# Synthesis, characterization and biological activity of new cyclometallated platinum(IV) complexes containing a para-tolyl ligand†

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**ABSTRACT:**

The synthesis of three new cyclometallated platinum(II) compounds containing a para-tolyl ligand and a tridentate [C,N,N'] (cm1) or a bidentate [C,N] ligand and an additional ligand such as SEt2 (cm2) or Ph3 (cm3) is reported. The X-ray molecular structure of platinum(II) compound cm3 is also presented. Intermolecular oxidative addition of methyl iodide or iodine upon cm1, cm2 and cm3 produced six novel cyclometallated platinum(IV) compounds. The cytotoxic activity against a panel of human adenocarcinoma cell lines (A-549 lung, MDA-MB-231 and MCF-7 breast, and HCT-116 colon), DNA interaction, topoisomerase I, IIα, and cathepsin B inhibition, and cell cycle arrest, apoptosis and ROS generation of the investigated complexes are presented. The best results for antiproliferative activity were obtained for platinum (IV) compounds cm1MeI and cm1I2 arising from oxidative addition of methyl iodide and iodine, respectively, to cm1. Cyclometallated platinum(IV) compounds cm1MeI and cm3MeI induce significant changes in the mobility of DNA and, in addition, cm1MeI, cm3MeI and cm1I2, showed considerable topoisomerase IIα inhibitory activity. Moreover, the compounds exhibiting the higher antiproliferative activity (cm1MeI and cm1I2) were found to generate ROS and to supress HCT-116 colon cancer cell growth by a mixture of cell cycle arrest and apoptosis induction. 1H NMR experiments carried out in a buffered aqueous medium (pH 7.40) indicate that compound cm1MeI is not reduced by common biologically relevant reducing agents such as ascorbic acid, glutathione or cysteine.

## INTRODUCTION

Metal containing anticancer drugs started to be relevant more than 40 years ago with the discovery of the therapeutic potential of cisplatin. More recently, platinum(IV) compounds have attracted great interest due to their advantages over platinum(II) analogues.1–6 Platinum(IV) compounds exhibit an octahedral coordination that permits the modification of some important physicochemical properties such as lipophilicity, stability and the reduction potential through the two extra coordination positions. Moreover, they are kinetically inert compared to platinum(II) analogues, which allows the possibility of oral administration.

On the other hand, platinum(II) cyclometallated compounds, especially those with nitrogen donor atoms, attract great interest due to their antitumour properties.7 They benefit from a strong σ(M–C) bond that improves their stability in front of biological reduction and labilises the trans ligands allowing the exchange in cellular uptake.

Surprisingly, very little attention has been devoted to cyclometallated platinum(IV) compounds although these species combine the properties imparted by the presence of a platinum(IV) centre and a cyclometallated ligand. We have recently reported the synthesis and biological studies of several cyclometallated platinum(IV) compounds that were prepared either by intramolecular C–X bond activation from an adequate platinum(II) substrate and a potentially tridentate [C,N,N′] ligand (method A in Scheme 1)8,9 or, more recently, by intermolecular oxidative addition of Y–Z reagents such as methyl iodide or iodine10 on a [C,N,N′]-cyclometallated platinum(II) precursor containing an additional ligand such as Cl, I or CH3 (method B in Scheme 1). This second method allows comparison of the biological properties of the platinum(IV) compounds with the parent platinum(II) precursors.

During these studies, it has been found that cyclometallated platinum(IV) compounds containing three Caryl-donor ligands (1a, 1a′, 1b′) and those containing two or three C-donor ligands including one axial methyl (2a–2c) displayed a remarkable cytotoxicity against several cancer cell lines in spite of their reluctance to be reduced.9,10 In view of these findings, we decided to further explore this type of cyclometallated platinum(IV) compound. In particular, the aim of this work is to study new cyclometallated platinum(IV) compounds obtained through oxidative addition of both methyl iodide and iodine (method B) on cyclometallated platinum(II) containing an additional aryl ligand. This aryl ligand places an additional C-donor ligand in the coordination sphere of platinum and increases the polarizability of the obtained compounds. In addition, the presence of an aryl ligand might favour intercalative binding to DNA through π–π stacking.

On the other hand, the presence of a triphenylphosphine ligand has been reported to increase the lipophilicity of cyclometallated palladium(II) and platinum(II) compounds leading to high cytotoxicity of these compounds.11–13 Stable platinum(IV) compounds containing triphenylphosphine ligand have been reported to be active against cancer cell lines.14 We therefore decided to include in the present study, in addition to [C,N,N′]-cyclometallated platinum(II) compound cm1, compounds containing a

[C,N]-platinacycle and an additional ligand such as SEt2 or PPh3 (cm2 and cm3 shown in Scheme 2, respectively) which should allow us to compare the effect of these additional ligands on the biological properties of these compounds. The platinum(II) precursors selected in this study are shown in Scheme 2.

## SYNTHESIS OF THE COMPOUNDS

### Synthesis of cyclometallated platinum(II) compounds

Cyclometallated platinum(II) compounds were prepared following known procedures from dinuclear platinum complex [Pt(4-CH3C6H4)2{μ-S(CH2CH3)2}]2 (A) and two different imines, L1 containing two nitrogen atoms and L2 containing one nitrogen atom.15 The reaction with L1 takes place through initial [N,N′] coordination of the imine to the platinum followed by an intramolecular C–H bond activation of the aryl ring of the imine, and elimination of a toluene molecule to produce a [C,N,N′]-cyclometallated platinum(II) compound cm1 shown in Scheme 3. Evidence of the tridentate coordination of the imine ligand is obtained from the 1H NMR spectrum in which the methylamino moiety, the imine and the aromatic proton adjacent to the metallated site (Hc) are coupled to 195Pt. The ortho protons of the para-tolyl group are also coupled to 195Pt. In addition, elemental analysis and mass spectrometry are consistent with the proposed structure.

In a similar process, the reaction with L2 produces a [C,N]-cyclometallated platinum(II) compound cm2 shown in Scheme 3. It is interesting to point out that for imine L2, C–H bond activation could take place at the 4-chlorophenyl or at the benzylic group. The former would give an endo-metallacycle (containing the imine group) while the latter would produce an exo-metallacycle. As previously observed for similar systems,15 the reaction takes place selectively at the 4-chlorophenyl ring leading to a more stable endo five-membered cycloplatinated compound. Compound cm2 is characterized by 1H NMR spectroscopy, elemental analyses and mass spectrometry. The imine and the aromatic proton adjacent to the metalation site are coupled to 195Pt, thus confirming the bidentate [C,N] coordination of the imine.

Compound cm3 (shown in Scheme 3) was prepared from reaction of cm2 with PPh3 in acetone for 2 h at room temperature that produced the substitution of the diethyl sulphide ligand by the PPh3 ligand. In the 1H NMR spectrum, the imine proton appears coupled to 195Pt, but the aromatic proton adjacent to the metallation site (Hc) could not be identified due to the higher complexity of the aromatic region arising from the presence of PPh3 protons. The 31P{1H} NMR spectrum displays one signal at 26.90 ppm for which the 1J (P–Pt) value (2201.3 Hz) is consistent with the presence of an aryl ligand trans to the P atom in a platinum(II) compound.15–18 Compound cm3 was also identified by mass spectrometry, elemental analysis and X ray diffraction analysis of suitable crystals grown from CH2Cl2/MeOH (1 : 1) solution.

The crystal structure is composed of discrete molecules held together by van der Waals interactions. The asymmetric unit contains two independent molecules with bond parameters equal within experimental error [3σ], one methanol and two water molecules. The molecular structure (molecule a) is shown in Fig. 1. The square-planar geometry around the platinum(II) is completed with a [C,N] ligand, a PPh3 and a para-tolyl which is tilted 84.52° from the mean coordination plane. The two C-donor ligands are mutually cis as expected from the high trans influence of these ligands and the PPh3 ligand is trans to the cyclometallated aryl. As expected, the [C,N] metallacycle exhibits an endo structure (including the

imine double bond). Bond lengths and angles are well within the range of values obtained for analogous compounds.15–18 In particular the imine CvN bond length lies in the usual range, resulting in a shorter distance than those reported for C–N bonds. The angles in the coordination sphere of platinum are close to 90° with the smallest angle corresponding to the metallacycle (C(8)–Pt–N(1) = 79.9(3)°).

**Synthesis of cyclometallated platinum(IV) compounds**

One of the most used methods for the preparation of octahedral platinum(IV) compounds from appropriate platinum(II)precursors proceeds via two-electron oxidation of the metal by the addition of a X–Y molecule to give products with an increased coordination number, due to the formation of two new bonds upon complete dissociation of the X–Y bond, and the corresponding evolution to an octahedral geometry.19–21

In this study, oxidative addition reactions were performed by the addition of I2 and CH3I molecules to platinum(II) compounds previously prepared. Different mechanisms have been proposed for these reagents,19,22 although leading in both cases to trans oxidative addition. Oxidative addition reactions of I2 and CH3I were carried out on the three previously prepared platinum(II) compounds resulting in the formation of six different platinum(IV) products (cm1I2, cm1MeI, cm2I2, cm2MeI, cm3I2, cm3MeI). These new compounds were characterized by 1H and 31P{1H} NMR spectroscopies (for cm3MeI), elemental analysis and mass spectrometry except for compounds cm2I2 and cm3I2 that have a low solubility in common solvents and were not studied further.

As shown in Table 1, the coupling constant 3J(H–Pt) value observed for the imine proton decreases when platinum(II) is oxidised to platinum(IV) compounds in agreement with previous studies for analogous compounds.23,24 Compounds arising from oxidative addition of methyl iodide display a methylplatinum resonance in the range 1.31–1.85 ppm for which the 2J(H–Pt) values (68–72 Hz) are in the range expected for platinum(IV) compounds.10,23 Moreover, addition of methyliodide in the axial positions of the platinum(II) precursors result in the loss of the symmetry plane and as a consequence non-equivalence of the methylamino protons (Hj) and the ortho protons of the para-tolyl (Ha) is observed for cm1MeI. Although we might expect that the non-equivalence of Ha protons depends upon the rate of rotation of the tolyl ligand around the Pt–C bond, the spectra taken in CDCl3 at 298 K or at 323 K did not show significant differences in the chemical shifts of these protons. For cm2MeI and cm3MeI, the higher complexity of the aromatic region did not allow unequivocal assignment of the ortho protons (Ha) of the para-tolyl ligand. However, non-equivalence of the methylene Hg protons could be observed for these compounds.

The changes observed in the signal corresponding to the ortho protons of the para-tolyl ligand (Ha) deserves some comment. This signal appears as a doublet at 7.39 ppm [3J (Pt–H) = 64.0 Hz] for compound cm1 and is considerably downfield shifted (δ = 8.43 ppm) for platinum(IV) compound cm1I2 as a result of the interaction of Ha with axial iodide ligands. For platinum(IV) compound cm1MeI, the Ha protons are non-equivalent and only one is downfield shifted to δ =8.64 ppm while the other appears

at 7.12 ppm. The para-tolyl ligand is expected to be nearly orthogonal to the plane containing the metallacycle in the solid state as observed for cm3, but it could undergo rotation in solution. At temperatures up to 323 K, the rate of rotation is slow so that a large separation in chemical shift of the diastereotopic Ha protons is observed.

The reaction of cm3 containing a PPh3 ligand with methyl iodide was followed by 31P{1H} NMR spectra. Initially, a signal at −12.40 ppm coupled to 195Pt (1J (P–Pt) = 983.2 Hz) is observed; both the chemical shift and the coupling constant which is consistently reduced from that of the platinum(II) precursor indicate formation of a platinum(IV) compound. After several hours a new signal appears at −9.47 ppm (1J (P–Pt) = 989.7 Hz) and after 48 hours at room temperature this new signal fully replaces the initial compound. These observations are fully consistent with previous observations for analogous compounds for which initial trans arrangement of the added methyl and iodido ligands is followed by isomerisation to place the bulky triphenylphosphine ligand in an axial position trans to the methyl ligand.25 This process (depicted in Scheme 4) reduces the steric effects arising from the PPh3 ligand while maintaining the stable fac-PtC3 configuration of the platinum(IV) compound.23,25

In order to complete the characterisation of the studied compounds, 195Pt NMR spectra were taken for the mostsoluble compounds cm1, cm1I2 and cm1MeI and the obtained values are shown in Table 1. As platinum increase its oxidation state and coordination number, the electronic density decreases, leading to a deshielding and higher frequency shifts. In particular, a higher deshielding is observed for cm1MeI versus cm1I2 in agreement with the presence of a more covalent Pt–Me bond.26,27

**Solution studies: stability and behaviour in the presence of ascorbic acid, glutathione and L-cysteine**

The stability of platinum(IV) compound cm1MeI in the aqueous biological media was evaluated recording the 1H NMR spectra of the compound (1 mM) in 50 mM phosphate buffer (in D2O, pD 7.40); 2 drops of deuterated DMSO were added to solubilise the compound in the media. The obtained spectra, shown in the ESI (Fig. S1†), were compared with those obtained at different storage periods. Compound cm1I2 was too insoluble as to carry out similar studies.

As previously reported for cyclometallated platinum(IV) compounds containing a fac-PtC3 geometry and a mer-[C,N,N′] arrangement of the tridentate ligand,9 compound cm1MeI display several singlet signals (δ = 8.58, 8.63 and 8.69 ppm) in the imine region. This result is consistent with the ‘quasilabile’ nature of platinum(IV) complexes containing three Pt–C bonds28 so that D2O or d6-DMSO can replace the more labile ligands (N-donor or iodido ligands) leading to a mixture of solvato complexes as shown in Scheme 5. Moreover, easy mer/fac isomerisation of the [C,N,N′] ligand has also been reported for this type of compounds.28,29 Interestingly, no doublet corresponding to Ha is observed at ca. 8.5 ppm, which might suggest that the iodido ligand dissociates from the platinum(IV). No further changes are observed in the 1H NMR after one week indicating that the solvato species remain stable.

Since it is generally accepted that platinum(IV) compounds are rapidly reduced under physiological conditions by biologically relevant reducing agents, the reactions of cm1MeI with ascorbic acid, glutathione and cysteine were also monitored by 1H NMR spectroscopy under analogous conditions and the obtained spectra are given in the ESI (Fig. S2–S4†). For the reaction with ascorbic acid, the imine region was most informative since in this region neither the solvents nor the ascorbic acid interfere with the products signals. In this case, only one signal is observed in the imine region at 8.61 ppm. The J(H–Pt) value obtained for this new species is 42.3 Hz which suggests that the platinacycle is not cleaved, and that the platinum(IV) is not reduced. The downfield shifted aromatic doublet is not observed, therefore we might deduce that the ascorbic acid can coordinate to the platinum replacing the iodido ligand as shown in Scheme 5. The newly formed compound is stable for 24 hours, however the spectrum taken after one week indicates that the intensity of the signal at 8.61 ppm decreases while a new resonance at 8.69 ppm [J = 45.2 Hz] appears. These values also correspond to a platinum(IV) metallacycle, therefore no reduction occurs and just minor changes in the coordination sphere of the platinum or the coordination mode of the ascorbic acid take place after one week (Fig. S2†).

Finally, for the reaction with glutathione (Fig. S3†) and L-cysteine (Fig. S4†) formation of novel species is clearly detected at the early stages of the process. For the reaction with glutathione, two resonances at 8.57 ppm [3J(H–Pt) = 43.0 Hz] and 8.55 ppm [3J(H–Pt) = 41.0 Hz] in the imine region and two resonances at 0.61 ppm [2J(H–Pt) = 64.0 Hz] and 0.58 ppm [2J(H–Pt) = 63.7 Hz] in the methyl region are observed. For the reaction with cysteine, two resonances at 8.60 ppm [3J(H–Pt) = 42.4 Hz] and 8.62 ppm [3J(H–Pt) = 40.9 Hz] in the imine region, and one resonance at 0.57 ppm [2J(H–Pt) = 70.0 Hz] in the methyl region are observed. In both cases, the observed values of the coupling constants are in the range expected for platinum(IV) compounds, thus suggesting coordination of the glutathione or the cysteine (see Scheme 5) rather than reduction of the platinum(IV). In particular, the values of 2J(H–Pt) for the axial methyl ligand are well within the range observed for methylplatinum(IV) with S-donor or N-donor ligands in trans.23 In agreement with the lower trans influence of O-donor ligands, slightly higher values of 2J(H–Pt) (75–77 Hz) have been reported for methylplatinum(IV) with O-donor ligands in trans.30,31 Although there is only a small difference in the 2J(H–Pt) values, we might tentatively suggest that coordination of cysteine and glutathione to platinum(IV) possibly takes place through either S or N donor atoms. While for cysteine, the new formed species are stable after one week, for glutathione the intensity of these signals decrease after 24 hours to produce rather complex spectra which suggest a fast decomposition of these species.

As a whole, in agreement with our previous studies concerning cyclometallated platinum(IV) compounds containing a fac-PtC3 geometry,9 these compounds are reluctant to be reduced while they display a high lability due to the presence of three Pt–C bonds.

The reduction of platinum(IV) complexes has been the subject of many studies, most of them involving compounds containing chlorido, hydroxido or carboxylato ligands in the axial positions, and several mechanisms have been proposed for the reductive elimination such as outer sphere, inner sphere and

platinum(II) catalysed reactions.32–35 The different axial ligands present in compound cm1MeI might define distinct reactivity patterns for this compound so that the presence of a methyl ligand favours substitution of the trans-iodido ligand as well as a higher stability of the oxidation state(IV) of the platinum.

## BIOLOGICAL STUDIES

### Antiproliferative assay

The antiproliferative activity of cyclometallated platinum(II) (cm1, cm2 and cm3) and cyclometallated platinum(IV) (cm1I2, cm1MeI, cm2MeI and cm3MeI) complexes along with cisplatin, as a positive control, was determined by the MTT assay. Compounds cm2I2 and cm3I2 were not considered due to their low solubility. The non-small A-549 lung, HCT-116 colon and MCF-7 and MDA-MB-231 breast adenocarcinoma cell lines were used in the study. The half-maximal inhibitory concentration (IC50) values of cisplatin and the investigated compounds evaluated after 72 h of drug exposure are depicted in Table 2 and Fig. 2.

Platinum(II) compounds cm1 and cm2 exhibited cytotoxicity in all the carcinoma cell lines selected in a similar range to that observed for previously reported compounds 1a–1c. Contrary to our expectations, compound cm3 containing a triphenylphosphine ligand did not exhibit cytotoxicity in these cell lines. This result suggests that the presence of a particular ligand per se may not imply cytotoxicity of the platinum compound. Interestingly, for palladium or platinum(II) derivatives giving good results the triphenylphosphine ligand is trans to a relatively labile N-donor and might increase its lability11,13 while in the present case the phosphine is trans to a non-labile C-donor.

The platinum(IV) compounds arising from oxidative addition of methyl iodide (cm1MeI) or iodine (cm1I2) to cm1 are the most potent. In particular, cm1MeI shows a special sensitivity for MDA-MB231 breast (IC50 = 1.56 μM) and HCT-116 colon (IC50 = 1.77 μM) cancer cells. The obtained IC50 values in all studied cell lines are in the same range than those previously reported by us for compounds 2b and 2c shown in Scheme 1.

Interestingly, compounds cm1MeI and cm1I2, as depicted in Fig. 3 and 4, showed a lower antiproliferative activity in normal human foreskin fibroblast cells (BJ) than that in the adenocarcinoma cell lines tested, indicating a desirable selectivity for tumour cells. For the most potent investigated compound cm1MeI this effect is seen at 5 and 10 μM concentration of compound, whereas for compound cm1I2 it is seen at 10, 25 and 50 μM concentration of compound.

These results confirm that [C,N,N′]-cyclometallated platinum(IV) compounds containing either a fac-PtC3 arrangement and one iodido ligand or a cis-PtC2 moiety and two iodide ligands are promising candidates as antitumor agents, while the nature of the C-donor ligand (methyl or aryl) is not relevant. In contrast, cyclometallated [C,N] platinum(IV) compounds cm2MeI and cm3MeI which also display a fac-PtC3 arrangement were considerably less potent than cyclometallated [C,N,N′] compound cm1MeI. As a whole, these results suggest that the presence of a particular ligand or the specific arrangement of the ligands may not produce the desired biological behaviour of a platinum compound, which is generally governed by an interplay of several factors.

## DNA interaction

The interaction of cyclometallated platinum(II) cm1, cm2 and cm3, and cyclometallated platinum(IV) complexes cm1MeI, cm1I2, cm2MeI and cm3MeI with DNA was studied by their ability to modify the electrophoretic mobility of the supercoiled closed circular (sc) and the open circular (oc) forms of pBluescript SK+ plasmid DNA. The sc form usually moves faster due to its compact structure. To provide a basis for comparison, incubation of DNA with cisplatin and ethidium bromide (EB) was also performed using the same conditions.

On the basis of the gel mobility shift assay (see Fig. 5) platinacycles cm1MeI and cm3MeI induce significant changes in the mobility of plasmid DNA, altering the DNA tertiary structure as the standard reference, cisplatin. Complex cm1MeI shows an interaction with plasmid DNA at concentrations greater than or equal to 100 μM, which are much higher than the concentrations for cisplatin. On the other hand, complex cm3MeI induces important changes at relatively low concentrations, as 25 μM. Platinacycles cm1, cm2, cm3, cm1I2 and cm2MeI were not efficient in removing the supercoils from DNA.

To evaluate the ability of the investigated platinum(II) and platinum(IV) complexes to intercalate into DNA, a topoisomerase-based gel assay was performed with the same complexes used in the previous assay. Supercoiled pBluescript plasmid DNA was incubated in the presence of topoisomerase I at 100 μM concentration of compounds under study.

The results are given in Fig. 6 and they show that none of the tested compounds prevent unwinding of DNA by the action of topoisomerase I, indicating that these compounds are neither intercalators nor topoisomerase I inhibitors, thus pointing out to another biological target.36

To study an alternative biomolecular target, a topoisomerase IIα-based gel assay was performed. This enzyme controls and alters the topologic states of DNA during transcription and catalyses the transient breaking and rejoining of two strands of duplex DNA, thus altering its topology. This enzyme is the target for several anticancer agents.37 Supercoiled pBluescript plasmid DNA was incubated at 37 °C in the presence of topoisomerase IIα at increasing concentrations of compounds under study. The gel mobility shift assay shows (see Fig. 7) that compounds cm1MeI, cm3MeI and cm1I2 were able to inhibit the action of topoisomerase IIα at low concentrations. Platinacycle cm2MeI was much less efficient in inhibiting the enzyme activity because its effective concentration is 100 μM. Platinum(II) complexes tested do not show any inhibition activity.

## Cathepsin B inhibition

Cathepsin B is a cysteine metalloprotease highly upregulated in a wide variety of cancers by mechanisms ranging from gene amplification to post-transcriptional modification. The exact role of cathepsin B in solid tumours has yet to be defined, but it has been proposed to participate in metastasis, angiogenesis, and tumour progression. Recently, compounds based on palladium, platinum, ruthenium, gold and tellurium were shown to be effective inhibitors of cathepsin B.9,11,38

In this study, compounds cm1, cm2, cm3, cm1I2, cm1MeI, cm2MeI and cm3MeI in a 50 μM and 100 μM concentrations were submitted to a cathepsin B inhibition assay. Results show that none of the studied compounds presents significant inhibitory activity against cathepsin B at both concentrations tested. The residual activity was in all cases greater than 50% (Table 3).

**Effect of compounds cm1MeI and cm1I2 on cell cycle distribution**

The cell cycle is a series of sequential and tightly regulated events that a cell must undergo before each division by mitosis. These events are classified into three distinct phases: first, G0/G1, in which a cell may be quiescent (G0) or preparing for its DNA replication (gap1 or G1); synthesis (S), in which the cell is duplicating its own genome; and G2/M (gap2 and mitosis), in which a cell actually generates an exact copy of itself. Given the importance of this whole process, it is strictly regulated by several checkpoints and signalling cascades that avoid uncontrolled cell proliferation or proliferation of damaged cells. Precisely, the dysfunction of these checkpoints is one of the key driving forces of oncogenic transformation39–41 and thus inhibiting cell proliferation through cell cycle arrest constitutes an attractive approach in cancer therapy research.

Compounds cm1MeI and cm1I2 were selected as representative of the set of platinum(IV) novel species, since they present higher efficacy in limiting cell proliferation of different cancer cell lines, especially in the highly-proliferative HCT-116 colon cancer model. Changes in cell cycle distribution of HCT-116 were evaluated after a 72 h incubation with half maximal inhibitory concentrations (IC50) of either cm1MeI or cm1I2, analysed by Fluorescence Activated Cell Sorting (FACS), staining DNA content with propidium iodide (PI). Our results (Fig. 8) show that both compounds cause a decrease in the percentage of cells in G0/G1 phase, while inducing an S and G2/M cell cycle arrest, indicating that both compounds inhibit cell proliferation by hindering cell cycle completion.42

**Effect of compounds cm1MeI and cm1I2 on apoptosis**

When cell cycle checkpoints are fully functional, damaged cells cannot progress through cell cycle phases. Instead, cell signalling cascades redirect those damaged cells into programmed cell death or apoptosis. However, as with cell cycle checkpoints, cancer cells are also able to counteract apoptotic stimuli by activating oncogenes that promote cell survival and proliferation.43 Thus, counteracting this anti-apoptotic oncogenic activation and selectively inducing apoptosis in cancer cells is also an appealing therapeutic window. For this reason, pro-apoptotic effect of compounds cm1MeI and cm1I2 on HCT-116 was also investigated. Cells were incubated for 72 h with either cm1MeI or cm1I2 at half maximal inhibitory concentration (IC50) and the relative populations of alive, preapoptotic and apoptotic/necrotic cells were measured by FACS, by simultaneously labelling the cells with fluorescein-annexin V (AV-FITC, annexin V-fluorescein isothiocyanate) and propidium iodide.

Annexin V-FITC is a fluorescent probe used to detect early apoptotic cells since it binds to phosphatidylserine (PS) residues on the outer membrane of the cell, process that only occurs as one of

the initial steps of the apoptotic program.44,45 On the other hand, propidium iodide is a fluorescent probe that binds to DNA and is able to stain all cells. However, the signal of the late apoptotic/necrotic cell population is much more intense than the one of alive or early apoptotic cells, since cell membrane integrity is lost at late stages of both cell death programs and larger amounts of PI can permeate the cell membrane.45 Flow cytometry analysis of cells stained with both probes allows us to relatively quantify three cell populations: alive cells (low PI/low annexin-V), early apoptotic cells (low PI/high annexin-V) and late apoptotic/necrotic cells (high PI).

Both cm1MeI and cm1I2 significantly trigger apoptosis in HCT-116 cells, as shown in Fig. 9. A 72 h incubation with cm1MeI at the IC50 dose caused a 50% decrease in the percentage of healthy cells, whereas early apoptotic and apoptotic/ necrotic cells presented 32% and 18% increases respectively. Compound cm1I2 incubation at the IC50 dose for 72 h resulted in a 15% decrease in healthy cell population and a consequent similar increase in late apoptotic or necrotic cells and a nonsignificant increase in early apoptotic cells at 72 h. These results highlight the potential suitability of both cm1MeI and cm1I2 as chemotherapeutic agents.

**Generation of reactive oxygen species (ROS)**

Reactive oxygen species (ROS) are oxidant by-products of cell metabolism, including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical (•OH) and singlet oxygen ($^1O_2$). In normal physiological conditions, ROS levels are low and they contribute to cell survival, proliferation, homeostasis and cell signalling.46–48 On the contrary, high ROS levels are linked to stress and pathological conditions and produce damage to DNA, proteins, and lipids, thus activating cell damage-responsive barriers that can lead to cell senescence or apoptosis triggered by cytochrome c release from the mitochondria.49,50 In particular, cancer cells are able to maintain higher ROS levels while evading these apoptotic programs. This feature permits sustained DNA damage and genomic instability and allows the constant evolution of tumour cell populations.51 Cisplatin mechanism of action in cancer cells involves further ROS production to an extent which cancer cells can no longer evade apoptosis.52,53

As part of the validation of the cytotoxic effect of compounds cm1MeI and cm1I2 on cancer cells, ROS generation on HCT-116 cell line was evaluated. HCT-116 cells were incubated with cm1MeI and cm1I2 at their half maximal inhibitory concentrations (IC50) for 24, 48 and 72 h and then analysed in a flow cytometer after exposure to DFCH-DA (2′,7′-dichlorofluorescein diacetate), a fluorescent probe that measures hydroxyl, peroxyl, and other ROS activities. Our findings suggest that significant increased ROS generation occurs for both compounds only after 72 h of incubation, as shown in Fig. 10. These results agree with previous studies that reported enhanced ROS generation in cancer cells as a response to platinum(IV) complexes.54,55

## CONCLUSIONS

New cyclometallated platinum(IV) compounds were obtained from intermolecular oxidative addition of either methyl iodide or iodine to platinum(II) precursors containing a para-tolyl ligand and a terdentate [C,N,N′] (cm1) or a bidentate [C,N] and an additional ligand such as SEt2 (cm2) or PPh3 (cm3). The compounds were characterized by mass-spectrometry, elemental analyses and NMR spectroscopy except for cm2I2 and cm3I2 that were too insoluble in common solvents and were not studied further. The molecular structure of platinum(II) compound cm3 was solved by X ray analyses.

The cytotoxic activity against a panel of human adenocarcinoma cell lines (A-549 lung, MDA-MB-231 and MC-7 breast, and HCT-116 colon) was determined for the new platinum(IV) compounds and the platinum(II) precursors. Most compounds exhibited a remarkable cytotoxicity in all the selected cancer cell lines, in particular compounds cm1I2 and cm1MeI containing a terdentate [C,N,N′] ligand are the most potent. The studies on electrophoretic mobility of DNA indicated that platinum(II) compounds cm1, cm2 and cm3 and platinum(IV) compounds cm1I2 and cm2MeI were not effective in removing the plasmid DNA supercoils. In contrast, platinacycles cm1MeI and cm3MeI induce significant changes in the mobility of DNA. Topoisomerase-based gel assays indicated that none of the studied compounds are intercalators or topoisomerase I inhibitors, but platinum(IV) compounds cm1MeI, cm3MeI and cm1I2, and to a lesser extent cm2MeI showed considerable topoisomerase IIα inhibitory activity. In contrast, none of the tested compounds inhibits cathepsin B.

Both cm1MeI and cm1I2 were found to supress HCT-116 colon cancer cell growth by a mixture of cell cycle arrest and apoptosis induction and to increase ROS levels. 1H NMR studies carried out in a buffered aqueous medium for platinum(IV) compound cm1MeI in the presence of biologically relevant reducing agents such as ascorbic acid, glutathione or cysteine indicated coordination of these molecules to platinum(IV) without reduction to a platinum(II) species. The capacity of cm1MeI to coordinate such molecules may explain its ability to induce ROS by capturing ROS-scavenging agents, preventing the cell of successful detoxification of oxidative damage, which may contribute to its cytotoxicity.

The multitarget nature and the low solubility of some of the investigated compounds may account for the difficulties encountered to establish reliable structure–activity relationships. The [C,N,N′]-cyclometallated platinum(IV) compound cm1MeI containing a fac-PtC3 arrangement and one iodide ligand can be considered a promising candidate as antitumor agent. This compound, more potent than the parent platinum(II) compound cm1, trigger antiproliferative activity by interacting with DNA (in a similar way than cisplatin but in a lesser extend) and inhibiting topoisomerase-IIα. In contrast, cyclometallated [C,N] platinum(IV) compounds cm2MeI and cm3MeI which also display a fac-PtC3 arrangement were considerably less potent than cm1MeI. Moreover, in spite of their similar structure with the potent antiproliferative [C,N,N′]-cyclometallated platinum(IV) compound cm1I2, the low solubility of cyclometallated [C,N] platinum(IV) compounds cm2I2 and cm3I2 prevent their study as antitumor agents. In addition, the obtained results also indicate that the presence of a triphenylphosphine

454 instead of a diethylsulfide ligand leads to decreased activity for both platinum(II) and platinum(IV)
455 compounds containing a [C,N] cyclometallated ligand.
456 As a whole, the studies here presented indicate that the new cyclometallated platinum(IV) compounds
457 cm1I2 and cm1MeI containing a terdentate [C,N,N′] ligand display a high potential to be used in cancer
458 chemotherapy in spite of their low proclivity to be reduced.
459
460

## EXPERIMENTAL SECTION

Chemistry

Microanalyses were performed at the Centres Científics I Tecnològics (Universitat de Barcelona) using a Carlo Erba model EA1108 elemental analyser. Electrospray mass spectra were performed at the Unitat d'Espectrometria de Masses (Universitat de Barcelona) in a LC/MSD-TOF spectrometer using H2O–CH3CN 1 : 1 to introduce the sample. NMR spectra were performed at the Unitat de RMN d'Alt Camp de la Universitat de Barcelona using a Mercury-400 (1H, 400 MHz) or a Bruker 400 Avance III (31P{1H} NMR, 161.98 MHz; 195Pt, 85.68 MHz) and referenced to SiMe4 (1H), to H3PO4 (31P) or to H2PtCl6 in D2O (195Pt). δ values are given in ppm and J values in Hz. Abbreviations used: s = singlet; d = doublet; t = triplet; m = multiplet.

Preparation of the complexes. All reagents were obtained from commercial sources and used as received. Ligands 4-ClC6H4CHN(CH2)3N(CH3)2 (L1) and 4-ClC6H4CHNCH2Ph (L2)16 and compound [Pt(4-CH3C6H4)2{μ-S(CH2CH3)2}]2 (A)56 were prepared as reported elsewhere.

### Cyclometallated platinum(II) compounds: synthesis and characterization

[Pt(4-CH3C6H4){(CH3)2N(CH2)3NCH(4-ClC6H3)}] (cm1). 0.200 g (0.21 mmol) of compound A and 0.096 g (0.43 mmol) of 4-ClC6H4CHNCH2CH2N(CH3)2 (L1) were dissolved in 25 mL of toluene and stirred at 90 °C for 6 h. The mixture was evaporated to dryness obtaining an orange oil. Addition of diethylether induced precipitation and the orange powder was filtered and dried. Yield: 0.131 g (60%). 1H NMR (400 MHz, CDCl3): δ 8.48 [s, 3J(H–Pt) = 56.0; 1H, Hf ], 7.39 [d, 3J(H–H) = 8.0; 3J(H–Pt) = 64.0; 2H, Ha], 7.14 [d, 3J(H–H) = 8.0; 1H, He], 6.92 [d, 3J(H–H) = 8.0; 2H, Hb], 6.88 [dd, 3J(H–H) = 8.0; 4J(H–H) = 2.0; 1H, Hd], 6.64 [d, 4J(H–H) = 1.6; 3J(H–Pt) = 66.8; 1H, Hc], 3.83 [td, 3J(H–H) = 5.2; 4J(H–H) = 1.6; 2H, Hg], 2.89 [m, 2H, Hh], 2.58 [s, 3J(H–Pt) = 23.2; 6H, Hj], 2.30 [s, 3H, Hk], 2.06 [m, 2H, Hi]. 195Pt NMR (85.68 MHz, CDCl3): δ −3699.1 (s). Anal.: calc. C19H23ClN2Pt (%): C, 44.75; H, 4.55; N, 5.49. Found (%): C, 44.18; H, 5.17; N, 5.24. MS-ESI(+): m/z: 509.12 [M]+, 419.06 [M-tolyl]+.

[Pt(4-CH3C6H4){(C6H5CH2)NCH(4-ClC6H3)}(S(CH2CH3)2)] (cm2). A 0.201 g (0.21 mmol) amount of compound A and 0.102 g (0.44 mmol) of 4-ClC6H4CHNCH2C6H4 (L2) were combined in 25 mL of toluene and stirred at room temperature for 24 h. The solvent was evaporated obtaining an orange oil. This residue was treated with hexane to yield a yellow solid that was filtered. Yield: 0.103 g (40%). 1H NMR (400 MHz, CDCl3): δ 8.44 [s, 3J(H–Pt) = 56.0; 1H, Hf ], 7.39–7.29 [m, 8H, Haromatic], 7.00 [d, 3J(H–H) = 8.0; 1H, Hd], 6.87 [d, 3J(H–H) = 8.0; 2H, Hb], 6.81 [d, 4J(H–H) = 2.0; 3J(H–Pt) = 72.0; 1H, Hc], 5.16 [s, 2H, Hg], 2.28 [q, 3J(H–H) = 7.6; 4H, Hl], 2.27 [s, 3H, Hk], 1.04 [t, J(H–H) = 7.6; 6H, Hm]. Anal.: calc. C25H28ClNPtS·H2O (%): C, 48.19; H, 4.85; N, 2.25; S, 5.15. Found (%): C, 47.42; H, 4.76; N, 2.48; S, 3.99. MS-ESI(+): m/z: 514.07 [M-tolyl + H]+, 531.10 [M-tolyl + H2O]+.

[Pt(4-CH3C6H4){(C6H5CH2)NCH(4-ClC6H3)}P(C6H5)3] (cm3). This compound was obtained mixing 0.050 g (0.08 mmol) of compound cm2 with 0.021 g (0.08 mmol) of PPh3 in 10 mL of acetone. The mixture was stirred at room temperature for 2 h and evaporated to dryness, obtaining a yellow oil. The residue was treated with diethyl ether and filtered to afford a crystalline yellow solid. Yield: 0.022 g (34%). 1H NMR (400 MHz, CDCl3): δ 8.10 [s, 3J(H–Pt) = 52.0; 1H, Hf ], 7.53–7.49 [m, 6H, Haromatic], 7.35–7.32 [m, 3H, Haromatic], 7.25–7.22 [m, 10H, Haromatic], {7.18 [dd, 3J(H–H) = 8.0; 4J(H–H) = 2.0; 1H]; 6.96 [dd, 3J(H–H) = 8.0; 4J(H–H) = 2.0; 1H] Hd,e}, 6.91 [d, 3J(H–H) = 8.0; 2H, Ha], 6.80–6.78 [m, 2H, Haromatic], 6.44 [d, 3J(H–H) = 8.0; 2H, Hb], 4.09 [s, 2H, Hg], 2.10 [s, 3H, Hk]. 31P{1H} NMR (161.98, CDCl3): δ 26.90 [1J (P–Pt) = 2201.31]. Anal.: calc. C39H33ClNPPt·H2O (%): C, 58.90; H, 4.43; N, 1.76. Found (%): C, 58.74; H, 4.73; N, 1.78. MS-ESI(+): m/z: 778.18 [M + H]+, 795.20 [M + H2O]+, 819.20 [M + CH3CN + H]+.

**Cyclometallated platinum(IV) compounds: synthesis and characterization**

[PtI2(4-CH3C6H4){(CH3)2N(CH2)3NCH(4-ClC6H3)}] (cm1I2). This compound was obtained from 0.051 g (0.10 mmol) of compound cm1 and 0.025 g (0.10 mmol) of I2 in 10 mL of acetone. The mixture was stirred at room temperature for 2 h and filtered giving an intense orange solid. Yield: 0.054 g (71%). 1H NMR (400 MHz, CDCl3): δ 8.43 [d, 3J(H–H) = 8.4; 3J(H–Pt) = 37.2; 2H, Ha], 8.13 [s, 3J(H–Pt) = 42.4; 1H, Hf ], 7.37 [d, 3J(H–H) = 8.4; 1H, He], 7.04 [d, 4J(H–H) = 1.6; 3J(H–Pt) = 36.8; 1H, Hc], 6.88 [dd, 3J(H–H) = 7.2; 4J(H–H) = 1.6; 1H, Hd], 6.85 [d, 3J(H–H) = 8.0; 2H, Hb], 4.14 [m, 2H, Hg], 3.14 [m, 2H, Hh], 3.04 [s, 3J(H–Pt) = 16.8; 6H, Hj], 2.35 [s, 3H, Hk], 2.27 [m, 2H, Hi]. 195Pt NMR (85.68 MHz, CDCl3): δ −3068.9 (s). Anal.: calc. C19H23ClI2N2Pt (%): C, 29.88; H, 3.04; N, 3.67. Found (%): C, 29.18; H, 2.87; N, 3.53. MS-ESI(+): m/z: 637.03 [M − I]+, 781.97 [M + NH4]+, 763.94 [M + H]+.

[PtCH3I(4-CH3C6H4){(CH3)2N(CH2)3NCH(4-ClC6H3)}] (cm1MeI). A 0.050 g (0.10 mmol) portion of cm1 and 1 mL of CH3I were combined in 10 mL of acetone, and the mixture was stirred at room temperature for 24 h. The solvent was eliminated and the residue was treated with diethyl ether and filtered to afford a yellow solid. Yield: 0.045 g (70%). 1H NMR (400 MHz, CDCl3): δ 8.64 [d, 3J(H–H) = 8.0; 3J(H–Pt) = 42.4; 1H, Ha], 8.42 [s, 3J(H–Pt) = 48.0; 1H, Hf ], 7.32 [d, 3J(H–H) = 8.0; 4J(H–Pt) = 8.0; 1H, He], 7.12 [d, 3J(H–H) = 8.0; 3J(H–Pt) = 38.0; 1H, Ha′], 6.99 [dd, 3J(H–H) = 8.0; 4J(H–H) = 2.0; 1H, Hd], 6.92 [d, 3J(H–H) = 8.0; 2H, Hb], 6.90 [d, 4J(H–H) = 2.0; 3J(H–Pt) = 48.4; 1H; Hc], {4.43 [t, 3J(H–H) = 14.0; 1H]; 4.00 [dt, 2J(H–H) = 12.0; 3J(H–H) = 4.0; 1H]; 3.82 [t, 3J(H–H) = 12.0; 1H]; 2.65 [dd, 2J(H–H) = 13.6; 3J(H–H) = 7.2; 1H]; 2.06 [m, 2H] Hg,h,i}, 2.80 [s, 3J(H–Pt) = 13.6; 3H, Hj], 2.47 [s, 3J(H–Pt) = 16.8; 3H, Hj′], 2.33 [s, 3H, Hk], 1.31 [s, 2J(H–Pt) = 68.0; 3H, Me-Pt]. 195Pt NMR (85.68 MHz, CDCl3): δ −2310.9 (s). Anal.: calc. C20H26ClIN2Pt (%): C, 36.85; H, 4.02; N, 4.30. Found (%): C, 36.54; H, 4.02; N, 4.07. MS-ESI(+): m/z: 433.08 [M− I-tolyl]+, 509.11 [M− Me − I]+, 651.04 (calc. 651.05) [M]+.

PtI2(4-CH3C6H4){(C6H5CH2)NCH(4-lC6H3)}(S(CH2CH3)2)] (cm2I2). The compound was obtained from 0.051 g (0.08 mmol) of cm2 and 0.026 g (0.10 mmol) of I2 in 10 mL of acetone. The mixture was

stirred at room temperature for 2 h and filtered, obtaining an intense orange solid. Yield: 0.052 g (72%). MS-ESI(+): m/z: 786.92 [M − SEt2 + H2O + H]+, 658.00 [M − SEt2 − I + H2O]+, 875.97 [M + NH4]+, 514.08 [M − SEt2 − 2I]+, 1555.80 [2M − 2SEt2 + NH4]+.

[PtCH3I(4-CH3C6H4){(C6H5CH2)NCH(4-ClC6H3)}(S(CH2CH3)2) (cm2MeI). A 0.052 g (0.09 mmol) portion of cm2 and 1 mL of CH3I were combined in 10 mL of acetone, and the mixture was stirred at room temperature for 24 h. The solvent was removed and the residue obtained was treated with hexane and filtered to afford a yellow solid. Yield: 0.046 g (72%).1H NMR (400 MHz, CDCl3): δ 7.85 [s,3J(H–Pt) = 45.6; 1H, Hf ], 7.47–7.36 [m, 6H, Haromatic], 7.13–7.08 [m, 2H, Haromatic], 7.04–7.01 [m, 1H], 6.94 [dd, 3J(H–H) = 8.0, 1.8, 1H, Haromatic], 6.91 [d, 3J(H–H) = 7.0, 2H], 5.78 [d, 3J(H–H) = 1.6; 1H, Hg], 5.76 [d, 3J(H–H) = 1,6; 1H, Hg′], 3.50 [m, 4H, Hl], 2.30 [s, 3H, Hk], 1.77 [s, 3J(H–Pt) = 72.0, Me-Pt], 1.45 [t, 3J(H–H) = 7.6; 6H, Hm]. Anal.: calc. C26H31ClINPtS (%): C, 41.80; H, 4.18; N, 1.88; S, 4.29. Found (%): C, 41.56; H, 4.18; N, 1.77; S, 4.07. MS-ESI(+): m/z: 528.09 [M-tolyl − I]+, 604.12 [M − Me − I]+.

[PtI2(4-CH3C6H4){(C6H5CH2)NCH(4-ClC6H3)}P(C6H5)3] (cm3I2). This compound was obtained from 0.040 g (0.05 mmol) of cm3 and 0.017 g (0.07 mmol) of I2 in 10 mL of acetone. The mixture was stirred for 2 h at room temperature, and the solvent was evaporated to dryness to obtain a brown oil. The residue was treated with diethyl ether and filtered, giving an orange solid. Yield: 0.030 g (57%). MS-ESI(+): m/z: 1049.00 [M + NH4]+, 904.07 [M − I]+, 776.16 [M − 2I]+.

[PtCH3I(4-CH3C6H4){(C6H5CH2)NCH(4-ClC6H3)}P(C6H5)3] (cm3MeI). A 0.071 g (0.09 mmol) amount of cm3 and 1 mL of CH3I were combined in 10 mL of acetone. The mixture was stirred at room temperature for 24 h. The solution was filtered to obtain a white solid. Yield: 0.028 g (33%). 1H NMR (400 MHz, CDCl3): δ 7.76 [d, 4J(H–H) = 1.2; 3J(H–Pt) = 49.2; 1H, Hf ], 7.47–7.38 [m, 8H, Haromatic], 7.32–7.28 [m, 9H, Haromatic], 7.12–6.95 [m, 5H, Haromatic], 6.80 [dd, 3J(H–H) = 8.0; 4J(H–H) = 2.8; Haromatic], 6.70 [dd, J(H–H) = 8.0; J(H–H) = 2.8, 2H, Haromatic], 6.45 [s, 3J(H–Pt) = 46.4; 1H, Hc], {5.39 [dd, 4J(H–H) = 18.0; 4J(H–H) = 2.4; 1H], 4.57 [dd, 4J(H–H) = 18.0; 4J(H–H) = 1.6; 1H], Hg}, 2.18 [s, 3H, Hk], 1.85 [d, 3J(H–P) = 8.0; 2J(H–Pt) = 72.0; 3H, Me-Pt]. 31P{1H} (161.98 MHz, CDCl3): δ −9.47 [1J (P–Pt) = 989.7]. Anal.: calc. C40H36ClINPPt (%): C, 52.27; H, 3.95; N, 1.52. Found (%): C, 51.94; H, 4.22; N, 1.61. MS-ESI(+): m/z: 792.20 [M − I]+.

**Stability and behaviour in presence of ascorbic acid, glutathione (GSH) and L-cysteine by NMR measurements**

The stability of the platinum(IV) compounds under investigation in aqueous solution was monitored by 1H NMR spectroscopy at ambient temperature. Samples were analysed in the Nuclear Magnetic Resonance Unit, Scientific and Technological Centres of the University of Barcelona (CCiTUB). Solutions of the complexes were prepared in 50 mM phosphate buffer (in D2O, pD 7,40) and minimum amount (2 drops) of d6-DMSO for solubilisation of the compound. Final concentration of the complex was 1 mM and 1H NMR spectra were recorded with a Varian 400 and a Bruker 400 spectrometer at time

periods between 0 h–1 week. For monitoring the reactivity of the studied compounds with ascorbic acid, GSH or L-cysteine, the samples were prepared in the same conditions described above with a final concentration of complex and ascorbic acid, GSH or L-cysteine of 1 mM and 25 mM, respectively. 1H NMR spectra were recorded over the same time period as above.

**Crystal data and structure refinement for cm3**

A yellow prism-like specimen of cm3, grown in dichloromethane–methanol at room temperature, was used for the X-ray crystallographic analysis. X-ray intensity data were collected on a D8 Venture system equipped with a multilayer monochromator and a Mo microfocus ($\lambda$ = 0.71073 Å) at 100 K. The structure was solved and refined at the Unitat de Difracció de RX (CCiTUB) using the Bruker SHELXT software package.57 Further information is given in Table 4.

**Biological studies**

Cell culture and cell viability assay. Human lung adenocarcinoma A-549 cells and human breast adenocarcinoma MDA-MB-231 cells were grown as a monolayer culture in minimum essential medium (DMEM (Dulbecco's Modified Eagle Medium) with L-glutamine, without glucose and without sodium pyruvate) with addition of 10% heat-inactivated Fetal Calf Serum (FCS), 10 mM D-glucose and 0.1% streptomycin/ penicillin, in standard culture conditions (humidified air with 5% CO2 at 37 °C). Human breast adenocarcinoma MCF-7 cells were cultured in MEM without phenol red, containing 10% Fetal Bovine Serum (FBS), 10 mM D-glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% streptomycin/penicillin, 0.01 mg ml−1 insulin, and 1% non-essential amino acids. Human colorectal carcinoma HCT116 cells were cultured in DMEM/HAM F12 (1 : 1 volume) mixture containing 10% FBS, 4 mML-glutamine, 12.5 mM D-glucose and 0.1% streptomycin/ penicillin.

For all viability assays, compounds were suspended in high purity DMSO at 20 mM as stock solution. To obtain final assay concentrations, they were diluted in DMEM (final concentration of DMSO was the same for all conditions, and was always lower than 1%). The assay was performed by a variation of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay described by Mosmann et al.58 and Matito and coworkers59 which is based on the ability of live cells to cleave the tetrazolium ring of the MTT thus producing formazan, which absorbs at 550 nm. In brief, the corresponding number of cells per well (2.5 × 103 A-549 cells per well, 5 × 103 MDA-MB-231 cells per well, 1 × 104 MCF-7 cells per well and 2 × 103 HCT-116 cells per well) were cultured in 96 well plates for 24 hours prior to the addition of different compounds at different concentrations, in triplicate. After incubation of the cells with the compounds for 72 h more, the media was aspirated and 100 μL of filtered MTT (0.5 mg mL−1) were added to each well. Following 1 h of incubation with the MTT, the supernatant was removed and the precipitated formazan was dissolved in 100 μL DMSO. Relative cell viability, compared to the viability of untreated cells, was measured by absorbance at 550 nm on an ELISA plate reader (Tecan

Sunrise MR20-301, TECAN, Salzburg, Austria). Concentrations that inhibited cell growth by 50% (IC50) after 72 h of treatment were subsequently calculated.

DNA migration studies. A stock solution (10 mM) of compounds cm1, cm2, cm3, cm1MeI, cm1I2, cm2MeI and cm3MeI was prepared in high purity DMSO. Then, serial dilutions were made in MilliQ water (1 : 1). Plasmid pBluescript SK + (Stratagene) was obtained using a QIAGEN plasmid midi kit as described by the manufacturer. Interaction of drugs with pBluescript SK + plasmid DNA was analysed by agarose gel electrophoresis. Plasmid DNA aliquots (40 μg mL−1) were incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) with different concentrations of compounds mentioned above ranging from 0 μM to 200 μM at 37 °C for 24 h. Cisplatin and ethidium bromide (EB) were used as a reference controls. Aliquots of 20 μL of the incubated solutions containing 0.3 μg of DNA were subjected to 1% agarose gel electrophoresis in TAE buffer (40 mM tris-acetate, 2 mM EDTA, pH 8.0). The gel was stained in TAE buffer containing ethidium bromide (0.5 mg mL−1) and visualized and photographed under UV light.

Topoisomerase I-based experiments were performed as described previously.60 Supercoiled pBluescript DNA, obtained as described above, was treated with topoisomerase I in the absence or presence of compounds mentioned previously. Assay mixtures contained supercoiled pBluescript DNA (0.3 μg), calf thymus topoisomerase I (3 units) and complexes cm1, cm2, cm3, cm1I2, cm1MeI, cm2MeI and cm3MeI (100 μM) in 20 μL of relaxation buffer Tris-HCl buffer (pH 7.5) containing 175 mM KCl, 5 mM MgCl2 and 0.1 mM EDTA. Reactions were incubated for 30 min at 37 °C and stopped by the addition of 2 μL of agarose gel loading buffer. Samples were then subjected to electrophoresis and DNA bands stained with ethidium bromide as described above.

Topoisomerase IIα activity was determined by incubating 0.3 μg of supercoiled pBluescript DNA with topoisomerase Iiα (3 units) in the absence or presence of increasing concentrations (5–100 μM) of compounds cm1I2, cm2MeI, cm1MeI and cm3MeI, and 200 μM of compounds cm1, cm2 and cm3 in 20 μL of topoisomerase II buffer for 40 min at 37 °C. The reaction was stopped by the addition of 2 μL of agarose gel loading buffer. Samples were then subjected to electrophoresis and DNA brands stained with ethidium bromide as described above.

Cathepsin B inhibition assay. The fluorimetric cathepsin B assay was performed following manufacturer's instructions (Sigma-Aldrich). Briefly, the reaction mixture contained 48 mM sodium phosphate (pH 6.0), 4.0 mM EDTA, 352 mM potassium phosphate buffer, 2.5 mM L-cysteine HCl solution 0.03% Brij 35 solution and 0.02 mM Nα-carbobenzoxy-Arg-Arg-7- amido-4-methylcoumarin as substrate. To test the inhibitory effect of the platinum compounds on cathepsin B, activity measurements were performed in duplicate using fixed concentrations of enzyme (0.5 units) and substrate (20 μM). The platinum compounds were used at two concentrations (50 μM and 100 μM). Before the addition of substrate, cathepsin B was incubated with the different compounds at 37 °C for 1 h. The cysteine proteinase inhibitor E-64 was used as a positive control of cathepsin B inhibition.

Complete inhibition was achieved at 10 μM concentration of E-64. Activity was measured over 5 min on a fluorescence spectrophotometer (excitation = 348 nm, emission = 440 nm).

Cell cycle analysis. Cell cycle was assessed by flow cytometry using a fluorescence activated cell sorter (FACS). For this assay, 2.5 × 104 HCT-116 cells were seeded in 6 well plates with 2 mL of growth medium. After 24 h of incubation, compounds cm1MeI or cm1I2 were added at their IC50 values 1.78 and 5.14 μM, respectively. Following 72 h of incubation, cells were harvested by mild tripsinization, collected by centrifugation and fixed in 70% ethanol and stored at −20 °C until measure. Right before measuring, fixed cells were incubated with phosphate buffer solution (PBS) containing 50 mg mL−1 PI and 10 mg mL−1 DNase-free RNase. The cell suspension was incubated for 1 h at room temperature to allow for the staining of the cells with the PI, and afterwards FACS analysis was carried out at 488 nm by employing a CyAn flow cytometer (Beckman Coulter). Data from 1 × 104 cells were collected and analysed using the FlowJo software.

**Apoptosis assay**

Apoptosis was assessed evaluating the annexin-V binding to phosphatidylserine (PS), which is externalized early in the apoptotic process. 2.5 × 104 HCT-116 cells per well were seeded in 6 well plates with 2 mL of medium and treated as described for the cell cycle analysis assay. After cell collection and centrifugation, cells were resuspended in 95 μL binding buffer (10 mM HEPES/NaOH, pH 7.40, 140 mM NaCl, 2.5 mM CaCl2). 3 μL of Annexin-V FITC conjugate (1 mg mL−1) were then added and the suspension was incubated in darkness for 30 min, at room temperature. The cell suspension was added to a vial containing 500 μL of binding buffer, stained with 20 μL of 1 mg mL−1 PI solution and analysed. Data from 1 × 104 cells were collected and analysed using the FlowJo software.

Determination of intracellular reactive oxygen species (ROS) levels. 2.5 × 104 HCT-116 cells per well were seeded in 6 well plates with 2 mL of growth medium and treated as described for the cell cycle analysis assay. Cells were collected and intracellular ROS was measured at 24, 48 and 72 h. First, cells were washed once with warm PBS, and incubated with 5 μM 2′,7′- dichlorofluorescein diacetate (DCFH-DA, Invitrogen) in PBS supplemented with 10 mM glucose and 2 mM glutamine for 30 min at 37 °C. Then, DCFH-DA solution in PBS was replaced with complete culture medium and the cells were incubated for another 30 min at 37 °C. Finally, cells were trypsinised and resuspended thoroughly in 0.4 mL of PBS containing DCFH-DA (50 μM) and PI (20 μg mL−1).61 Intracellular internalized probe reacts with ROS and emits fluorescence when excited at 492 nm. Emitted fluorescence was recorded by flow cytometry at 520 nm using a CyAn flow cytometer (Beckman Coulter). Data of DCF fluorescence concentrations from 1 × 104 PI negative cells were collected and analysed using FlowJo software.

**Data analysis**

For each compound, a minimum of three independent experiments with triplicate values were conducted to measure cell viability. A minimum of two independent experiments in triplicates were performed for cell cycle analysis, assessment of apoptosis and ROS. Significant differences compared to control were assessed by Student's t-test where $p < 0.05$(*), $p < 0.01$(**) or $p < 0.001$(***) were taken into consideration. Data are given as the mean ± standard deviation (SD).

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**Legends to figures**

**Scheme 1** Previously studied cyclometallated platinum(IV) compounds

**Scheme 2** New cyclometallated platinum(II) compounds used as precursors in this work.

**Scheme 3** Synthesis of the cyclometallated platinum(II) and platinum(IV) compounds. (i) Toluene, 90 °C, 6 h; (ii) toluene, RT, 24 h; (iii) +PPh3, acetone, 2 h; (iv) +CH3I, acetone, RT, 24 h; (v) +I2, acetone, RT, 2 h (the numbering scheme used in the Experimental section and in Table 1 is shown).

**Scheme 1.** Synthesis of Platinum(II) Compounds[a]

**Figure. 1**. Molecular structure of compound cm3 (molecule a). Selected bond lengths (Å) and angles (°) with estimated standard deviations: Pt1a–C1a, 1.998(5); Pt1a–C8a, 2.068(4); Pt1a–N1a, 2.154(4); Pt1a–P1a, 2.2996(11); N1a–C14a, 1.295(5); N1a–C15a, 1.475(6); C8a–C13a, 1.434(7); C13a–C14a, 1.427(6); C1a–Pt1a–C8a, 89.33(19); C8a–Pt1a–N1a, 79.91(17); C1a–Pt1a–P1a, 89.38(12); N1a–Pt1a–P1a, 101.50(10). Hydrogens are omitted for clarity.

**Scheme 4** Oxidative addition of methyl iodide followed by isomerisation.

**Scheme 5** Proposed species formed in solution (the charges of the ionic species are omitted).

**Figure. 2**. Antiproliferative activity of cyclometallated platinum(II) cm1 and cm2 and cyclometallated platinum(IV) compounds cm1I2, cm1MeI and cm2MeI, and cisplatin (IC50 μM) against A-549 lung, MDA-MB-231 and MCF-7 breast, and HCT-116 colon human cancer cell lines. Compounds cm3 and cm3MeI with high IC50 values or even IC50 values >100 in several cancer cell lines are not shown.

**Figure. 3**. Antiproliferative activity of cyclometallated platinum(IV) compound cm1I2 (IC50 μM) against BJ fibroblast human normal cells line and A-549 lung, MDA-MB-231 and MCF-7 breast, and HCT-116 colon human cancer cells lines.

**Figure. 4** Antiproliferative activity of cyclometallated platinum(IV) compound cm1MeI (IC50 μM) against BJ normal cells and A-549 lung, MDA-MB-231 and MCF-7 breast, and HCT-116 colon human cancer cell lines.

**Figure. 5**. Interaction of pBluescript SK+ plasmid DNA (0.3 μg) with ethidium bromide (EB), cisplatin and increasing concentrations of compounds under study. Lane 1: DNA only. Lane 2: 0.5 μM. Lane 3: 1 μM. Lane 4: 2.5 μM. Lane 5: 5 μM. Lane 6: 10 μM. Lane 7: 25 μM. Lane 8: 50 μM. Lane 9: 100 μM. Lane 10: 200 μM; sc = supercoiled closed circular DNA; oc = open circular DNA..

**Figure. 6**. Analysis of compounds under study as putative DNA intercalators or topoisomerase I inhibitors. Conversion of supercoiled pBluescript plasmid DNA (0.3 μg) incubated at 37 °C to relaxed DNA by
the action of topoisomerase I (3 units) in the absence or in the presence
of compounds in a 100 μM concentration was analysed by agarose gel.
Lane P: scDNA only. Lane T: Topoisomerase I (3 units) + 0 μM drug.
Lane 1: cm1. Lane 2: cm2. Lane 3: cm3. Lane 4: cm1I2. Lane 5: cm1MeI.
Lane 6: cm2MeI. Lane 7: cm3MeI; sc = supercoiled closed circular DNA;
oc = open circular DNA..

**Figure 7.** Analysis of compounds under study as topoisomerase IIα inhibi-tors. Conversion of supercoiled pBluescript plasmid DNA (0.3 μg) incubated at 37 °C to relaxed DNA by the action of topoisomerase Iiα (3 units) in the absence or in the presence of increasing amount of compounds was analysed by agarose gel. Lane E: topoisomerase IIα + 0 μM drug. Lane 1: scDNA only. Lane 2: 5 μM. Lane 3: 10 μM. Lane 4: 25 μM. Lane 5: 50 μM. Lane 6: 100 μM. Lane 7: 200 μM; sc = supercoiled closed circular DNA; oc = open circular DNA

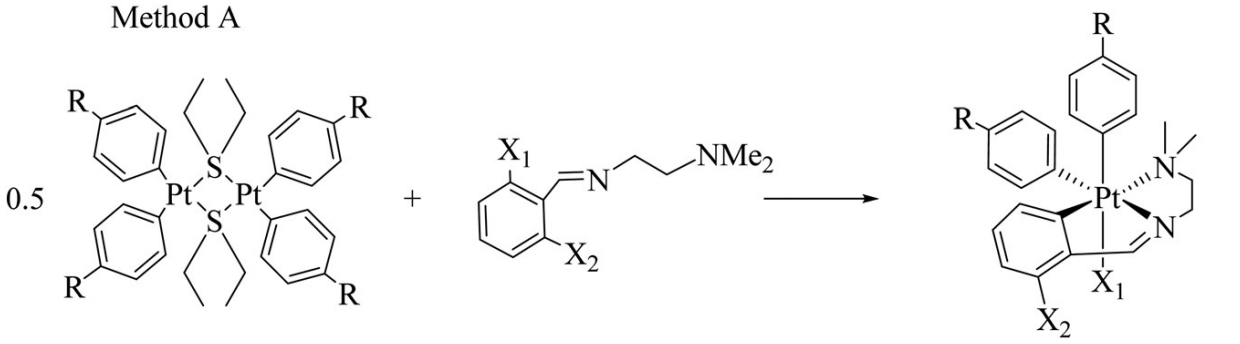
**Figure 8.** Cell cycle phase distribution at 72 h incubation with compounds cm1MeI and cm1I2 at their IC50 concentration in HCT-116 colon cancer cell line. Cells were stained with propidium iodide (PI) and their DNA content was analysed by flow cytometry.

**Figure 9**. Percentage variations of alive, early apoptotic and late apoptotic/ necrotic cell populations at 72 h incubation with compounds cm1MeI and cm1I2 at their IC50 concentration in HCT-116 colon cancer cell line. Cells were stained with propidium iodide (PI) and FITC-annexin and were analysed by flow cytometry.

**Figure 10** ROS levels after 24, 48 and 72 h incubation with compounds cm1MeI and cm1I2 at their IC50 concentration in HCT-116 colon cancer cell line.

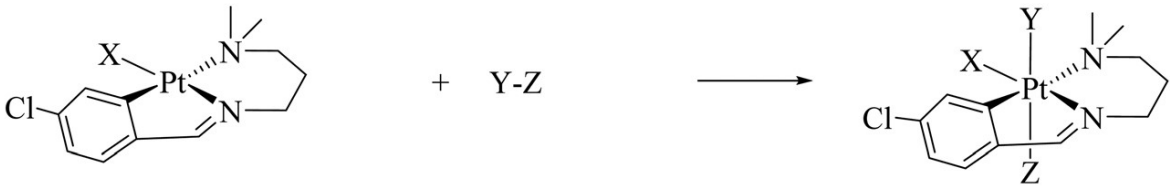
**SCHEME 1**

Method A




**1a**, R = F; $X_1$= Br, $X_2$ = H
**1a'**, R = $CH_3$; $X_1$ = Br; $X_2$ = H
**1b'**, R = $CH_3$; $X_1$= Cl; $X_2$ = Cl

Method B




**2a**, X = Cl; Y = $CH_3$; Z = I
**2b**, X = $CH_3$; Y = $CH_3$; Z = I
**2c**, X = I; Y = $CH_3$; Z = I
**3a**, X = Cl; Y = I; Z = I
**3b**, X = $CH_3$; Y = I; Z = I
**3c**, X = I; Y = I; Z = I

878 **SCHEME 2**

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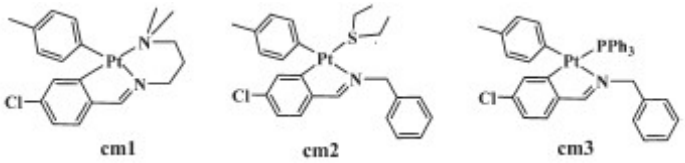
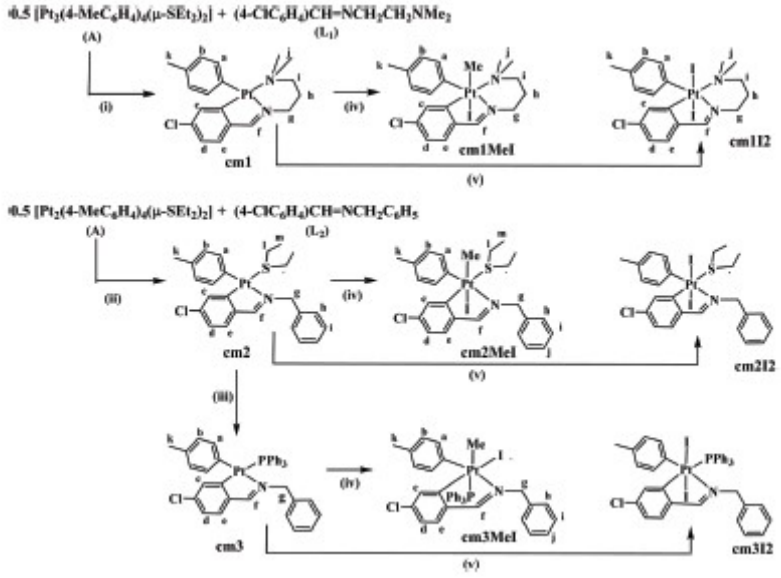



cm1 cm2 cm3

881

882

**SCHEME 3**


0.5 [Pt2(4-MeC6H4)4(μ-SEt2)2] + (4-ClC6H4)CH=NCH2CH2NMe2
(A)
(L1)
(i)
cm1
(iv)
cm1Mel
cm1I2
(v)
0.5 [Pt2(4-MeC6H4)4(μ-SEt2)2] + (4-ClC6H4)CH=NCH2C6H5
(A)
(L2)
(ii)
cm2
(iv)
cm2Mel
cm2I2
(v)
(iii)
cm3
(iv)
cm3Mel
cm3I2
(v)

888 **FIGURE 1**

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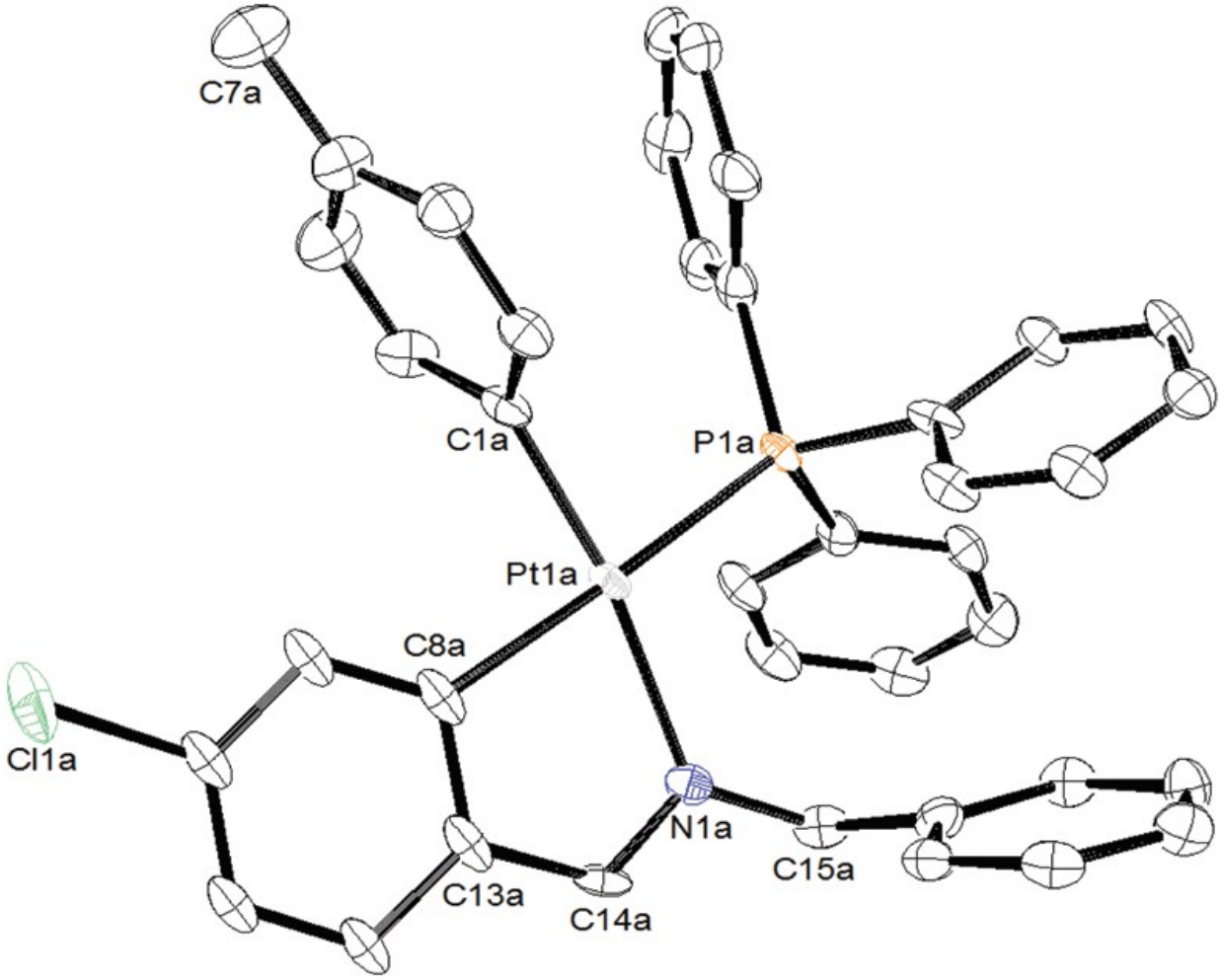


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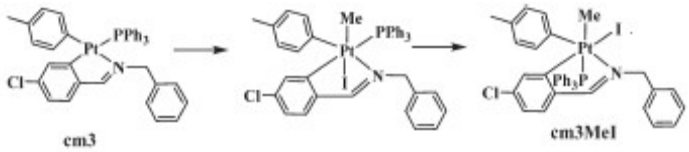
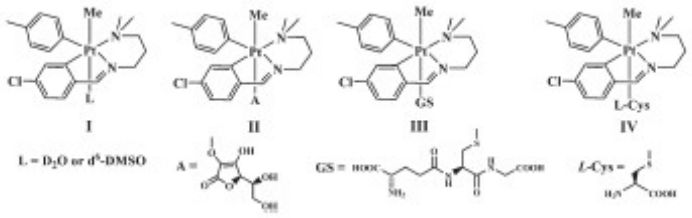
**SCHEME 4**



**SCHEME 5**



905 **FIGURE 2**

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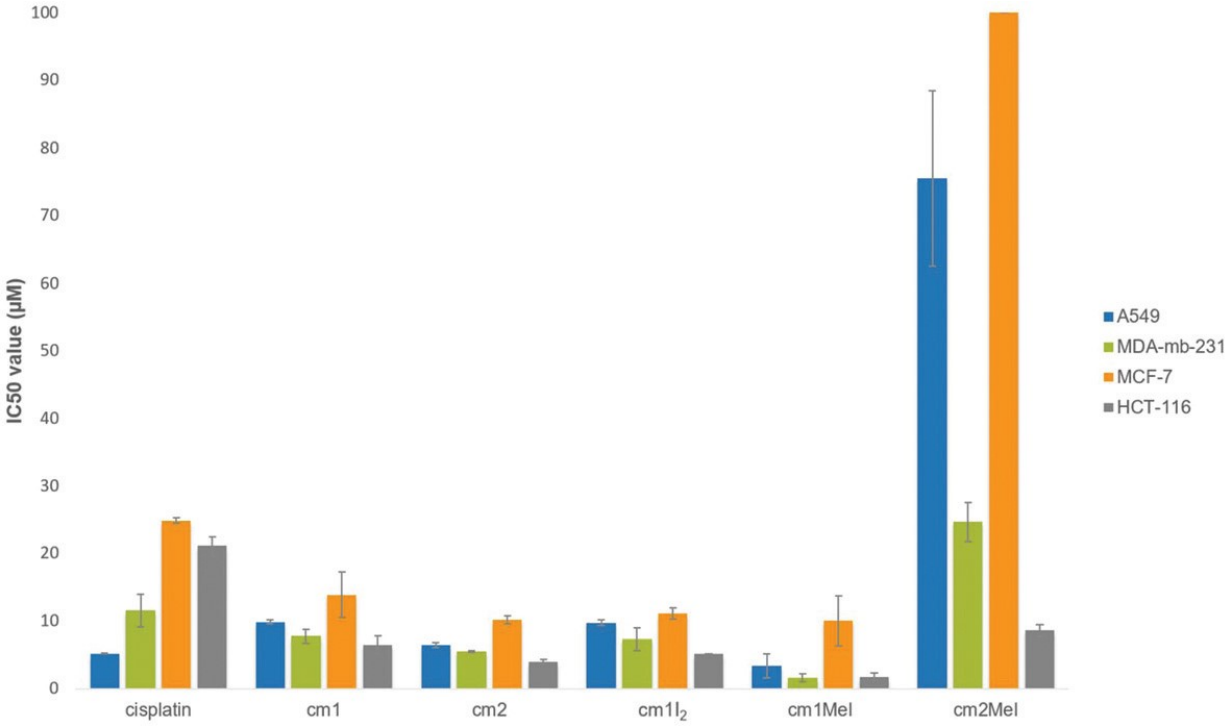


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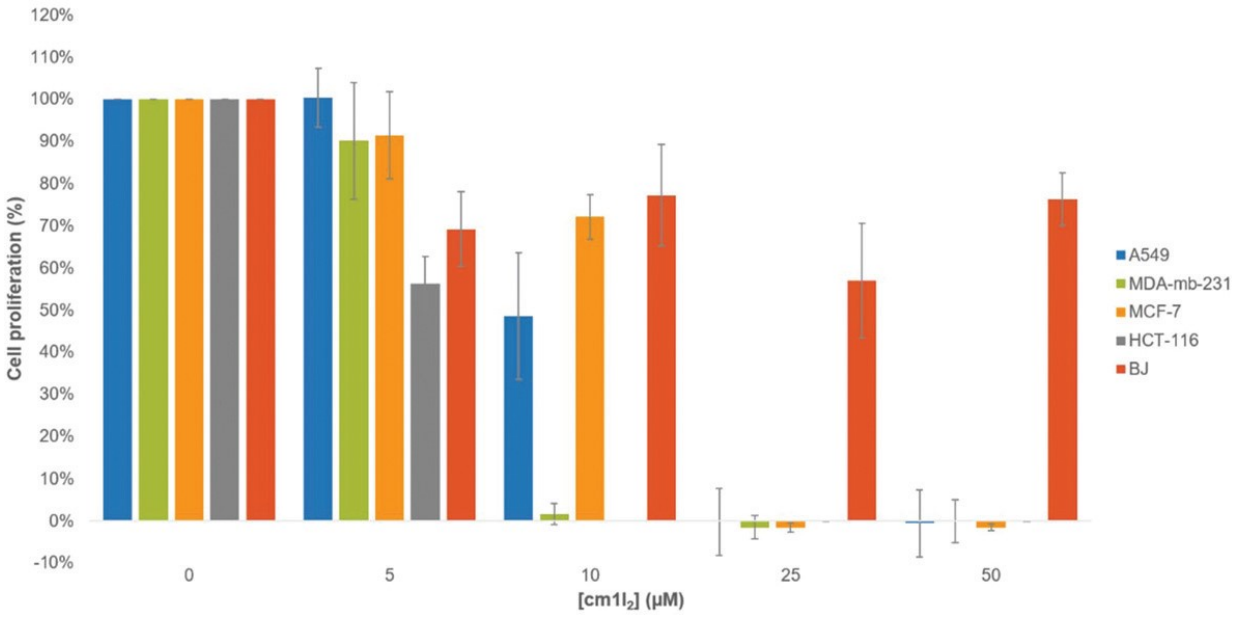
**FIGURE 3**


120%
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
Cell proliferation (%)
0
5
10
25
50
[cm1I2] (μM)
A549
MDA-mb-231
MCF-7
HCT-116
BJ

915 **FIGURE 4**

916

917

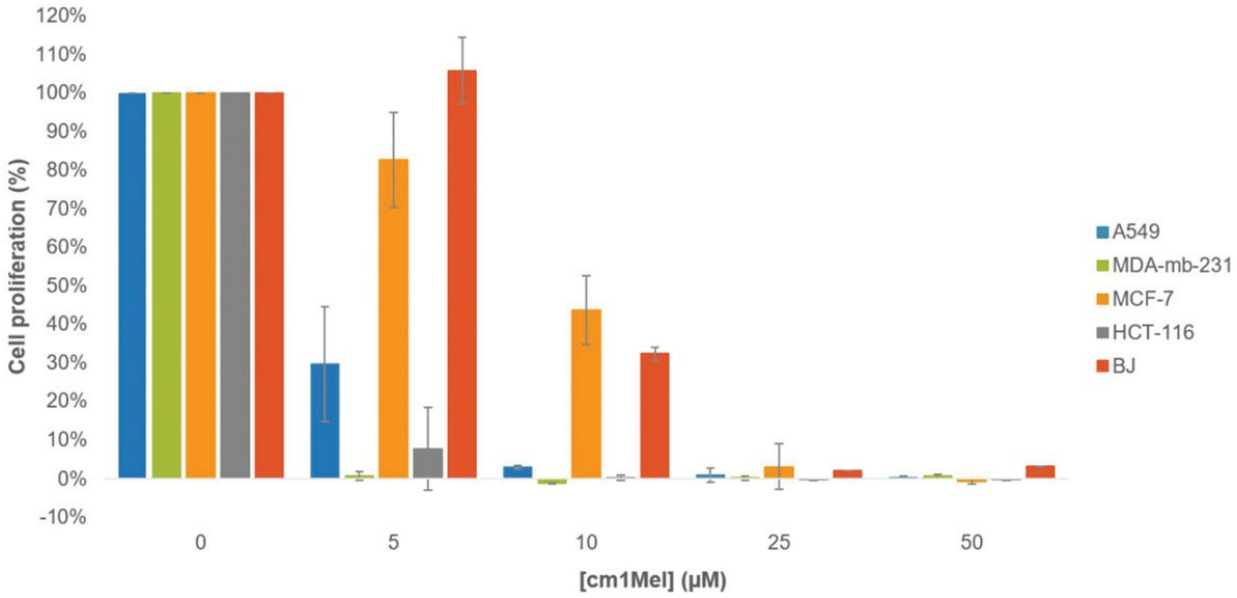



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**FIGURE 5**

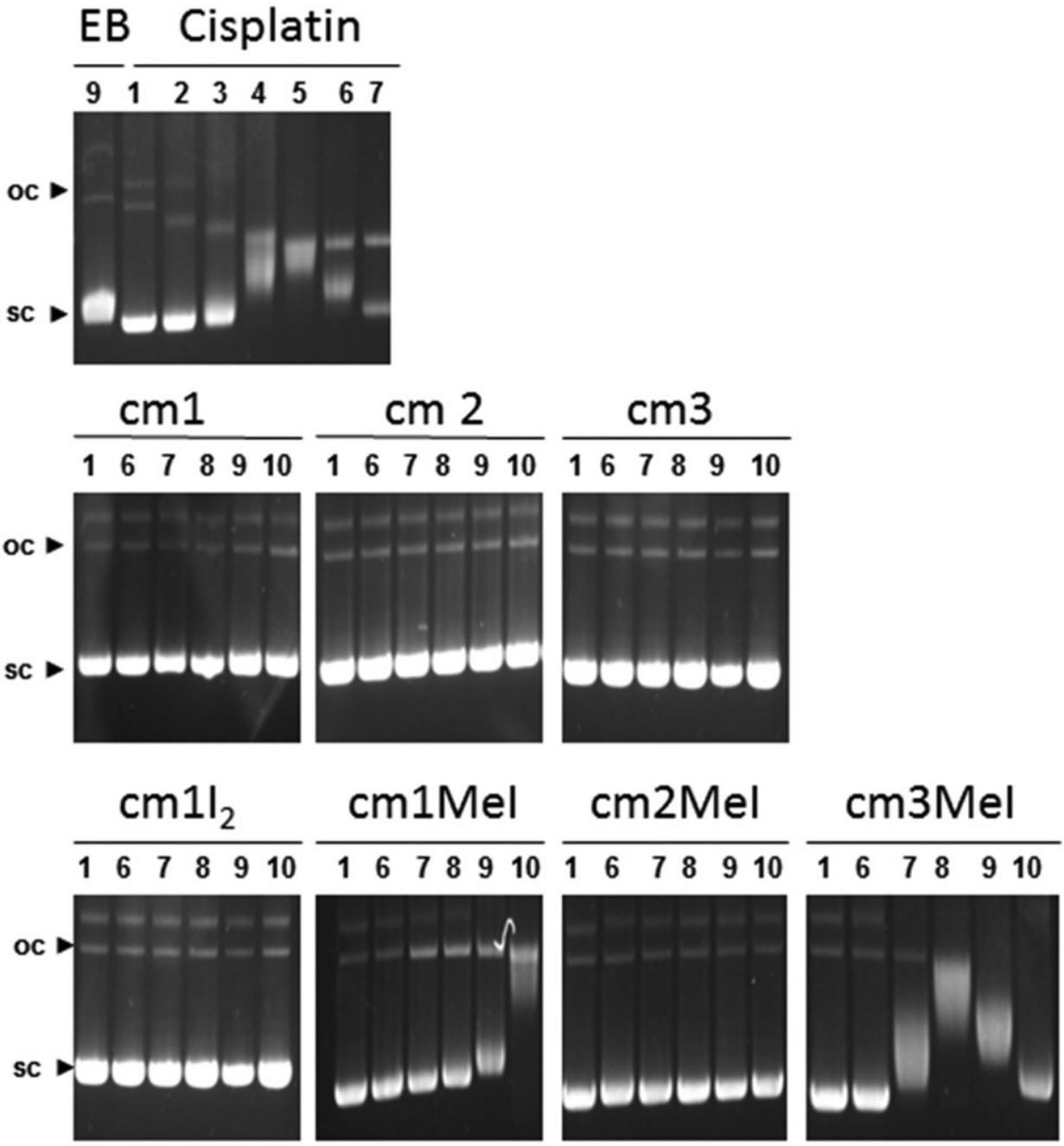

EB
Cisplatin
9 1 2 3 4 5 6 7
oc
sc
cm1
cm 2
cm3
1 6 7 8 9 10
cm1I2
cm1MeI
cm2MeI
cm3MeI

**FIGURE 6**

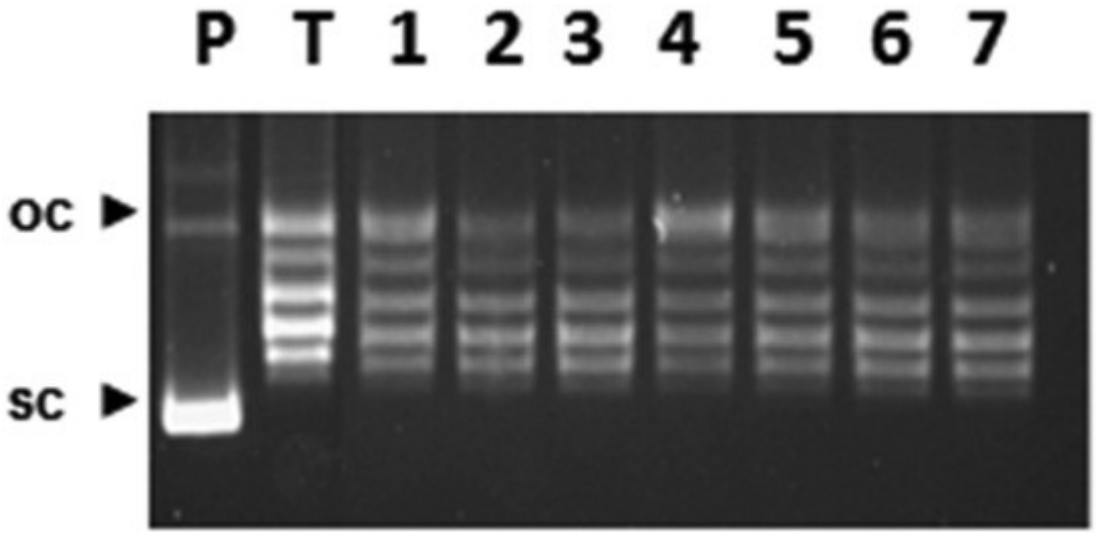


**FIGURE 7**

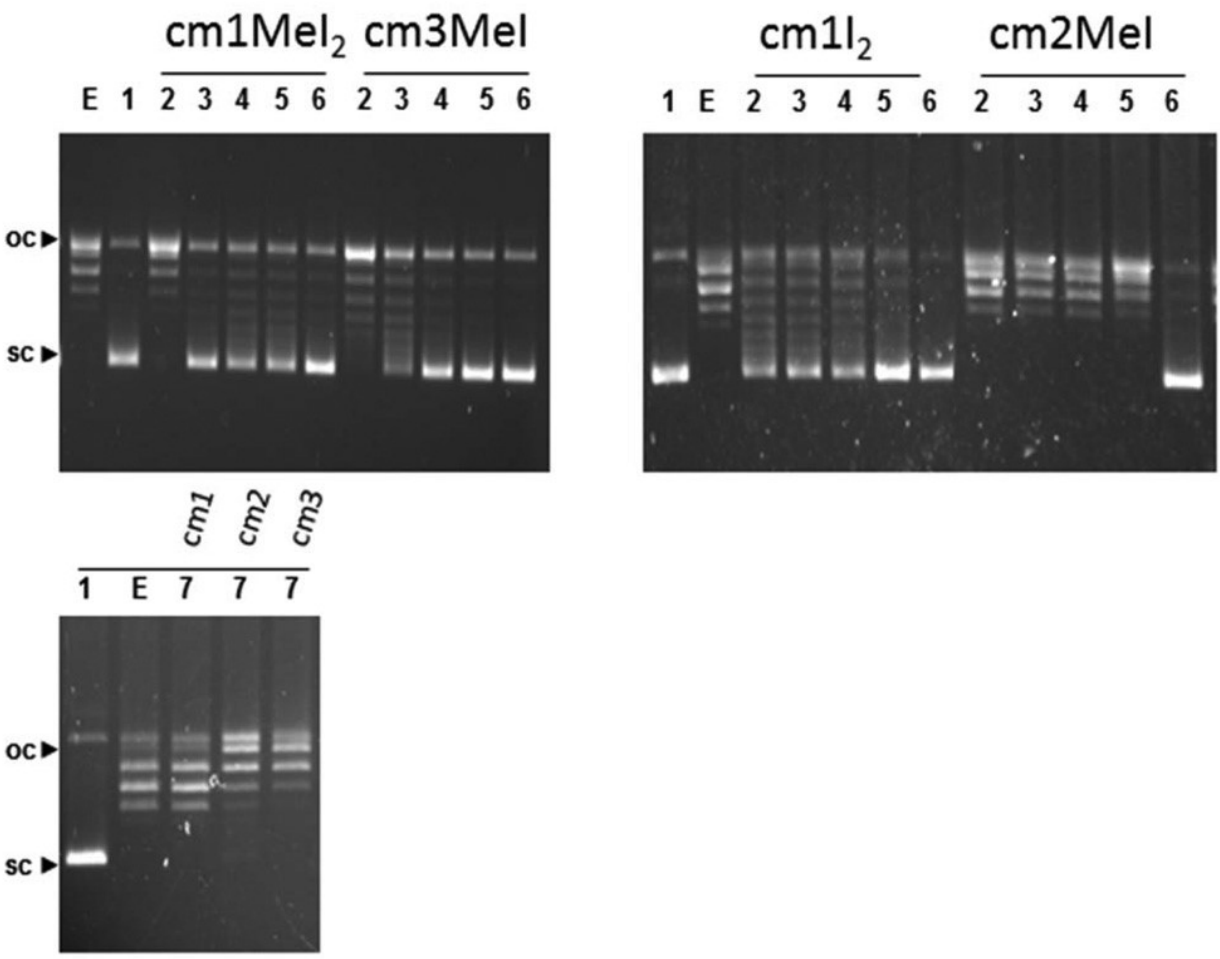
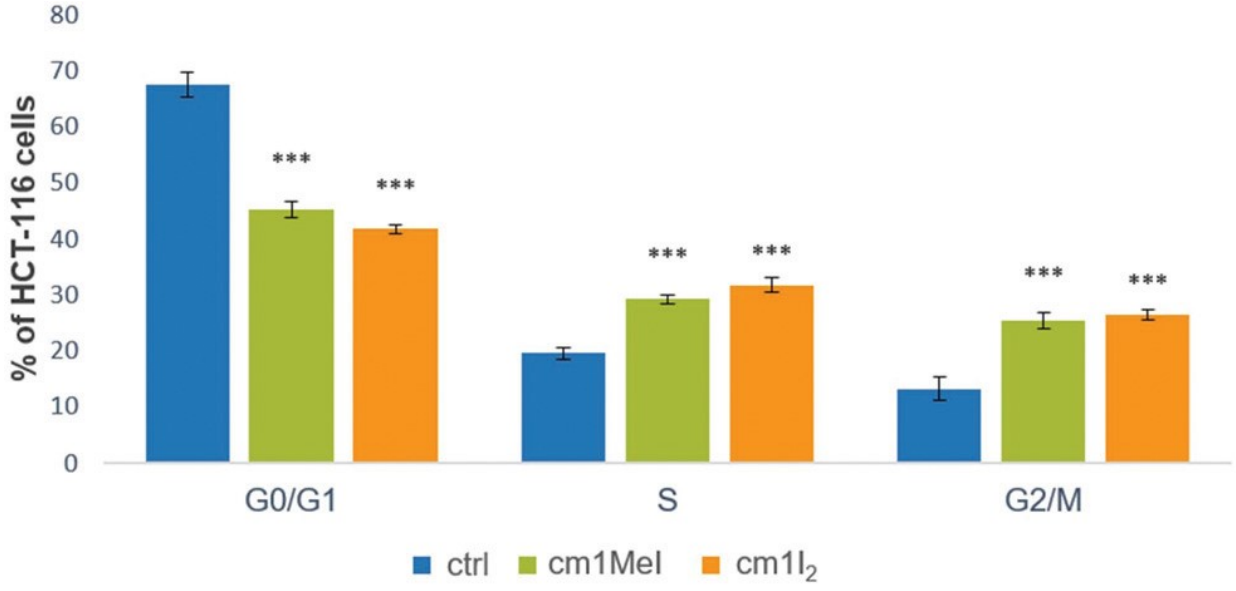

cm1MeI2 cm3MeI
E 1 2 3 4 5 6 2 3 4 5 6
oc
sc
cm1I2 cm2MeI
1 E 2 3 4 5 6 2 3 4 5 6
cm1 cm2 cm3
1 E 7 7 7
oc
sc

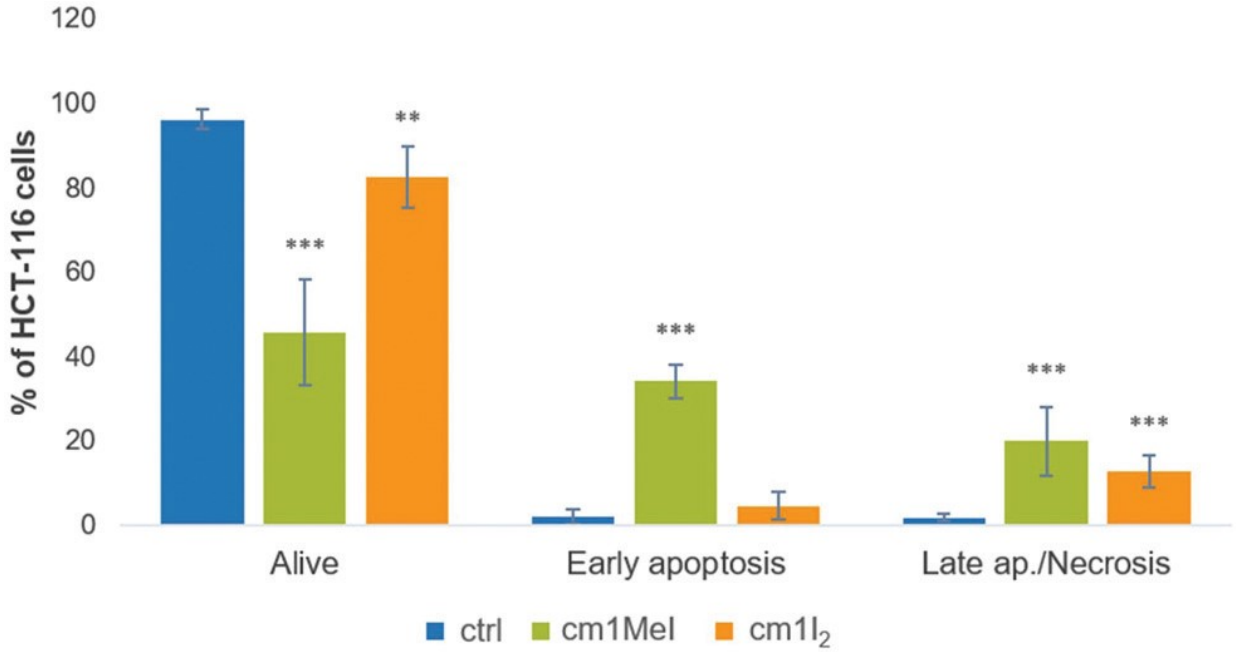
**FIGURE 8**


80
70
60
50
40
30
20
10
0
% of HCT-116 cells
***
***
***
***
***
***
G0/G1
S
G2/M
ctrl
cm1MeI
cm1I2

940 **FIGURE 9**

941

942 .

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945 **FIGURE 10**

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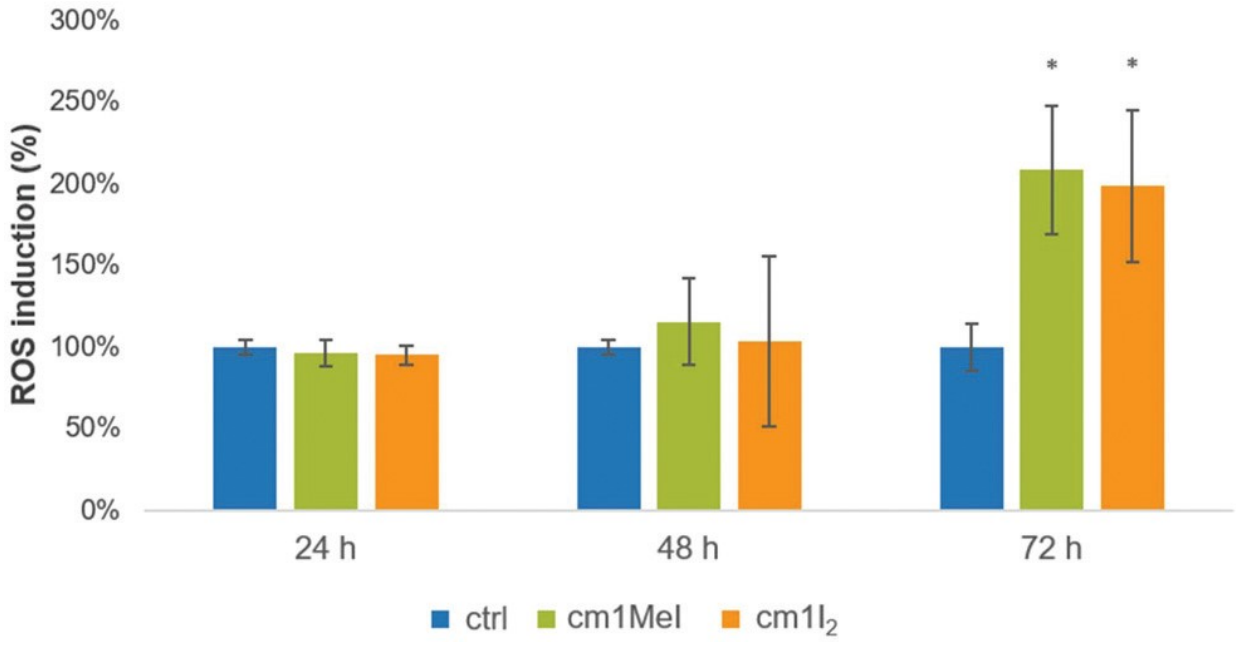


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**Table 1** Selected NMR data for the studied compoundsa

| | $\delta(H^f)$ [$^3J$(Pt–H)] | $\delta(H^c)$ [$^3J$(Pt–H)] | $\delta(H^a)$ [$^3J$(Pt–H)] | $\delta(CH_3)$ [$^2J$(Pt–H)] | $\delta$(P) [$^4J$(Pt–H)] | $\delta(^{195}Pt)$ |
|---|---|---|---|---|---|---|
| **cm1** | 8.48 [56.0] | 6.64 [66.8] | 7.39 [64.0] | — | — | −3699.1 |
| **cm2** | 8.44 [56.0] | 6.81 [72.0] | —[b] | — | — | —[c] |
| **cm3** | 8.10 [52.0] | —[b] | —[b] | — | 26.90 [2201.3] | —[c] |
| **cm1I$_2$** | 8.13 [42.4] | 7.04 [36.8] | 8.43 [37.2] | — | — | −3068.9 |
| **cm1MeI** | 8.42 [48.0] | 6.90 [48.4] | 8.64 [42.4]<br>7.12 [38.0] | 1.31 [68.0] | — | −2310.9 |
| **cm2MeI** | 7.85 [45.6] | —[b] | —[b] | 1.77 [72.0] | — | —[c] |
| **cm3MeI** | 7.76 [49.2] | 6.45 [46.4] | —[b] | 1.85 [72.0] | −9.47 [989.7] | —[c] |

[a] In $CDCl_3$, $\delta$ in ppm, $J$ in Hz. [b] Overlapped by other signals in the aromatic region. [c] No data available (see text). Labels as indicated in Scheme 3.

954 **Table 2** Antiproliferative activity (IC50 μM) on A-549 lung, MDA-MB-231 and MCF-7 breast, and
955 HCT-116 cancer cell lines for the studied compounds and cisplatina
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| Compound | A-549 | MDA-MB-231 | MCF-7 | HCT-116 |
|---|---|---|---|---|
| **cm1** | 9.80 ± 0.32 | 7.71 ± 1.00 | 13.83 ± 3.37 | 6.48 ± 1.27 |
| **cm2** | 6.45 ± 0.33 | 5.52 ± 0.16 | 10.16 ± 0.58 | 3.99 ± 0.38 |
| **cm3** | >100 | >100 | >100 | >100 |
| **cm1I$_2$** | 9.69 ± 0.43 | 7.25 ± 1.68 | 11.08 ± 0.83 | 5.13 ± 0.03 |
| **cm1Mel** | 3.40 ± 1.74 | 1.58 ± 0.58 | 10.02 ± 3.69 | 1.77 ± 0.59 |
| **cm2Mel** | 75.47 ± 12.97 | 24.65 ± 2.92 | >100 | 8.57 ± 0.87 |
| **cm3Mel** | >100 | 38.29 ± 7.44 | >100 | 30.54 ± 6.87 |
| **Cisplatin**[b] | 5.19 ± 0.08 | 11.5 ± 2.4 | 24.84 ± 0.40 | 21.1 ± 1.34 |

[a] Data are shown as the mean values of two experiments performed in triplicate with the corresponding standard deviations. [b] Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) is taken as reference compound.
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**Table 3** Percentages of residual activity and of inhibition of cathepsin B for both concentrations tested of the compounds under study

| Compound | Concentration (μM) | % of residual activity | % of inhibition |
|---|---|---|---|
| **cm1** | 50 | 89 ± 5.5 | 11 ± 5.5 |
| | 100 | 69 ± 3.4 | 31 ± 3.4 |
| **cm2** | 50 | 90 ± 8.2 | 10 ± 8.2 |
| | 100 | 72 ± 0.9 | 28 ± 0.9 |
| **cm3** | 50 | 100 ± 0.7 | — |
| | 100 | 82 ± 0.1 | 18 ± 0.1 |
| **cm1I$_2$** | 50 | 92 ± 5.7 | 8 ± 5.7 |
| | 100 | 80 ± 1.9 | 20 ± 1.9 |
| **cm1MeI** | 50 | 100 ± 0.9 | — |
| | 100 | 84 ± 1.7 | 16 ± 1.7 |
| **cm2MeI** | 50 | 83 ± 1.3 | 17 ± 1.3 |
| | 100 | 76 ± 1.0 | 24 ± 1.0 |
| **cm3MeI** | 50 | 100 ± 0.3 | — |
| | 100 | 82 ± 0.2 | 18 ± 0.2 |

No inhibition (—).

966 **Table 4** Crystallographic and refinement data for compound cm3

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| Formula | $C_{79}H_{74}Cl_2N_2O_2P_2Pt_2$ |
|---|---|
| Fw | 1622.42 |
| Temp. (K) | 100(2) |
| $\lambda$ (Å) | 0.71073 |
| Crystal system | Triclinic |
| Space group | $P\bar{1}$ |
| $a$ (Å) | 14.4066(6) |
| $b$ (Å) | 15.0604(6) |
| $c$ (Å) | 18.6565(8) |
| $\alpha$ (°) | 76.586(2) |
| $\beta$ (°) | 79.459(2) |
| $\gamma$ (°) | 61.663(2) |
| $V$ (Å$^3$); $Z$ | 3453.1(3); 2 |
| $D$ (calcd), (Mg m$^{-3}$) | 1.560 |
| Abs coeff. (mm$^{-1}$) | 4.220 |
| $F$(000) | 1612 |
| Rflns coll./independent | 73 583/14 087 [$R$(int) = 0.0794] |
| Data/restraint/parameters | 14 087/1/705 |
| GOF on $F^2$ | 0.982 |
| Final $R$ index ($I > 2\sigma(I)$) | $R_1$ = 0.0313, w$R_2$ = 0.0699 |
| $R$ index (all data) | $R_1$ = 0.0528, w$R_2$ = 0.0763 |
| Peak and hole (e Å$^{-3}$) | 2.426 and −1.506 |
| CCDC | 1830967 |

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