Cytotoxic performances of new anionic cyclometalated Pt(II) complexes bearing chelated O^O ligands

Andreea Ionescu,*[a,b] Rossella Caligiuri,[a] Nicolas Godbert,[a,b] Loredana Ricciardi,[b] Massimo La Deda,[a,b] Mauro Ghedini,[a,b] Nicola Ferri,[c] Maria Giovanna Lupo,[c] Giorgio Facchetti,[d] Isabella Rimoldi [d], Iolinda Aiello*[a,b]

a. *MAT-InLAB, LASCAMM CR-INSTM, Unità INSTM della Calabria,Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Ponte Pietro Bucci Cubo 14C, 87036 Arcavacata di Rende (CS), Italy; e-mail: andreea.ionescu@unical.it, iolinda.aiello@unical.it*

b. *CNR NANOTEC-Istituto di Nanotecnologia U.O.S. Cosenza, 87036 Arcavacata di Rende (CS), Italy*

c. *Università degli Studi di Padova, Dipartimento di Scienze del Farmaco, Via Marzolo 5, 35131, Padua, Italy.*

d. *Università degli Studi di Milano, Dipartimento di Scienze Farmaceutiche, Via Venezian 21, 20133 Milan, Italy.*

The *in vitro* biological activity on the MDA-MB-231 triple-negative breast cancer (TNBC) cell line of two different series of anionic Pt(II) organometallic complexes was tested. For the first time, the cytotoxic activity of anionic Pt(II) complexes has been observed. The anionic compounds of general formula NBu₄[(C^N)Pt(O^O)], where (C^N) stands for the cyclometalated form of 2-phenylpyridine (H(PhPy)), 2-thienylpyridine (H(Thpy)) or 2-benzo[*h*]quinoline (H(Bzq)) feature two different (O^O) chelated ligands: the tetrabromocatechol [BrCat]²⁻ (**1-3**) or alizarine [Aliz]²⁻ (**4-6**). Complexes **1-6** displayed a significant cytotoxic effect on the studied cell line (1.9-52.8 μM IC₅₀ range). For BrCat containing complexes, the biological activity was indipendent of the nature of the coordinated (C^N) ligand. In the case of **4-6** instead, the cytotoxicity (significantly low for **4**) was concomintantly induced by the presence of either the PhPy and the [Aliz]²⁻ ligands. Since complexes **1-6** are emissive in solution, the potential use of complex **4** as theragnostic agent was investigated by confocal analysis. The fluorescence signal from MDA-MB-231 cells incubated with **4** indicated the localization of the compound into the cytosol region.

Introduction

Owe to the numerous side effects, long term resistance and clinical limitation of the well-known *first generation* Pt(II) anticancer complex cisplatin (**Chart 1a**),[1] numerous DNA binding platinum drugs have been developed over the years.[2,3] The so called *second generation* platinum drugs include Pt(II) complexes which deviate from the cisplatin template as the metal centre

experiences a different ligand field, leading to a modulation of the electronic, steric hindrance and basicity features.[4,5] Among these, the most effective carboplatin (**Chart 1b**), presents higher solubility and stability than cisplatin and lower side effects.[6] The *third generation* Pt(II) antitumor drugs, incorporate, instead, different ammines and leaving groups, and among this category emerges as the most popular example, like the water soluble oxaliplatin (**Chart 1c**), embedding two chelating ligands and exhibiting a broader spectrum of activity than cisplatin or carboplatin.[7-9] Differently from the mechanism of action of cisplatin and its previously described derivatives, covalently binding Pt(II) complexes, incorporating a planar aromatic ligand, such as 1,10-phenanthroline, anthraquinone, acridine etc., may intercalate between two adjacent DNA base pairs stabilized through $\pi-\pi$ stacking stabilization,[10] and/or may subsequently coordinately bind to DNA by losing a labile leaving group.[11] Intercalation causes thus DNA to lose its regular helical structure.[14-16] As evidenced by a comprehensive study on a series of positively charged Pt(II) complexes bearing aromatic (N^N) ligands,[17-21] (**Chart 2a-c**) the cytotoxicity is influenced by the extension of the aromatic core, as well as by the charge (positive in the examined cases), solubility and effective Pt(II) complex cellular uptake, following a mechanism not yet fully elucidated, but different from the one followed by cisplatin.[13,17-18,22-23] Several studies have indeed suggested an activity of these Pt(II) complex at a mitochondrial level, while X-ray structures[24] have demonstrated that the aromatic ligands insert between the base pairs of DNA.[25-27]

Due to their capability of forming repair resistant lethal Pt-DNA crosslinks, bis-chelated Pt(II) complexes have been found more efficient than their counterparts embedding monodentate ligands.[13] Following such an approach, Mitra *et al*. synthesized the catecholate containing Pt(II) neutral complexes [(N^N)Pt(O^O)][28] (**Chart 2b**) based on carboplatin and oxaliplatin archetypes (**Chart 1b,c**) and by performing time-dependent UV-visible spectral studies they demonstrated the breakage of the Pt-O bond in the presence of an excess of glutathione (GSH), which can be at the basis of the observed activity triggered by the thus generated free owing to the generated free coordinating site.

Regarding the "orphan" tumours, one of the most aggressive is the triple-negative breast cancer (TNBC), which displays evinces a negative response to drugs due to the lack in the main three receptors, common targets of the therapy against breast cancer.[29,30] Indeed, TNBC does not respond to hormonal therapy medicines or medicines that target HER2 protein receptors. For this reason, it is necessary to introduce a new detecting methodology and treatment for TNBC. In the last years, many research groups have attempted to overcome this limit by focalizing their attention on the development of new therapeutic agents, characterized by the capability to allow a

targeted imaging together with the induction of a cytotoxic effect in TNBC tumour cells.[29,30] Taking into consideration this premise, we tested the *in vitro* biological activity on the MDA-MB-231 cell line.

To the best of our knowledge, anionic Pt(II) complexes have never been tested for their biological properties. Herein, we have considered a series of ionic complexes of general formula $NBu_4[(C^{\wedge}N)Pt(O^{\wedge}O)]$, featuring the tetrabutylammonium cation and the Pt(II) organometallic anion $[(C^{\wedge}N)Pt(O^{\wedge}O)]$, where $(C^{\wedge}N)$ stands for the cyclometalated form of 2-phenylpyridine (H(PhPy)), 2-thienylpyridine (H(Thpy)) or 2-benzo[*h*]quinoline (H(Bzq)), while $(O^{\wedge}O)$ represents either the dianion of the tetrabromocatechol (H_2(BrCat), (**Chart 3**, complexes **1**-**3**)[31] or the dianionic form of the alizarine (H_2(Aliz)). (**Chart 3**, complexes **4**-**6**) To complete the series in order to probe the eventual activity of the newly introduced $[Aliz]^{2-}$ molecular fragment, the neutral complex **7** (**Chart 3**), parent of the cisplatin archetype, was synthesized and tested. The Pt(II) complexes were selected taking into account the peculiar molecular structure, ionic in nature, whose anion contains a Pt(II) centre bonded to a cyclometalated $(C^{\wedge}N)$ ligand[32-34] and coordinated to a chelating $(O^{\wedge}O)$ fragment. In particular, it has previously been documented that a series of cycloplatinated complexes showed promising IC_{50} values (μM) against Sarcoma 18 cells.[35,36] Instead, both the H_2(BrCat) and H_2(Aliz) ligands were chosen due to the fact that they may be prone to Pt-O bond breaking in intracellular medium, favouring DNA covalent binding. Finally, compared to $[BrCat]^{2-}$, the $[Aliz]^{2-}$ fragment presents a more extended aromatic core combined with a chemical structure similar to those of the already tested antitumor intercalating aromatic molecules doxorubicin (**Chart 2e**) [37-42] and mitoxantrone (**Chart 2f**).[43]

In addition, besides potential anticancer activity, the extended aromatic system and the acidic character of the hydroxyl substituents could confer to the anthraquinone-derived molecule a fluorescent behaviour.[44] Accordingly, its incorporation into the Pt(II) complexes is expected to contribute to the development of new compounds as cellular labels or probes for imaging.

Results and discussion

Synthesis

The cyclometalated anionic Pt(II) complexes **1**-**3** derived from H_2(BrCat) were synthesized as previously reported.[33] Complexes **4**-**6** were prepared starting from the respective cycloplatinated precursors **I**-**III** (**Scheme 1**) by adapting an already reported procedure.[45] Complexes bearing the cyclometalated ligands PhPy (**4**) and ThPy (**5**) were prepared starting from a mononuclear intermediate, obtained by microwave assisted synthesis, as previously reported,[45] while for

complex **6** incorporating the Bzq fragment, a chloro-bridged binuclear cycloplatinated precursor was used (**Scheme 1**).[46] The treatment of **I-III** with H_2(Aliz) in basic media (NBu$_4$OH)[47] afforded the anionic square-planar Pt(II) complexes **4-6**, in moderate to high yields.

Complex **7** was synthesized starting from cisplatin, which was prepared according to an already described microwave assisted procedure,[48] followed by treatment with AgNO$_3$ as halogen scavenger and subsequent coordination of [Aliz]$^{2-}$ in basic media (**Scheme 2**).

The newly synthesized **4-7** complexes were fully characterized by FT-IR and ^{1}H-, ^{13}C-NMR spectroscopies, ESI-MS, melting points and elemental analysis. Considering the IR spectra of complexes **4-6**, the coordination of the [Aliz]$^{2-}$ group was confirmed by the shift of the C=O and C-O stretching bands from 1663 cm^{-1} and 1460 cm^{-1}, respectively, in the free H_2(Aliz) ligand to *ca.* 1635 and *ca.* 1442 cm^{-1} for complexes **4-6**. The C-H aliphatic stretching bands (*ca.* 2900 cm^{-1}) associated to the presence of the [NBu$_4$]$^+$ counterion confirmed the negative charge of the Pt(II) thus synthesized complexes.

However, due to the asymmetrical structure of the H_2(Aliz) ligand, upon complexation to the Pt(II) metal centre, two *cis/trans* geometrical isomers (with respect to the relative position of the N-coordinated atom and the more sterically hindered O-chelated atom) could be expected.[32] The ^{1}H-NMR spectra were characterized by the splitting of the signal associated with the proton in *alfa* position with respect to the cyclometalated nitrogen atom, so accounting for the presence of the two *cis/trans* isomers that, according to the corresponding signal integration, resulted in 1:0.7 (**4**), 1:1 (**5**), 1:1(**6**) *cis/trans* ratios. Moreover, the signals associated to the aliphatic protons in the upfield region of the spectrum confirmed the presence of the [NBu$_4$]$^+$ counterion in stoichiometric ratio with respect to the aromatic protons, evidence of the ionic nature of complexes **4-6**.

The formation of **4-6** was also confirmed by ^{13}C-NMR spectroscopy. ESI-MS analyses of complexes, carried out via direct infusion in the negative mode, showed an isotope cluster, centred at *m/z* 587.55 (**4**), 593.28 (**5**), 611.68 (**6**) due to the natural abundances, and corresponding to the [(C^N)Pt(Aliz)]$^-$ complex anion. The formation of complex **7**, instead, was confirmed by the analysis of the FT-IR spectrum, displaying the signal associated to the NH$_3$ groups at 3434 cm^{-1}. Considering the signals relative to the coordinated [Aliz]$^{2-}$ fragment, the C=O and C-O stretching are shifted at 1625 and 1490 cm^{-1}, respectively, in complex **7,** compared with 1663 and 1460 cm^{-1} in the free ligand. The ^{1}H-NMR spectrum is consistent with the chemical structure of complex **7**, although showing a broad single signal accounting for both NH$_3$ groups.

Photophysical properties

The photophysical properties in solution of **1-3** have been extensively investigated previously.[33] For a direct comparison, the absorption and emission spectra were re-proposed in the ESI (**Figures SI-1,2**) and the data summarized in **Table 1**.

The UV-Vis absorption spectra in DMSO solution of the Aliz series **4-7** are shown in **Figure 1**.

All the cyclometalated complexes **4-6** displayed intense bands in the ultraviolet region between 280 and 330 nm assigned to the allowed $^1LC(\pi\text{-}\pi^*)$ transitions localized respectively on the PhPy, ThPy and Bzq ligand[49-51] The low-energy absorption bands between 330 and 650 nm of **4-7** were instead attributed to a mixed (MLCT-LC) character, which predominantly involved the chelating ligand, in agreement with the experimental and theoretical electronic spectra of the Aliz molecule.[52,53]

All the new anionic compounds displayed emissive properties in solution in the red region of the electromagnetic spectrum (**Figure 2** and **Table 1**), showing a structured band arising from the MLCT-LC transition.

Evaluation of *in vitro* cytotoxic activity

It has been reported that the Pt(II) anticancer drug cycloplatam, important for overcoming drug resistance to cisplatin (**Chart 4**)[54] exists under two isomeric *cis/trans* forms, the biological activity of any single isomeric form remained first unknown, but recently, being still unknown. Recently, the two bioactive isomers have been separated and tested showing equal anticancer activity.[55,56]

For the NBu4[(C^N)Pt(Aliz)] **4-6** complexes, NMR spectroscopy indicate the presence of geometrical isomers. Separation of the two isomers through HPLC, both with chiral and achiral columns under reverse phase conditions, have been attempted but was not successful. Accordingly, in the present study, we preliminary tested the cytotoxic properties of the *cis*/*trans* mixture for the **4**-**6** alizarin containing complexes.

As shown in **Table 2**, all the complexes exerted a significant cytotoxic effect on triple-negative breast cancer cell line MDA-MB-231, with IC$_{50}$ values ranging from 1.9 to 127 µM.

As exhibited by **Table 2**, all the complexes **1**-**3** with dianion [BrCat]$^{2-}$ showed a good cytotoxic activity on MDA-MB-231 with values of IC$_{50}$ around 40 µM, independently from the ligand used in the complex. On the contrary, when Aliz was used as dianion as in species **4-6**, significant differences were evinced in function of the cyclometalated (C^N) ligand employed in the

coordination sphere of the Pt(II) centre. In particular, the cytotoxic activity of **4**, in which the PhPy was bound to the Pt(II), resulted in a very low value of IC_{50} (IC_{50} equal to 1.9±1.6 μM). Complex **4**, showed somewhat significant cytotoxic effect on MDA-MB-231 tumour cells compared to normal not transformed vascular smooth muscle cells, showing a ten-fold higher IC_{50} value (12.5±1.0 µM *vs* 1.9±1.6 µM). With the aim to evaluate the capability of H_2(Aliz) ligand to influence alone the cytotoxic activity of a complex, compound **7** was synthetized. The potency of the cytotoxic effect decreased in comparison to cisplatin's value on MDA-MB-231 (IC_{50} equal to 126.9±2.7 μM for **7** and 59.4 μM for cisplatin).[23] This data confirmed that the matching between the cyclometalated H(PhPy) moiety and $[Aliz]^{2-}$ ligand, in the complexes, was necessary to contribute to cytotoxic activity of the compound. In addition, we evaluated the cytotoxicity effect of ligands utilized for the synthesis of the complexes. As shown in Table 2, BrCat was the only ligand that showed a significant cytotoxic effect with an IC_{50} value similar to the complexes, while Aliz, ThPy, PhPy and Bzq were inactive. These data suggest a more potent cytotoxic effect of complexes compared to single ligands.

In order to investigate the molecular mechanisms underlying the cytotoxic effect of these complexes, we analysed the effect of **4** on cell-cycle progression in MDA-MB-231 cell line. Incubation of MDA-MB-231 with 10% FCS shows a 15.9% of cells in S phase (**Figure 3**). The addition of 5 μM of complex **4** to the medium determined an accumulation of cells in S phase (20.4%) associated to a concomitant reduction of cells in G2/M phase, from 33.7% to 22.7%. Taken together, these results indicated that complex **4** exerts an antiproliferative/cytotoxic action by blocking the progression of the cells through the S phase of the cell cycle.

The potential use of complex **4** as theragnostic agent was investigated by measuring the fluorescence signal from MDA-MB-231 cells incubated with 2.5 and 5 μM concentration of the compound. As shown in **Figure 2**, we detected a positive signal after 3h exposure of the cells with 5 μM of complex **4**. Moreover, confocal analysis indicated that the majority of the compound is localized into the cytosol in the perinuclear region (**Figure 4**).

Conclusions

A series of cyclometalated anionic complexes of general formula $NBu_4[(C^\wedge N)Pt(O^\wedge O)]$ were synthesized, spectroscopically characterized and tested for their cytotoxicity against MDA-MB-231 triple negative breast cancer cell line. A 2-phenylpyridine (H(PhPy)), 2-thyenylpyridine (H(Thpy)) or 2-benzo[*h*]quinoline (H(Bzq)) were respectively coordinated as (C^N) ligands, while alizarine (Aliz) and tetrabromocatechol (BrCat) were used as a (O^O) chelated ligand.

The Aliz based complexes NBu$_4$[(C^N)Pt(Aliz)] exhibited an important cytotoxic effect on the studied cell line (1.9-127 μM IC$_{50}$ range), more efficient compared with the catecholate containing series. By studying the effect of the best performing **4** (with a IC$_{50}$ of 1.9 μM) onto the MDA-MB-231 cycle progression, it was evidenced that the Pt(II) species exert an antiproliferative/cytotoxic effect by blocking the progression of the cell cycle in its S phase.

Since all the investigated complexes are emissive in solution, the most efficient complex NBu$_4$[(PhPy)Pt(Aliz)] was studied by confocal analysis. The fluorescence signal observed from the MDA-MB-231 cells incubated with NBu$_4$[(PhPy)Pt(Aliz)] indicated the presence of the Pt(II) compound into the cytosol region, for which its potential use as a theragnostic agent could be considered.

Experimental

General considerations

All commercially available chemicals were purchased from Sigma Aldrich or Alfa Aesar and were used without further purification. Cycloplatinated precursors **I-III** and complexes **1-3** were synthesized as already reported.[33] Microwave assisted syntheses were carried out using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). Melting points were determined with a Leica DMLP polarising microscope equipped with a Leica DFC280 camera and a CalCTec (Italy) heating stage. Elemental analyses were performed with a PerkinElmer 2400 analyzer CHNS/O.

Spectroscopy and spectrometry

IR spectra (KBr pellets) were recorded on a Spectrum One Perkin-Elmer FT-IR spectrometer. ^{1}H-NMR spectra were recorded on a Brucker WH-300 spectrometer in deuterated solvents with TMS as internal standard. MS analyses were performed by using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electronspray ionisation source and an 'Ion Trap' mass analyser. The MS spectra were obtained by direct infusion of a sample solution in MeOH under ionisation, ESI negative. Spectrofluorimetric grade DMSO was used for the photophysical investigations in solution, at room temperature. A Perkin Elmer Lambda 900 spectrophotometer was employed to obtain the UV/Vis absorption spectra, using quartz cuvettes of 1 cm path length. Steady-state emission spectra were recorded on a Horiba Jobin Yvon Fluorolog 3 spectrofluorimeter, equipped with a Hamamatsu R-928 photomultiplier tube. The emission

quantum yields of **4-7** were obtained using the optical dilution method[57] using cresyl violet perchlorate in methanol as reference standard (Φ=0.54).[58]

Synthesis

Preparation of NBu4[(PhPy)Pt(O^O)] (4). Two equivalents of H_2(Aliz) (22 mg, 0.093 mmol) were added to a solution of **I** (50 mg, 0.093 mmol) in 20 ml of degassed ethanol. In this solution, NBu_4OH (0.18 ml, 0.186 mmol) was added and the mixture was stirred for 24 hours under reflux. The crude product was concentrated, and water was added. The solid was isolated by filtration and washed with water and diethyl ether. Dark blue solid, yield 61 %; m.p. 139°C; ^1H-NMR (d_6-DMSO, 300 MHz, TMS) δ = 9.22 (d, J_{H-H}= 5.43 Hz, 1H), 8.96 (d, J_{H-H}= 5.43 Hz, 0.7H), 8.11-8.08 (m, 1H), 8.01-7.98 (m, 2.1H), 7.94-7.89 (d, 0.7H), 7.84-7.82 (m, 3.5H), 7.65-7.53 (m, 6.4H), 7.39-7.36 (m, 2H), 7.30-7.27 (m, 0.7H), 7.18-7.17 (m, 1.3H), 7.05-6.94 (m, 2.7H), 6.51 (d, J_{H-H}= 3.21 Hz, 0.7H), 6.48 (d, J_{H-H}= 3.06 Hz, 1H), 3.07 (s, 13.6H), 1.47 (s, 13.6H), 1.21 (s, 13.6H), 0.84 (s, 20.4H); ^{13}C-NMR ($CDCl_3$, 300 MHz, TMS) δ =182.88, 182.78, 182.26, 181.65, 176.75, 176.65, 170.42, 170.40, 167.39, 167.35, 144.44, 144.20, 144.11, 143.92, 136.74, 136.58, 136.54, 136.30, 134.76, 134.74, 134.67, 133.15, 132.25, 132.12, 132.00, 131.99, 131.64, 131.46, 130.28, 129.43, 129.34, 129.28, 126.50, 126.44, 126.16, 126.09, 125.92, 122.99, 122.89, 122.60, 122.13, 122.02, 121.75, 121.50, 121.29, 119.81, 118.81, 118.52, 118.03, 117.80, 58.26, 23.83, 19.42, 13.53; FT-IR (KBr) ν (cm^{-1}) = 2959, 2931, 2872, 1633, 1511, 1443, 1339, 1301, 1281, 1257, 761, 721; E.A. calculated (%) for $C_{37}H_{42}N_2O_4Pt$: C, 57.43; H, 5.47; N, 3.62; found: C, 57.83; H, 5.39; N, 3.49; MS (ESI) m/z 587.55 [M]$^-$

Preparation of NBu4[(ThPy)Pt(Aliz)] (5). Following the same procedure described for the synthesis of **4**, the reaction was conducted using **II** (100 mg, 0.18 mmol), H_2(Aliz) (43 mg, 0.18 mmol) and NBu_4OH (0.40 ml, 0.36 mmol). Dark blue solid, yield 72 %; m.p. 108°C; ^1H-NMR (d_6-DMSO, 300 MHz, TMS) δ = 9.18 (d, J=5.46 Hz, 1H), 8.91 (d, J=5,46 Hz, 1H), 8.18-8.13 (m, 1H), 8.07-8.05 (m, 3H), 7.87-7.82 (m, 1H), 7.72-7.65 (m, 6H), 7.45-7.42 (m, 2H), 7.36-7.34 (m, 1H), 7.28-7.27 (m, 2H), 7.16-7.10 (m, 3H), 7.05-7.02 (m, 2H), 6.60 (d, 1H), 3.14 (s, 16H), 1.54 (s, 16H), 1.29 (s, 16H), 0.91 (s, 24 H); ^{13}C-NMR ($CDCl_3$, 300 MHz, TMS) δ =182.76, 182.70, 182.17, 181.67, 177.17, 173.37, 170.89, 168.02, 163.39, 163.29, 150.25, 149.69, 147.99, 147.70, 137.74, 137.66, 137.39, 137.36, 136.50, 136.23, 134.65, 134.61, 133.41, 133.10, 132.56, 132.20, 132.09, 131.69, 131.53, 131.38, 128.02, 127.02, 126.48, 126.19, 126.03, 125.93, 122.69, 121.44,

121.25, 119.70, 118.81, 118.64, 117.88, 116.72, 116.66, 116.44, 57.82, 23.64, 19.26, 13.46; FT-IR (KBr) ν (cm^{-1}) = 2959, 1635, 1601, 1513, 1475, 1444, 1257, 720; E.A. calculated (%) for C$_{35}$H$_{40}$N$_2$O$_4$PtS: C, 53.90; H, 5.17; N, 3.59; found: C, 54.15; H, 5.29; N, 3.19; MS (ESI) m/z 593.28 [M]$^-$

Preparation of NBu$_4$[(Bzq)Pt(Aliz)] (6). Following the same procedure described for the synthesis of **4**, the reaction was conducted using **III** (100 mg, 0.12 mmol), H$_2$(Aliz) (59 mg, 0.24 mmol) and NBu$_4$OH (0.48 ml, 0.48 mmol). Purple solid, yield 55 %; m.p. 196 °C; ^{1}H-NMR (d$_6$-DMSO, 300 MHz, TMS) δ = 9.46 (d, 1H), 9.24 (d, 1H), 8.62-8.58 (m, 2H), 8.20-8.18 (m, 2H), 8.10-8.06 (m, 2H), 7.88-7.83 (m, 2H), 7.76-7.71 (m, 9H), 7.61-7.53 (m, 4H) 7.49-7.45 (m, 2H), 7.39-7.36 (m, 1H), 6.66 (d, 1H), 6.63 (d, 1H), 3.14 (s, 16H), 1.53 (s, 16H), 1.27 (s, 16H), 0.91 (s, 24H); ^{13}C-NMR (CDCl$_3$, 300 MHz, TMS) δ =182.94, 182.86, 182.82, 182.36, 182.35, 182.34, 182.32, 182.31, 181.77, 181.74, 181.71, 176.85, 174.13, 174.16 174.10, 175.09, 174.06, 174.03, 168.83, 148.95, 148.58, 148.32, 148.29, 141.95, 141.91, 141.08, 140.52, 136.36, 135.54, 135.41, 134.79, 134.72, 133.29, 133.19, 132.15, 132.02, 131.64, 131.47, 130.92, 130.29, 129.15, 129.03, 126.56, 126.20, 126.11, 125.96, 122.87, 121.48, 120.90, 120.73, 119.81, 119.63, 118.09, 116.79, 57.99, 23.67, 19.21, 13.31; FT-IR (KBr) ν (cm^{-1}) =2958, 2871, 1634, 1512, 1443, 1339, 1298, 1280, 1256, 821, 717; E.A. calculated (%) for C$_{39}$H$_{42}$N$_2$O$_4$Pt: C, 58.71; H, 5.31; N, 3.51; found: C, 58.73; H, 5.25; N, 3.63; MS (ESI) m/z 611.68 [M]$^-$

Preparation of [(NH$_3$)$_2$Pt(Aliz)] (7). Cisplatin (40 mg, 0,13 mmol) and AgNO$_3$ (43 mg, 0,26 mmol) were suspended in 1 ml of dimethylformamide and stirred at r.t. for 12 h. The precipitated AgCl was filtered off and to the filtrated solution H$_2$(Aliz) (13 mg, 0,33 mmol) and 5 eq. of NaOH solubilized in 4 mL of distilled water were added. The reaction mixture was stirred at r.t. for 5h under nitrogen atmosphere. The dark solid was filtered, washed with distilled water, ethanol and dichloromethane. Dark blue solid, yield 85%; m.p. >250°C. ^{1}H-NMR (d$_6$-DMSO, 300 MHz, TMS) δ = 8.16-8.08 (m,2H), 7.74-7.72 (m, 2H), 7.43 (d, J=8,46 Hz, 1H), 6.42 (d, J=8,13 Hz, 1H), 3.64 (s, 6H) ; FT-IR (KBr) ν (cm^{-1}) = 3434, 1626, 1490, 1384, 1286, 1208, 715. E.A. calculated (%) for C$_{14}$H$_{12}$N$_2$O$_4$Pt: C, 35.98; H, 2.59; N, 5.99; found: C, 36.03; H, 2.55; N, 5.63.

Biological studies

Reagents

Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA, penicillin/streptomycin, sodium pyruvate, non-essential amino acid solution, fetal calf serum (FCS), plates and Petri dishes were purchased from EuroClone. The compounds were dissolved in dimethyl sulfoxide (DMSO) before performing each experiment. The maximal concentration utilized was 200 μM; cisplatin was tested up to 100 µM. The amount of DMSO did not exceed the 0.25% of the culture media volume.

Cell culture

Human triple negative cancer cell line MDA-MB-231 were cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids 100X, 1% penicillin/streptomycin 100X, 1% L-glutamine 200 mM and 1% sodium pyruvate at 37°C in a humidified atmosphere (5% CO_2 and 95% air).

Cell viability assay

Sulphorhodamine B (SRB) assay was performed to assess the cell viability after treatments. 5×10^3 cells/well were seeded in a 96-well tray in triplicate. After 24 h of incubation, the cells were treated with different concentrations of compounds. SRB assay were performed after 48h as previously described.[24]

Confocal microscopy analysis

4×10^4 cells/well were seeded in a 24-well tray in duplicate. After 24h of incubation, the cells were treated with 2.5 and 5 μM of complex **4**. After 3h of incubation, the cells were washed with PBS, fixed with paraformaldehyde (PFA) 4% at r.t. for 10 min. After fixation, free aldehyde groups were removed by incubate the cells with NH_4Cl 50 mM for 10 min at r.t.. Finally, the cells were washed three times with PBS and stored in PBS at 4°C until the confocal analysis.

The confocal microscopy analysis has been performed with Zeiss LSM 800 by exciting the cells with $\lambda = 405$ nm. Fluorescence has been collected at $\lambda = 590\text{-}670$ nm.

Statistical analysis

Experimental data are expressed as mean $\pm$ S.D. The effects of the complexes versus control were analysed by two-tailed Student's t test for unpaired data. The concentration of compounds

required to reduce by 50% of cell viability (IC$_{50}$) was calculated by 4-PL nonlinear regression curve (GraphPad Prism, Version 5.01).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Table 1.   Photophysical data of compounds **1-7** in DMSO solution ($1·10^{-5}$M) at 298K.

Complex	Absorption λ_{max}/nm (ε/M^{-1}cm^{-1})	Emission λ_{max}/nm	Φ(%)
1[a]	293(21072)-314(13937)-328(11313)-417(sh)-442(9324)	593	0.02
2[a]	309(27950)-349(sh)-421(10075)-448(10303)	581, 606, 529	0.19
3[b]	319(21070)-354(11237)-378(10444)-430(sh)-464(9141)	621	0.04
4	287(32152)-422(5248)-535(sh)-571(11707)-612(sh)	670, 725, 790 (sh)	0.40
5	295(27715)-347(sh)-423(7221)-532(sh)-569(13975)-609(sh)	670, 725, 790 (sh)	0.25
6	278(28036)-321(sh)-360(8281)-436(sh)-532(sh)-571(15800)-612(sh)	670, 725, 790 (sh)	0.31
7	357(5770)-421(sh)-480(sh)-557(4178)	670, 780	0.20

[a] Complex **1-3** are reproduced from ref. 33.

Table 2. Cytotoxicity effect of anionic Pt(II) complexes **1-7** and their ligands on MDA-MB-231 cell line.
N.A.: not active

Complex	IC$_{50}$ (µM)
1	52.8±3.9
2	46.1±3.1
3	31.7±0.6
4	1.9±1.6
5	18.3±2.3
6	6.8±0.6
7	126.9±2.7
Aliz	N.A.
BrCat	32.7±7.3
ThPy	N.A.
PhPy	N.A.
Bzq	N.A.