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Research paper

Toward overcoming cisplatin resistance via sterically hindered platinum(II) complexes

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ABSTRACT

A number of platinum(II) complexes with steric hindrance derived from (1*R*,2*R*)-N^1-benzylcyclohexane-1,2-diamine derivatives were designed and prepared. Biological assay indicated that most complexes showed antitumor activity against the tested cancer cell lines, especially those with chloride anions as leaving groups had compatible or superior activity to cisplatin and oxaliplatin. Complex **2a**, as the most potent agent, is also sensitive to cisplatin resistant SGC7901/CDDP cancer cell line, which has been subsequently studied by cellular uptake, flow cytometry, gel electrophoresis and western blot assays. The steric hindrance resulting from a pending 2-fluorobenzyl moiety of the ligand might be the key factor for its ability to overcome cisplatin resistant cancer cells.

1. Introduction

Since the discovery of cisplatin by Rosenberg in 1965, the application and research of platinum-based anticancer drugs have achieved rapid development [1–3]. Cisplatin as a broad-spectrum anticancer agent has shown great advantages in the treatment of some solid tumors, especially in the treatment of cervix, ovary, testicle, neck and head [4,5]. However, some side effects, including acquired or intrinsic drug resistance, serious toxicity and low solubility in water, have greatly limited its clinical application [6]. Thus, the non-classical antitumor platinum(II) complexes such as sterically hindered platinum(II) complexes [7], trans-platinum complexes [8], bi- and multi-nuclear platinum(II) complexes [9,10] have been investigated. So far, several mechanisms have been involved in cisplatin resistance, including reduced accumulation of cellular Pt and increased repair of DNA damage [11–13]. The interaction of cisplatin with biological molecules has been found to be one of the key reasons for the drug resistance [14,15] due to its high affinity to the sulfur-containing binding sites. It is well known that sulfur-containing biomolecules are correlated with the resistance of platinum-based drugs [16–18]. In order to overcome the resistance of cisplatin and reduce its side effects, different carrier ligands have been designed and applied to platinum complexes. Oxaliplatin, a third-generation platinum drug with 1*R*,2*R*-diaminocyclohexane (DACH) as carrier ligand, has shown great activity against many tumors including those resistant to cisplatin [19–21]. The high stability of the DACH-platinum fragment in oxaliplatin compared with the ammine in cisplatin leads to a different reaction behavior [22–24], thus, researchers have made great efforts on designing and synthesis of oxaliplatin derivatives [21–27]. Recent studies demonstrated that the introduction of steric hindrance to amine carrier ligand has a strong influence on the activity of the resulting platinum complexes [28]. Therefore, by changing the nature of the amine ligand, it is possible to obtain platinum(II) complexes with potent antitumor activity, which may be active toward cisplatin resistant cell lines as well.

In our recent research, a number of different alkyl groups as sterically hindered moieties have been introduced to DACH skeleton, which were used as carrier ligands to prepare several kinds of platinum(II) complexes [29–32]. The related studies indicated that the platinum(II) complexes containing such ligands showed potent antitumor activity and possessed somewhat different mechanism from cisplatin and oxaliplatin in reaction with DNA, and exhibited distinct cell cycle blocking and cell apoptosis. However, the *N*-monosubstituted alkyl group is flexible, which seems not feasible to offer fixed spatial resistance and may also result in the formation of stereo isomers [29]. Hence, a rigid benzyl group has been introduced to the DACH framework by connecting to one of the nitrogen

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atoms (Scheme 1) [33–35], and several new ligands as shown in Fig. 1 have been obtained, which have been used to prepare a series of platinum(II) complexes bearing the steric hindrance(Scheme 2). Considering the significant feature of fluorine atom in drugs, we selected it as a substituent on the phenyl ring. Herein reported are these platinum(II) complexes (Fig. 2) of the ligands and their biological evaluation against a few human cancer cell lines including the typical compounds against cisplatin resistant cancer cell lines. Moreover, chemical and biological properties of the typical compounds have been studied to explicit their preliminary mechanism of action as well as the function of spatial resistance caused by the ligand.

2. Results and discussion

2.1. Synthesis of ligands and their complexes

The preparation of the ligand (**L**) was carried out starting from 1*R*, 2*R*-diaminocyclohexane via several synthetic steps (Scheme 1), in which *N*-mono Boc protected DACH (**I**) was used as the starting material. The unprotected amino group first underwent Schiff base condensation with benzaldehyde, followed by *in situ* reduction with sodium boronhydride, and then the Boc group was removed through hydrolysis in the presence of hydrochloric acid to give **L** in its hydrochloride salts. **LF^1**–**LF^3** were prepared by the same way except *o*-fluorobenzaldehyde, *m*-fluorobenzaldehyde and *p*-fluorobenzaldehyde were used as starting materials, respectively.

All ligands (**L**, **LF^1**, **LF^2** and **LF^3**) were characterized by elemental analysis, IR, ESI-MS, ^{1}H and ^{13}C NMR spectra, which were in agreement with the chemical structure proposed. The specific optical rotations of these ligands were as left-handed as that of DACH.

Complexes **1a**–**4a** were directly prepared by the reaction of the corresponding ligand and K_2PtCl_4. The rest complexes were obtained by using complexes **1a**–**4a** as starting materials to react with the corresponding silver dicarboxylate, respectively [29]. As expected, a chiral center at N^1 position of DACH can be formed after coordination of the ligand with a metal atom in the synthesized platinum(II) complexes. The diastereoisomeric mixture could be produced with one pendant benzyl moiety (at N^1 position) in the pseudo axial position above or below the resulting PtN_2O_2 square plane. Like the pending alkyl group we reported formerly [29], *S* configuration at N^1 atom of the complex with a pendant benzyl group is more thermodynamically stable than the corresponding *R* configuration resulting from a steric effect of the ligand by molecular calculation, which has also been confirmed by the crystallographic data of analogous metal complexes [29,36,37].

In the infrared spectra of the complexes, the strong absorption at 3100 cm^{-1} was attributed to the N–H stretching vibration that was significantly red shifted in comparison to the corresponding ligand. The C=O stretching vibration of complexes appeared between 1568 and 1616 cm^{-1}, blue shifting compared with the single carboxylate group, which is characteristic of coordinated carboxylate ligands. The absorption near 1250 cm^{-1} was attributed to the Ar–F bonding which is an obvious sign to distinguish complexes **1a**, **1b**, **1c**, **1d** and **1e** from others.

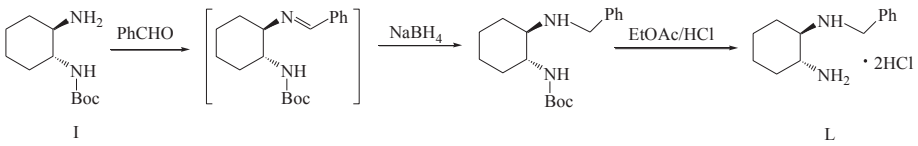
NMR spectroscopy has been applied to determine the framework of the synthesized metal complexes. In the ^{1}H NMR spectra, the signals of hydrogen atoms in DACH appeared in a range of 1.24–2.50 ppm, and the chemical shifts of NH*CH* and NH_2*CH* on DACH of the complexes moved to highfield area compared with the corresponding ligand. ^{13}C NMR spectra were in agreement with the proposed chemical structure of the complexes, particular those of complexes with a fluorine atom showed splitting carbon peaks in the area of aromatic carbons as expected. Besides, a representative ^{195}Pt NMR spectrum (complex **2a**) was recorded.

All the ESI-MS spectra of the complexes gave main peaks corresponding to $[M+H]^+$, $[M-H]^-$ or $[M-Cl]^-$ ions, which were composed of a few isotopic peaks owing to the presence of platinum isotopes, suggesting the bonding between Pt(II) ions and the ligands.

2.2. In vitro cytotoxicity assay

The cytotoxicity of all metal complexes against three human cancer cell lines was evaluated by MTT method with cisplatin and oxaliplatin as positive control. As shown in Table 1, complexes **1a**–**4a** with chloride anions as leaving groups showed strong anticancer activity comparable or even superior to cisplatin and oxaliplatin, whereas other complexes with dicarboxylates as leaving groups, except individual cases, exhibited moderate or low cytotoxicity against HepG2, SGC7901, and A2780 cell lines. Among all compounds, complex **2a** exhibited the strongest activity against all the tested three cancer cell lines, remarkably superior to cisplatin and oxaliplatin. It was 3.71 ± 0.48-fold, 1.56 ± 0.25-fold and 3.66 ± 0.56-fold as potent as cisplatin and 34.34 ± 3.49-fold, 9.63 ± 1.54-fold, and 8.64 ± 1.34-fold as potent as oxaliplatin towards HepG2, SGC7901, and A2780, respectively. In contrast to complexes **2a**–**4a** with a ligand containing a fluoro substituent on the phenyl ring, complex **1a** was less active, which was about one fifth as effective as cisplatin on SGC7901 and A2780, and one tenth as active as cisplatin on HepG2. Although weaker than those of complex **2a** and cisplatin, the cytotoxicity of complexes **3a** and **4a** was stronger than that of oxaliplatin against almost all the tested cancer cell lines, demonstrating the importance and unique function of the fluorine atom of the ligand in our complexes. However, complexes **1b**-**4e** which substituted the leaving group from chloride anions to dicarboxylate species would result in the conspicuous decrease of the antitumor activity of the complexes compared with their parent compounds. This can be due to the slow dissociation ability of chelating dicarboxylate from the metal atom in comparison to chloride anions.

In terms of their strong inhibiting ability on the above cancer cell lines, complexes **1a**–**4a** were further investigated against cisplatin resistant cancer cell line (SGC7901/CDDP) and human normal liver cell line (LO2). As showed in Table 2, complex **2a** has the potential to overcome cisplatin resistance, as its resistance index (RF) can reach 1.39, which is better than cisplatin and oxaliplatin. While complexes **1a**, **3a** and **4a** were less effective against SGC7901/CDDP cell than complex **2a**, even junior to that of oxaliplatin. These results indicated that the position of the fluoro



Scheme 1. Synthesis of ligand **L**.

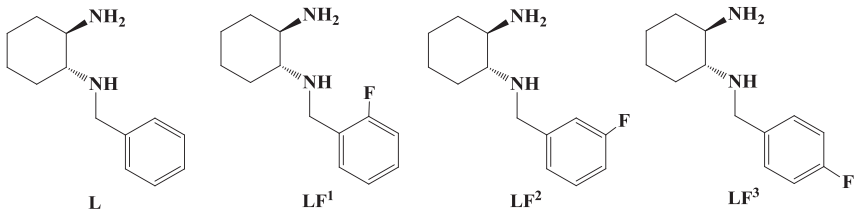
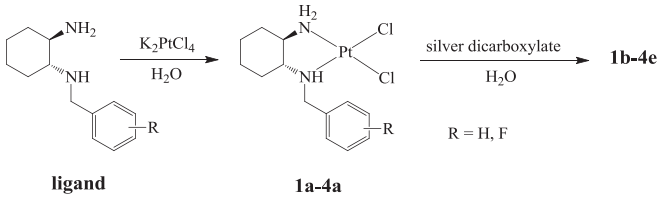


Fig. 1. Chemical structures of the ligands.



Scheme 2. Synthetic route to prepare platinum(II) complexes **1a**–**4e**.

substituent has played an important role in the nature of the complex overcoming cisplatin resistance. It is noticed that complexes **2a**–**4a** are more toxic toward LO2 cell line than complex **1a** and oxaliplatin, but they are less toxic than cisplatin on normal human cell. Upon the remarkable antitumor feature of complex **2a** as our partner reported [38], it was selected as a representative compound for the subsequent study.

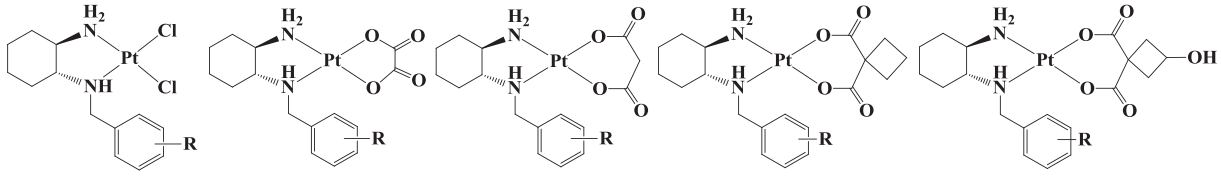


Fig. 2. Chemical structures of the synthesized platinum(II) complexes.

Table 1
In vitro cytotoxicity of complexes **1a**–**4e** against human cancer cell lines.

Complex	IC_{50} (μM)[a]		
	HepG2[b]	SGC7901[c]	A2780[d]
cisplatin	0.70 ± 0.04	1.35 ± 0.12	4.08 ± 0.26
oxaliplatin	6.49 ± 0.32	8.29 ± 0.76	9.48 ± 0.71
1a	7.15 ± 0.64	6.72 ± 0.53	23.96 ± 2.14
2a	0.19 ± 0.01	0.87 ± 0.06	1.11 ± 0.09
3a	3.42 ± 0.29	2.90 ± 0.12	11.42 ± 0.92
4a	4.30 ± 0.32	3.02 ± 0.21	6.52 ± 0.53
1b	97.35 ± 7.56	193.48 ± 13.11	>200
2b	11.89 ± 0.99	40.03 ± 3.87	1.56 ± 0.12
3b	6.59 ± 0.52	28.11 ± 2.17	8.33 ± 0.74
4b	25.38 ± 2.08	39.42 ± 3.76	25.57 ± 1.96
1c	22.70 ± 1.98	35.25 ± 2.95	36.22 ± 3.19
2c	19.11 ± 1.03	28.36 ± 1.99	23.77 ± 1.98
3c	44.17 ± 3.85	>200	8.09 ± 0.62
4c	15.27 ± 1.28	42.94 ± 3.87	9.82 ± 0.74
1d	44.07 ± 4.06	116.42 ± 10.23	>200
2d	35.18 ± 2.79	34.46 ± 3.12	33.67 ± 2.79
3d	140.34 ± 10.21	194.91 ± 13.23	>200
4d	40.71 ± 3.89	39.72 ± 2.98	38.48 ± 2.91
1e	>200	156.64 ± 1.25	>200
2e	>200	106.53 ± 9.35	>200
3e	39.36 ± 2.75	76.77 ± 4.23	120.84 ± 10.13
4e	35.54 ± 3.08	30.50 ± 2.94	30.92 ± 3.02

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 72 h drug exposure. Each value is the mean of three independent experiments.
[b] HepG2: human hepatocellular carcinoma cell line.
[c] SGC7901: human gastric cancer cell line.
[d] A2780: human ovarian cancer cell line.

Table 2
Cytotoxicity of complexes **1a**–**4a** against SGC7901/CDDP and LO2 cell lines.

Complex	IC_{50} (μM)[a]			
	SGC7901[b]	SGC7901/CDDP[c]	Resistant factor[d]	LO2[e]
cisplatin	1.35 ± 0.12	11.59 ± 1.01	8.59	3.67 ± 0.31
oxaliplatin	8.29 ± 0.76	12.46 ± 0.93	1.50	11.18 ± 0.87
1a	6.72 ± 0.53	28.71 ± 1.85	4.27	10.19 ± 0.98
2a	0.87 ± 0.06	1.21 ± 0.08	1.39	6.55 ± 0.43
3a	2.90 ± 0.12	16.73 ± 1.27	5.77	7.54 ± 0.65
4a	3.02 ± 0.21	19.74 ± 0.94	6.54	6.27 ± 0.59

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 72 h drug exposure. Each value is the mean of three independent experiments.
[b] SGC7901: human gastric cancer cell line.
[c] SGC7901/CDDP: human gastric cancer cisplatin resistant cell line.
[d] Resistant factor (RF) is defined as IC_{50} in SGC7901/CDDP/IC_{50} in SGC7901.
[e] LO2: normal human liver cell line.

2.3. Cellular uptake

Since it has the most potent antitumor activity in the cytotoxicity assay, complex **2a** was selected for the intracellular uptake test. The cellular uptake was measured by treating SGC7901 and SGC7901/CDDP cells with 50 μM of complex **2a**, cisplatin and oxaliplatin for 12 h, respectively. The content of platinum in the tumor cells was determined by the technique of ICP-MS. It can be seen from Table 3 that complex **2a** had the highest intracellular accumulation of platinum both in SGC7901 and in SGC7901/CDDP cells, while oxaliplatin had the lowest cell uptake values among

Table 3
Cellular uptake of complex **2a** on SGC7901 and SGC7901/CDDP cells after 12 h incubation.[a]

Complex	Pt content (ng/10^6 cells)	
	SGC7901	SGC7901/CDDP
complex **2a**	578 ± 19	620 ± 24
cisplatin	360 ± 21	344 ± 16
oxaliplatin	222 ± 16	110 ± 12

[a] Intracellular accumulation of complex **2a** cisplatin and oxaliplatin in SGC7901 and SGC7901/CDDP cells (per 10^6 cells) after 12 h incubation at a concentration of 50 μM. Results are expressed as the mean ± SD for three independent experiments.

three compounds. The content of complex **2a** taken up by the SGC7901 cells was 2.6 fold as much as that of oxaliplatin and the uptake of complex **2a** in SGC7901/CDDP cells was 5.6 fold greater than that of oxaliplatin. The uptake values of cisplatin in the tested two cells are in the middle of those of complex **2a** and oxaliplatin. Based on the results from the cytotoxicity and cellular uptake tests, it is found that complex **2a** has a positive correlation between these two tests, namely, the enhanced cellular uptake can result in the increase of the cytotoxicity, and so do cisplatin and oxaliplatin. The platinum accumulation of complex **2a** in the tested cells was highly compatible to its lipophilicity when compared with cispaltin and oxaliplatin, although there have been reports about a unclear correlation between cellular uptake and lipophilicity of the platinum compounds [39,40].

2.4. Flow cytometry study

2.4.1. Apoptosis study with complex *2a*

To study the apoptosis induced by complex **2a**, Annexin V/PI staining assay was used. The tested complexes were incubated with SGC7901 and HepG2 cell lines for 24 h at the concentration of 50 μM. Q1−Q4 represents four different cell states: necrotic cells, late apoptotic or necrotic cells, living cells and early apoptotic cells, respectively (Fig. 3). As shown in Fig. 3a and c, increased apoptotic rates of SGC7901 cells were detected after cells being treated with complex **2a**, cisplatin and oxaliplatin as positive controls. Compared with untreated cells as 6.49 ± 0.3% apoptotic rate, cells treated with complex **2a**, cisplatin and oxaliplatin showed 79.97 ± 1.9%, 28.44 ± 0.8% and 10.72 ± 0.7% apoptotic rates, respectively, including both the early and the late apoptotic cells. The results in Fig. 3b and c showed that the apoptotic rate of HepG2 cells for complex **2a** (26.61%) was slightly lower than cisplatin (28.79%), but better than oxaliplatin (21.74%). In contrast to the apoptotic rate of the HepG2 cell line, SGC7901 cell line was relatively sensitive to complex **2a**. These results indicated that complex **2a** could induce cancer cell death based on an apoptotic pathway.

2.4.2. Cell cycle analysis of complex *2a*

The effects of complex **2a** on both SGC7901 and HepG2 cell cycle progression were investigated by 24 h incubating at a concentration of 50 μM, cisplatin and oxaliplatin were used as positive controls. As shown in Fig. 4, G1 phase, S phase and G2/M phase represent early synthesis of DNA, DNA synthesis and late synthesis of DNA, respectively. For SGC7901 cells, cisplatin arrested cell cycle majors in G1 and S phases, whereas complex **2a** majors in S and G2 phases compared with control. For HepG2 cells, the cell cycle arrest induced by cisplatin and oxaliplatin majors in G2 phase, while the cell cycle of complex **2a** was arrested in G1 phase. This indicated that the mechanism of cell cycle arrest induced by complex **2a** was clearly different from cisplatin.

2.5. Interaction with pET22b plasmid DNA

To understand the interaction of our complexes with DNA, the DNA binding ability of complex **2a** was investigated by gel electrophoresis, in which cisplatin and oxaliplatin were taken as positive controls and plasmid DNA pET22b was used as target. DNA chain can generally be divided into closed circular DNA or supercoil DNA, linear DNA and open circular DNA. As shown in Fig. 5, it mainly contains closed circular DNA and open circular DNA which are defined as Form I and Form II, respectively.

After the plasmid was incubated with cisplatin at a concentration of 40 μM, the density of Form II and Form I was decreased compared with the blank control. When the concentration of cisplatin increased (160, 640 μM), a gradual increase in the mobility of covalently closed circular DNA (Form I) was observed, accompanied by the obvious decrease in density at the same time, indicating that cisplatin has an interaction with pET22b plasmid DNA in a high concentration. When treated with different concentrations of oxaliplatin, the migration of pET22b plasmid DNA increased slightly and stripe density was reduced only a little, exhibiting that oxaliplatin could cause DNA cleavage with a weak ability compared with cisplatin. As for complex **2a**, both Form I and Form II changed slightly at a low concentration. With the increase of the concentration, there was a significantly migration and an obvious decrease in density in Form I. As a result, the effect of complex **2a** on pET22b plasmid DNA is similar to cisplatin and oxaliplatin, and the ability of the effect is cisplatin > complex **2a** > oxaliplatin.

2.6. Western blot analysis

In order to character complex **2a** in intrinsic mitochondrial pathway to induce apoptosis, the expression levels of Bax, Bcl-2 and Procaspase-3 proteins were investigated in SGC7901 cells by Western blot method, meanwhile cisplatin and oxaliplatin were used as positive control. As shown in Fig. 6, obvious increase of Bax expression was observed in cells incubated with cisplatin and complex **2a**, while decrease of Bcl-2 and Procaspased-3 expression was detected in cells treated with cisplatin, oxaliplatin and complex **2a**. Thus, the complex **2a** can induce cell apoptosis by intrinsic mitochondrial pathway.

3. Conclusion

In this study, a number of sterically hindered platinum(II) complexes, with (1*R*,2*R*)-N^1-benzylcyclohexane-1,2-diamine derivatives as carrier ligands and chloride anions or dicarboxylates as leaving groups, were designed and prepared. The in vitro antitumor activity of all compounds against HepG2, SGC7901, and A2780 cancer cell lines revealed that complexes **1a**–**4a** with chloride anions showed strong anticancer activity comparable or even superior to cisplatin and oxaliplatin, whereas other complexes (**1b**–**4e**) with dicarboxylates, except individual cases, exhibited moderate or low cytotoxicity. Further evaluation on complexes **1a**–**4a** against cisplatin resistant SGC7901/CDDP cancer cell line indicated complex **2a** is the most potent agent to overcome cisplatin resistance, and less toxic than cisplatin to human normal liver LO2 cells. It was found that the platinum accumulation of complex **2a** in the tested cells was highly compatible to its lipophilicity, which had a positive correlation between its cellular uptake and cytotoxicity values. Apoptosis study on HepG2 and SGC7901 cell lines indicated that complex **2a** could induce cancer cell death based on an apoptotic pathway, but the mechanism of cell cycle arrest induced by complex **2a** was different from cisplatin. However, the interaction of complex **2a** with DNA showed that its effect on pET22b plasmid DNA was similar to cisplatin and oxaliplatin, and western blot

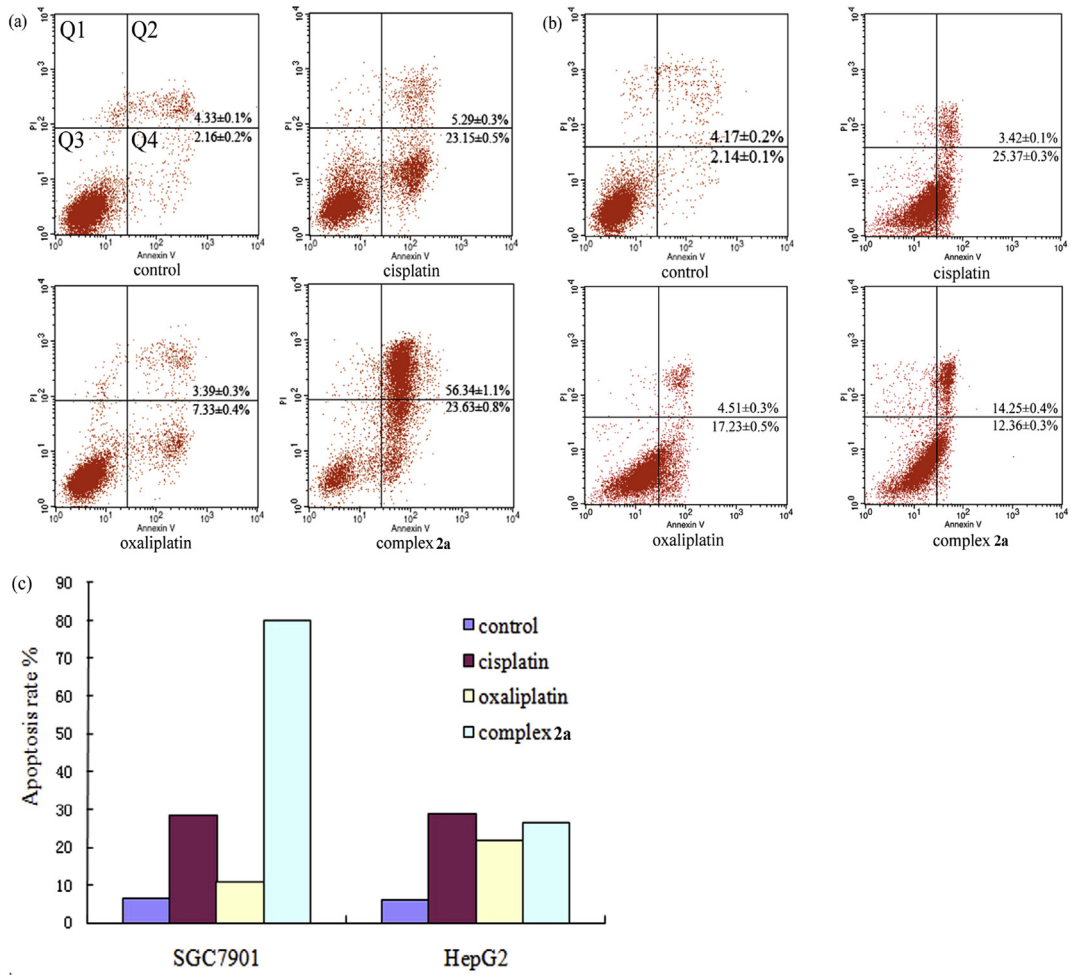


Fig. 3. Apoptosis induced by tested complexes in SGC7901 and HepG2 cells. Cells were treated with 50 μM of complex **2a**, cisplatin and oxaliplatin, respectively, for 24 h. Annexin V/PI double-staining assays of (a) SGC7901 and (b) HepG2 cells were analyzed by flow cytometry. (c) The apoptotic rates of cells induced by tested complexes.

analysis indicated that the induced apoptosis by complex **2a** was to some extent caused by intrinsic mitochondrial pathway. Overall, suitable steric hindrance in the platinum(II) complex can play an important role in overcoming cisplatin resistance, and complex **2a** can be a drug candidate for further research.

4. Experimental section

4.1. Materials and measurements

All chemicals and solvents were of analytical reagent grade and were used without further purification. Potassium tetrachloroplatinate(II) was obtained from a local chemical company (Lingfeng Chemical Ltd.). Human cancer cell lines including SGC7901/CDDP were obtained from Nanjing KeyGEN BioTECH company. Elemental analyses for C, H, and N were made on a Vario MICRO CHNOS Elemental Analyzer (Elementar). The specific optical rotations were measured on a WZZ-2A Automatic Polarimeter. Infrared spectra were recorded on KBr pellets on a Nicolet IR200 FT-IR spectrometer in the range of 4000–400 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded in D_2O or d_6-DMSO with a Bruker 300 or 500 MHz spectrometer. ^{195}Pt NMR spectrum of a typical compound was recorded on a Bruker DRX500 spectrometer. Mass spectra were measured on an Agilent 6224 TOF LC/MS instrument. Platinum contents were measured on an Optima 5300DV ICP-MS instrument.

4.1.1. Synthesis of ligands

*4.1.1.1. (1R,2R)-N^1-benzyl-1,2-diaminocyclohexane (**L**).* To a solution of Boc protected 1*R*, 2*R*-cyclohexanediamine (8.56 g, 40 mmol) in methanol (150 mL), benzaldehyde (8.48 g, 80 mmol) was added. The reaction mixture was kept stirring for 2 h at room temperature, then it was cooled in an ice water bath, followed by adding $NaBH_4$ (4.54 g, 120 mmol) in portions, and kept stirring overnight at 50 °C. Then, 10 mL of water was added to quench the reaction, white solids were obtained by concentrating the solution. The aqueous phase was extracted with EtOAc (50 mL × 3). Organic extracts were combined and washed by saturated NaCl solution and dried by anhydrous Na_2SO_4. By concentrating the solution in vacuum, the intermediate N^1-monobenzyl, N^2-Boc-(1*R*, 2R)-diaminocyclohexane was obtained. Then the intermediate was dissolved in absolute ether (75 mL) and a solution of HCl/EtOAc (1 mol/L, 50 mL) was added slowly with stirring. The mixture was kept stirring for 12 h at room temperature, then the resulting white solid was filtered off, washed with absolute ether and dried in vacuum. The product was obtained as dihydrochloride salt (**L**·2HCl). Yield: 56%. $[\alpha]_D^{25} = -52.4$ (c 0.44, H_2O). Anal. calcd for $C_{13}H_{20}N_2 \cdot 2HCl$: C, 56.32; H, 8.00; N, 10.10. Found: C, 56.43; H, 8.25; N, 9.81. IR (KBr cm^{-1}): 3402, 2942,

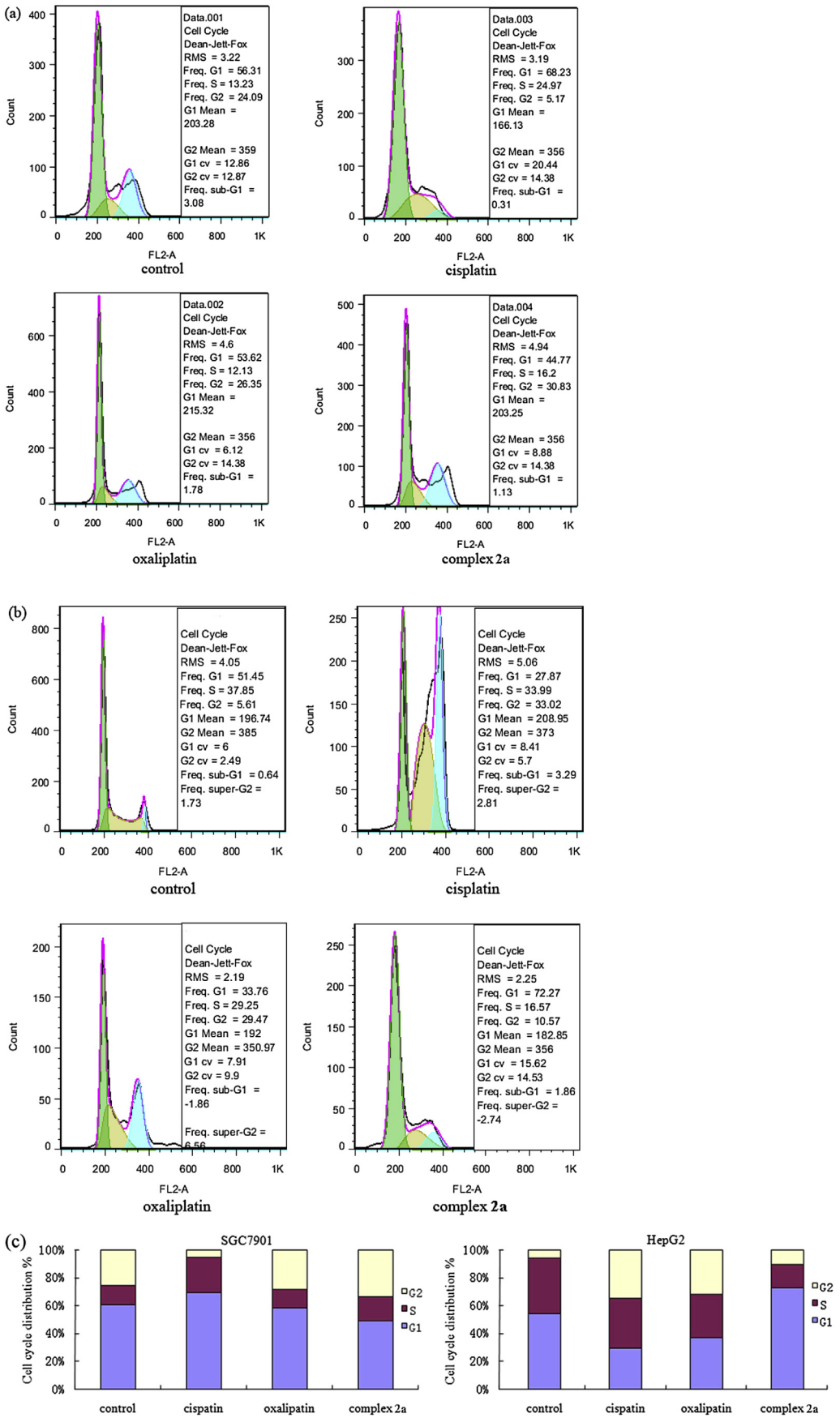


Fig. 4. Cell cycle arrest induction effects of tested complexes on SGC7901 and HepG2 cells. Cell cycle analysis of (a) SGC7901 and (b) HepG2 cells treated with cisplatin, oxaliplatin and complex **2a** at 50 μM for 24 h was performed by flow cytometry. (c) The percentages of G1, G2 and S phase cells following tested complexes treatment for 24 h are shown.

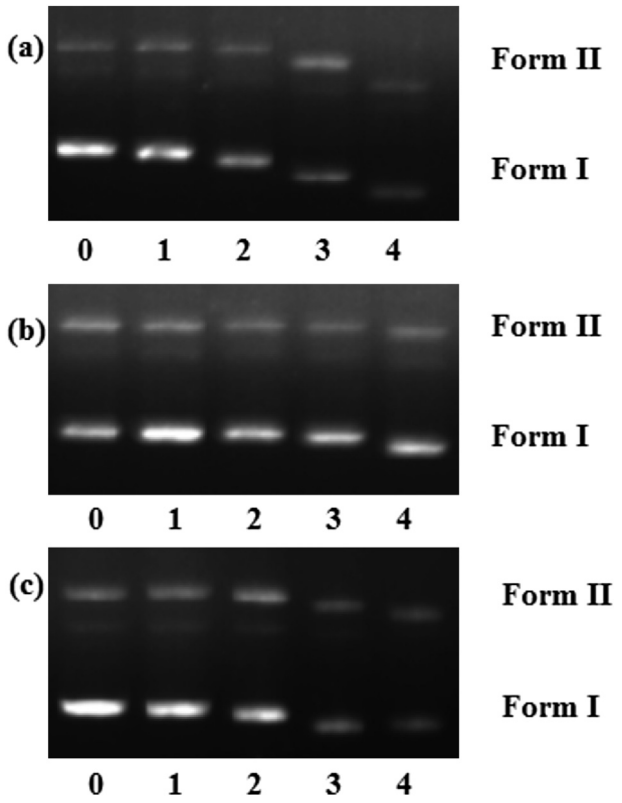


Fig. 5. Gel electrophoretic mobility pattern of pET22b plasmid DNA incubated with different concentrations of platinum(II) complexes, (a) cisplatin, (b) oxaliplatin, (c) complex **2a**. Lanes 0–4 expressed concentrations of 0, 10, 40, 160, and 640 μM of the tested compounds incubated with pET22b plasmid DNA. Form I and Form II represent closed circular DNA and open circular DNA, respectively.

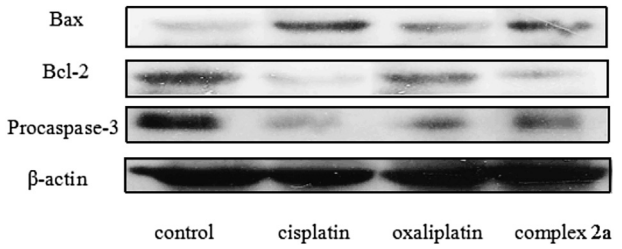


Fig. 6. SGC7901 cells treated with cisplain, oxaliplatin and complex **2a** at 50 μM for 24 h. Equal loading was testified by the detection of β-actin. Results were obtained similarly from three independent experiments.

2727, 2525, 2395, 1621, 1585, 1508, 1448, 1033, 762, 701. ^{1}H NMR (300 MHz, D_2O): δ = 1.24–1.48 (m, 2H, *CH_2* of DACH), 1.58–1.65 (m, 2H, *CH_2* of DACH), 1.82–1.88 (m, 2H, *CH_2* of DACH), 2.18–2.22 (d, *J* = 12 Hz, 1H, *CH_2* of DACH), 2.44–2.48 (d, *J* = 12 Hz, 1H, *CH_2* of DACH), 3.47–3.58 (m, 2H, NH*CH* and NH_2*CH*), 4.22–4.63 (dd, 2H, NH*CH_2*), 7.41–7.51 (m, 5H, *ArH*); ^{13}C NMR (D_2O, ppm): δ = 24.9, 25.0, 28.7, 32.0, 51.5, 53.9, 61.0, 131.9, 132.4, 132.5, 132.9. ESI-MS: *m/z* $[M+H]^+$ = 205.2 (100%).

4.1.1.2. (1R,2R)-N^1-(2-fluorobenzyl)-1,2-diaminocyclohexane ***(LF^1)***. The synthetic procedure was similar to that of **L** except using *o*-fluorobenzaldehyde instead, white solid powder, yield: 55%. $[\alpha]_D^{25}$ = −36.2 (c 0.39, H_2O). Anal. calcd for $C_{13}H_{19}FN_2 \cdot 2HCl$: C, 52.89; H, 7.17; N, 9.49; Found: C, 53.03; H, 7.33; N, 9.26. IR (KBr cm^{-1}): 3427, 2944, 1618, 1557, 1494, 1459, 1105, 1034, 784, 763. ^{1}H NMR (300 MHz, D_2O): δ = 1.27–1.42 (m, 2H, *CH_2* of DACH), 1.54–1.60 (m, 2H, *CH_2* of DACH), 1.84–1.76 (m, 2H, *CH_2* of DACH), 2.13–2.17 (d, *J* = 12 Hz, 1H, *CH_2* of DACH), 2.38–2.43 (d, *J* = 15 Hz, 1H, *CH_2* of DACH), 3.38–3.39 (m, 2H, NH*CH_2*), 4.17–4.44 (m, 2H, NH*CH*), 7.09–7.40 (m, 4H, *ArH)*; ^{13}C NMR (D_2O, ppm): δ = 24.9, 25.0, 28.7, 31.9, 45.3, 53.7, 61.4, 118.4, 118.6, 127.7134.5–135.0135.1, 161.7–164.7 (J_{CF} = −225 Hz). ESI-MS: *m/z* $[M+H]^+$ = 223.3 (100%).

4.1.1.3. (1R,2R)-N^1-(3-fluorobenzyl)-1,2-diaminocyclohexane ***(LF^2)***. The synthetic procedure was analogous to that of **L** except using *m*-fluorobenzaldehyde instead, white solid powder, yield: 64%. $[\alpha]_D^{25}$ = −39.2 (c 0.42, H_2O). Anal. Calcd (%) for $C_{13}H_{19}FN_2 \cdot 2HCl$: C, 52.89; H, 7.17; N, 9.49; Found: C, 52.78; H, 7.32; N, 9.35. IR (KBr cm^{-1}): 3434, 2947, 2867, 1617, 1591, 1491, 1455, 1257, 1152, 1028, 875, 792, 690. ^{1}H NMR (300 MHz, D_2O): δ = 1.43–1.46 (m, 2H, *CH_2* of DACH), 1.64 (s, 2H, *CH_2* of DACH), 1.86–1.92 (m, 2H, *CH_2* of DACH), 2.16–2.25 (d, *J* = 27 Hz, 1H, *CH_2* of DACH), 2.46–2.50 (d, *J* = 12 Hz, 1H, *CH_2* of DACH), 3.54–3.56 (m, 2H, NH*CH* and NH_2*CH*), 4.25–4.57 (dd, 2H, NH*CH_2*), 7.24–7.56 (m, 4H, *ArH*); ^{13}C NMR (D_2O, ppm): δ = 24.9, 25.1, 28.7, 32.0, 50.8–50.9, 53.9, 62.2, 119.0–119.8, 128.3, 133.7–133.8, 135.0–135.1, 137.6, 163.5–166.8 (J_{CF} = −247.5 Hz). ESI-MS: *m/z* $[M+H]^+$ = 223.1 (100%).

4.1.1.4. (1R,2R)-N^1-(4-fluorobenzyl)-1,2-diaminocyclohexane ***(LF^3)***. The synthetic procedure was the same as that of **L** except using *p*-fluorobenzaldehyde instead, white solid powder, yield: 58%. $[\alpha]_D^{25}$ = −33.1 (c 0.33, H_2O). Anal. Calcd (%) for $C_{13}H_{19}FN_2 \cdot 2HCl$: C, 52.89; H, 7.17; N, 9.49; Found: C, 52.81; H, 7.28; N, 9.23. IR(KBr cm^{-1}): 3437, 2944, 1604, 1513, 1451, 1231, 1162, 1024, 827, 776. ^{1}H NMR (300 MHz, D_2O): δ = 1.28–1.43 (m, 2H, *CH_2* of DACH), 1.56 (s, 2H, *CH_2* of DACH), 1.77–1.86 (m, 2H, *CH_2* of DACH), 2.13–2.17 (d, *J* = 12 Hz, 1H, *CH_2* of DACH), 2.38–2.43 (d, *J* = 15 Hz, 1H, *CH_2* of DACH), 3.34–3.36 (m, 2H, NH*CH* and NH_2*CH_2*), 3.97–4.36 (dd, 2H, NH*CH_2*), 7.04–7.40 (m, 4H, *ArH*). ^{13}C NMR (D_2O, ppm): δ = 22.4, 22.5, 26.2, 29.4, 48.2, 51.3, 58.4, 116.1–116.2, 126.4, 132.0, 162.3–163.9 (J_{CF} = −240 Hz). ESI-MS: *m/z* $[M+H]^+$ = 223.3 (100%).

4.1.2. Preparation of complexes ***1a–4a***

Under nitrogen and protection from light, an aqueous solution (75 mL) of K_2PtCl_4 (2.07 g, 5 mmol) was added to the ligand (5 mmol) in water (5 mL). The reaction mixture was then stirred at room temperature for 10 h and yellow solids deposited. The products were filtered off, washed with ethanol and distilled water, and then dried in vacuum. Greater than 95% purity of complexes **1a–4a** was confirmed by elemental analysis.

4.1.2.1. Cis-dichloro[(1R,2R)-N^1-benzyl-1,2-diaminocyclohexane-N,N′]platinum(II) (Complex ***1a****): yield: 86%.* $[\alpha]_D^{25}$ = 142.9 (c 0.13, DMF). Anal. calcd for $C_{13}H_{20}Cl_2N_2Pt$: C, 33.20; H, 4.29; N, 5.96. Found: C, 33.41; H, 4.43; N, 5.69. IR (KBr, cm^{-1}): 3466 (br), 3120, 2935, 2860, 1580, 1452, 751, 702. ^{1}H NMR (300 MHz, DMSO-d_6): δ1.12–1.16 (m, 1H, *CH_2* of DACH), 1.19–1.21 (m, 2H, *CH_2* of DACH), 1.24–1.26 (m, 1H, *CH_2* of DACH), 1.49–1.56 (m, 2H, *CH_2* of DACH), 1.90–1.93 (m, 2H, *CH_2* of DACH), 2.12–2.25 (m, 2H, NH*CH* and NH_2*CH*), 3.91–4.54 (m, 2H, NH*CH_2*Ph), 6.11–6.16 (dd, 2H, CH*NH_2*), 7.29–7.31 (m, 1H, CH_2*NH*), 7.38–8.11 (m, 5H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ24.4, 24.7, 28.0, 31.8, 48.9, 61.9, 65.7, 128.3, 128.6, 131.1, 134.9. ESI-MS: *m/z* $[M-Cl]^+$ = 435 (100%).

4.1.2.2. Cis-dichloro[(1R,2R)-N^1-(2-fluorobenzyl)-1, 2-diaminocyclohexane-N, N′]platinum(II) (Complex ***2a****): yield: 97%.* $[\alpha]_D^{25}$ = 130.0 (c 0.13, DMF). Anal. calcd for $C_{13}H_{19}Cl_2FN_2Pt$: C, 31.98; H, 3.92; N, 5.74. Found: C, 32.20; H, 4.23; N, 5.49. IR (KBr, cm^{-1}): 3138(br), 2933, 2862, 1579, 1494, 1451, 1229, 1187, 1139, 761. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.76–0.87 (m, 1H, *CH_2* of DACH),

0.95–1.06 (m, 2H, *CH*$_2$ of DACH), 1.13–1.20 (m, 1H, *CH*$_2$ of DACH), 1.39–1.48 (m, 2H, *CH*$_2$ of DACH), 1.75–1.82 (m, 2H, *CH*$_2$ of DACH), 2.11–2.23 (m, 2H, NH*CH* and NH$_2$*CH*), 3.75–4.55 (m, 2H, NH*CH*$_2$Ar), 4.96–5.47 (dd, 2H, CH*NH*$_2$) 6.75–7.45 (m, 4H, *ArH*), 8.89–8.93 (m, 1H, CH*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 24.6, 24.7, 28.2, 31.9, 42.0, 62.4, 66.6, 115.3–115.6, 121.9–122.1, 124.9–125.0, 130.6–130.7, 134.1–134.2, 160.0–163.3 (J_{CF} = −248 Hz). ^{195}Pt NMR (DMSO-d_6, ppm): δ = −2349.2. ESI-MS: *m*/*z* [M−Cl]$^+$ = 453 (100%).

*4.1.2.3. Cis-dichloro[(1R,2R)-N^1-(3-fluorobenzyl)-1,2-diaminocyclohexane-N,N′]platinum(II) (Complex **3a**): yield: 85%.* $[\alpha]_D^{25}$ = 135.2 (c 0.24, DMF) Anal. calcd for $C_{13}H_{19}Cl_2FN_2Pt$: C, 31.98; H, 3.92; N, 5.74. Found: C, 32.31; H, 4.20; N, 5.36. IR (KBr, cm^{-1}): 3267(br), 3188, 3100, 2938, 1587, 1451, 1258, 1147, 789, 754. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.76–0.87 (m, 1H, *CH*$_2$ of DACH), 0.95–1.06 (m, 2H, *CH*$_2$ of DACH), 1.13–1.39 (m, 1H, *CH*$_2$ of DACH), 1.41–1.75 (m, 2H, *CH*$_2$ of DACH), 1.77–1.90 (m, 2H, *CH*$_2$ of DACH), 1.98–2.23 (m, 2H, NH*CH* and NH$_2$*CH*), 3.67–4.58 (m, 2H, NH*CH*$_2$Ar), 5.01–5.48 (dd, 2H, CH*NH*$_2$), 6.72–7.86 (m, 4H, *ArH*), 8.25–8.27 (m, 1H, CH$_2$*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 24.4, 24.6, 28.0, 31.7, 48.2, 61.8, 65.8, 115.2–115.5, 117.7–117.9, 127.3–127.3, 130.3–130.4, 137.5–137.6, 160.7–163.9 (J_{CF} = −240 Hz). ESI-MS: *m*/*z* [M−Cl]$^+$ = 453 (100%).

*4.1.2.4. Cis-dichloro[(1R,2R)-N^1-(4-fluorobenzyl)-1,2-diaminocyclohexane-N,N′]platinum(II) (Complex **4a**): yield: 79%.* $[\alpha]_D^{25}$ = 133.1 (c 0.17, *DMF*). Anal. calcd for $C_{13}H_{19}Cl_2FN_2Pt$: C, 31.98; H, 3.92; N, 5.74. Found: C, 32.25; H, 4.19; N, 5.31. IR (KBr, cm^{-1}): 3142(br), 3142, 2939, 2866, 1598, 1509, 1452, 1220, 1152, 976, 823, 773. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.76–0.87 (m, 1H, *CH*$_2$ of DACH), 0.95–1.06 (m, 2H, *CH*$_2$ of DACH), 1.13–1.39 (m, 1H, *CH*$_2$ of DACH), 1.41–1.75 (m, 2H, *CH*$_2$ of DACH), 1.77–1.90 (m, 2H, *CH*$_2$ of DACH), 2.12–2.25 (m, 2H, NH*CH* and NH$_2$*CH*), 3.67–4.58 (m, 2H, NH*CH*$_2$Ar), 5.01–5.48 (dd, 2H, CH*NH*$_2$), 6.72–7.86 (m, 4H, *ArH*), 8.25–8.27 (m, 1H, CH$_2$*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 24.4, 24.6, 27.9, 31.8, 47.9, 61.9, 65.5, 115.2–115.5, 131.1, 133.3–133.4, 160.7–163.9 (J_{CF} = −240 Hz). ESI-MS: *m*/*z* [M−Cl]$^+$ = 453 (100%).

*4.1.3. Preparation of complexes **1b**–**4b***

Complex **1a**, **2a**, **3a** or **4a** (1 mmol) was suspended in distilled water (100 mL) and silver nitrate (0.34 g, 2 mmol) was added. The mixture was heated to 38 °C and stirred for 12 h in the lighting shielding condition. Subsequently, AgCl deposits were filtered off, the filtrate was mixed with an aqueous solution (15 mL) of sodium oxalate (1.00 mmol) and heated to 35 °C in the dark for 12 h. After the reaction terminated, the solution was filtered through celite and washed with water repeatedly, dried in vacuum, pale yellow powders were obtained. Greater than 95% purity of complexes ***1b***–***4b*** was confirmed by elemental analysis.

*4.1.3.1. [(1R,2R)-N^1-benzyl-1,2-cyclohexanediamine-N,N′](oxalate-O,O′)platinum(II) (Complex **1b**): yield: 65%.* $[\alpha]_D^{25}$ = 121.3 (c 0.12, DMF). Anal. calcd for $C_{15}H_{20}N_2O_4Pt$: C, 36.96; H, 4.14; N, 5.75. Found: C, 36.55; H, 4.32; N, 5.53. IR (KBr, cm^{-1}): 3433(br), 3237, 3048, 2933, 2859, 1621, 1447, 1291, 780, 745. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.04–1.07 (m, 2H, *CH*$_2$ of DACH), 1.22–1.23 (m, 2H, *CH*$_2$ of DACH), 1.61 (s, 2H, *CH*$_2$ of DACH), 1.93 (s, 1H, *CH*$_2$ of DACH), 2.15 (s, 1H, *CH*$_2$ of DACH), 1.93–2.15 (m, 2H, *CH*NH$_2$ and *CH*NH), 2.89 (m, 2H, NH*CH*$_2$Ar), 4.01–4.13 (dd, 2H, CH*NH*$_2$), 5.72 (m, 1H, CH$_2$*NH*), 6.93–7.51 (m, 5H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.6, 24.4, 28.9, 34.4, 57.2, 61.1, 66.8, 121.3, 124.3, 125.4, 133.6, 165.2. ESI-MS: *m*/*z* [M−H]$^-$ = 486 (100%).

*4.1.3.2. [(1R,2R)-N^1-(2-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](oxalate-O,O′)platinum(II) (Complex **2b**): yield: 48%.* $[\alpha]_D^{25}$ = 89.5 (c 0.10, DMF). Anal. calcd for $C_{15}H_{19}FN_2O_4Pt$: C, 35.65; H, 3.79; N, 5.54. Found: C, 35.15; H, 3.92; N, 5.23. IR (KBr, cm^{-1}): 3439 (br), 3439, 3239, 2935, 2861, 1601, 1451, 1384, 1292, 1230, 1124, 1062, 1037, 875, 778, 714. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.04–1.13 (m, 2H, *CH*$_2$ of DACH), 1.23–1.25 (m, 1H, *CH*$_2$ of DACH), 1.41–1.43 (m, 1H, *CH*$_2$ of DACH), 1.60 (s, 2H *CH*$_2$ of DACH), 1.92–1.94 (m, 1H, *CH*$_2$ of DACH), 2.16–2.18 (m, 1H, *CH*$_2$ of DACH), 2.65–2.89 (m, 2H, *CH*NH$_2$ and *CH*NH), 3.97–4.32 (m, 2H, NH*CH*$_2$Ar), 5.24–5.78 (dd, 2H, CH*NH*$_2$), 6.82–7.33 (m, 4H, *ArH*), 7.83 (m, 1H, CH$_2$*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.2, 28.8, 34.4, 51.7, 61.9, 67.4, 124.2, 127.5–127.9, 129.9, 138.6, 139.9, 155.1–156.8 (J_{CF} = −255 Hz), 164.4. ESI-MS: *m*/*z* [M−H]$^-$ = 504 (100%).

*4.1.3.3. [(1R,2R)-N^1-(3-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](oxalate-O,O′)platinum(II) (Complex **3b**): yield: 24%.* $[\alpha]_D^{25}$ = *109*.9 (c 0.14, DMF). Anal. calcd for $C_{15}H_{19}FN_2O_4Pt$: C, 35.65; H, 3.79; N, 5.54. Found: C, 35.28; H, 4.01; N, 5.31. IR (KBr, cm^{-1}): 3445 (br), 3234, 2133, 2935, 2861, 1616, 1450, 1384, 1295, 1229, 778. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.04–1.12 (m, 2H, *CH*$_2$ of DACH), 1.23 (s, 1H, *CH*$_2$ of DACH), 1.45 (s, 1H, *CH*$_2$ of DACH), 1.61 (s, 2H *CH*$_2$ of DACH), 1.92 (s, 1H, *CH*$_2$ of DACH), 2.15 (s, 1H, *CH*$_2$ of DACH), 2.65–2.92 (m, 2H, *CH*NH$_2$ and *CH*NH), 4.09–4.28 (m, 2H, NH*CH*$_2$Ar), 5.17–5.94 (dd, 2H, CH*NH*$_2$), 6.77–7.49 (m, 4H, *ArH*), 7.68 (m, 1H, CH$_2$*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.8, 24.2, 27.6, 31.6, 49.5, 61.1, 64.8, 115.2–115.3, 117.5–117.7, 127.1, 130.4, 136.6–136.7, 164.0–165.4 (J_{CF} = −210 Hz), 166.4. ESI-MS: *m*/*z* [M−H]$^-$ = 504 (100%).

*4.1.3.4. [(1R,2R)-N^1-(4-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](oxalate-O,O′)platinum(II) (Complex **4b**): yield: 28%.* $[\alpha]_D^{25}$ = 108.7 (c 0.15, DMF). Anal. calcd for $C_{15}H_{19}FN_2O_4Pt$: C, 35.65; H, 3.79; N, 5.54. Found: C, 35.47; H, 3.94; N, 5.22. IR (KBr, cm^{-1}): 3435(br), 3314, 3243, 2936, 1622, 1591, 1449, 1384, 1293, 770. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.95–1.11 (m, 2H, *CH*$_2$ of DACH), 1.23 (s, 1H, *CH*$_2$ of DACH), 1.43 (s, 1H, *CH*$_2$ of DACH), 1.59 (s, 2H *CH*$_2$ of DACH), 1.92 (s, 1H, *CH*$_2$ of DACH), 2.11–2.13 (m, 1H, *CH*$_2$ of DACH), 2.65–2.83 (m, 2H, *CH*NH$_2$ and *CH*NH), 3.97–4.19 (m, 2H, NH*CH*$_2$Ar), 5.32–5.78 (dd, 2H, CH*NH*$_2$), 6.81–7.29 (m, 4H, *ArH*), 7.71–7.92 (m, 1H, CH$_2$*NH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.8, 24.2, 27.6, 31.6, 49.5, 61.2, 64.5, 115.2–115.4, 130.3, 133.2–133.3, 161.3–162.9 (J_{CF} = −240 Hz), 165.5, 166.4. ESI-MS: *m*/*z* [M−H]$^+$ = 504 (100%).

*4.1.4. Preparation of complexes **1c**–**4c***

The synthetic procedure was similar to that of complexes **1b**–**4b** except sodium malonate was used to take the place of sodium oxalate.

*4.1.4.1. [(1R,2R)-N^1-benzyl-1,2-cyclohexanediamine-N,N′](malonato-O,O′)platinum(II) (Complex **1c**): yield: 67%.* $[\alpha]_D^{25}$ = 127.5 (c 0.16, DMF). Anal. calcd for $C_{16}H_{22}N_2O_4Pt$: C, 38.32; H, 4.42; N, 5.59. Found: C, 38.55; H, 4.74; N, 5.34. IR (KBr, cm^{-1}): 3417(br), 3241, 3140, 3048, 2931, 2858, 1581, 1445, 1347, 745. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.14–1.21 (m, 2H, *CH*$_2$ of DACH), 1.40 (s, 2H, *CH*$_2$ of DACH), 1.65 (m, 2H, *CH*$_2$ of DACH), 1.91–1.94 (m, 1H, *CH*$_2$ of DACH), 2.16–2.18 (m, 1H, *CH*$_2$ of DACH), 2.16–2.60 (m, 2H, *CH*NH and *CH*NH$_2$), 2.74 (s, 2H, (OOC)$_2$*CH*$_2$), 3.98–4.17 (m, 2H, NH*CH*$_2$Ar), 4.83 (m, 1H, CH$_2$*NH*), 5.43 (dd, 2H, CH*NH*$_2$), 6.93–7.60 (m, 5H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.3, 28.9, 34.5, 38.9, 56.9, 61.5, 67.4, 121.7, 124.6, 125.5, 133.9, 171.6. ESI-MS: *m*/*z* [M+H]$^+$ = 502 (100%).

*4.1.4.2. [(1R,2R)-N^1-(2-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](malonato-O,O′)platinum(II) (Complex **2c**): yield: 46%.*

$[\alpha]_D^{25} = 93.4$ (c 0.13, DMF). Anal. calcd for $C_{16}H_{21}FN_2O_4Pt$: C, 37.00; H, 4.08; N, 5.39. Found: C, 37.35; H, 4.22; N, 5.17. IR (KBr, cm^{-1}): 3237 (br), 2934, 2860, 1596, 1450, 1348, 1231, 1161, 1124, 1037, 875, 778, 716. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.15–1.21 (m, 2H, CH_2 of DACH), 1.39–1.47 (m, 2H, CH_2 of DACH), 1.64–1.71 (m, 2H, CH_2 of DACH), 1.92–1.98 (m, 1H CH_2 of DACH), 2.06–2.13 (m, 1H, CH_2 of DACH), 2.22–2.64 (m, 2H, *CH*NH and *CH*NH_2), 2.75 (s, 2H, $(OOC)_2CH_2$), 3.86–4.33 (m, 2H, NHCH_2Ar), 4.92–5.03 (dd, 2H, CHNH_2), 5.49 (m, 1H, CH_2NH), 6.84–7.32 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.2, 28.7, 34.4, 38.9, 51.7, 61.8, 67.4, 111.0–111.2, 127.6, 129.9, 138.3, 139.9, 155.1–156.8 (J_{CF} = −212.5 Hz), 171.6. ESI-MS: *m*/*z* $[M+H]^+$ = 520 (100%).

4.1.4.3. [(1R,2R)-N^1-(3-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](malonato-O,O')platinum(II) (Complex ***3c****): yield: 35%.* $[\alpha]_D^{25} = 115.9$ (c 0.18, DMF). Anal. calcd for $C_{16}H_{21}FN_2O_4Pt$: C, 37.00; H, 4.08; N, 5.39. Found: C, 37.25; H, 4.36; N, 5.25. IR (KBr, cm^{-1}): 3445 (br), 3241, 3133, 2934, 2861, 1718, 1593, 1450, 1384, 1278, 1230, 1214, 778. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.11–1.13 (m, 2H, CH_2 of DACH), 1.22 (s, 1H, CH_2 of DACH), 1.43–1.62 (m, 1H, CH_2 of DACH), 1.70 (m, 2H CH_2 of DACH), 1.88–1.91 (m, 1H, CH_2 of DACH), 2.07 (s, 1H CH_2 of DACH), 2.17–2.61 (m, 2H, *CH*NH and *CH*NH_2), 2.74 (s, 2H, $(OOC)_2CH_2$), 4.00–4.25 (m, 2H, NHCH_2Ar), 4.92–5.66 (dd, 2H, CHNH_2), 7.61 (m, 1H, CH_2NH), 6.71–7.51 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.0, 28.6, 33.8, 38.9, 57.2, 61.2, 67.5, 109.0–109.1, 112.0–112.1, 112.6–112.8, 126.5, 135.1, 164.9–166.5 (J_{CF} = −240 Hz), 171.5. ESI-MS: *m*/*z* $[M+H]^+$ = 520 (100%).

4.1.4.4. [(1R,2R)-N^1-(4-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](malonato-O,O')platinum(II) (Complex ***4c****): yield: 19%.* $[\alpha]_D^{25} = 95.7$ (c 0.15, DMF). Anal. calcd for $C_{16}H_{21}FN_2O_4Pt$: C, 37.00; H, 4.08; N, 5.39. Found: C, 37.39; H, 4.19; N, 5.13. IR (KBr, cm^{-1}): 3434(br), 3240, 2935, 2860, 1590, 1449, 1384, 1350, 1243, 1174. H NMR (300 MHz, DMSO-d_6): δ = 1.14–1.20 (m, 2H, CH_2 of DACH), 1.31–1.40 (m, 2H, CH_2 of DACH), 1.60–1.63 (m, 2H, CH_2 of DACH), 1.71–1.74 (m, 1H CH_2 of DACH), 1.87–1.90 (m, 1H, CH_2 of DACH), 1.93–2.19 (m, 2H, *CH*NH and *CH*NH_2), 2.74 (s, 2H, $(OOC)_2CH_2$), 3.98–4.19 (m, 2H, NHCH_2Ar), 4.88 (s, 2H, CHNH_2), 5.46 (s, 1H, CH_2NH), 6.81–7.30 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.3, 28.8, 34.3, 38.9, 56.3, 61.6, 67.4, 110.9–111.1, 119.9, 120.0–120.3, 123.1, 149.1, 158.6–160.2 (J_{CF} = −240 Hz), 171.6. ESI-MS: *m*/*z* $[M+H]^+$ = 520 (100%).

4.1.5. Preparation of complexes ***1d****–****4d***

The synthetic procedure was similar to that of complexes **1b**–**4b** except sodium 1, 1-cyclobutanedicarboxylate was used to take the place of sodium oxalate.

4.1.5.1. [(1R,2R)-N^1-benzyl-1,2-cyclohexanediamine-N,N′](1,1-cyclobutanedicarboxylato-O,O′) platinum(II) (Complex ***1d****): yield: 59%.* $[\alpha]_D^{25} = 140.1$ (c 0.13, DMF). Anal. calcd for $C_{19}H_{26}N_2O_4Pt$: C, 42.14; H, 4.84; N, 5.17. Found: C, 42.35; H, 5.02; N, 4.88. IR (KBr, cm^{-1}): 3419(br), 3232, 2935, 2860, 1648, 1579, 1361, 1448, 1379, 1237, 1119, 747. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.07–1.20 (m, 3H, CH_2 of DACH), 1.40 (s, 1H, CH_2 of DACH), 1.60–1.62 (m, 2H, CH_2 of DACH), 1.89–1.91 (m, 2H, CH_2 of DACH), 1.93–2.06 (m, 2H CH_2 of cdba), 2.13–2.67 (m, 4H CH_2 of cdba), 2.32–2.78 (m, 2H, NH*CH* and NH_2CH), 4.01–4.17 (m, 2H, NHCH_2Ar), 6.75–7.07 (m, 5H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 16.5, 23.4, 24.3, 28.9, 29.0, 34.4, 45.8, 56.9, 61.5, 67.4, 68.3, 121.8, 124.6, 125.5, 133.9, 177.2. ESI-MS: *m*/*z* $[M+H]^+$ = 542 (100%).

4.1.5.2. [(1R,2R)-N^1-(2-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex ***2d****): yield: 63%.* $[\alpha]_D^{25} = 97.6$ (c 0.12, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_4Pt$: C, 40.79; H, 4.50; N, 5.01. Found: C, 41.24; H, 4.69; N, 4.88. IR (KBr, cm^{-1}): 3436(br), 3237, 3140, 2938, 2862, 1595, 1563, 1451, 1381, 1228, 778. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.17 (s, 3H, CH_2 of DACH), 1.40 (s, 1H, CH_2 of DACH), 1.65 (s, 2H, CH_2 of DACH), 1.88–1.90 (m, 2H, CH_2 of DACH), 1.95–1.98 (m, 2H CH_2 of cdba), 2.23–2.28 (m, 4H CH_2 of cdba), 2.65–2.86 (m, 2H, NH*CH* and NH_2CH), 3.97–4.36 (m, 2H, NHCH_2Ar), 4.97 (dd, 2H, CHNH_2), 5.53 (m, 1H, CH_2NH), 6.85–7.42 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 16.2, 23.4, 24.2, 28.7, 29.0, 34.3, 48.8, 51.7, 61.8, 67.4, 69.4, 111.0–111.1, 127.5, 129.9, 138.6–138.7, 140.1, 155.1–156.8 (J_{CF} = −255 Hz), 177.4. ESI-MS: *m*/*z* $[M+H]^+$ = 560 (100%).

4.1.5.3. [(1R,2R)-N^1-(3-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex ***3d****): yield: 59%.* $[\alpha]_D^{25}$ = 128.6 (c 0.18, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_4Pt$: C, 40.79; H, 4.50; N, 5.01. Found: C, 40.65; H, 4.86; N, 4.86. IR (KBr, cm^{-1}): 3444(br), 3248, 3133, 2938, 2863, 1721, 1592, 1450, 1384, 1277, 1230, 1213, 778. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.10 (s, 3H, CH_2 of DACH), 1.46 (s, 1H, CH_2 of DACH), 1.61 (s, 2H, CH_2 of DACH), 1.89–1.94 (m, 2H, CH_2 of DACH), 2.15–2.26 (m, 2H CH_2 of cdba), 2.28–2.50 (m, 4H CH_2 of cdba), 2.63–2.85 (m, 2H, NH*CH* and NH_2CH), 4.03–4.27 (m, 2H, NHCH_2Ar), 5.13 (dd, 2H, CHNH_2), 5.74 (m, 1H, CH_2NH), 6.74–7.51 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 16.5, 23.4, 24.2, 28.2, 28.7, 29.0, 34.2, 48.8, 51.5, 61.8, 67.3, 111.0–111.2, 127.5, 129.9, 138.6, 140.1, 155.1–156.8 (J_{CF} = −240 Hz), 177.3. ESI-MS: *m*/*z* $[M+H]^+$ = 560 (100%).

4.1.5.4. [(1R,2R)-N^1-(4-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex ***4d****): yield: 28%.* $[\alpha]_D^{25}$ = 110.7 (c 0.19, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_4Pt$: C, 40.79; H, 4.50; N, 5.01. Found: C, 40.93; H, 4.69; N, 4.77. IR (KBr, cm^{-1}): 3420(br), 3240, 2938, 2862, 1568, 1459, 1383, 1243, 1204. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.23 (s, 3H, CH_2 of DACH), 1.41 (s, 1H, CH_2 of DACH), 1.62 (s, 2H, CH_2 of DACH), 1.88–1.92 (m, 2H, CH_2 of DACH), 1.96–1.99 (m, 2H CH_2 of cdba), 2.13–2.28 (m, 4H CH_2 of cdba), 2.30–2.38 (m, 2H, NH*CH* and NH_2CH), 3.98–4.19 (m, 2H, NHCH_2Ar), 6.83–7.60 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 16.5, 23.4, 24.4, 28.9, 31.3, 34.8, 45.0, 48.9, 56.3, 61.8, 67.6, 110.9–111.0, 119.9–120.0, 123.0, 139.6, 149.0, 158.7–160.3 (J_{CF} = −240 Hz), 177.3. ESI-MS: *m*/*z* $[M+H]^+$ = 560 (100%).

4.1.6. Preparation of complexes ***1e****–****4e***

The synthetic procedure was similar to that of complexes **1b**–**4b** except sodium 3-hydroxy-1,1-cyclobutanedicarboxylate was used to take the place of sodium oxalate.

4.1.6.1. [(1R,2R)-N^1-benzyl-1,2-cyclohexanediamine-N,N′](3-hydroxy-1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex ***1e****): yield: 50%.* $[\alpha]_D^{25}$ = 131.2 (c 0.14, DMF). Anal. calcd for $C_{19}H_{26}N_2O_5Pt$: C, 40.93; H, 4.70; N, 5.02. Found: C, 40.75; H, 4.59; N, 4.89. IR (KBr, cm^{-1}): 3418(br), 3200, 3100, 2934, 2860, 1582, 1446, 1382, 1036, 747, 662. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.13 (s, 3H, CH_2 of DACH), 1.36 (s, 1H, CH_2 of DACH), 1.62 (s, 2H, CH_2 of DACH), 1.90 (s, 2H, CH_2 of DACH), 2.06–2.18 (m, 2H CH_2 of hcdba), 2.24–2.49 (m, 3H CH_2 of hcdba), 2.69–2.63 (m, 2H, NH*CH* and NH_2CH), 3.99–4.22 (m, 2H, NHCH_2Ph), 4.65–4.85 (m, 1H, *CH*OH), 6.80–7.49 (m, 5H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.8, 24.1, 28.0, 28.9, 31.6, 32.2, 41.9, 61.6, 62.8, 63.1, 72.5, 128.4, 128.7, 130.2, 133.9, 177.0, 177.5. ESI-MS: *m*/*z* $[M-H]^-$ = 556 (100%).

4.1.6.2. [(1R,2R)-N^1-(2-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](3-hydroxy-1,1-cyclobutanedicarboxylato-O,O′)platinum(II)

*(Complex **2e**): yield: 23%.* $[\alpha]_D^{25} = 96.8$ (c 0.17, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_5Pt$: C, 39.65; H, 4.38; N, 4.87. Found: C, 39.95; H, 4.58; N, 4.47. IR (KBr, cm^{-1}): 3241(br), 2936, 2861, 1713, 1599, 1452, 1383, 1231, 1126, 1038, 874, 779. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.14–1.21 (m, 3H, CH_2 of DACH), 1.39 (s, 1H, CH_2 of DACH), 1.65 (s, 2H, CH_2 of DACH), 1.81 (s, 2H, CH_2 of DACH), 1.91 (s, 1H CH_2 of hcdba), 1.95–2.07 (m, 1H CH_2 of hcdba), 2.22–2.39 (m, 3H CH_2 of hcdba), 2.50–2.66 (m, 2H, NHCH and NH_2CH), 4.30–4.37 (m, 2H, $NHCH_2Ar$), 4.65 (m, 1H, CHOH), 6.84–7.40 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.5, 24.2, 27.9, 28.7, 31.7, 34.3, 40.9, 48.9, 61.3, 61.7, 66.4, 115.1–115.3, 121.0, 124.0, 130.3, 133.6, 160.4–162.1 (J_{CF} = −255 Hz), 177.2, 177.5. ESI-MS: *m*/*z* $[M-H]^-$ = 574 (100%).

*4.1.6.3. [(1R,2R)-N^1-(3-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](3-hydroxy-1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex 3**e**): yield: 23%.* $[\alpha]_D^{25} = 89.8$ (c 0.11, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_5Pt$: C, 39.65; H, 4.38; N, 4.87. Found: C, 39.40; H, 4.64; N, 4.51. IR (KBr, cm^{-1}): 3308(br), 2936, 2861, 1709, 1590, 1449, 1382, 1211, 1146, 1038, 780, 666. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.05–1.08 (m, 3H, CH_2 of DACH), 1.45 (s, 1H, CH_2 of DACH), 1.61 (s, 2H, CH_2 of DACH), 1.89–2.07 (m, 2H, CH_2 of DACH), 2.15–2.20 (m, 2H CH_2 of hcdba), 2.23–2.38 (m, 3H CH_2 of hcdba), 2.40–2.63 (m, 2H, NHCH and NH_2CH), 4.03–4.27 (m, 2H, $NHCH_2Ar$), 4.45 (m, 1H, CHOH), 6.70–7.51 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.5, 24.1, 28.7, 28.8, 33.8, 34.2, 41.4, 56.6, 57.9, 61.7, 67.7, 109.0, 112.5–112.7, 118.7, 126.3–126.4, 155.3, 164.9–166.5 (J_{CF} = −240 Hz), 177.7. ESI-MS: *m*/*z* $[M-H]^-$ = 574 (100%).

*4.1.6.4. [(1R,2R)-N^1-(4-fluorobenzyl)-1,2-cyclohexanediamine-N,N′](3-hydroxy-1,1-cyclobutanedicarboxylato-O,O′)platinum(II) (Complex **4e**): yield: 30%.* $[\alpha]_D^{25} = 119.3$ (c 0.15, DMF). Anal. calcd for $C_{19}H_{25}FN_2O_5Pt$: C, 39.65; H, 4.38; N, 4.87. Found: C, 39.86; H, 4.51; N, 4.55. IR (KBr, cm^{-1}): 3401 (br), 2936, 2861, 1569, 1453, 1383, 1242, 1206, 1037, 881. ^1H NMR (300 MHz, DMSO-d_6): δ = 1.15–1.22 (m, 3H, CH_2 of DACH), 1.38 (s, 1H, CH_2 of DACH), 1.66 (s, 2H, CH_2 of DACH), 1.83 (s, 2H, CH_2 of DACH), 1.91 (s, 1H CH_2 of hcdba), 1.93–2.07 (m, 1H CH_2 of hcdba), 2.20–2.28 (m, 3H CH_2 of hcdba), 2.41–2.64 (m, 2H, NHCH and NH_2CH), 3.97–4.28 (m, 2H, $NHCH_2Ar$), 4.27–4.30 (m, 1H, CHOH), 6.76–7.86 (m, 4H, *ArH*). ^{13}C NMR (DMSO-d_6/TMS, ppm): δ = 23.4, 24.3, 28.0, 28.7, 33.8, 34.3, 41.2, 56.3, 61.7, 67.4, 68.3, 110.9–111.0, 119.9, 123.0–123.1, 139.9, 149.1, 158.7–160.3 (J_{CF} = −240 Hz), 177.1, 177.6. ESI-MS: *m*/*z* $[M-H]^-$ = 574 (100%).

4.2. Biological studies

4.2.1. Cell culture

Four human solid tumor cell lines including HepG2 (human hepatocellular carcinoma cell line), A2780 (human ovarian cell line), SGC7901 (human gastric cancer cell line) and SGC7901/CDDP (human gastric cancer cisplatin resistant cell line) as well as LO2 (normal liver cell line) were selected for the cytotoxicity study of all synthesized platinum(II) complexes. The human solid tumor cell lines were incubated carefully in RPMI-1640 medium or Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and ampicillin sodium (100 μg/mL) in an atmosphere of 5% CO_2 and 95% air at 37 °C.

4.2.2. Cytotoxicity analysis (IC_{50})

The toxicity studies of all synthesized platinum(II) complexes (IC_{50} values) were determined by MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT, Sigma). About 5000–10000 $cells^{-1}$ were seeded in 96-well cell culture plates and treated for 72 h with the diluted solution of the complexes, which were obtained by dissolving the compounds in DMF and diluting with culture medium (DMF final concentration <0.4%). Cisplatin and oxaliplatin were used as positive controls. After that, cells were stained with MTT (5 mg/mL) for 4 h and dissolved with DMSO. The UV absorption intensity was measured with an automatic microplate ELISA reader at 490 nm. The IC_{50} values were calculated by the SPSS software.

4.2.3. Cellular uptake test

SGC7901 and SGC7901/CDDP cells were cultured in 6-well plates until the cells reached about 90% confluence. Cisplatin, oxaliplatin and complex **2a** were added at a concentration of 50 μM, respectively. After incubated for 12 h, cells were collected and washed 3 times with cold PBS. 1 μL suspended cells were taken to measure the cell density. The rest of the cells was spun down and digested at 65 °C in 200 μL 65% HNO_3 for 10 h. The concentrations of platinum were detected by ICP-MS.

4.2.4. Apoptosis assay analysis

The cultured SGC-7901 cells were washed with PBS (phosphate-buffered saline), digested by trypsin solution and the suspension of cells were diluted with medium to a certain concentration of 1×10^5 cells/mL. Cells were plated into 6-well culture plates (2 mL/well) and cultured in 5% CO_2 at 37 °C overnight. A series of tested reagents were added into each well and incubated for 24 h at 37 °C in 5% CO_2, cisplatin and oxaliplatin were used as positive controls. The apoptosis induced by platinum complexes was determined by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (Keygen, China). The detailed operation as follows: cells were collected and washed twice with cold PBS, then stained with 5 μL Annexin V-FITC for 5 min in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Propidium Iodide was added to cells with 5 μL before incubated at room temperature for 15 min. Fluorescence of cells was measured by flow cytometer (FAC Scan, Becton Dickenson, USA). The results were analyzed using Cell Quest software and appeared as percentage of normal and apoptotic cells at various stages. FL1 and FL2 channel measured FITC and PI fluorescence, respectively. The treatment of HepG2 cells was in the similar way as mentioned above.

4.2.5. Cell cycle measurement

SGC7901 cells (1×10^6) were seeded in 6-well plate and incubated at 37 °C in 5% CO_2 overnight. Then, cells were treated with the platinum drugs (50 μM) for 24 h and cells were harvested from adherent cultures by trypsin and washed with PBS. After that, cells were fixed in cold 70% ethanol and stored at 4 °C overnight. Then cells were centrifuged and treated with 100 μg/mL RNase for 30 min at 37 °C, resuspended in 50 μg/mL propidium iodide in PBS for nucleic acids staining for 30 min at 4 °C. The cells (1×10^4) acquired for each sample using the Cell Quest software and recording propidium iodide (PI) in FL2 channel. Cell cycle profiles were performed with ModFit software. The treatment of HepG2 cells was in an analogous way as mentioned above.

4.2.6. Interaction with pET22b plasmid DNA

The interaction of complex **2a** with pET22b plasmid DNA was studied by agarose gel electrophoresis, cisplatin and oxaliplatin were used as positive controls. Solution of pET22b DNA (0.20 μg, 5 μL) was mixed with the tested complex to achieve a set of concentrations ranging from 0 to 640 μM. The mixture was incubated in a water bath at 37 °C for 24 h. 10 μL aliquots of drug-DNA mixtures were loaded on agarose gel (ultra pure grade, Bio-Rad, made up to 1% w/v) which was prepared using TA buffer (45 mM Tris-acetate, pH 7.5) containing ethidium bromide (1 mg/mL) and underwent electrophoresis under TAE buffer (0.05 M Tris base, 0.05 M glacial acetic acid, 1 mM EDTA, pH = 8.0) for 60 min at 100 V. The

DNA bands were photographed under a Molecular Imager (Tanon, China) under UV light.

4.2.7. Western blot analysis

SGC7901 cells were cultured in culture flasks until the cell density reached 80% and incubated with 50 μM platinum complexes for 24 h at 37 °C. Proteins were extracted by lysis buffer and concentration was determined with protein assay (Thermo, Waltham, MA) by BCA (bicinchoninic acid) as well as adjusted to an equal concentration. Protein samples (20 mg/lane) were separated in the polyacrylamide gel which was prepared by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, protein samples were transferred to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Bio-Rad, USA) by transblot apparatus (Bio-Rad, USA). Then, the membrane was socked with 5% nonfat milk for 1 h in TBST buffer and incubated at 4 °C with primary antibodies diluted in PBST (1:2000 β-actin, Santa Cruz, USA; 1:500 for Bax, BD Pharmagin, USA; 1:500 for Bcl-2, Cell Signal, USA) overnight. 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).

4.2.8. Statistical analysis

All the values are expressed as mean ± SD of not least than three different determinations. $P < 0.05$ was defined as statistically significant by using the Student's test.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.02.060.

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