the proteasome. We also hypothesize that species **1**, already containing a labile cobalt(II) species will not remain intact intracellularly in order to reach the targeted proteasome. The possibility of using redox-active metals that can be intracellularly bioreduced opens a stimulating window of opportunity to explore proteasome inhibition, both by metal activation as demonstrated here and as suggested by Scarpellini [10] as well as by using metal ions as carriers for drug delivery [12]. Such studies are currently under investigation in our group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMC	7-amino-4-methylcoumarin
COSY	homonuclear correlation spectroscopy
CT	chymotrypsin
DEPT	distortionless enhancement by polarization transfer
DMF	dimethylformamide
ESI-MS	electron spray ionization mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
HL¹	2,4-diiodo-6-((pyridine-2-ylmethylamino)methyl)phenol
H₂L²	6,6 -((ethane-1,2-diylbis((pyridin-2-ylmethyl)azanediyl))bis(methylene))bis(2,4-diiodophenol)
HMQC	heteronuclear multiple quantum coherence
MTT	3(4,5dimethylthiazol-2-yl)2,5diphenyltetrazolium bromide
NOE	nuclear Overhauser effect
RPMI	1640 (Roswell Park Memorial Institute) cell culture medium
SDSPAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
CDCl₃	deuterated chloroform
PARP	poly (ADP-ribose) polymerase
PC-3	prostate cancer cell line
NHE	normal hydrogen electrode
TBAPF₆	tetrabutylammonium hexafluorophosphate electrolyte

Fig. 1. Isotopic distribution for complex **2** (left) and **3** (right). The bars indicate the experimental results and the continuous spectra indicate the simulated results.

Fig. 2. ORTEP diagram at 50% probability level for **2**. Selected bond lengths include Co(1)-O(1) = 2.003(3), Co(1)-N(1) = 2.139(3), Co(1)-N(2) = 2.234(3) Å. Selected angles include N(1)-Co(1)-N(2) = 76.57(12), O(1)-Co(1)-N(1) = 90.32(12), O(1)-Co(1)-N(2) = 89.65(11). Goodness of fit is given by $R(F)(\%) = 3.13$.

Fig. 3.
NMR spectroscopic measurements for **3**; (a) ^1H -NMR spectrum and (b) HMQC spectra.

Fig. 4.
UV-visible spectra of complexes 1–3 in N,N-dimethylformamide, 1.0×10^{-4} M.

Fig. 5.
MTT, PC-3 cells after 18 h treatment. Control is DMSO, for each concentration from 10 to 50 μ M, compound **1** is indicated in the left column and **3** is indicated in the right column.

Fig. 6. Chymotrypsin-activity inhibition in human purified 20S proteasome; DMSO and Co^{II}(ClO₄)₂ are controls. Top: comparison between **1** (left column) and **2** (right column); bottom comparison between **1** (left column) and **3** (right column).

Fig. 7.

Chymotrypsin-activity inhibition in PC-3 cell lysates after 18 h treatment. Compound 1 was measured at 40 and 50 μ M and is shown in the left column. Compound 3 is shown as a single column for 1–30 μ M and in the right column for 40 and 50 μ M. DMSO is the control.

Fig. 8.

Comparison between **1** (left column) and **3** (right column) for Caspase-3 (apoptosis) Induction in PC-3 cell lysates after 18 h treatment. DMSO is the control.

Fig. 9.
Western blot for PC-3 cell lysates after 18 h treatment.

Scheme 1.
Cobalt complexes.

Scheme 2.
Synthesis of the H_2L^2 ligand.

Table 1Crystal data and structure refinement results for [Co^{II}L²] (2).

	[Co ^{II} (L ²)] (2)
Formula	C ₂₈ H ₂₄ CoI ₄ N ₄ O ₂
<i>M</i>	1015.04
Space group	C2/c
<i>a</i> / Å	13.8089(4)
<i>b</i> / Å	9.9958(4)
<i>c</i> / Å	22.0818(9)
/ °	
/ °	96.013(2)
/ °	
<i>V</i> / Å ³	3031.20(19)
<i>Z</i>	4
<i>T</i> / K	100(2)
/ Å	0.71073
<i>D</i> _{calc} / g cm ⁻³	2.224
<i>μ</i> / mm ⁻¹	4.673
<i>R</i> (<i>F</i>) (%)	3.13
<i>Rw</i> (<i>F</i>) (%)	6.82

$$^a R(F) = \frac{\sum |F_o| - |F_c|}{\sum |F_o|} \text{ for } h^2 + k^2 + l^2 \leq 1; R_w(F) = \left[\frac{\sum w(F_o^2 - F_c^2)^2}{\sum w(F_o^2)^2} \right]^{1/2} \text{ for } h^2 + k^2 + l^2 \leq 1.$$

Table 2¹H NMR assignment for **HL**¹ and [Co(L¹)₂]ClO₄ (**3**) in DMSO-*d*₆.

Proton H	HL ¹ (ppm)	[Co ^{III} (L ¹) ₂ ClO ₄] (ppm)
H1	8.54 (d)	8.99 (dd)
H2	7.29 (dd)	7.46 (t)
H3	7.8 br (dt)	7.95 (t)
H4	7.37 (d)	7.57 (d)
H5 H5	3.83 (s)	4.39(d) 5.58 (dd)
H6 H6	3.87 (s)	3.48(dd) 3.66 (d)
H7	7.33 (d)	7.39 (s)
H8	7.78 (d)	7.34 (s)
H9	6.60–7.10 br (s)	8.33 (s)
H10	6.60–7.10 br (s)	No peak