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Synthesis and Cytotoxic Study of a Platinum(IV) Anticancer Prodrug with Selectivity toward Luteinizing Hormone-Releasing Hormone (LHRH) Receptor-Positive Cancer Cells

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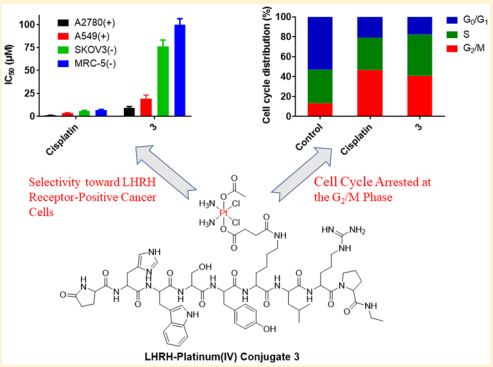
Supporting Information

ABSTRACT: Platinum drugs including cisplatin are widely used in clinics to treat various types of cancer. However, the lack of cancer-cell selectivity is one of the major problems that lead to side effects in normal tissues. Luteinizing hormone-releasing hormone (LHRH) receptors are overexpressed in many types of cancer cells but rarely presented in normal cells, making LHRH receptor a good candidate for cancer targeting. In this study, we report the synthesis and cytotoxic study of a novel platinum(IV) anticancer prodrug functionalized with LHRH peptide. This LHRH−platinum(IV) conjugate is highly soluble in water and quite stable in a PBS buffer. Cytotoxic study reveals that the prodrug selectively targets LHRH receptor-positive cancer cell lines with the cytotoxicities 5−8 times higher than those in LHRH receptor-negative cell lines. In addition, the introduction of LHRH peptide enhances the cellular accumulation in a manner of receptor-mediated endocytosis. Moreover, the LHRH−platinum(IV) prodrug is proved to kill cancer cells by binding to the genomic DNA, inducing apoptosis, and arresting the cell cycle at the G_2/M phase. In summary, we report a novel LHRH−platinum(IV) anticancer prodrug having largely improved selectivity toward LHRH receptor-positive cancer cells, relative to cisplatin.



INTRODUCTION

Since being approved by the U.S. Food and Drug Administration (FDA) for the treatment of cancer in 1978, cisplatin (*cis*-diamminedichloroplatinum(II), cDDP) has been widely used as a chemotherapeutic drug for more than 40 years.[1] The major action mechanism of cisplatin to kill cancer cells is binding at the N_7 positions of two guanines after activation to form intrastrand and interstrand cross-links with genomic DNA and inducing apoptosis.[2−8] However, because of the lack of cancer-cell selectivity, cisplatin has serious side effects, including nephrotoxicity, myelotoxicity, neurotoxicity, vomiting, and nausea.[9] Therefore, great efforts have been devoted to develop cancer-cell-targeted platinum complexes with the aim to decrease the side effects while improving efficacy.

Unlike square-planar cisplatin, which faces the challenge of deactivation by human serum albumin in blood,[10] platinum(IV) compounds with a six-coordinate octahedral geometry are much more inert during the circulation, thus minimizing undesired premature activation prior to the entrance of cancer cells. Certain types of platinum(IV) compounds are regarded as prodrugs because once inside the cancer cells, they will be reduced to form the original active platinum(II) species by the reducing agents such as glutathione (GSH) and ascorbic acid.[11−17] Along with platinum(II), in some cases, the axial ligands or the equatorial ligands will also be released after the reduction.[15,18] Therefore, the axial positions are ideal for installing cancer-targeting moieties such as glucose, estrogen, folate, biotin, and RGD peptide.[19−38] However, the selectivity of these Pt(IV) compounds to cancer cells is not always significant, because of the fact that normal cells also express the receptors, although the level is not as high as that in cancer cells.[21,27,34]

Luteinizing hormone-releasing hormone (LHRH), also known as gonadotropin releasing hormone (GnRH), is a decapeptide hormone released from GnRH neurons in the hypothalamus.[39] It specifically targets LHRH receptors that are overexpressed in many types of tumors, including ovarian, lung, breast, cervical, and endometrial. In contrast, LHRH receptors are not expressed or expressed at a very low level in most visceral organs.[40] Overexpression of LHRH receptors in cancer cells and their rare presence in normal cells make the LHRH receptor a good candidate for cancer targeting.[41−44] Previous works demonstrated that replacing the glycine in LHRH with D-lysine and attaching cytotoxic compounds or

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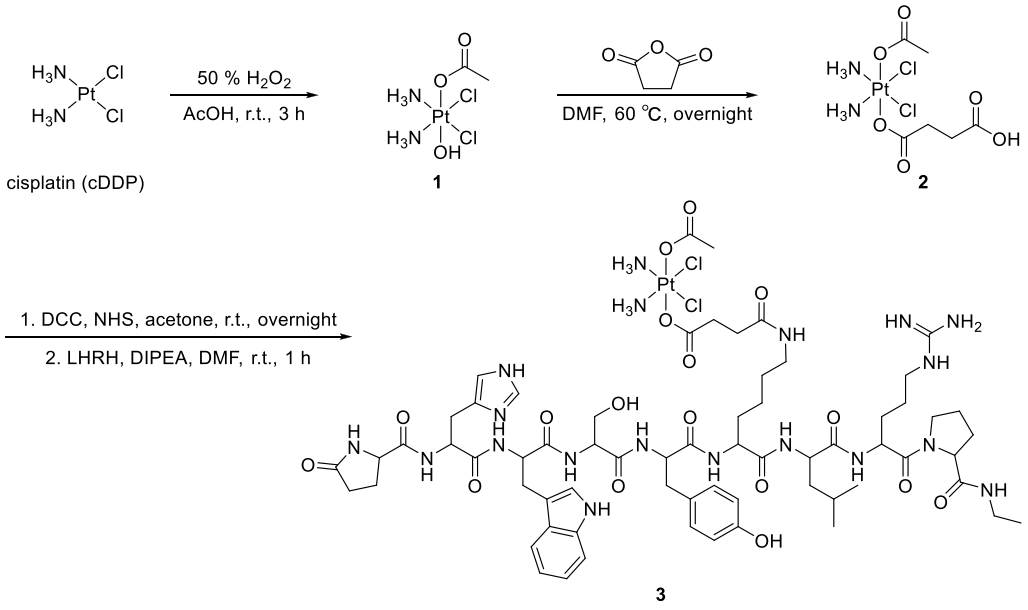
Scheme 1. Synthesis of the LHRH−Platinum(IV) Conjugate[a]



[a]Reagents: H_2O_2, hydrogen peroxide; AcOH, acetic acid; DMF, *N*,*N*′-dimethylformamide; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DIPEA, *N*,*N*′-diisopropylethylamine; LHRH, Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt.

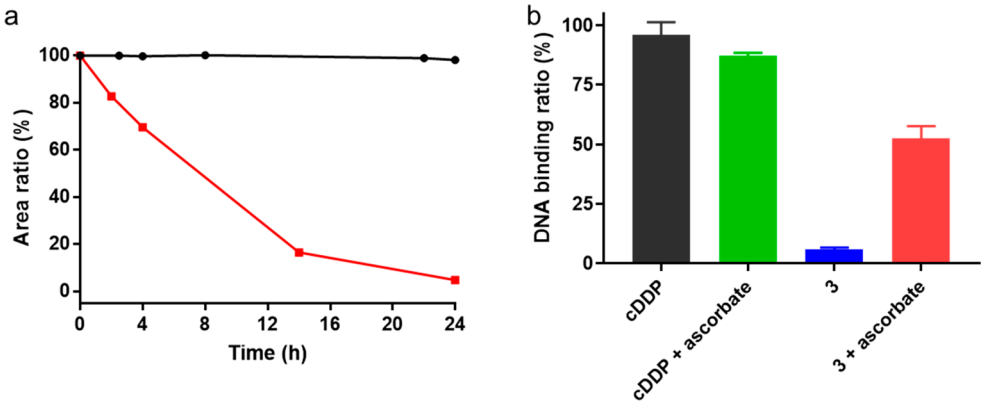


Figure 1. Stability and reduction of the LHRH−platinum(IV) conjugate. (a) HPLC results of 100 μM LHRH−platinum(IV) conjugate in a PBS buffer (pH 7.4) without (black) or with 2 mM sodium ascorbate (red) at 37 °C. (b) The ct-DNA binding ratio of cisplatin and complex **3** (50 μM) in a PBS buffer (pH 7.4) with or without 2 mM sodium ascorbate at 37 °C for 24 h.

nanoparticles to the amine group of lysine do not change the high binding affinity of the peptide to its receptors.[45−47] LHRH−doxorubicin conjugates have shown high binding affinity and much less toxicity in vivo than doxorubicin.[48]

Herein, we report the design, synthesis, characterization, and cytotoxic study of a novel platinum(IV) anticancer prodrug functionalized with LHRH peptide. The water solubility, stability, and reduction property of the conjugate were examined. The selectivity of the conjugate toward LHRH receptor-positive cancer cells and the possible relationship between selectivity and cellular accumulation were illustrated. Furthermore, the action mechanism of this anticancer prodrug was scrutinized through analysis of the Pt binding level in the genomic DNA, apoptosis inducing effect, and cell cycle arresting ability.

RESULTS AND DISCUSSION

Synthesis of the LHRH−platinum(IV) conjugate (complex **3**) started from the oxidation of cisplatin (Scheme 1). Cisplatin was oxidized by H_2O_2 in a large amount of acetic acid, to avoid the side reaction with H_2O in a 50% H_2O_2 solution.[49] The resulting Pt(IV) compound **1** (Figures S1 and S2 in the Supporting Information) further reacted with succinic anhydride in DMF at 60 °C to obtain compound **2** (Figures S3−S6 in the Supporting Information).[50] After activation by *N*-hydroxysuccinimide (NHS) (see Figures S7 and S8 in the Supporting Information), the carboxyl group of compound **2** reacted with D-lysine of the LHRH peptide (Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt) under basic conditions without adding any coupling reagents to yield the final complex **3**. After purification by semipreparative HPLC, the new complex **3** was characterized by NMR spectroscopy (^{1}H, ^{13}C, and ^{195}Pt NMR) and electrospray ionization mass spectrometry (ESI-MS) (see Figures S9−S12 in the Supporting Information). The purity of the conjugate was confirmed to be >98% via analytical high-performance liquid chromatography (HPLC) (see Figure S13 in the Supporting Information).

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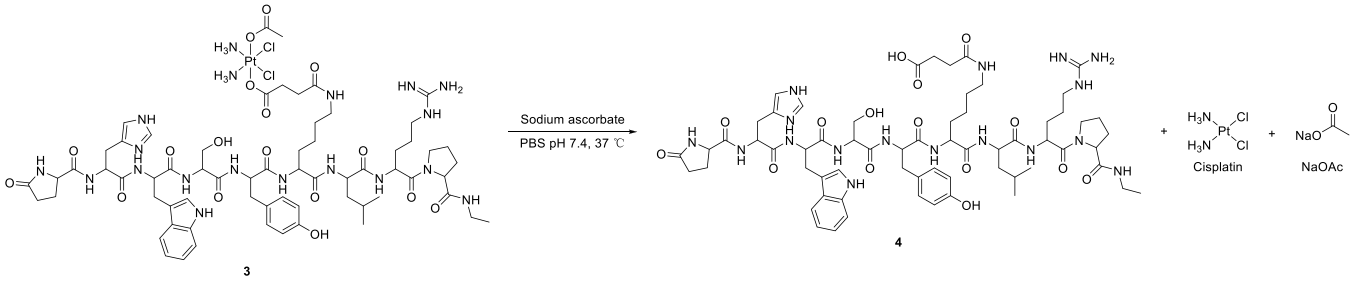
Scheme 2. Reduction of the LHRH−Platinum(IV) Conjugate by Sodium Ascorbate to Form Compound 4, Cisplatin, and Sodium Acetate (NaOAc) in a PBS Buffer (pH 7.4)



From the ^{1}H NMR spectrum of complex **3** (see Figure S9), the peaks between 6.83 and 8.57 ppm are assigned to the 11 protons in the aromatic heterocycles of histidine, tryptophan, and tyrosine, along with the three CH_3 peaks at 1.07, 0.92, and 0.85 ppm demonstrating the existence of LHRH peptide. The typical singlet at 2.10 ppm is assigned to the acetyl group at the axial position of the Pt(IV) complex (Table S1 in the Supporting Information). In the ^{13}C NMR spectrum (see Figure S10), the 14 carbons with chemical shifts between 156 ppm and 183 ppm belong to the two ester carbons, 11 amide carbons, and the −C═N group in arginine. The 17 carbons of the aromatic heterocycles in histidine, tryptophan, and tyrosine appear between 108 ppm and 155 ppm. Because of the electron-withdrawing effect of the OH group, the signal of CH_2 in serine down-shifts to 61 ppm. The other nine carbons between 50 ppm and 60 ppm are the nine tertiary carbons. The remaining 24 carbons in the high field are assigned to the 20 secondary carbons and the four primary carbons (Table S2 in the Supporting Information). Because of the coupling of the two N that linked to Pt, the Pt peak splits into a pentet at ∼1080 ppm in the ^{195}Pt NMR spectrum (see Figure S11).

The solubility of the LHRH−platinum(IV) conjugate in water was determined to be >32.2 mM, which is more than 6.1 times higher than that of cisplatin (5.3 mM), possibly due to the high aqueous solubility of LHRH peptide (see Table S3 in the Supporting Information). The stability of the LHRH−platinum(IV) conjugate in PBS was examined by HPLC. Complex **3** was dissolved in a PBS buffer (pH 7.4) and incubated at 37 °C. A portion of the solution was injected into HPLC for analysis at different time points. As shown in Figure 1a, complex **3** is stable in PBS within 24 h; more than 98% of the complex remains unchanged in the solution after 24 h (see Figure S14 in the Supporting Information). Supposedly, the LHRH−platinum(IV) prodrug will be reduced to cisplatin to kill cancer cells. Therefore, we tested its reduction property in the presence of sodium ascorbate by HPLC. Complex **3** was dissolved in a PBS buffer (pH 7.4) containing 2 mM sodium ascorbate and incubated at 37 °C. After 24 h, almost all of the complex was reduced (see Figure 1a, as well as Figure S15 in the Supporting Information). A new peak appeared at 16.5 min, representing the reduced product **4**, which is confirmed by ESI-MS (see Scheme 2, as well as Figure S16 in the Supporting Information). The peak of the formed cisplatin is difficult to observe in the HPLC chromatogram, because of its low absorbance at 254 nm. Therefore, we monitored the reduction of complex **3** using NMR spectroscopy. A quantity of 1 mM of complex **3** was incubated in a PBS/D_2O buffer (pH 7.4) containing 10 mM ascorbate at 37 °C. The ^{1}H NMR and DOSY data prove the formation of sodium acetate (Figures S17 and S18 in the Supporting Information). From the ^{195}Pt NMR spectra, we can clearly identify a new group of peaks formed at ∼2149.9 ppm, which are the same as cisplatin and also literature reports,[51] while the peaks of complex **3** at ∼1080 ppm disappeared after 18 h (Figures S19 and S20 in the Supporting Information). After the NMR tests, this sample was also examined by ESI-MS and HPLC. From the ESI-MS and HPLC spectra, we can also identify the peaks of cisplatin (see Figures S21 and S22 in the Supporting Information). These data confirm that the reduced Pt species is cisplatin. Moreover, the reduced product compound **4** (Scheme S1 in the Supporting Information) did not react with cisplatin (see Figures S23 and S24 in the Supporting Information). All together, the LHRH−platinum(IV) prodrug is reduced to cisplatin, compound **4**, and sodium acetate in the presence of ascorbate (see Scheme 2).

The reduction property of the LHRH−platinum(IV) conjugate was further tested by a calf thymus (ct)-DNA binding assay. In the presence of sodium ascorbate, complex **3** will be reduced and then release the platinum(II) cisplatin that will bind to ct-DNA. By testing the amount of platinum in ct-DNA, we can monitor the reduction level of the platinum(IV) complex. Thus, complex **3** was incubated with ct-DNA in a PBS buffer (pH 7.4) with or without sodium ascorbate at 37 °C for 24 h. Cisplatin was used as a positive control. As shown in Figure 1b, cisplatin binds very efficiently in the ct-DNA, with or without the presence of sodium ascorbate. Without ascorbate, complex **3** is not able to bind to ct-DNA, and the binding ratio is as low as 6% (see Table S4 in the Supporting Information). However, in the presence of sodium ascorbate, 52% of Pt binds to ct-DNA, which is much higher than the ratio in the group without ascorbate. The reduction rate from the ct-DNA binding assay is lower than that from HPLC (95%). One possible reason is that the LHRH−platinum(IV) conjugate has been reduced to cisplatin, but some of cisplatin did not bind to ct-DNA yet. After precipitating ct-DNA, the contents in the supernatants were detected using HPLC and inductively coupled plasma−mass spectrometry (ICP-OES). Most of complex **3** was found to be reduced to compound **4** by ascorbate from the HPLC chromatogram (see Figure S25 in the Supporting Information). Besides, the platinum content in the supernatant, together with that in the ct-DNA, is close to 100% in each group (Table S4). These results demonstrate that the LHRH−platinum(IV) conjugate can be reduced to compound **4** and cisplatin efficiently by the reducing agent.

The cytotoxicity of LHRH−platinum(IV) conjugate was examined by a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Two pairs of cell lines were utilized: A2780 (human ovarian carcinoma) and A549 (human lung adenocarcinoma) are LHRH receptor-positive cell lines, and SKOV3 (human ovarian carcinoma) and MRC-5 (human

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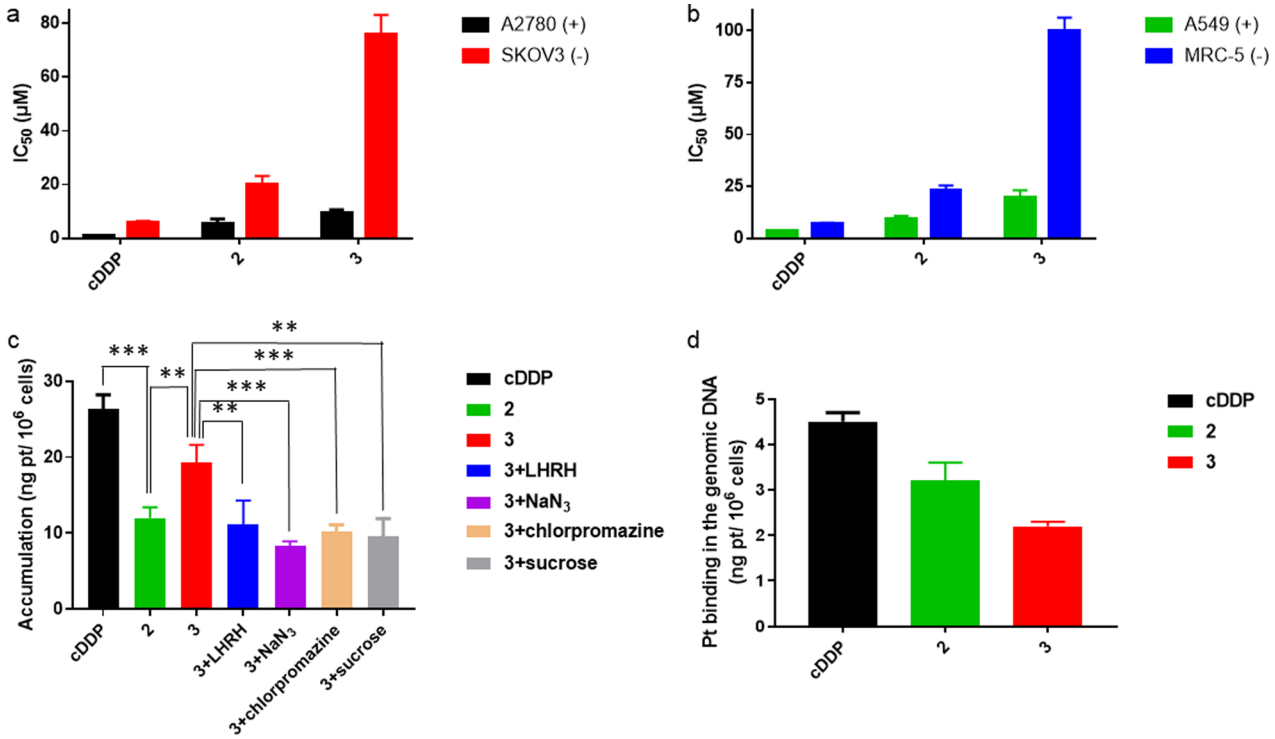

Figure 2. Cytotoxicity, cellular accumulation, and Pt binding level in the genomic DNA of the LHRH−platinum(IV) conjugate. (a) IC_{50} of cisplatin, compound **2**, and complex **3** in LHRH receptor-positive cell line A2780 and LHRH receptor-negative cell line SKOV3. (b) IC_{50} of cisplatin, compound **2**, and complex **3** in LHRH receptor-positive cell line A549 and LHRH receptor-negative cell line MRC-5. (c) Cellular accumulation of 10 μM cisplatin, compound **2**, and complex **3** in A2780 cells after 8 h. For the **3** + LHRH group, cells were pretreated with 1 mM LHRH for 2 h; for the **3** + NaN_3 group, cells were pretreated with 10 mM NaN_3 for 2 h; for the **3** + chlorpromazine group, cells were pretreated with 50 μM chlorpromazine for 0.5 h; for the **3** + sucrose group, cells were pretreated with 500 mM sucrose for 0.5 h before complex **3** was added. [Legend: (**) $P < 0.01$, (***) $P < 0.001$.] (d) Pt binding level in the genomic DNA of 10 μM cisplatin, compound **2**, and complex **3** in A2780 after 8 h.

lung normal) are the corresponding LHRH receptor-negative cell lines.[40,43,52] Cells were treated with cisplatin, compound **2**, complex **3**, and LHRH peptide at different concentrations for 24 h, because most of the complex **3** accumulated in the cells at this time point (see Figure S26 in the Supporting Information). Cells were then cultured in fresh medium for another 48 h. As shown in Figures 2a and 2b, compared with that of cisplatin and compound **2**, the cytotoxicity of complex **3** is comparable in the LHRH receptor-positive cancer cells, but the cytotoxicity decreases a lot in LHRH receptor-negative cell lines. For example, the half-maximal inhibitory concentration (IC_{50}) of complex **3** in SKOV3 cells is 76.1 μM, while that value for cisplatin is as low as 5.7 μM. The selectivity index (SI), which is defined as

$$\mathrm{SI} = \frac{IC_{50} \text{ in the LHRH receptor-negative cell line}}{IC_{50} \text{ in the LHRH receptor-positive cell line}}$$

increases from 5.7 for cisplatin to 8.2 for complex **3** in SKOV3/A2780 cells. The IC_{50} values of cisplatin in A549 and MRC-5 cells are 3.6 and 6.9 μM, respectively, while the values for complex **3** are 19.4 and 100 μM, respectively. The SI increases from 1.9 for cisplatin to 5.2 for complex **3** in this pair of cells. The IC_{50} values of LHRH peptide are >50 μM in these cell lines, indicating that the cytotoxicity of complex **3** is not from the LHRH moiety (see Table S5 in the Supporting Information). Collectively, the introduction of the LHRH peptide improves the selectivity of Pt toward LHRH receptor-positive cells.

Mechanistic investigations were performed to reveal the possible reasons why complex **3** has increased selectivity. We first tested the entire cell accumulation of cisplatin, compound **2**, and complex **3** in A2780 cells. Cells were treated with 10 μM of compounds for 8 h before washing by PBS and measuring the concentration of Pt by an inductively coupled plasma-mass spectrometry (ICP-MS). The level of Pt in the cells treated with cisplatin is 26.3 ng Pt/10^6 cells (Figure 2c). The accumulation level of compound **2** is much lower than that of cisplatin, which is likely due to the negative charge from the carboxyl group of compound **2**, preventing it from diffusing passively through the cell membrane. Compared with compound **2**, the introduction of LHRH peptide increases the accumulation of complex **3** to 19.3 ng Pt/10^6 cells (see Figure 2c, as well as Table S6 in the Supporting Information). Pretreatment with LHRH peptide to block the LHRH receptors competitively decreases the accumulation of complex **3** to 11.3 ng Pt/10^6 cells. Besides, pretreatment with energy inhibitor NaN_3 that blocks endocytosis also dramatically decreases the accumulation level of complex **3** to 8.4 ng Pt/10^6 cells. The accumulation of complex **3** is also inhibited by chlorpromazine (an inhibitor of clathrin-mediated endocytosis), hypertonic sucrose (an inhibitor of clathrin-mediated endocytosis), and wortmannin (an inhibitor of clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis), rather than methyl-β-cyclodextrin (an inhibitor of lipid raft) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (a micropinocytosis inhibitor),[53,54] indicating that the accumulation process of complex **3** is most likely through

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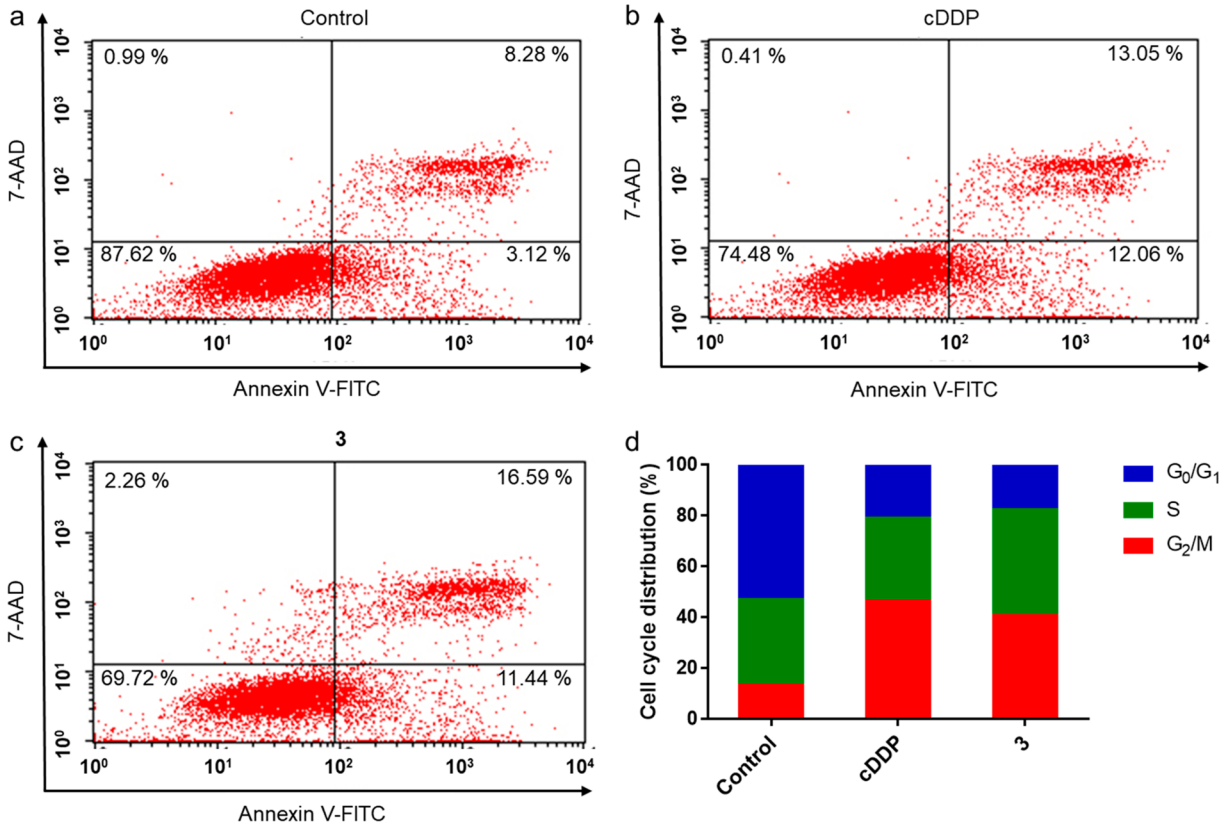

Figure 3. Apoptosis-inducing and cell-cycle-arresting effects of the LHRH−platinum(IV) conjugate. (a−c) Apoptotic level induced by cisplatin and complex **3** in A2780 cells. Cells were treated at IC_{70} (2.5 μM for cisplatin; 20 μM for complex **3**) for 48 h. Cells with medium only were used as the control group. (d) Cell cycle distribution of A2780 cells treated with cisplatin and complex **3** at IC_{70} (2.5 μM for cisplatin, 20 μM for complex **3**) for 24 h. Cells with medium only were used as the control group.

clathrin-mediated endocytosis (see Figure 2c, as well as Table S6). In addition, the accumulation of complex **3** in the LHRH receptor-negative cell line SKOV3 is lower than that in the LHRH receptor-positive cell line A2780 (see Table S6). These results indicate that the uptake of the LHRH−platinum(IV) conjugate is mainly in a manner of LHRH receptor-mediated endocytosis. The lower accumulation of complex **3**, relative to cisplatin, partially leads to the lower cytotoxicity of the platinum(IV) conjugate.

The binding level of Pt in the genomic DNA was also examined in A2780, A549, SKOV3, and MRC-5 cells. Cells were treated with 10 μM cisplatin, compound **2**, and complex **3** for 8 h before washing by PBS. The Pt level in the genomic DNA was determined via ICP-MS. Complex **3** binds to the genomic DNA in the cells at a level of 2.2 ng Pt/10^6 A2780 cells, which is slightly lower than that for cisplatin and compound **2** (see Figure 2d, as well as Table S7 in the Supporting Information). The cellular accumulation of complex **3** is higher than that of compound **2**, but the Pt level in the genomic DNA is lower at 8 h, indicating that complex **3** may be slower to be reduced to cisplatin than compound **2**, leading to a lower level in the genomic DNA at this time point. This hypothesis is further corroborated by comparing the reduction rate of compound **2** and complex **3**, using HPLC (see Figures S27 and S28 in the Supporting Information). The Pt levels in the genomic DNA of LHRH receptor-positive cancer cells (A2780 and A549) treated with complex **3** are higher than those in the LHRH receptor-negative cancer cells (SKOV3 and MRC-5; see Figure S29 and Table S7 in the Supporting Information), proving that this LHRH−platinum(IV) selectively targets LHRH receptor-positive cancer cell lines.

We next tested the ability of the LHRH−platinum(IV) conjugate to induce apoptosis. A2780 cells were treated with cisplatin and complex **3** at IC_{70} (2.5 μM for cisplatin and 20 μM for complex **3**). Then, all the cells were collected, and a double-staining assay using Annexin V-FITC conjugate and 7-aminoactinomycin D (7-AAD) was performed. The apoptotic level was determined by a flow cytometer. The total apoptosis induced by complex **3** is 28% in A2780 cells, which is comparable to the cisplatin-treated group (Figure 3). Last, but not least, the effect of the LHRH−platinum(IV) conjugate on the cell cycle distribution was tested. A2780 cells were treated with cisplatin and complex **3** at IC_{70}. Cells were fixed in ethanol overnight and stained with propidium iodide (PI), then the cell cycle distribution of the samples was analyzed by a flow cytometer. In the control group, 13.4% of cells were at the G_2/M phase, whereas, in the group treated with cisplatin, the percentage of cells arrested at the G_2/M phase increased to 46.6% (see Figure 3d, as well as Figure S30 in the Supporting Information). Similar to cisplatin, complex **3** also arrested cells at the G_2/M phase, with a percentage of 40.9%. These results show that the LHRH−platinum(IV) conjugate induces apoptosis and arrests the cell cycle at the G_2/M phase to kill cancer cells and the reduced product cisplatin is the main protagonist for these effects.

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CONCLUSION

In summary, we report the first example of a LHRH−platinum(IV) conjugate for the purpose of targeting platinum drug to cancer cells that overexpress LHRH receptors. The cancer-targeting peptide LHRH is attached to compound **2** through an amide bond. The resulting LHRH−platinum(IV) conjugate **3** is quite stable and can be reduced in the presence of reducing agent to release the LHRH moiety and cisplatin that binds to DNA. This conjugate with high aqueous solubility possesses a comparable cytotoxicity but a much higher selectivity toward LHRH receptor-positive cancer cells, compared with cisplatin. The selectivity of the conjugate is, at least in part, due to the selective cellular accumulation via LHRH receptor-mediated endocytosis pathway and higher levels of Pt in the genomic DNA of cancer cells that overexpress LHRH receptors. Furthermore, the action mechanism of the conjugate is proved to be similar to cisplatin, i.e., inducing apoptosis and arresting the cell cycle at the G_2/M phase. These results indicate the possibility of selectively transporting platinum anticancer drugs into LHRH-receptor positive cancer cells while leaving the normal cells unaffected.

EXPERIMENTAL SECTION

Materials and Instruments. All the reagents were used as received without further purification. Cisplatin was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. LHRH peptide (Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt) was purchased form GL Biochem (Shanghai), Ltd. All the reactions were performed in darkness. ^{1}H, ^{13}C, and ^{195}Pt NMR spectra were obtained on a Bruker AVANCE III 400 MHz NMR spectrometer or a Bruker Ascend AVANCE III HD 600 MHz spectrometer with a BBO probe. ESI-MS tests were performed on an Agilent API-3200 Q-Trap mass spectrometry. High-performance liquid chromatography (HPLC) experiments were conducted on a Shimadzu Prominence HPLC system. For analysis, a Phenomenex column (Gemini, 5 μm, C18, 110 Å, 250 mm × 4.6 mm) was used. For semipreparation, a Waters column (Xbridge BEH C18 OBD Prep Column, 130 Å, 5 μm, 250 mm × 10 mm) was utilized. Pt contents were measured by an inductively coupled plasma−optical emission spectrometry (ICP-OES) system (Optima 8000 spectrometer) or an inductively coupled plasma mass spectrometry (ICP-MS) system (PE Nexion 2000). DNA concentrations were determined by a NanoDrop Spectrophotometer (ND-1000). Apoptosis and cell cycle analyses were conducted on a flow cytometer (BD Bioscience FACS Calibur).

Synthesis of Compound 1. Compound **1** was synthesized following the procedure reported previously.[49] Cisplatin (1 g, 3.3 mmol, 1 equiv) was suspended in 400 mL of acetic acid before hydrogen peroxide (50% in H_2O, 10 mL, 176.4 mmol, 53 equiv) was added. The mixture was stirred at room temperature for 3 h, and the solution became clear. The reaction mixture was centrifuged to remove unreacted cisplatin. The supernatant was concentrated under reduced pressure. Excess ethyl ether was added to the residue to precipitate a yellow solid. The formed solid was collected by centrifugation and washed by acetone, dichloromethane (DCM), and ethyl ether, respectively, to obtain 980 mg of yellow solid **1**. Yield: 78%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.21−5.72 (m, 6H, $2NH_3$), 1.87 (s, 3H, CH_3). ESI-MS: m/z $[M-H]^-$ calculated for $C_2H_9Cl_2N_2O_3Pt$: 375.1, found 375.3.

Synthesis of Compound 2. Compound **2** was synthesized following the procedure reported in the literature with modification.[50] Compound **1** (300 mg, 0.8 mmol, 1 equiv) was suspended in 2 mL of DMF. Succinic anhydride (480 mg, 4.8 mmol, 6 equiv) was then added. The mixture was stirred at 60 °C overnight. Two milliliters (2 mL) of DCM and 90 mL of ethyl ether were added to the reaction mixture to form a light yellow solid. The solid was collected by centrifugation and washed by 2 mL of DCM and 10 mL of ethyl ether three times to obtain 260 mg of light yellow solid **2**. Yield: 68%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.05 (s, 1H, COOH), 6.51 (s, 6H, $2NH_3$), 2.48−2.29 (m, 4H, $2CH_2$), 1.90 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 179.6 (C_2), 178.2 (C_3), 173.8 (C_6), 30.5 (C_4), 29.9 (C_5), 22.8 (C_1). ESI-MS: m/z $[M-H]^-$ calculated for $C_6H_{13}Cl_2N_2O_6Pt$: 475.2, found 475.2. These spectra data are same as that reported in the literature.[50]

Synthesis of Compound 3. Compound **2** (100 mg, 0.21 mmol, 1 equiv) was dissolved in 6 mL of acetone. *N*-hydroxysuccinimide (NHS, 37 mg, 0.31 mmol, 1.5 equiv) and dicyclohexylcarbodiimide (DCC, 52 mg, 0.25 mmol, 1.2 equiv) were added. The mixture was stirred at room temperature overnight. The formed white solid was removed by filtration. The filtrate was concentrated under reduced pressure. One milliliter (1 mL) of DCM and 10 mL of ethyl ether were added to the residue to form a light yellow solid. The solid was collected by centrifugation and washed by DCM and ethyl ether three times to obtain 102 mg of pale yellow solid. Yield: 85%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.51 (s, 6H, $2NH_3$), 2.85−2.78 (m, 6H, $3CH_2$), 2.63 (t, J = 6.8 Hz, 2H, CH_2), 1.91 (s, 3H, CH_3). ESI-MS: m/z $[M-H]^-$ calculated for $C_{10}H_{16}Cl_2N_3O_8Pt$: 572.2, found 572.4. LHRH (30 mg, 0.024 mmol, 1.1 equiv) was dissolved in 1 mL of DMF before *N*,*N*′-diisopropylethylamine (DIPEA, 19 μL, 0.11 mmol, 5 equiv) was added. After 5 min, the NHS ester of compound **2** (13 mg, 0.022 mmol, 1 equiv) synthesized above was added. The solution was stirred at room temperature for 2 h. Two milliliters (2 mL) of DCM and 10 mL of ethyl ether were added to the reaction mixture to form a white solid. The solid was collected by centrifugation and washed by DCM and ethyl ether three times. The crude product was dissolved in Milli-Q water at a concentration of 10 mg/mL and purified via semipreparative HPLC. A quantity of 100 μL of sample was injected into the HPLC system each time. Mobile phase A was H_2O with 0.1% TFA, and mobile phase B was acetonitrile (ACN) with 0.1% TFA. The absorbance wavelengths were set at 254 and 360 nm. The flow rate was 2 mL/min. The HPLC program was set as follows: 10%−40% phase B (linear increased from 0 to 30 min). The fraction with pure product was lyophilized to obtain 10 mg of white solid **3**. Yield: 27%. ^{1}H NMR (600 MHz, D_2O) δ: 8.57 (d, J = 1.2 Hz, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.25 (t, J = 7.2 Hz, 1H), 7.21 (s, H), 7.19 (s, H), 7.13 (t, J = 7.2 Hz, 1H), 7.10 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 4.72 (d, J = 7.8 Hz, 1H), 4.66 (dd, J = 8.4, 6.6 Hz, 1H), 4.48 (dd, J = 8.4, 5.4 Hz, 1H), 4.44−4.40 (m, 1H), 4.38−4.29 (m, 2H), 4.24−4.16 (m, 2H), 4.13 (dd, J = 9.0, 4.8 Hz, 1H), 3.78−3.68 (m, 2H), 3.68−3.59 (m, 1H), 3.51 (dd, J = 16.8, 6.8 Hz, 1H), 3.27−3.02 (m, 10H), 2.98 (dd, J = 13.8, 7.2 Hz, 1H), 2.91 (dd, J = 13.6, 8.4 Hz, 1H), 2.67 (t, J = 6.6 Hz, 2H), 2.48 (t, J = 6.6 Hz, 2H), 2.40−2.23 (m, 3H), 2.21−2.14 (m, 1H), 2.10 (s, 3H), 1.99 (dt, J = 12.0, 4.6 Hz, 1H), 1.92−1.80 (m, 2H), 1.78 (dd, J = 14.4, 6.0 Hz, 1H), 1.69−1.60 (m, 4H), 1.59−1.48 (m, 5H), 1.45−1.36 (m, 2H), 1.07 (t, J = 7.2 Hz, 3H), 1.04−0.94 (m, 2H), 0.92 (d, J = 6.0 Hz, 3H), 0.85 (d, J = 6.0 Hz, 3H). ^{13}C NMR (151 MHz, D_2O) δ 182.1, 181.7, 181.0, 174.9, 174.6, 174.2, 173.9, 173.7, 173.6, 173.4, 172.9, 171.2, 171.1, 170.8, 156.6, 154.7, 136.2, 133.6, 130.6, 128.3, 127.5, 127.0, 124.5, 122.0, 119.4, 118.2, 117.3, 117.2, 115.6, 115.4, 111.9, 108.7, 61.1, 60.8, 56.5, 55.7, 55.5, 54.4, 53.8, 52.3, 52.2, 51.0, 47.9, 40.6, 39.6, 39.1, 34.4, 31.4, 31.1, 30.6, 29.4, 29.2, 27.8, 27.6, 26.2, 25.1, 24.6, 24.3, 24.0, 22.3, 22.2, 21.7, 20.6, 13.5. ^{195}Pt NMR (129 MHz, D_2O) δ: 1084.8−1075.6 (m). ESI-MS: m/z $[M+H]^+$ calculated for $C_{65}H_{98}Cl_2N_{19}O_{17}Pt$: 1683.6, found 1683.4.

Synthesis of Compound 4. LHRH (5 mg, 0.004 mmol, 1 equiv) was dissolved in 0.1 mL DMF before *N*,*N*′-diisopropylethylamine (DIPEA, 2.8 μL, 0.016 mmol, 4 equiv) was added. After 5 min, succinic anhydride (0.8 mg, 0.008 mmol, 2 equiv) was added. The solution was stirred at room temperature for 2 h. Ethyl ether (2 mL) was added to the reaction mixture to form a white solid. The solid was collected by centrifugation and washed by DCM and ethyl ether three times. The crude product was dissolved in Milli-Q water and purified by semipreparative HPLC. The fraction with pure product was lyophilized to get 3 mg of white solid **4**. Yield: 57%. ESI-MS: m/z $[M+H]^+$ calculated for $C_{63}H_{90}N_{17}O_{15}$: 1325.5, found 1325.3.

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Solubility in Water. Cisplatin (5 mg), LHRH peptide, and complex **3** were added into 100 μL of Milli-Q water. LHRH peptide and complex **3** was dissolved immediately. The mixtures were subjected to ultrasonic treatment for 10 min and then stored at room temperature overnight. The mixtures were centrifuged at 14 000 rpm for 5 min. The Pt concentrations of the supernatants were determined by ICP-OES after digestion by HNO_3 and dilution by Milli-Q water.

HPLC Analyses: Stability and Reduction. The powders of compound **2** and complex **3** were dissolved in Milli-Q water to prepare the stock solutions at a concentration of 5 mM. For the stability test, 100 μM complex **3** was dissolved in 500 μL of PBS buffer (pH 7.4) and incubated at 37 °C in darkness. For the reduction test, 2 mM sodium ascorbate was added into the PBS buffer (pH 7.4) of compound **2** and complex **3** (100 μM). A quantity of 20 μL of sample was injected into HPLC for analysis at different time points. Phase A is H_2O with 0.1% TFA, and phase B is acetonitrile (ACN) with 0.1% TFA. The absorbance wavelengths were set at 254 and 220 nm. The flow rate was 1 mL/min. HPLC programs were set as follows: isocratic 5% phase B in 10 min for compound **2** and isocratic 20% phase B in 30 min for complex **3**. For the stability test, the area ratio of complex **3** at 0 h was set as 100%. For the reduction of compound **2**, after rejecting the peak of reducing agent (sodium ascorbate), the area ratio of compound **2** at 0 h was set as 100%. For the reduction of complex **3**, as the peak of reducing product was too close to the peak of complex **3**, the area ratio of the left half of the peak of complex **3** at 0 h was set as 100%.

Reduction of Complex 3 Analyzed by NMR Spectroscopy. The powder of complex **3** was dissolved in 500 μL PBS/D_2O buffer (pH 7.4) at a concentration of 1 mM. Sodium ascorbate (10 mM) was added into the mixture. The resulting solution was incubated at 37 °C in darkness. The reduction of complex **3** was monitored by ^{1}H NMR and diffusion NMR. After 18 h, ^{195}Pt NMR spectra were recorded by a 600 MHz NMR spectroscopy. The powder of cisplatin was dissolved in the same solution as the standard for ^{195}Pt NMR tests. After NMR tests, these samples were further analyzed by an ESI-MS and a HPLC. Phase A is H_2O with 0.1% TFA, and phase B is acetonitrile (ACN) with 0.1% TFA. The absorbance wavelengths were set at 254 and 211 nm. The flow rate was 1 mL/min. HPLC programs were set as follows: isocratic 0% phase B in 7 min; gradient increased to 20% phase B in 1 min and isocratic 20% phase B until 30 min.

Compound 4 Incubated with Cisplatin. 0.1 mM compound **4** was incubated with 0.1 mM cisplatin in a phosphate buffer (12.3 mM NaH_2PO_4, 37.7 mM Na_2HPO_4, pH 7.4) at 37 °C for 48 h. The reactivity of compound **4** with cisplatin was monitored by HPLC and ESI-MS.

Ct-DNA Binding Assay. 0.075 mg DNA sodium salt from calf thymus (ct-DNA, Sigma D1501) was incubated with 50 μM complex **3** or cisplatin in 150 μL PB buffer (1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.15 mM $NaClO_4$, pH 7.4) containing 2 mM sodium ascorbate at 37 °C. Mixtures without ascorbate were set as negative controls. After 24 h, DNA was precipitated by adding 15 μL sodium acetate solution (3 M, pH 5.2) and 375 μL cold ethanol solution. The mixtures were stored at −80 °C for 2 h. The white solids were collected by centrifugation (14 000 rpm, 4 °C, 20 min). The contents in the supernatants were analyzed by HPLC and ICP-OES. The pellets were then washed by 500 μL 70% ethanol solution twice. The solids then were redissolved in a Tris-HCl buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA and Pt concentrations were determined by a Nanodrop spectrophotometer and ICP-OES, respectively.

Cell Lines and Cell Culture Conditions. A2780 (human ovarian carcinoma) cells were cultured in RPMI 1640 with 12% FBS, 1% L-glutamine, and 100 units of penicillin and streptomycin. SKOV3 (human ovarian carcinoma) and A549 (human lung adenocarcinoma) cells were cultured in DMEM containing 12% FBS and 100 units of penicillin and streptomycin. MRC-5 (human lung normal) cells were cultured in MEM supplemented with 12% FBS, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 100 units of penicillin and streptomycin. All cells were incubated at 37 °C in 5% CO_2.

Cytotoxicity and Selectivity. Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. LHRH receptor-positive cells A2780 and A549 and LHRH receptor-negative cells SKOV3 and MRC-5 were seeded in 96-well plates at a density of 2000 or 3000 cells per well. After 24 h, compound **2**, complex **3**, LHRH peptide, and cisplatin at different concentrations were added. Cells treated with media only were used as the controls. Cells were cultured at 37 °C for 24 h, the media with compounds were replaced by fresh media, and cells were then cultured for another 48 h. Media were removed and 200 μL of MTT (1 mg/mL in serum-free media) solution was added. Cells were cultured for another 4 h in darkness. MTT solution was removed carefully. Then, 200 μL of DMSO was added to dissolve the crystals. A microplate reader (Biotek Powerwave XS) was used to read the absorbances at 570 and 630 nm.

absorbance, A:

$$A = A_{570} - A_{630}$$

viability, V:

$$V = \frac{\text{mean of } A_{\text{compound}} - \text{mean of } A_{\text{blank}}}{\text{mean of } A_{\text{control}} - \text{mean of } A_{\text{blank}}}$$

IC_{50} values were calculated from the dose−response curves by the Graphpad Prism 7 software.

Whole Cell Accumulation. A2780 and SKOV3 cells (2×10^5 per well) were seeded in six-well plates and incubated at 37 °C overnight. The cells were then treated with 10 μM compound **2**, complex **3**, and cisplatin for 8 h. For the pretreatment groups, cells were pretreated with 1 mM LHRH, 10 mM NaN_3 for 2 h, or 50 μM chlorpromazine, 500 mM sucrose, 10 mM methyl-β-cyclodextrin, 50 μM 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), or 50 μM wortmannin for 30 min, the media were replaced by fresh media containing 10 μM complex **3**; cells then were cultured for another 8 h. Afterward, cells were washed with ice-cold PBS three times and harvested by trypsinization. The suspensions were spun at 500 g for 5 min, and the cell pellets were washed by PBS three times. After counting the cell numbers, cells were digested by 65% nitric acid at 70 °C overnight. The lysates were diluted by Milli-Q water to a final volume of 1 mL, and the Pt concentrations were measured via ICP-MS.

Binding of Pt in Genomic DNA. A2780, A549, SKOV3, and MRC-5 cells (2×10^5 per well) were seeded in six-well plates and incubated at 37 °C overnight. The cells were then treated with 10 μM compound **2**, complex **3**, and cisplatin for 8 h. The media were removed and the cells were washed by PBS three times. The cells then were harvested by trypsinization and centrifugation (500 g, 5 min) followed by washing with ice-cold PBS three times. The cell numbers were counted. The genomic DNA was extracted and purified by the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). First, cells were resuspended in 200 μL of PBS. 200 μL of Lysis solution, and 20 μL of Proteinase K solution (20 mg/mL) were added, and the suspensions were vortexed and then incubated at 56 °C for 10 min. Afterward, 20 μL of RNase A Solution was added, and the mixtures were vortexed and incubated at room temperature for 10 min. Next, 400 μL of 50% ethanol was added, and the mixtures were vortexed and transferred to GeneJET Genomic DNA Purification Columns inserted in collection tubes and centrifuged at 6000 g for 1 min. The columns were put into new collection tubes and then washed by 500 μL of Wash Buffer I (centrifugation at 8000*g* for 1 min) and Wash Buffer II (centrifugation at 14 000*g* for 3 min) respectively. Finally, 200 μL Elution Buffer was added to the center of the columns. After 2 min, the elution buffers were collected by centrifugation at 8000*g* for 1 min. Then, the DNA contents in the elution buffers were determined using a NanoDrop spectrophotometer. After digestion in 40% HNO_3 at 70 °C overnight, Pt concentrations of the elution buffers were tested by an ICP-MS.

Apoptosis Assay. A2780 cells (2×10^5 per well) were seeded in six-well plates and incubated at 37 °C overnight. The cells were then treated with complex **3** and cisplatin at IC_{70} (20 μM for complex **3**;

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2.5 μM for cisplatin) for 48 h. The media were collected and the cells were washed by PBS three times. The cells were collected by trypsinization, centrifugation (700*g*, 3 min), and then washing with cold PBS once and annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) twice gently. Cells were resuspended in 1 mL of binding buffer and 100 μL of suspension was extracted, respectively. A total of 5 μL of Annexin V-FITC conjugate (Thermo Fisher Scientific) and 1 μL of 7-aminoactinomycin D (7-AAD, 1 mg/mL in DMSO, Thermo Fisher Scientific) solutions were added to stain the cells for 15 min at room temperature. Afterward, 400 μL of binding buffer was added. The samples were analyzed by a flow cytometer (BD Bioscience FACS Calibur) with excitation at 488 nm.

Cell Cycle Arrest. A2780 cells (2×10^5 per well) were seeded in six-well plates and incubated at 37 °C overnight. The cells were then treated with complex **3** and cisplatin at IC_{70} (20 μM for complex **3**; 2.5 μM for cisplatin) for 24 h. The media were removed and the cells were washed by PBS three times. The cells then were harvested by trypsinization and centrifugation (1500 rpm, 5 min) followed by washing twice with ice-cold PBS. Cells were resuspended in 0.5 mL of PBS (pH 7.4) and fixed in 5 mL of 70% ethanol at 4 °C overnight. The cells then were spun down (3000 rpm, 5 min), washed twice by PBS, and stained with 1 mL propidium iodide (PI) solution (20 μg/mL PI, 0.1% Triton X-100 and 200 μg/mL RNase A, pH 7.4) for 15 min at 37 °C. Finally, samples were analyzed by a flow cytometer (BD Bioscience FACS Calibur) with excitation at 488 nm. The data were analyzed by Modifit 1.2 software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.9b01583.

NMR and ESI-MS spectra, purity, stability and reduction, solubility, ct-DNA binding ratio, cytotoxicity, cellular accumulation, Pt binding level in the genomic DNA and cell cycle distribution (PDF)

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The manuscript was written through contributions of H.Y. and G.Z. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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