In vitro anticancer activity evaluation of new cationic platinum(II) complexes based on imidazole moiety.

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Abstract. *The development and the synthesis of cationic platinum(II) complexes were realized and their cytotoxic activity was tested on triple negative breast cancer MDA-MB-231 cell line and in two cell lines poorly responsive to cisplatin (DLD-1 and MCF-7). The complex **2c** resulted the most potent cytotoxic agent in MDA-MB-231 (IC$_{50}$=61.9 μM) and more effective than cisplatin on both DLD-1 (IC$_{50}$=57.4 μM) and MCF-7 (IC$_{50}$=79.9 μM) cell lines. **2c** showed different cellular uptake and pharmacodynamic properties than cisplatin, interfering with the progression of the M phase of the cell cycle. Thus, **2c** represents a lead compound of a new class of cytotoxic agents with promising antitumor activity*

1. Introduction

Platinum based-drugs currently used in therapy, i.e. cisplatin[1, 2] and its second and third generation derivatives carboplatin[3] and oxaliplatin,[4] were established to elicit their cytotoxic effect through the formation of bifunctional intra- and interstrand DNA adducts at the guanosine residues.[5, 6] These lesions are known for being responsible of transcription inhibition thus resulting in triggering apoptosis and finally causing cell death. Although cisplatin and its analogues still proved effective in treating a variety of cancers, their heavy side effects along with their limited activity towards certain types of tumor evoke the need of new platinum chemotherapeutics endowed with structural features that by violating the structure-activity relationships summarized by Cleare and Hoeschele[7, 8] could offer the possibility to explore alternative cellular targets besides nuclear DNA.

Platinum-based anticancer drugs have attracted some attention as potential breast cancer therapies especially for triple-negative breast cancer (TNBC, hereafter).[9, 10] TNBC, clinically defined as tumors that doesn't express estrogen receptors (ER), progesterone receptors (PgR), or Her-2, remains a major therapeutic challenge due to the lack of available targeted agents and the high risk of disease recurrence. Indeed, the addition of platinum agents to neoadjuvant treatment of TNBC is clearly associated with significantly higher rates of pathologic complete response.

Breaking the assumption that bifunctionality is necessary for antiproliferative activity, monofunctional platinum complexes are emerging as a valid approach for circumvent the shortcomings of bifunctional platinum based drugs.[11, 12]

Since the discovery of the potent antiproliferative activity of the platinum triamine complex phenanthriplatin and of its analogues,[13] the mechanism of action of monofuntional platinum compounds as anticancer agents have been deeply investigated.[14-16]

This class of platinum complexes bearing only one labile ligand is able to form only a single covalent bond at the N7 position of guanine residues without significantly bending or unwinding the double helix but indeed forming lesions of such a shape that result able to elude cellular repair responses and to potentially interact with other-than-DNA targets, thus affording a novel activity profile.[17, 18] Moreover, depending on their cationic nature, monofunctional platinum compounds represent an ideal substrate for the Organic Cation Transporters (OTCs),[19, 20] recently recognized as determinant of the oxaliplatin specificity of action.[21] Indeed, the development of novel anticancer compounds targeted to OCTs offer the possibility to exploit a selective delivery to those tumors known for over expressing these channels.

In a previous work[22] we reported the synthesis and cytotoxicity studies on a series of platinum dichloro-complexes based on imidazole ligands functionalised at the *N*1 of the imidazole ring with differently-long saturated and unsaturated chains with compounds bearing a butyl and an hexyl moiety resulted endowed with a biological profile more similar to the oxaliplatin than cisplatin one. Starting from those encouraging findings, we synthesised a series of cationic triamine platinum compounds of general formula [Pt(N-N')N'Cl]X⁻ where N-N' is an aminomethylimidazole ligand and the N' an imidazole ring, both bearing the same alkyl group at the *N*1 position. The resulting platinum complexes were analytically characterized and biologically evaluated on different cancer cell lines known for their aggressive behaviour (triple negative breast cancer) and for their poor sensitivity to the common cisplatin chemotherapy. Moreover, several *in vitro* assays were performed in order to better define their antitumor activity and target selectivity.

2. Results and discussion

Starting from the reported platinum(II) complexes[22, 23] the synthesis of platinum(II) **2 a-c** as the other square-planar d⁸ complexes proceed by associative ligand substitution in dimethyl formamide in presence of 1 equivalent of opportunely substituted *N*-alkyl imidazole at 55°C.[11] The stereochemistry of the resulting products is dictated by relative *trans* effect of the ligands within the complex.
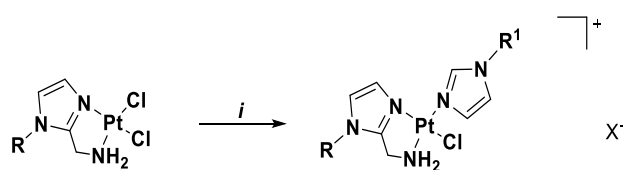
Reagents and conditions: *i)* N-alkyl imidazole, DMF, 55°C

1a R= -CH₃
1b R= -C₄H₉
1c R= -C₆H₁₃

2a R=R¹= -CH₃   X= Cl⁻
3a R=R¹= -CH₃   X= NO₃⁻
2b R=R¹= -C₄H₉   X= Cl⁻
**2c** R=R¹= -C₆H₁₃   X= Cl⁻

Scheme 1. General procedure for the synthesis of cationic platinum(II) complexes.

Moreover, water solubility (and thus applicability) can be achieved for cationic Pt(II) complexes by selecting specific counter ions and/or introducing charged groups onto the ligands' molecular structure.[13] For the substitution of leaving group literature procedures will be employed. In **3a** the substitution of Cl$^-$ with NO$_3^-$ as counterion was realized by an exchanging reaction in presence of AgNO$_3$. The obtained complexes were purified through a celite pad after their dissolution in methanol.

This new complexes' series was also synthesized in order to get platinum-based drugs more soluble in water improving pharmacokinetics and their clinical use. For the platinum(II) complexes here reported the determination of lipophilicity parameter was evaluated by RP-HPLC equipped with C18 ODS at 25 °C with water/methanol in ratio 70/30 as eluents and using commercially available platinum complexes as references.[24-26]

The lipophilicity increased along the series: log P_{ow} values are -1.09 for **2a**, 0.383 for **2b** and 1.54 for **2c**, respectively in accordance with the increase of lipophilicity due to the length of aliphatic chains on the amine ligands.

To begin to investigate the potential cytotoxicity of the new cationic Pt(II) complexes we carried out a series of MTT analyses on the triple-negative breast cancer cell line MDA-MB-231 and colorectal cancer cell line HCT-116. Complexes **2a** and **3a** did not show any significant cytotoxic effect at concentration range of 50-150 μM, while **2b** and **2c** very efficiently and potently reduced the viability of both MDA-MB-231 and HCT-116 (Figure 1). In particular, the calculated IC$_{50}$ values on MDA-MB-231 cell viability was 81.6 μM and 61.9 μM for **2b** and **2c**, respectively. Similar IC$_{50}$ values were observed on HCT-116 cells (IC50 values equal to 55.6 μM and 49.9 μM for **2b** and **2c**, respectively). The potency of the cytotoxic effect was very similar to that observed with cisplatin on the same cell lines (IC$_{50}$ equal to 59.4 μM and 33.5 μM for MDA-MB-231 and HCT-116, respectively).
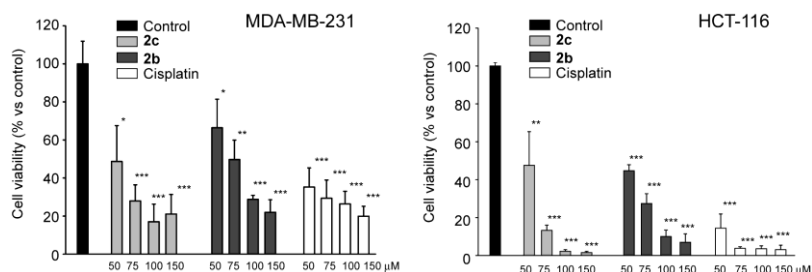
Figure 1. Cytotoxic effect of **2c, 2b** and cisplatin on MDA-MB-231 and HCT-116 cell lines. Cells were seeded (40,000/well of 48 well tray) and incubated with DMEM supplemented with 10% FCS; 24h later the medium was replaced with one containing 10% FCS and the reported concentrations of complexes and cisplatin and the incubation was continued for a further 48h. At the end of this incubation period, the cell viability was determined by MTT assay. Each bar represents the mean ± SD of a representative of three independent experiments. Compounds versus control: *p<0.05; **p<0.01; ***p<0.001.

Thus, complex **2c** was chosen for additional pharmacological evaluations. The first step was to extend the cytotoxicity evaluation of **2c** on two cell lines considered to be particularly unresponsive to cisplatin: the DLD-1 (colorectal tumor) and MCF-7 (mammary adenocarcinoma). As shown in Figure 2, **2c** reduced the cell viability of both cell lines with IC$_{50}$ values of 57.4 μM and 79.9 μM for DLD-1 and MCF-7, respectively. On the contrary, at the same concentrations, cisplatin didn't reduce the cell viability of MCF-7

and DLD-1 by more than 50% and 30% (Figure 2) confirming their partial resistance. These data suggest that complex **2c** overcomes the cisplatin resistance mechanism in both cell lines.
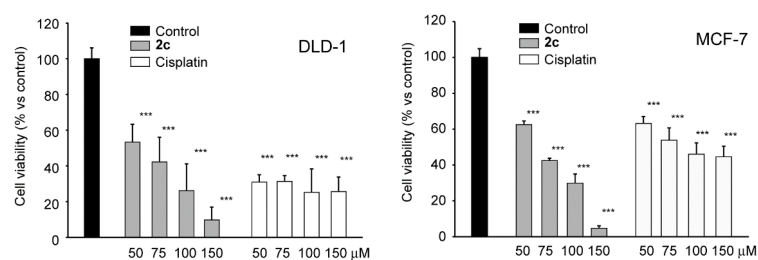
Figure 2. Cytotoxic effect of **2c** and cisplatin on DLD-1 and MCF-7 cell lines. The experimental conditions are the same than figure 1. Each bar represents the mean ± SD of a representative of three independent experiments. Compounds versus control: ***p<0.001.

The cytotoxic effect of cisplatin and **2c** were then evaluated in non-tumor cell line, such as the primary human smooth muscle cells, in order to calculate the in vitro therapeutic index. **2c** showed similar cytotoxic effect on smooth muscle cells than that observed for tumor cell lines, with an IC_{50} equal to 48.7 .µM, while cisplatin was more effective reducing by more than 95% the cell viability at 50µM concentration (Figure 3). Thus, **2c** did not show a selective cytotoxic effect towards cancer cell lines, (therapeutic index ≈ 1) but cisplatin appeared to be even more toxic on normal cells than cancer cells.
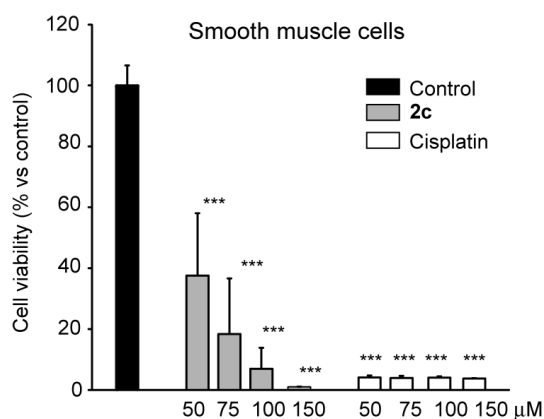
Figure 3. Cytotoxic effect of **2c** and cisplatin on human smooth muscle cells. The experimental conditions are the same than figure 1. Each bar represents the mean ± SD of a representative of three independent experiments. Compounds versus control: ***p<0.001.

Since membrane transporters and channels, collectively known as the transportome, are increasingly recognized as important determinants of tumor cell chemosensitivity and chemoresistance,[27-30] we therefore investigated the potential lack of interaction of **2c** with the copper transporters (CTRs) involved in cellular uptake and potential platinum sensitivity/resistance of tumors. Determination of the physical interaction of **2c** with the Met7 peptide mimicking the CTR1 transporter was realized as previously reported by our research group.[22] As expected, after 5 days only a little amount of mono charged species [Mets7+Pt(N-N-N)] corresponding to a monocoordinated complex in a 1:1 stoichiometry between **2c** and Mets7 was

evinced by ESI spectroscopy. As evinced, the prevalent species in solution remained the same found just at the beginning of reaction underlining the presence of coordinately stable triamine complex (Figure 4). This result suggests that, differently from cisplatin, complex **2c** does not interact with CTR-1.
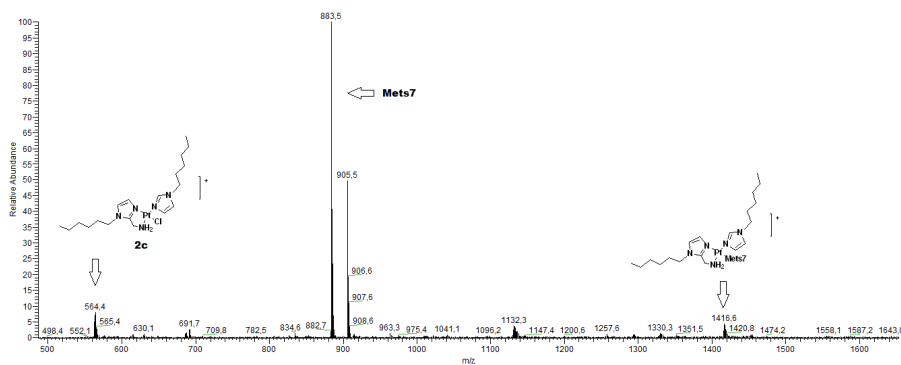

Figure 4. ESI-MS spectra of peaks due to the lack of interaction between Mets7 and complex **2c** after 5 days 1:1 mixture

To further investigate the potential involvement of the CTR-1 transporters on complex **2c** cellular uptake, we analyzed their mRNA levels in MDA-MB-231 cell line incubated for 6h with cytotoxic concentrations of **2c**. Cisplatin significantly induced the mRNA levels of CTR-1, ATP7a and ATP7b, while **2c** did not affect their expression (Figure 5).[31, 32] These results suggest, indirectly, that **2c** does not interact with any of these transporters that, instead, plays a pivotal role on cisplatin sensitivity/resistance. Nevertheless, additional and more specific experiments need to be performed in order to better define the mechanism of cellular uptake of **2c**.

Additional evidences indicate that membrane transporters of platinum compounds, including solute carriers and in particular organic cation transporters (OCTs) belonging to the SLC22 subfamily, markedly affect critical pharmacokinetic parameters, and determine the severity of platinum-associated adverse events.

The cellular uptake was then investigated by monitoring the platinum concentrations by ICP-MS from a total cell extract or DNA isolated from MDA-MB-231 incubated for 6h with either **2c** or cisplatin. Interestingly, **2c** was incorporated by the cells two times more efficiently than cisplatin (Figure 6B). However, differently from cisplatin, the nuclear DNA isolated from cells incubated with **2c** did not show any significant accumulation of platinum (Figure 6A).
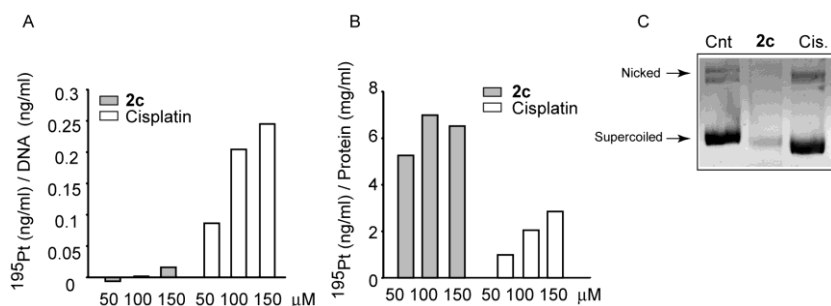

Figure 6. DNA-bound and total intracellular concentrations of ^{195}Pt after incubation of MDA-MB-231 cell line with complex **2c** and cisplatin. Cells were seeded (250,000/35 mm petri dish) and incubated with
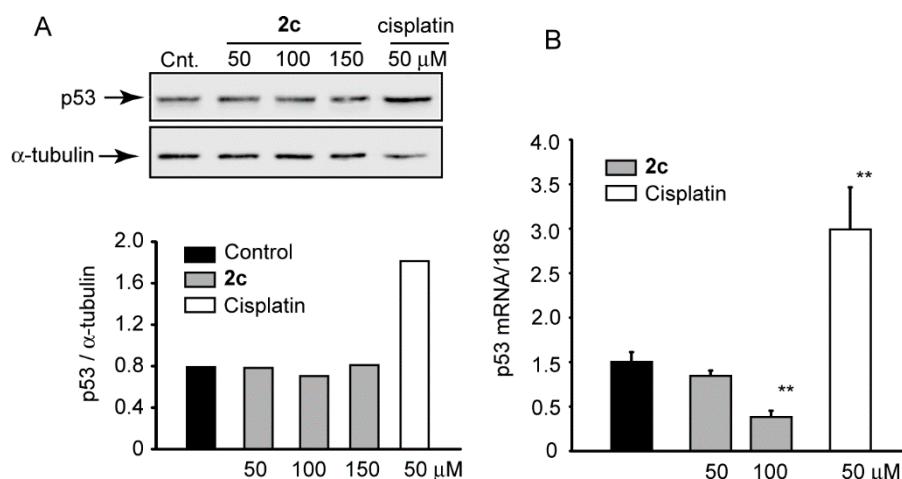
DMEM supplemented with 10% FCS; 24 h later the medium was replaced with one containing 10% FCS and **3c** or cisplatin at indicated concentrations. The incubation was continued for 3 h at 37°C. At the end of this incubation period, the DNA was extracted and cell homogenates prepared. DNA-bound (A) and total (B) ^{195}Pt concentrations were then determined by ICP-MS and the values corrected for DNA and protein contents. (C) Circular plasmid DNA was incubated for 2 h at 37°C with cisplatin (Cis.) or complex **2c** at the final concentration of 100 µM. The DNA samples were then loaded on agarose gel containing ethidium bromide.

These results suggest that the nuclear DNA is not the cellular target of complex **2c**, thus its potent cytotoxicity action cannot be the result of a molecular mechanism similar to cisplatin. In accordance to this hypothesis, **2c** did not affect the expression of p53, at both protein and mRNA levels, while cisplatin significantly upregulates both levels (Figure 67A and 67B). Nevertheless, the incubation of plasmid DNA with complex **2c** significantly affected the electrophoretic mobility on agarose gel, suggesting a direct interaction between the complex **2c** and the DNA in a cell-free assay. As previously described, also cisplatin changed the DNA electrophoretic mobility, although in a different manner compared to complex **2c** (Figure 6C).[33]

To begin to investigate the molecular mechanism of the cytotoxic effect of **2c** on MDA-MB-231 cells, we performed a cell cycle analysis by flow cytometry after propidium iodide labelling of nuclear DNA. As shown in Figure 8, complex **2c** induced a concentration-dependent accumulation of cells into G2/M phase, while cisplatin increase the percentage of cells in S phase. Thus, compound **2c** appeared to interfere with a cellular event different for cisplatin.
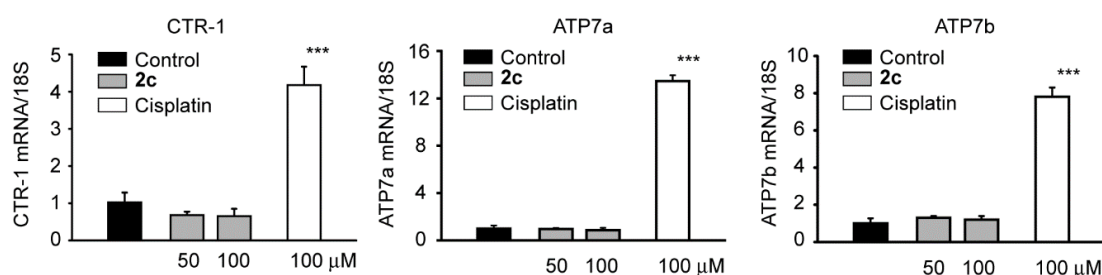
Figure 5. Effect of complex **2c** and cisplatin on mRNA expression of CTR-1, ATP7a and ATP7b in MDA-MB-231 cell line. Cells were seeded (40,000/well of 48 well tray) and incubated with DMEM supplemented with 10% FCS; 24h later the medium was replaced with one containing 10% FCS and the reported concentrations of **2c** and cisplatin and the incubation was continued for a further 24h. At the end of this incubation period, the total RNA were extracted and the real-time PCR performed with specific primers for CTR-1, ATP7a, and ATP7b. Each bar represents the mean ± SD of a representative of three independent experiments. Cisplatin versus control: ***p<0.001.

Figure 7. Effect of **2c** and cisplatin on p53 expression in MDA-MB-231 cell line. Cells were seeded (250,000/35 mm petri dish) and incubated with DMEM supplemented with 10% FCS; 24 h later the medium was replaced with one containing 10% FCS and the reported concentrations of complex **2c** and cisplatin and the incubation was continued for a further 24 h. At the end of this incubation period the total cell lysates were prepared and p53 expression evaluated by Western blotting analysis (A). The quantification of fluorescence signal was determined by using Image Studio software (LI-COR Biosciences) and normalized with □-tubulin. (B) Under the same experimental conditions described for panel A, p53 mRNA levels were determined by real-time PCR reaction and normalized with 18S. Cisplatin versus control: **$p<0.01$.
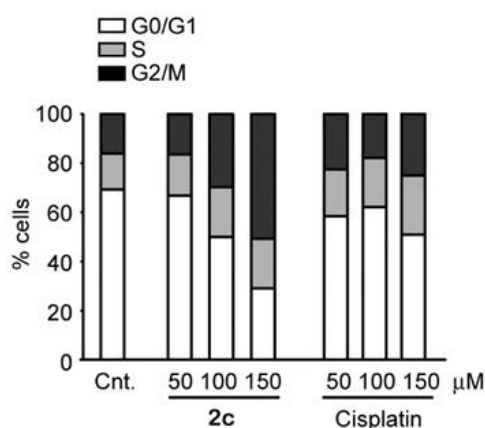
Figure 8. Effect of **2c** and cisplatin on cell cycle distribution of MDA-MB-231 cell line. Cells were seeded (250,000/35 mm petri dish) and incubated with DMEM supplemented with 10% FCS; 24 h later the medium was replaced with one containing 10% FCS in the presence or absence (control) of indicated concentrations of **2c** and cisplatin and the incubation was continued for a further 24 h. At the end of this incubation period the cell cycle analysis was performed.

In conclusion, the new cationic platinum (II) complex **2c** showed a very effective and potent cytotoxic effect in triple-negative breast cancer cells and in cell lines partially resistant to cisplatin. Surprisingly, **2c** showed a completely different pharmacodynamic and cellular uptake behaviour than cisplatin. Indeed, **2c**

did not seem to interact with the cellular transporters involved in the cisplatin uptake, such as CTR-1, ATP7a and ATP7b, and did not reach the nuclear DNA. The fact that **2c** does not interact with the nuclear DNA is further supported by the absence of any significant induction of p53 after 24h exposure to the compound. Nevertheless, in a cell-free assay, complex **2c** was shown to be able to interact with plasmid DNA and alter its electrophoretic mobility. Thus, it is conceivable to conclude that complex **2c** did not interact with the nuclear DNA simply because it didn't reach the nucleus. The different mechanism of action of **2c** and cisplatin was confirmed by the results of the cell cycle analysis that clearly indicated a different phase of accumulation of cells. Based on this evidence, it is conceivable to hypothesize that complex **2c** may elicit its cytotoxic effect via a p53-independent activation of the mitochondrial apoptotic pathway, a mechanism previously shown for bipyridine gold(III) dithiocarbamate-containing complexes.[34] Nevertheless, the actual mechanism of action of this new cationic platinum(II) complex still needs to be determined.

3. Experimental section

All manipulations involving air sensitive materials were carried out in an inert atmosphere glove box or using standard Schlenk line techniques under an atmosphere of nitrogen or argon in oven-dried glassware. All solvents were used anhydrous. ^{1}H-NMR, ^{13}C-NMR and ^{195}Pt-NMR spectra were recorded on a Bruker DRX Avance 300 MHz equipped with a non-reverse probe and also on a Bruker DRX Avance 400 MHz. MS analyses were performed by using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electrospray 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 positive. Elemental analyses were performed using a Perkin Elmer SeriesII/CHNS/O 2400 Analyzer. Log P_{ow}'s values and purity were evaluated with Partisil C18-ODS reversed-phase HPLC column with Merck-Hitachi L-7100 equipped with Detector UV6000LP. ICP-MS data were recorded with BRUKER aurora M90 ICP-MS (MA, USA).

Synthesis of compounds **1a**, **1b**, **1c**: the synthesis and characterization of these compounds is described elsewhere.[22, 23]

General procedure for the synthesis of compounds 2a, 2b, 2c: To a stirred solution in anhydrous DMF (1 mL) of **1** (1 mmol) the appropriate substituted *N*-alkyl imidazole (0.9 mmol) was added and the reaction mixture was warmed at 55°C overnight under nitrogen atmosphere. The solvent was then removed *in vacuo* and the oily residue was dissolved in dry methanol. After filtration of any eventually formed precipitate, the solution was concentrated to give the crude product as an oil that upon washing with diethyl ether afforded the desired complex as a differently coloured powder.

[Pt((1-methyl-1H-imidazol-2-yl) methanamine)(1-methyl-1H-imidazole)Cl]Cl **2a**: Brown solid. Yield: 35%. MS (ESI) m/z 424.1 [M+H]+. ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.37 (t, J = 1.47 Hz, 1H), 7.29 (t, J = 1.52 Hz, 1H), 7.21 (t, J = 1.48 Hz, 1H), 7.10 (t, J = 1.48 Hz, 1H), 6.95 (t, J = 1.51 Hz, 1H), 6.51-6.48 (br, 2H), 3.85 (t, J = 4.46 Hz, 2H), 2.99 (s, 3H), 2.85 (s, 3H) ppm. ^{13}C NMR (75 MHz, CD$_3$OD) δ 156.30, 152.52, 129.12, 128.30, 123.03, 122.69, 42.34, 35.78, 34.20 ppm. ^{195}Pt NMR (300 MHz, DMF-d$_7$) δ -3.228 ppm. Elemental analysis for C$_9$H$_{15}$Cl$_2$N$_5$Pt: calculated C, 23.54; H, 3.29; N, 15.25; found C, 23.34; H, 3.34; N, 15.01.

[Pt((1-butyl-1H-imidazol-2-yl) methanamine)(1-butyl-1H-imidazole)Cl]Cl **2b**: Grey solid. Yield: 63%. MS (ESI) m/z 507.3 [M]+. ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.35 (s, 1H), 7.30 (s, 1H), 7.22 (s, 1H), 7.18 (s, 1H), 6.88 (s, 1H), 6.16-5.91 (br, 2H), 4.32-4.19 (m, 2H), 4.13-3.91 (m, 4H), 1.84-1.67 (m, 4H), 1.43-1.26 (m, 4H), 0.99-0.89 (m, 6H) ppm. ^{13}C NMR (75 MHz, CD$_3$OD) δ 155.51, 128.49, 128.40, 128.28, 123.09, 120.11, 51.56, 42.94, 32.58, 32.42, 31.86, 19.52, 19.31, 12.73, 12.61 ppm. ^{195}Pt NMR (300 MHz, DMF-d$_7$) δ -3.133 ppm. Elemental analysis for C$_{15}$H$_{27}$Cl$_2$N$_5$Pt: calculated C, 33.16; H, 5.01; N, 12.89; found C, 32.87; H, 4.94; N, 12.92 .

[Pt((1-hexyl-1H-imidazol-2-yl) methanamine) (1-hexyl-1H-imidazole)Cl]Cl **2c**: Light brown solid. Yield: 46%. MS (ESI) m/z 564.3 [M+H]+. ^{1}H NMR (200 MHz, CD$_3$OD) δ 7.68 (s, 1H), 7.59 (s, 1H), 7.18 (s, 1H), 7.14 (s, 1H), 6.91 (s, 1H), 6.08-5.95 (br, 2H), 4.32-4.13 (m, 2H), 4.12-3.87 (m, 4H), 1.91-1.59 (m, 4H), 1.46-1.12 (m, 12H), 1.00-0.77 (m, 6H) ppm. ^{13}C NMR (50 MHz, DMSO) δ 156.46, 134.75, 129.26, 127.09, 124.63, 120.79, 48.59, 31.32, 31.23, 30.70, 30.63, 30.03, 29.90, 26.04, 25.99, 22.58, 22.51, 14.49, 14.25 ppm. ^{195}Pt NMR (300 MHz, DMF-d7) δ -3.028 ppm. Elemental analysis for C$_{19}$H$_{35}$Cl$_2$N$_5$Pt: calculated C, 38.07; H, 5.88; N, 11.68; found C, 38.39; H, 5.22; N, 11.15

Synthesis of [Pt((1-methyl-1H-imidazol-2-yl)methanamine)

(1-methyl-1H-imidazole)Cl]NO$_3$$^-$ 3a: To a solution of **1c** (50 mg, 0.13 mmol) in 1 mL of dry DMF was added AgNO$_3$ (22.6 mg, 0.13 mmol) and the resulting mixture was stirred in the dark under a nitrogen atmosphere at 55 °C for 16 h. After removing the AgCl formed over the reaction by filtration, the supernatant was diluted with additional 2 mL of dry DMF and 1-methyl-1*H*-imidazole (9.8 mg, 0.12 mmol, 0.9 equiv) was added. The reaction solution was stirred for 16 h at 55 °C, thereafter the solvent was removed under reduced pressure and the residue was dissolved in 5 mL of dry methanol. The unreacted precipitate of **1c** was removed by filtration. The dropwise addition of cold diethyl ether to the vigorously stirred methanolic solution afforded the desired product **3a**. The product was collected as a greenish solid. Yield: 67%. MS (ESI) *m/z* 424.1 [M+H]$^+$. ^{195}Pt NMR (300 MHz, DMF-*d$_7$*) δ -3.264 ppm. Elemental analysis for C$_9$H$_{15}$ClN$_6$O$_3$Pt: calculated C, 22.25; H, 3.11; N, 17.30; found C, 22.15; H, 3.02; N, 17.06

Log P_{ow} determination. RP-HPLC analysis were performed to correlate the hydrophobicity of the platinum(II) complexes with their retention time. The chromatograms were registered using Partisil C18-ODS reversed-phase HPLC column at 25 °C and with water/methanol in ratio 70/30 as mobile phase with KI as internal standard (flow rate of 1 mL/min, λ= 210 nm). The calibration curve was realized in comparison with reference compounds chosen in commercially available platinum compounds series.[35]

Biological evaluation procedures

Reagents and antibodies. MEM, trypsin-EDTA, penicillin, streptomycin, non-essential amino acid solution, fetal calf serum (FCS), disposable culture flasks and petri dishes were purchased from Euroclone S.p.A. (Pero, Milan, Italy). For western blot analysis, the following antibodies were used: mouse monoclonal anti-p53 (Sigma-Aldrich, Milan, Italy, clone DO7), mouse monoclonal anti-a-tubulin (Sigma-Aldrich, Milan, Italy), IRDye800 Goat anti-mouse (Carlo Erba reagents, Cornaredo Milan, Italy).

Cell culture. The following human cell lines have been utilized in the study: DLD1 (colorectal cancer), MCF-7,MDA-MB-231 (breast cancer), human primary smooth muscle cells. Cells were cultured in DMEM supplemented with penicillin (10,000 U/mL), streptomycin (10 mg/mL), nonessential amino acid and 10% Fetal Calf Serum (FCS). Cells were incubated with newly synthesized complexes dissolved in DMSO. The same volume of solvent were added to control conditions and did not exceed 0.5% v/v.

MTT-assay. The determination of the conversion of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) to formazan was determined as previously described.[23]

Determination of DNA-bound ^{195}Pt concentration. For the DNA-bound ^{195}Pt concentrations, the nuclear DNA was extracted by incubating cell monolayers with digest buffer (50 mM Tris HCl 1 M, 100 mM NaCl, 100 mM EDTA, 1% SDS) then transferred to 1.5 mL microcentrifuge tubes and a saturated NaCl solution was added. The samples were then clear by centrifugation for 15 min at 13,000 rpm and the supernatant transferred to new microcentrifuge tubes and DNA precipitated by isopropanol. DNA was then washed by centrifugation two times with 70% ethanol and resuspended in TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA). The ^{195}Pt concentrations were then determined by ICP-MS and normalized by total DNA. For the determination of total intracellular ^{195}Pt concentrations, cells were washed twice with PBS and lysed by incubation with 1% Triton X100/0.1% SDS for 5 min at room temperature. Cell lysates were then cleared by centrifugation at 14,000g for 10 min, and the ^{195}Pt concentrations determined by ICP-MS. The data were normalized with the protein concentrations determined using the BCA protein assay (Thermo scientific, Rockford, IL USA).

Electrophoresis Mobility Assay. Cisplatin (100 µM) and compound 2C (100 µM) were incubated respectively with 250ng of plasmid DNA pBM-IRES-PURO for 2 hour at 25°C [36], allowing the intercalation of complexes in the molecules of DNA. For the electrophoresis, the Thermo Scientific 6X DNA Loading Dye was added to the samples and then seeded on a 1% agarose gel in the presence of Ethidium bromide. Migration of different configuration of DNA was analyzed with Image Lab™ Software (Bio-Rad Laboratories).

Western blot analysis. Cells were washed twice with PBS and lysed by incubation with a solution of 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet-P40, containing a protease and phosphatase inhibitor cocktails (Sigma Aldrich, Milan, Italy) for 30 minutes on ice. Cell lysates were then cleared by centrifugation at 14,000g for 10 min, and protein concentrations were determined using the BCA protein assay (Thermo scientific, Rockford, IL USA). Equal amount of total protein per sample were separated by SDS–PAGE under reducing conditions, transferred to nitrocellulose membrane (GE Healthcare Little Chalfont, Buckinghamshire, UK) and subsequently immunoblotted with primary antibody following appropriate secondary fluorescently labeled antibody and acquired with the Odissey FC system (LI-COR). Quantitative densitometric analyses was performed with Image Studio software (LI-COR).[37, 38]

RNA preparation and quantitative real-time PCR. Total RNA was extracted with the iScript Sample Preparation Buffer cDNA synthesis preparation reagents (BIO-RAD laboratories) and reverse transcription performed with the Maxima 1st strand DNA RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real time PCR (qPCR) was performed with Kit Thermo SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems; Life Technologies Europe BV, Milan, ITALY) as previously described (Ruscica JBC 2016). The primer sequences used for qPCR analysis are: CTR1 Forward 5'-GGGCAACAGATGCTGAGCTT-3' Reverse 5'-GCTTATGAC CACCTGGATGATG-3'; ATP7a Forward 5'-TCTCCGATTGT GTGAGCTTTGT-3' Reverse 5'-TTCCTCATTACATTCCTGG TTTC TC-3'; ATP7b Forward 5'-CAGGCGCTTCTCCAATGC-3' Reverse 5'-GGGATGTGTGAGCAAGGAA-3'; p53 Forward 5′-GGAGGTTGTGAGGCGCTGG-3′ Reverse 5′-CACGCACCT CAAAGCTGTTC-3′; 18S Forward 5'-CTCAACACGGGAAAC CTCAC-3' Reverse 5'-CGCTCCACCAACTAAGAACG-3'.

Statistical Analysis. Experimental data are expressed as mean ± S.D. The effects of the tested drugs versus control on the different parameters were analyzed by two-tailed Student's *t* test for unpaired data. The

concentration of compounds required to inhibit 50% of cell proliferation (IC_{50}) was calculated by nonlinear regression curve (SigmaPlot software; Systat Software, Inc., Point Richmond, CA).

Author Contributions

[±]These authors equally contributed to this work.

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