On the stability and biological behavior of Pt(IV) complexes with halido and aryl ligands in the axial positions

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Abstract

A series of cyclometallated platinum(IV) compounds (**3a**, **3a'** and **3b'**) with a meridional [C,N,N'] terdentate ligand, featuring an halido and an aryl group in the axial positions has been evaluated for electrochemical reduction and preliminary biological behavior against a panel of human adenocarcinomas (A549 lung, HCT-116 colon, and MCF-7 breast) cell lines and the normal bronquial epithelial BEAS-2B cells. Cathodic

reduction potentials (shifting from -1.463 to -1.570 V) reveal that the platinum(IV) compounds under study are highly reluctant to be reduced in a biological environment. The three assayed platinum(IV) complexes exhibit a remarkable cytotoxicity effectiveness (with IC_{50} values in the low micromolar range), even greater than that of cisplatin and the IC_{50} for A-549 lung cells and clog *P* values were found to follow the same trend: **3b'** > **3a'** > **3a**. However, no correlation was observed between reduction potential and *in vitro* activity. As a representative example, cyclometallated platinum(IV) compound **3a'**, exercise its antiproliferative activity directly over non-microcytic A549 lung cancer cells through a mixture of cell cycle arrest (13% arrest at G1 phase and 46% arrest at G2 phase) and apoptosis induction (increase of early apoptosis by 30 times with regard to control). To gain further insights into the mode of action of the investigated platinum(IV) complexes, drug uptake and cathepsin B inhibition were also evaluated.

Keywords: platinum(IV), platinum(II), cyclometallated compounds, cytotoxicity, redox potential, anticancer drugs.

Abbreviations

CDK: Cyclic-dependent kinases

DMEM: Dulbecco's Modified Eagle Medium

DTT: Dithiothreitol

E: Etoposide

EB: Ethidium bromide

FACS: Fluorescence-Activated Cell Sorting

FCS: Fetal calf serum

FITC: Fluorescein Isothiocyanate

5’-GMP: Guanosine-5’-monophosphate

ICP-MS: Inductively coupled plasma mass spectrometry

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PI: Propidium Iodide

PBS: Phosphate buffered saline

PS: Phosphatidylserine

TAE: Tris-acetate/EDTA buffer

TBS: Tris-buffered saline

TE: Tris/EDTA buffer

1. Introduction

Platinum(IV) complexes are considered promising candidates as new anticancer agents capable of overcoming some of the problems associated with the existing platinum(II)- chemotherapeutics. The two additional coordination sites of octahedral Pt(IV) complexes may provide several advantages *versus* the corresponding square-planar Pt(II) compounds, such as: i) a greater cellular uptake, due to higher lipophilicity [1,2], (ii) enough stability as to be administered orally [3], lower reactivity toward biological substrates and diminished side effects [2], due to their higher kinetic inertness, and (iii) the possibility to link pharmacological active molecules in the axial positions in order to obtain synergistic [4-9] targeting [10-11] and delivery [12-15] effects. Based on these advantages, hundreds of Pt(IV) compounds have been synthesized and evaluated in the last decade. Nevertheless, just four complexes (tetraplatin, iproplatin, satraplatin and LA-12) have entered clinical trials (Chart 1) and still there is no platinum(IV) complex approved for clinical use [16].

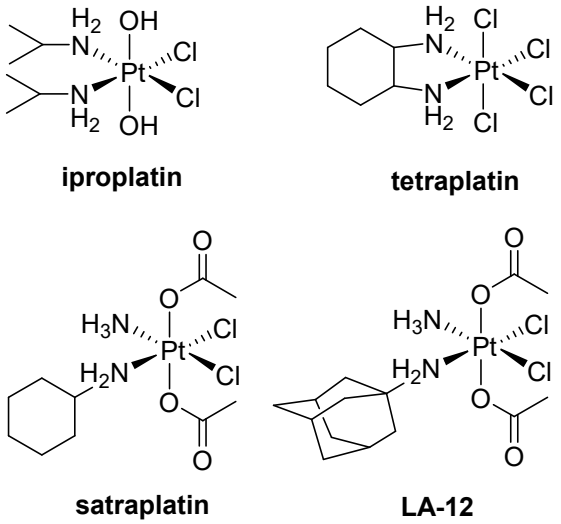


Chart 1. Platinum(IV) complexes involved in clinical trials

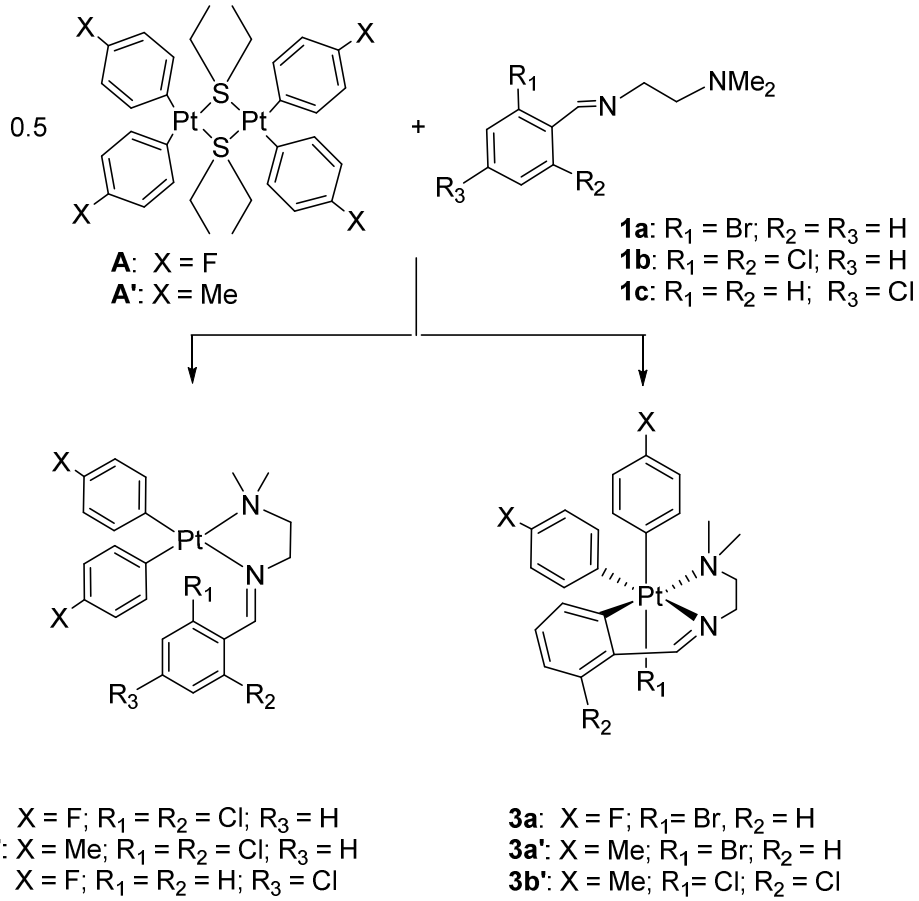
Most evidence to date indicates that platinum(IV) complexes exhibiting symmetrical axial ligands (Cl, OH, and OAc) are reduced under physiological conditions by biologically relevant reducing agents (ascorbic acid, glutathione and sulfhydryl group proteins) to release two axial ligands and yield the cytotoxic cisplatin-like platinum(II) analogs [2,17,18]. The series of three model Pt(IV) complexes with axial chlorido, acetato, and hydroxo ligands have reduction potentials such that the ease of reduction follows the trend Cl > OAc > OH [19,20], although a general relationship between reduction potential of Pt(IV) complexes and their anticancer activity (*in vitro* or *in vivo*) has not been established. Current efforts have been directed toward the development of platinum(IV) complexes containing two different axial ligands on the same scaffold [21-24] or two chelating dicarboxylato ligand [25] displaying several advantages in terms of solubility, lipophilicity, redox behavior, etc. However it was found recently that redox potential does not always correlate with the rate of reduction of the platinum(IV) complexes [26]. Besides, the influence of the equatorial ligands [27,28] and the precise mechanisms of reduction are not always fully understood [29,30].

Hitherto none of the octahedral platinum(IV) complexes clinically trialed have revealed activity in humans significantly greater than that of cisplatin. The problems with tetraplatin, satraplatin and LA-12 could be related with the rapid reduction to Pt(II) species with the consequent loss in lipophilicity [17]. Although iproplatin, with a more negative reduction potential, was abandoned because of the lack of advantage over cisplatin, it was postulated that the development of complexes with very negative reduction potentials could provide drugs that arrive at the target side intact thus exhibiting lower toxicity [2,31-33]. In addition, some other investigations have reported

that platinum(IV) complexes may bind to nucleobases/nucleotides [19,34-36] and DNA [37,19] and hence could achieve anti-cancer activity without prior reduction.

We have been involved in the use of diarylplatinum(II) complexes as precursors in the synthesis of [C,N,N′] cyclometallated platinum(II) and platinum(IV) compounds [38,39] and the study of the mechanisms involved in these processes [40-42]. The platinum(IV) compound **3a** (Scheme 1), synthesized in previous studies [43], is a cyclometallated platinum(IV) compound with a *fac*-PtC_3 arrangement and a meridional [C,N,N′] terdentate ligand, thus leaving one bromido and one aryl ring as axial ligands. This compound exhibits a great antiproliferative activity against non-small lung cancer cells (A549) with IC_{50} value (11.6 μM) very close to that of cisplatin. In addition **3a** induced some modification of the plasmid DNA migration in an agarose gel electrophoretic assay [43].

In line with this task, and to shed more light on the mode of action of antitumor platinum(IV) complexes, in the present study we aimed to explore the redox behavior of unsymmetrical monomeric Pt(IV) complexes featuring a halido and a phenyl group as axial ligands (**3a**, **3a'** and **3b'**). The presence of such ligands in mutually *trans* positions has been confirmed by crystallographic characterization of **3a** [43] and **3b'** [40].



Scheme 1. Synthesis of coordination and cyclometallated platinum compounds from precursors **A** or **A'** [39, 43].

For these compounds, their antiproliferative activity by means of the MTT colorimetric assay in a panel of human adenocarcinama (A549 lung, HCT-116 colon, and MCF-7 breast) cell lines and normal bronquial epithelial cells (BEAS-2B), the corresponding mechanism involved in A-549 lung cancer cells (induction of cell cycle arrest and/or apoptosis), and the identification of plausible biomolecular targets (DNA migration studies, inhibition of cathepsin B and topoisomerase I) were investigated. Evaluation of other relevant physicochemical properties like lipophilicity and the influence of this parameter on Pt cell accumulation are also reported. For comparison

purposes biological parallel studies have been carried out for the previously synthesized platinum(II) chelated compounds **2b**, **2c** and **2b'** containing a bidentate [N,N'] ligand.

2. Results and discussion

2.1. Electrochemistry

Since the studies concerning the reduction of platinum(IV) anti-cancer drugs are important in understanding their biological activity, the ability of the platinum(IV) complexes to be reduced was studied by cyclic voltammetry. As the compounds do not dissolve in water, a highly polar solvent such as acetonitrile was chosen as the solvent in electrochemical studies. ^{1}H NMR spectra of these compounds in CD_3CN indicated that they are stable in this solvent for several days (see experimental section).

As shown in Table 1 and Fig. 1, cyclic voltammograms obtained with 10^{-3} M solutions of the compounds in acetonitrile (0.1 M $(Bu_4N)[PF_6]$) showed in the reduction scan either three major irreversible reduction potentials (**3b'**) or two major irreversible reduction potentials and a shoulder (**3a** and **3a'**). Low intensity oxidation peaks at -1.403 and -0.533 V (**3a**), -1.308 and -0.633 V (**3a'**), and -1.345 and -0.551 V (**3b'**), possibly associated with re-oxidation of the products formed were also observed.

For all the cyclometallated platinum(IV) compounds, the first reduction potential is in the region between -1.45 and -1.57 V, while the remaining reductions are only slightly shifted from those of related ligands (see Table S1, supporting information). The obtained values for the first reduction are in the range recently reported [33] for platinum(IV) organoamide complexes, that display good biological activity in spite of their large negative reduction potentials. In the present case, as stated in previous electrochemical studies of cyclometallated platinum(IV) compounds [44-46] the first

reduction of the platinum(IV) compounds is probably followed by a fast decomposition reaction with involvement of the axial ligands and formation of platinum(II) species.

According to the obtained results, all three compounds are reluctant to be reduced, and, on the basis of the first reduction potential, the following order is obtained: **3a** < **3b'** < **3a'**. Therefore, compound **3a** containing electron withdrawing fluorine substituents in the aryl ligands is the least prone to be reduced. The comparison of the two compounds containing para-tolyl ligands (**3a'** and **3b'**) indicates that the presence of the two chlorine atoms in **3b'** makes this compound more difficult to be reduced. As a whole, only very small differences are observed for the reduction potentials of the three studied compounds and it can be concluded that the reduction of these cyclometallated platinum(IV) compounds to platinum(II) species is a difficult process. A comparative study of the reduction potentials for the studied compounds with those previously reported in the literature is presented in Table 2. Although different solvents were used, some conclusions might be drawn; i) The obtained values are much lower than that reported for iproplatin for which reduction in the body to produce an active platinum(II) species is too slow [4]; ii) The obtained values are in the range reported for complexes with polyfluoroaryl ligands that greatly stabilise the platinum(IV) oxidation state [31] indicating an analogous behavior for the studied cyclometallated platinum(IV) compounds.

Since $\delta(^{195}Pt)$ values may also give valuable information about the electronic properties of the compounds, ^{195}Pt NMR spectra were also recorded for the studied cyclometallated platinum(IV) complexes (see Table 1). It was observed that the $\delta(^{195}Pt)$ values follow the trend **3b'** < **3a** < **3a'**. This order does not match with the first potential trend indicated above. However, this result is in line with the fact that $\delta(^{195}Pt)$ values are highly sensitive to the nature of the binding atoms [47], and the presence of a bromido

(**3a** and **3a'**) versus a chlorido ligand (**3b'**) increases the covalency and leads to a high field shift of the signal. Anyway, the observed differences are small and the obtained values for the three compounds are in the range expected for octahedral platinum(IV) compounds with a [C_3N_2X] donor atoms set [47].

Table 1. Electrochemical data[a] and $\delta(^{195}Pt)$[b] for the platinum(IV) compounds.

Compound	**Reduction potentials**			**$\delta(^{195}Pt)$**
	E Red (1)	**E Red (2)**	**E Red (3)**	
3a	-1.570	-2.155	-2.407 (sh)	-1929.6
3a'	-1.463	-2.126	-2.365 (sh)	-1934.5
3b'	-1.523	-2.078	-2.508	-1836.6

[a] From cyclic voltammetry (reduction scan) in CH_3CN (0.1M $(Bu_4N)[PF_6]$) at 298 K, potentials in V versus Fc/Fc^+ couple, scan rate 0.1 Vs^{-1}; sh indicates a shoulder. [b] In $CDCl_3$ at room temperature.

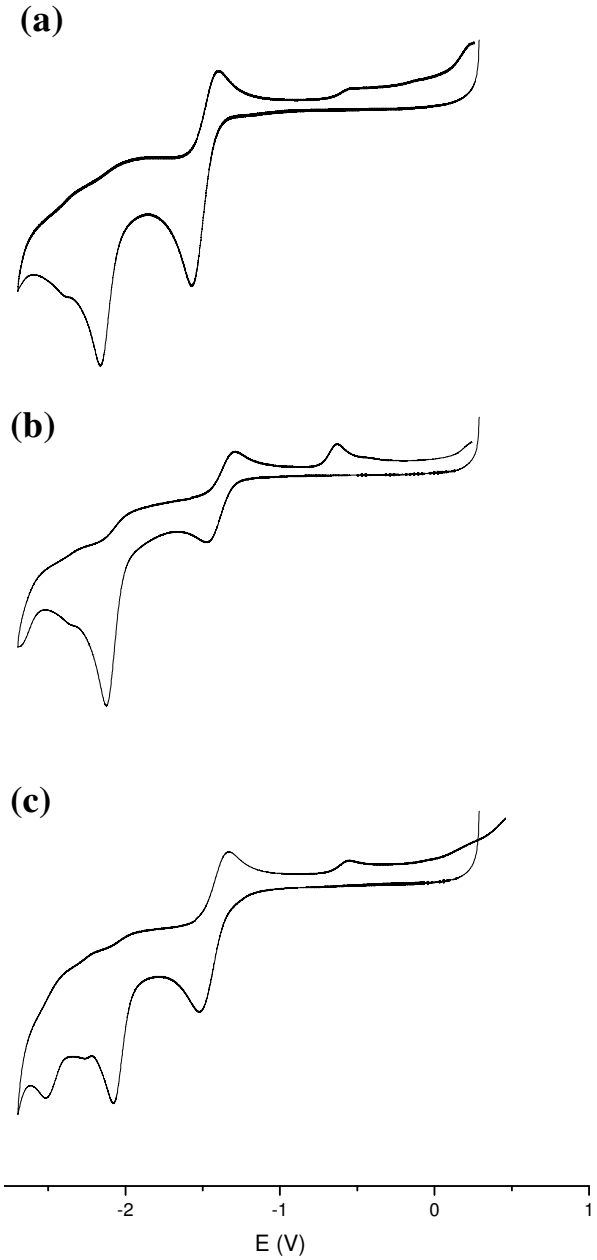


Fig. 1. Cyclic voltammograms obtained at 298 K for compounds **3a** (a), **3a'** (b) and **3b'** (c) in acetonitrile (0.1 M (Bu_4N)[PF_6]), at scan rate 0.1 Vs^{-1}, versus the Fc/Fc^+ couple.

Table 2. A comparative study of the Pt(IV)/Pt(II) reduction potentials.

Compound	**E (V vs. Fc/Fc^+)**	**E (V vs. Ag/AgCl)**	**Solvent**
3a	-1.570[a]		CH_3CN
3a'	-1.463[a]		CH_3CN
3b'	-1.523[a]		CH_3CN
$[PtCl_2(O_2CMe)_2(en)]$	-1.006[b]	-0.546[c]	H_2O
$[PtCl_2(O_2CEt)_2(en)]$	-0.981[b]	-0.521[c]	H_2O
$[PtCl_2(O_2C^nPr)_2(en)]$	-0.953[b]	-0.493[c]	H_2O
$[Pt(p\text{-}HC_6F_4)_2(O_2CMe)_2(en)]$	-1.676[b]		CH_2Cl_2
$[Pt(p\text{-}HC_6F_4)_2(O_2CEt)_2(en)]$	-1.701[b]		CH_2Cl_2
$[Pt(p\text{-}HC_6F_4)_2(O_2C^nPr)_2(en)]$	-1.730[b]		CH_2Cl_2
tetraplatin	-0.550[d]	-0.090[e]	H_2O
satraplatin	-0.710[d]	-0.250[e]	H_2O
iproplatin	-1.190[d]	-0.730[e]	H_2O

[a] This work. [b] Data from Hambley et al. (ref. 31). [c] Data from Ellis et al. (ref.19). [d] Estimated using the conversion factor $E_{Fc/Fc+}$ = 0.46 V vs. $E_{Ag/AgCl}$, as used in ref. 31. [e] Data from Choi et al. (ref. 20).

2.2. Biological studies

2.2.1. Antiproliferative assay

The inherently cisplatin-resistant A549 human adenocarcinoma cell line was used first to test the cytotoxic activity of the platinum(II) complexes **2b'** and Pt(IV) metallacycles **3a'** and **3b'**. Cisplatin, as positive control, was evaluated under identical experimental conditions. Inhibition of cell proliferation by the assayed platinum compounds, observed in A549 cells after 72 h, is represented in the histogram that depicts the toxicity of the compounds at 50% cell viability (IC_{50}). It should be noted that the IC_{50} values against the non-small cell lung cancer A549 have been previously reported by our research group for platinum(II) compounds (**2b** and **2c**), platinum(IV) metallacycles **3a** and the free ligands (**1a**-**1c**) [43]. The obtained values for **2b**, **2c** and **3a** are included in Fig. 2 while those for compounds **1a**, **1b** and **1c** were higher than 100 μM and are not shown.

The cytotoxic activity for the platinum(IV) compounds (**3a**, **3a'**, **3b'**) was also tested in HCT-116 colon and MCF-7 breast adenocarcinoma cells, and normal bronquial epithelial (BEAS-2B) cells, The obtained IC_{50} values for all the cellular lines assayed are listed in Table 3. The corresponding clog *P* values for the studied platinum compounds are also given in Table 3. Most of the studied complexes showed greater cytotoxicity effectiveness than cisplatin against each cancer cell lines. Metallacycle **3a'** was up to 2-, 17- and 2.5-fold more potent than cisplatin in A-549 lung, HCT-116 colon and MCF-7 breast adenocarcinoma cells, respectively.

In A-549 lung cancer cell line, metallacycle **3b'** was the more potent compound, up to 3-fold more potent than cisplatin itself. Interestingly for the platinum(IV) compounds the same trend was observed for both potency and lipophilicity [4]; namely:

3b’ > **3a’** > **3a**. The more lipophilic compounds (**3a’** and **3b’**) were more potent than the fluorinated derivative **3a** *versus* the three adenocarcinoma cells lines tested. It should be noted that fluoro substituents have been proposed as an excellent choice to modify the electronic properties and hydrophobicity of platinum(II) metallacycles in order to increase cytotoxic effectiveness[48]. Interestingly compound **3a** and **3b’** showed less cytotoxicity in normal bronquial epithelial BEAS-2B cells than that of cisplatin

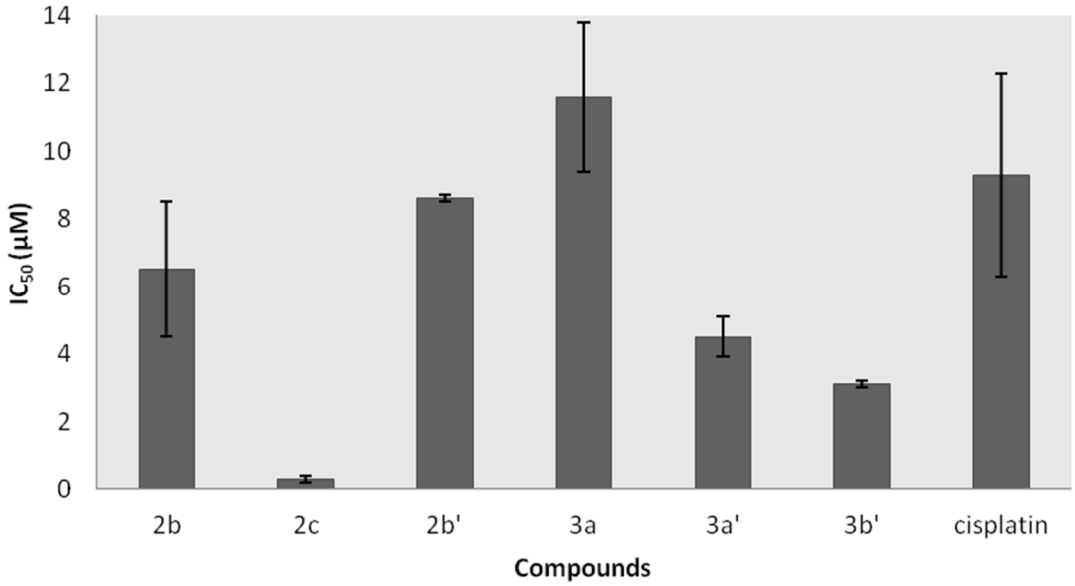


Fig. 2. Cytotoxicity of platinum(II) compounds **2b**, **2c** and **2b’**, platinum (IV) complexes **3a**, **3a’** and **3b’**, and cisplatin (IC_{50} µM) against A549 lung adenocarcinoma cell line.

Table 3. Cytotoxic activities on A-549 lung, HCT-116 colon, MCF-7 breast human cancer cell lines, and normal bronquial epithelial (BEAS-2B) cells, and clog *P* values for the studied compounds and cisplatin.

	IC_{50} (μM) [a]				
compound	**A-549**	**HCT-116**	**MCF-7**	**BEAS-2B**	**clog *P***
3a	11.6 ± 2.2[b]	11.8 ± 0.5	19.3 ± 2.2	15.9 ± 0.5	6.74
3a'	4.5 ± 0.6	2.3 ± 0.2	7.3 ± 0.9	2.3 ± 0.1	7.84
3b'	3.1 ± 0.1	8.1 ± 0.1	14.6 ± 2.4	13.4 ± 0.2	8.56
Cisplatin[c]	9.3 ± 3.0	40 ± 4.4	19 ± 4.5	11.7 ± 0.5[d]	-2.5

[a] Data are shown as the mean values of two experiments performed in triplicate with the corresponding standard deviations. [b] Ref. 43. [c] Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) is taken as reference compound. [d] Ref. 49

*2.2.2. Effect of compounds **2b'** and **3a'** on cell cycle distribution*

Cell cycle is a tightly regulated process with checkpoints at some intervals that allow the cells to multiply in a defined manner. Proteins such as cyclin-dependent kinases (CDKs) play a critical role in controlling the cell cycle and any disruption causes oncogenesis. These critical proteins have been proposed as targets against tumor [50-52]. The compounds **2b'** and **3a'** were tested against the A549 lung cancer cell line and the effects were represented in 3 main phases of the cell cycle distribution: quiescent and gap1 (G0 and G1), synthesis (S), gap2 and mitosis (G2 and M) phases. A549 cells were incubated with these compounds at concentrations at which they are toxic at 50% of cell viability (IC_{50}) for 72 hours and the results were analyzed by Fluorescence Activated Cell Sorting (FACS) using propidium iodide (PI) staining to quantify their DNA content. From the results (Fig. 3), we see that both compounds exhibit cell cycle arrests under different mechanisms. The compound **3a'** shows about 13% arrest at G1 phase (where the cells are effectively arrested stopping them from

entering DNA synthesis) and 46% arrest at G2 phase (where they are arrested before mitosis). While the compound **2b'** shows about 44% arrest at S phase, at which the cells supposedly stop with DNA replication, and 46% arrest at G2 phase.

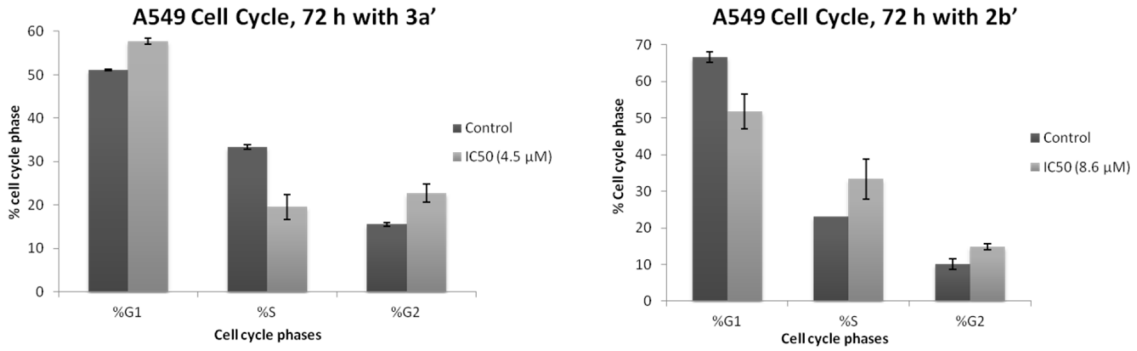


Fig. 3. The percentages of cell cycle distributions in A549 cells are depicted in the histogram and represented in the cell number vs. DNA content plot. The conditions include untreated cells (control) and those treated with compounds **2b'** and **3a'** at concentrations equal to their IC_{50} values (4.5 µM and 8.6 µM, respectively) for 72 h. The harvested cells were stained with propidium iodide (PI) and their DNA content analyzed by flow cytometry.

*2.2.3. Effect of compounds **2b'** and **3a'** on apoptosis induction*

Apoptosis is a process of self-destruction by the cells when there is DNA damage. By using fluorescein-labeled annexin V (AV–FITC, annexin V- fluorescein isothiocyanate) and PI, the apoptosis-inducing properties of **2b'** and **3a'** in A549 cells were examined by performing flow cytometry. In the early phase of apoptosis the plasma membrane symmetry is lost, followed by phosphatidylserine (PS) translocation from the inner to the outer membrane [53]. Thus the externally exposed PS, in the outer environment of the cell, can bind to the annexin V-FITC conjugate with a high affinity [53-54]. In the late apoptotic and necrotic stages, the cell membranes lose their integrity, allowing PI to access the nucleus and intercalate between DNA bases. To differentiate between the non-apoptotic cells (annexin V- and PI-) and early apoptotic (annexin V+ and PI-) and necrotic or later apoptotic (PI+) cells, FACS analysis was used with annexin V-FITC staining and PI accumulation. The apoptotic effects of these

compounds against the A549 cells (Fig. 4) were studied and it was observed that compound **3a'** demonstrates a very high apoptotic effect, while the compound **2b'** shows a very high effect in inducing both apoptosis and necrosis. In particular, compound **3a'** at its IC_{50} concentration (4.5 μM), generated early apoptosis in 33% of the total cells population, as compared to the 1% of the early apoptotic cells in the untreated controls, and **2b'** at its IC_{50} concentration (8.6 μM) generated early apoptosis and late apoptosis/necrosis in 55 and 28% of the total cell population, as compared to the 1 and 4% of the early apoptosis and late apoptotic/necrotic cells, respectively in the untreated controls.

All these results strongly suggest that compounds **2b'** and **3a'** show potent antiproliferative effects against A549 cells and the mechanism is a mixture of cell cycle arrest and apoptosis induction, with a high efficiency in inducing apoptosis and necrosis, thus illustrating the potential of this family of compounds against the tumor cell.

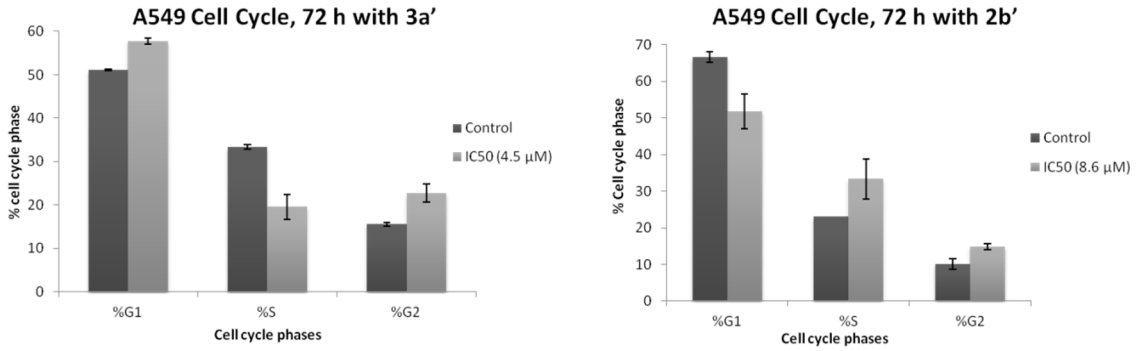


Fig. 4. The histogram shows the percentage variations of the quadrants depicting alive (Q4), early apoptotic (Q3) and late apoptotic/necrotic (Q2/Q1) phases in A549 cells. The conditions include untreated cells (control) and those treated with compounds **2b'** and **3a'** at concentrations equal to their IC_{50} values (4.5 μM and 8.6 μM, respectively) for 72 h.

2.2.4. DNA interaction

The interaction of the platinum(II) complexes **2b'** and Pt(IV) metallacycles **3a'** and **3b'** 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 concentrations and conditions. It should be noted that the interaction of the platinum(II) complexes **2b** and **2c** and Pt(IV) metallacycle **3a** were previously studied in our research group [43].

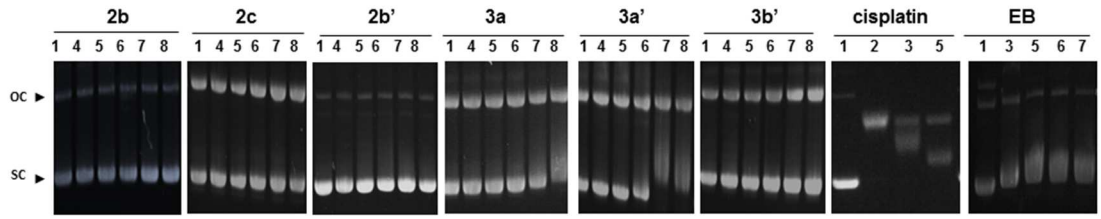


Fig. 5. Interaction of pBluescript SK+ plasmid DNA (0.8 μg) with increasing concentrations of compounds under study, cisplatin and ethidium bromide (EB). Lane 1: DNA only. Lane 2: 2.5 μM. Lane 3: 5 μM. Lane 4: 10 μM. Lane 5: 25 μM. Lane 6: 50 μM. Lane 7: 100 μM. Lane 8: 200 μM; sc = supercoiled closed circular DNA; oc = open circular DNA.

On the basis of the gel mobility shift assay, it is hypothesized that platinum(IV) metallacycles **3a** and **3a'** alter the DNA tertiary structure as the standard reference, cisplatin, although at much higher concentrations (Fig. 5). A decrease in mobility of the supercoiled form was observed in parallel with elongation and loss of brightness of the band just at the higher concentrations tested (up to 100 μM for **3a'** and up to 200 μM for **3a**). In contrast with these findings, the platinum(IV) metallacycle **3b'** was not effective at all for removing the supercoils of plasmid DNA. No modification on the electrophoretic DNA mobility was also observed for the chelated platinum(II) compounds **2b**, **2c** and **2b'** (Fig. 5) pointing to a different mechanism of action or an alternative biomolecular target.

Studies of binding to plasmid DNA (using BamH1 digestion) in the absence and presence of glutathione have been carried out previously by other authors [19] to investigate whether platinum(IV) complexes (with Cl^-, OH^- and carboxylato as axial ligands) can bind to DNA. Given that the mobility of the plasmid DNA did not shift as much as would be expected if platinum(II) would be involved, the results pointed out that the studied platinum(IV) complexes bind directly to DNA following loss of one of the axial ligands. Molecular modeling studies were used by the same authors [19] to assess whether there is a substantial steric barrier for the formation of monofunctional platinum(IV) – DNA adducts, and it was shown that this binding is feasible through a N-7(Guanine). On the other hand, it has been shown using NMR spectroscopy that the nucleotide guanosine-5'-monophosphate (5'-GMP) can directly attack a platinum(IV) complex giving rise to an adduct that is stable over several weeks without reduction in detectable amounts under physiological conditions [36]. Further studies upon DNA have found that oxoplatin [the platinum(IV) analog of cisplatin] preferentially binds to guanine residues, it can form DNA intrastrand and interstrand cross-links containing platinum(IV), and these adducts can inhibit in vitro transcription by a prokaryotic DNA-dependent RNA polymerase [37].

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 complexes **2c**, **2b'** and **3b'**. Supercoiled pBluescript plasmid DNA was incubated in the presence of topoisomerase I at increasing concentrations of compounds under study. The results are given in Fig. 6 and they show that ethidium bromide (EB), used as an intercalator control, prevent the shift of supercoiled DNA into a relaxed state. Complexes **2c**, **2b'** and **3b'** did not 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 [55].

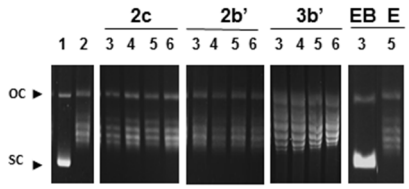


Fig. 6. Analysis of **2c, 2b'** and **3b'** as putative DNA intercalators or topoisomerase I inhibitors. Conversion of supercoiled pBluescript plasmid DNA (0.8 μg) to relaxed DNA by the action of topoisomerase I (3 units) in the absence or in the presence of increasing amounts of compounds **2c, 2b'** and **3b'** was analyzed by agarose gel. Ethidium bromide (EB) was used as a control of intercalating agent and etoposide (E) as a control of nonintercalating agent. Lane 1: *(-)* scDNA only. Lane 2: 0 μM drug. Lane 3: 10 μM drug. Lane 4: 25 μM drug. Lane 5: 100 μM drug. Except for lane 1, all lanes included topoisomerase I; sc = supercoiled closed circular DNA; oc = open circular DNA.

2.2.5. 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 tumors has yet to be defined, but it has been proposed to participate in metastasis, angiogenesis, and tumor progression. Recently, compounds based on palladium, platinum, ruthenium, rhenium, gold and tellurium were shown to be effective inhibitors of cathepsin B [56]. In addition, an excellent correlation between cathepsin B inhibition and cytotoxicity for some dinuclear biphosphine palladacycles [57] and mononuclear platinacycles containing a fluorinated phosphine [48] has been reported. A notable potency for a highly cytotoxic coordination compound containing two non-cyclometallated amine ligands [58] has been observed in our research group. On the other hand, we have also reported that a series of

cyclopalladated and cycloplatinated benzophenone imines were not efficient inhibitors of cathepsin B, although showing *in vitro* high cytotoxicity [59].

Table 4. IC_{50} (μM) and % of residual activity of cathepsin B at 100 μM for the indicated compounds.[a]

Compound	IC_{50} (μM) vs. cathepsin B	% of residual activity at 100 μM
2c	> 50	60 ± 1.7
2b'	> 100	100 ± 0.6
3a'	> 50	38 ± 0.9
3b'	> 100	100 ±.1.8

[a] The enzyme was preincubated for 2 h with each compound (from 5 to 100 μM). The activity is given as a percentage of the enzyme activity determined in the absence of the test compound. Data are shown as the mean values of the experiment performed in triplicate with the corresponding standard deviation.

None of compounds tested in this study (**2c**, **2b', 3a'** and **3b'**) had a high efficiency as cathepsin B inhibitor. Among those, only compound **2c** and **3a'** inhibited more than 50% the enzyme activity at 100 μM concentration (Table 4).

2.2.6. Cell accumulation

A high cellular uptake of structurally different palladium [60,61] and platinum [61-63] compounds by several human cancer cell lines has been detected. Furthermore, within a series of complexes the highest cellular accumulation is in line with the highest cytotoxic activity [61-64].

The cellular accumulation of platinum (mole of Pt/cell × 10^{-16}) was used as a measure of the cellular accumulation of the platinum(IV) metallacycles (**3a'** and **3b'**), the chelated platinum(II) compounds (**2c** and **2b'**) and cisplatin was used as the

reference compound. Table 5 illustrates cellular accumulation of the four tested compounds and cisplatin over 4 h, as applied to lung cancer cell line A549. Accumulation of the studied platinum complexes in A549 lung cancer cells was considerably greater than that of cisplatin (Table 5) for all the compounds tested and follows the order:

3a' > 2b' > 3b' > 2c > cisplatin

The platinum(IV) metallacycle **3a'** was associated with the highest cellular accumulation and this data was in line with the notable potency assessed for this compound (IC_{50} = 4.5 μM). The accumulation of complexes **2b'**, **3b'** and **2c** was about 10, 7 and 6 times higher than that of cisplatin, respectively. No correlation was observed between the antiproliferative efficacy in A549 lung cancer cells and the Pt cellular uptake. These data are in agreement with previous studies reporting that cell uptake *per se*, may not necessarily give an indication of the level of the antitumor activity [65,66].

Table 5. Platinum accumulation in the A549 cell line

Compound	**Cell accumulation[a]**
2c	0.83 ± 0.04
2b'	1.44 ± 0.30
3a'	1.96 ± 0.34
3b'	1.06 ± 0.04
cisplatin	0.15 ± 0.006

[a] Cell accumulation for compounds **2c, 2b', 3a', 3b'** and for cisplatin is given as (mol of Pt per cell ± SD) × 10^{-16}, and was measured by ICP-MS after 4 h of treatment at 50 μM with the indicated compounds. Experiments were performed in duplicate. SD = standard deviation

3. Conclusions

Three cyclometallated platinum(IV) compounds (**3a**, **3a'** and **3b'**) featuring an unsymmetrical coordination sphere, leaving one halido and one aryl ring as axial ligands, were tested as new antitumor drugs. For comparative purposes, three chelated (N, N') platinum(II) complexes (**2b**, **2c** and **2b'**) were included in the study. Cytotoxicity studies by the MTT assay against non-microcytic A549 lung, HCT-116 and MCF-7 breast cancer cells, revealed the high effectiveness of cycloplatinated platinum(IV) complexes. For instance, metallacycle **3b'** was up to 3-fold more potent than cisplatin (IC_{50} = 3.1 μM) in adenocarcinoma A-549 lung cells. Interestingly for platinum(IV) compounds the trend in cytotoxicity in this cancer cell line, was the same as observed for the lipophilicity of the compounds: **3b'** > **3a'** > **3a**. On the other hand, metallacycle **3a'** was up to 2-, 17- and 2.5-fold more potent than cisplatin in A-549 lung, HCT-116 colon and MCF-7 breast adenocarcinoma cells, respectively.

For all the cyclometallated platinum(IV) compounds, the first reduction potential is in the region between -1.45 and -1.57 V, the same range as recently reported for platinum(IV) organoamide complexes, that display good biological activity in spite of their large negative reduction potentials. According to the obtained results, all three compounds are very reluctant to be reduced, and, on the basis of the first reduction potential, the following order is observed: **3a** < **3b'** < **3a'**. Therefore, compound **3a** containing electron withdrawing fluorine substituents in the aryl ligands is the least prone to be reduced.

The electrophoretic mobility of cyclometallated platinum(IV) complexes **3a** and **3a'** indicates alteration of the DNA tertiary structure in a similar way as cisplatin, although at higher concentrations, which is in line with previously published results for the electrophoretic behavior of platinum(IV) compounds. In the present study platinum(IV) complex **3a'**, as a representative example, was shown to exercise its

intrinsic antiproliferative activity through a mechanism which is a mixture of cell cycle arrest and apoptosis induction. At its IC_{50} concentration (4.5 μM), compound **3a'** shows about 13% arrest at G1 and 46% arrest at G2 phase and generated early apoptosis in 33% of the total cells population as compared to the control. Furthermore the mechanism observed for the platinum(IV) compound was clearly different from that of the platinum(II) complex **2b'** assayed in the study as a representative example. At its IC_{50} concentration (8.6 μM), the compound **2b'** shows about 44% arrest at S phase, and 46% arrest at G2 phase, and generated early apoptosis and late apoptosis/necrosis by 55 and 7% of the total cell population, as compared to the controls.

Further studies are in progress to gain insights into the molecular mechanism of action of the cyclometallated platinum(IV) complex under investigation. A thorough understanding of these mechanisms will have implications on future anticancer platinum(IV) drug design.

4. Experimental

4.1. Chemistry

General. ^{1}H NMR spectra in CD_3CN (99.8% D) were obtained with a Mercury 400 spectrometer and ^{195}Pt NMR spectra were recorded in $CDCl_3$ (99.8% D) with a Bruker 400 Avance III HD spectrometer. Chemical shifts are given in δ values (ppm) relative to $SiMe_4$ (^{1}H) and H_2PtCl_6 in D_2O (^{195}Pt), and coupling constants are given in Hz.

*4.1.1. Synthesis of the compounds***.** Compounds **2b**, **2b'**, **2c**, **3a**, **3a'**, and **3b'** were prepared following previously reported procedures [39, 43].

*4.1.1.1 Compound **3a***. **^{1}H NMR** (400 MHz, CD_3CN), δ = 8.69 (s, $^{3}J_{H\text{-}Pt}$= 48.0, 1H, CH=N), {7.57 (t, $^{3}J_{H\text{-}Pt}$= 32.0, $^{3}J_{H\text{-}H}$ = $^{4}J_{H\text{-}F}$ = 6.8, 2H), 7.44 (d, $^{3}J_{H\text{-}H}$ = 6.8, 1H), 7.22 (d, $^{3}J_{H\text{-}H}$ = 7.6, 1H), 7.16-7.12 (m, 2H), 7.04 (t, $^{3}J_{H\text{-}H}$ = 6.8, 1H), 6.83 (t, $^{3}J_{H\text{-}H}$ = 8.0, 2H), 6.71 (m, 1H), 6.56 (t, $^{3}J_{H\text{-}H}$ = 8.0, 2H), aromatics}, {4.42 (m, 1H), 4.25 (m, 2H), 2.91 (m, 1H), CH_2}, 2.84 (s, $^{3}J_{H\text{-}Pt}$ = 11.6, 3H, NMe_2), 2.49 (s, $^{3}J_{H\text{-}Pt}$ = 15.6, 3H, NMe_2). **^{195}Pt NMR** (85.68 MHz, $CDCl_3$), δ = -1929.6 (s).

*4.1.1.2. Compound **3a'***. **^{1}H NMR** (400 MHz, CD_3CN), δ = 8.69 (s, $^{3}J_{H\text{-}Pt}$= 48.0, 1H, CH=N), {7.47 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), 7.45-7.38 (m, 2H), 7.13-6.98 (m, 2H), 6.90 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), 6.64 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), 6.59 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), aromatics}, {4.52-4.43 (m, 1H), 4.33-4.23 (m, 2H), 2.97-2.22 (m, 1H), CH_2}, 2.81 (s, $^{3}J_{H\text{-}Pt}$ = 12.0, 3H, NMe_2), 2.54 (s, $^{3}J_{H\text{-}Pt}$ = 16.0, 3H, NMe_2), 2.31 (s, Me), 2.09 (s, Me). **^{195}Pt NMR** (85.68 MHz, $CDCl_3$), δ = -1934.5 (s).

*4.1.1.3. Compound **3b'**.* **^{1}H NMR** (400 MHz, CD_3CN), δ = 9.12 (s, $^{3}J_{H\text{-}Pt}$= 48.0, 1H, CH=N), {7.40 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), 7.17 (d, $^{3}J_{H\text{-}H}$ = 8.0, 1H), 7.08 (t, $^{3}J_{H\text{-}H}$ = 8.0, 1H), 7.01 (dd, $^{3}J_{H\text{-}H}$ = 8.0, 1.2, 1H), 6.92 (d, $^{3}J_{H\text{-}H}$ = 8.4, 2H), 6.72 (d, $^{3}J_{H\text{-}H}$ = 8.4, 2H), 6.63 (d, $^{3}J_{H\text{-}H}$ = 8.0, 2H), aromatics}, {4.61-4.52 (m, 1H), 4.34 (dd, $^{2}J_{H\text{-}H}$ = 16.0, $^{3}J_{H\text{-}H}$ = 4.0, 1H), 4.18 (td, $^{2}J_{H\text{-}H}$ = 14.0, $^{3}J_{H\text{-}H}$ = 6.0, 1H), 2.94-2.83 (m, 1H), CH_2}, 2.71 (s, $^{3}J_{H\text{-}Pt}$ = 12.0, 3H, NMe_2), 2.26 (s, $^{3}J_{H\text{-}Pt}$ = 16.8, 3H, NMe_2), 2.32 (s, Me), 2.13 (s, Me). **^{195}Pt NMR** (85.68 MHz, $CDCl_3$), δ = -1836.6 (s).

4.1.2. Electrochemistry

Electrochemical data for compounds under study were obtained by cyclic voltammetry under N_2 at 298 K using acetonitrile (HPLC-grade) as solvent and $(Bu_4N)[PF_6]$ (0.1 M) as supporting electrolyte. A M263A potentiostat from EG&G instruments and a conventional three-electrode configuration consisting of two platinum wires as the auxiliary and reference electrodes, and a working glassy carbon (GC) electrode were used. Prior to voltammetric experiments, the working electrode was polished with alumina, rinsed thoroughly with water and acetone and dried. The scanning rate was 0.1 Vs^{-1}. All potentials are referred to the ferrocene/ferrocenium (Fc/Fc^{+}) couple. In these experimental conditions the standard error of the measured potentials is $\pm 5\times10^{-3}$ V.

4.2. Biological studies

4.2.1. Cell culture and cell viability assay

Human lung adenocarcinoma A549 cells were grown as a monolayer culture in minimum essential medium (DMEM with L-glutamine, without glucose and without sodium pyruvate) in the presence 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% CO_2 at 37 ºC).

For A549 cell 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 (Dulbecco's Modified Eagle Medium) (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.* [67] and Matito and coworkers[68] 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, 2.5×10^3 A549 cells/wells were cultured in 96 well plates for 24 hours prior to the addition of different compounds at different concentrations, in triplicate. After incubation for 72 h more, the media was aspirated and 100 µL of filtered MTT (0.5 mg/mL) 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 % (IC_{50}) after 72 h of treatment were subsequently calculated.

4.2.2. Cell cycle analysis. Cell cycle was assessed by flow cytometry using a fluorescence activated cell sorter (FACS). For this assay, 4×10^4 A549 cells were

seeded in 6 well plates with 2 mL of medium. After 24 h of incubation, **3a'** or **2b'** was added at their IC_{50} values (9.3, 1.2 and 4.6 μM, respectively). Following 72 h of incubation, cells were harvested by mild tripsinization, collected by centrifugation and resuspended in Tris buffered saline solution (TBS) containing 50 mg/mL PI, 10 mg/mL DNase-free RNase and 0.1% Igepal CA-630. 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 in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL). Data from 1×10^4 cells were collected and analyzed using the Multicycle program (Phoenix Flow Systems, San Diego, CA).

*4.2.3. Apoptosis assay***.** Apoptosis was assessed evaluating the annexin-V binding to phosphatidylserine (PS), which is externalized early in the apoptotic process. 4×10^4 A549 cells per well were seeded in 6 well plates with 2 mL of medium and treated as described above for the cell cycle analysis assay. After cell collection and centrifugation, cells were resuspended in 95 μL binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 3 μL of Annexin-V FITC conjugate (1 mg/mL) were then added and the suspension was incubated in darkness for 30 min, at room temperature. Just before FACS analysis, the cell suspension was added to a vial containing 500 μL of binding buffer, and then stained with 20 μL of 1 mg/mL PI solution. Data from 1×10^4 cells were collected and analyzed.

Data analysis. For each compound, a minimum of three independent experiments with triplicate values to measure cell viability and a minimum of two independent experiments for cell cycle analysis and assessment of apoptosis were conducted. Data are given as the mean ± standard deviation (SD).

4.2.4. DNA migration studies.

A stock solution (10 mM) of each compound was prepared in high purity DMSO. Then, serial dilutions were made in MilliQ water (1:1). Plasmid pBluescript SK^+ (Stratagene) was obtained using QIAGEN plasmid midi kit as described by the manufacturer. Interaction of drugs with pBluescript SK^+ plasmid DNA was analysed by agarose gel electrophoresis following a modification of the method described by Abdullah *et al*. [69] Plasmid DNA aliquots (40 μg / mL) were incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) with different concentrations of compounds **2b'**, **3a'** and **3b'** ranging from 0 μM to 200 μM at 37 ºC for 24 h. Final DMSO concentration in the reactions was always lower than 1%. For comparison, cisplatin and ethidium bromide (EB) were used as reference controls. Aliquots of 20 μL of the incubated solutions containing 0.8 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) and visualized and photographed under UV light.

Topoisomerase I-based experiments were performed as described previously [70]. Supercoiled pBluescript DNA, obtained as described above, was treated with topoisomerase I in the absence or presence of increasing concentrations of compounds **2b'**, **3a'** and **3b'**. Assay mixtures contained supercoiled pBluescript DNA (0.8 μg), calf thymus topoisomerase I (3 units) and complexes **2b'**, **3a'** and **3b'** (0 - 100 μM) in 20 μL of relaxation buffer Tris-HCl buffer (pH 7.5) containing 175 mM KCl, 5 mM $MgCl_2$ and 0.1 mM EDTA. Ethidium bromide (EB, 10 μM) was used as a control of intercalating agents and etoposide (E, 100 μM) as a control of non-intercalating agent. Reactions were incubated for 30 min at 37 ºC 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.

4.2.5. Cathepsin B inhibition assay

The colorimetric cathepsin B assay was performed as described by Casini *et al.* [71] with few modifications. Briefly, the reaction mixture contained 100 mM sodium phosphate (pH 6.0), 1 mM EDTA and 200 μM sodium *N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester as substrate. To have the enzyme catalytically active before each experiment the cysteine in the active site was reduced by treatment with dithiothreitol (DTT). For this purpose, 5 mM DTT was added to cathepsin B sample, before dilution, and incubated 1 h at 30 ºC. To test the inhibitory effect of the platinum compounds on cathepsin B, activity measurements were performed in triplicate using fixed concentrations of enzyme (500 nM) and substrate (200 μM). The platinum compounds were used at concentrations ranging from 5 to 100 μM. Previous to the addition of substrate, cathepsin B was incubated with the different compounds at 25ºC for 2 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 90 s at 326 nm on a UV- spectrophotometer.

4.2.6. Cell accumulation

Cell accumulation of platinum compounds was measured in lung adenocarcinoma A549 cells. A total of 1×10^6 cells were seeded in 6 mm tissue culture dishes for 16 h in DMEM-high glucose plus 10% FCS (fetal calf serum). Then, compounds were added at 50 μM for 4 h at 37 ºC, 5% CO_2. Cisplatin was used as a positive control at same concentration, and DMSO as a vehicle control. After treatment,

cells were washed twice with PBS, tripsinized and harvested in PBS. Cell suspension was centrifuged and pellets were digested with 12 M HCl and diluted to 1.2 M HCl. Each treatment was done in duplicate. The samples were analyzed by ICP-MS using an ICP-MS Perkin Elmer (Elan 6000). Results are expressed as mols Pt/cell.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at …

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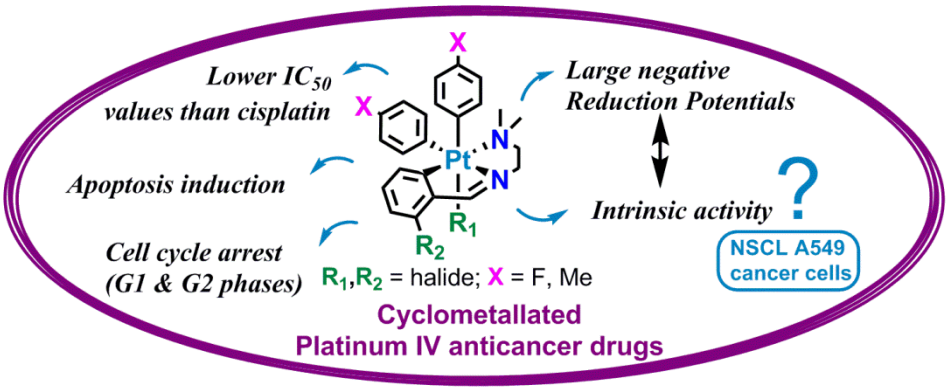
Graphical abstract



Highlights

- Cyclometallated Pt(IV) compounds with halido and aryl as axial ligands
- Based on very negative reduction potentials, high difficulty of the compounds for being reduced.
- Low IC_{50} values in human A-549 lung, HCT-116 colon and MCF-7 breast cancer cells.
- Antiproliferative activity in NSCL A-549 by cell cycle arrest and apoptosis