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In vitro biological evaluation of platinum(II) complexes with 1-(methoxy substituted benzyl) azetidine-3,3-dicarboxylato ligands

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ABSTRACT

A number of platinum(II) complexes with ammine or 1*R*,2*R*-diaminocyclohexane as carrier ligands and 1-(methoxy-substituted benzyl) azetidine-3,3-dicarboxylate as leaving groups were synthesized and spectrally characterized. Biological evaluation in vitro showed that some of compounds showed positive antitumor activity. In particular, complex **3a**, (1*R*,2*R*-diaminocyclohexane)[1-(3-methoxylbenzyl) azetidine-3,3-dicarboxylato)-*O*,*O*′] platinum(II), possessed a potent antitumor effect comparable to cisplatin and/or oxaliplatin, and very low toxicity in vivo. Preliminary antitumor mechanism of **3a** has been investigated by cell apoptosis assays compared with cisplatin and oxaliplatin.

1. Introduction

Cisplatin is one of the most important antitumor agents, but severe toxic side effects[1–3] and intrinsic or acquired drug resistance[4–7] limit its applicability and chemotherapeutic efficacy. Moreover, poor aqueous solubility makes the use of cisplatin inconvenient in clinical practice. Stimulated by this, intensive efforts have been made to the discovery of new platinum anticancer agents with improved pharmacological properties.[8] However, over the past 40 years, only carboplatin and oxaliplatin have been in routine use so far. Another four (including nedaplatin, lobaplatin, heptaplatin (SKI2053R) and miriplatin) have gained regionally limited approval. Up to now, about 35 platinum complexes entered clinical trials in order to circumvent the side-effects and the problem of tumor resistance to cisplatin and thousands of platinum complexes have been in the preclinical stage of research.[5,9–15] This situation is due to the limitation of cisplatin mentioned above.

Recently, major approach to overcome the impediment has been made by modifications in either the carrier ligands or the leaving ligands in order to improve efficacy or reduce toxicity. In this strategy, numerous cisplatin analogues have been designed according to the classical structure–activity relationship. Besides, some attention turned to the synthesis of novel platinum complexes, including sterically hindered platinum(II) complexes,[16–20] *trans*-platinum complexes, bi- and multi-nuclear platinum complexes, oral platinum(IV) complexes, platinum(II) oxadiazoline complexes, etc.[21–23] These complexes appear to have different mechanisms of action from those of the classical platinum complexes and to overcome the tumor resistance. However, none of them has been approved for clinical application so far. Therefore, it is still necessary to investigate new platinum complexes with anticipation to possess broad spectra of activity, improved clinical efficacy, reduced toxicity and the ability to overcome drug resistance. We have recently designed and prepared a class of 1-(substituted benzyl) azetidine-3,3-dicarboxylate derivatives as ligands to obtain a series of platinum(II) complexes with mixed ammine/cyclohexylamine as carrier ligands.[24] In our initially biological screening against several human tumor cells in vitro, platinum(II) complexes with above mentioned dicarboxylates containing a methoxy group in the benzyl moiety were proved to promote the antitumor activity of the corresponding compounds.[24] For further research, those ligands with methoxy groups have been applied to prepare a number of new platinum complexes with ammine or 1*R*,2*R*-diaminocyclohexane as carrier ligands. Herein reported are such platinum(II) compounds together with their biological activity.

2. Experimental section

2.1. General considerations

All the commercially available chemicals were of reagent grade and used as received without further purification. Analyses for carbon, hydrogen and nitrogen were performed on a Perkin–Elmer

Abbreviations: PBS, phosphate-buffered saline; w/v, weight per volume; DACH, 1,2-diaminocyclohexane; GS, glucose solution; TSP, sodium trimethylsilylpropionate; TMS, tetramethyl silane; ESI-MS, eletrospray ionization-mass spectrometry; RP-HPLC, reverse phase high-performance liquid chromatography; FITC, fluorescein isothiocyanate.

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1400C analyzer. Infrared spectra (4000–400 cm^{-1}) were measured with a Nicolet 200 FT-IR spectrophotometer on KBr disks. Electrospray ionization (ESI) mass spectra were recorded on a Finnigan MAT SSQ 710 mass spectrometer in a scan range of 100–1200 amu. ^1H NMR and ^{13}C NMR spectroscopic measurements were performed on a Bruker AM-500 NMR spectrometer, using TSP and TMS as internal references at 298 K, respectively. A549 human non-small-cell lung cancer, HCT116 human colon carcinoma, MCF7 human breast cancer, LS174T human colon carcinoma, SGC7901 human gastric cancer and HL60 human promyelocytic leukemia cell lines were purchased from American type Cell Culture (ATCC, Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics. Mice were purchased from the Shanghai Laboratory Animal Centre of the Chinese Academy of Sciences. All the compounds in the in vitro or in vivo assays were dissolved at the specific concentration in 5% (w/v) glucose solution (GS), and these solutions were freshly prepared just before the assay started. All the animal assays were approved to perform in China. The general method for the preparation of ligands H_2L1, H_2L11, H_2L12 and H_2L13 with their spectral data has been reported previously.[24] The corresponding disilver 1-(benzyl/substitude benzyl) azetidine-3,3-dicarboxylate was prepare by the reaction of disodium 1-(benzyl/substituted benzyl) azetidine-3,3-dicarboxylate related and silver nitrate in water.

2.2. Preparation of silver dicarboxylate

To a solution of Na_2L (3 mmol) in distilled water (20 mL) was added silver nitrate (6 mmol) in distilled water (10 mL). The reaction mixture was then stirred at 25 °C for 15 min in the dark. White precipitate was filtered off, washed with water, and then dried in vacuum to give Ag_2L, yield 80–89%.

2.3. General synthesis of Pt(II) complexes 1–4

cis-Diammine diiodo platinum(II) was prepared by the method from the literature.[25] *cis*-Diammine diiodo platinum(II) complex (0.97 g, 2.0 mmol) was first suspended in 100 mL water, and next an aqueous solution (10 mL) of silver nitrate (0.68 g, 4.0 mmol) was added. After stirring at 40 °C for 24 h in the dark, the mixture was cooled down and filtered off. Then the filtrate was blended with disodium 1-benzylazetidine-3,3-dicarboxylate (0.56 g, 2.0 mM) or disodium 1-(methoxy-substituted benzyl) azetidine-3,3-dicarboxylate (0.62 g, 2.0 mmol) in 50 mL water, and stirred at 50 °C for 24 h. The solution was concentrated about to 5 mL by rotavapor and then cooled to 4 °C. Light yellow solids were collected, washed with a small amount of water several times and dried in vacuo.

2.3.1. *cis*-Diammine [1-benzylazetidine-3,3-dicarboxylato-*O,O′*] platinum(II) (1)

Yield 63%. IR (cm^{-1}) 3372 (br), 3169 (s), 3075 (m), 2967 (m), 2884 (w), 1646 (s), 1578 (s), 1456 (m), 1409 (m), 1361 (s), 1289 (m), 1187 (m), 890 (w), 850 (w), 770 (m). ^1H NMR (D_2O, ppm) δ 7.11–7.50 (m, 5H, ArH), 4.35–4.37 (m, 2H, $-CH_2Ar$), 3.87–3.91 (m, 4H, $-CH_2NCH_2-$). ^{13}C NMR (DMSO, ppm) δ 51.6, 52.2 (C2 and C3), 54.5 (C1), 59.4 (NCH_2Ph), 125.5, 126.2, 127.1, 135.8 (Ph), 176.7, 178.3 (C=O). MS (ESI) *m/z* 486.3 (100%) [M+Na^+]. Anal. Calcd for $C_{12}H_{17}N_3O_4Pt$ C, 31.17; H, 3.71; N, 9.09; Pt, 42.19%. Found: C, 31.12; H, 3.62; N, 9.39; Pt, 41.68%.

2.3.2. *cis*-Diammine [1-(2-methoxylbenzyl))azetidine-3,3-dicarboxylato-*O,O′*]platinum(II) (2)

Yield 53%. IR (cm^{-1}) 3423 (br), 3223s, 2937 (m), 2865 (m), 1619 (s), 1597 (s), 1450 (w), 1360 (s), 861 (w), 756 (m), 726 (w). ^1H NMR (D_2O) δ 7.39–7.50 (m, 2H, ArH), 7.06–7.11 (m, 2H, ArH), 4.39–4.42 (m, 2H, $-CH_2Ar$), 3.94–4.39 (m, 4H, $-CH_2NCH_2-$), 3.89 (s, 3H, $-OCH_3$). ^{13}C NMR (DMSO, ppm) δ 52.3, 53.0 (C2 and C3), 55.4 (C1), 59.6, 61.5 (NCH_2Ph and OCH_3), 125.8, 126.9, 127.5, 132.3, 135.8, 153.6 (Ph), 177.2, 178.9 (C=O). MS (ESI) *m/z* (%) 492.8 (100%) $[M+H]^+$. Anal. Calcd for $C_{13}H_{19}N_3O_5Pt$ C, 31.71; H, 3.89; N, 8.53; Pt, 39.62%. Found: C, 31.86; H, 3.78; N, 8.87; Pt, 40.12%.

2.3.3. *cis*-Diammine [1-(3-methoxylbenzyl))azetidine-3,3-dicarboxylato-*O,O′*]platinum(II) (3)

Yield 55%. IR (cm^{-1}) 3421 (br), 3226 (s), 2937 (m), 2865 (m), 1625 (s), 1597 (s), 1468 (w), 1367 (s), 861 (w), 756 (m), 726 (w). ^1H NMR (D_2O) δ 7.46 (m, 1H, ArH), 7.08–7.17 (m, 3H, ArH), 4.37 (m, 2H, $-CH_2Ar$), 4.06–4.37 (m, 4H, $-CH_2NCH_2-$), 3.88 (s, 3H, $-OCH_3$). MS (ESI) ^{13}C NMR (DMSO, ppm) δ 51.6, 52.3 (C2 and C3), 56.1 (C1), 61.5, 62.3 (NCH_2Ph and OCH_3), 121.7, 123.0, 125.5, 128.3, 134.0, 154.8 (Ph), 174.2, 176.0 (C=O). *m/z* (%) 492.8 (100%) $[M+H]^+$. Anal. Calcd for $C_{13}H_{19}N_3O_5Pt$ C, 31.71; H, 3.89; N, 8.53; Pt, 39.62%. Found: C, 31.62; H, 3.50 ; N, 8.32; Pt, 40.25%.

2.3.4. *cis*-Diammine [1-(4-methoxylbenzyl))azetidine-3,3-dicarboxylato-*O,O′*]platinum(II) (4)

Yield 57%. IR (cm^{-1}) 3420 (br), 3254 (s), 3106 (m), 2882 (w), 1659 (s), 1611 (s), 1558 (w), 1516 (s), 1364 (s), 860 (m), 770 (w), 735 (m). ^1H NMR (D_2O) δ 7.30–7.31 (m, 2H, ArH), 6.96–6.97 (m, 2H, ArH), 4.32–4.34 (m, 2H, $-CH_2Ar$), 3.83–3.94 (m, 4H, $-CH_2NCH_2-$), 3.73 (s, 3H, $-OCH_3$). ^{13}C NMR (DMSO, ppm) δ 53.0, 53.8 (C2 and C3), 55.2 (C1), 61.5, 63.0 (NCH_2Ph and OCH_3), 123.5, 125.3 130.8, 150.6 (Ph), 176.7, 177.8 (C=O). MS (ESI) *m/z* (%) 492.9 (100%) $[M+H]^+$. Anal. Calcd for $C_{13}H_{19}N_3O_5Pt$ C, 31.71; H, 3.89; N, 8.53; Pt, 39.62%. Found: C, 31.53; H, 3.622; N, 8.27; Pt, 39.28%.

2.4. General synthesis of Pt(II) complexes 1a–4a

cis-(1*R*,2*R*-Diaminocyclohexane) dichlorido platinum(II) was prepared according to the reported literature.[26] A suspension of *cis*-(1*R*,2*R*-diaminocyclohexane) dichlorido platinum(II) (0.76 g, 2.0 mM) and disilver 1-benzylazetidine-3,3-dicarboxylate (0.90 g, 2.0 mM) or disilver 1-(methoxy-substituted benzyl) azetidine-3,3-dicarboxylate (0.96 g, 2.0 mM) in H_2O (100 mL) was stirred at 50 °C in the dark overnight. The resulting silver chloride was filtered off through a pad of celite. The filtrate was concentrated under a reduced pressure about to 5 mL and then cooled to 4 °C. Light yellow solids were collected, washed with a small amount of water several times and dried in vacuo.

2.4.1. (1*R*,2*R*-Diaminocyclohexane)[1-benzylazetidine-3,3-dicarboxylato-*O,O′*]platinum(II) (1a)

Yield 41%. IR (cm^{-1}) 3424 (br), 3208 (m), 3101 (m), 2937 (m), 2861 (w), 1606 (s), 1497 (w), 1452 (m), 1354 (s), 848 (w), 769 (w). ^1H NMR (D_2O) δ 7.35–7.44 (m, 4H, ArH), 4.32–4.34 (m, 2H, $-CH_2Ar$), 3.90–4.05 (m, 4H, $-CH_2NCH_2-$), 2.33–2.35 (m, 2H, CH of DACH), 1.97–1.99 (m, 1H, CH_2 of DACH), 1.87–189 (m, 1H, CH_2 of DACH), 1.49 (m, 2H, CH_2 of DACH), 1.06–1.19 (m, 4H, CH_2 of DACH). ^{13}C NMR (DMSO, ppm) δ 24.0 (C7 and C8), 31.3 (C6 and C9), 44.5, 45.2 (C4 and C5), 51.4, 52.1 (C2 and C3), 54.2 (C1), 58.6 (NCH_2Ph), 124.7, 125.5, 126.1, 134.6 (Ph), 172.7, 177.2 (C=O). MS (ESI) *m/z* (%) 543.2 (100%) $[M+H]^+$. Anal. Calcd for $C_{18}H_{25}N_3O_4Pt$ C, 39.85; H, 4.64; N, 7.75; Pt, 35.69%. Found: C, 39.57; H, 4.39; N, 7.63; Pt, 36.08%.

2.4.2. (1*R*,2*R*-Diaminocyclohexane)[1-(2-methoxylbenzyl)) azetidine-3,3-dicarboxylato-*O,O′*]platinum(II) (2a)

Yield 31%. IR (cm^{-1}) 3429 (s), 3192 (m), 3091 (m), 2939 (m), 2862 (w), 1648 (s), 1495 (s), 1455 (w), 1384 (s), 1369 (m), 827 (m), 715 (w). ^1H NMR (D_2O) δ 7.50–7.53 (m, 1H, ArH), 7.39–7.40 (m, 1H, ArH), 7.09–7.17 (m, 2H, ArH), 4.31–4.32 (m, 2H, $-CH_2Ar$), 3.91–4.05 (m, 4H, $-CH_2NCH_2-$), 3.89 (s, 3H, $-OCH_3$), 2.45–2.47

(m, 2H, CH of DACH), 2.02–2.09 (m, 2H, CH_2 of DACH), 1.59 (m, 2H, CH_2 of DACH), 1.17–1.30 (m, 4H, CH_2 of DACH). ^{13}C NMR (DMSO, ppm) δ 24.1 (C7 and C8), 31.4 (C6 and C9), 43.8, 44.5 (C4 and C5), 52.3, 55.4 (C2 and C3), 56.8 (C1), 60.5, 61.8 (N*CH*$_2$Ph and O*CH*$_3$), 111.6, 119.2, 120.5, 130.9, 134.3, 158.6 (Ph), 170.9, 176.7 (C=O). MS (ESI) *m*/*z* (%) 573.0 (100%) $[M+H]^+$. Anal. Calcd for $C_{19}H_{27}N_3O_5Pt$ C, 39.86; H, 4.75; N, 7.34; Pt, 34.07%. Found: C, 39.51; H, 4.67; N, 7.12; Pt, 34.50%.

2.4.3. (1*R*,2*R*-Diaminocyclohexane)[1-(3-methoxylbenzyl)) azetidine-3,3-dicarboxylato-*O*,*O*′]platinum(II) (3a)

Yield 43%. IR (cm^{-1}) 3428 (s), 3253 (m), 3193 (w), 2933 (m), 2859 (w), 1635 (s), 1517 (s), 1490 (w), 1449 (w), 1385 (s), 825 (m), 774 (w). ^1H NMR (D_2O) δ 7.37 (m, 1H, ArH), 6.94–7.00 (m, 3H, ArH), 4.24–4.32 (m, 2H, –CH_2Ar), 3.89–3.97 (m, 4H, –CH_2NCH_2–), 3.77 (s, 3H, –OCH_3), 2.31 (m, 2H, CH of DACH), 1.94 (m, 2H, CH_2 of DACH), 1.47 (m, 2H, CH_2 of DACH), 1.08 (m, 4H, CH_2 of DACH). ^{13}C NMR (DMSO, ppm) δ 24.0 (C7 and C8), 31.3 (C6 and C9), 44.8, 45.3 (C4 and C5), 51.5, 54.2 (C2 and C3), 55.9 (C1), 61.5, 62.8 (N*CH*$_2$Ph and O*CH*$_3$), 115.4, 121.1, 122.7, 131.6, 135.3, 157.4 (Ph), 174.2, 177.2 (C=O). MS (ESI) *m*/*z* (%) 573.0 (100%) $[M+H]^+$, 595.2 (45%) $[M+Na]^+$. Anal. Calcd for $C_{19}H_{27}N_3O_5Pt$ C, 39.86; H, 4.75; N, 7.34; Pt, 34.07%. Found: C, 39.65; H, 4.71; N, 7.15; Pt, 34.61%.

2.4.4. (1*R*,2*R*-Diaminocyclohexane)[1-(4-methoxylbenzyl)) azetidine-3,3-dicarboxylato-*O*,*O*′]platinum(II) (4a)

Yield 40%. IR (cm^{-1}) 3365 (s), 3125 (m), 3058 (m), 2940 (m), 2863 (w), 1637 (s), 1609 (s), 1514 (s), 1456 (w), 1409 (w), 1374 (m), 824 (m), 708 (w). ^1H NMR (D_2O) δ 7.30–7.32 (m, 2H, ArH), 7.00–7.02 (m, 2H, ArH), 4.25–4.26 (m, 2H, –CH_2Ar), 3.83–3.97 (m, 4H, –CH_2NCH_2–), 3.78 (s, 3H, –OCH_3), 2.35–2.37 (m, 2H, CH of DACH), 1.98–2.01 (m, 1H, CH_2 of DACH), 1.91–1.94 (m, 1H, CH_2 of DACH), 1.50 (m, 2H, CH_2 of DACH), 1.08–1.21 (m, 4H, CH_2 of DACH). ^{13}C NMR (DMSO, ppm) δ 24.3 (C7 and C8), 31.4 (C6 and C9), 43.9, 45.1 (C4 and C5), 52.4, 55.2 (C2 and C3), 55.5 (C1), 62.4, 64.9 (N*CH*$_2$Ph and O*CH*$_3$), 121.4, 125.1, 136.3, 156.2 (Ph), 172.6, 176.0 (C=O). MS (ESI) *m*/*z* (%) 573.2 (100%) $[M+H]^+$, 595.1 (35%) $[M+Na]^+$. Anal. Calcd for $C_{19}H_{27}N_3O_5Pt$ C, 39.86; H, 4.75; N, 7.34; Pt, 34.07%. Found: C, 39.82; H, 4.54; N, 7.63; Pt, 33.38%.

2.5. Preparation of ligand L14 and complex 5a

Disodium 1-(3,5-dimethoxylbenzyl) azetidine-3,3-dicarboxylate (H_2L14)*:* $KHCO_3$ (2.0 g, 0.02 mol) and formaldehyde (15.4 g, 37% aqueous solution) were mixed, and stirred at 30 °C until the solution turned to be a colorless and transparent liquid. To the mixture was added diethyl malonate (16.0 g, 0.1 mol). After stirring for 3 h at room temperature, $(NH_4)_2SO_4$ (17.0 g) in 50 mL water was added slowly to the resulting white turbid solution. The aqueous phase was extracted twice with ethyl (30 mL × 2). The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. Fifty milliliters of isopropyl ether was added to the residue and the mixture was kept cool at −17 °C overnight, giving colorless crystals as the intermediate, diethyl 2,2-bis(hydroxymethyl) malonate.

To 100 mL of anhydrous acetonitrile was added diethyl 2,2-bis(hydroxymethyl) malonate (22.0 g, 0.1 mol) obtained in the first step. The resulting solution was stirred until the dissolution was complete. Then the solution was cooled to −20 °C, trifluoromethanesulfonic anhydride (59.5 g, 0.21 mol) was added drop wise with stirring for 40 min. To the mixture was added *N*-ethyldiisopropylamine (65.0 g, 0.5 mol) drop by drop at −10 °C for 1 h, followed by adding 3,5-dimethoxylbenzylamine (20.0 g, 0.12 mol). The mixture was stirred and refluxed for 2 h, and then cooled to the room temperature. 150 mL of toluene was added and the organic phase was washed twice with 150 mL of water. The solvent was evaporated in vacuo and the residue was added to 50 mL of methanol. Thirty milliliters of sodium hydroxide solution (10 mol/L) was carefully added to the stirred mixture at room temperature and the mixture was kept stirring for 2 h at 50 °C. After cooling to the room temperature, the resulting white solids were filtered off and washed twice with methanol. The white product was dried at 30 °C in vacuo. Yield 80%. IR (cm^{-1}) 2953 (m), 2838 (m), 1606 (s), 1489 (w), 1436 (m), 1333 (s), 1266 (m), 1152 (m), 1047 (m), 789 (w), 689 (w). ^1H NMR (D_2O) δ 3.54 (m, 4H, –CH_2NCH_2–), 3.58 (s, 2H, –CH_2Ar), 3.77 (s, 6H, 2OCH_3), 6.47 (s, 1H, ArH), 6.52 (s, 2H, ArH). MS (ESI) *m*/*z* (%) 294.1 (100%) $[M-2Na+H]^-$. Anal. Calcd for $C_{14}H_{15}NO_6Na_2$ C, 49.56; H, 4.46; N, 4.13. Found: C, 49.76; H, 4.39; N, 4.18%.

2.5.1. (1*R*,2*R*-Diaminocyclohexane)[1-(3,5-dimethoxylbenzyl) azetidine-1,1-dicarboxylato-*O*,*O*′]platinum(II) (5a)

A suspension of *cis*-(1*R*,2*R*-diaminocyclohexane) dichlorido platinum(II) (0.76 g, 2.0 mmol) and disilver 1-(3,5-dimethoxylbenzyl) azetidine-3,3-dicarboxylate (1.02 g, 2.0 mmol) in H_2O (100 mL) was stirred at 50 °C in the dark overnight. The resulting silver chloride deposit was filtered off through a pad of celite. The filtrate was concentrated under a reduced pressure about to 5 mL and then cooled to 4 °C. Light yellow solids were collected, washed with a small amount of water several times and dried in vacuo. Yield 43%. IR (cm^{-1}) 3417 (m), 3223 (m), 2936 (m), 2860 (w), 1595 (s), 1457 (w), 1431 (w), 1384 (s), 1347 (m), 1151 (m), 1064 (m), 830 (m), 737 (w). ^1H NMR (D_2O) δ 6.57 (m, 1H, ArH), 6.47 (m, 1H, ArH), 6.12–6.30 (m, 1H, ArH), 4.20–4.34 (m, 2H, CH_2Ar), 3.80–3.94 (m, 4H, –CH_2NCH_2–), 3.45–3.75 (m, 6H, 2OCH_3), 2.35–2.37 (m, 2H), 1.93–2.22 (m, 4H, CH_2 of DACH and 2CH of DACH), 1.55 (m, 2H, CH_2 of DACH), 1.08–1.10 (m, 4H, CH_2 of DACH). ^{13}C NMR (DMSO, ppm) δ 24.1 (C7 and C8), 31.4 (C6 and C9), 43.5, 44.3 (C4 and C5), 52.1, 53.0 (C2 and C3), 55.5 (C1), 61.2, 62.0 (N*CH*$_2$Ph and O*CH*$_3$), 105.5, 110.2, 111.5, 132.9, 158.6, 159.4 (Ph), 175.5, 178.3 (C=O). MS (ESI) *m*/*z* (%) 602.5 (100%) $[M+H]^+$. Anal. Calcd for $C_{20}H_{29}N_3O_6Pt$ C, 39.86; H, 4.85; N, 6.98; Pt, 32.38%. Found: C, 39.96; H, 4.79; N, 7.25; Pt, 32.80%.

2.6. In vitro assays

2.6.1. Antiproliferation activity of complexes

The in vitro cytotoxicity of all platinum compounds against A549, HCT116, MCF7, LS174T, SGC7901 or HL60, was measured by the MTT assays as described in our previous work.[27] Briefly, the cells were seeded in 96-well tissue cultured plates at a density of 5000 cells/well. After overnight incubation (16 h), the cells were treated with the platinum complexes. After 48 h of incubation 10 μL of a freshly diluted 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/mL) were added to each well and the plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. At the end of the incubation period the medium was removed and the formazan product was dissolved in 150 μL of DMSO. The cell viability was evaluated by measurement of the absorbance at 490 nm, using an Absorbance Reader (Bio-Rad). IC_{50} values (compound concentration that produces 50% of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in quintuplication. The reading values were converted to the percentage of control (% cell survival). Cytotoxic effects were expressed as IC_{50} values.

2.7. Induction of cell apoptosis

HCT116 was grown in culture as described in the part of Section 2.1. Cell apoptosis assays were performed as follows. Briefly, cells

were washed with PBS and digested by trypsin solution. A cell suspension was made with culture medium, and the concentration was adjusted 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 overnight. A series of indicated doses of test reagents were added into each well and incubated with cells for 24 h at 37 °C in 5% CO_2. 5%GS was used as a negative control; cisplatin and oxaliplatin were used as positive controls for HCT116. The apoptosis of cells was measured by Flow cytometery using annexin V-FITC/PI apoptosis kit (Biouniquer, Nanjing, China) according to the manufacturer's instructions as below. Cells were harvested and washed in cold PBS, then stained with annexin V-FITC (100 ng/mL) and propidium iodide (2 μg/mL) in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). After 15 min incubation at room temperature, the fluorescence of cells was measured using the flow cytometer (FAC Scan, Becton Dickenson, USA). The results were analyzed using Cell Quest Pro software and represented as percentage of normal and apoptotic cells at various stages.[28] FITC and PI fluorescences were measured in FL1 and FL2 channel, respectively.

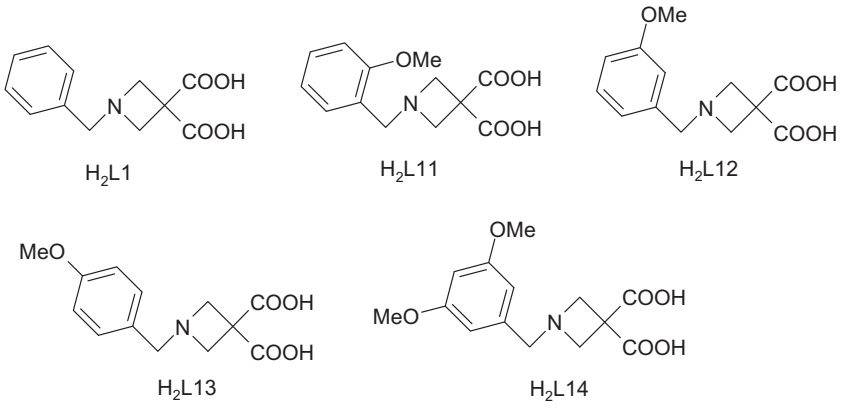
2.8. In vivo assay

The in vivo toxicity of complex **3a** compared with cisplatin was measured by acute toxicity assay. In this work, acute toxicity tests were used to evaluate the safety of these complexes. We first assessed the range of dose of **3a** that caused 0% and 100% death rates for mice in a pre-test. ICR mice were randomly divided into different groups (5 female mice and 5 male mice for each group) to study the acute toxicity of complexes. The doses of **3a** and cisplatin were 115.2, 144, 180, 225, 281.25 mg/kg body wt. and 3.34, 4.0, 5.0, 5.44, 6.4 mg/kg body wt., respectively. All drugs were administered i.v. once. After administration, all external morphological, behavioral changes, numbers of dead and time to death, as well as some other toxic effects were recorded continuously in 2 w. According to the mortality of mice observed within 2 w, the LD_{50} values for each drug were calculated using SPSS software with the Bliss method.[29]

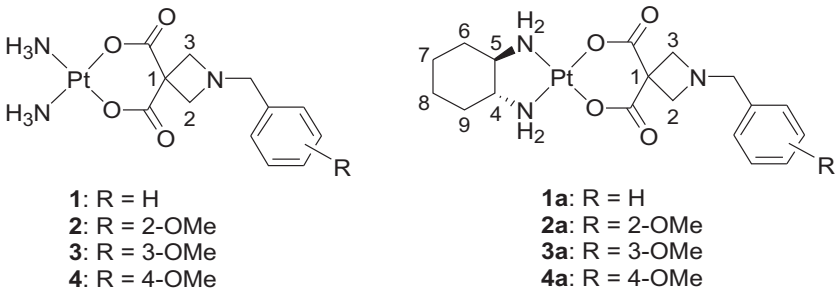
3. Results and discussion

In our last report, a number of 1-substitutedbenzyl azetidine-3,3-dicarboxylates have been designed and synthesized as ligands. This kind of ligands is somewhat similar to 1,1-cyclobutyldicarboxylate that may improve aqueous solubility of the resulting platinum(II) compounds, which has been used in carboplatin. Among these ligands, those containing methoxybenzyl moieties were selectively used for the present study due to their good performance in our former study on the antitumor activity of the platinum(II) complexes with them (Scheme 1: H_2L11, H_2L12 and H_2L13, while H_2L1 is used for the contrast study). In the mean time, ammine and 1*R*,2*R*-diaminocyclohexane (DACH) were selected as carrier ligands since they have been successfully applied to develop the clinically used platinum drugs in cisplatin (ammine) and oxaliplatin (DACH). With H_2L1, H_2L11, H_2L12 and H_2L13, two classes of the corresponding platinum(II) complexes have been prepared (Scheme 2), which are found to have good solubility both in water and organic solvents like alcohols and chloroform. For these platinum(II) compounds, 1-substituted azetidine-3,3-dicarboxylate serves as a bidentate ligand to chelate the metal atom through an *O*,*O* mode, analogous to that in carboplatin.

All the compounds together with their corresponding ligands were characterized by Infrared, electro-spray ionization MS and ^1H NMR spectroscopy techniques in addition to elemental analysis. From the IR data, it can be seen that N–H stretching vibrations of resulting platinum(II) complexes exhibit, in the range 3058–3254 cm^{-1}, red shifting compared with the amino group of the corresponding metal-free ligands (NH_3 and 1*R*,2*R*-DACH). The



Scheme 1. Chemical structures of the ligands.



Scheme 2. Chemical structures of platinum(II) complexes, DACH stands for *1R*, 2*R*-diaminocyclohexane.

ν_{as}(C–O) vibration of the complexes appears between 1619 and 1659 cm^{-1} which is characteristic of coordinated carboxylate ligands. D_2O was used as a solvent to measure the ^1H NMR spectra of both the ligands and the corresponding platinum complexes. The expected chemical signals were found in ^1H NMR spectra. As for complexes **1a**–**5a**, the signals of C–H protons connected to the amino groups occur in the spectra of 2.22–2.47 ppm as a multiplet, shifting highfield compared with the corresponding signal (2.81 ppm) in the free ligand (1*R*,2*R*-DACH), due to the amino group coordinating with Pt(II) ions. The signals of C–H protons belonging to azetidine group for complexes **1**–**4** and **1a**–**5a** were located in the range of 3.80–4.39 ppm, shifting downfield compared with the corresponding signals (3.40–3.59 ppm) of the metal-free leaving groups owing to the deshielding effect caused by the coordination of the ligands with platinum(II). All the platinum complexes were characterized by ESI-MS, showing the presence of $[M+H]^+$ and/or $[M+Na]^+$ peaks which also display the isotopic distribution of the platinum element. Elemental analysis confirms the elemental composition of target compounds.

Biological activities of these complexes have been investigated. For in vitro assay, all the compounds were determined for their cytotoxic effect in the human non-small-cell lung cancer A549, the human colon carcinoma HCT116, and the human breast cancer MCF7 cell lines by MTT assay. It is well known that oxaliplatin is the first-line drug in the colorectal cancer therapy, so we choose oxaliplatin as a positive drug for the study about human colorectal cancer cell lines HCT116 and LS-174T. While cisplatin usually shows more potent effect of treatment than other platinum drugs in clinic use, it is used as the positive control in our study about other cancer cell lines. According to the results, it can be concluded that two complexes for MCF7 and three complexes for A549 have good cytotoxicity including complex **3a** (IC_{50} <50 μM). And only one compound, **3a**, performed significant activity toward HCT116 cells, and indeed better than the positive contrast oxaliplatin (Table 1). It is noticed that both complexes **1** and **1a** without a methoxy group in the ligand ($L1^{2-}$) showed no cytotoxicity against tumor cells tested. By summarizing the structure-activity relationship, we found that the position of the methoxy group on the aromatic ring can remarkably affect the antitumor activity of the compounds (Table 2, Figs. 1 and 2). It turned out that the ligand with a methoxy group on 3-position relative to the methylene group of the phenyl ring has greatly promoted the biological activity of the platinum(II) complex. Moreover, compound **3a** showed pretty good antitumor activity in other cancer cell lines such as LS174T (human colon carcinoma), SGC7901 (human gastric cancer), and HL60 (human promyelocytic leukemia) (Table 2). Based on these findings, **3a** was discovered to exhibit higher cytotoxicity and possess broader antitumor spectrum than any other compounds obtained.

Table 1
IC_{50} (μM) values of complexes **1**–**4** and **1a**–**4a** at inhibiting the growth of A549 human non-small-cell lung cancer, HCT-116 human colon carcinoma and MCF-7 human breast cancer cell lines

Complex	IC_{50} (μM)		
	A549	HCT116	MCF7
1	568.490 ± 26.862	419.026 ± 28.737	3.629E7 ± 813.479
2	36.8 ± 3.6	116.899 ± 9.627	45.3 ± 5.3
3	63.8 ± 6.2	766.155 ± 51.538	125.884 ± 6.117
4	240.878 ± 22.490	2.205E4 ± 109.742	246.729 ± 6.765
1a	50.004 ± 6.625	224.526 ± 24.122	52.845 ± 4.493
2a	44.4 ± 7.1	213.579 ± 15.872	72.3 ± 2.7
3a	5.1 ± 0.7	13.3 ± 2.5	4.9 ± 1.2
4a	482.519 ± 39.198	166.809 ± 11.832	427.406 ± 35.439
Cisplatin	7.9 ± 0.8	Not measured	19.9 ± 1.3
Oxaliplatin	20.268 ± 4.350	26.3 ± 4.3	4.7 ± 0.9

Table 2
Antitumor activities of complex **3a**, oxaliplatin and cisplatin against tumor cells in vitro

Cell line	Complex	IC_{50} (μM)
LS-174T	**3a**	2.4 ± 0.1
	Oxaliplatin	1.0 ± 0.1
SGC-7901	**3a**	4.8 ± 0.5
	Cisplatin	2.4 ± 0.3
HL-60	**3a**	15.7 ± 1.7
	Cisplatin	2.3 ± 0.6

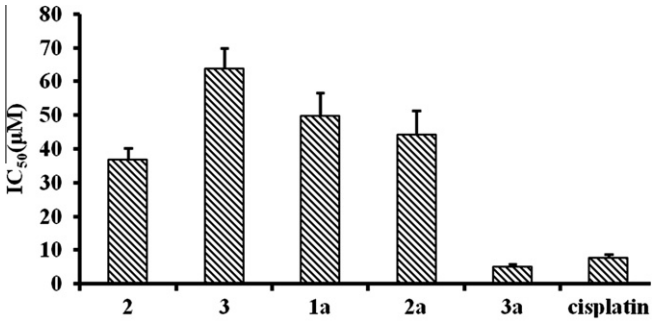


Figure 1. Cytotoxicity of complexes **2**, **3**, **1a**, **2a** and **3a** toward A549 human non-small-cell lung cancer cell line in MTT assay.

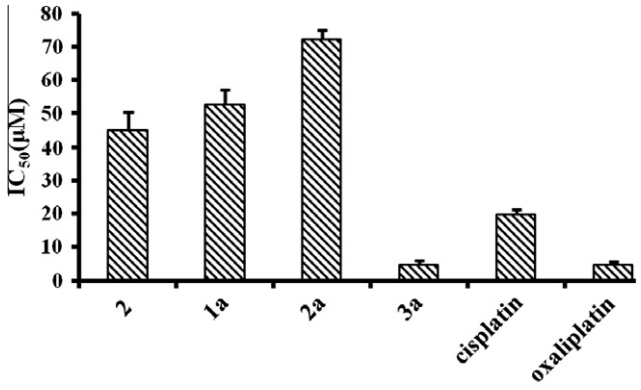


Figure 2. Cytotoxicity of complexes **2**, **1a**, **2a** and **3a** toward MCF7 human breast cancer cell line in MTT assay.

In the following study, exposure of HCT116 cells to complex **3a** assumed a great degree of apoptosis (Fig. 3). Compound **3a** at 50 μM dose induced apoptosis of HCT116 cells in 23.6% (Fig. 4), which behaves like double-dosed oxaliplatin (35%). However, cisplatin could not obviously induce cells apoptosis at the dosage of 100 μM. The results indicated that complex **3a** showed a larger degree of apoptosis induction than oxaliplatin towards cisplatin-resistant cancer, which might be caused by eluding cell drug-resistant mechanism such as intracellular detoxification by glutathione which was attributed to the chelate effect of the DACH ring[30] and increasing cell uptake caused by adequate balance of liposolubility and aqueous solubility.

In agreement with these pharmacological results, we assumed that the substituted methoxy group of the aromatic ring had a great influence on the interaction of the compound with DNA. When the substituted group hindered the interaction, the activity was low. Otherwise, when the substituted group benefited the interaction, the activity became high. Complex **3a** had high antitumor activity possibly because the 3-methoxybenzyl group could take a suitable position and promoted the interaction of the

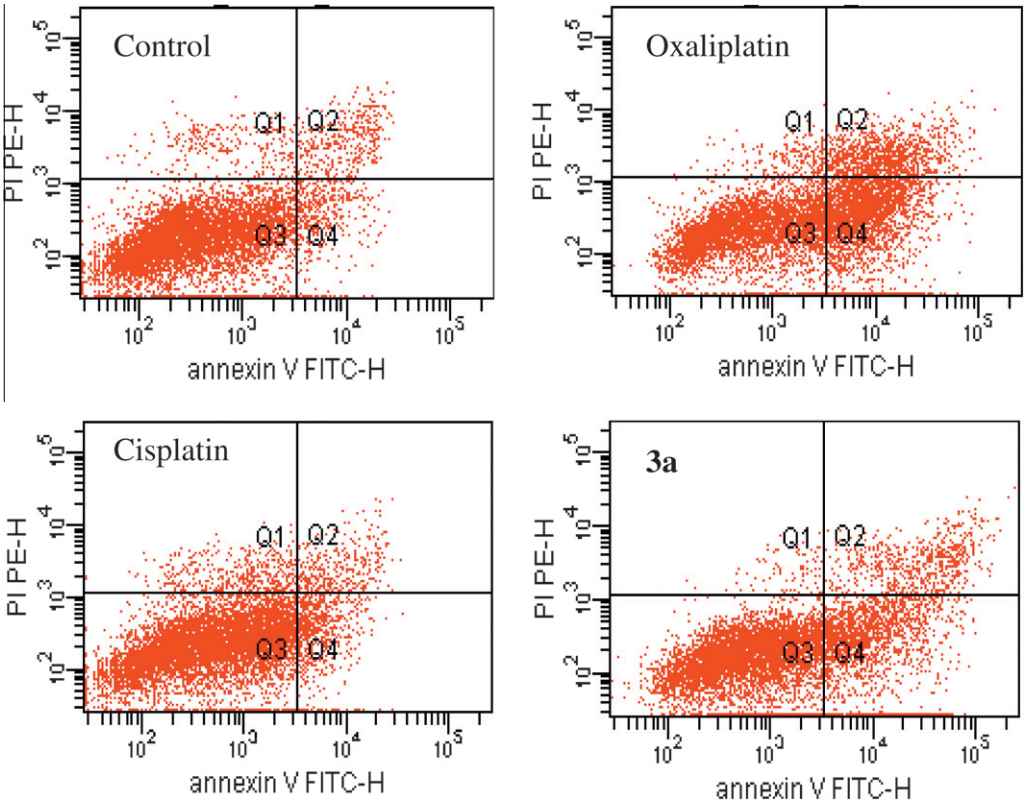


Figure 3. The exposure of HCT116 cells to the test compounds assumed various degrees of apoptosis. The dot plot shows three distinct populations: (a) the viable cells which have low Annexin V-FITC and low PI signal (Q3 in panel); (b) the apoptotic cells which have high Annexin V-FITC and low PI signal (Q4 in panel); (c) late stage apoptotic/secondary necrotic cells with compromised membranes exhibiting high Annexin V-FITC and high PI signal (Q2 in panel). In some cases, a fourth population corresponding to damaged viable cells with low Annexin V-FITC and high PI signal may be observed (Q1 in panel).

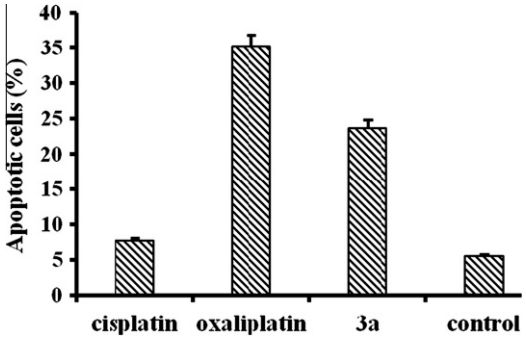
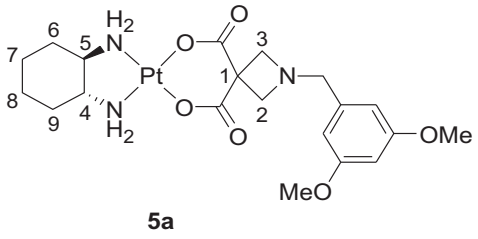


Figure 4. Percentage of apoptotic HCT116 cells in total cells following treatment with complexes **3a** at 50 μM, compared with cisplatin (50 μM) and oxaliplatin (100 μM).



5a

Scheme 3. Chemical structure of complex **5a**.

Table 3
Antitumor activities of complexes **3a**, **5a** and cisplatin against tumor cells

Complex	IC_{50} (μM)	
	A549	MCF-7
3a	5.9 ± 0.76	5.6 ± 1.4
5a	7.7 ± 1.4	4.2 ± 0.6
Cisplatin	7.3 ± 0.4	4.4 ± 0.3

compound with DNA. For comparison, in our early study, several alkyl substituents like isopropyl and *n*-butyl groups have been connected with the nitrogen atom of 1-azetidine-3,3-dicarboxylate to prepare the analogous ligands. However, the resulting platinum(II) complexes of these ligands containing *N*-alkyl groups did not exhibit much cytoxicity against the same human cancer cell lines.

Based on the above discussion, we further designed and synthesized ligand H_2L14 and its platinum(II) complex **5a**, whose structure is similar to **3a** (Scheme 3). This compound was evaluated for its in vitro cytotoxicity against A549 and MCF7 cell lines compared with those of **3a** and cisplatin. The difference between their chemical structures was that **5a** has two methoxy groups located in 3- and 5-position of the benzyl moiety, while **3a** only has one located in 3-position of the benzyl species. But it is noted that all the methoxy groups in two compounds are on the meso position relative to the methylene group in the phenyl ring. We hoped this structure modification could have comparable or increased the interaction of the compound with the target. The biological results showed that **5a** exerted comparable activity to those of **3a** and cisplatin (Table 3).

In view of the importance of toxic and side effect of platinum drugs for their clinical application, we examined acute toxicity of **3a** in vivo compared with cisplatin. Based on the comparison of LD_{50} values of these compounds, **3a** showed much lower toxicity

Table 4
Acute toxicity of complex **3a** and cisplatin in vivo[a]

Complex	LD50 (mg kg^{-1})	95% FL (mg kg^{-1})
3a	181.3	165.0–199.3
Cisplatin	4.3	3.7–4.9
Carboplatin[29]	139.0	

[a] FL: fiducial limits.

than cisplatin in this assay (Table 4); even lower than carboplatin according to the literature report.[29]

4. Conclusions

A number of ammine or *1R*,*2R*-diaminocyclohexane platinum(II) complexes with 1-methoxy-substituted benzyl azetidine-3,3-dicarboxylates as ligands have been designed, prepared and studied about their antitumor activity. Among these complexes, **3a**, (*1R*,*2R*-diaminocyclohexane)[(1-(3-methoxybenzyl) azetidine-3,3-dicarboxylato)-*O*,*O*′] platinum(II), had a potent antitumor effect relative to both cisplatin and oxaliplatin, and extremely low toxicity in vivo, even much lower than carboplatin. By summarizing the structure–activity relationship of the complexes, we found that when the tertiary nitrogen atom of the ligand was linked with a 3-methoxybenzyl group, the corresponding complex displayed better activity in cytotoxicity and ability of inducing cell apoptosis.

Acknowledgments

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