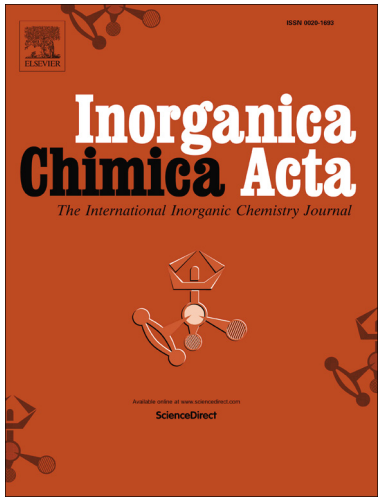


Synthesis, characterization, and *in vitro* cytotoxicity of a Kiteplatin-Ibuprofen Pt(IV) prodrug

Alessandra Curci, Nunzio Denora, Rosa Maria Iacobazzi, Nicoletta Ditaranto, James D. Hoeschele, Nicola Margiotta, Giovanni Natile

PII: S0020-1693(17)30801-0
DOI: http://dx.doi.org/10.1016/j.ica.2017.07.019
Reference: ICA 17742

To appear in: *Inorganica Chimica Acta*

Received Date: 23 May 2017
Revised Date: 3 July 2017
Accepted Date: 5 July 2017

Please cite this article as: A. Curci, N. Denora, R.M. Iacobazzi, N. Ditaranto, J.D. Hoeschele, N. Margiotta, G. Natile, Synthesis, characterization, and *in vitro* cytotoxicity of a Kiteplatin-Ibuprofen Pt(IV) prodrug, *Inorganica Chimica Acta* (2017), doi: http://dx.doi.org/10.1016/j.ica.2017.07.019

Synthesis, characterization, and *in vitro* cytotoxicity of a Kiteplatin-Ibuprofen Pt(IV) prodrug.

Alessandra Curci,[1] *Nunzio Denora,*[2] *Rosa Maria Iacobazzi,*[3] *Nicoletta Ditaranto,*[1] *James D. Hoeschele,*[4] *Nicola Margiotta,*[1,*] *Giovanni Natile.*[1,*]

[1]Dipartimento di Chimica, Università degli Studi di Bari Aldo Moro, via E. Orabona 4, 70125 Bari (Italy);

[2]Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari Aldo Moro, via E. Orabona 4, 70125 Bari (Italy);

[3]Istituto Tumori IRCCS Giovanni Paolo II, viale O. Flacco 65, 70124 Bari (Italy).

[4]Department of Chemistry, Eastern Michigan University, Ypsilanti, MI, USA 48197.

***Corresponding Authors**

Phone: +39 080 5442759 (N.M.); +39 080 5442774 (G.N.). E-mail: nicola.margiotta@uniba.it (N.M.); giovanni.natile@uniba.it (G.N.).

Keywords: cisplatin, Kiteplatin, *cis*-1,4-DACH, Ibuprofen, Pt(IV) drugs, NSAID, colon cancer.

Abstract

The Pt(IV) prodrug of kiteplatin, *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)], having in the axial positions two molecules of Ibuprofen, has been synthesised, characterized and tested *in vitro*. The aim was to potentiate the cytotoxic effect of kiteplatin with the anti-inflammatory activity of Ibuprofen. The reduction potential of the conjugate resulted comparable to those of other reported Pt(IV) carboxylate complexes, ensuring *in vivo* stability in blood during transport and intracellular reduction with release of the active species. The cytotoxic activity of the complex resulted remarkably potentiated reaching nanomolar activity. It is possible that the coordinated

Ibuprofen molecules promote the transport and the accumulation of the complex in tumour cells by increasing its lipophilicity. In addition to the increased uptake, Ibuprofen could also exert an anti-inflammatory action mediated by inhibition of the COX enzymes which are overexpressed in tumors. The use of a conjugate with Pt-bound Ibuprofen can ensure similar cellular uptake and biodistribution for both the anti-inflammatory and the cytotoxic drugs in the exact ratio of 2:1.

Introduction

Cisplatin (Chart 1) has become one of the most widely used chemotherapeutic agents and is mainly used for the treatment of testicular, ovarian, bladder, and lung cancers, melanoma, lymphomas, and myelomas.[1],[2] The clinical efficacy demonstrated by the three platinum-based drugs currently approved by the FDA (cisplatin, carboplatin, and oxaliplatin; Chart 1) has prompted an intense research aiming to discover new metal-based anticancer drugs with enhanced efficacy, lower toxicity, and a broader spectrum of activity.[3]

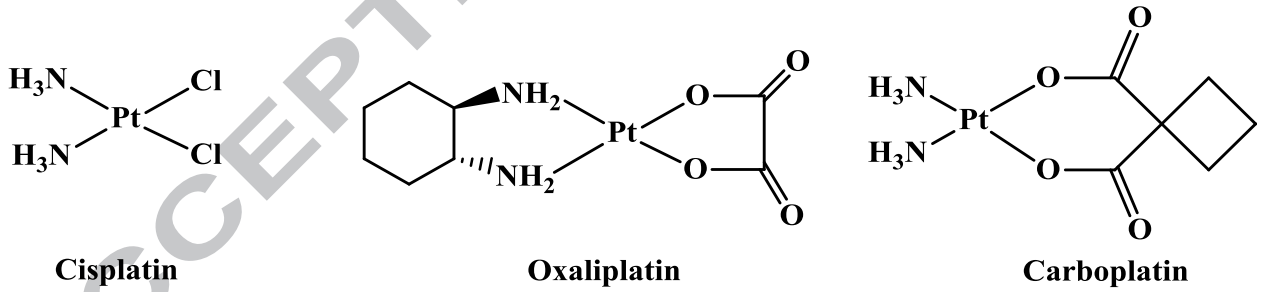


Chart 1. Platinum drugs currently approved by FDA.

Several advantages can be obtained by the use of targeted and/or controlled drug delivery systems that can improve dramatically the degree and duration of the clinical outcome.[4],[5] Moreover, the synthesis of prodrugs has been exploited to lessen the limitations of conventional platinum(II) anticancer drugs.[4] A recent strategy in the development of platinum-based chemotherapeutic drugs is the assembly in a single complex of two different pharmacologically active

agents.[6],[7],[8],[9] This is possible especially in Pt(IV) prodrugs that are known to be rapidly reduced intracellularly by biological reducing agents, such as glutathione and ascorbic acid, to release the active ligands and the cytotoxic Pt(II) species.[10],[11]

NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) have been recently used as axial ligands in Pt(IV) complexes as a consequence of the generally accepted correlation between cancer and inflammation processes.[12],[13],[14] Indeed, recent work has demonstrated that the inflammation processes correlated to cancer are responsible for the evolution of the tumor mass with consequent formation and spreading of metastases. For instance, the occurrence of prostate, colon, and pancreatic cancer is highly frequent in patients experiencing the inflammation processes prostatitis, ulcerative colitis, and pancreatitis, respectively. NSAIDs have as major targets the cyclooxygenases (COX) enzymes, which are involved in the biosynthesis of prostaglandins and are expressed in two isoforms, COX-1 and COX-2.[15] COX is also implicated in cisplatin resistance with COX-2 being overexpressed in many tumors and involved in tumor initiation and progression. For this reason COX inhibitors and, in particular, COX-2 selective inhibitors are used as cancer-preventive and as adjuvant agents in chemotherapeutic regimens.[16] As evidenced in clinical and tissue culture investigations, a problem associated with the combination therapy of antitumor drugs and COX inhibitors, is the different pharmacokinetic profile and biodistribution of the two drugs administered separately. To obtain concerted transport into the tumor cells, different groups have pursued the conjugation of the antiproliferative agent with the anti-inflammatory drug and have prepared platinum(II) [17] and platinum(IV) [16],[18],[19],[20] prodrugs containing NSAIDs such as aspirin, diclofenac, indomethacin, and Ibuprofen.

We are currently investigating the cisplatin fourth generation derivative [$PtCl_2$(*cis*-1,4-DACH)] (DACH = diaminocyclohexane), also dubbed Kiteplatin (Chart 2), a new candidate platinum anticancer drug that contains an isomeric form of the diamine ligand present in oxaliplatin (1*R*,2*R*-DACH).[21],[22],[23],[24],[25],[26],[27],[28],[29] Our interest in this compound stems from its

activity against cisplatin (2008/C13* ovarian cancer cells) and oxaliplatin (LoVo/LoVo-OXP colon cancer cells) sensitive/resistant cell lines. The cross-resistance profiles indicate that kiteplatin is as good as oxaliplatin and significantly better than cisplatin (particularly in the case of colon cancer cells) toward the sensitive sublines. Furthermore, despite sharing similar lipophilicity with oxaliplatin and similar propensity for passive diffusion, the accumulation of kiteplatin is not reduced in oxaliplatin-resistant cells lines. Similarly to cisplatin and oxaliplatin, also Pt(IV) derivatives of kiteplatin have been prepared (particularly with benzoate or long-chain carboxylates in axial positions) with the aim of improving the drug uptake and, hence, the pharmacological activity in cancer cells (Chart 2).[30],[31]

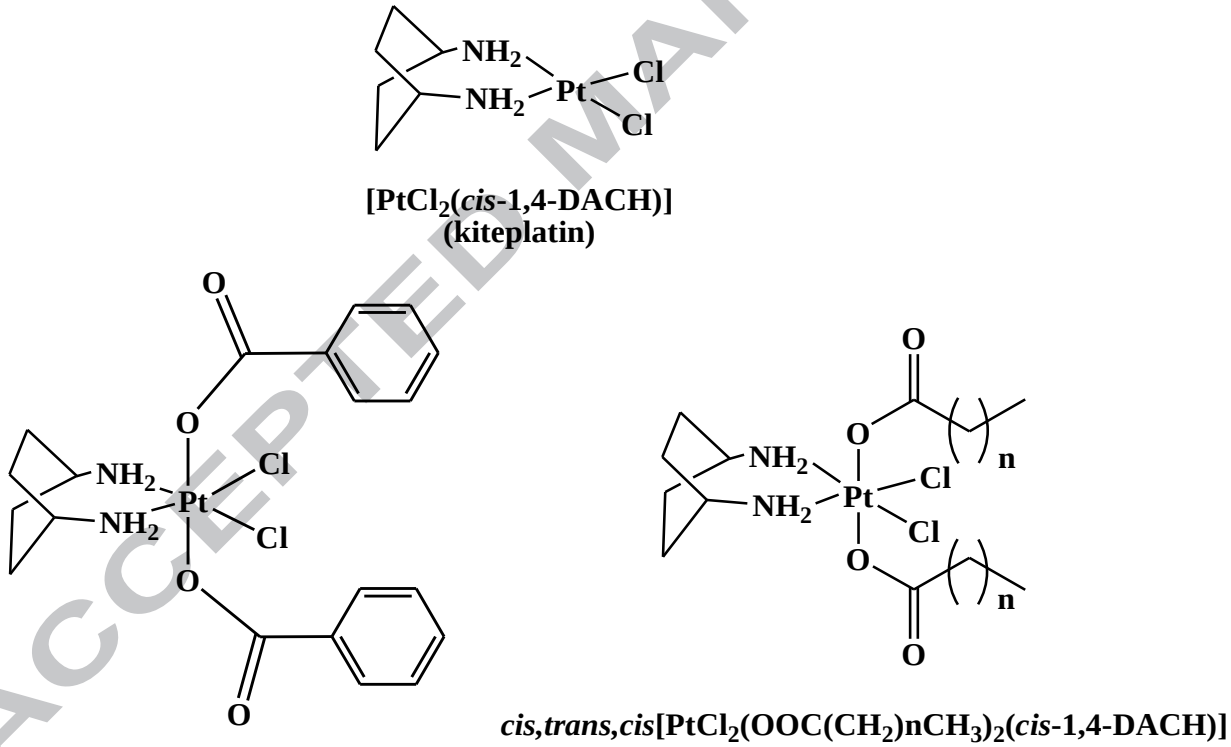


Chart 2. Structures of [$PtCl_2$(*cis*-1,4-DACH)] (kiteplatin) and its Pt(IV) lipophilic derivatives.

Noteworthy, the cytotoxic activity of the Pt(IV) kiteplatin-benzoate derivative resulted to be remarkably higher (ca. 45 times) than that of kiteplatin, reaching nanomolar concentrations against

a wide panel of human cancer cells, including cisplatin- and oxaliplatin-resistant colon-cancer cells.[31]

S-Ibuprofen belongs to the 2-arylpropionic acid group of NSAIDs and is a potent COX-1 and COX-2 inhibitor while the *R* isomer is *ca*. 2-3 orders of magnitude less potent.[32] Although the mechanism of action is not fully understood,[33] we were attracted by the ability of Ibuprofen to inhibit tumor initiation and proliferation in prostate and breast cancer and, in particular, in colon cancer cell lines such as HCA-7, HCT-116, and HCT-15, this latter expressing only COX-1 and producing nearly no prostaglandin E_2.

With the aim of potentiating the activity of kiteplatin against colon cancer cells, not only by enhancing the lipophilicity of the platinum compound but also by exerting an additional anti-inflammatory effect on the tumor cells, in this investigation we have prepared a Pt(IV) prodrug of kiteplatin having Ibuprofen in the axial positions (Scheme 1). We also hoped that the combination of an anti-inflammatory and a chemotherapeutic drug would be more effective if the two drugs are delivered in the form of a single prodrug. Racemic Ibuprofen was extracted by a commercial anti-inflammatory drug and its acyl chloride was reacted with the dihydroxido Pt(IV)-derivative of kiteplatin. The Pt(IV) conjugate was fully characterized and its cathodic potential was determined by cyclic voltammetry. Finally, the new Pt(IV) derivative of kiteplatin was tested *in vitro* against HCT-15 and HCT-116 human colon-cancer cell lines.

Experimental

Materials and methods

Commercial reagent grade chemicals and solvents were used as received without further purification.

^{1}H-NMR and [^{1}H-^{13}C] HSQC spectra were recorded on a Bruker Avance III 700 MHz instrument.

^{1}H and ^{13}C chemical shifts were referenced using the internal residual peak of the solvent (Acetone-d_6: 2.05 ppm for ^{1}H and 29.92 ppm for ^{13}C). ^{195}Pt-NMR spectra were recorded on Bruker Avance DPX 300 MHz instrument. ^{195}Pt NMR spectra were referenced to K_2PtCl_4 (external standard placed at -1620 ppm with respect to $Na_2[PtCl_6]$).[34]

Electrospray ionisation mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap system Agilent, Palo Alto, CA).

Elemental analyses were carried out with an Eurovector EA 3000 CHN instrument.

Kiteplatin [21] and *cis,trans,cis*-[$PtCl_2(OH)_2$(*cis*-1,4-DACH)],[27] were prepared according to already reported procedures and all analytical data were in good agreement with the given formulation (data not shown).

Extraction of Ibuprofen from BRUFEN® 600mg expired tablets

Ibuprofen ((*RS*)-2-[4-(2-methylpropyl) phenyl] propanoic acid) was isolated from two commercial Brufen® 600 mg expired tablets. The tablets were finely ground and suspended in 40 mL of 1M NaOH. The mixture was stirred at room temperature for few minutes until a white and foamy suspension was formed. The suspension was extracted with 40 mL of ethyl ether and the aqueous phase was collected and treated again with 40 mL of Et_2O for a second extraction. The aqueous phase was separated and filtered through a plug of Celite® to remove the undissolved solids.

Then, the colorless filtrate was acidified with 5M HCl until pH ~1-2 leading to the formation of a white dispersed solid. The acidic suspension was extracted using 40 mL of ethyl acetate (1:1, v/v with the aqueous phase) for three times. The organic phase was separated from the aqueous phase, treated with anhydrous sodium sulfate and then filtered. The filtrate was evaporated to dryness using a rotary evaporator until the formation of a white solid which was subsequently dried under vacuum. Obtained: 1.11 g (92% yield). *^{1}H-NMR* (acetone-d_6): 10.66 (s broad, 1H, C(1)OOH), 7.25 (d, $J_{H\text{-}H}$ = 7.87 Hz, 2H, C(4/5)H), 7.13 (d, $J_{H\text{-}H}$ = 7.87 Hz, 2H, C(6/7)H), 3.71 (m, $J_{H\text{-}H}$ = 7.10 Hz, 1H, C(2)H), 2.45 (d, $J_{H\text{-}H}$ = 7.16 Hz, 2H, C(8)H_2), 1.85 (m, $J_{H\text{-}H}$ = 6.02 Hz, 1H, C(9)H), 1.42 (d, $J_{H\text{-}H}$

= 7.18 Hz, 3H, C(3)H_3) and 0.88 (d, $J_{H\text{-}H}$ = 6.82 Hz, 6H, C(10/11)H_3) ppm. *^{13}C NMR* (acetone-d_6): 129.7 (C(4/5)), 128.0 (C(6/7)), 45.40 (C(8)), 45.18 (C(2)), 30.9 (C(9)), 22.4 (C(10/11)) and 18.9 (C(3)) ppm. (See Figure 1 for numbering of atoms)

Synthesis of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] (*RS*-Ibuprofen-H = 2-[4-(2-metylpropyl)phenyl]propanoate; $^-$OOC-CH(CH_3)-C_6H_4-CH_2-CH(CH_3)$_2$).

The Pt(IV) complex was prepared according to already reported procedures with slight modifications.[16],[31] Ibuprofen (0.500 g, 2.41 mmol) was solubilized in 2 mL of $SOCl_2$ and the solution was stirred at room temperature for 18 hours. Then the solution was concentrated by rotary evaporation until formation of a yellow-brown oil which was diluted with 1.5 mL of acetone. The acetone solution of Ibuprofen acyl chloride was added to a suspension containing *cis,trans,cis*-[$PtCl_2(OH)_2$(*cis*-1,4-DACH)] (0.050 g, 0.120 mmol) and pyridine (390 μL; 4.828 mmol) in acetone (1.5 mL). The reaction mixture was stirred at 75 °C for 48 h in the dark in a capped round-bottom pressure flask. After cooling the obtained suspension to room temperature, the white precipitate was eliminated by filtration. An excess of *n*-pentane was added to the filtrate to yield a precipitate that was isolated by filtration of the mother liquor and washed with water and diethyl ether. The obtained solid was dissolved in acetone and treated with water which caused the precipitation of a white solid which was separated and dried under vacuum. Obtained 0.032 g (32% yield).

Anal. Calculated for $C_{32}H_{48}Cl_2N_2O_4Pt$: C, 48.61; H, 6.12; N, 3.54 %. Found: C, 48.28; H, 6.12; N, 3.45 %. *ESI-MS* (MeOH): calc. for $C_{32}H_{48}Cl_2N_2O_4PtNa$, $[M+Na]^+$: 813.70. Found: $^m/_z$ 813.26. *^{1}H-NMR* (acetone-d_6): 8.41, 8.04, 7.62 and 7.22 (4H, broad, NH_2), 7.28 (d, $J_{H\text{-}H}$ = 7.47 Hz, 4H, C(4/5)H), 7.06 (d, $J_{H\text{-}H}$ = 7.47 Hz, 4H, C(6/7)H), 3.68 (m, $J_{H\text{-}H}$ = 6.84 Hz, 2H, C(2)H), 3.26 (s, $J_{H\text{-}Pt}$ = 80.02 Hz, 2H, C(a)H), 2.43 (d, $J_{H\text{-}H}$ = 7.18 Hz, 4H, C(8)H_2), 1.82 (m, $J_{H\text{-}H}$ = 6.54 Hz, 2H, C(9)H), 1.56 (br, 4H, C(b/c)H_2), 1.47 (br, 4H, C(b/c)H_2), 1.37-1.35 (m, 6H, C(3)H_3), 0.87 (d, $J_{H\text{-}H}$ = 6.65 Hz, 12H, C(10/11)H_3) ppm. *^{13}C NMR* (acetone-d_6): 129.4 (C(4/5)), 128.1 (C(6/7)), 50.9 (C(a)), 48.1

(C(2)), 45.5 (C(8)), 31.0 (C(9)), 22.4 (C(10/11)), 20.09 (C(b/c)), 20.09 (C(b/c)), 19.4 (C(3)) ppm. *^{195}Pt NMR* (acetone-d_6): 1099 ppm. (See Scheme 1 for numbering of atoms).

Electrochemical measurements

A CHI 1140B electrochemical analyzer (CH Instruments, Inc.) was used for the cyclic voltammetry (CV) measurements performed with a standard three-electrode cell. The working electrode was a glassy carbon electrode (GC), the reference electrode was KCl-saturated Ag/AgCl, and the auxiliary electrode was a platinum lamina. The GC working electrode was polished with alumina and then rinsed with distilled water and dried, to obtain a reproducible surface for all the experiments. The platinum complex was dissolved in DMSO containing 0.1M $[NBu_4][PF_6]$ as supporting electrolyte, to a final concentration of 1.0 mM. Nitrogen was bubbled through the solution to remove oxygen. Peak potentials were measured at different scan rates (0.10, 0.15, 0.20, and 0.25 V s^{-1}) and reported vs KCl-saturated Ag/AgCl. The stability of the reference electrode in DMSO was verified frequently by measuring the potential vs SCE.

Cell viability assay

The human colorectal carcinoma cell lines (no COX-2 expression) HCT-116 and HCT-15 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37 °C. All materials for cell culturing were purchased from EuroClone, Italy. The antitumor activity of the Pt(IV)–Ibuprofen complex, *cis-trans-cis*-$[PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] and of kiteplatin, Ibuprofen, and cisplatin, used as reference compounds, was assessed by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) test performed as described elsewhere with some modifications.[35] Cells were seeded in 96-well plates with a density of 5000 cells/well and 24 h post seeding, the medium was removed and replaced with drug-

containing medium. In particular, the Pt(IV) conjugate, kiteplatin, and cisplatin were tested in the concentration range 0.01-100 µM, while Ibuprofen was tested in the range 0.01-800 µM. After 72 h of incubation, 10 µL of MTT were added to each well and the cells were incubated at 37 °C for further 2 h. Absorbance at 570 nm was measured using a Perkin Elmer VictorX3 microplate reader and Prism software (ver. 6d; 2013, GraphPad, USA) was used to calculate the half maximal inhibitory (IC_{50}) values for each cell line.

Results and Discussion

Extraction of Ibuprofen from BRUFEN® 600mg expired tablets and its characterization

Two Brufen® 600 mg expired tablets were used to extract the sodium salt of Ibuprofen by treatment with NaOH after removing the lipophilic excipients with an organic phase. The aqueous phase was acidified to protonate Ibuprofen, which was subsequently extracted twice with ethyl acetate.

Ibuprofen was characterized by NMR spectroscopy. The ^{1}H-NMR spectrum of Ibuprofen in acetone-d_6 (Figure 1) shows a broad peak falling at 10.66 ppm assigned to the carboxylic proton (not shown). The doublets falling at 7.25 and 7.13 ppm, were assigned to the aromatic protons. This doublet of doublets is typical of a phenyl system disubstituted in para positions (H(4/5) and H(6/7) in Figure 1). The quartet integrating for one proton at 3.71 ppm and the doublet at 1.42 ppm were assigned, respectively, to the proton and to the methyl group bound to the chiral carbon (H(2) and H(3) in Figure 1). The doublet integrating for two protons at 2.45 ppm was assigned to the methylene protons (H(8) in Figure 1). The multiplet at 1.85 ppm integrating for one proton was assigned to H(9) while at 0.88 ppm resonates a doublet assigned to the isopropylic methyls (H(10/11) in Figure 3).

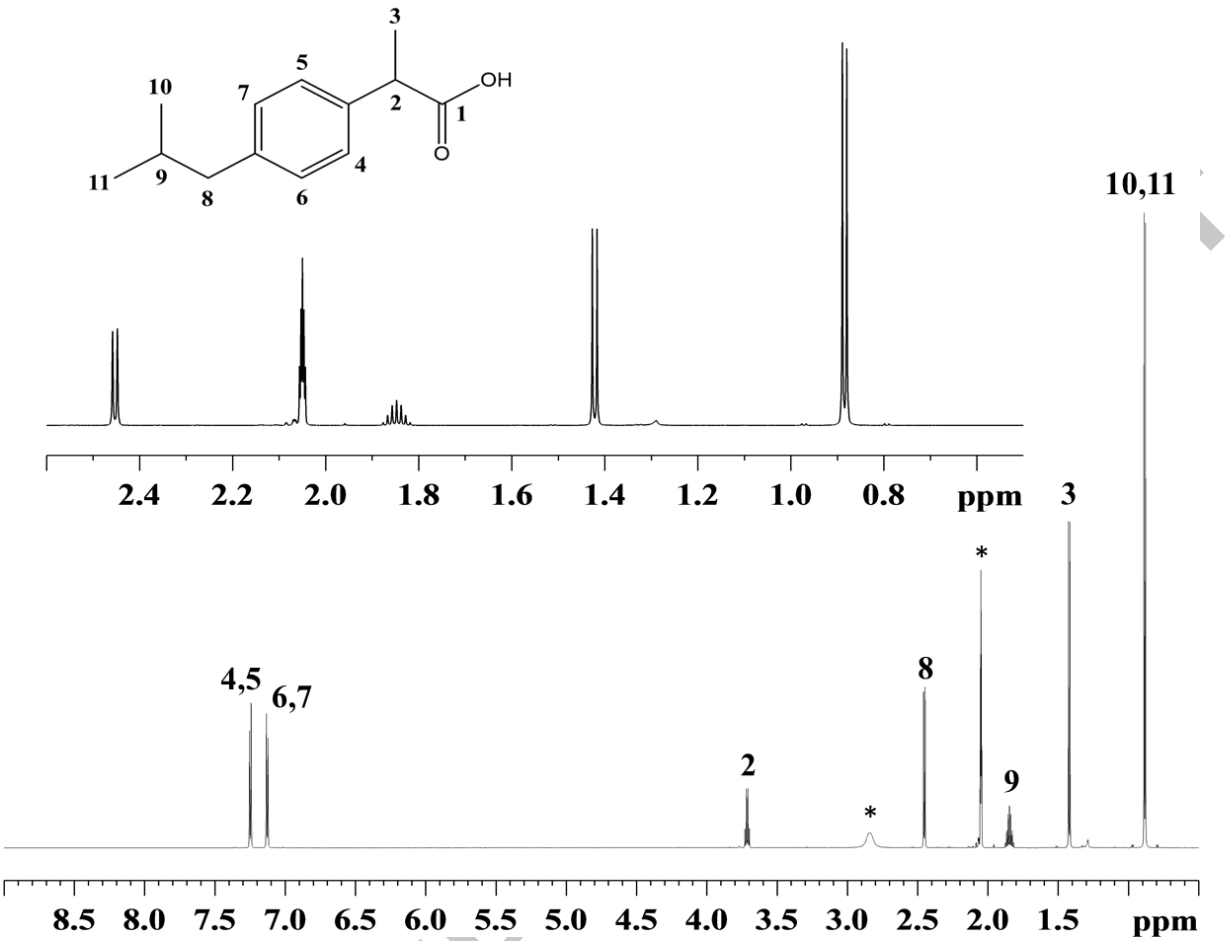


Figure 1. ^{1}H-NMR (700 MHz) of Ibuprofen in acetone-d_6. The asterisks indicate the residual solvent peaks.

The NMR characterization of the carbon atoms was obtained by a [^{1}H-^{13}C]-HSQC 2D NMR experiment also performed in acetone-d_6 (Figure 2). The cross peaks falling at 7.25/128.0 and 7.13/129.7 ppm (^{1}H/^{13}C) were attributed to the aromatic C(4/5)H and C(6/7)H. The cross peaks at 2.45/45.40, 1.85/30.9 and 0.88/22.4 ppm (^{1}H/^{13}C) were assigned to C(8), C(9) and C(10/11), respectively (see Figure 1 for numbering of atoms). The chiral carbon C(2) resonates at 45.18 ppm while the methyl carbon C(3) falls at 18.9 ppm (Figure 2).

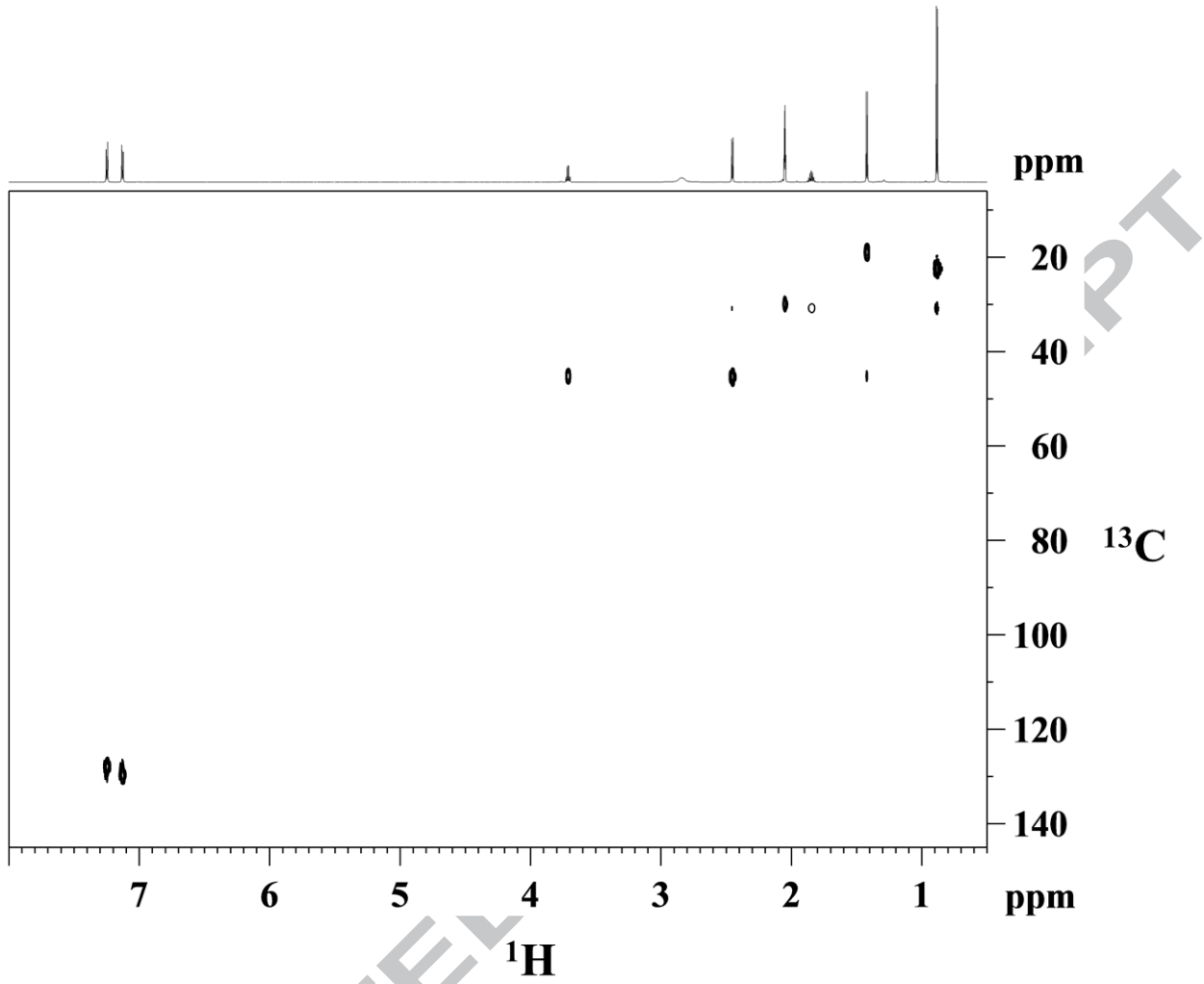
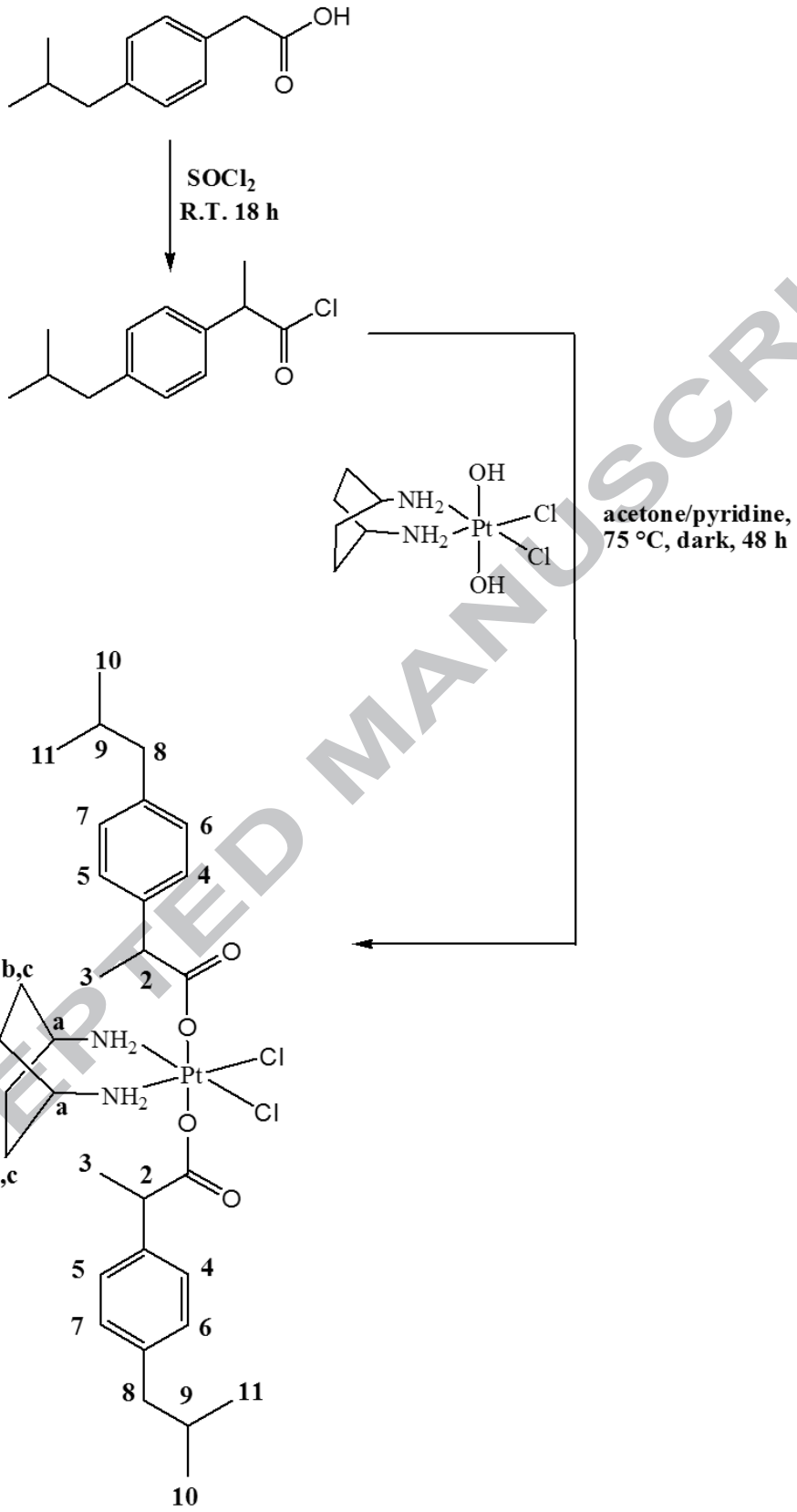


Figure 2. [^{1}H-^{13}C] HSQC 2D (176.05 MHz, ^{13}C) spectrum of Ibuprofen in acetone-d_6.

Synthesis and characterization of *cis*-*trans*-*cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)]

The Pt(IV) conjugate *cis*-*trans*-*cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] was synthesized by esterification of the dihydroxido platinum(IV) precursor *cis,trans,cis*-[$PtCl_2(OH)_2$(*cis*-1,4-DACH)] with the acyl chloride of Ibuprofen, prepared starting from Ibuprofen and $SOCl_2$ (Scheme 1). The reaction was carried out in the presence of pyridine to neutralize the released HCl and in the dark to avoid the possible spontaneous solvent-assisted reduction of *cis,trans,cis*-[$PtCl_2(OH)_2$(*cis*-1,4-DACH)] to kiteplatin.[27,36–38]



Scheme 1. Synthesis of the conjugate Pt(IV) complex *cis*-*trans*-*cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)].

The ESI-MS spectrum of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] showed the presence of a peak at *m/z* = 813.26 corresponding to $[M+Na]^+$ as also confirmed by the isotopic pattern (data not shown).

The ^{1}H NMR spectrum of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] in acetone-d_6 is reported in Figure 3. The doublets falling at 7.28 and 7.06 ppm were assigned to the aromatic protons of Ibuprofen H(4/5) and H(6/7), respectively. The multiplet at 3.68 ppm was attributed to the chiral -CH of the coordinated Ibuprofen. This signal appears as a superimposition of two or more quartets, probably due to the presence of different diasteroisomers in solution (we indeed used a racemic Ibuprofen). The singlet at 3.68 ppm shows a cross-peak with the signals in the region 1.35-1.37 ppm in the COSY-spectrum (cross-peak **a** in Figure 4). These latter signals at 1.35-1.37 ppm confirm the presence of more diasteroisomers in solution and were attributed to the protons of methyl groups bound to the chiral carbons. The singlet with Pt satellites at 3.26 ppm, assigned to the methynic protons of coordinated DACH, has a cross-peak with the broad signals at 1.56-1.47 ppm in the 2D COSY spectrum (cross-peak **b** in Figure 4). The latter two broad signals were assigned to the methylene protons of *cis*-1,4-DACH. The multiplet falling at 1.82 ppm was assigned to the H(9) protons (see Scheme 1 for the numbering of protons). Indeed, the cross-peak **d** in the 2D COSY spectrum (Figure 4) correlates the signal of C(9)H with that of the methyl groups C(10/11)H_3. The doublet at 2.43 ppm, integrating for two protons, was attributed to C(8)H_2 of Ibuprofen and it was correlated to C(9)H through a cross-peak (cross-peak **c** in Figure 4). The doublet integrating for twelve protons at 0.87 ppm was assigned to the isopropyl methyl groups of Ibuprofen. Finally, the ^{1}H-NMR spectrum shows four very low broad signals at 8.41, 8.04, 7.62 and 7.22 ppm attributed to the aminic protons of coordinated *cis*-1,4-DACH. In particular, the signal at 7.22 ppm not visible in the ^{1}H-NMR spectrum was assigned with the help of the 2D-COSY experiment (data not shown).

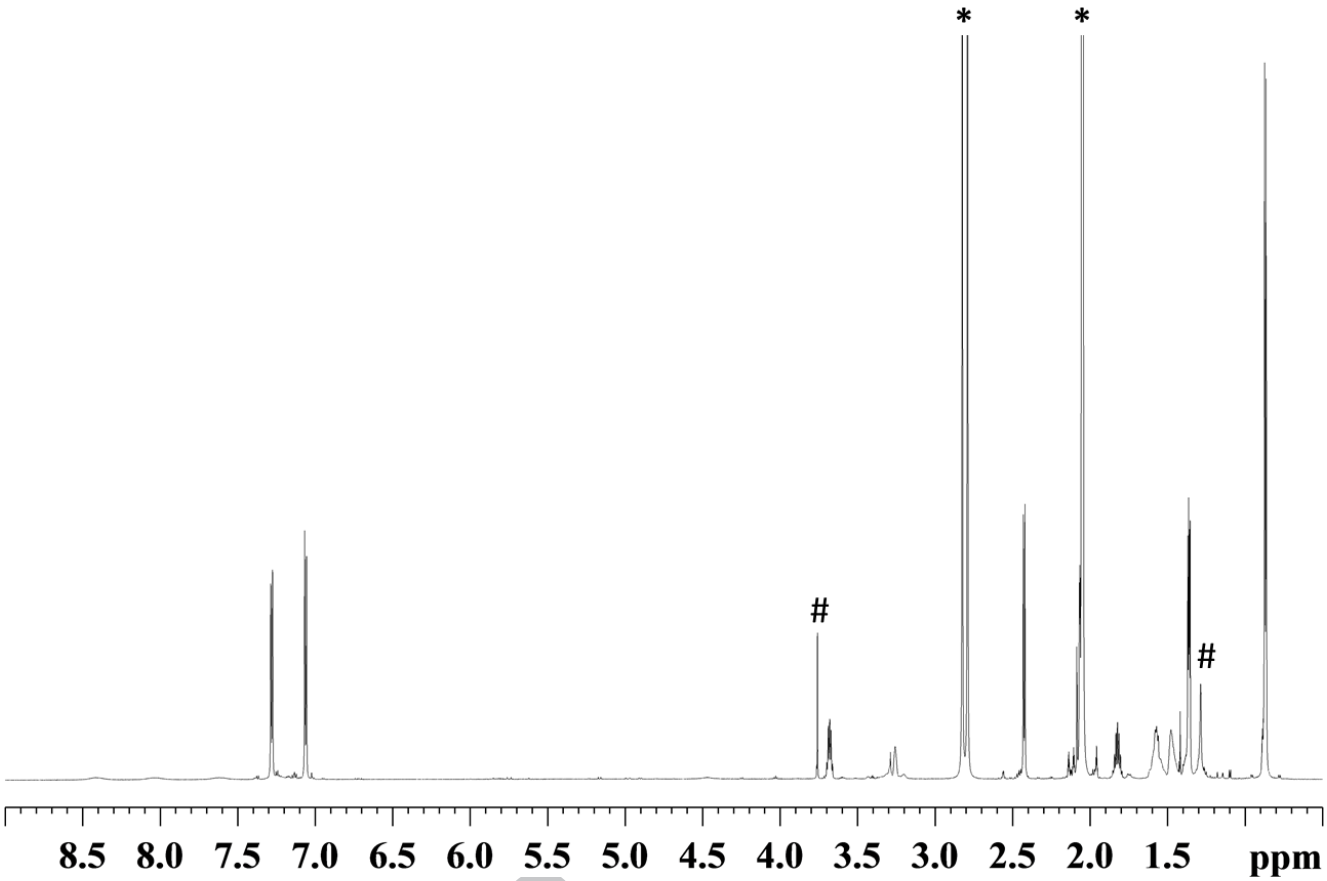


Figure 3. ^{1}H NMR (700 MHz) spectrum of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] in acetone-d_6. (* indicate the residual solvent peaks, # indicate impurities of the solvent).

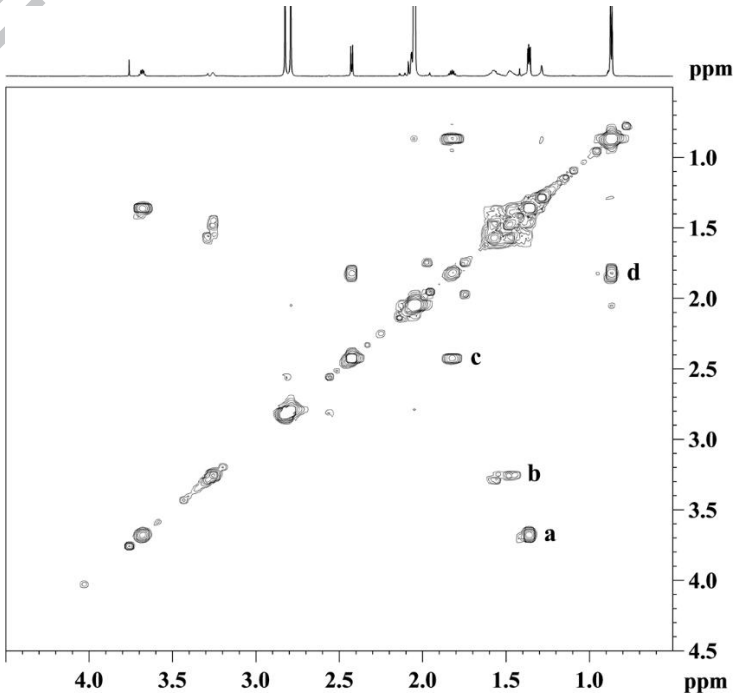


Figure 4. Selected region of the 2D COSY (700 MHz) spectrum obtained for *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] in acetone-d_6.

The NMR characterization of the carbon atoms of the complex was obtained by a [^{1}H-^{13}C]-HSQC 2D NMR experiment also performed in acetone-d_6 (Figure 5). The cross peaks falling at 3.26/50.9, 1.56/20.09 and 1.47/20.09 ppm (^{1}H/^{13}C), were attributed to the methynic and methylenic groups of DACH, respectively. The aromatic carbons of Ibuprofen resonate at 128.1 and 129.4 ppm. The ^{13}C signals at 48.1 and 19.4 ppm were assigned to the chiral carbons C(2) and to the methyl carbons C(3), respectively.

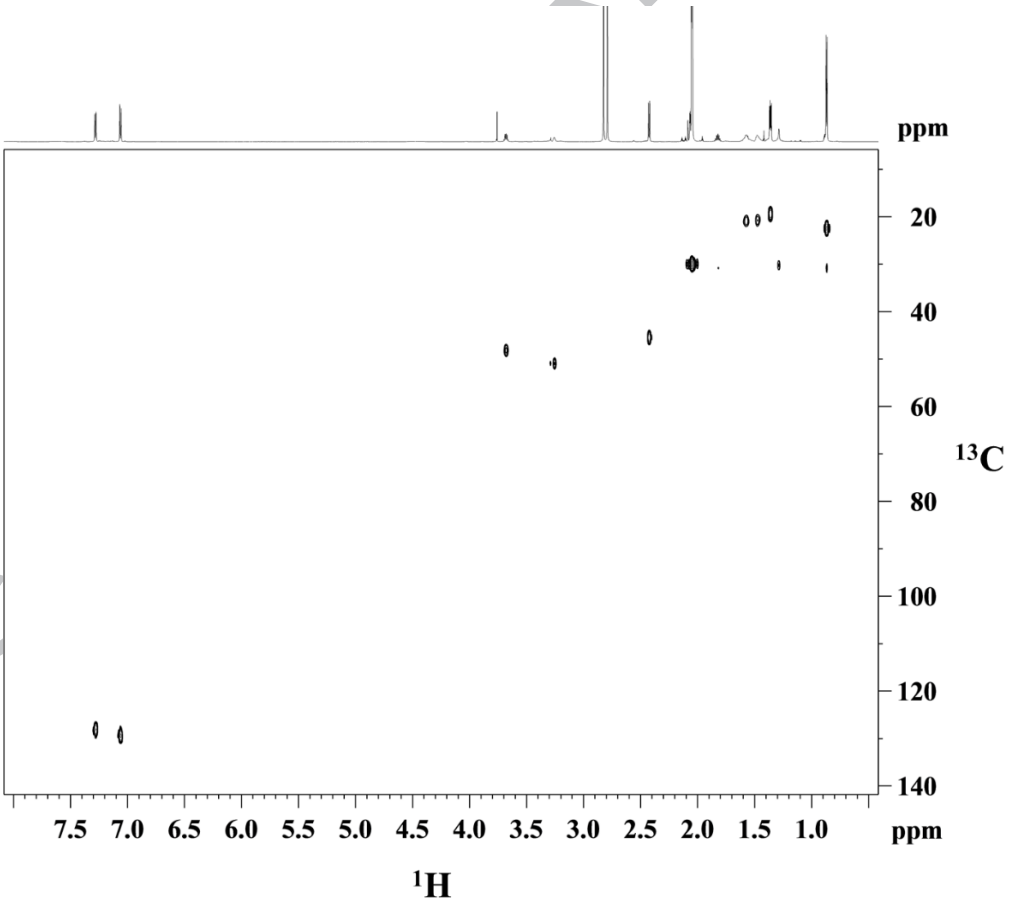


Figure 5. [^{1}H-^{13}C] HSQC 2D (176.05 MHz, ^{13}C) spectrum of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] in acetone-d_6.

The ^{195}Pt-NMR of the Pt(IV) compound is reported in Figure 6. It shows a single peak at 1099 ppm which is in the range typical for a Pt(IV) atom in a $Cl_2O_2N_2$ coordination environment.[39,40]

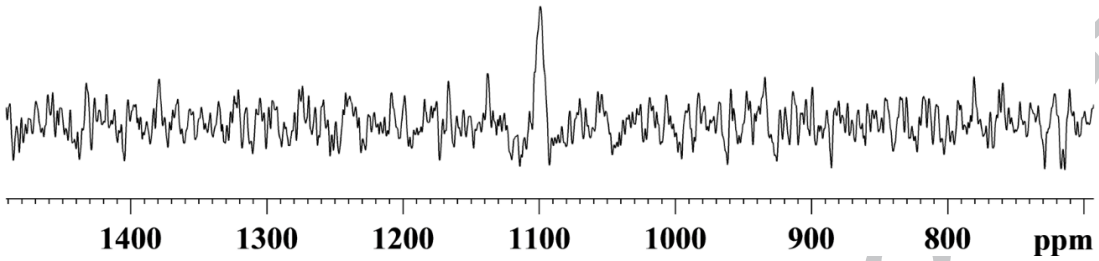


Figure 6. ^{195}Pt-NMR (64.52 MHz) spectrum of *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] in acetone-d_6.

The ^{1}H, ^{13}C and ^{195}Pt chemical shifts are in good agreement with those already reported for the analogous Pt(IV)-Ibuprofen derivative *cis,trans,cis*-[$PtCl_2$(Ibuprofen)$_2$(NH_3)$_2$].[19]

Electrochemical measurements

The reduction potential of Pt(IV) complexes plays a key role in their activity. Therefore, CV electrochemical measurements of blank and Pt(IV) complex solutions were recorded on a glassy carbon electrode in DMSO solvent. Contrary to the expectations, the CV plot of the electrolyte solution showed the electro activity of a couple peak with Ep^c = -0.83 V (vs SCE). Experiments were then carried out to exclude solvent and electrolyte impurities, along with water traces due to the well-known hygroscopic nature of the alkyl ammonium salt. Moreover, the scan rate was varied from 0.10 to 0.25 Vs^{-1} and it was determined that the ip^c was directly proportional to the square root of the scan rate (Figure S1 in Supporting Information), meaning that the electro-activity was due to a dissolved interfering compound rather than to an adsorbate. It was not possible to fully elucidate the nature of the interfering compound, most likely it derives from partial degradation of the ammonium salt to the amine, but its peak was taken into proper account in the analysis of the Pt(IV) complex voltammogram.

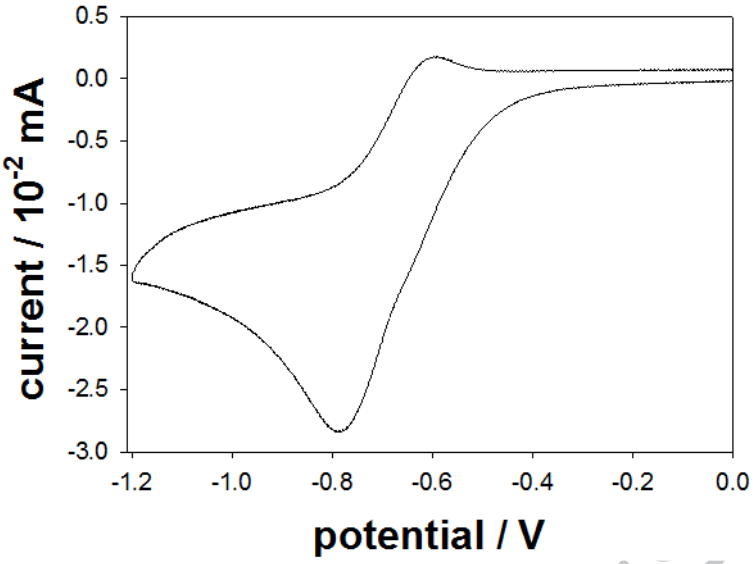


Figure 7. Cyclic voltammogram recorded at a glassy carbon electrode on a DMSO solution containing 0.1M $[NBu_4][PF_6]$ as supporting electrolyte and 1.0 mM of Pt(IV) complex. Scan rate 0.20 V s^{-1}.

The CV plot (Figure 7) clearly shows that the cathodic peak area is larger than the anodic one, this can be a consequence of the activity of the analyte being superimposed to that of the electrolyte. By subtracting the contribute of the latter, the Ep^c of the platinum complex was unequivocally measured and found equal to -0.93 V (corrected vs SCE) (see also Figure S2 in Supporting Information). This value is compatible with the reduction potential of similar Pt(IV) complexes [41] and with a chemically irreversible $2e^-$ reduction due to the detachment of the two axial ligands upon reduction of the octahedral Pt(IV) complex to the square-planar Pt(II) species.[27] Moreover, it was observed that the kinetics of the process is highly affected by the steric hindrance of the axial ligands. The electrochemical behaviour at low scan rates indicated a slow transfer process to the electrode causing hysteresis phenomena. For this reason, the experimental scan rate was set unusually high.

In conclusion, the CV investigation shows that the Ep^c measured for the Pt(IV) conjugate with Ibuprofen is suitable for the intracellular reduction necessary to activate this kind of prodrugs into the pharmacologically active species.

Cytotoxicity assay

In order to evaluate the antitumor activity of the Pt(IV)–Ibuprofen prodrug, *in vitro* cytotoxic assays were performed on two colorectal carcinoma cell lines both characterized by no expression of COX-2 protein [19,32,42]: HCT-116 and HCT-15. Data from MTT assay after 72 h of incubation were compared with those obtained for Ibuprofen, kiteplatin, and cisplatin used as reference compounds. The IC_{50} values calculated from the dose-response curves reported in Figure 8 are shown in Table 1. According to already reported data,[32] Ibuprofen is not cytotoxic in the range of concentrations analyzed, with IC_{50} values >800 and >708 μM for HCT-15 and HCT-116 cell lines, respectively. Interestingly, the Pt(IV) conjugate *cis-trans*-cis-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] is more active than the precursor kiteplatin and the reference compound cisplatin, with IC_{50} values of 0.45 and 0.26 μM for HCT-15 and HCT-116, respectively. Specifically, the *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] conjugate is *ca*. 25 times more effective than its Pt(II) precursor kiteplatin (IC_{50} of 11 and 7 μM for HCT-115 and HCT-116, respectively) and *ca*. 40 times more active than cisplatin (IC_{50} of 11 and 17 μM for HCT-116 and HCT-15 cell lines, respectively). In a previous work,[31] the Pt(IV) derivative of kiteplatin with the biologically inactive hydroxido ligands, *cis-trans-cis*-[$PtCl_2(OH)_2$(*cis*-1,4-DACH)], was found to possess an IC_{50} value of 10.32 μM (MTT test; 72 h) against HCT-15 tumor cell line. This finding indicates that the higher cytotoxicity found in the present investigation for *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] is likely due to the presence of ibuprofen ligands in the axial positions.

Although the *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)] complex had very marked activity, the mechanism responsible for the greater activity of the conjugate with respect to the

precursor complex is difficult to identify. It cannot be related to an additional inhibition of COX-2 by Ibuprofen, since both cell lines do not express this enzyme. A possible explanation could be a better cellular uptake of the conjugate Pt(IV) complex with respect to both kiteplatin and cisplatin. In fact, the two Ibuprofen axial ligands would confer a greater lipophilicity to the Pt(IV) complex with consequent facilitate transport through the cell membrane. The axial ibuprofen ligands are benzoate-like ligands for which in a previous paper was demonstrated that the loss of the axial ligands is slow ($t_{1/2}$ of *ca.* 26 h).[31] Hence, we are quite confident that also the hydrolysis of ibuprofen is slow. Moreover, also the reduction potential measured by cyclic voltammetry are compatible with an intracellular reduction of the Pt(IV) complex by biological reductants. However, we cannot exclude the intervention of some other type of synergistic effect between kiteplatin and Ibuprofen within the tumor cells and, to clarify this point, other experiments such as cell uptake and cytotoxicity tests with combinations of kiteplatin and Ibuprofen (administered separately and at a dose of 1:2) are planned. The resulted combination index will clarify whether there is a synergistic action between the two compounds.

Table 1. IC_{50} values [µM] determined in cell viability assays against human colorectal cancer cell lines HCT-15 and HCT-116 (incubation times of 72 h). Data are mean ± SD (n=3).

Compounds	**IC_{50} (µM)**	
	HCT15	**HCT116**
cis-*trans*-*cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)]	0.45 ± 0.04	0.26 ± 0.03
Kiteplatin	11±1	7 ±1
Cisplatin	17±2	11±1
Ibuprofen	>800	708 ± 8

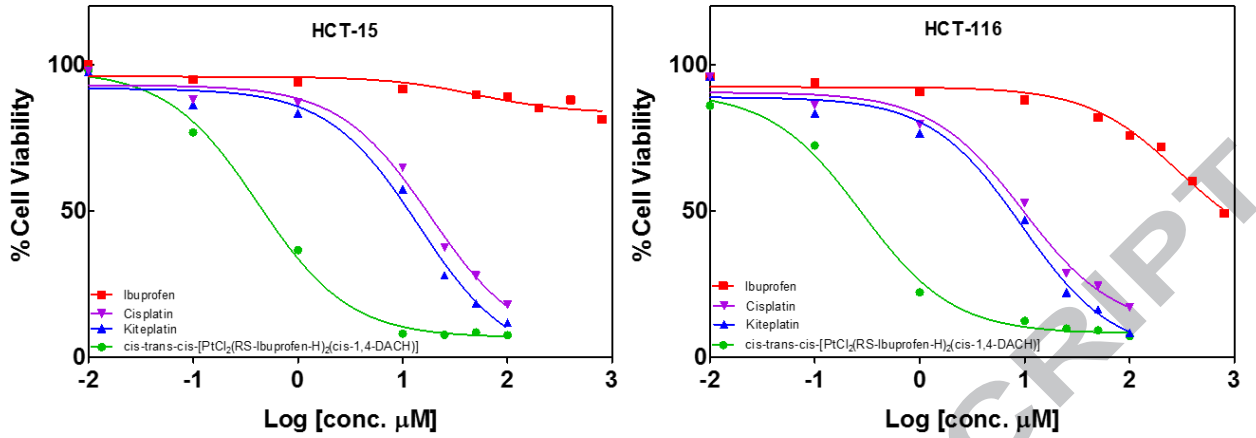


Figure 8. Cell viability profiles of HCT-15 and HCT-116 cells determined after 72 h of incubation with *cis-trans-cis*-[$PtCl_2$(RS-Ibuprofen-H)$_2$(*cis*-1,4-DACH)], Kiteplatin, Cisplatin and Ibuprofen. Data are mean ± SD (n=3).

Conclusions

In this work we have synthesised, characterized and tested *in vitro* a Pt(IV) prodrug of kiteplatin, *cis-trans-cis*-[$PtCl_2$(*RS*-Ibuprofen-H)$_2$(*cis*-1,4-DACH)], having in the axial positions two molecules of Ibuprofen. The complex was designed with the aim of potentiating the cytotoxic effect of kiteplatin by exploiting the increased intracellular uptake of the compound (due to the high lipophilicity of the axial ligands) and the potential dual cytotoxic and anti-inflammatory activity.

In order to evaluate the propensity of the Pt(IV) prodrug to undergo intracellular activation to the corresponding Pt(II) active species by cytoplasmic reducing agents (such as glutathione and ascorbic acid), the reduction potential of the conjugate was measured electrochemically. The reduction potential of the conjugate resulted comparable to those of other reported Pt(IV) carboxylate complexes, ensuring *in vivo* stability in blood during transport but enabling its intracellular reduction and consequent activation.

The cytotoxic activity of the complex resulted remarkably potentiated by the presence of the lipophilic axial ligands reaching nanomolar activity. Thus, the Pt(IV) conjugate showed a markedly increased cytotoxicity compared to both cisplatin and the Pt(II) precursor kiteplatin. It is possible

that the coordinated Ibuprofen molecules promote the transport and the accumulation of the complex in tumour cells. In addition to the increased uptake, Ibuprofen could also exert an anti-inflammatory action mediated by inhibition of the enzymes COX-1 and COX-2, which are overexpressed in tumors. Moreover, the use of a conjugate with Pt-bound Ibuprofen could also ensure the same cellular uptake and biodistribution for both the anti-inflammatory and the cytotoxic drugs in the exact ratio of 2:1. Experiments with tumor cells overexpressing COX-1 and/or COX-2 enzymes will tell if an anti-inflammatory action of Ibuprofen can further increase the antitumor efficacy.

In conclusion, the nanomolar *in vitro* cytotoxicity of this new Pt(IV)-Ibuprofen prodrug of kiteplatin makes this compound clinically attractive as antitumor agent for its potential capability to overcome the side effects of the conventional Pt-based drugs and to act as a dual acting drug. Extension to other tumor cells and investigation of drug uptake by cells and anti-inflammatory properties are under way.

Acknowledgments

We acknowledge the University of Bari (Italy), the Italian Ministero dell'Università e della Ricerca, the Inter-University Consortium for Research on the Chemistry of Metal Ions in Biological Systems (C.I.R.C.M.S.B.), and the European Union (COST CM1105: Functional metal complexes that bind to biomolecules) for support. We are grateful to Prof. Giovanni Lentini (Dipartimento di Farmacia-Scienze del Farmaco, Università di Bari, Italy) for his precious assistance in the extraction of Ibuprofen from expired commercial tablets and to Ms. Ilaria Orlando for the preparation and analyses of the Pt compounds.

References

[1] L. Kelland, The resurgence of platinum-based cancer chemotherapy., Nat. Rev. Cancer. 7 (2007) 573–584. doi:10.1038/nrc2167.

[2] N.J. Wheate, S. Walker, G.E. Craig, R. Oun, The status of platinum anticancer drugs in the clinic and in clinical trials., Dalton Trans. 39 (2010) 8113–8127. doi:10.1039/c0dt00292e.

[3] M. Galanski, M. Jakupec, B. Keppler, Update of the Preclinical Situation of Anticancer Platinum Complexes: Novel Design Strategies and Innovative Analytical Approaches, Curr. Med. Chem. 12 (2005) 2075–2094. doi:10.2174/0929867054637626.

[4] T.C. Johnstone, K. Suntharalingam, S.J. Lippard, The Next Generation of Platinum Drugs: Targeted Pt(II) Agents, Nanoparticle Delivery, and Pt(IV) Prodrugs, Chem. Rev. 116 (2016) 3436–3486. doi:10.1021/acs.chemrev.5b00597.

[5] E. Gabano, M. Ravera, D. Osella, The drug targeting and delivery approach applied to pt-antitumour complexes. A coordination point of view., Curr. Med. Chem. 16 (2009) 4544–4580. doi:10.2174/092986709789760661.

[6] X. Wang, Z. Guo, Targeting and delivery of platinum-based anticancer drugs, Chem. Soc. Rev. (2013) 202–224. doi:10.1039/c2cs35259a.

[7] R.G. Kenny, S.W. Chuah, A. Crawford, C.J. Marmion, Platinum(IV) Prodrugs - A Step Closer to Ehrlich's Vision?, Eur. J. Inorg. Chem. 2017 (2017) 1596–1612. doi:10.1002/ejic.201601278.

[8] D. Gibson, Platinum(IV) anticancer prodrugs - hypotheses and facts, Dalt. Trans. 45 (2016) 12983–12991. doi:10.1039/C6DT01414C.

[9] E. Gabano, M. Ravera, D. Osella, Pros and cons of bifunctional platinum(iv) antitumor prodrugs: two are (not always) better than one, Dalt. Trans. 43 (2014) 9813. doi:10.1039/c4dt00911h.

[10] M.D. Hall, H.R. Mellor, R. Callaghan, T.W. Hambley, Basis for Design and Development of Platinum(IV) Anticancer Complexes, J. Med. Chem. 50 (2007) 3403–3411. doi:10.1021/jm070280u.

[11] M.D. Hall, T.W. Hambley, Platinum(IV) antitumour compounds: their bioinorganic chemistry, Coord. Chem. Rev. 232 (2002) 49–67. doi:10.1016/S0010-8545(02)00026-7.

[12] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, Inflammation, and Cancer, Cell. 140

(2010) 883–899. doi:10.1016/j.cell.2010.01.025.

[13] A.M. De Marzo, E.A. Platz, S. Sutcliffe, J. Xu, H. Grönberg, C.G. Drake, et al., Inflammation in prostate carcinogenesis, Nat. Rev. Cancer. 7 (2007) 256–269. doi:10.1038/nrc2090.

[14] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature. 420 (2002) 860–867. doi:10.1038/nature01322.

[15] J.C. Frölich, A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes., Trends Pharmacol. Sci. 18 (1997) 30–4. http://www.ncbi.nlm.nih.gov/pubmed/9114728.

[16] W. Neumann, B.C. Crews, M.B. Sárosi, C.M. Daniel, K. Ghebreselasie, M.S. Scholz, et al., Conjugation of Cisplatin Analogues and Cyclooxygenase Inhibitors to Overcome Cisplatin Resistance, ChemMedChem. 10 (2015) 183–192. doi:10.1002/cmdc.201402353.

[17] F.P. Intini, J. Zajac, V. Novohradsky, T. Saltarella, C. Pacifico, V. Brabec, et al., Novel Antitumor Platinum(II) Conjugates Containing the Nonsteroidal Anti-inflammatory Agent Diclofenac: Synthesis and Dual Mechanisms of Antiproliferative Effects, Inorg. Chem. 56 (2017) 1483–1497. doi:10.1021/acs.inorgchem.6b02553.

[18] R.K. Pathak, S. Marrache, J.H. Choi, T.B. Berding, S. Dhar, The Prodrug Platin- A : Simultaneous Release of Cisplatin and Aspirin, Angew. Chemie Int. Ed. 53 (2014) 1963–1967. doi:10.1002/anie.201308899.

[19] W. Neumann, B.C. Crews, L.J. Marnett, E. Hey-Hawkins, Conjugates of Cisplatin and Cyclooxygenase Inhibitors as Potent Antitumor Agents Overcoming Cisplatin Resistance, ChemMedChem. 9 (2014) 1150–1153. doi:10.1002/cmdc.201402074.

[20] Q. Cheng, H. Shi, H. Wang, Y. Min, J. Wang, Y. Liu, The ligation of aspirin to cisplatin demonstrates significant synergistic effects on tumor cells, Chem. Commun. 50 (2014) 7427. doi:10.1039/c4cc00419a.

[21] N. Margiotta, C. Marzano, V. Gandin, D. Osella, M. Ravera, E. Gabano, et al., Revisiting [PtCl2(cis-1,4-DACH)]: An underestimated antitumor drug with potential application to the treatment of oxaliplatin-refractory colorectal cancer, J. Med. Chem. 55 (2012) 7182–7192. doi:10.1021/jm3006838.

[22] J. Kasparkova, T. Suchankova, A. Halamikova, L. Zerzankova, O. Vrana, N. Margiotta, et al., Cytotoxicity, cellular uptake, glutathione and DNA interactions of an antitumor large-ring PtII chelate complex incorporating the cis-1,4-diaminocyclohexane carrier ligand, Biochem. Pharmacol. 79 (2010) 552–564. doi:10.1016/j.bcp.2009.09.019.

[23] V. Brabec, J. Malina, N. Margiotta, G. Natile, J. Kasparkova, Thermodynamic and mechanistic insights into translesion DNA synthesis catalyzed by Y-family DNA polymerase across a bulky double-base lesion of an antitumor platinum drug, Chem. - A Eur. J. 18 (2012) 15439–15448. doi:10.1002/chem.201202117.

[24] M. Lelli, N. Roveri, C. Marzano, J.D. Hoeschele, A. Curci, N. Margiotta, et al., Hydroxyapatite nanocrystals as a smart, pH sensitive, delivery system for kiteplatin, Dalt. Trans. 45 (2016) 13187–13195. doi:10.1039/C6DT01976E.

[25] N. Margiotta, E. Petruzzella, J.A. Platts, S.T. Mutter, R.J. Deeth, R. Ranaldo, et al., DNA fragment conformations in adducts with Kiteplatin, Dalt. Trans. 44 (2015) 3544–3556. doi:10.1039/c4dt01796j.

[26] E. Petruzzella, A. Curci, N. Margiotta, G. Natile, J.D. Hoeschele, Kiteplatin: Differential binding between GSH and GMP, Inorganica Chim. Acta. 452 (2016) 130–136. doi:10.1016/j.ica.2016.04.004.

[27] E. Petruzzella, N. Margiotta, M. Ravera, G. Natile, NMR investigation of the spontaneous thermal- and/or photoinduced reduction of trans dihydroxido Pt(IV) derivatives, Inorg. Chem. 52 (2013) 2393–2403. doi:10.1021/ic302100x.

[28] R. Ranaldo, N. Margiotta, F.P. Intini, C. Pacifico, G. Natile, Conformer distribution in (cis-1,4-DACH)bis(guanosine-5′-phosphate) platinum(II) adducts: A reliable model for DNA adducts of antitumoral cisplatin, Inorg. Chem. 47 (2008) 2820–2830. doi:10.1021/ic702202x.

[29] S.T. Mutter, N. Margiotta, P. Papadia, J.A. Platts, Computational evidence for structural consequences of kiteplatin damage on DNA, J. Biol. Inorg. Chem. 20 (2015) 35–48. doi:10.1007/s00775-014-1207-5.

[30] N. Margiotta, S. Savino, N. Denora, C. Marzano, V. Laquintana, A. Cutrignelli, et al., Encapsulation of lipophilic kiteplatin Pt(<scp>iv</scp>) prodrugs in PLGA-PEG micelles, Dalt. Trans. 45 (2016) 13070–13081. doi:10.1039/C6DT00763E.

[31] N. Margiotta, S. Savino, C. Marzano, C. Pacifico, J.D. Hoeschele, V. Gandin, et al.,

Cytotoxicity-boosting of kiteplatin by Pt(IV) prodrugs with axial benzoate ligands, J. Inorg. Biochem. 160 (2016) 85–93. doi:10.1016/j.jinorgbio.2015.11.028.

[32] A. Janssen, T.J. Maier, S. Schiffmann, O. Coste, M. Seegel, G. Geisslinger, et al., Evidence of COX-2 independent induction of apoptosis and cell cycle block in human colon carcinoma cells after S- or R-ibuprofen treatment, Eur. J. Pharmacol. 540 (2006) 24–33. doi:10.1016/j.ejphar.2006.04.030.

[33] A. Janssen, S. Schiffmann, K. Birod, T.J. Maier, I. Wobst, G. Geisslinger, et al., p53 is important for the anti-proliferative effect of ibuprofen in colon carcinoma cells, Biochem. Biophys. Res. Commun. 365 (2008) 698–703. doi:10.1016/j.bbrc.2007.11.051.

[34] P.S. Pregosin, Platinum-195 nuclear magnetic resonance, Coord. Chem. Rev. 44 (1982) 247–291. doi:10.1016/S0010-8545(00)80523-8.

[35] V. Laquintana, N. Denora, A. Lopalco, A. Lopedota, A. Cutrignelli, F.M. Lasorsa, et al., Translocator Protein Ligand–PLGA Conjugated Nanoparticles for 5-Fluorouracil Delivery to Glioma Cancer Cells, Mol. Pharm. 11 (2014) 859–871. doi:10.1021/mp400536z.

[36] H.A. Wee, I. Khalaila, C.S. Allardyce, L. Juillerat-Jeanneret, P.J. Dyson, Rational design of platinum(IV) compounds to overcome glutathione-S- transferase mediated drug resistance, J. Am. Chem. Soc. 127 (2005) 1382–1383. doi:10.1021/ja0432618.

[37] V. Gandin, C. Marzano, G. Pelosi, M. Ravera, E. Gabano, D. Osella, *trans* , *cis* , *cis* -Bis(benzoato)dichlorido(cyclohexane-1 *R* ,2 *R* -diamine)platinum(IV): a Prodrug Candidate for the Treatment of Oxaliplatin-Refractory Colorectal Cancer, ChemMedChem. 9 (2014) 1299–1305. doi:10.1002/cmdc.201400061.

[38] M. Galanski, B.K. Keppler, Carboxylation of Dihydroxoplatinum(IV) Complexes via a New Synthetic Pathway, Inorg. Chem. 35 (1996) 1709–1711. doi:10.1021/ic9509490.

[39] W. Geiger, Organometallic radical processes, Trogler, W. C., Ed.; Elsevier Amsterdam, Netherlands. (1990).

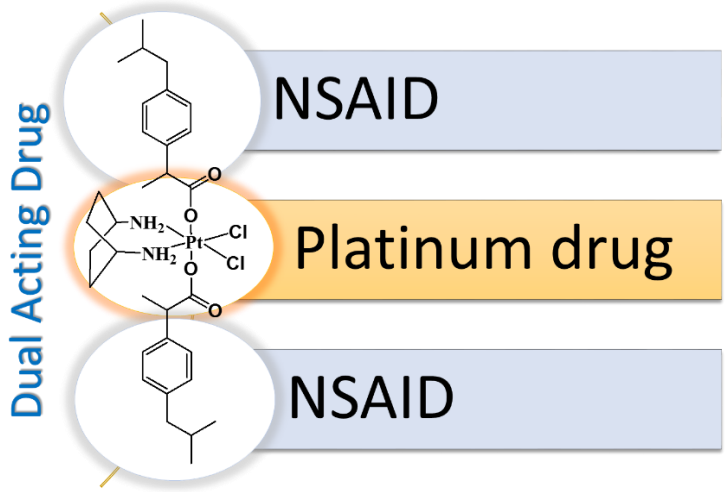
[40] S.J. Berners-Price, L. Ronconi, P.J. Sadler, Insights into the mechanism of action of platinum anticancer drugs from multinuclear NMR spectroscopy, Prog. Nucl. Magn. Reson. Spectrosc. 49 (2006) 65–98. doi:10.1016/j.pnmrs.2006.05.002.

[41] S. Choi, C. Filotto, M. Bisanzo, S. Delaney, D. Lagasee, J.L. Whitworth, et al., Reduction

and Anticancer Activity of Platinum(IV) Complexes, Inorg. Chem. 37 (1998) 2500–2504. doi:10.1021/ic971047x.

[42] B.R. Subramaniya, G. Srinivasan, S.S. Mohammed Sadullah, N. Davis, L. Baddi Reddi Subhadara, D. Halagowder, et al., Apoptosis Inducing Effect of Plumbagin on Colonic Cancer Cells Depends on Expression of COX-2, PLoS One. 6 (2011) e18695. doi:10.1371/journal.pone.0018695.

Table of Contents (TOC)



Highlights

A Pt(IV) prodrug of kiteplatin having in the axial positions two molecules of Ibuprofen has been synthesised.

The reduction potential of the conjugate ensures *in vivo* stability in blood during transport and intracellular reduction with release of the active species.

The cytotoxic activity of the complex results remarkably potentiated reaching nanomolar activity.