Anti-tumor platinum (IV) complexes bearing the anti-inflammatory drug naproxen in the axial position

Dina A. Tolan | Yasser K. Abdel-Monem | Mohamed A. El-Nagar

Department of Chemistry, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt

Correspondence
Dina A. Tolan, Department of Chemistry, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt.
Email: d_tolan2005@yahoo.com

The role of inflammation in cancer generation is gaining importance in the field of cancer research. The chemo-anti-inflammatory strategy that involves using non-steroidal anti-inflammatory drug compounds as effective anti-tumor agents is being acceded globally. In the present study, seven new Pt (IV) complexes based on cisplatin, carboplatin and oxaliplatin scaffold bearing the anti-inflammatory drug naproxen in the axial position were synthesized and characterized by elemental analysis, ESI-MS, Fourier transform-infrared, $^1$H- and $^{195}$Pt-NMR spectroscopy. The reduction behavior in the presence of ascorbic acid was studied using high-performance liquid chromatography. The cytotoxicity against two human breast cell lines and the anti-inflammatory properties were evaluated. All the complexes are able to promote a comparable activity, with average three- and 13-fold more cytotoxic than cisplatin against MCF7 and MDA-MB-231 cell lines, respectively. The complexes show remarkable anti-inflammatory effects, which indicated their potential in treating cancer associated with inflammation and reducing side-effects of chemotherapy.

KEYWORDS
anti-cancer, inflammation, naproxen, platinum (IV), prodrugs

1 | INTRODUCTION

Breast cancer is the most common cancer among women.[1] The invasive breast tumors with estrogen and progesterone and HER-2-negative expressions are known as ‘triple-negative breast cancer’ (TNBC), and constitute 10–20% of the breast cancer population.[1,2] Treatment of patients with TNBC has been challenging due to the heterogeneity of the disease and the lack of well-defined molecular targeting.[3] Common treatments like hormone therapy and drugs that target estrogen, progesterone and HER-2 are ineffective, while using chemotherapy is still an effective option.[1–3] Therefore, finding new medications that can treat this kind of breast cancer is a critical demand. Chemotherapy remains the primary clinical treatment for TNBC in both the early and advanced stages of the disease; however, the absence of recognized molecular targets is considered a big challenge in the treatment of TNBC.[4] Despite the multiple studies performed to improve the clinical treatment of TNBC, none has been succeeded as a standard therapy.[5] TNBC is not sensitive to conventional targeted therapy, but the studies done to date have shown that TNBC is highly sensitive to certain classes of DNA-damaging agents.[6,7] Platinum (II) complexes (cisplatin, carboplatin, oxaliplatin) are one of the most effective groups of anti-cancer drugs that target DNA (Figure 1). Platinum drugs bind to purine bases in DNA to form DNA adducts, resulting in double-strand DNA breaks that induce cancer cell death. Despite their success in treating different tumors, Pt (II) drugs have some drawbacks that limit their usage, such as their high toxicity, severe side-effects and...
resistance.\textsuperscript{[8–14]} Cancer cells might become resistant to these Pt (II) drugs due to detoxification, decreased cellular uptake, DNA repair, increased drug efflux and diminished apoptosis. Platinum (IV) prodrugs are considered one of the approaches developed to overcome the drawbacks of the Pt (II) drugs.\textsuperscript{[14–18]} The octahedral Pt (IV) complexes are activated by reduction inside the cell to the active platinum (II) counterparts before binding to their target DNA (Figure 2).\textsuperscript{[19–22]} The axial ligands in Pt (IV) complexes that are released upon reduction can be used to control important properties of the drug, such as stability, reduction potential and lipophilicity.\textsuperscript{[22–26]} Lipophilicity plays a vital role in the activity of the platinum drugs.\textsuperscript{[27]} The improvement of lipophilicity decreases the drug resistance by elevating passive diffusion through the cell membrane.\textsuperscript{[28]} Moreover, the axial coordination site can serve as a binding site for other biologically active ligands to form a single molecule that combines two biologically active compounds called dual-threat pharmaceutical agents.\textsuperscript{[29–34]}

On the other hand, a large number of studies have showed the effect of chronic inflammation in tumorgenesis,\textsuperscript{[35,36]} and the role of using non-steroidal anti-inflammatory drugs (NSAIDs) as anti-cancer agents.\textsuperscript{[37–40]} Although the use of NSAIDs can cause some problems, such as gastrointestinal bleeding and increased cardiovascular (CV) issues,\textsuperscript{[41]} the anti-inflammatory drug naproxen (Figure 1) has fewer CV effects with a possible cardioprotective role in humans. It was found that naproxen was safe and effective in treating progressive prostate cancer,\textsuperscript{[42]} and also it showed anti-cancer properties against colon cancer.\textsuperscript{[43]} In addition, Deb et al. reported that some naproxen derivatives have powerful anti-inflammatory and anti-tumor properties as they induce an appreciable amount of apoptosis in the TNBC cell line.\textsuperscript{[40]}

The combination of NSAIDs with Pt (II) drugs in the form of a single Pt (IV) prodrug to increase the efficiency and reduce side-effects of chemotherapy can be an attractive strategy to treat a variety of cancer tumors. Pt (IV) compounds that have been investigated in this context include complexes with ibuprofen and indomethacin as the axial ligands.\textsuperscript{[44]} The Pt (IV) derivative of cisplatin with one axial aspirin and one hydroxido ligand was shown to exhibit a potent cytotoxic activity and anti-inflammatory properties.\textsuperscript{[29]}

The conjugation of naproxen with cisplatin, carboplatin and oxaliplatin could be a promising strategy for improving the efficiency of the complexes and reducing their side-effects. Here we describe the synthesis, characterization, cytotoxicity and anti-inflammatory properties of novel platinum (IV) complexes with naproxen as the biologically active axial ligand. The cytotoxicity of the complexes was evaluated against breast tumors MCF-7 and MDA-MB-231 cells (TNBC cells) by MTT assay.

\section*{2 | EXPERIMENTAL}

\subsection*{2.1 | Materials and methods}

All reactions were carried out under normal atmospheric conditions. Solvents were used as received without additional drying or purification. K\textsubscript{2}PtCl\textsubscript{4} was obtained from ARCOS. Ligands, naproxen, succinic anhydride and acetic anhydride were obtained from Aldrich; benzoic anhydride was purchased from TCI; diaminocyclohexane and 1,1-cyclobutanedicarboxylic acid were obtained from ARCOS, and all were used as received. NHS (N-hydroxysuccinimide) and DCC (N,N′-dicyclohexylcarbodiimide) were purchased from Sigma Aldrich. The ultrapure water used was purified by a Milli-Q UV purification system. All other solvents and chemicals are of analytical grade or high-performance liquid chromatography (HPLC) grade obtained from commercial sources.

\subsection*{2.2 | Physical measurements}

\textsuperscript{1}H-NMR spectra were recorded on a Jeol ECX-400. Chemical shifts (δ) were reported in parts per million (ppm) and referenced internally using the residual solvent signals relative to tetramethylsilane [δ (\textsuperscript{1}H-NMR) = 0 ppm]. One-dimensional \textsuperscript{195}Pt-NMR spectra were
recorded on a Varian 500 AR spectrometer in DMF with insert D₂O. K₂PtCl₆ in D₂O was used as an external standard. Mass spectra were measured using a Waters LCT Premiere XE with electron spray ionization and time of flight mass analyzer. Elemental analyses (carbon, nitrogen and hydrogen) were performed by an Exeter Analytical CE-440. Fourier transform-infrared (FT-IR) spectra were recorded on a PerkinElmer FT-IR spectrometer fitted with an ATR accessory. The HPLC studies were performed using an Agilent 1200 series DAD analytical HPLC instrument.

2.3 | Reduction reaction studied by HPLC

The reduction of complex 1 with ascorbic acid was followed by HPLC using a Phenomenex Luna C18 (5 μm, 100 Å, 250 mm × 4.60 mm i.d.) column at a flow rate of 1.0 mL min⁻¹ with 280 nm UV detection at room temperature (rt). The mobile phase was 80:20 acetonitrile (1% trifluoroacetic acid):water (1% trifluoroacetic acid). The complex was dissolved in DMF (0.5 mL) and added to a 5 mM solution of ascorbic acid in 2 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7), and diluted to a final concentration of 0.5 mM using acetonitrile. The process was followed at 37°C until complete reduction.

2.4 | Reduction reaction studied by ¹⁹⁵Pt NMR spectroscopy

Complex 1 (10 mM) was incubated at 37°C in the presence of 10 mM ascorbic acid in 12 mM HEPES buffer at pH 7. Complex 1 was dissolved in DMF, and then ascorbic acid and aqueous buffer solution were added (the concentrations given are the final concentrations). The reaction was followed until complete reduction of the complexes. The ¹⁹⁵Pt-NMR spectra were recorded by inserting a tube with D₂O into the NMR tube.

2.5 | Cell culture and treatments

Human breast cell lines MCF-7 and MDA-MB-231 (TNBC cells), and RAW murine 264.7 macrophage were purchased from ATCC, Rockville, MD, USA, and were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2% penicillin/streptomycin, 1.25% 1-Glut and 1% sodium pyruvate. Cells were maintained in humidified air, 5% CO₂ and 37°C. All experiments were repeated independently (n = 8). Cells were harvested after brief trypsinization.

2.6 | Cytotoxicity

To evaluate the cytotoxic effect of the prepared conjugates on RAW 264.7 macrophage, MCF-7 (ER⁺, PR⁺) and MDA-MB-231 cells (ER⁻, PR⁻, HER2/Neu⁻), the 3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) technique was used. The untreated cells were considered 100% viability.

2.7 | Apoptosis and necrosis staining

To study the effect of cytotoxic conjugates on the cell death mode in both MCF-7 and MDA-MB-231 cells, apoptosis and necrosis were analyzed by ethidium bromide/acridine orange (EB/AO) DNA staining.

2.8 | Cell lysate preparation

For ELISA, the cells were lysed by ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 100 mM Na₃VO₄, 20 mM NaF, 0.5% NP40, 1% Triton X-100, 10 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 mg/mL leupeptin; pH 7.4). The cell lysate was passed through a needle (21-gage) and then centrifuged for 15 min at 14,000 g at 4°C.

2.9 | Cyclin D1 level

Cyclin D1 levels were measured in the cell lysate of both of MCF-7 and MDA-MB-231 cells using a commercial quantitative ELISA kit (Abcam # ab218793; USA) following manufacturer's protocol. The levels of cyclin D1 in the cell lysate were expressed as ng mL⁻¹.

2.10 | Estimation of NO

The nitrite accumulation, which is an indicator of nitric oxide (NO) synthesis, was measured in the macrophages' culture medium using a microplate assay based on the Griess reaction. Bacterial lipopolysaccharide (LPS) was used to treat the macrophages 20 hr as an efficient induced of NO to treat the macrophages before being treated with the tested conjugates (10 μg mL⁻¹). In each well of a flat-bottom 96-well microliter plate, 40 μL freshly prepared Griess reagent was mixed with 40 μL macrophages culture supernatant or different concentrations of sodium nitrite. After 10 min in the dark, the absorbance of the mixture at 540 nm was determined. A standard curve relating the different concentrations of sodium nitrite in μM to their respected absorbance was
constructed, from which the nitrite levels (index of NO production) in the supernatant samples were calculated.

2.11 Synthetic procedures

2.11.1 Synthesis of naproxen NHS ester

The carboxylic acid (0.49 g, 2.17 mmol) and NHS (0.25 g, 2.17 mmol) were dissolved in THF (10 mL). A solution of DCC (0.45 g, 2.17 mmol) in THF (5 mL) was added dropwise. After stirring for 5 hr at rt, the precipitated dicyclohexylurea was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was dissolved in ethyl acetate (30 mL) and left in the fridge overnight. The insoluble material formed was removed by filtration. The organic layer was washed with NaHCO₃ solution (4%; 60 mL) and with water (30 mL). The product was precipitated by addition of diethyl ether. The solid was collected by centrifugation and diethylether. The precipitate was filtered off, washed with cold water, ethanol and ether, then dried under vacuum. Yield: 397 mg (72%). IR (cm⁻¹): 3510 s (νO-H); 3250 m (νN-H); 2870 br; 2722 m, 1586 s, 1360 m (Pt-OH bend).

2.11.2 Synthesis of cisplatin, cis,cis,trans-[Pt (NH₃)₂Cl₂(OH)₂]

Oxoplatin was synthesized as reported. Cisplatin (500 mg) was suspended in 12 mL water, and 18 mL 30% H₂O₂ was added. The mixture was stirred for 1 hr at 50°C, and a pale yellow powder was formed. The product was filtered off, washed with cold water, ethanol and ether, then dried under vacuum. Yield: 397 mg (72%). IR (cm⁻¹): 3510 s (νO-H); 3250 m (νN-H); 2870 br; 2722 m, 1586 s, 1360 m (Pt-OH bend).

2.11.3 Synthesis of oxoplatin, cis,cis,trans-[Pt (NH₃)₂Cl₂(OH)₂]

The naproxen-NHS ester (43 mg, 0.131 mmol) was added to oxoplatin, cis,cis,trans-[Pt (NH₃)₂Cl₂(OH)₂] (50 mg, 0.149 mmol) in DMSO (5 mL). The reaction mixture was stirred at 50°C for 20 hr, and then filtered to remove the unreacted oxoplatin. The solvent was evaporated using a freeze-dryer and the residue was dissolved in DMF (2 mL). The desired product was precipitated by adding diethyl ether. The solid was collected by centrifugation, washed several times with dichloromethane and diethylether to remove the residual DMF, and finally dried under vacuum.

Complex 1 (white): yield: 39 mg (48%). ¹H-NMR (400 MHz, CDCl₃): δ = 7.72 (d, 1H, Ar-H), 7.70 (s, 1H, Ar-H), 7.67 (s, 1H, Ar-H), 7.40 (d, 1H, Ar-H), 7.21 (d, 1H, Ar-H), 7.10 (d, 1H, Ar-H), 3.89 (s, 3H, -OCH₃), 3.82 (q, 1H, -CHCH₃), 2.8 (s, 4H, CO-CH₃-CH₂-CO) ppm. ¹³C-NMR (107.6 MHz, DMF (D₂O)): δ = 992 ppm. IR (cm⁻¹): 3507 br (νO-H); 3203 br (νN-H); 3067 m, 3003 m (νC-H(AR)); 2936 w (νCH(CH₃)); 1699 m (νC=O); 1630 s (νC=C); 1604 s; 1329; 1265 s, 1227 s, (νC=C); 1211 s (νO-OR); 1024 m (δC-H); 855 m (δC-H); ESI-MS (negative ion mode): m/z = 545.04 [M – H⁻]⁻, 581.02 [M + Cl]⁻. Anal. calcd for C₁₄H₂₀Cl₂N₂O₄Pt: C, 30.70; H, 3.29; N, 13.00%; found: C, 30.57; H, 3.45; N, 12.40%.

2.11.5 Synthesis of cis,cis,trans-[Pt (NH₃)₂Cl₂(RCO₂)(C₆H₅CO₂)] (2), cis,cis,trans-[Pt (NH₃)₂Cl₂(RCO₂)(O₂CC₂H₄COOH)] (3) and cis,cis,trans-[Pt (NH₃)₂Cl₂(RCO₂)(O₂CC₃H₆COOH)] (4)

Excess of the corresponding anhydride and the monocarboxylato complex 1 were suspended in DMF. The reaction mixture was stirred overnight at 60°C. The solution was then concentrated under reduced pressure. The desired product was precipitated by addition of diethylether. The solid was collected by centrifugation and washed with 4 mL of dichloromethane and diethylether to remove the residual DMF.
Complex 2: benzoic anhydride (82.8 g, 0.882 mmol), 50 mg (0.091 mmol) of 1 in DMF (5 mL); yellow color; yield: 25 mg (42.22%). ¹H-NMR (400 MHz, DMF-d₇): δ 7.90 (d, 1H, Ar-H), 7.85 (d, 1H, Ar-H), 7.55–7.37 (m, 4H, Ar-H), 7.26 (d, 1H, Ar-H), 7.08 (d, 1H, Ar-H), 6.62 (br, 6H, NH₃), 3.82 (s, 3H, -OCH3), 3.74 (m, 1H, -CH₂CH₃), 1.37 (s, 3H, CH₃) ppm.

Complex 3: succinic anhydride (36.67 mg, 0.366 mmol), 50 mg (0.091 mmol) of 1 in DMF (3 mL); white color; yield: 35 mg (59.5%). ¹H-NMR (400 MHz, DMF-d₇): δ 12.05 (s, 1H, COOH), 7.72 (d, 1H, Ar-H), 7.70 (s, 1H, Ar-H), 6.77 (d, 1H, Ar-H), 7.43 (d, 1H, Ar-H), 7.23 (d, 1H, Ar-H), 7.10 (d, 1H, Ar-H), 6.49 (br, 6H, NH₃), 3.89 (s, 3H, -OCH3), 3.82 (q, 1H, -CH₂CH₃), 2.39–2.49 (m, 4H, COCH₂CH₂CO), 1.38 (S, 3H, CH₃) ppm. ¹³C-NMR (107.6 MHz, DMF (D₂O)): δ 1716 IR (cm⁻¹): 3439 br (ν(OH)), 3289 br (ν(OH)), 3083 m, 3003 m (ν(C-H)), 2936 w (ν(C-C)), 1716 s, 1648 s (ν(C=C)), 1625 s (ν(C=O)), 1604 s, 1581 s; 1373; 1266 s, 1232 s, (ν(C=C)), 1210 s (ν(O=O)), 1028 m (δ(C=C)), 855 s, 816 s (ν(C=O)). ESI-MS (negative ion mode): m/z = 696.05 [M + Cl]⁻.

Complex 4: glutaric anhydride (41.7 mg, 0.366 mmol), 50 mg (0.091 mmol) of 1 in DMF (3 mL); yellow color; yield: 32 mg (53%). ¹H-NMR (400 MHz, DMF-d₇): δ 11.98 (s, 1H, COOH), 7.72 (d, 1H, Ar-H), 7.69 (s, 1H, Ar-H), 7.67 (d, 1H, Ar-H), 7.43 (d, 1H, Ar-H), 7.23 (d, 1H, Ar-H), 7.10 (d, 1H, Ar-H), 6.5 (br, 6H, NH₃), 3.82 (s, 3H, -OCH3), 3.81 (q, 1H, -CH₂CH₃), 2.3 (m, 1H, -COOCH₂), 1.68 (m, 2H, -CH₂), 1.43 (S, 3H, CH₃) ppm. ¹⁵N-[¹H]-NMR (107.6 MHz, DMF (D₂O)): δ 1180 ppm. IR (cm⁻¹): 3513 br (ν(OH)), 3197 br (ν(OH)), 3075 m, (ν(C-H)), 2976 w (ν(C-H)), 1704 s, 1640 (ν(C=O)), 1628 s (ν(C=O)), 1604 s, 1505, 1371; 1262 s, 1230 s (ν(C=O)), 1207 s (ν(O=O)), 1024 m (δ(C=C)), 854 s, 811 s (ν(OH)). ESI-MS (negative ion mode): m/z = 569.07 [M – H]⁻, 695.05 [M + Cl]⁻. Anal. calcd for C₁₈H₂₃Cl₂N₂O₄Pt: C, 32.3; H, 3.71; N, 4.23%; found: C, 32.3; H, 3.8; N, 4.00%.

2.11.6 | Synthesis of cis,cis,trans-[Pt(NH₃)₂Cl₂(RCOO)(CH₃COO)] (5)

The monocarboxylato complex 1 (50 mg) was stirred at rt in acetic anhydride (5 mL) for 20 hr. The reaction mixture was lyophilized and washed with diethyl ether (2 × 5 mL) to yield complex 5 as a white precipitate.

Complex 5: yield: 26 mg (48.5%). ¹H-NMR (400 MHz, DMSO-d₆): δ 7.73 (d, 1H, Ar-H), 7.69 (s, 1H, Ar-H), 7.67 (d, 1H, Ar-H), 7.44 (d, 1H, Ar-H), 7.22 (d, 1H, Ar-H), 7.11 (d, 1H, Ar-H), 6.51 (br, 6H, NH₃), 3.82 (s, 3H, -OCH3), 3.81 (q, 1H, -CH₂CH₃), 1.88 (s, 3H, CH₃COO), 1.42 (s, 3H, CH₃) ppm. ¹⁹⁵Pt[¹H]-NMR [107.6 MHz, DMF (D₂O)]: δ 1177 ppm. IR (cm⁻¹): 3206 br (ν(OH)), 3067 m, 3007 (ν(C=O)), 2928 w (ν(C=O)), 1650 s (ν(C=O)), 1629 s (ν(C=O)), 1604 s; 1505; 1361; 1220 s (ν(C=O)); 1207 s (ν(O)), 1017 m (δ(C=O)), 854 s, 812 s (ν(C=O)). ESI-MS (negative ion mode): m/z = 587.04 [M – H]⁺, 623.02 [M + Cl]⁻. Anal. calcd for C₁₆H₂₂Cl₂N₂O₅Pt: C, 32.63; H, 3.73; N, 4.75%; found: C, 32.32; H, 3.80; N, 4.60%.

2.11.7 | Synthesis of [(IR,2R-DACH) (oxalato) Pt (II)] (oxaliplatin)

Oxaliplatin was synthesized as described in patent PCT/EP2010/003753[51] with slight modifications. K₂PtCl₄ (2 g, 4.8 mmol) was dissolved in 40 mL of water. To this solution, diaminocyclohexane (DACH; 5.4 g, 4.8 mmol) in 20 mL ethanol was added and the mixture was allowed to react for 6 hr at rt. During the reaction, the product (DACH)PtCl₂ precipitated. It was filtered off, washed with water, methanol and acetone, and dried under vacuum overnight. The (DACH)PtCl₂ was suspended in 60 mL of water, and 1.43 g Ag₂SO₄ was added to this solution and stirred at rt for 20 hr under light exclusion. This caused the formation of the soluble (DACH)Pt(H₂O)₂(II)-sulfate complex accompanied by the formation of poorly soluble AgCl, which was filtered off. To the filtrate containing the (DACH)Pt(H₂O)₂(II)-sulfate complex, 0.46 g (3.7 mmol) of oxalic acid dehydrate and 0.5 mL NaOH were added until the pH reached 6. The mixture was stirred at rt for a further 20 hr. The solution was concentrated in a rotavapor, until the final product [(IR,2R-DACH)(oxalato) platinum (II)] precipitated. This was filtered off, washed with water and acetone, and dried under vacuum. Yield: 0.668 g (35%). Purity was confirmed by IR-spectroscopy and elemental analysis. IR (cm⁻¹): 3211 m, 3160 m, 3081 s (ν(OH)), 2960 w (ν(C=O)), 2929 m; 2864 m; 1969 s, 1660 s, 1653 m (ν(C=O)); 1699 m; 1373 s; 1266 s; 1069 s; 808 s, 742 (ν(C=O)). Anal. calcd for C₁₆H₁₀N₂O₅Pt: C, 32.19; H, 3.71; N, 4.01; found: C, 32.3; H, 3.80; N, 4.00%.

2.11.8 | Synthesis of dihydroxyoxaliplatin, [(IR,2R-DACH)(oxalato) Pt (IV)(OH)₂]
solution was then concentrated on a rotavapor, and the desired product was precipitated by adding ethanol, the white precipitate formed was filtered off and washed with ethanol and diethyl ether, and dried under vacuum. Yield: 0.49 g, 90%. $^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 10.2 (s, 2H, OH), 7.62 (2H, NH$_2$), 6.85 (2H, NH$_2$), 1.95 (d, 2H, DACH), 1.44 (m, 4H, DACH), 1.04 (m, 4H, DACH) ppm. IR (cm$^{-1}$): 3427 m ($\nu$ Pt–OH); 3268 m, 3211 m, 3150 br ($\nu$ N–H); 2952 m ($\nu$ C–H); 2816 m; 1716 s, 1655 s ($\nu$ C=O); 1609 m; 1554 m; 1449 m; 1390 s; 1221 s; 1065 m; 805 s, 770 ($\gamma$ C–H). ESI-MS (negative ion mode): m/z = 430.26 [M–H]$^–$.

2.11.9 | Synthesis of trans-[Pt (CO$_2$R)(OH) (oxalato)(DACH)] (1x)

Naproxen-NHS ester (30.36 mg, 0.093 mmol) was added to dihydroxyoxaliplatin (50 mg, 0.116 mmol) in DMSO (5 mL). The reaction was stirred at 50°C for 20 hr. The solvent was evaporated and the residue was dissolved in DMF (2 mL). The solution was centrifuged to remove the insoluble part from the excess of dihydroxyoxaliplatin. The desired product was precipitated by adding diethyl ether. The solid was collected by centrifugation. The solid was washed several times with dichloromethane and diethylether to remove the residual DMF, and finally dried under vacuum.

Complex 1x (yellow color): yield: 33 mg (44.23%). $^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.52–7.08 (m, 4 H, NH$_2$), 7.73 (d, 1H, Ar–H), 7.67 (s, 1H, Ar–H), 7.64 (d, 1H, Ar–H), 7.55 (d, 1H, Ar–H), 7.22 (d, 1H, Ar–H), 7.09 (d, 1H, Ar–H), 3.82 (s, 3H, $\text{OC}_3$H$_3$), 3.80 (q, 1H, $\text{CHCH}_3$), 1.38 (s, 3H, CH$_3$), 1.05–2.2 (m, 10H, DACH) ppm. $^{195}$Pt-NMR [107.6 MHz, DMF (D$_2$O)]: $\delta$ 1389 ppm. IR (cm$^{-1}$): 3523 s, 3400 m, 3164 m ($\nu$ N–H); 2952 w ($\nu$ C–H); 1625 s, 1600 s ($\nu$ C=O); 1464 w; 1371 s; 1345 s; 1280 m; 1118 m; 900 m; 764 w. ESI-MS (negative ion mode): m/z = 430.26 [M–H]$^–$.

2.11.10 | Synthesis of carboplatin [Pt (NH$_3$)$_2$(CBDCA)]

Carboplatin was synthesized as described in the literature.$^{[53,54]}$ cis-(NH$_3$)$_2$PtI$_2$ was synthesized as shown previously in cisplatin synthesis. To a solution containing 2.1 g of cis-(NH$_3$)$_2$PtI$_2$ in 15 mL deionized distilled water, 15 mL of a solution containing 1.54 g of silver nitrate in deionized distilled water was added. The resulting solution was stirred overnight in the dark. After that the AgI formed was removed by filtration, to the filtrate 0.685 g (4.77 mmol) of 1,1-cyclobutanedicarboxylic acid was added, and the pH of the resulting solution was adjusted to 5.0 using 10 M KOH. After stirring for 3 hr, the solvent was removed in a vacuum and the white solid was washed with 5 mL of cold water, ethanol and ether, and dried to yield 0.9 g (50.3%). The $^1$H-NMR spectrum of carboplatin in D$_2$O exhibits signals at $\delta$ 2.70 (t, 4H) and 1.72 (q, 2H) ppm. IR (cm$^{-1}$): 3357 s, 3150 br ($\nu$ N–H); 2952 w ($\nu$ C–H); 1625 s, 1600 s ($\nu$ C=O); 1464 w; 1371 s; 1345 s; 1280 m; 1118 m; 900 m; 764 w. ESI-MS (negative ion mode): m/z = 370.03 [M–H]$^–$. Anal. calcd for carboplatin (C$_6$H$_{12}$N$_2$O$_4$Pt): C, 19.41; H, 3.26; N, 7.55%; found: C, 19.15; H, 3.01; N, 7.32%.

2.11.11 | Synthesis of dihydroxycarboplatin, cis,cis,trans-[Pt (NH$_3$)$_2$(CBDCA)(OH)$_2$]

Carboplatin (0.5 g, 1.16 mmol) was oxidized with 18 mL of 30% H$_2$O$_2$ in 12 mL water at rt for half an hour, then it was heated with stirring at 50°C for 1 hr. The solution was centrifuged to remove the insoluble part from the excess of dihydroxyoxaliplatin. The desired product was precipitated by adding diethyl ether. The solid was collected by centrifugation. The solid was washed several times with dichloromethane and diethylether to remove the residual DMF, and finally dried under vacuum.

Complex 1x (yellow color): yield: 33 mg (44.23%). $^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 8.52–7.08 (m, 4 H, NH$_2$), 7.73 (d, 1H, Ar–H), 7.67 (s, 1H, Ar–H), 7.64 (d, 1H, Ar–H), 7.55 (d, 1H, Ar–H), 7.22 (d, 1H, Ar–H), 7.09 (d, 1H, Ar–H), 3.82 (s, 3H, $\text{OC}_3$H$_3$), 3.80 (q, 1H, $\text{CHCH}_3$), 1.38 (s, 3H, CH$_3$), 1.05–2.2 (m, 10H, DACH) ppm. $^{195}$Pt-NMR [107.6 MHz, DMF (D$_2$O)]: $\delta$ 1389 ppm. IR (cm$^{-1}$): 3523 s, 3400 m, 3164 m ($\nu$ N–H); 2952 w ($\nu$ C–H); 1625 s, 1600 s ($\nu$ C=O); 1464 w; 1371 s; 1345 s; 1280 m; 1118 m; 900 m; 764 w. ESI-MS (negative ion mode): m/z = 642.14 [M–H]$^–$. Anal. calcd for dihydroxycarboplatin (C$_{22}$H$_{28}$N$_2$O$_8$Pt): C, 41.05; H, 4.35; N, 4.28%; found: C, 39.81; H, 4.45; N, 7.32%.

FIGURE 3 Structures of the seven Pt (IV) complexes described in this paper
was concentrated; the white precipitate formed was filtered off, washed with cold water then ethanol, and dried under vacuum.\(^{[55]}\) Yield: 0.47 g (86.5%). The \(^1\)H-NMR spectrum of dihydroxy carboplatin in D\(_2\)O exhibits signals at \(\delta\) 2.59 (t, 4H) and 1.96 (q, 2H) ppm. IR (cm\(^{-1}\)): 3444 br (\(\nu\)Pt-OH); 3220 br, 3090 br (\(\nu\)N-H); 2950 m (\(\nu\)C-H); 1610 s; 1570 s (\(\nu\)C=O); 1455 w; 1350 s; 1345 s; 1219 m; 1102 m; 921 m; 778 m.

2.11.12 | Synthesis of cis,cis,trans-[Pt (NH\(_3\))\(_2\)(CBDCA)(CO\(_2\)R)OH], 1c

Naproxen-NHS ester (32 mg, 0.098 mmol) was added to cis,cis,trans-[Pt (NH\(_3\))\(_2\)(CBDCA)(OH)\(_2\)] (50 mg, 0.123 mmol) in DMSO (4 mL). The reaction was stirred at 50°C for 24 hr and then filtered. The solvent was evaporated, and the residue was dissolved in DMF (2 mL) and centrifuged to remove the unreacted [Pt (NH\(_3\))\(_2\)(CBDCA)(OH)\(_2\)]. The desired product was precipitated by adding diethyl ether to the solution. The solid was collected by centrifugation. The solid was washed several times with diethylether to remove the residual DMF and finally dried under vacuum.

Complex 1c (yellow color): yield: 40 mg (52%). \(^1\)H-NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 7.69 (d, 1H, Ar-H), 7.65 (s, 1H, Ar-H), 7.62 (d, 1H, Ar-H), 7.38 (d, 1H, Ar-H), 7.20 (d, 1H, Ar-H), 7.05 (d, 1H, Ar-H), 5.87 (br, 6H, NH\(_3\)), 3.81 (s, 3H, -OCH\(_3\)), 3.72 (q, 1H, -CHCH\(_3\)), 1.37 (s, 3H, CH\(_3\)) ppm. \(^{195}\)Pt\(^{[1]}\)NMR [107.6 MHz, DMF (D\(_2\)O)]: \(\delta\)

---

**FIGURE 4** High-performance liquid chromatography (HPLC) chromatograms of the reaction of complex 1 with 10 equiv. ascorbic acid (AsA) at 37°C. The chromatogram of the free ligand (naproxen) is shown for comparison purposes.

**FIGURE 5** \(^{195}\)Pt-NMR spectra of (a) complex 1 and (b) after reacting 1 with ascorbic acid for 3 hr at 37°C. Zooms of the 600 to 1400 and −2300 to −2000 ppm regions are shown.
1737 ppm. IR (cm⁻¹): 3448 br (ν(O-H)); 3217 br (ν(N-H)); 3083 m, 3003 m (ν(C=H)); 2940 w (ν(C-H)); 1690 w (ν(C=O)); 1630 s (ν(C=O)); 1565 s; 1538 s; 1230 s (ν(C=O)); 1211 s (ν(C=C)); 1028 m (δ(C-H)); 854 s (ν(C=H)). ESI-MS (negative ion mode): m/z = 616.12 [M – H]⁻. Anal. calcld for C₂₀H₂₆N₂O₈Pt: C, 38.89; H, 4.21; N, 4.53%; found: C, 38.64; H, 4.39; N, 4.37%.

3 | RESULTS AND DISCUSSION

3.1 | Synthesis and characterization

Seven naproxen Pt (IV) complexes have been successfully synthesized and their structures are shown in Figure 3. Cisplatin, carboplatin and oxaliplatin were synthesized according to previously reported procedures. The monodentate derivatives were obtained by oxidation of cisplatin, carboplatin and oxaliplatin with H₂O₂. The monocarboxylato complexes 1, 1x, 1c were prepared in a single step by reacting oxoplatin, c,c,t-[Pt(NH₃)₂Cl₂(OH)₂], dihydroxyoxaplatin, [1R,2R-DACH] (oxalato) Pt (IV)(OH)₂ and dihydroxyoxaplatin, c,c,t-[Pt(NH₃)₂(CBDCA)(OH)₂], respectively, with 0.8 equiv. of the activated naproxen NHS-ester (see Experimental section). The unsymetrically substituted complexes (2–5) were prepared by reacting the monocarboxylate complex 1 with the corresponding anhydrides. We chose four different anhydrides (benzoic, acetic, succinic and glutaric anhydride) to vary the lipophilicity of the resulting Pt (IV) complexes. The synthesized Pt (IV) complexes were characterized using elemental analysis, ESI-MS, FT-IR, ¹H- and ¹⁹⁵Pt-NMR spectroscopy (Figures S1–S14). The purity of the complexes had been confirmed through elemental analysis. Elemental analyses of the complexes are in good agreement with their expected values. The mass spectra of the complexes show a peak in the negative mode with the typical platinum isotope pattern corresponding to M – H⁻ or M + Cl⁻. The IR spectra displayed characteristic C=O stretching frequencies ranging from 1630 to 1680 cm⁻¹, and N-H stretching frequencies of the ammine ligands at about 3200 cm⁻¹ for all complexes. Complexes 3 and 4 have additional peaks at 1716 and 1704 cm⁻¹, respectively, attributed to the free carbonyl group (Figures S8–S14). The proton NMR spectra of the complexes in DMSO-d₆ show the typical signal of the NH₃ protons, which is a multiplet at about 6 ppm for the monocarboxylato complexes 1 and 1c, while there is a broad signal at about 6.5 ppm for the di-substituted complexes (2–5; Figures S1–S5 and S7). The proton resonances of the coordinated ammine ligands appear in complex 1x as multiplet peaks from 8.52 to 7.08 ppm. The ¹⁹⁵Pt-NMR spectra of 1 in D₂O gave a quintet peak due to the spin to spin coupling between ¹⁹⁵Pt and two ¹⁴N nuclei at 992 ppm, which is consistent with reported data.[34] These resonances were shifted downfield to 1170–1180 ppm in the dicarboxylato complexes (2–5; Figures S2–S5). The presence of an additional electron-withdrawing carbonylate ligand caused a greater deshielding effect on the platinum (IV) metal center in the dicarboxylato complexes than the monocarboxylato ones that cause the observed downfield shift on their ¹⁹⁵Pt-NMR resonances.[20] The ¹⁹⁵Pt-NMR spectra of 1x and 1c in D₂O display a single resonance at 1389 and 1737 ppm, respectively (Figures S6 and S7).

The reduction reaction of monocarboxylato complex 1 and the release of naproxen were analyzed by HPLC using ascorbic acid as the reductant at 37°C and pH 7

### TABLE 1 | Cytotoxicity of the complexes in comparison to cisplatin in MCF-7 and MDA-MB-231 cancer cell lines by IC₅₀ (µM)

<table>
<thead>
<tr>
<th>IC₅₀ (µM) ± SD</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.40 ± 0.79</td>
<td>17.05 ± 1.09</td>
</tr>
<tr>
<td>2</td>
<td>3.92 ± 0.42</td>
<td>6.14 ± 0.45</td>
</tr>
<tr>
<td>3</td>
<td>7.65 ± 0.84</td>
<td>18.45 ± 1.72</td>
</tr>
<tr>
<td>4</td>
<td>8.73 ± 0.89</td>
<td>14.96 ± 0.76</td>
</tr>
<tr>
<td>5</td>
<td>8.90 ± 1.06</td>
<td>19.12 ± 1.34</td>
</tr>
<tr>
<td>1x</td>
<td>9.47 ± 0.75</td>
<td>17.22 ± 0.89</td>
</tr>
<tr>
<td>1c</td>
<td>9.12 ± 0.63</td>
<td>10.93 ± 0.71</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>16.46 ± 1.03</td>
<td>184 ± 4.24</td>
</tr>
<tr>
<td>Naproxen</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Data taken from ref. 40. IC₅₀ values were calculated by a four-parameter logistic model (P < 0.05). SD, standard deviation.

**FIGURE 6** A comparative cytotoxicity (IC₅₀, µM) between MCF-7 (black bars) and MDA-MB-231 (white bars) after the incubation with different conjugates for 72 hr, using MTT assay. IC₅₀ Values were calculated by a four-parameter logistic model (P < 0.05). SD, standard deviation.
(Figure 4). The HPLC chromatograms of the reduction reaction of the complex were compared with the chromatograms of the pure sample of naproxen ligand. The results indicated that complex 1 released the naproxen ligand upon reduction from Pt (IV) to Pt (II). Complex 1 was completely reduced after 3 hr incubation with 10 equiv. of ascorbic acid. We further studied the nature of the Pt (II) species produced upon the reduction of 1 by $^{195}$Pt-NMR. Figure 5 shows that the reaction of 1 with 10 equiv. of ascorbic acid resulted in a strong signal for cisplatin at −2094 ppm.$^{[34]}$ Complex 1 was completely reduced after 3 hr of incubation with ascorbic acid at 37°C, in line with the HPLC analyses.

### 3.2 Cytotoxicity

The cytotoxicities of the complexes and cisplatin were evaluated by the MTT assay on breast tumors MCF-7 and MDA-MB-231. The IC$_{50}$ values were calculated from the dose–survival curves obtained after 72 hr of drug treatment, and the results were summarized in Table 1. The naproxen proved to be hardly effective against the tested cell lines,$^{[40]}$ as shown in Table 1, while the newly synthesized Pt (IV) complexes were able to promote a significant cancer cell-killing effect. All the complexes exhibited a significant cancer cell-killing effect, with average threefold more cytotoxic than cisplatin against MCF-7 (Figure 6). The IC$_{50}$ value of cisplatin in MDA-MB-231 is 184 μM, while the average IC$_{50}$ of the complexes decreased to 14 μM, which is a 13-fold shift in IC$_{50}$. Among all the complexes, the dicarboxylato complex bearing the most lipophilic benzoate ligand 2 showed the highest in vitro anti-proliferative activity (mean IC$_{50}$ value equal to 5.03 μM). The dicarboxylato complexes bearing acetate, succinate, glutarate ligand showed similar activity with mean IC$_{50}$ values of 8.4 μM and 17.4 μM against MCF-7 and MDA-MB-231 cell lines, respectively. The monocarboxylato complexes 1, 1x and 1c showed similar cytotoxicity against MCF-7 with a mean IC$_{50}$
value of 9.16 μM. While against MDA-MB-231 cells, complex 1c showed higher activity with IC50 value of 10 μM, while the IC50 values for complexes 1 and 1x are about 17 μM. The higher potency of the complexes compared with cisplatin and the free naproxen may be attributed to their higher accumulation into cells. It is expected that conjugation of naproxen to Pt (IV) affords significant enhancement in intracellular accumulation of both relative to either cisplatin or free naproxen as the Pt (IV) complexes are provided with synergistic accumulation where cisplatin and naproxen are prodrugs of each other.[56]

### 3.3 | Estimation of NO

Nitric oxide is a signaling molecule that plays a key role in the pathogenesis of inflammation. It is considered as a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations. Therefore,
NO inhibitors represent an important therapeutic advance in the management of inflammatory diseases. The anti-inflammatory properties of the newly synthesized complexes were assessed through NO inhibition. The nitrite accumulation, which is an indicator of NO synthesis, was measured in the macrophages culture medium using a microplate assay based on the Griess reaction. Bacterial LPS was used as an efficient induced medium using a microplate assay based on the Griess reaction. The calculated concentrations of IC50 were tested in vitro for 24 hr with LPS treated macrophages resulted in high extents of NO inhibition, as shown in Figure 7. The most potent NO inhibitors were 1c, 2 and 1x, with inhibitory percentages of 72.17%, 70.50% and 66.69% of the LPS-induced NO, respectively. These potent NO inhibition properties of the complexes indicated their potential in reducing tumor-associated inflammation.

3.4 | Evaluation of cell death mode

The calculated concentrations of IC50 were tested in another independent experiment, resulting in a cell death percentage ranging from 47.2% to 54.1%. The IC50 values of 2 in MCF-7 cells and MDA-MB-231 were used in another experiment to screen the mode of the induced cell death. The results indicated that 2 had lead to a remarkable elevation (P < 0.01) in the necrotic and apoptotic cell death percentages in MCF-7 into 30.56% and 22.68%, respectively, as shown in Figures 4a and b, and 5a. Similarly, 2 treatment of MDA-MB-231 enhanced cell death due to a significant enhancement in both of the populations of early and late apoptotic (36.21%; P < 0.001) as well as necrotic cells (12.98%; P < 0.01), as shown in Figures 8c and d, and 9a.

3.5 | Cyclin D1 level

Cyclin D1 is one of the main regulatory molecules of the cell cycle. Overexpression of cyclin D1 protein has been observed in many types of tumors.157-159 To define the influence of 2 of the cell cycle, cyclin D1 concentration was investigated in the lysate of both of the cell lines by ELISA. As shown in Figure 9b, cyclin D1 showed a significant decrease in 2-treated MCF-7 cells into 64.34% of the control (P < 0.05), while in 2-treated MDA-MB-231 cells (Figure 9c) there was a dramatic decrease in cyclin D1 into 45.18% of the control (P < 0.01).

4 | CONCLUSIONS

In conclusion, we have reported the synthesis of seven new Pt (IV) complexes based on cisplatin, carboplatin and oxaliplatin scaffold bearing the anti-inflammatory drug naproxen as an axial ligand. A favorable reduction pattern for complex 1 to the active Pt (II) drug was observed with the release of the anti-inflammatory drug naproxen. In vitro studies showed that the synthesized Pt (IV) complexes were up to three- and 13-fold more cytotoxic than cisplatin against MCF7 and the TNBC MDA-MB-231 cell lines, respectively. Complex 2 with the most lipophilic benzoate binder showed the highest in vitro anti-proliferative activity and demonstrated a significant decrease in cyclin D1 concentration. Furthermore, the Pt (IV) complexes showed anti-inflammatory properties with remarkable NO inhibition, which indicated their potential in reducing cancer associated with inflammation.

REFERENCES


Dina A. Tolan https://orcid.org/0000-0003-3917-2822

**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Tolan DA, Abdel-Monem YK, El-Nagar MA. Anti-tumor platinum (IV) complexes bearing the anti-inflammatory drug naproxen in the axial position. Appl Organometal Chem. 2019:e4763. https://doi.org/10.1002/aoc.4763