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Development of a series of 4-hydroxycoumarin platinum(IV) hybrids as antitumor agents: Synthesis, biological evaluation and action mechanism investigation

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ABSTRACT

A series of new 4-hydroxycoumarin platinum(IV) complexes were designed, synthesized and evaluated as antitumor agents. All the title compounds display moderate to effective antitumor activities toward the tested cell lines and two prominent compounds were screened out with activities comparable to cisplatin and oxaliplatin. The mechanism investigation demonstrates that the platinum(IV) compounds could be reduced to bivalence and exert significant genotoxicity to tumor cells. Meanwhile the coumarin moiety endows the title compounds with cyclooxygenase inhibitory competence which might favour the reduction of tumor-related inflammation and further influence tumor proliferation. The coumarin platinum(IV) complex could effectively induce apoptosis of SKOV-3 cells through up-regulating the expression of caspase3 and caspase9. Furthermore, the conversion of platinum(II) drugs to platinum(IV) form *via* the conjunction with 4-hydroxycoumarin enhances the drug uptake in whole cells and DNA simultaneously. Moreover, the 4-hydroxycoumarin platinum(IV) complex could combine with human serum albumin *via* van der Waals force and hydrogen bond, which would influence their transport and bioactivities *in vivo*.

1. Introduction

Platinum drugs are extensively applied in chemotherapeutic treatment of a wide spectrum of human malignancies [1–3]. Platinum complexes exert anticancer activities principally by forming intra- and interstrand cross-links with DNA helix which induce remarkable DNA damage to tumor cells. However, the small proportion of drugs targeted DNA and the high amount of off-target portion lead serious side effects of platinum(II) drugs such as nephrotoxicity, hepatotoxicity, ototoxicity and neurotoxicity. Moreover, the increasingly serious drug resistance of tumor cells which is mainly induced by the self-repair and enhanced tolerance to DNA damage has badly affected the clinical performance of platinum(II) drugs [4]. Therefore, the development of new platinum drugs with novel structure possessing improved tumor selectivity and reduced toxicity has become an urgent task for medical and pharmaceutical researchers.

Platinum(IV) compounds as the prodrugs of classical platinum(II) drugs exhibit promising anticancer properties and have attracted great attention for their potential in improving therapeutic index (TI) and decreasing toxic effects [5–8]. Several prominent complexes including satraplatin (JM216), ormaplatin, iproplatin (JM9) and LA-12 which have stepped into clinical trials display excellent antitumor activities and show rather low side toxicities in comparison with platinum(II) drugs [9]. Inspired by these situations, the discovery of new drugs based on platinum(IV) scaffold by introducing various functional moieties to construct novel multi-functional drugs has become a hot topic in cancer therapy field [10–13].

Cancer-associated inflammatory responses play decisive roles at different phases of tumor development, including initiation, promotion, malignant conversion, invasion and metastasis, *etc.* [14–16] The cyclooxygenase-2 (COX-2) as the most important inflammatory factor is overexpressed in vast malignancies such as colon, breast, lung, womb

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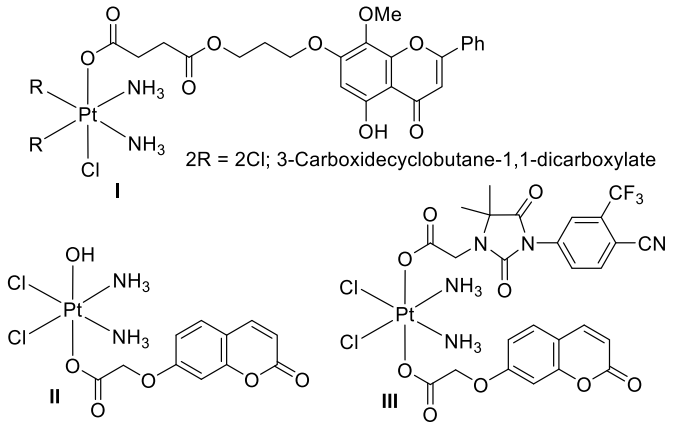

Fig. 1. Structure of 7-hydroxycoumarin platinum(IV) compounds.

and cervical cancers. It has been certified that nonsteroidal anti-inflammatory drugs (NSAIDs) which primarily targeted COX could reduce the incidence and proliferation of colon cancers *in vivo* [17,18]. The platinum(IV) compounds bearing NSAIDs ligands such as aspirin, indomethacin and ibuprofen were reported to exhibit remarkable antitumor activities with pronounced anti-inflammatory features [19–21]. Coumarin derivatives as an important class of natural benzopyrene compounds have been widely investigated as new COX inhibitors and possessed promising anti-inflammatory activities [22]. Recently, 7-hydroxycoumarin platinum(IV) hybrids **I–III** (Fig. 1) were disclosed to display multiple anticancer effects in literatures [23–26]. Especially complex **I** not only inherited the genotoxicity from platinum(II) drug, but also obtained the COX inhibitory properties from the coumarin moiety. The synergistic antitumor mechanism enabled the coumarin platinum(IV) prodrugs to be an effective framework to promote the anticancer potency of platinum drugs.

The 4-hydroxycoumarin is an important COX inhibitory scaffold possessing satisfactory anti-inflammatory efficacy [22] and has great potential as an antitumor agent [27–30]. Inspired by the observations above and in constitution of our ongoing interest in the development of new antitumor agents [31–33], herein a series of 4-hydroxycoumarin suspended platinum(IV) hybrids **1–4** were designed, prepared and evaluated for antitumor activities (Fig. 2). The conjunction of 4-hydroxycoumarin with platinum(IV) was expected not only to exhibit effective antitumor properties *via* the released platinum(II) complex, but also to exert COX inhibitory efficacy from coumarin which would inhibit the tumor-associated inflammation and further influence antitumor activities. To explore the impacts of platinum core on antitumor competence, the cisplatin and oxaliplatin derived compounds were designed and synthesized. Then the target compounds with different linkers were also prepared to evaluate their influence on activities. The antitumor activities of the target compounds were evaluated and the likely action mechanism was investigated.

2. Results and discussion

2.1. Chemistry

Coumarin platinum(IV) compounds **1–4** were prepared by the condensation of oxoplatin **B1** and **B2** with acids **A1** and **A2** (Scheme S1). Compounds **A** and **B** were synthesized starting from 4-hydroxycoumarin **C**, cisplatin and oxaliplatin according to literature procedures (presented in ESI). A two-fold amount of acid **A** was added to the solution of oxoplatin **B** in dry *N,N*-dimethylformamide (DMF) in the presence of *N,N,N′,N′*-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) and *N,N,N*-triethylamine (TEA). The resultant mixture was stirred under N_2 protection for 48 h in dark at 50 °C. The target compounds **1–4** were obtained in yields of 27%–34% after silica gel column chromatography purification.

The structures of all the title compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{195}Pt NMR, HRMS and elemental analysis. The HRMS and elemental analysis results of compounds **1–4** are in accordance with the assigned structures and prove the condensation of oxoplatins **B1**, **B2** with coumarin acids **A1**, **A2**. The singlets above 1000 ppm in the ^{195}Pt NMR spectra of compounds **1–4** evidence the platinum(IV) cores in their structures. Compounds **1** and **2** with cisplatin cores display relatively upfield shifts (1226 and 1229 ppm) in comparison with the oxaliplatin derived compounds **3** and **4** (1620 and 1640 ppm). In ^{1}H NMR spectra, the broad peaks at about 6.6 ppm for compounds **1** and **2** are assigned to the protons of amino groups on the cisplatin cores. Meanwhile the cyclohexyl moieties on oxaliplatin cores of compounds **3** and **4** give typical peaks below 3.0 ppm. Then, the protons in downfield of 5.8–8.0 ppm indicate the presence of the coumarin group. Moreover, all the carbons of compounds **1–4** give ^{13}C NMR peaks in the expected regions.

2.2. Antitumor activities in vitro

All the coumarin platinum(IV) derivatives **1–4** were evaluated for antitumor activities against ovarian cancer (SKOV-3), lung cancer

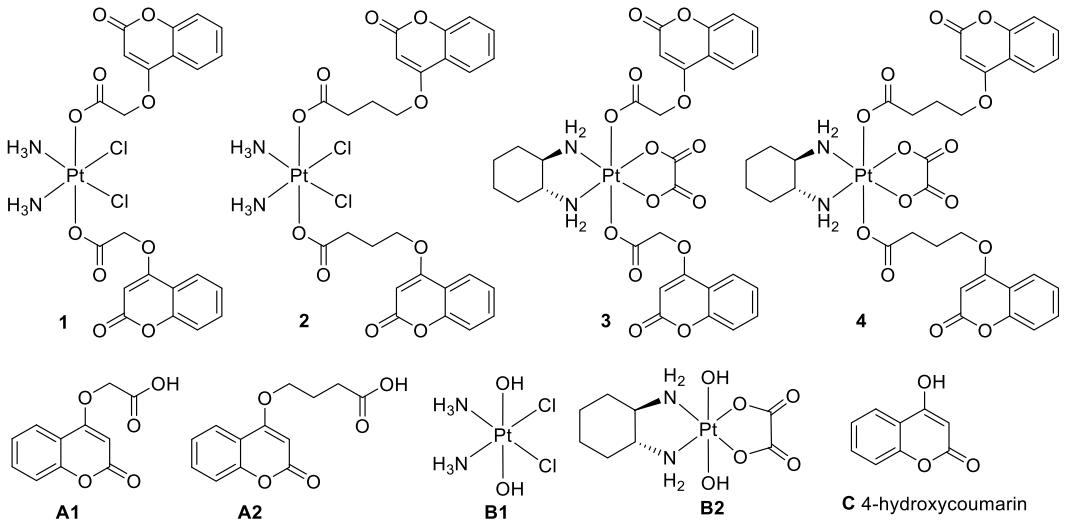

Fig. 2. Structure of 4-hydroxycoumarin platinum(IV) compounds **1–4** and their precursor compounds **A1, A2, B1**, **B2** and 4-hydroxycoumarin **C**.

Table 1
Cytotoxicity profiles of coumarin platinum(IV) complexes toward five human carcinoma cell lines and a normal human cell line expressed as IC_{50} (μM).

Compd.	SKOV-3 (48 h)	SKOV-3 (72 h)	A549 (48 h)	A549R (48 h)	RF (A549R)[a]	HeLa (48 h)	HeLa/DDP (48 h)	RF (HeLa/DDP)[a]	293 T (48 h)
1	5.6 ± 1.1	3.2 ± 0.7	7.4 ± 1.5	21.0 ± 3.0	2.8	4.7 ± 0.7	14.7 ± 2.5	3.2	2.4 ± 0.9
2	0.7 ± 0.2	0.4 ± 0.2	1.4 ± 0.4	7.0 ± 1.7	4.9	1.1 ± 0.2	4.7 ± 2.2	4.4	0.6 ± 0.4
3	0.9 ± 0.2	1.8 ± 0.6	6.1 ± 1.1	5.0 ± 1.0	0.8	3.0 ± 0.4	2.8 ± 0.7	0.9	2.0 ± 0.5
4	2.0 ± 0.6	3.5 ± 0.8	10.7 ± 2.2	15.2 ± 2.1	1.4	7.6 ± 1.0	6.5 ± 1.0	0.7	21.2 ± 3.1
A1	56.4 ± 9.8	/[c]	94.7 ± 14.7	> 100	NC[d]	> 100	> 100	NC[d]	/[c]
B1	20.4 ± 7.1	/[c]	31.3 ± 3.6	50.6 ± 15.4	NC[d]	26.2 ± 6.5	29.2 ± 4.6	NC[d]	/[c]
B2	48.6 ± 4.6	/[c]	94.3 ± 5.5	84.0 ± 7.7	NC[d]	68.4 ± 4.2	41.0 ± 7.9	NC[d]	/[c]
A1-B1[b]	31.8 ± 7.7	/[c]	33.6 ± 10.6	46.8 ± 6.4	NC[d]	32.7 ± 6.6	27.4 ± 6.9	NC[d]	/[c]
A1-B2[b]	46.9 ± 9.5	/[c]	50.1 ± 12.5	74.4 ± 15.8	NC[d]	57.0 ± 11.4	52.0 ± 8.5	NC[d]	/[c]
Cisplatin	1.3 ± 0.1	1.2 ± 0.2	9.5 ± 1.5	32.7 ± 8.8	3.4	3.0 ± 0.8	12.2 ± 3.8	4.1	0.9 ± 0.2
Oxaliplatin	2.8 ± 0.5	2.5 ± 0.6	11.7 ± 3.8	17.6 ± 5.2	1.5	10.4 ± 3.5	19.5 ± 1.6	1.9	10.9 ± 1.5

[a] RF: Resistant factor. RF (A549) = IC_{50}(A549R)/IC_{50}(A549); RF (HeLa) = IC_{50}(HeLa/DDP)/IC_{50}(HeLa).
[b] **A1-B1/B2**: 2 equivalents of acid **A1** mixed with 1 equivalent of oxoplatin **B1** or **B2**.
[c] /: not tested.
[d] NC: not calculated.

(A549), cervical cancer (HeLa), and two cisplatin resistant cells A549R and HeLa/DDP as well as human embryonic kidney cell (293T) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To evaluate the effects of incubation time, the half maximal inhibitory concentration values (IC_{50}) of compounds **1–4** against SKOV-3 after 48 h and 72 h incubation were tested and given in Table 1. It could be observed that the incubation time prolonging from 48 h to 72 h induces no dramatic change of the IC_{50} values. Thereby, the 48 h incubation time was selected and applied in the following evaluation.

The antitumor results indicate that all the target compounds show moderate to effective antitumor activities toward all tested cell lines with IC_{50} values below 15.2 μM which are significantly more effective than the oxoplatins **B1**, **B2** and the acid **A1**. To further confirm the influence of the combination of coumarin moiety with platinum(IV) core on activities, the mixture of **A1** with **B1** and **B2** (**A1-B1/B2**) were also evaluated. The results reveal that no significant enhancements of the bioactivities are observed in comparison with the free oxoplatins and acids, and these facts evidence the favourable effects of the conjunction of coumarin with platinum(IV) on the antitumor activities.

Compounds with different platinum core and linkage exhibit different antitumor properties. For compounds with cisplatin core, the complex **2** with three carbon linker is more effective than compound **1** bearing one carbon linker and exhibits prominent activities against all the tested tumor cell lines, especially to SKOV-3, A549 and HeLa ($IC_{50} \leq 1.4$ μM) which are over 1.8-fold more potent than the reference drugs cisplatin and oxaliplatin. Meanwhile, the oxaliplatin derived compound **3** with one carbon linker is relatively superior to the three carbon linker one **4**. Compound **3** display effective activities to the tested cell lines ($IC_{50} \leq 6.1$ μM) which are more potent than oxaliplatin.

The coumarin platinum(IV) compounds with oxaliplatin core are of much potential in overcoming the drug resistance of cisplatin. The target compounds **1–4** give rather no higher IC_{50} values for resistant cells A549R and HeLa/DDP in contrast to A549 and HeLa. Especially the oxaliplatin derived compounds **3** and **4** exert promising resistance overcoming properties by reducing the resistant factors (RF) to 0.8, 1.4 for A549R and 0.9, 0.7 for HeLa/DDP respectively, which are lower than cisplatin and oxaliplatin.

Additionally, the toxicities of these compounds to normal human cell 293 T were also tested. The results display that compounds **1–4** exert much influence on the proliferation of 293 T which are similar to that of the platinum(II) drugs cisplatin and oxaliplatin. It seems that these coumarin platinum(IV) complexes exhibit no lower toxicities to normal cells *in vitro* in comparison with the platinum(II) drugs.

The facts mentioned above manifest that all the coumarin platinum (IV) compounds exert moderate to effective antitumor abilities and the oxaliplatin derived compounds exhibit much competence in overcoming resistance of platinum(II) drugs. Compounds **2** and **3** with prominent activities to all tested cell lines were selected for further investigation in the following experiments.

2.3. The antitumor mechanism detection

2.3.1. Reduction and DNA damage properties of compound 3

It has been extensively proven that platinum(IV) compounds as prodrugs of platinum(II) drugs exhibit their antitumor efficacy after being reduced to platinum(II) complexes. Thus the reduction potential of platinum(IV) compounds is a key factor influencing their bioactivities. Herein, the reduction of complex **3** in the presence of ascorbic acid (AsA) was tested by high performance liquid chromatography (HPLC). It is observed that the compound **3** keeps stable for at least 96 h in phosphate buffer saline (PBS) (Fig. S1) and undergoes no significant reduction in the presence of 5′-guanosine monophosphate (5′-GMP) as a model of DNA (Fig. S4). The HPLC spectra in Fig. 3 reflect that compound **3** could mainly be reduced by AsA in 96 h, accompanied by the release of coumarin acid. Following such a reductive activation, the emergence of platined-GMP peak demonstrates the generation of platinum(II) complex and its abilities to conjunct with 5′-GMP as well as DNA [34]. Summarily, the coumarin platinum(IV) compounds as prodrugs would cause dramatic DNA damage *via* the reduction to platinum (II) complexes upon the activation of the reductants.

2.3.2. The cyclooxygenase inhibition

The cyclooxygenase, especially COX-2, is related to the occurrence of inflammatory and the development of tumor. The COX has become an attractive target for cancer prevention and treatment. The recombinant human COX-2 (rhCOX-2) inhibitory experiments of compounds **2** and **3** at different concentrations ranging from 5 μM to 250 μM were carried out with celecoxib as a positive reference drug. The results in Fig. 4 reveal that 4-hydroxycoumarin **C** and acids **A1** and **A2** exhibit COX inhibition in a concentration dependent manner. The 4-hydroxycoumarin **C** shows significant inhibitory capacity of 75% at high concentration of 500 μM but low ability at 62 μM, and the acids **A1** and **A2** show moderate inhibitory activities at 500 μM. For the platinum (IV) complexes **2** and **3**, they retain the COX inhibitory competence from 4-hydroxycoumarin. Especially platinum(IV) complex **2** with cisplatin core could effectively inhibit the activity of rhCOX-2 at concentration above 31 μM, which is superior to the precursor 4-hydroxycoumarin **C**. The oxaliplatin derived compound **3** shows relatively weaker COX inhibition than compound **2** at same concentration. Consequently, the title coumarin platinum(IV) compounds could exert rhCOX-2 inhibitory competence to some extent which should be attributed to the coumarin fragment, and are of much potential to decrease the tumor-related inflammation. Their antitumor activities might be associated with the COX inhibition.

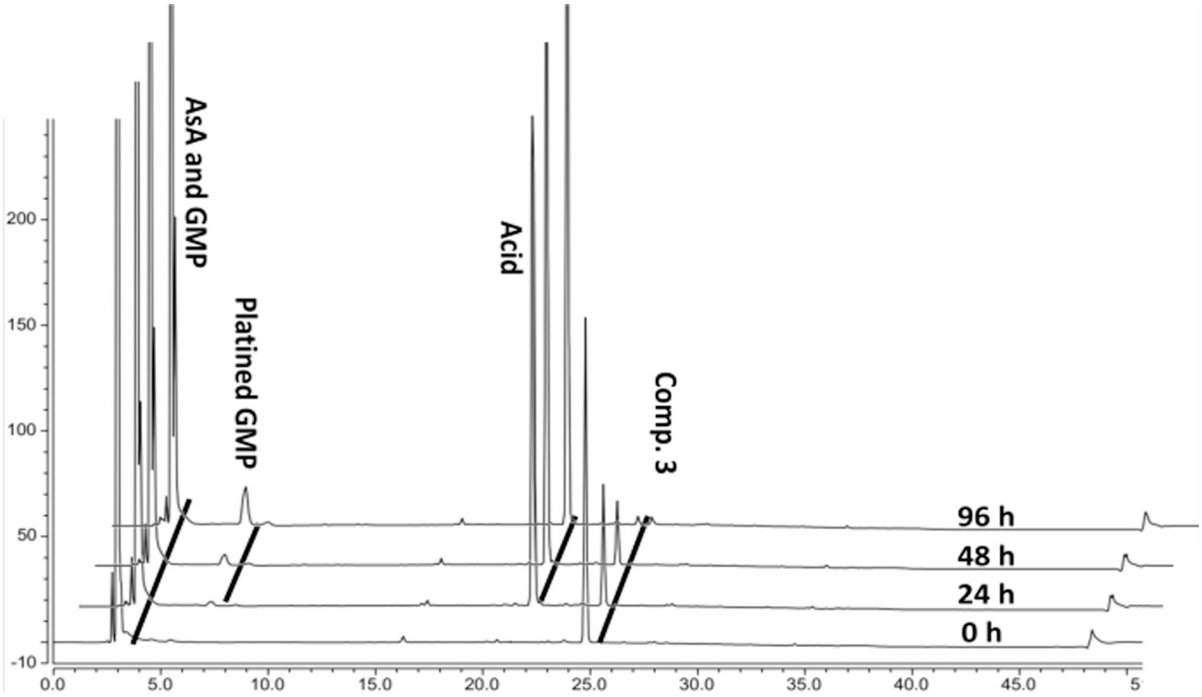

Fig. 3. HPLC spectra of compound **3** in the presence of AsA and 5′-GMP.

2.4. Apoptosis experiments

Since compound **2** exerted prominent antitumor activities and COX inhibitory competence, it was selected for further mechanism investigation as an optional candidate. To detect if the inhibitory effects of the coumarin platinum(IV) complexes to tumor cells was related to apoptosis, the apoptosis inducing properties of compound **2** were evaluated with an annexin V/propidium iodide (PI) staining assay in SKOV-3 cells. The results in Fig. 5 display that after 24 h incubation, compound **2** induces comparable populations (41.94%) of cells undergoing apoptosis in contrast to cisplatin (40.84%) and more populations than oxaliplatin (20.86%) at the same concentration (20 μM), which is in agreement with the trend of IC_{50} values. The activities of coumarin platinum(IV) complexes to tumor cells are associated with the apoptosis inducing properties.

2.5. Western blot results

To better elucidate the mechanisms of the antitumor activity, western blot assay was used to investigate the influence of coumarin

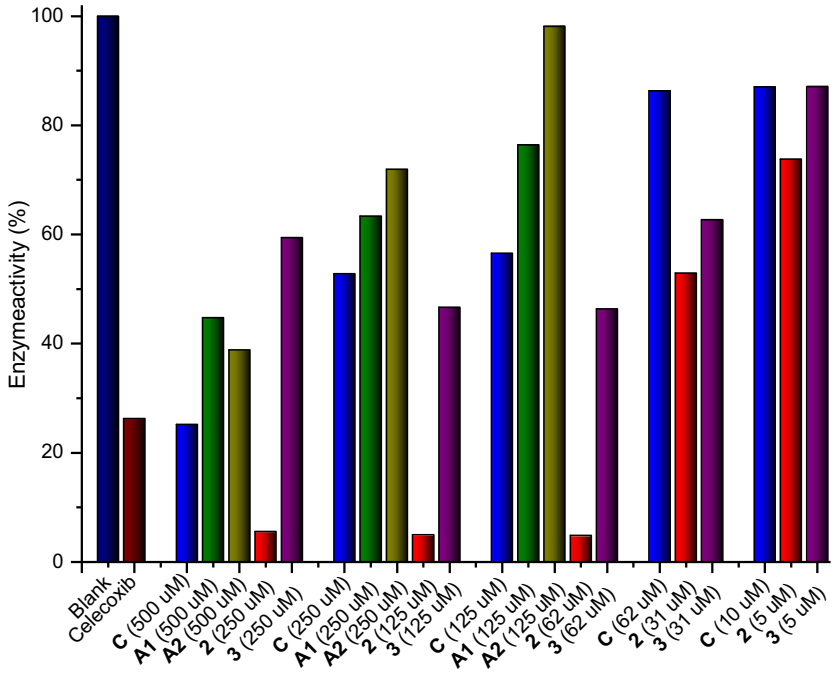

Fig. 4. The inhibition of the selected compounds at different concentrations to rhCOX-2. Compound **2/3**: 5, 31, 62, 125, 250 μM. Compound **A1/A2** and 4-hydroxycoumarin **C**: 10, 62, 125, 250, 500 μM.

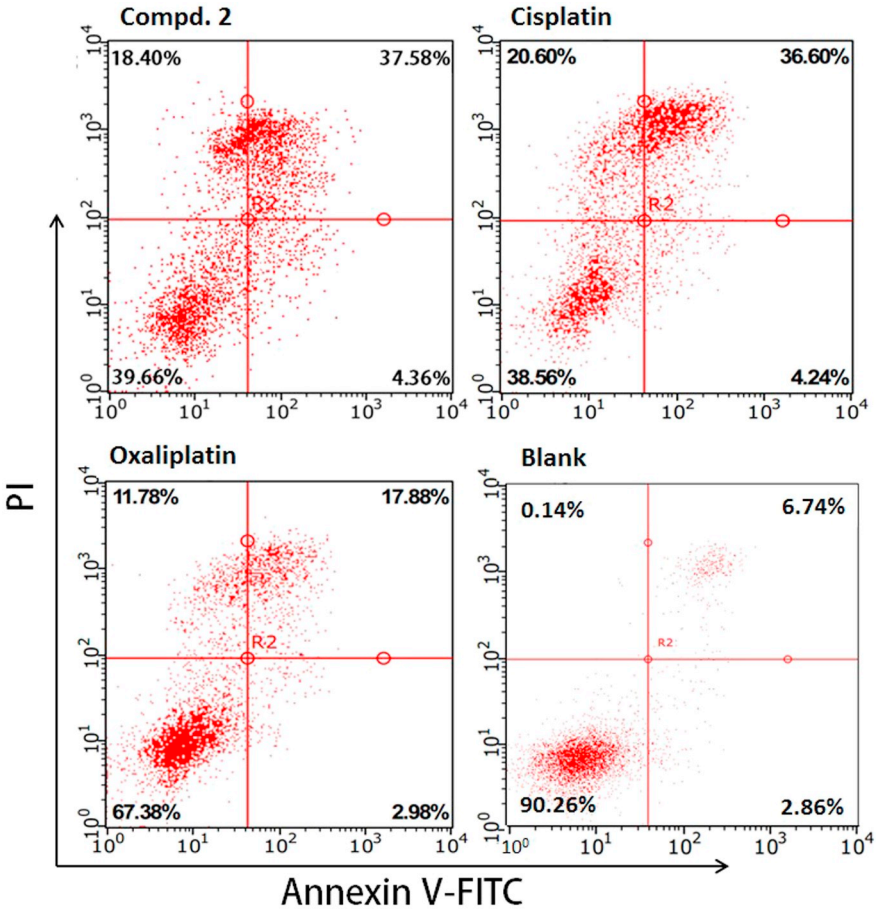

Fig. 5. Quantification of apoptosis in SKOV-3 cells using an annexin V/PI assay. The SKOV-3 cells were treated with platinum compounds for 24 h at 37 °C in incubator (blank: no compound; cisplatin, oxaliplatin, compound **2**: 20 μM).

platinum(IV) complexes on the expression of the apoptotic proteins caspase3 and caspase9. Compound **2** was tested as a candidate. The results in Fig. 6 manifest that compound **2** could upregulate the expression both of pro-caspase9 and pro-caspase3 in SKOV-3 cells in comparison with the untreated cells, which is similar to the reference drugs cisplatin and oxaliplatin, and further induce higher expression of cleaved-caspase3. Accordingly, compound **2** could activate the apoptotic pathway and ultimately induce the apoptosis of SKOV-3 cells.

Taken the cellular accumulation results (see Section 2.7) in consideration, these results may be ascribed to the enhanced drug accumulation in cells.

2.6. HSA binding studies

The human serum albumin (HSA) is the most abundant protein constituent of blood plasma. It has been reported that the drug-protein

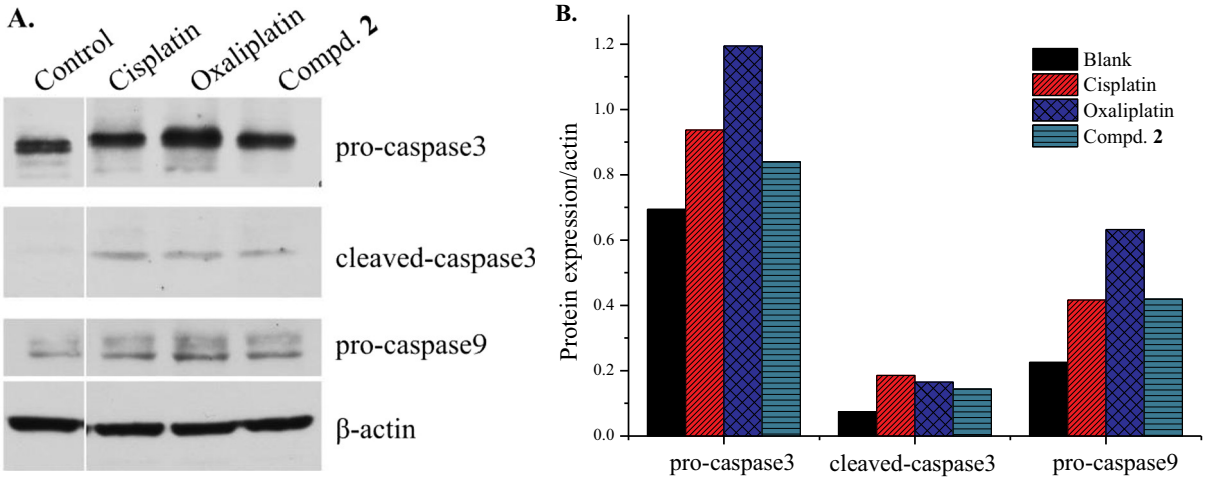

Fig. 6. Cell apoptotic pathway Western blot analysis of SKOV-3 cells incubated with compound **2**, cisplatin and oxaliplatin. SKOV-3 cells were incubated with and without platinum complexes for 24 h at 37 °C in incubator (blank: no compound; cisplatin, oxaliplatin, compound **2**: 20 μM). (A) Blots. (B) Relative gray intensity analysis (Relative gray intensity = (gray intensity of indicated protein)/(gray intensity of β-actin)).

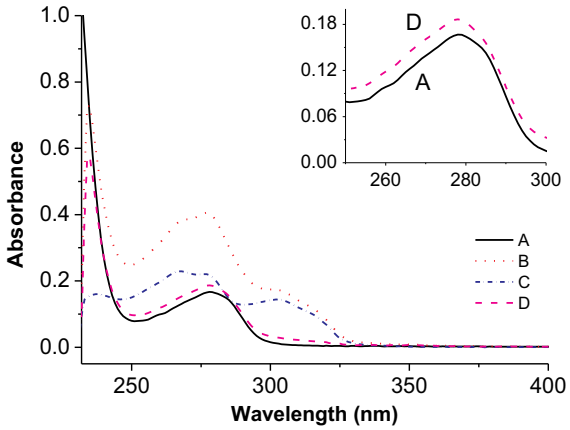

Fig. 7. UV–vis spectra of HSA with and without compound **3**. (A) Absorption spectrum of HSA, *c*(HSA) = 10 μM; (B) Absorption spectrum of compound **3**-HSA system, *c*(HSA) = 10 μM, *c*(compound **3**) = 15 μM; (C) Absorption spectrum of compound **3**, *c*(compound **3**) = 15 μM; (D) Subtracting spectrum of (B) and (C). Inset: the curves (A) and (D) for the wavelength ranging from 250 to 300 nm.

binding may influence their pharmacokinetic and pharmacodynamic properties such as drug solubility, biodistribution, half-life in blood and would further affect their bioactivity, toxicity, and metabolism and so on [35,36]. The HSA binding study does many favors for gaining fundamental insights into the behavior of small molecular drugs in blood [37–39]. Thereby, the HSA binding properties of compound **3** was evaluated *via* multiple spectral methods.

2.6.1. UV–vis spectroscopy

The UV–vis spectrum is widely applied in the detection of the interaction of small molecules with HSA. Herein, the UV–vis method was employed to validate the complexation of compound **3** with HSA. The Fig. 7 depicts that the subtraction spectrum (Curve D) of the mixed solution **3**-HSA (Curve B) and compound **3** (Curve C) is not coincident to the absorption curve of HSA (Curve A) in the range of 250–300 nm. These facts prove that compound **3** could bind to HSA in solution.

2.6.2. The fluorescence analyses

The fluorescence spectra of HSA in the absence and presence of platinum(IV) complex **3** (Fig. 8) reveal that the HSA displays strong fluorescence emission at 343 nm under the excitation of 280 nm. The typical fluorescence emission of HSA quenches gradually accompanied with a slight blue shift from 343 nm to 340 nm with the increasing

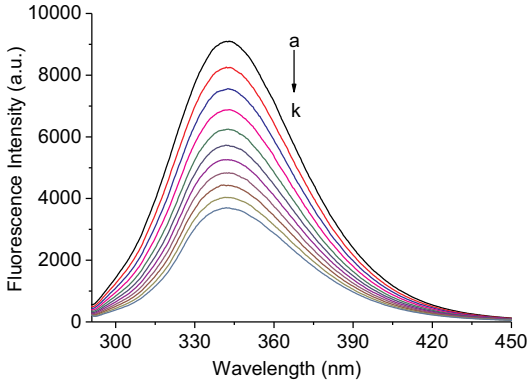

Fig. 8. Fluorescence spectra of HSA in the absence and presence of platinum (IV) complex **3** (λex = 280 nm, *T* = 298 K). a–k: *c*(HSA) = 4.0 μM, *c*(complex **3**) = 0.0–25.0 μM, at increments of 2.5 μM.

Table 2
Stern-Volmer quenching constants for the interaction of compound **3** with HSA at different temperatures.

T (K)	K_{sv} (M^{-1})	K_q ($M^{-1}S^{-1}$)	SD[a]	K_b (M^{-1})	*n*
298	5.32×10^4	5.32×10^{12}	0.15	2.622×10^5	1.15
301	4.59×10^4	4.59×10^{12}	0.12	2.281×10^5	1.15
304	4.51×10^4	4.51×10^{12}	0.11	1.785×10^5	1.12

[a] SD: standard deviation.

concentration of platinum(IV) compound (0.0–25.0 μM) in solution. These facts evidence the conjunction of complex **3** with HSA which are in consistent to the results obtained above by the UV–vis spectra.

With the aim of further identifying the interaction mode, the fluorescence spectra at other temperatures 301 K and 304 K were also recorded and given in Fig. S5. The quenching trends at three temperatures are in agreement with Stern-Volmer equation. The Stern-Volmer quenching constants K_{SV} are calculated and established in Table 2. It is indicated that the K_{SV} decreases with the rise of temperature from 298 K to 304 K. Then the quenching rate constants K_q are obtained in the range of 4.51–5.32 × 10^{12} $M^{-1}S^{-1}$ which are higher than the maximum scatter collision quenching constant of the biomolecule (2.0 × 10^{10} $M^{-1}S^{-1}$). It could be implied that the tested coumarin platinum(IV) complex quenches the fluorescence emission of HSA in a static quenching manner. Then, the binding constant K_b and binding site *n* at three temperatures were also calculated. The binding constants are moderate and the temperature leads weak impacts on K_b. The K_b decreases with the raise of temperature, which is in consistent to that of K_{SV}. The binding sites calculation demonstrates that one high affinity binding site is involved in the HSA interaction of compound **3**.

It has been widely approved that four types of interactions including hydrogen bond, van der Waals interaction, electrostatic interaction and hydrophobic interaction are mainly involved in the interaction of small molecular drugs with HSA. The thermodynamic parameters enthalpy change Δ*H*, entropy change Δ*S* and Gibbs free energy change Δ*G* could be used for the identification of the type of interaction forces. From the thermodynamic standpoint, $\Delta H > 0$ and $\Delta S > 0$ indicate a hydrophobic interaction, $\Delta H < 0$ and $\Delta S < 0$ imply the van der Waals force and hydrogen bond formation, and $\Delta H < 0$ and $\Delta S > 0$ are characteristics for electrostatic interactions [40,41]. To verify the interaction mode of compound **3** with HSA, the thermodynamic parameters were calculated by Van't Hoff equation and shown in Table 3. The detail calculation procedures are given in the ESI. The negative Δ*G* reflects the spontaneous complexation of platinum(IV) complex **3** with HSA. Then the negative Δ*H* and Δ*S* indicate that the binding process is driven by enthalpy, and the van der Waals force and hydrogen bond are associated with the above HSA conjunction process. Summarily, the tested coumarin platinum(IV) complex quenches the fluorescence emission of HSA in a static procedure *via* van der Waals force and hydrogen bond. These features might enable the target compounds to be transported by HSA in blood and further influence their bioactivities *in vivo*.

2.6.3. CD spectroscopy

Circular dichroism (CD) spectroscopy is a sensitive and useful technique to monitor the secondary structural changes of protein upon

Table 3
Thermodynamic parameters of compound **3**-HSA interaction at different temperatures (298 K, 301 K and 304 K).

T (K)	Δ*H* (kJ/mol)	Δ*G* (kJ/mol)	Δ*S* (J/mol K)
298	−48.20	−30.96	−57.85
301		−30.78	
304		−30.61	

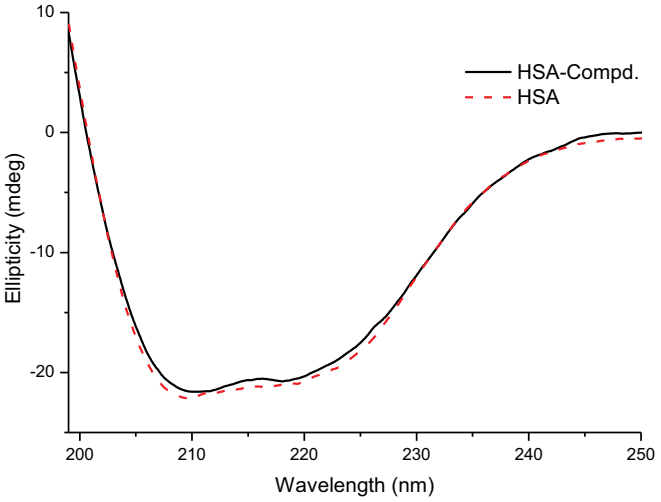

Fig. 9. CD spectra of HSA in the absence and presence of the platinum(IV) compound **3**. HSA: *c*(HSA) = 2 μM, HSA-Comp.: *c*(HSA) = 2 μM, *c*(compound **3**) = 2 μM.

the interactions with small molecules and has been extensively applied as a useful strategy in HSA binding investigation. The HSA solution shows two negative bands at about 208 and 220 nm respectively, which reflect the transition of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ of α-helix structure of protein [42]. The results in Fig. 9 demonstrate that the CD signals of HSA decrease at 208 nm and 220 nm with the addition of compound **3**. These facts manifest the change of the secondary structure of HSA which is probably attributed to the conjunction of platinum(IV) compound with HSA.

2.7. Cellular uptake and DNA platination of compounds 2 and 3

Cellular uptake of drug plays a key role in the tumor inhibition, and the DNA platination of platinum drug influences activities crucially. Thereby, the cellular uptake and DNA platination levels of compounds **2**, **3** and reference drugs cisplatin, oxaliplatin were evaluated by treating SKOV-3 with 100 μM of the platinum complexes at 37 °C for 10 h (Fig. 10). It is proven that the uptakes of the tested coumarin platinum(IV) compounds **2** and **3** in SKOV-3 cells are 2.9–3.8 times higher than platinum(II) drugs cisplatin and oxaliplatin, meanwhile the selected platinum(IV) compounds also display 3.1–4.1 folds higher accumulation level in DNA than the reference drugs. Consequently, the transformation of platinum(II) drugs to the title coumarin platinum(IV) complexes by incorporation of coumarin moiety exhibits significantly positive impacts on the uptakes in whole tumor cells and DNA which are probably ascribed to the enhanced lipophilicity.

3. Conclusions

Summarily, a series of new 4-hydroxycoumarin platinum(IV) complexes were designed, prepared and evaluated for antitumor activities in this work. The biological results *in vitro* demonstrate that all the target compounds exhibit moderate to potent antitumor activities, especially compounds **2** and **3** which are comparable to that of the reference drugs cisplatin and oxaliplatin. It is demonstrated that the platinum core and linkage in the 4-hydroxycoumarin platinum(IV) system influence their activities remarkably, and the oxaliplatin derived compounds exert much potential in overcoming the drug resistance of cisplatin. Furthermore, the incorporation of 4-hydroxycoumarin fragment to platinum(IV) system leads to higher platinum uptake in both the whole tumor cells and DNA than the corresponding platinum(II) drugs. The mechanism investigation manifests that the 4-hydroxycoumarin platinum(IV) compounds exhibit significant genotoxicity after being reduced to platinum(II) complexes in reducing microenvironment, meanwhile their activities are also associated with COX inhibition attributed to the coumarin ligand which would influence the tumor-related inflammation and further impact their activities. In addition, the target complexes could combine with HSA by van der Waals force and hydrogen bond which would influence their delivery and bioactivities *in vivo*. Eventually, in view of the results obtained above, the coumarin platinum(IV) compounds are of great value for further investigation as new platinum antitumor agents.

4. Materials and methods

4.1. Chemistry

4.1.1. General

All reactions were carried out under an atmosphere of nitrogen in flame-dried glassware with magnetic stirring unless otherwise indicated. Cisplatin and oxaliplatin were purchased from Yurui chemical Co. Ltd. (Shanghai, China). Other reagents were obtained from Alfa Aesar, Sigma, Aladdin, and GL Biochem Ltd. ^{1}H NMR, ^{13}C NMR and ^{195}Pt NMR spectra were recorded on a Varian (400 MHz). The ^{1}H and ^{13}C NMR chemical shifts were referenced to residual solvent peaks or to TMS as an internal standard. All coupling constants *J* were quoted in Hz. ^{195}Pt NMR spectra were referenced externally using a standard of K_2PtCl_4 in D_2O ($\delta = -1628$ ppm). High resolution mass spectra (HRMS) were obtained on an IonSpec QFT mass spectrometer with ESI ionization. The HPLC analyses were performed on a Thermo Ultimate 3000 RS equipped with an Agilent Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm). All cells were kindly donated by Prof. Peng George Wang (College of Pharmacy, Nankai University, Tianjin, China). The cyclooxygenase 2 inhibitor screening kit and Genomic DNA mini preparation kit were purchased from Beyotime, China. The HSA was purchased from Sigma. The annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased

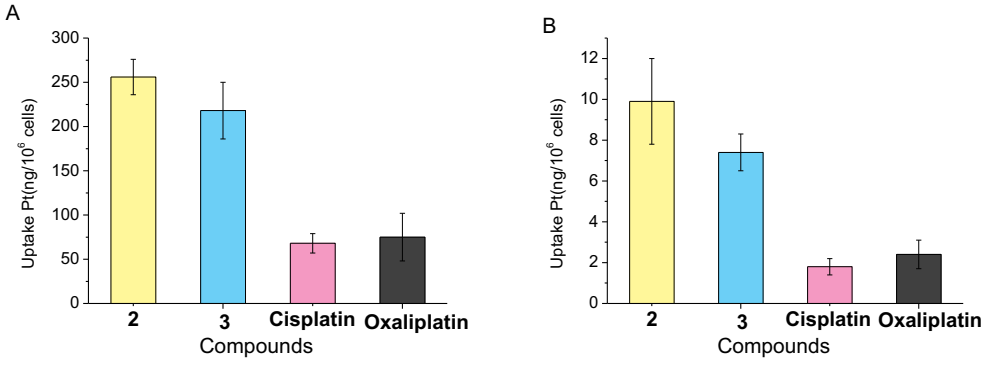

Fig. 10. Cellular uptakes and DNA platinations of complexes **2**, **3**, cisplatin and oxaliplatin in SKOV-3 cells. (A) Platinum in whole cells; (B) Platinum in DNA.

from KeyGen BioTech, China. UV–vis spectra were measured on a Scinco S-3100 UV–vis spectrophotometer. Fluorescence spectroscopic data were recorded on a Hitachi F-7000 spectrofluorometer. CD spectra were recorded on a Jasco *J*-810 spectropolarimeter.

4.1.2. Synthetic procedures

The detail methods for the synthesis of the coumarin acids **A1**, **A2** and the oxoplatin **B1**, **B2** are supplied in supporting information based on the literature procedures.

*4.1.2.1. Preparation of compound **1***. Coumarin acid **A1** (165 mg, 0.75 mmol) and TBTU (241 mg, 0.75 mmol) were dissolved in dry DMF 5 mL. The mixture was stirred for about 10 min after injection of TEA (104 μL, 0.75 mmol). Then the dry oxoplatin **B1** (100 mg, 0.30 mmol) was added and the resulting mixture was vigorously stirred for 48 h at 50 °C. Upon completion of the reaction, the solvent was evaporated under vacuum, and the crude product was obtained as solid. Subsequently, the pure compound **1** (60 mg, 27%) was obtained as pale yellow solid after purification by silica gel column chromatography (DCM:MeOH 30:1).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.82 (d, J = 7.9 Hz, 2H), 7.65 (t, J = 7.7 Hz, 2H), 7.38 (dd, J = 15.7, 8.1 Hz, 4H), 6.58 (br, 6H), 5.97 (s, 2H), 4.93 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 173.73, 164.69, 162.04, 153.17, 133.20, 124.66, 123.33, 116.88, 115.61, 92.04, 65.42. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ (ppm) 1226. HRMS: Calcd. for $[M + H]^+$: 738.0216 ($M = C_{22}H_{20}Cl_2N_2O_{10}Pt$), found: 738.0241. Anal. Calcd for $C_{22}H_{20}Cl_2N_2O_{10}Pt$: C, 35.79; H, 2.73; N, 3.79; found: C, 35.21; H, 2.89; N, 3.68.

*4.1.2.2. Preparation of compound **2***. Coumarin acid **A2** (186 mg, 0.75 mmol) and TBTU (241 mg, 0.75 mmol) were dissolved in dry DMF 5 mL. The mixture was stirred for about 10 min after injection of TEA (104 μL, 0.75 mmol). Then the dry oxoplatin **B1** (100 mg, 0.30 mmol) was added and the resulting mixture was vigorously stirred for 48 h at 50 °C. Upon completion of the reaction, the solvent was evaporated under vacuum, and the crude product was obtained as solid. Subsequently, the pure compound **2** (71 mg, 30%) was obtained as pale yellow solid after purification by silica gel column chromatography (DCM:MeOH 50:1).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.80 (d, J = 6.5 Hz, 2H), 7.68–7.59 (m, 2H), 7.41–7.31 (m, 4H), 6.53 (br, 6H), 5.85 (s, 2H), 4.24 (t, J = 6.4 Hz, 4H), 2.52–2.41 (m, 4H), 2.10–1.94 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 180.19, 165.35, 162.09, 153.20, 133.19, 124.66, 123.28, 116.90, 115.70, 90.97, 69.26, 32.05, 24.93. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ (ppm) 1229. HRMS: Calcd. for $[M + H]^+$: 794.0842 ($M = C_{26}H_{28}Cl_2N_2O_{10}Pt$), found: 794.0830. Anal. Calcd for $C_{26}H_{28}Cl_2N_2O_{10}Pt$: C, 39.31; H, 3.55; N, 3.53; found: C, 39.19; H, 3.78; N, 3.44.

*4.1.2.3. Preparation of compound **3***. Coumarin acid **A1** (129 mg, 0.58 mmol) and TBTU (187 mg, 0.58 mmol) were dissolved in dry DMF 5 mL. The mixture was stirred for about 10 min after injection of TEA (80 μL, 0.58 mmol). Then the dry oxoplatin **B2** (100 mg, 0.23 mmol) was added and the resulting mixture was vigorously stirred for 48 h at 50 °C. Upon completion of the reaction, the solvent was evaporated under vacuum, and the crude product was obtained as solid. Subsequently, the pure compound **3** (56 mg, 29%) was obtained as pale yellow solid after purification by silica gel column chromatography (DCM:MeOH 40:1).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.84 (d, J = 7.7 Hz, 2H), 7.69–7.63 (m, 2H), 7.42–7.24 (m, 4H), 5.84 (s, 2H), 4.94 (d, J = 7.7 Hz, 4H), 2.87–2.54 (m, 2H), 2.16–1.95 (m, 2H), 1.49–1.19 (m, 4H), 1.08–0.82 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 172.84, 169.03, 164.78, 161.91, 153.20, 133.32, 129.98, 124.74, 123.37, 116.91, 115.52, 91.64, 66.19, 31.21, 24.18. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ (ppm) 1644. HRMS: Calcd. for $[M + H]^+$: 836.1261 ($M = C_{30}H_{28}N_2O_{14}Pt$), found: 836.1248. Anal. Calcd for $C_{30}H_{28}N_2O_{14}Pt$: C, 43.12; H, 3.38; N, 3.35; found: C, 43.03; H, 3.65; N, 3.31.

*4.1.2.4. Preparation of compound **4***. Coumarin acid **A2** (144 mg, 0.58 mmol) and TBTU (187 mg, 0.58 mmol) were dissolved in dry DMF 5 mL. The mixture was stirred for about 10 min after injection of TEA (80 μL, 0.58 mmol). Then the dry oxoplatin **B2** (100 mg, 0.23 mmol) was added and the resulting mixture was vigorously stirred for 48 h at 50 °C. Upon completion of the reaction, the solvent was evaporated under vacuum, and the crude product was obtained as solid. Subsequently, the pure compound **4** (70 mg, 34%) was obtained as pale yellow solid after purification by silica gel column chromatography (DCM:MeOH 60:1).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.81 (d, J = 7.8 Hz, 2H), 7.66 (t, J = 7.8 Hz, 2H), 7.49–7.34 (m, 4H), 5.87 (s, 2H), 4.22 (t, J = 5.8 Hz, 4H), 2.61–2.54 (m, 2H), 2.14–1.94 (m, 8H), 1.55–1.38 (m, 6H), 1.11–1.04 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 180.23, 165.36, 164.06, 162.15, 153.14, 133.24, 124.72, 123.31, 116.87, 115.62, 90.88, 69.05, 61.49, 32.35, 30.78, 24.74, 24.06. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ (ppm) 1620. HRMS: Calcd. for $[M + H]^+$: 892.1887 ($M = C_{34}H_{36}N_2O_{14}Pt$), found: 892.1874. Anal. Calcd for $C_{34}H_{36}N_2O_{14}Pt$: C, 45.79; H, 4.07; N, 3.14; found: C, 45.68; H, 3.96; N, 3.02.

4.2. Biological evaluation

4.2.1. In vitro cellular cytotoxicity assays

Dulbecco's Modified Eagle Medium (DMEM), RPMI1640, 0.25% trypsin/EDTA solutions, fetal bovine serum (FBS) and penicillin-streptomycin solutions were purchased from Gibco. MTT was purchased from Sigma. The cells were maintained in either DMEM (for HeLa, HeLa/DDP, 293 T cells) or RPMI1640 (for SKOV-3, A549, A549R cells) medium containing 10% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. The A549R and HeLa/DDP cells were maintained with 2 μg/mL cisplatin. Phosphate buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 (pH 7.4). MTT solution at concentration of 5 mg·mL^{-1} for MTT assays was prepared before it used.

The stock solutions of the tested compounds in DMF were prepared at concentration of 20 mM. Cells were seeded in 96-well plates at 5000 cells per well in 100 μL of complete medium, and incubated in a 5% CO_2 atmosphere at 37 °C for 24 h. Then 100 μL of freshly prepared culture medium containing drugs at gradient concentrations were added. The working concentration of the investigated compounds was set to 100 μM, 45.9 μM, 21.1 μM, 9.7 μM, 4.5 μM, 2.0 μM, 0.9 μM and 0.4 μM. The cells were further incubated for another 48 h, and then freshly prepared MTT solution (5 mg·mL^{-1}) 20 μL was added. The resultant mixtures were incubated for 3 h to allow viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. After removal of the medium, formazan was dissolved in DMSO (150 μL) and quantified by a microplate reader (570 nm). The IC_{50} values were calculated using GraphPad Prism 6 based on three parallel experiments.

4.2.2. The antitumor mechanism detection

4.2.2.1. Reduction of platinum(IV) complexes by AsA. The reduction of platinum(IV) complexes and the further DNA binding properties of the reduced platinum(II) compounds were detected by HPLC. HPLC analyses were performed on Thermo Ultimate 3000 RS equipped with an Agilent Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm) with flow rate of 1 mL/min.

The linear gradient was given in Table 4.

To confirm the stability of platinum(IV) complexes, the solution of compound **3** (250 μM) in PBS was evaluated for 96 h. The reduction potential was confirmed by the evaluation of compound **3** (250 μM) in the presence of AsA (1 mM, same concentration as in tumor cells). The

Table 4
The linear gradient for HPLC.

Time (min)	A (0.01% aqueous trifluoroacetic acid)	B (methanol)
0	90	10
5	90	10
35	0	100
45	0	100

DNA interaction abilities were testified with the addition of 5′-GMP (3 mM) which was often applied as a model of DNA base. With the aim of confirming if the reduction of platinum(IV) compound was essential for DNA combination, the solution containing compound **3** (250 μM) and 5′-GMP (3 mM) without AsA was also detected as negative experiment.

4.2.2.2. The cyclooxygenase inhibitory experiment. The cyclooxygenase inhibitory experiment was carried out according to the manufacturer's protocol (Cyclooxygenase 2 inhibitor screening kit, Beyotime, China). The rhCOX-2 was applied in the experiments. The coumarin platinum (IV) compounds **2** and **3** were evaluated at concentrations of 5, 31, 62, 125 and 250 μM as rhCOX-2 inhibitor, meanwhile the precursor coumarin acids **A1** and **A2** at the corresponding concentrations 10, 62, 125, 250 and 500 μM were also tested. The sample with no rhCOX-2 inhibitor was evaluated as blank, and the celecoxib was used at the advised concentration in the protocol (100 nM) as positive reference sample.

4.2.3. Apoptosis experiments

The apoptosis experiment was carried out according to the manufacturer's protocol (Annexin V-FITC/PI Apoptosis Detection Kit, KeyGEN, China). SKOV-3 cells were incubated for 24 h in incubator with or without the test compounds (blank: no compound; cisplatin, oxaliplatin, compound **2**: 20 μM). Then the cells were harvested and strained by annexin V-FITC and PI for 15 min at room temperature. The apoptosis-inducing properties were measured using a flow cytometric assay.

4.2.4. Western blot experiments

The influence of platinum(IV) complex **2**, cisplatin and oxaliplatin on the expression of the apoptotic proteins caspase3 and caspase9 were evaluated. The SKOV-3 cells were treated with or without platinum compounds for 24 h in incubator (blank: no compound; cisplatin, oxaliplatin, compound **2**: 20 μM). Proteins were extracted with lysis buffer, and their concentrations were measured by bicinchoninic acid (BCA) assay using a BCA protein assay kit (Servicebio). The proteins (20 mg/Lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Servicebio) and transferred onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore). The β-actin was used as a loading control. The blots were blocked with 5% nonfat milk in TBST (Trisbuffered saline plus 0.1% Tween 20) for 1 h. The membrane was incubated with primary antibodies overnight at 4 °C. Then, the membrane was washed with PBST for three times and incubated with secondary antibody for 1 h at 37 °C. After that, the membrane was treated with an ECL western blotting substrate kit (Servicebio). Finally, the detection was performed by a scanning system (EPSON).

4.2.5. HSA binding experiments

4.2.5.1. UV–vis spectra assay. The UV–vis spectroscopy is employed to detect the HSA combination of the coumarin platinum(IV) compound. The experiments were performed in Tris-HCl buffer (10 mM Tris-HCl/10 mM NaCl, pH 7.4). The spectra of free compound **3** (15.0 μM) and HSA (10.0 μM) were recorded, and the mixed solution compound **3**-HSA containing compound **3** (15.0 μM) and HSA (10.0 μM) was also measured. To judge the interaction of compound **3** with HSA, the subtraction spectrum of the mixed solution compound **3**-HSA and free compound **3** was calculated and drawn to compare with HSA.

4.2.5.2. Fluorescence spectra assay. The experiments were performed in Tris-HCl buffer (10 mM Tris-HCl/10 mM NaCl, pH 7.4). The quenching spectra of HSA solution (4.0 μM) by compound **3** (0.0–25.0 μM, at increments of 2.5 μM) were recorded at three temperatures (298 K, 301 K and 304 K). A 1.00 cm quartz cell was used for the measurements. The well-mixed solutions were holding for 15 min for equilibrium, and the fluorescence emission spectra were then tested from 280 to 500 nm (λex = 280 nm) at scan rate of 1200 nm·min^{-1}. The widths of both the excitation and emission slits were 5.0 nm.

4.2.5.3. CD spectrum assay. The solution of HSA (2 μM) with and without platinum(IV) complex **3** (2 μM) in buffer solution (10 mM Tris-HCl/10 mM NaCl, pH 7.4) were incubated for 48 h in dark. The CD spectrum were recorded at room temperature in the range of 190 and 320 nm with a scan speed of 100 nm·min^{-1} and 1 s response time. Each CD spectrum was recorded as an average of three scans. The final spectra were background-corrected by subtracting the corresponding buffer spectra.

4.2.6. Cell uptakes and DNA platinations

The SKOV-3 cells were seeded in 6-well cell culture plate and incubated for 3 h at 37 °C. The cells were treated with platinum compounds including compounds **2**, **3** and reference drugs cisplatin, oxaliplatin at concentration of 100 μM for 10 h in incubator. Then all the cells were harvested, collected and washed with PBS for three times. *Ca.* 1 million cells were mineralized with 70% HNO_3 (LC), and the platinum in cells was determined with inductively coupled plasma mass spectrometry (ICP-MS). Results were presented as the mean of 3 determinations for each data point.

To test the DNA platination of the tumor cells, the DNA of *ca.* 1 million cells was isolated with a Genomic DNA Mini Preparation Kit. Then the DNA was mineralized with 70% HNO_3 and measured by ICP-MS. The results were calculated based on 3 determinations for each data point.

Abbreviations

AsA	ascorbic acid
BCA	bicinchoninic acid
COX	cyclooxygenase
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
FITC	fluorescein isothiocyanate
GMP	guanosine monophosphate
HPLC	high performance liquid chromatography
HSA	human serum albumin
IC_{50}	half maximal inhibitory concentration
ICP-MS	inductively coupled plasma mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSAIDs	nonsteroidal anti-inflammatory drugs
PBS	phosphate buffer saline
PVDF	polyvinylidene difluoride
RF	resistant factor
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBTU	*N,N,N′,N′*-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate
TEA	*N,N,N*-triethylamine
TI	therapeutic index

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.02.011.

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