Full Paper

Synthesis, Cytotoxicity, and Apoptosis Induction Study of Antitumor Dinuclear Platinum(II) Complexes

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Five novel dinuclear platinum(II) complexes with a new chiral ligand, 3-(2-amino-cyclohexylamino)-propionic acid (HP), were designed, prepared and spectrally characterized. The *in vitro* cytotoxicities of these compounds were evaluated against the HepG-2, MCF-7, A549, and HCT-116 cell lines. The results indicated that all compounds showed cytotoxicity towards the HepG-2 cell line. Particularly, complex **X5**, which has SO_4^{2-} as a bridge, exhibited better cytotoxicity than carboplatin or oxaliplatin against all selected cell lines. Moreover, double dyeing flow cytometric resection indicated that the target compounds inhibited tumor cell growth by inducing apoptosis.

Keywords: 3-(2-Amino-cyclohexylamino)-propionic acid / Apoptosis induction / Cytotoxicity / Dinuclear platinum complex

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Introduction

Platinum-based anticancer drugs are widely used for the treatment of various types of solid tumors. Cisplatin, carboplatin, and oxaliplatin, the three global drugs, are used to treat 40–80% of all cancer patients. However, their application is severely limited by their cumulative toxicities such as nephrotoxicity, ototoxicity, and peripheral neuropathy. Additionally, both intrinsic and acquired resistance can develop [1, 2]. These drawbacks have stimulated the search for other platinum-based anticancer agents not obeying the classical structure–activity relationship (SAR) [3].

Multinuclear platinum complexes have been reported increasingly as an important type of non-classical anticancer platinum complexes, which could probably overcome the drug resistance/cross resistance arising from cisplatin and its analogs [4]. This is partly due to the fact that their DNA binding mode is different from cisplatin. BBR3464, a trinuclear platinum complex with 1,6-hexyldiamine as a bridge, is a typical example as a lead compound for its potent cytotoxicity and different anticancer mechanism from cisplatin [5–9].

Initially, Pt(II) ions in multinuclear antitumor platinum complexes were reported to be linked by amines such as flexible aliphatic polyamines and aromatic amines with rigid ring structures [10, 11]. Recently, there have been several reports on antitumor dinuclear platinum(II) complexes, in which dicarboxylate and amino acidic anions as well as halide anions were used as bridges to connect two metal ions [12–14]. And the related study indicated that the bridges were found to have an important effect on the cytotoxicity of the resulting dinuclear complexes.

In view of an essential role of 1*R*,2*R*-diaminocyclohexane (DACH) in the success of oxaliplatin [15], we have designed a class of chiral ligands in which carboxylato groups were introduced to bind one of the nitrogen atoms of 1*R*,2*R*-DACH to generate amino acidic derivatives. In our early work, two different series of dinuclear platinum(II) complexes of 2-(((1*R*,2*R*)-2-aminocyclohexyl)amino)acetic acid and 2-(((1*R*,2*R*)-2-aminocyclohexyl)amino)propanoic acid, with dicarboxylates or sulfate as bridges were prepared. *In vitro* evaluations showed that the dinuclear complexes with succinate (complex **S0** and **S2**) and sulfate (complex **S1**, Fig. 1) as bridges exhibited better or comparable activities against HCT-116,

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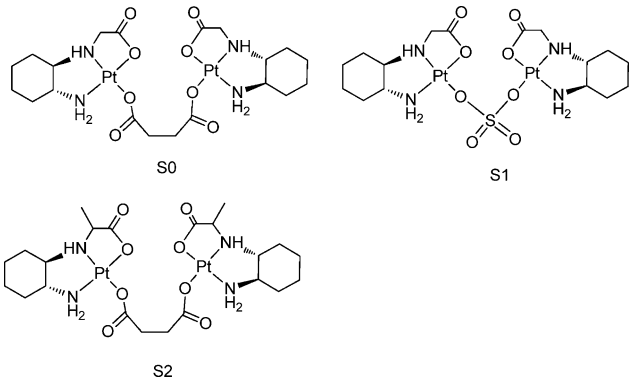


Figure 1. Chemical structures of complexes **S0** and **S1**.

MCF-7, and HepG-2 cell lines compared with positive controls [16, 17].

Inspired by the result, we introduced another aliphatic acid, namely propionic acid, to DACH and got a new chiral ligand, 3-(2-amino-cyclohexylamino)-propionic acid (abbreviated as HP), to evaluate the relationship between the substituting carboxylato groups and biological activities of the resulting compounds. With HP as a carrier group and different dicarboxylates as bridges, five dinuclear platinum complexes were prepared and characterized by IR, MS, and ^{1}H NMR spectra. It is noted that all dinuclear platinum complexes showed good aqueous solubility. The results of *in vitro* cytotoxicity assays showed that all compounds exhibited affirmative activity to selected human cell lines; especially some of them surpassed that of oxaliplatin against HCT-116. The results of flow cytometry (FCM) tests showed that they inhibited the proliferation of tumor cells by inducing cell apoptosis.

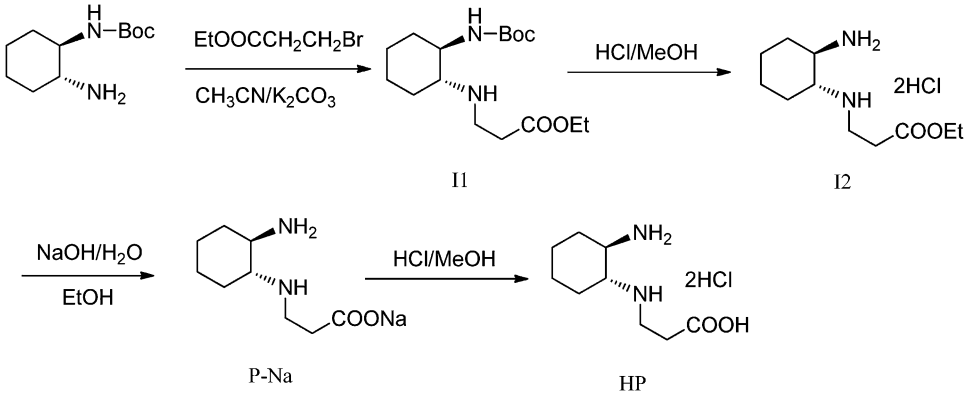
Results and discussion

Mono-Boc protected 1*R*,2*R*-DACH was used as the starting material, since it was hard to get an N-monosubstituted derivative directly due to the equivalent reactivity of the two amino groups in DACH. The ligand was synthesized via several synthetic steps shown in Scheme 1.

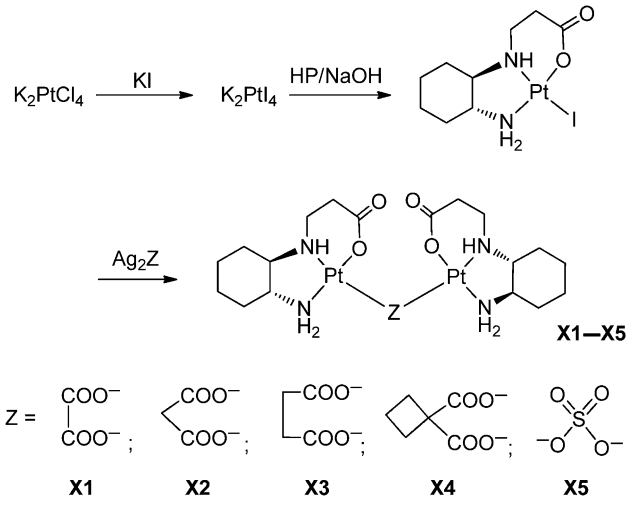
When preparing the dinuclear platinum complexes, we first synthesized the intermediate [PtPI] by adopting a similar method described literaturally [17]. And then silver dicarboxylate/sulfate were used to remove the iodide anions of [PtPI] to give complexes **X1**–**X5** (Scheme 2).

All dinuclear platinum(II) complexes were characterized by IR, ^{1}H NMR, ESI-MS spectra, and microanalyses. The elemental analysis results are in good agreement with the calculated values. The IR spectra of these complexes are analogous, Pt–N coordination bonds were confirmed by the examination of ν_{NH_2}/ν_{NH} shifting to lower frequencies compared with their free amino groups. On the other hand, the shifts of the C=O absorption from free carboxylic acids near 1700 cm^{-1} to a band near 1541–1647 cm^{-1} proved that carboxylate anions were coordinated to Pt(II) ions. The ^{1}H NMR spectra of the complexes are consistent with their corresponding protons both in the chemical shifts and the number of hydrogens. All prepared complexes showed a peak of $[M+Na]^+$ or $[M+H]^+$ in their positive ESI mass spectra, which are consistent with the expected molecular formula weights. The mass spectra of the compounds exhibited four main molecular ion peaks due to the isotopes of ^{194}Pt (33%), ^{195}Pt (34%), ^{196}Pt (25%), and ^{198}Pt (7%). It is noted that a new chiral center was generated at the substituted nitrogen atom of 1*R*,2*R*-DACH after coordination. As stated in our former study including crystal structure analysis and theoretical calculations [18, 19], we proposed that the *S* configuration was preferable in the nitrogen atom.

The aqueous solubility of the resulting dinuclear compounds was measured, because it is a very important factor for anticancer platinum drugs. Compared with cisplatin (1 mg/mL) and oxaliplatin (8 mg/mL), all prepared platinum compounds have rather good aqueous solubility (ranging from 18 to 47 mg/mL).



Scheme 1. Synthetic chart for ligand HP.



Scheme 2. Synthetic route of dinuclear platinum(II) complexes **X1**–**X5**.

The *in vitro* cytotoxicities of dinuclear platinum compounds were evaluated by MTT assay using HepG-2 human hepatocellular carcinoma cells, MCF-7 human breast cancer cells, A549 human lung cancer cells and HCT-116 human colorectal cancer cells, with carboplatin or oxaliplatin as positive control. As we can see from Table 1, most complexes showed cytotoxicity to the selected cell lines.

Complex **X5** owning sulfate as a bridge showed good activity to all the four selected cancer cell lines. When tested against A549 and MCF-7 cells, complex **X5** had much better activity than other dinuclear compounds in which dicarboxylates served as bridges, and even is twofold more potent than that of the positive control (carboplatin), which indicated that sulfate was probably a good choice as linking ligand.

Table 1. Cytotoxicity of complexes **X1**–**X5** against four tumor cell lines.

Compound	IC_{50} [μM][b]			
	A549	**MCF-7**	**HEPG-2**	**HCT-116**
X1	40	40	32	5.1
X2	29.4	>50	4	17.8
X3	>50	>50	43.9	43.3
X4	30	34.2	18	>50
X5	6.4	8.1	9.1	4.2
S0	>50[a]	9.9	8.2	5.8 [13]
S1	>50	7.1	6.8	5.1 [13]
S2	Not tested	7.9	>50	4.0
Carboplatin	11.2	15.3	9.7	Not tested
Oxaliplatin	Not tested	Not tested	Not tested	4.3

[a] The cytotoxicities of complexes **S0** and **S1** were tested but not reported in [13].
[b] All IC_{50} values (drug concentration giving 50% survival) calculated based on the Pt content are means ± SD (SD <12% of the mean value) from at least three separate experiments.

When tested against HepG-2 cells, all complexes showed antitumor cytotoxicity. Complex **X2** that has malonate as a bridge gave the best performance and its activity was 2.5 times that of carboplatin, while complex **X5** also showed very similar activity to carboplatin.

For HCT-116 cells, complex **X5** also exhibited better activity than oxaliplatin and complex **X1** showed very close activity to the positive control. Interestingly, when HCT-116 was employed, the longer the dicarboxylate bridge is, the lower the antitumor activity is. Especially, when a cyclobutyl moiety was introduced to the dicarboxylate bridge, the antitumor activity significantly decreased, indicating the cyclobutyl fragment had a negative impact on the activity.

The main difference between complexes **S0**/**S1** and **X3**/**X5** is the alkyl length of N-substituted carboxylates which leads to the different size of the PtNO ring resulted from coordination. The former complex has a N-acetate 1*R*,2*R*-DACH ligating group to generate a five-membered ring, while the latter has a N-propionate 1*R*,2*R*-DACH moiety to generate a six-membered ring. As we could conclude from the IC_{50} data, no dramatic changes were observed between these two series of complexes. Complexes **S1** and **X5**, bridged by sulfate, had almost the same activities towards MCF-7, HepG-2, and HCT-116 cell lines, but the cytotoxicity of **X5** against A549 was much better than its counterpart. Although complexes **X3**, **S0**, and **S2** were all connected by succinate, the anticancer activity of **X3** was much decreased compared with **S0** and **S2** in all the four tested cancer cell lines. It seemed that N-acetate 1*R*,2*R*-DACH was a slightly better choice than the N-propionate one as a carrier ligand, and sulfate was a good linker in these two series of complexes.

In summary, complex **X5** showed much better activity than other dinuclear compounds, and its activity surpassed the positive controls against all selected human cell lines.

Complexes **X1** and **X5**, which have shown favorable cytotoxicity in MTT assay, were chosen to test their capability to induce apoptosis of HCT-116 cells. The results are shown in Fig. 2.

In Fig. 2, we can see that the apoptosis rate is very low under 10% in the control (cells without drugs), while complexes **X1**, **X5**, and the positive control inhibited HCT-116 cell proliferation by inducing apoptosis obviously. It is noted that the apoptosis rate of complex **X5** surpassed oxaliplatin, which is consistent with the results of the MTT method.

Conclusion

We have prepared a new chiral ligand of HP derived from 1*R*,2*R*-DACH. Using HP as a carrier ligand, five novel

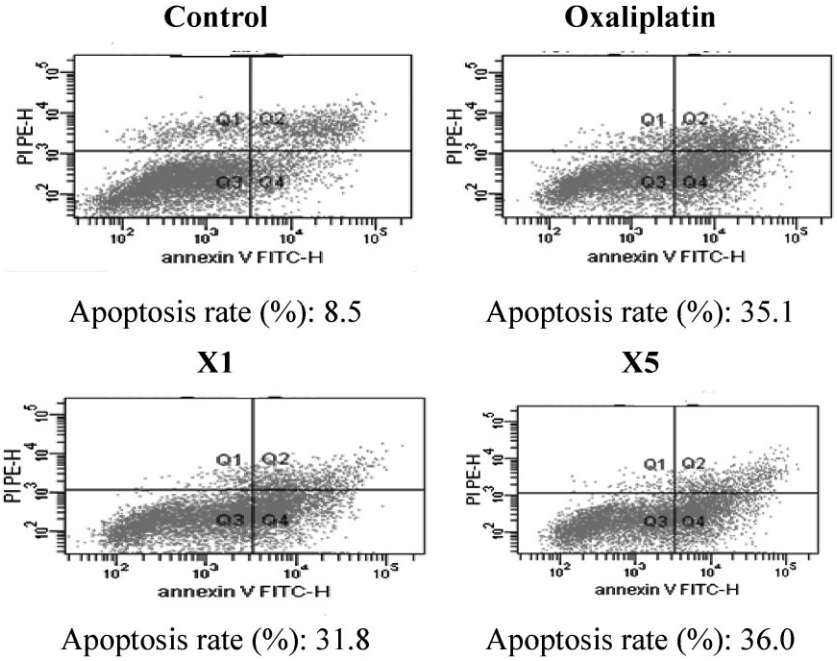


Figure 2. Induction of apoptosis (HCT-116) of complexes **X1** and **X5**. Q1, unnatural death; Q2, late apoptosis and/or necrosis; Q3, normal; Q4, early apoptosis.

dinuclear platinum(II) complexes were designed and synthesized with dicarboxylates or sulfate as bridges. The results of *in vitro* cytotoxicity assays suggested that complex **X5** owning sulfate as a bridge showed better cytotoxicity against all selected human cell lines than the positive controls. Typical platinum(II) complex indicated that it can inhibit tumor growth by induction of apoptosis. Consequently, the prepared dinuclear platinum(II) complexes, especially **X5**, may be promising leading compounds for further research.

Experimental

Materials and instruments

K_2PtCl_4 was purchased from Sino-Platinum Co., Ltd. [17, 20]; *mono*-Boc protected DACH was used as the starting material and prepared according to the procedure reported [21].

All reagents were of high purity and used without any further purification. Elemental analysis for C, H, and N was performed with a Perkin-Elmer 1400C instrument. ESI-MS spectra were carried out in a Finnigan MAT SSQ 710 (120–1000 am) apparatus and IR spectra were scanned by a Nicolet IR200 spectrophotometer in the range of 4000–400 cm^{-1} in KBr pellet. ^1H NMR spectra were recorded on a Bruker DRX-500 spectrometer at 500 MHz in D_2O using sodium tetramethylsilylpropanoate (TSP) as an internal reference.

Synthesis

Ligand HP: 3-(2-amino-cyclohexylamino)-propionic acid

Fifty millimolar of *mono*-Boc protected 1*R*,2*R*-DACH, 70 mmol K_2CO_3 and 50 mmol ethyl 3-bromopropionate were dissolved in 300 mL of acetonitrile, and refluxed for 12 h. Pale yellow oil (intermediate I1) was separated by silica gel column chromatography (PE(60–90)/EtOAc/methanol = 5:1:1). The intermediate 1 was then dissolved in 200 mL of methanol and excess HCl/methanol (5 mol/L) was added, leading to the formation of intermediate I2, which was finally hydrolyzed under a basic condition to give the sodium salt of HP, 3.1 g. Yield, 30%. M.W.: 208.0. Found (calcd. for $C_9H_{17}N_2O_2Na$), C 51.89 (51.92), H 8.26 (8.17), N 13.55 (13.46). IR (cm^{-1}): 3330s (br), 2963s, 2926s, 2858s, 1590vs (sh), 1443vs, 1338m, 1300m, 1140m, 1076w, 916m, 879m, 769m, 438m; ^1H NMR (D_2O/TSP, ppm): δ 1.02–1.24 (m, 4H, 2$\underline{CH_2}$ of DACH), 1.65–2.20 (m, 4H, 2$\underline{CH_2}$ of DACH), 2.22–2.24 (m, 1H, $NH_2\underline{CH}$ of DACH), 2.35–2.46 (m, 2H, $CH_2\underline{CH_2}COO$), 2.69–2.74 (m, 1H, $NH\underline{CH}$ of DACH), 2.90–3.35 (m, 2H, $NH\underline{CH_2}CH_2COO$); ESI-MS *m*/*z*: $[M+H]^+ = 209.0$ (100%).

Intermediate [PtPI]

K_2PtCl_4 (10 mmol) was added to a stirring aqueous solution (50 mL) of KI (70 mmol). The solution was stirred at 25°C for 30 min under a nitrogen atmosphere to obtain a black solution of K_2PtI_4, into which an aqueous solution of 10 mmol sodium salt of HP in 15 mL water was added dropwise with stirring. After 24 h at 25°C in the dark, a yellow precipitate was filtered, washed sequentially and thoroughly with water, ethanol and ether, and finally dried in vacuum. Data for [PtPI]: 2.8 g, yield: 56%, dark yellow solid. Found (calcd. for $C_9H_{17}IN_2O_2Pt$), C 21.23 (21.30), H 3.44 (3.35), N 5.37 (5.52), Pt 38.30 (38.47). IR (cm^{-1}): 3432s (br), 3179s, 3106s, 2928m, 2854m, 1594vs, 1443m, 1383s, 1322m, 1255m, 1067m, 1020m, 930w, 884m, 643w, 486m; ^1H NMR (D_2O/TSP, ppm): δ 1.11–1.66 (m, 6H, 3$\underline{CH_2}$ of DACH), 2.01–2.78 (m, 6H, 2$\underline{CH}$ of DACH and $\underline{CH_2}$ of DACH and $NH\underline{CH_2}CH_2COO$), 3.27–3.66 (m, 2H, $NHCH_2\underline{CH_2}COO$); ESI-MS *m*/*z*: $[M+H]^+ = 508$ (100%), $[M+Na]^+ = 530$ (50%).

Complex ***X1***

A suspension of silver oxalate (10 mmol) and [PtPI] (20 mmol) in 200 mL water was stirred at 60°C under a nitrogen atmosphere in the dark for 24 h, the resulting AgI precipitate was filtered off and washed with water for two times. The filtrate was evaporated to nearly dryness and to give a white solid, which was washed with cool water and ethanol for several times, and then dried in vacuum. Yield 31%. Found (calcd. for $C_{20}H_{34}N_4O_8Pt_2$), C 28.25 (28.30), H 4.09 (4.01), N 6.53 (6.60), Pt 45.92 (46.01). IR (cm^{-1}): 3479s (br), 3111s, 2937m, 2864m, 1647vs (sh), 1392s, 1322m, 1271m, 1078w, 1030w, 903w, 806m, 572m, 499m; ^{1}H NMR (D_2O/TSP, ppm): δ 1.11–1.63 (m, 12H, 6$\underline{CH_2}$ of 2DACH), 1.92–2.65 (m, 12H, 2$\underline{CH_2}$ of 2DACH and 4$\underline{CH}$ of 2DACH and 2NH$\underline{CH_2}CH_2COO$), 3.15–3.29 (m, 4H, 2NHCH$_2\underline{CH_2}$OO); ESI-MS m/z: $[M+Na]^+ = 871.5$ (80%). The synthetic procedures for complexes **X2**–**X5** were similar to **X1**.

Complex ***X2***

Yield 26%. Found (calcd. for $C_{21}H_{36}N_4O_8Pt_2$), C 29.31 (29.23), H 4.26 (4.18), N 6.58 (6.50), Pt 45.10 (45.27). IR (cm^{-1}): 3423s (br), 3228s, 2937s, 2859s, 1615vs, 1453s, 1355vs, 1253m, 1174m, 1079m, 1031m, 960m, 890m, 722m, 653w, 563m, 499m. ^{1}H NMR (D_2O/TSP, ppm): δ 1.14–1.61 (m, 12H, 6$\underline{CH_2}$ of 2DACH), 2.02–2.66 (m, 12H, 2$\underline{CH_2}$ of 2DACH and 4CH of 2DACH and 2NH$\underline{CH_2}CH_2COO$), 3.21–3.28 (m, 6H, 2NHCH$_2\underline{CH_2}$COO and COO$\underline{CH_2}$COO). ESI-MS m/z: $[M+H]^+ = 863.5$ (30%), $[M+K]^+ = 901.6$ (50%).

Complex ***X3***

Yield 22%. Found (calcd. for $C_{22}H_{38}N_4O_8Pt_2$), C 29.99 (30.14), H 4.36 (4.34), N 6.56 (6.39), Pt 44.31 (44.52). IR (cm^{-1}): 3443s (br), 3232s, 2938s, 2857m, 1616vs (sh), 1387vs, 1261s, 1174m, 1079m, 1030w, 888w, 807m, 640m (br), 547m. ^{1}H NMR (D_2O/TSP, ppm): δ 1.11–1.62 (m, 12H, 6$\underline{CH_2}$ of 2DACH), 2.02–2.67 (m, 16H, 2$\underline{CH_2}$ of 2DACH and 4$\underline{CH}$ of 2DACH and 4H of COO$\underline{CH_2}\underline{CH_2}$COO and 2NH$\underline{CH_2}CH_2COO$), 3.09–3.29 (m, 4H, 2NHCH$_2\underline{CH_2}$COO). ESI-MS m/z: $[M+H]^+ = 877.6$ (30%), $[M+Na]^+ = 899.5$ (30%).

Complex ***X4***

Yield 28%. Found (calcd. for $C_{24}H_{40}N_4O_8Pt_2$), C 31.79 (31.93), H 4.50 (4.43), N 6.33 (6.21), Pt 43.09 (43.28). IR (cm^{-1}): 3444vs (br), 3232vs, 2944s, 2865s, 1615vs, 1374vs, 1253m, 1154m, 1117m, 1079w, 1031w, 890m, 787m, 704m, 654w, 563m, 499m. ^{1}H NMR (D_2O/TSP, ppm): δ 1.15–1.83 (m, 14H, 6$\underline{CH_2}$ of 2DACH and 2H of (COO)$_2$C(CH$_2$)$_2\underline{CH_2}$), 1.88–2.86 (m, 16H, 2$\underline{CH_2}$ of 2DACH and 4$\underline{CH}$ of 2DACH and 4H of (COO)$_2$C($\underline{CH_2}$)$_2$CH$_2$ and 2NH$\underline{CH_2}CH_2COO$), 2.90–3.30 (m, 4H, 2NHCH$_2\underline{CH_2}$COO). ESI-MS m/z: $[M+H]^+ = 903.5$ (25%), $[M+Na]^+ = 925.5$ (33%).

Complex ***X5***

Yield 35%. Found (calcd. for $C_{18}H_{34}N_4O_8SPt_2$), C 25.31 (25.23), H 4.05 (3.97), N 6.50 (6.54), Pt 45.39 (45.57). IR (cm^{-1}): 3459m (br), 3114m, 2938m, 2856m, 1716m, 1541s, 1455m, 1399m, 1332m, 1190vs, 1110vs, 1048sm, 871m, 658m, 618s (sh). ^{1}H NMR (D_2O/TSP, ppm): δ 1.14–1.62 (m, 12H, 6$\underline{CH_2}$ of 2DACH), 2.04–2.69 (m, 12H, 2$\underline{CH_2}$ of 2DACH and 4$\underline{CH}$ of 2DACH and 2NH$\underline{CH_2}CH_2COO$), 3.08–3.25 (m, 4H, 2NHCH$_2\underline{CH_2}$OO). ESI-MS m/z: $[M+Na]^+ = 879.5$ (70%).

In vitro **cytotoxicity**

In vitro cytotoxicity was evaluated against four different human carcinoma cell lines: HepG-2 human hepatocellular carcinoma cells, MCF-7 human breast cancer cells, A549 human lung cancer cells and HCT-116 human colorectal cancer cells. Among them, MCF-7 and HepG-2 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin; HCT-116 was cultured in McCoy's 5A (Sigma–Aldrich), which contained 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The experimental procedures of MTT assay were described by Mosmann [22]. Complexes **X1**–**X5**, oxaliplatin and carboplatin were added in final concentrations ranging from 0 to 100 μM. After 48 h, 10 μL MTT in PBS (5 mg/mL) was added to each well and the plates were incubated for 3 h at 37°C. Remove the liquid and add DMSO (150 μL) to dissolve the formazan. The OD for each well was measured on a microplate reader at a wavelength of 490 nm. All cytotoxicity tests were carried out three times parallelly, IC_{50} values were calculated from curves constructed by plotting the inhibitory rate of compounds against tumor cells (%) versus the logarithm of compounds concentrations.

Induction of cell apoptosis

Apoptosis of tumor cells induced by complexes **X1**, **X5**, or oxaliplatin (positive control) was measured by FCM using annexin V-FITC/PI apoptosis kit according to the instructions. HCT-116 cells were washed in cold phosphate-buffered saline (PBS) and then centrifuged (5 min, 2000 rpm). Cells were stained with annexin V-FITC and propidium iodide (PI) in the binding buffer. After 15 min of incubation at room temperature, the fluorescence was tested using a flow cytometer (FACS Calibur, BD Corp, USA) and the excitation wavelength was 488 nm. Results were analyzed using Cell Quest Pro software and represented as percentage of normal and apoptotic cells at various stages. FITC and PI fluorescences were measured in the FL1 and the FL2 channel, respectively.

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The authors have declared no conflict of interest.

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