Non-classical anticancer agents: synthesis and biological evaluation of zinc(II) heteroleptic complexes†

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New heteroleptic complexes (**1–8**) containing Zn(II) ion coordinated to an N,N-chelating ligand (the 4,4′-dinonyl-2,2′-bipyridine, bpy-9) and to diketonates L such as tropoloids (Tropolone and Hinokitiol) or 1-phenyl-3-methyl-4-R-5-pyrazolones have been synthesized by using different stoichiometric ratio with respect to the L ancillary ligand. The molecular structure of the bis-tropolonate derivative [(bpy-9)Zn(L)$_2$] **5** has been determined by single-crystal X-ray diffraction. The antitumour activity of all Zn(II) complexes was tested *in vitro* against three different human prostate cancer cells: DU145, LNCaP and PC-3. Moreover, their effect on cell survival signalling and/or inhibitors of the PC-3 cell cycle have been analyzed. The results indicate that **1–8** exhibit strong cytotoxic activity against all cell lines affecting key molecules such as p-AKT and p21 waf, involved in the cell proliferation and/or arrest. Zinc(II) is thus a promising alternative to Pt(II) ion in the design of new, better performing antitumour agents.

Introduction

New inorganic complexes have been recently proposed as effective alternatives to antitumor agents currently used in chemotherapy, in order to overcome toxicity and drug-resistance phenomena and to achieve higher activity and better selectivity than platinum-based anticancer drugs. Indeed non-platinum antitumor therapeutics can show various geometries and coordination numbers, various oxidation states, better solubility properties and various substitution kinetics or mechanism pathways, all factors which may induce a pharmacological profile different than those of platinum-drugs.[1–3]

The development of new metal-based therapeutics is changing rapidly,[4] and gold(I and III),[5] ruthenium(II and III)[6] and titanium(IV)[7] derivatives have received intense interest as the most promising candidates among the new generation of cytotoxic complexes. However, despite the variety of physiological roles of the zinc(II) ion[8–10] and the wide repertoire of Zn(II) complexes utilized in many fields (binder complexes at DNA sites,[11-14] radioprotective agents,[15] tumor photosensitizers,[16] antidiabetic insulin-mimetic[17,18] and antibacterial or antimicrobic activities[19–21]) very little data on the cytotoxicity of zinc-based compounds against human cancer cell lines are as yet available.[22–30] The role of this metal in the growth and survival of cells, and its versatile coordination states and geometries preserving the same oxidation state, prompted us to engineer heteroleptic Zn(II) complexes as potential anticancer agents with low *in vivo* toxicity and perhaps new modes of action and cellular targets with respect to the classical metallodrugs.

Our recent works have been focused on biologically active square-planar complexes containing two different chelating organic frameworks tethered to Pt(II) or Pd(II) ions: an aromatic N,N-ligand and a biologically active O,O-ligand, both able to induce synergistic effects in the resulting derivatives.[31–34]

In particular, we have synthesised mononuclear ionic complexes for which the presence of the dihexadecyl 2,2′-bipyridine-4,4′-dicarboxylate (bpy-16) and tropolonate around the metal ion, led them to be relatively inert towards ligand substitutions and remarkably cytotoxic *in vitro* against the human prostate DU145 and LNCaP cell lines.[33]

Based on these evidences, in this study we explored the possibility of using the same strategy but taking advantage of both the bioavailability and versatile coordination ability of Zn(II) and the better solubility of a different bipyridine ligand such as the 4,4′-dinonyl-2,2′-bipyridine (bpy-9). Moreover, by using this metal ion we can investigate the impact of the presence of one or two diketonate ligands around the metal ion on the antiproliferative activity of the resulting complexes. Hence, here we report on the synthesis and characterization of a novel series of heteroleptic complexes involving Zn(II) ion in two different coordination environments, depending on the amount of the diketonate ancillary ligand used. The O,O-ligands (L) chosen are tropolones such as Tropolone

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† Electronic supplementary information (ESI) available: Antiproliferative effects of organic precursors in all cell lines, IC50 values of cisplatin, complex **1** and Pd(II) and Pt(II) derivatives, UV/vis spectra of ligands and complexes **1–8**. CCDC reference number 752117. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b922101h

(Trop) and Hinokitiol (Hkt, also called β-thujaplicin) or 1-phenyl-3-methyl-4-acyl-5-pyrazolones such as HQw and HQ_T, all shown in Fig. 1. Tropolone and its derivatives, natural products with a seven-membered aromatic ring and various side groups, have powerful antibacterial and antifungal activity, particularly against antibiotic-resistant bacteria. Many other biological functions such as antiviral, antioxidant, antiinflammatory, insecticidal and antidiabetic activities are associated with tropolones.[35] Moreover, even though tropolone and hinokitiol exhibit an inhibitory effect on the growth of a range of tumor cell lines,[36–39] very few studies have been performed on tropolone-based metal complexes as antineoplastic agents.[22,33,40] As regards acylpyrazolones, while their coordination chemistry has been extensively studied and the corresponding metal derivatives applied in many fields,[41,42] their biological properties have been scarcely investigated up to now.[43–45]

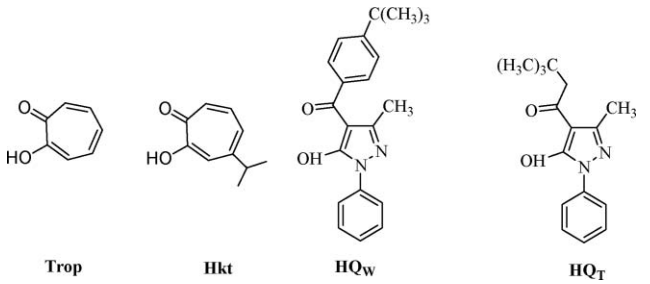


Fig. 1 Structures of the ancillary ligands L.

All the new Zn(II) complexes **1–8** in Scheme 1 have been screened for their *in vitro* effect on cell proliferation of three human prostate cell lines: hormone-resistant DU145, hormone-sensitive LNCaP and PC-3. Moreover a study of the expression of p-AKT and p21 waf proteins of the PC-3 cells has been performed in order to understand the role of these new Zn(II) species in the control of the balance of cell cycle.

Results and discussion

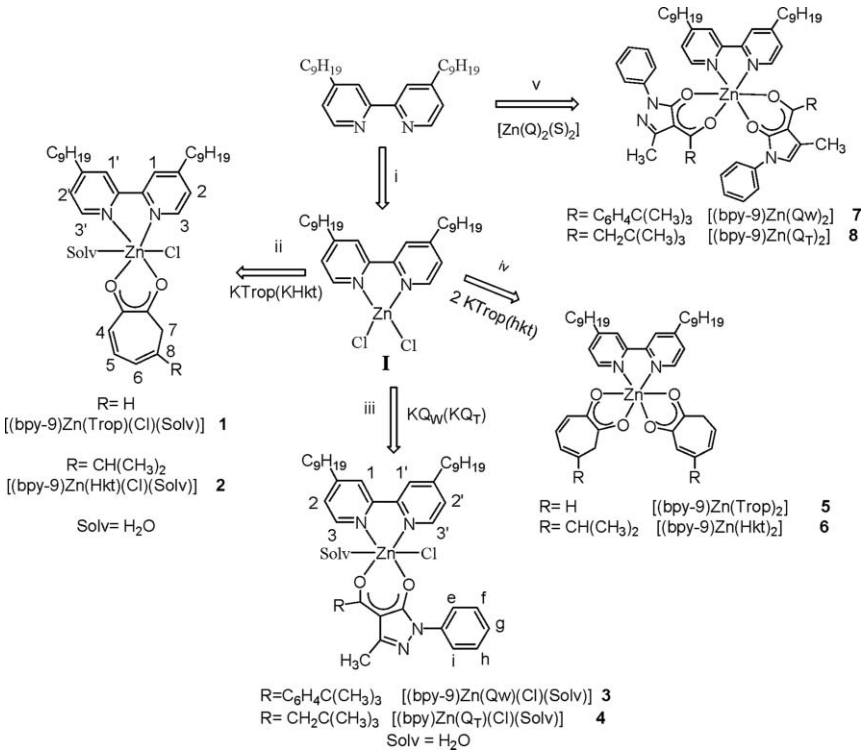
Synthesis and characterization

All Zn(II) complexes **1–8** were prepared starting from the same N,N-chelating ligand, the 4,4′-dinonyl-2,2′-bipyridine (bpy-9), as summarized in Scheme 1.

The dichloro precursor [(bipy-9)$ZnCl_2$] **I** was prepared by reaction of an equimolar amount of the main ligand bpy-9 with $ZnCl_2$, in dichloromethane, at room temperature. The corresponding mono-diketonate derivatives **1–4** were obtained through reaction with one equivalent of the appropriate KL salt in dichloromethane, at room temperature. The versatility of coordination of the Zn(II) ion allowed to synthesize also the bis-diketonate species **5–8** through two different synthetic strategies, depending on the nature of the ancillary ligand, as shown in Scheme 1.

In particular, the reaction of the dichloro complex **I** with two equivalents of the potassium tropolonates, in ethanol, gave rise to the formation of complexes **5** and **6** within 24 h. On the other hand, the synthesis of the acylpyrazolonate derivatives **7** and **8**, was carried out in two steps, involving the reaction of the appropriate $[Zn(Q)_2(S)_2]$ precursor[46] with the bpy-9 ligand, in methanol.

The structures of all complexes have been assigned on the basis of elemental analysis, IR and ^{1}H NMR spectroscopies. In



Scheme 1 Synthesis of complexes **1–8**. *Reagents and conditions*: (i) $ZnCl_2$, CH_2Cl_2, r.t., 96 h; (ii) CH_2Cl_2–EtOH, r.t., N_2, 48 h; (iii) CH_2Cl_2, r.t, N_2, 48 h; (iv) EtOH, 80 °C, 24 h; (v) $CHCl_3$, r.t, 5 days.

particular, in the IR spectra of derivatives **1–8**, the carbon–oxygen bands of the ancillary ligands are shifted by about 10–15 cm^{-1} with respect to the corresponding O,O-free ligands, confirming that the coordination to the Zn(II) ion took place. Moreover, a broad band at *ca.* 2700 cm^{-1} indicates the presence of water in the mono-diketonate complexes **1–4**.

In the ^{1}H NMR spectra of **1–8** the upfield shift of the $H_{3,3'}$, $H_{1,1'}$, H_6 and H_{e-i} resonances (Scheme 1) is consistent with the presence of chelated systems around the Zn(II) ion. Moreover, in the ^{1}H NMR spectra of complexes **5–8** the intensities of the signals corresponding to the protons of the ancillary ligands are in agreement with the 1 : 2 (metal : O,O-ligand) stoichiometry proposed in Scheme 1.

Conductivity measurements have been performed in dichloromethane solution, confirming the neutral nature of all species, at least in solution. Therefore, we can assume that the coordination sphere, apart from the N,N-ligand grafted around the Zn(II) ion, is saturated by a chloride ion and a solvent molecule for complexes **1–4**, [(bpy-9)Zn(L)(Cl)(Solv)], and by two O,O-chelating units for complexes **5–8**, [(bpy-9)Zn$(L)_2$].

In order to test their stability in solution, the behaviour of all complexes has been investigated by UV/VIS spectroscopy in various solvents and in physiological pH conditions. Measurements conducted over the time have proved the good stability of these systems, since both the shape and the absorption maximum positions remain unchanged after 24, 48 and 96 h (see ESI†). Only in the case of complex **4**, significant variations in the UV/VIS spectra are observed, indicating a certain level of instability due to both a loss of ligands and/or a rearrangement of ligands around the metal ion.

The molecular structure of **5** has been obtained by single-crystal X-ray analysis (Fig. 2). Selected bond length and angles are given in Table 1. The central metal ion is six-coordinated by four oxygen atoms of two bidentate tropolonate and two nitrogen atoms of the bipyridine ligands. The coordination around the zinc(II) ion yields a distorted octahedral geometry, with the largest deviations represented by the O(2)–Zn(1)–O(3) and O(1)–Zn(1)–N(2) angles with values of 164.7(1) and 163.2(1)°, respectively, and the bite angle of the bipyridine ligand at 75.3(1)°. The "bite" angles as well as the Zn–O bond distances of the two chelated tropolonate ligands are comparable with those of the only molecular structures of tropolonate Zn(II) derivatives reported up to now.[47,48]

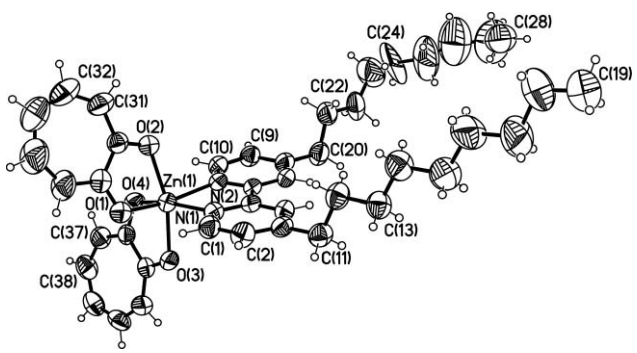


Fig. 2 Perspective view of complex **5** with atomic numbering scheme (ellipsoids at the 40% level).

Table 1 Selected bond lengths (Å) and angles (°) for complex **5**

Zn(1)–N(1)	2.150(3)	Zn(1)–N(2)	2.131(3)
Zn(1)–O(1)	2.083(3)	Zn(1)–O(2)	2.090(3)
Zn(1)–O(3)	2.099(3)	Zn(1)–O(4)	2.060(3)
O(1)–Zn(1)–N(1)	94.1(1)	O(3)–Zn(1)–N(2)	99.5(1)
O(1)–Zn(1)–N(2)	163.1(1)	O(4)–Zn(1)–N(1)	164.4(1)
O(1)–Zn(1)–O(2)	76.5(1)	O(4)–Zn(1)–N(2)	95.5(1)
O(1)–Zn(1)–O(3)	93.8(1)	O(4)–Zn(1)–O(1)	97.6(1)
O(2)–Zn(1)–N(1)	100.6(1)	O(4)–Zn(1)–O(2)	92.2(1)
O(2)–Zn(1)–N(2)	92.5(1)	O(4)–Zn(1)–O(3)	77.1(1)
O(2)–Zn(1)–O(3)	164.7(1)	N(2)–Zn(1)–N(1)	75.2(1)
O(3)–Zn(1)–N(1)	91.8(1)		

Table 2 IC50 values of complexes **1–8** against DU145 and LNCaP cells

	IC50 ± SD/μM	
Complex	DU145	LNCaP
Cisplatin	33 ± 1.0	34 ± 0.4
[(bpy-9)Zn(Trop)(Cl)(Solv)], **1**	4.5 ± 0.1	17 ± 1.0
[(bpy-9)Zn(Hkt)(Cl)(Solv)], **2**	38 ± 5.0	23 ± 2.0
[(bpy-9)Zn(Q_w)(Cl)(Solv)], **3**	20 ± 5.0	25 ± 5.0
[(bpy-9)Zn(Q_T)(Cl)(Solv)], **4**	4.8 ± 0.2	20 ± 0.2
[(bpy-9)Zn$(Trop)_2$], **5**	3.3 ± 0.1	18 ± 0.6
[(bpy-9)Zn$(Hkt)_2$], **6**	80 ± 100	70 ± 10
[(bpy-9)Zn$(Qw)_2$], **7**	>100	>100
[(bpy-9)Zn$(Q_T)_2$], **8**	10 ± 0.6	>100

Biological evaluation

The antiproliferative activity of all organic ligands and the corresponding new Zn(II) complexes has been investigated, *in vitro*, towards human prostate hormone-resistant DU145 and hormone-sensitive LNCaP cell lines,[49,50] using a colorimetric assay (MTS).

As regards as the precursors, only Hkt and HQw exhibit toxic effects with an IC50 of about 20 and 60 μM, respectively, in DU145 and LNCaP cells, while bpy-9, Trop and HQ_T do not have any toxicity in the dose range used (see ESI†).

On the contrary, the coordination of the organic ligands to the Zn(II) ion induces synergistic effects leading the novel corresponding complexes **1–8** to show relevant cytotoxic activity, as shown in Table 2.

All complexes **1–4** show an interesting cytotoxic activity toward both cells when compared to cisplatin (Table 2). In particular, the tropolonate and acylpyrazolonate Q_T derivatives **1** and **4** are the most active species on DU145 cells.

On the contrary, the presence of a further unit of L in complexes **5–8** causes remarkable differences in their biological behaviour. In both cell lines, while the cytotoxic activity of the tropolonate derivative **5** is the same as that observed in the corresponding mono-diketonate complex **1**, the Hkt and Qw-based Zn(II) complexes **6** and **7**, appeared to be only active at higher concentration (IC50 >60 μM or inactive) with respect to the corresponding mono-diketonate species **2** and **3**, respectively. On the contrary, the bis-acylpyrazolonate Q_T **8** shows selectivity towards both cancer cells used, preserving its activity in the hormone-resistant DU145 cells but losing it towards the hormone-sensitive LNCaP cells.

In order to evaluate the cell selectivity of these novel Zn(II) species, we tested **1–8** additionally against the PC-3 prostatic cell line, intermediately differentiated between DU145 and LNCaP,

Table 3 IC50 values of complexes **1–8** against PC-3 cell line

Complex	IC50 ± SD/μM
[(bpy-9)Zn(trop)(Cl)(S)], **1**	5.0 ± 0.8
[(bpy-9)Zn(hkt)(Cl)(S)], **2**	5.0 ± 0.3
[(bpy-9)Zn(Q_w)(Cl)(S)], **3**	4.2 ± 0.4
[(bpy-9)Zn(Q_T)(Cl)(S)], **4**	3.2 ± 0.1
[(bpy-9)Zn(trop)$_2$], **5**	4.5 ± 0.2
[(bpy-9)Zn(hkt)$_2$], **6**	4.2 ± 0.3
[(bpy-9)Zn(Qw)$_2$], **7**	3.9 ± 0.2
[(bpy-9)Zn(Q_T)$_2$], **8**	2.8 ± 0.1

in the same range of concentration where **1–8** show significant activity in the other cell lines.

While the organic precursors are able to decrease only slightly the cellular growth of PC-3 cells (see ESI†), complexes **1–8** reveal a significant antiproliferative effect, with IC50 values between 2.8 and 5 μM (Table 3).

The improved activity showed in the interaction with this cell line is particularly evident in the case of compounds **6** and **7**, which are inactive in the other two cell types.

Comparing the obtained results with the analogous studies conducted in similar systems, we can establish important conclusions about the use of the Zn(II) species as antitumour drugs. Indeed, a strong increase of cytotoxicity can be evidenced on going from the tetracoordinated Pd(II) and Pt(II) tropolonate derivatives[33] to the Zn(II) homologues **1** and **5** (ESI†), suggesting that the nature of the metal ion plays a crucial role in determining the biological activities of the corresponding complexes. Keeping the same metal atom, the addition of an N,N-chelating fragment greatly enhances the antiproliferative activity when the heteroleptic species **1–8** are compared to the few examples of homoleptic Zn(II) bis-diketonates investigated up to now.[22,45]

Since the net cell growth rate depends on a fine balance between the cell proliferation rate and the cell death rate, we have examined whether complexes **1–8** might affect the key transductional signals mainly involved in the regulation of cell survival. Thus we have evaluated, in untreated and treated PC-3 cells, the expression levels of phospho-AKT (pAkt), an important pro-survival protein involved in the cell survival, and of cyclin inhibitor p21 waf, a signalling protein implicated in apoptosis and growth arrest.[51–53] As shown in Fig. 3, a down-regulation of phospho-AKT levels occurs after 48 h, in the prostatic cells treated with all Zn(II) complexes, mainly evident in the case of **1** and **6**. At the same time, for **1** and **6** an increasing of level of the p21 waf, is observed (Fig. 3).

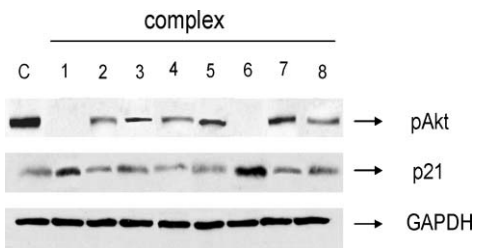


Fig. 3 Immunoblots of pAkt and p21 from PC-3 cell extracts treated for 48 h with Zn(II) complexes **1–8**. GAPDH was used as loading control. The panel is representative of three independent experiments.

These results indicate that these new zinc(II) complexes have an antiproliferative activity in the prostatic tumoral cells studied, which reflects their effects also on the key protein signalings involved in the control of the balance of cell proliferation and/or cell arrest. Indeed complexes **1–8** are able to target the pro-survival-signal phospho-AKT, which plays an important role in mediating drug resistance and stress-induced apoptosis inhibition in tumoral cells.[54–56] At the same time, the activation of the signalling protein involved in cell mitotic block reflects an ongoing stress signalling.[57,58]

Conclusions

Two series of heteroleptic Zn(II) complexes containing 4,4′-dinonyl-2,2′-bipyridine (bpy-9) as main ligand and tropolones or 1-phenyl-3-methyl-4-R-5-pyrazolones (L) as ancillary ligands, have been synthesized by using different stoichiometric ratios with respect to the diketonates L. In the series **1–4** and **5–8**, the same molecular fragment (bpy-9)Zn(L), obtained through chelation of both bpy-9 and L is present. In [(bpy-9)Zn(L)(Cl)(Solv)] derivatives, **1–4**, the coordination sphere of the Zn(II) ion is saturated by a solvent molecule and a chloride ion. In **5–8** the presence of a second chelated L unit, leading to a general formula [(bpy-9)Zn(L)$_2$], has been confirmed through single-crystal X-ray diffraction analysis performed on derivative **5**.

The uncoordinated ligands as well as the corresponding Zn(II) complexes have been tested *in vitro* towards three different human prostatic cancer cell lines: DU145, LNCaP and PC-3. While almost all ligands are inactive, most of the Zn(II) complexes show promising anticancer properties.

On the whole, all Zn(II) complexes synthesized show selectivity toward cancer cells, being more active on DU145 and PC-3 cells with respect to LNCaP cells. Moreover, while the mono-diketonate derivatives **2** and **3** in DU145 and LNCaP cells exhibit cytoxicity higher than the corresponding bis-diketonates **6** and **7**, the activity of all Zn(II) complexes towards PC-3 cells is not strongly related to the stoichiometric ratio of the ancillary ligands, being almost the same for the mono- and the bis-diketonate species.

Moreover, from this study it is possible to confirm that the nature of the central metal ion as well as its coordination environment induce significant changes in the biological activities of the resulting complexes. Indeed, comparing complexes **1–8** with Pd(II) and Pt(II) heteroleptic mono-tropolonate derivatives or with homoleptic Zn(II) bis-diketonates, we can conclude that the simultaneous presence of flat chelating ligands around the Zn(II) ion as metal scaffold has been a winning choice for endowing new non-platinum complexes with good antitumour activity.

Finally, preliminary studies of protein expression implicated in apoptosis and growth arrest indicate a key role played by these Zn(II) complexes in the control of cell proliferation and/or cell arrest. Further studies are needed to completely understand the mechanism of action and the structure–cellular activity of this interesting class of metal complexes.

Experimental

Materials and measurements

All commercially available starting materials were used as received without further purification while the ligands HQ_w and HQ_T

and the corresponding $[Zn(Q)_2(S)_2]$ derivatives were synthesised according to the procedure previously described.[46]

The tropolone and hinokitiol potassium salts have been prepared by reacting, in ethanol, one equivalent of potassium hydroxide with the appropriate tropolone ligand. DMEM/Ham's F-12, RPMI 1640, L-glutamine, penicillin/streptomycin, foetal bovine serum, bovine serum albumin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), isopropanol, hydrochloric acid were purchased from Sigma (Milan, Italy), the ECL system come from Amersham Biosciences. Anti-p21 WAF, *anti*-GAP-DH pAbs, anti-mouse IgG were procured from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-phospho-Akt (Ser473) from Cell Signaling Technology (Beverly, MA, USA). Nylon membranes were provided by Roche diagnostics Corporation (Indianapolis, USA).

Instrumentation

The ^{1}H NMR spectra were recorded on a Bruker Avance AC-300 spectrometer in $CDCl_3$ solution, using tetramethylsilane (TMS) as internal standard. Elemental analyses (CHN) were performed with a Perkin Elmer 2400 microanalyzer by the Microanalytical Laboratory at the University of Calabria. Infrared spectra (KBr) were recorded on a Spectrum One FT-IR Perkin Elmer spectrometer. A Perkin-Elmer Lambda 900 spectrophotometer was used to record absorbtion spectra of complexes **1–8** on 6% EtOH–phosphate buffered saline solutions at a concentration of 6×10^{-4} M.

Synthesis of complexes

[(bpy-9)ZnCl$_2$]. 1.5 equiv of $ZnCl_2$ (0.250 g, 1.83 mmol) was added to a solution of 4,4′-dinonyl-2,2′-bipyridine (0.500 g, 1.22 mmol) in CH_2Cl_2 (20 ml). After stirring for 6 days (r.t.) the solution was filtered through Celite and the solvent was distilled under reduced pressure and then the product was recrystallized from $CHCl_3$–Et_2O (0.523 g, 78% yield). Mp 150 °C. IR (ν_{max}/cm^{-1}: 2921, 2852 (CH), 1617, 1556 (C=C, C=N). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.68 (d, 2H, J(H,H) = 5.3 Hz, $H_{3,3'}$), 8.01 (s, 2H, $H_{1,1'}$), 7.51 (d, 2H, J(H,H) = 5.3 Hz, $H_{2,2'}$), 2.83 (m, 4H, $CH_2(CH_2)_7CH_3$), 1.71 (m, 4H, CH_2CH_3), 1.27 (m, 24H, $(CH_2)_6CH_2CH_3$), 0.88 (t, 6H, J(H,H) = 6.6 Hz, CH_3). Anal. Calc. for $C_{28}H_{44}N_2ZnCl_2$: C, 61.71; H, 8.14; N, 5.14. Found: C, 61.31; H, 8.31; N, 5.31%.

[(bpy-9)Zn(Trop)(Cl)(Solv)], 1. A hot solution of potassium tropolonate (0.029 g, 0.183 mmol) in EtOH (5 ml) was added to a solution of [(bpy-9)$ZnCl_2$] (0.100 g, 0.183 mmol) in CH_2Cl_2 (10 ml). The resulting solution was stirred under nitrogen (4 days, r.t.). After removal of the solvent under vacuum the remaining residue was dissolved in EtOH and was filtered through Celite. EtOH was distilled under reduced pressure and the product was dissolved in $CHCl_3$ and recrystallized from *n*-hexane (0.081 g, 70% yield). Mp 130 °C. IR (ν_{max}/cm^{-1}: 2921, 2852 (CH), 1609, 1595 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 9.04 (d, 2H, J(H,H) = 3.3 Hz, $H_{3,3'}$), 7.93 (s, 2H, $H_{1,1'}$), 7.48 (d, 2H, J(H,H) = 4.9 Hz, $H_{2,2'}$), 7.33 (m, 4H, $H_{4,7,5,8}$), 6.82 (t, 1H, J(H,H) = 8.5 Hz, H_6), 2.78 (m, 4H, $CH_2(CH_2)_7CH_3$), 1.70 (m, 4H, CH_2CH_3), 1.23 (m, 24H, $(CH_2)_6CH_3$), 0.88 (t, 6H, J(H,H) = 6.6 Hz, CH_3). Anal. Calc. for $C_{35}H_{51}N_2O_3ZnCl$: C, 64.82; H, 7.92; N, 4.32. Found: C, 64.67; H, 7.85; N, 4.39%.

[(bpy-9)Zn(Hkt)(Cl)(Solv)], 2. A hot solution of potassium inokitiolate (0.111 g, 0.550 mmol) in EtOH (50 ml) was added to a solution of [(bpy-9)$ZnCl_2$] (0.300 g, 0.550 mmol) in CH_2Cl_2 (30 ml). The resulting solution was stirred under nitrogen (3 days, r.t.). After removal of the solvent under vacuum the remaining residue was dissolved in Et_2O and was filtered through Celite. Et_2O was removed under reduced pressure and the product was dissolved in $CHCl_3$ and recrystallized from petroleum ether (0.253 g, 68% yield). Mp 128 °C. IR (ν_{max}/cm^{-1}: 2921, 2852 (CH), 1610, 1590 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 9.05 (d, 2H, J(H,H) = 4.6 Hz, $H_{3,3'}$), 7.92 (s, 2H, $H_{1,1'}$), 7.45 (d, 2H, J(H,H) = 5.0 Hz, $H_{2,2'}$), 7.39 (s, 1H, H_4), 7.24 (s, 2H, $H_{5,7}$), 6.76 (t, 1H, J(H,H) = 5.0 Hz, H_6), 2.82 (m, 5H, $CH(CH_3)_{2hkt}$, $CH_2(CH_2)_7CH_3$), 1.70 (m, 4H, CH_2CH_3), 1.29 (m, 30H, $(CH_3)_{2hkt}$, $(CH_2)_6CH_2CH_3$), 0.88 (t, 6H, J(H,H) = 6.6 Hz, CH_3). Anal. Calc. for $C_{38}H_{57}N_2O_3ZnCl$: C, 66.08; H, 8.32; N, 4.05. Found: C, 65.92; H, 8.18; N, 3.92%.

[(bpy-9)Zn(Q$_W$)(Cl)(Solv)], 3. To a solution of [(bpy-9)$ZnCl_2$] (0.150 g, 0.275 mmol) in 20 ml of CH_2Cl_2 was added KQ_W, (0.102 g, 0.275 mmol). The resulting yellow solution was stirred under nitrogen for 3 days at room temperature. The solution was filtered through Celite and evaporated to dryness under reduced pressure. The product was then dissolved in Et_2O, filtered through Celite, and evaporated to dryness under reduced pressure giving the pure product as a yellow solid (0.093 g, 40% yield). IR (ν_{max}/cm^{-1}: 2921–2852 (CH), 1610 (C=O). ^{1}H NMR (300 MHz, $CDCl_{3)}$: δ_H 9.03 (d, 2H, J(H,H) = 5.13 Hz, $H_{3,3'}$), 7.99 (d, 2H, J(H,H) = 8.08 Hz, $H_{e,i}$), 7.95 (s, 2H, $H_{1,1'}$), 7.54 (d, 2H, J(H,H) = 8.43 Hz, $H_{a,d}$), 7.46 (m, 6H, $H_{2,2',c,d,h,f}$), 7.18 (t, 1H, J(H,H) = 7.34 Hz, $H_{g,g'}$), 2.75 (t, 4H, J(H,H) = 7.45 Hz, $CH_2(CH_2)_7CH_3$), 1.85 (s, 3H, CH_{3QW}), 1.66 (m, 4H, CH_2CH_3), 1.37 (s, 9H, $C(CH_3)_{3QW}$), 1.29 (m, 24H, $(CH_2)_6CH_2CH_3$), 0.87 (t, 6H, J(H,H) = 6.6 Hz, CH_3). Anal. Calc. for $C_{49}H_{67}N_4O_3ZnCl$: C, 68.36; H, 7.84; N, 6.50. Found: C, 68.22; H, 8.12; N, 6.26%.

[(bpy-9)Zn(Q$_T$)(Cl)(Solv)], 4. To a solution of [(bpy-9)$ZnCl_2$] (0.100 g, 0.183 mmol) in 20 ml of CH_2Cl_2 was added KQ_T, (0.057 g, 0.183 mmol). The solution was filtered through Celite, evaporated to dryness under reduced pressure, the product was dissolved in Et_2O, filtered through Celite, and evaporated to dryness under reduced pressure giving the pure product as an brown solid (0.100 g, 70% yield). Mp 200 °C. IR (ν_{max}/cm^{-1}: 2926–2855 (CH), 1612 (C=O). ^{1}H NMR (300 MHz, $CDCl_{3)}$: δ_H 9.05 (2H, d, J(H,H) = 5.48 Hz, $H_{3,3'}$), 7.98 (2H, s, $H_{1,1'}$), 7.95 (2H, s, $H_{e,i}$), 7.40 (4H, m, $H_{h,f}$; $H_{2,2'}$), 7.18 (1H, t, J(H,H) = 7.14 Hz, $H_{g,g'}$), 2.80 (t, 4H, J(H,H) = 7.68 Hz, $CH_2(CH_2)_7CH_3$), 2.67 (s, 2H, CH_2), 2.49 (s, 3H, CH_{3QT}), 1.72 (m, 4H, CH_2CH_3), 1,32 (m, 24H, $(CH_2)_6CH_2CH_3$),1.15 (s, 9H, $C(CH_3)_{3QT}$), 0.87 (t, 6H, J(H,H) = 6.6 Hz, CH_3). Anal. Calc. for $C_{44}H_{65}N_4O_3ZnCl$: C, 66.19; H, 8.20; N, 7.05. Found: C, 66.50; H, 8.50; N, 6.95%.

[(bpy-9)Zn(Trop)$_2$], 5. 2 equiv. of potassium tropolonate (0.059 g, 0.366 mmol) was dissolved in EtOH (15 ml) and 1 equiv. [(bpy-9)$ZnCl_2$] (0.100 g, 0.183 mmol) was added. The resulting mixture was stirred at 80 °C over a period of 24 h. Then the reaction mixture was filtered and the solvent containing the product was evaporated *in vacuo*. The product was dissolved in

$CHCl_3$, filtered through Celite, then concentrated and precipitated by *n*-hexane (0.098 g, 75% yield). Mp 170 °C. IR (ν_{max}/cm^{-1}: 2926–2855 (CH), 1611, 1592 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.71 (d, 2H, *J*(H,H) = 5.1 Hz, $H_{3,3'}$), 7.87 (s, 2H, $H_{1,1'}$), 7.23 (m, 10H, $H_{2,2',4,4',7,7',5,5',8,8'}$), 6.68 (t, 2H, *J*(H,H) = 8.3 Hz, $H_{6,6'}$), 2.69 (m, 4H, $CH_2(CH_2)_7CH_3$), 1.68 (m, 4H, CH_2CH_3), 1.37 (m, 24H, $(CH_2)_6CH_2CH_3$), 0.86 (t, 6H, *J*(H,H) = 6.1 Hz, CH_3). Anal. Calc. for $C_{42}H_{54}N_2O_4Zn$: C, 70.43; H, 7.60; N, 3.91. Found: C, 70.31; H, 7.49; N, 4.31%.

[(bpy-9)Zn(Hkt)$_2$], 6. Two equiv. of potassium inokitiolate (0.232 g, 1.147 mmol) was dissolved in EtOH (100 ml) and 1 equiv. of [(bpy-9)$ZnCl_2$] (0.250 g, 0.459 mmol) was added. The resulting mixture was stirred at 80 °C over a period of 24 h. Then the reaction mixture was filtered and the solvent containing the product was evaporated *in vacuo*. The product was dissolved in $CHCl_3$, filtered through Celite, then concentrated and precipitated by *n*-hexane (0.265 g, 72% yield). Mp 180 °C. IR (ν_{max}/cm^{-1}: 2921, 2852 (CH), 1611, 1587 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.73 (d, 2H, *J*(H,H) = 5.2 Hz, $H_{3,3'}$), 7.86 (s, 2H, $H_{1,1'}$), 7.29 (d, 2H, *J*(H,H) = 1.3 Hz, $H_{2,2'}$), 7.24 (d, 2H, *J*(H,H) = 6.5 Hz, H_4), 7.14 (t, 4H, *J*(H,H) = 3.7 Hz, $H_{5,5',7,7'}$), 6.62 (m, 2H, $H_{6,6'}$), 2.72 (m, 6H, $CH(CH_3)_{2Hinok}$, $CH_2(CH_2)_7CH_3$), 1.64 (m, 4H, CH_2CH_3), 1.24 (m, 24H, $(CH_2)_6CH_2CH_3$), 1.20 (d, 12H, *J*(H,H) = 6.7 Hz, $(CH_3)_{2hkt}$), 0.87 (t, 6H, *J*(H,H) = 6.7 Hz, CH_3). Anal. Calc. for $C_{48}H_{66}N_2O_4Zn$: C, 72.02; H, 8.31; N, 3.50. Found: C, 71.81; H, 8.00; N, 3.81%.

[(bpy-9)Zn(Q$_W$)$_2$], 7. To a solution of complex [Zn(Q$_W$)$_2$(S)$_2$] (0,400 g, 0.542 mmol) in 30 ml of $CHCl_3$ was added an equivalent of bpy-9 (0.222 g, 0.542 mmol). The resulting yellow solution was stirred at room temperature for five days. Then it was evaporated to dryness under reduced pressure on a rotavapor. To a residue was added *n*-hexane (20 ml). After concentration of the solvent a yellow precipitate was afforded which was filtered off and dried to constant weight under reduced pressure (0.451 g, 73% yield). Mp 180 °C. IR (ν_{max}/cm^{-1}: 2926–2854 (C–H), 1617 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.92 (d, 2H, *J*(H,H) = 5.15 Hz, $H_{3,3'}$), 7.90 (d, 4H, *J*(H,H) = 1.21 Hz, $H_{e,e'}$;$H_{i,i'}$), 7.88 (d, 2H, *J*(H,H) = 0.85 Hz, $H_{1,1'}$), 7.34 (m, 4H, $H_{a,a'}$, $H_{d,d'}$), 7.31 (s, 2H, $H_{2,2'}$), 7.25 (d, 4H, *J*(H,H) = 8.43 Hz, $H_{b,b'}$, $H_{c,c'}$), 7.16 (t, 4H, *J*(H,H) = 8.01 Hz, $H_{f,f'}$; $H_{h,h'}$), 6.99 (t, 2H, *J*(H,H) = 6.86 Hz, $H_{g,g'}$), 2.75 (t, 4H, *J*(H,H) = 7.68, $CH_2(CH_2)_7CH_3$), 1.63 (m, 10H, CH_{3Qw}, CH_2CH_3), 1.32 (m, 42H, $(CH_2)_6CH_2CH_3$, $(CH_3)_3$), 0.87 (t, 6H, *J*(H,H) = 6.67 Hz, CH_{3L1}). Anal. Calc. for $C_{70}H_{86}N_6O_4Zn$: C, 73.69; H, 7.60; N, 7.37. Found: C, 73.44; H, 7.35; N, 7.42%.

[(bpy-9)Zn(Q$_T$)$_2$], 8. To a solution of [Zn(Q$_T$)$_2$(S)$_2$] (0,150 g, 0.223 mmol) in 30 ml of $CHCl_3$ was added an equivalent of bpy-9 (0.091 g, 0.223 mmol). The resulting colourless solution was stirred at room temperature for five days. Then it was evaporated to dryness under reduced pressure on a rotavapor. To a residue was added *n*-hexane (20 ml), from which a crystalline precipitate slowly formed at room temperature (0,227 g, 50% yield). Mp 121 °C. IR (ν_{max}/cm^{-1}: 2926–2856 (CH), 1635 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.78 (2H, d *J*(H,H) = 5.28 Hz $H_{3,3'}$), 7.93 (6H, d, *J*(H,H) = 8.41 Hz, $H_{1,1'}$; $H_{e,e'}$; $H_{i,i'}$), 7.30 (2H, d, *J*(H,H) = 5.28 Hz, $H_{2,2'}$), 7.19 (4H, t, *J*(H,H) = 7.92 $H_{f,f'}$; $H_{h,h'}$), 7.00 (2H, t, *J*(H,H) = 7.14 $H_{g,g'}$), 2.76 (4H, t, *J*(H,H) = 7.53 Hz, $CH_2(CH_2)_7CH_3$), 2.42 (4H, s, $CH_2C(CH_3)_3$), 2.38 (6H, s, CH_{3QT}), 1.70 (4H, m, CH_2CH_3), 1.28 (24H, m, $(CH_2)_6CH_2CH_3$), 0.87 (24H, m, CH_{3L1}; $(CH_3)_3$). Anal. Calc. for $C_{60}H_{82}N_6O_4Zn$: C, 70.88; H, 8.13; N, 8.26. Found: C, 70.76; H, 8.13; N, 8.28%.

Table 4 Crystallographic data for complex **5**

	5
Empirical formula	$C_{42}H_{54}N_2O_4Zn$
M_r	716.24
Cryst system	Triclinic
Space group	$P\bar{1}$
a/Å	10.862(2)
b/Å	13.476(2)
c/Å	14.342(2)
α/°	77.235(5)
β/°	86.019(6)
γ/°	75.809(5)
V/Å^3	1984.8(5)
Z	2
D_c/g cm^{-3}	1.198
μ/mm^{-1}	0.66
F(000)	764
θ/°	1.92–24.61
No. measured reflns	34037
No. unique reflns	6749 [*R*(int) = 0.0753]
Reflns with $I > 2\sigma(I)$	3967
Refined params	496
Goodness-of-fit	1.0052
R indices, $I > 2\sigma(I)^{a,b}$	$R_1 = 0.0573$, w$R_2 = 0.1482$
R indices (all data)	$R_1 = 0.1110$, w$R_2 = 0.1738$

^{a} $R1 = \sum(|F_o| - |F_c|)/\Sigma|F_o|$. ^{b} $wR2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2}$.

X-Ray crystallography

Single crystals of [(bpy-9)Zn(Trop)$_2$], **5** suitable for X-ray analysis were obtained by slow diffusion of ethanol into a chloroform solution. X-Ray crystal data for **5** were collected at room temperature on a Bruker-Nonius X8 Apex CCD area detector equipped with graphite monochromator and Mo-Kα radiation (λ = 0.71073), and data reduction was performed using the SAINT programs; absorption corrections based on multiscans were obtained by SADABS.[60] The structure was solved by the Patterson method (SHELXS/L program in the SHELXTL-NT software package)[61] and refined by full-matrix least squares based on F^2. All non-hydrogen atoms were refined anisotropically. Due to the severe disorder found on one aliphatic chain (carbon atoms from C(22) to C(28) refined in two positions with occupancy factors of 0.6 and 0.4) the SIMU constraint has been applied on their displacement parameters. Hydrogen atoms were included as idealized atoms riding on the respective carbon atoms with C–H bond lengths appropriate to the bond. Details of the crystal data collection are listed in Table 4.

Cell lines and cytotoxic assay

Human prostate cancer cell lines (DU145 and LNCaP) were grown in RPMI-1640 (Gibco) supplemented with 10% of fetal bovine serum (GIBCO), 5% of L-glutamine (GIBCO) and antibiotics, under standard conditions (37 °C temperature, 5% CO_2 in a humidified atmosphere). All the experiments have been performed at the earlier passage of the cell lines. For cytotoxic assays (MTS) and IC50 evaluation, cells were plated in 96-well plates (Falcon,

CA) in 100 µl of culture medium. For each experiment precursor or complexes were serially diluted in cell culture medium to the desired concentrations and an equal volume of the diluted solution (100 µl/well) was added to the cells which were continuously exposed to the compounds for 72 h. Each treatment was performed in triplicate in three independent experiments. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay using CellTiter 96 AQueous One Solution Proliferation Assay System (Promega). This assay measures the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron coupling reagent phenazine methosulfate. The plates were incubated 2 h at 37 °C and then the absorbance at 490 nm was measured using Sirio-S (SEAC, Radim Group), results are expressed as mean ± standard deviation (SD) of the percentage of viable cells at each drug concentration compared to the untreated cells. Then IC50 values were calculated by using the GraFit32 program.

PC3 cells were maintained in RPMI 1640 medium enriched with 10% of FBS, 1% penicillin/streptomycin and 1% glutamine and were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air. Sub-confluent cell cultures, synchronized for 24 h in DMEM without phenol red and serum (PRF-SFM-DMEM), were used for all experiments. The effect of all species on proliferation of the human cancer cells PC3 was measured with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay.[59] Briefly, 80 000 viable cells were plated in a 12-well format and became attached to the bottom of the well overnight. On the second day of the procedure, the original medium was removed and 1 ml new phenol red and fetal calf serum-free medium containing the test substances was added. After an incubation period of 72 h, the living cells were assayed by the addition of 50 ul 5 mg ml^{-1} MTT solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed and the precipitated crystals were dissolved in isopropanol–0.04 M HCl. Finally, the reduced MTT was monitored at 570 nm using a spectrophotometer with untreated cells being taken as the control.

Western blotting

PC3 cells were grown in 100 mm dishes to 70–80% confluence, synchronized in serum-free medium for 24 h and treated with test substances at a concentration of 1×10^{-5} M for 48 h.

PC3 cells, following treatments were harvested and lysed in 500 µl of lysis buffer, containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM EGTA pH 7.5, 10% glycerol, 1% Triton X-100 and protease inhibitors (2 µM Na_3VO_4, 1% PMSF, 20 µg ml^{-1} aprotinin).

The expression of different proteins was tested by Western Blot in 30 µg of protein lysates. Proteins were transferred to a nitrocellulose membrane, probed with primary antibody and then stripped and reprobed with the appropriate secondary antibodies. The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with a peroxidase-coupled *anti*-IgG antibody and revealed using the ECL system.

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