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Research paper

Design, synthesis and biological evaluation of demethylcantharidate-linked platinum(II) complexes of *N*-monoalkyl-1*R*,2*R*-diaminocyclohexane derivatives

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ABSTRACT

Nine platinum(II) complexes with *N*-monoalkyl 1*R*,2*R*-DACH derivatives as carrier ligands and demethylcantharidate as a leaving group were synthesized and spectrally characterized. All the complexes showed considerable cytotoxicity against tested human cancer cell lines: A549, HCT116 HepG-2 and MCF7 cell lines. Especially, complex **2** exhibited potent cytotoxicity against A549 (1.01 μM) and HCT116 (0.83 μM) cell lines, and showed no cross-resistance to cisplatin against SGC7901/CDDP cell line (RF = 1.44). In addition, the typical compounds were further studied by flow cytometric analysis and western blot method. The results indicated that they induced apoptosis by a mitochondrial-dependent pathway, which were similar to cisplatin.

1. Introduction

Cisplatin has been widely used in the treatment of testicular, ovarian, bladder and head/neck tumors for decades since its first approval by FDA in 1978 [1]. After the success of cisplatin, a large number of anticancer platinum complexes have been studied. Among them, carboplatin and oxaliplatin have been accepted worldwide, along with five other platinum(II) complexes gained approval regionally in different nations (nedaplatin and miriplatin in Japan, lobaplatin and dicycloplatin in China, heptaplatin in Korea) [2]. However, these clinical used platinum drugs possess similar drawbacks, including severe side effects and intrinsic and/or acquired resistance [3–5]. Several strategies have been employed in order to overcome these drawbacks, among which application of bioactive ligands is a promising one in the design of novel platinum complexes. Such resulting complexes may have dual mechanisms of action, i.e., one from the proper biologically active ligand and the other from Pt pharmacophore [6–12].

Traditional Asian medicine, especially traditional Chinese medicine (TCM), is a treasure yet to be efficiently explored [13,14]. Artemisinin [15] as antimalarial agent and arsenic trioxide [16] for the use in leukaemia is one of modern drugs successfully mined from traditional Chinese medicine. The platinum-TCM conjugates may generate a novel strategy to combine the two kinds of old drugs together synergistically [17]. The platinum(II) complexes containing camphorato, derivative of long used TCM camphor, showed promising *in vitro* and *in vivo* anticancer activity [18,19]. Cantharidin is another TCM used for the treatment of liver, lung and intestinal cancers for a long time. Demethylcantharidin is a synthetic analogue with similar biological activity and much less toxic than cantharidin. Combination platinum(II) moieties with demethylcantharidate as a leaving group have resulted in a series of novel complexes. The relative platinum complexes showed more potent antitumor activity against some cancer cell lines than cisplatin/oxaliplatin and had no cross-resistance with cisplatin. Moreover, they possessed a dual antitumor mechanism, namely, inhibition of PP2A (demethylcantharidin) and platination of DNA (platinum moiety) [20–22].

So far, numerous platinum complexes containing 1*R*,2*R*-diaminocyclohexane (1*R*,2*R*-DACH) and its derivatives as carrier ligands have been reported after oxaliplatin was approved worldwide. In our previous research [23–28], a number of platinum(II) complexes of *N*-monoalkyl 1*R*,2*R*-DACH as carrier ligands have been investigated and some of these compounds showed better anticancer activity towards some cancer cell lines than their counterparts with 1*R*,2*R*-DACH as ligand. To continue our research, a series of platinum(II) complexes were obtained in this study with the above mentioned ligands and demethylcantharidate as a leaving ligand. The *in vitro* cytotoxicity of the synthesized complexes

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against six human solid cancer lines was studied. Additionally, the mechanism of cellular death of typical complexes was also investigated in this paper.

2. Results and discussion

2.1. Synthesis and characterization

Complexes **1**–**9** were synthesized *via* Scheme 1. The intermediates **M1–M9** were obtained by a routine method described by our previous study [23–25]. Demethylcantharidate was prepared by a Diels-Alder reaction between furan and maleic anhydride, followed by hydrogenation using Pd/C as catalyst [20]. All intermediates were characterized by ^{1}H NMR, FT-IR, and ESI-MS spectra together with microanalysis. Platinum(II) complexes **1**–**9** were spectrally characterized by ^{1}H NMR, ^{13}C NMR (complexes **2** and **7**), FT-IR and ESI-MS spectra as well as element analyses.

In the IR spectra of all platinum complexes, the N—H stretching vibrations appeared between 3202 and 3440 cm^{-1}, shifting to lower frequencies than those of the free alkyl amine. The ν_{as}(C=O) signal of the complexes appears in the range from 1650 to 1622 cm^{-1}, characteristic signals of coordinated dicarboxylates, and the C—O signal appeared in the range of 1311–1325 cm^{-1}. All ^{1}H NMR and ^{13}C NMR data are compatible to the molecular structures proposed in Scheme 1. In the ^{1}H NMR spectra of complexes **1**–**9**, the broad signals of hydrogen atoms belonging to amino groups appear in the range of δ 6.17–6.50 ppm due to amine coordination with metal ions, shifting to high-field compared with the metal-free ligands in the range of δ 8.80–9.40 ppm (appeared only when d_6-DMSO was used as NMR solvent, complexes **2** and **7**). Moreover, the signals of C—H protons connected to the amino groups were observed between 2.48 and 2.94 ppm, clearly shifting high-field relative to the corresponding signals ($\delta \approx 3.5$ ppm) in the free ligands. Besides, all the complexes showed 100% of $[M+H]^+$ or $[M+Na]^+$ peaks in the ESI-MS spectroscopy, which have three main ion peaks due to the existence of ^{194}Pt (33%), ^{195}Pt (34%) and ^{196}Pt (25%) isotopes. Elemental analysis data of C, H and N for each complex were in good fit with the empirical formula proposed.

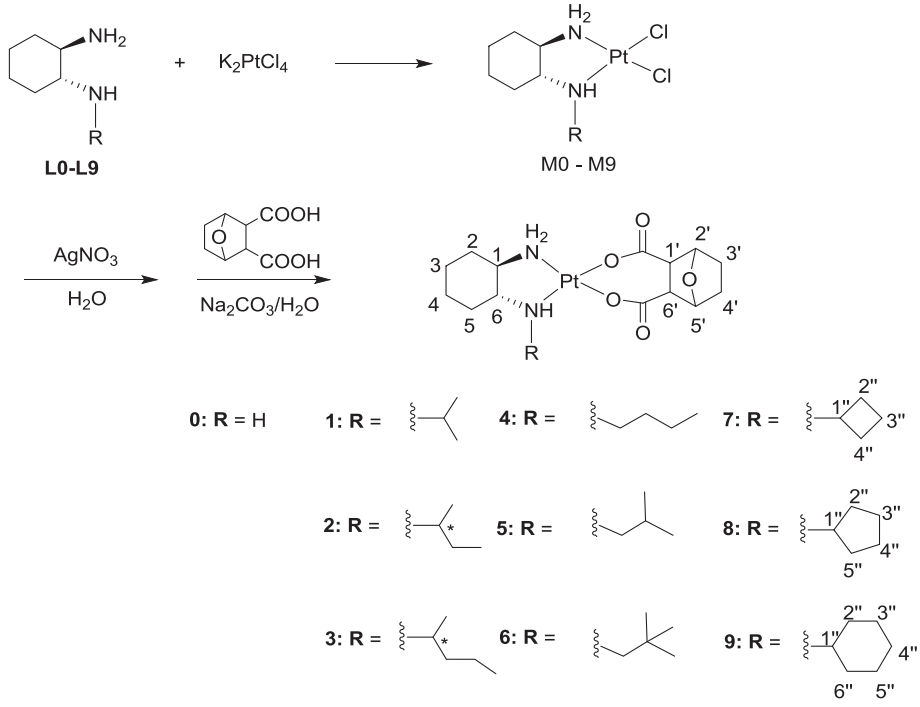
Notably, complexes **2** and **3** had an extra stereogenic carbon center along with other three stereogenic centers [24]; however, no effort was devoted to isolating the pure enantiomers in this study. Therefore, both *R* and *S* configurations at the α-carbon atom are present in the final compounds.

2.2. In vitro cytotoxicity studies

The cytotoxicity of complexes **0**–**9** was determined by MTT assays against A549, HCT116, HepG-2 and MCF7 human cancer cell lines. The *in vitro* and *in vivo* suppression of growth of cancer cells (especially hepatocellular carcinoma cells) by complex **0** were reported before by Yee-Ping Ho's group [20], and the *in vitro* cytotoxicity of complex **0** was measured in this paper as another positive control, along with cisplatin and oxaliplatin. The result of cytotoxicity against four cancer cell lines are expressed as IC_{50} values listed in Table 1.

According to the IC_{50} values, most of the synthesized complexes showed comparable or better cytotoxicity to cisplatin and oxaliplatin against A549, HCT116 and MCF7 cancer cell lines. However, only complex **2** possessed comparable cytotoxicity against HepG-2 cell line with positive controls.

It is noted that the antiproliferative properties of the synthesized platinum complexes with *N*-monoalkylsubstituted 1*R*,2*R*-DACH as carrier ligands against A549 and HCT116 cancer cells were enhanced compared with complex **0**. Significantly, complex **2** were 3–4 times more potent than cisplatin/oxaliplatin against A549 cell line and 2–3 folds more potent than cisplatin/oxaliplatin against HCT116 cell line. Furthermore, complexes **5** and **7** had better cytotoxicity against MCF7 cell line than complex 0, and complex 5 was slightly more potent than cisplatin against MCF7 cell line. However, the HepG-2 cell line was much less responsive to our complexes than complex **0**, which indicated that the steric hinderance in the carrier ligands might reduce the cytotoxicity of the platinum complex against HepG-2 cell line.



Scheme 1. The synthetic method and related chemical complexes studied in this work.

Table 1
In vitro cytotoxicity of complexes **1**–**9** and positive controls against human tumor cell lines.

Complex	IC_{50} (μM)[a]			
	A549[b]	HCT116[c]	HepG-2[d]	MCF7[e]
Cisplatin	3.65 ± 0.38	1.72 ± 0.19	1.22 ± 0.10	1.18 ± 0.22
Oxaliplatin	3.98 ± 0.51	2.21 ± 0.24	1.42 ± 0.12	2.61 ± 0.18
0	21.2 ± 1.7	8.23 ± 0.78	0.52 ± 0.03	9.30 ± 1.01
1	11.41 ± 0.98	> 100	84.38 ± 5.11	25.7 ± 1.87
2	1.01 ± 0.05	0.83 ± 0.04	7.55 ± 1.08	11.44 ± 2.11
3	42.12 ± 3.21	9.22 ± 1.23	55.60 ± 4.30	31.61 ± 2.88
4	> 100	16.28 ± 2.01	99.04 ± 7.44	9.10 ± 4.40
5	15.77 ± 1.34	3.41 ± 0.23	> 100	0.85 ± 0.07
6	8.17 ± 0.96	> 100	67.82 ± 4.33	22.81 ± 1.86
7	20.81 ± 1.76	5.21 ± 1.03	> 100	2.41 ± 0.19
8	9.32 ± 0.84	6.62 ± 0.44	13.82 ± 1.09	> 100
9	32.12 ± 2.22	18.37 ± 1.26	29.60 ± 1.83	13.39 ± 1.34

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 48 h drug exposure; Values represent the mean ± SD from three intendent experiments.
[b] Human non-small-cell lung cancer cell line.
[c] Human colorectal carcinoma cell line.
[d] Human hepatocellular carcinoma cell line.
[e] Human breast carcinoma cell line.

Intrinsic and/or acquired resistance is the main drawback of platinum-based drugs. It is reported that there are three main mechanisms of Pt resistance, namely, (i) reduced drug uptake and/or increased drug efflux; (ii) detoxification by intracellular thiols such as glutathione; (iii) increased repair and/or tolerance of DNA–cisplatin adducts [29,30]. The drug resistance may arise from one or more of the above-mentioned mechanisms.

The cytotoxicity of complex **2** against cisplatin-sensitive and cisplatin-resistant gastric carcinoma cell lines (SGC7901 and SGC7901/CDDP) was further investigated with cisplatin as positive control, in order to find out whether our platinum complexes can overcome the cisplatin resistance. The IC_{50} data are listed in Table 2. The result showed that complex **2** was devoid of cross-resistance to cisplatin with a remarkable decrease in RF value (1.44) compared with cisplatin (10.00), and complex **2** had better cytotoxicity against SGC7901/CDDP cell lines than cisplatin.

2.3. Apoptosis study

Apoptosis is a programmed cell death. The apoptotic analysis of complex **2**, complex **7**, cisplatin and oxaliplatin was carried out using an AnnexinV-FITC/PI assay. The tested complexes were incubated with HCT-116 cells for 24 h at 50 μM, and the results are shown in Figs. 1 and 2. Q1 (top left quarter), Q2 (top right quarter), Q3 (lower left quarter) and Q4 (lower right quarter) of the diagram stand for the unnatural death cells, late apoptotic/necrotic cells, normal cells, and early apoptotic cells, respectively.

As shown in Figs. 1 and 2, complex **2** and complex **7** greatly increased the population of apoptotic cells compared with negative control, while complex **2** showed the highest population of apoptotic cells (39.6%) among all tested complexes, about 1.1-fold higher than cisplatin (35.2%) and 1.5-fold higher than oxaliplatin (25.6%). The result of apoptosis study is consistent with that of *in vitro* cytotoxicity assay against HCT116 cells, which indicates that the tested compounds may cause cancer cell death through an apoptotic mechanism.

Table 2
Further *in vitro* assay of complexes **2** and **7** with cisplatin as positive controls against human cancer cell lines.

Complex	IC_{50} (μM)[a]		
	SGC7901[b]	SGC7901/CDDP[c]	RF[d]
Cisplatin	3.83 ± 0.18	38.32 ± 1.77	10.00
2	8.77 ± 0.81	12.69 ± 1.21	1.44

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 48 h drug exposure.
[b] Human gastric carcinoma cell lines.
[c] Cisplatin-resistant human gastric carcinoma cell lines.
[d] RF (resistance factor), equals the IC_{50} of the resistant cells divided by the sensitive ones.

2.4. Western blot analysis

In order to explore more about the apoptotic mechanism, western blot assay was employed to determine the expressions of four apoptotic-related proteins (Bax, Bcl-2, Procapase-3 and Cleaved-caspase-3) of HCT116 and MCF7 cell lines treated with complex **2**, complex **7** and cisplatin. As shown in Fig. 3, remarkable increase of Bax expression and decrease of Bcl-2 expression were observed in both HCT116 and MCF7 cells treated with cisplatin, complex **2** and complex **7** compared with negative control. Furthermore, the expressions of Procaspase-3 in two cancer cell lines were significantly decreased, while the expressions of Cleaved-caspase-3 were up-regulated. The result of western bolt analysis suggested that platinum(II) complex **2** and **7** could induce cell apoptosis through an intrinsic mitochondrial-dependent pathway similar to cisplatin.

3. Conclusion

In the present study, a series of platinum(II) complexes with *N*-monoalkylsubstituted 1*R*,2*R*-DACH as carrier ligands and demethylcantharidate as a leaving ligand were synthesized and characterized. According to the result of *in vitro* cytotoxicity, almost all the synthesized complexes showed considerable cytotoxicity against tested human cancer cell lines, especially A549, HCT116 and MCF7 cell lines. Notably, complex **2** was the most promising one among these complexes, which showed 2–4 times higher cytotoxicity against A549 and HCT116 cells than cisplatin. Moreover, complex **5** and complex **7** exhibited slightly potent or similar cytotoxicity against MCF7 compared to positive controls, while most of the complexes only had moderate responses to HepG-2 cell lines. Further *in vitro* assay against SGC7901 and SGC7901/CDDP indicated that complex 2 had no cross-resistance to cisplatin with a smaller RF value than 2.00. The flow cytometric analysis suggested that complex **2** and complex **7** cause cell death by inducing apoptosis, which were similar to cisplatin and oxali-

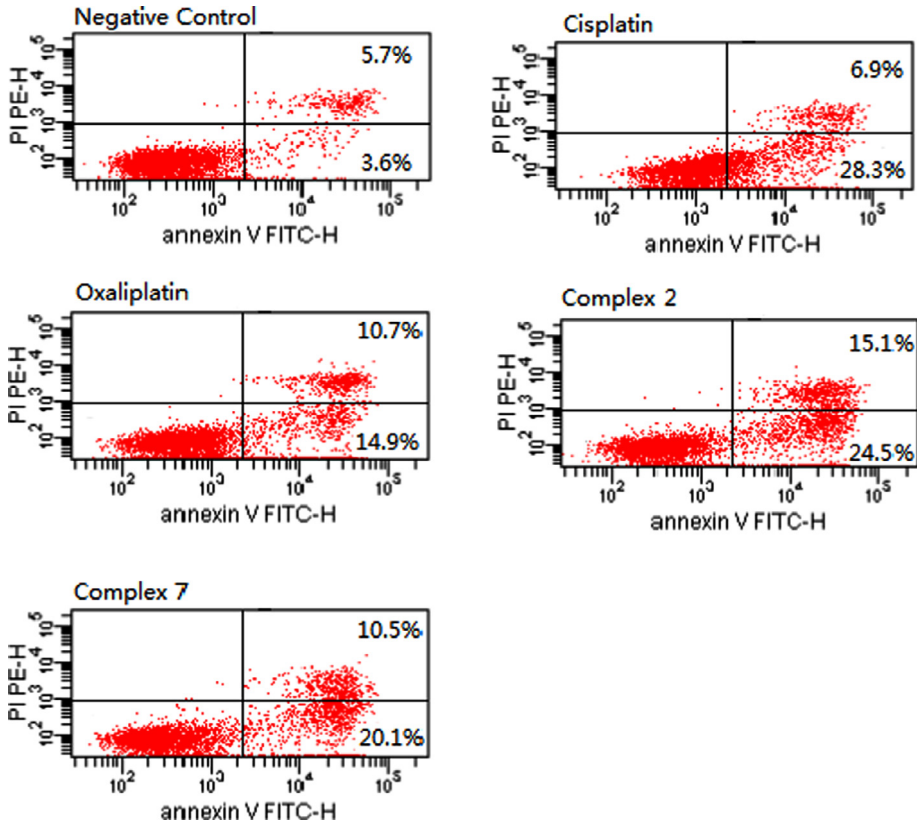


Fig. 1. Flow cytometric analysis of the distribution of HCT116 cell lines untreated or treated with cisplatin, oxaliplatin, complexes **2** and **7** at 50 μm for 24 h.

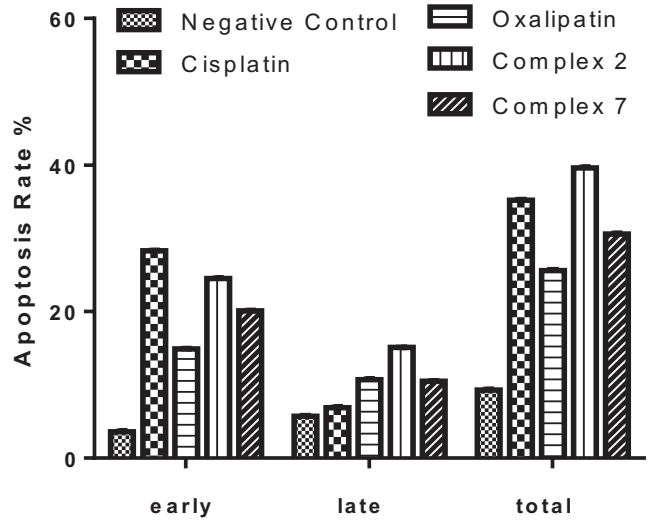


Fig. 2. Results of flow cytometric analysis of the distribution of HCT116 cell lines untreated or treated with cisplatin, oxaliplatin, complexes **2** and **7** at 50 μm for 24 h.

platin. Furthermore, western blot analysis indicated that both complexes had a similar intrinsic mitochondrial apoptotic pathway to cisplatin. In summary, the results indicated that the steric hindrance of carrier ligand has an impact on the biological activity of the platinum(II) complexes, and both *N*-(2-butyl)-1*R*,2*R*-DACH and *N*-cyclobutyl-1*R*,2*R*-DACH are proper carrier ligands in this type of anticancer platinum(II) complexes, and complex **2** is a promising candidate worth further investigation.

4. Experimental details

4.1. Chemistry

All solvents and reagents were of analytical purity and used without further purification. Potassium tetrachloroplatinate(II) and 1*R*,2*R*-diaminecyclohexane were obtained from Shangdong Boyuan Chem. Co. Ltd., China. IR spectra were carried out on KBr pellet in the range of 4000–400 cm^{-1} with a Nicolet IR200 FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker DRX500 spectrometer. C, H and N elemental analyses were performed on a Vario MICRO CHNOS Elemental Analyze (Elementar). ESI-MS analysis was performed with an Agilent Accurate Mass 6224 TOF LC/MS system.

4.1.1. Preparation of complexes ***1*–*9***

4.1.1.1. General procedure. A solution of $AgNO_3$ (2 mmol, 5 mL deionized water) was added into a suspension of the corresponding intermediates **M1–M9** (1 mmol) in 50 mL water, and the suspension was vigorously stirred at 50 °C under a nitrogen atmosphere in darkness for 16 h. The AgCl deposit was filtered off, then a solution of sodium demethylcantharidate (1 mmol) in 5 mL water was added into the filtrate and stirred for 24 h at 40 °C in darkness. The volume of water was reduced down to 2 mL approximately, and the product slowly precipitated.

Complex **1**: White powder; Yield 55%; IR (KBr): 3434 s (br), 2942 s, 2871 s, 2426 s, 2356 s, 1634vs, 1455 s, 1384vs, 1324 s, 1256 s, 1261 s, 1144 m, 1055 m, 1040 m, 992 s, 932 s, 820 s, 807 s, 668 m, 584 m cm^{-1}; ^{1}H NMR (D_2O/TSP, ppm): 1.04–1.10 (m, 6H, $CH(CH_3)_2$), 1.16–2.17 (m, 12H, 4CH_2 of DACH and 2CH_2 of $C_8H_8O_5$), 2.49–3.02 (m, 3H, NH*CH* and 2*CH* of DACH), 3.58–3.71 (m, 2H, *CHOCH*), 4.72 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 22.0 ($2CH_3$), 23.9, 24.4 (C3 and C4), 30.6, 31.5, 32.4 (C2, C5

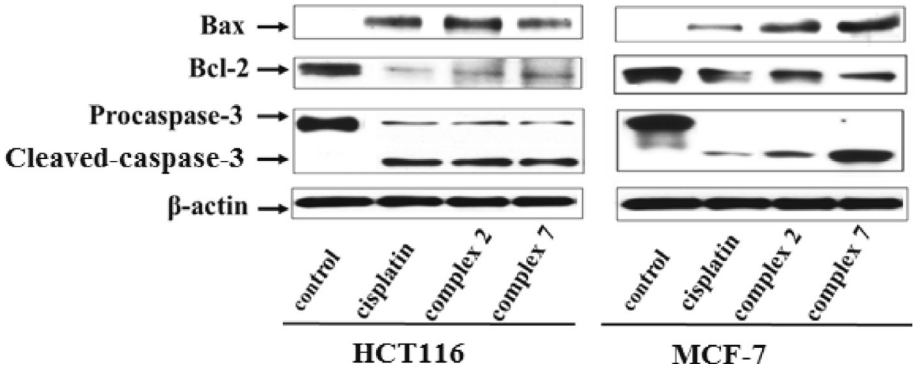


Fig. 3. The expression of apoptosis-related proteins was determined by western-blot assay. HCT116 and MCF7 cells treated with cisplatin, complex **2** and complex **7** at 50 μM for 12 h, and equal loading was testified by the detection of β-actin. Results were obtained similarly from three independent experiments.

and C3′/C4′), 56.8, 58.1 (C1′/C6′ and NHCH$(CH_3)_2$), 61.2, 63.7 (C1 and C6), 83.1 (C2′/C5′), 184.4 (*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 536.1 (100%), $[M+Na]^+$ = 558.2 (45%); Anal. calcd. for $C_{17}H_{28}N_2O_5Pt$ (535.1): C 38.13, H 5.27, N 5.23; found: C 38.01, H 5.29, N 5.08.

Complex **2**: White powder; Yield 33%; IR (KBr): 3431 s (br), 3202 s, 2939 s, 2873 s, 2360 s, 2340 s, 1629vs, 1453 s, 1384vs, 1323vs, 1259 s, 1213 m, 1172 m, 1145 m, 1060 m, 1033 m, 993 s, 933 s, 902 m, 867 m, 820 s, 668 m, 581 s cm^{-1}; 1H NMR (d_6-DMSO/TMS, ppm): 0.97–1.16 (m, 6H,*CH*$_3$CHCH$_2$*CH*$_3$), 1.22–2.12 (m, 14H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$ and 2H of CH$_3$CH*CH*$_2$-CH$_3$), 2.49–2.86 (m, 3H, NH*CH* and 2CH of DACH), 3.58–3.70 (m, 2H, *CHOCH*), 4.66–4.75 (m, 2H, 2*CH*COO), 6.17–6.36 (m, 3H, CH*NH*$_2$ and CH*NH*); ^{13}C NMR (d_6-DMSO, ppm): 11.1 (CH_2CH_3), 18.2 (CH*CH*$_3$), 24.2, 24.5 (C3 and C4), 26.4, 28.8, 31.0, 31.9 (C2, C5, C3′/C4′ and *CH*$_2$CH$_3$), 56.3 (C1′/C6′), 59.1, 60.7, 66.7 (C1, C6 and NHCHCH$_3$), 81.6 (C2′/C5′), 183.8 (*C*=*O*); ESI-MS *m*/*z*: [M +H]$^+$ = 550.2 (21%), $[M+Na]^+$ = 572.0 (100%); Anal. calcd. for $C_{18}H_{30}N_2O_5Pt$ (549.2): C 39.34, H 5.50, N 5.10; found: C 39.13, H 5.29, N 4.97.

Complex **3**: White powder; Yield 75%; IR (KBr): 3424 s (br), 2943 s, 2871 s, 2360 s, 2340 s, 1629vs, 1454 s, 1384vs, 1323 s, 1259 s, 1213 m, 1175 m, 1144 m, 1059 m, 1031 m, 993 s, 933 s, 820 s, 808 s, 668 m, 576 s cm^{-1}; ^{1}H NMR (D_2O/TSP, ppm): 0.81–0.97 (m, 6H,*CH*$_3$CHCH$_2$CH$_2$*CH*$_3$), 1.06–2.15 (m, 16H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$ and 4H of CH$_3$CH*CH*$_2$*CH*$_2$CH$_3$), 2.49–2.79 (m, 3H, NH*CH* and 2CH of DACH), 3.42–3.70 (m, 2H, *CHOCH*), 4.72–4.74 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 14.1 (CH_2CH_3), 19.2, 21.1 (*CH*$_2$CH$_3$ and CH*CH*$_3$), 23.8, 24.2 (C3 and C4), 29.3, 31.0, 31.9, 32.4 (C2, C5, C3′/C4′ and *CH*$_2$CH$_2$CH$_3$), 56.1 (C1′/C6′), 59.4, 60.1, 63.4 (C1, C6 and NHCHCH$_3$), 79.7 (C2′/C5′), 180.9 (*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 564.2 (34%), $[M+Na]^+$ = 586.0 (100%); Anal. calcd. for $C_{19}H_{32}N_2O_5Pt$ (563.2): C 40.49, H 5.72, N 4.97; found: C 39.99, H 5.57, N 4.78.

Complex **4**: Pale yellow powder; Yield 46%; IR (KBr): 3435 s (br), 3119 s, 2940 s, 2870 s, 2426 m, 2360 m, 1650vs, 1384vs, 1336 s, 1256 s, 1212 s, 1144 m, 1059 m, 1040 m, 993 s, 932 s, 902 m, 821 s, 666 m, 582 s cm^{-1}; ^{1}H NMR (D_2O/TSP, ppm): 0.88–0.95 (t, 3H,CH$_2$*CH*$_3$), 1.04–2.12 (m, 16H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$ and CH$_3$CH$_2$CH$_2$), 2.49–2.80 (m, 4H, NH*CH*$_2$ and 2*CH* of DACH), 3.59–3.68 (m, 2H, *CHOCH*), 4.57–4.72 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 13.6 (CH$_2$*C*H$_3$), 19.5 (*C*H$_2$CH$_3$), 25.1, 25.4 (C3 and C4), 29.1, 30.7, 31.2, 33.2 (C2, C5, C3′/C4′ and NHCH$_2$-*C*H$_2$), 49.5 (NHCH$_2$), 57.1 (C1′/C6′), 60.2, 63.5 (C1, C6), 78.6 (C2′/C5′), 177.5 (*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 550.1 (100%), [M +Na]$^+$ = 572.2 (83%); Anal. calcd. for $C_{18}H_{30}N_2O_5Pt$ (549.2): C 39.34, H 5.50, N 5.10; found: C 39.09, H 5.59, N 4.98.

Complex **5**: Pale yellow powder; Yield 42%; IR(KBr): 3435 s (br), 2946 s, 2870 s, 2360 s, 2341 s, 1634vs, 1455 s, 1383vs, 1322 s, 1258 s, 1213 s, 1142 m, 994 s, 932 s, 901 s, 820 s, 807 s, 667 m, 580 s cm^{-1}; 1H NMR (D_2O/TSP, ppm): 0.81–0.95 (m, 6H, CH(*CH*$_3$)$_2$), 1.03–2.12 (m, 13H, 4*CH*$_2$ of DACH, 2*CH*$_2$ of $C_8H_8O_5$ and *CH* of CH$(CH_3)_2$), 2.49–2.69 (m, 4H, NH*CH*$_2$ and 2*CH* of DACH), 3.61–3.69 (m, 2H, *CHOCH*), 4.72–4.73 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 21.8, 24.1, 24.6 (2*C*H$_3$, C3 and C4), 28.7, 29.6, 30.4, 33.4 (*C*H$(CH_3)_2$, C2, C5 and C3′/C4′), 55.7, 58.9 (C1′/C6′ and NH*C*H$_2$), 62.7, 66.8 (C1 and C6), 81.9 (C2′/C5′), 180.3 (*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 550.1 (75%), $[M+Na]^+$ = 572.1 (100%); Anal. calcd. for $C_{18}H_{30}N_2O_5Pt$ (549.2): C 39.34, H 5.50, N 5.10; found: C 39.18, H 5.54, N 4.85.

Complex **6**: Pale yellow powder; Yield 46%; IR (KBr):3434 s (br), 2945 s, 2868 s, 2359 s, 1622vs, 1464 s, 1384vs, 1311 s, 1253 m, 1213 m, 1142 m, 1056 m, 1036 m, 995 s, 932 s, 901 m, 820 s, 788 m, 657 m, 573 s cm^{-1}; ^{1}H NMR (D_2O/TSP, ppm): 0.87 (s, 9H, C(*CH*$_3$)$_3$), 1.05–2.12 (m, 12H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$), 2.50–2.87 (m, 4H, NH*CH*$_2$ and 2*CH* of DACH), 3.44–3.89 (m, 2H, *CHOCH*), 4.54–4.71 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 23.8, 24.3 (C3 and C4), 27.6, 29.1, 30.6, 31.5, 33.4 (3*C*H$_3$, C2, C5, C3′/C4′ and *C*$(CH_3)_3$), 54.4 (C1′/C6′), 60.2, 61.4, 67.6 (NH*C*H$_2$, C1 and C6), 80.1 (C2′/C5′), 178.6 (*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 564.0 (100%); Anal. calcd. for $C_{19}H_{32}N_2O_5Pt$ (563.2): C 40.49, H 5.72, N 4.97; found: C 40.15, H 5.81, N 4.69.

Complex **7**: White powder; Yield 71%; IR (KBr): 3434 s (br), 2942 s, 2360 s, 1622vs, 1384vs, 1322 s, 1258 s, 1213 m, 1142 m, 1056 m, 1036 m, 995 s, 930 s, 900 m, 820 s, 791 m, 665 m, 582 s cm^{-1}; ^{1}H NMR (d_6-DMSO/TMS): 1.05–2.12 (m, 18H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$ and 6H of cyclobutyl), 2.48–2.85 (m, 3H, NH*CH* and 2*CH* of DACH), 3.62–3.70 (m, 2H, *CHOCH*), 4.70–4.73 (m, 2H, 2*CH*COO), 6.25–6.50 (m, 3H, CH*NH*$_2$ and CH*NH*); ^{13}C NMR (d_6-DMSO, ppm): 14.1(C3″), 24.2, 24.5 (C3 and C4), 27.6, 29.2, 31.0, 32.3/32.5 (C2, C5, C3′/C4′ and C2″/C4″), 58.1, 60.4, 66.7, 68.9 (C1′/C6′, C1, C6 and C1″), 80.9 (C2′/C5′), 183.1(*C*=*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 548.1 (100%), $[M+Na]^+$ = 570.0 (58%); Anal. calcd. for $C_{18}H_{28}N_2O_5Pt$ (547.2): C 39.49, H 5.15, N 5.12; found: C 39.12, H 5.07, N 4.97.

Complex **8**: White powder; Yield 78%; IR (KBr): 3434 s(br), 3209 s, 2944 s, 2869 s, 2360 s, 2340 s, 1634vs, 1448 m, 1383vs, 1323 s, 1258 s, 1213 m, 1175 m, 820 s, 806 s, 668 m, 581 s cm^{-1}; ^{1}H NMR (D_2O/TSP, ppm): 1.01–2.17 (m, 20H, 4*CH*$_2$ of DACH and 2*CH*$_2$ of $C_8H_8O_5$ and 8H of cyclopentyl), 2.57–2.79 (m, 3H, NH*CH* and 2*CH* of DACH), 3.58–3.66 (m, 2H, *CHOCH*), 4.62–4.70 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 22.4, 22.6, 24.1, 24.3 (C3″, C4″, C3 and C4), 27.9, 30.0, 31.9, 34.8, 35.0 (C2, C5, C3′/C4′, C2″ and C5″), 57.5, 59.4, 61.7, 66.6 (C1′/C6′, C1, C6 and C1″), 77.9 (C2′/C5′), 178.2(*C*=*O*);ESI-MS *m*/*z*: $[M+H]^+$ = 562.2(88%), [M +Na]$^+$ = 583.9(88%); Anal. calcd. for $C_{19}H_{30}N_2O_5Pt$ (561.2): C 40.64, H 5.39, N 4.99; found: C 40.22, H 5.31, N 4.73.

Complex **9**: White powder; Yield 66%; IR (KBr): 3440 s(br), 3105 s, 2934 s, 2857 s, 2360 s, 2341 s, 1635vs, 1449 s, 1382vs, 1322 s, 1258 s, 1212 m, 1168 m, 1059 m, 1031 m, 993 s, 933 s, 899 m, 866 m, 820 s, 806 s, 667 m, 582 s cm^{-1}; ^1H NMR (D_2O/TSP): 1.02–2.16 (m, 22H, 4CH_2 of DACH and 2CH_2 of $C_8H_8O_5$ and 10H of cyclohexyl), 2.62–2.94 (m, 3H, NH*CH* and 2*CH* of DACH), 3.60–3.71 (m, 2H, *CHOCH*), 4.72–4.73 (m, 2H, 2*CH*COO); ^{13}C NMR (d_6-DMSO, ppm): 24.0, 24.3, 25.1, 26.0, 26.2 (C3″, C4″, C5″, C3 and C4), 29.9, 31.8, 32.8, 34.9, 35.2 (C2, C5, C3′/C4′, C2″ and C6″), 59.8, 61.3, 63.4, 66.9 (C1′/C6′, C1, C6 and C1″), 78.6 (C2′/C5′), 180.3(*C*═*O*); ESI-MS *m*/*z*: $[M+H]^+$ = 576.2 (100%); Anal. calcd. for $C_{20}H_{32}N_2O_5Pt$ (575.2): C 41.74, H 5.60, N 4.87; found: C 41.33, H 5.51, N 4.66.

4.2. Biological studies

4.2.1. Cell culture

Four human solid tumor cell lines including human non-small cell lung cancer cell line (A549, ATTC No.: CCL-185), human colorectal carcinoma cell line (HCT-116, ATCC No.: CCL-247), human hepatocellular carcinoma cell line (HepG-2, ATCC No.: HB-8065) and human breast carcinoma cell line (MCF7, ATCC No.: HTB-22; MDA-MB-231, ATCC No.: HTB-26) were used in the cytotoxicity test of all the synthesized platinum(II) complexes. Furthermore, complex **2** and complex **7** were selected to determine the cytotoxicity against gastric carcinoma cell line (SGC-7901, KG026) and cisplatin-resistant gastric carcinoma cell line (SGC-7901/CDDP, KG309), and both of these two cancer cell lines were purchased from Keygen Biotec Co., Ltd. (China). The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 μg/mL of streptomycin, and 100 μg/mL of penicillin in an atmosphere of 5% CO_2 at 37 °C.

4.2.2. In vitro cytotoxicity test

Cytotoxicity of all the compounds including cisplatin and oxaliplatin were determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma). The standard procedure of MTT assay is described briefly as follows: the suspension of 5000 cells per well was plated in 96-well culture plates with culture medium and incubated for 24 h in a water-atmosphere (5% CO_2) at 37 °C. Then the diluted solution of platinum complexes with desired concentration (dissolving in water and diluting with culture medium) was added to the wells. 10 μL of a freshly diluted MTT solution (5 mg/mL in PBS) were added to each well after 48 h of incubation and the plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for another 4 h. The media with MTT solution were removed with DMSO solution (100 μL). The absorbance was measured at 490 nm using an Absorbance Reader (BioRad). The IC_{50} value was calculated from the chart of cell viability (%) versus drug concentration. All experiments were carried out independently in three times.

4.2.3. Flow cytometry analysis

Apoptosis of HCT116 cell line effected by complex **2**, complex **7** and oxaliplatin/cisplatin at the concentration of 50 μM were measured by doubledyeing flow cytometric resection according to the manufacturer's manual. In brief, HCT-116 cells were washed in cold phosphate-buffered saline (PBS) and digested by trypsin solution, and then the suspension of cells were diluted with medium to 1×10^5 cells/mL. Cells were plated into 6-well culture plates (2 mL/well) and incubated at 37 °C in 5% CO_2 for 16 h, and the tested complexes were added into each well and incubated with cells at 37 °C in 5% CO_2 for 24 h. The apoptotic percentage induced by platinum complexes was determined by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (Keygen, China). According to the manufacturer's instructions, cells were stained with annexin V/FITC and propidium iodide (PI) in binding buffers (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH = 7.4). After 5 min incubation at room temperature, the fluorescence was monitored with a flow cytometer (FAC Scan, Becton Dickenson, USA) at an excitation wavelength of 488 nm. Results were analyzed with CellQuest Pro software and are represented as the percentage of normal and apoptotic cells at various stages. Results were analyzed with CellQuest Pro software and are represented as the percentage of normal and apoptotic cells at various stages.

4.2.4. Western blot

HCT116 and MCF7 cells were cultured in culture flasks to reach the cell density of 80%. 50 μM of platinum complex was added and incubated at 37 °C for 12 h. Proteins were extracted by lysis buffer and concentration was determined with protein assay (Thermo, Waltham, MA) by BCA (bicinchoninic acid) and then adjusted to same concentration. The samples (20 μg/lane) were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Bio-Rad, USA) by a transblot apparatus (Bio-Rad, USA). The membrane was blocked with 5% nonfat milk in TBST buffer for 1 h, followed by overnight incubation at 4 °C with primary antibodies diluted in PBST (1:500 for Bax, BD Pharmagin, USA; 1:500 for Bcl-2, Cell Signal, USA; 1:2000 β-actin, Santa Cruz, USA). Membrane was washed with PBST for 3 times and incubated with IRDye 800 conjugated secondary antibody which was diluted in PBST with 1:30,000 for 1 h. Labeled proteins were measured by Odyssey Scanning System (LiCOR., Lincoln, Nebraska, USA).

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