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Combination of 7-hydroxycoumarin in a platinum(IV) complex derived from cisplatin enhanced cytotoxicity with multiple mechanisms of action

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ABSTRACT

A novel compound, Cou-platin, composed of 7-hydroxycoumarin and a platinum(IV) moiety derived from cisplatin was designed and synthesized. Significantly, Cou-platin exhibited more potent in vitro antitumor activity against all tested cancer cell lines than that of cisplatin, which was mainly attributed to the liberation of cisplatin and 7-hydroxycoumarin upon reduction with a biomolecular agent. Besides, cellular accumulation of Cou-platin was dramatically increased among several cancer cells in contrast to cisplatin. Flow cytometry study revealed that Cou-platin arrested cell cycle at G2 phase and induced cell apoptosis. Western blots results indicated that it not only activated cell apoptosis pathway, but also inhibited extracellular regulated protein kinases/mitogen-activated protein kinase pathway. In vivo tests showed that Cou-platin, at equimolar dose to cisplatin, could inhibit tumor growth in nude mouse HCT116 tumor xenograft models almost as cisplatin and oxaliplatin, but with less toxicity.

1. Introduction

Cisplatin has become one of the most popular agents in cancer chemotherapy since it was approved by FDA in 1978. After that, other cisplatin analogues, carboplatin and oxaliplatin have been successively approved (Fig. 1). Principal indications for cisplatin are metastatic testicular cancer, metastatic ovarian cancer, and transitional bladder cancer [1]. By cross-linking DNA and inhibiting DNA transcription [2], cisplatin can kill cancer cells in a relative high level and cure more patients compared with the drugs used before [3,4]. However cisplatin faces some embarrassments, such as drug resistance and side effects. Meanwhile, it has negligible effect on some certain kinds of cancer in vivo. Therefore, development of new platinum based anticancer agents with higher anticancer activity but less toxicity is very meaningful.

Platinum(IV) complexes with an octahedral geometry showed great promising due to their improved cytotoxicity and relatively lower toxicity [5–10]. Moreover, it has been reported that some platinum(IV) complexes were sensitive to some cisplatin-resistant cancer cell lines [11,12], indicating that platinum(IV) complexes can overcome cisplatin resistance. Usually, platinum(IV) complexes were generally designed as prodrugs by modification at the axial position with different functional groups, which may improve the anticancer efficacy, kinetic performance or selectivity of the platinum(IV) complexes against certain cancer cells [13–17].

The extracellular regulated protein kinases (ERK)/mitogen-activated protein kinase (MAPK) pathway plays a central role in regulating cell growth, proliferation and survival. Hence, ERK/MAPK pathway has emerged as a promising target for tumor therapy [18,19]. Coumarins, a wide class of natural compounds, are well known for their various biological functions, inclusive of antiviral [20], anti-inflammatory [21] and anticancer activities [22]. It has been found that some 7-hydroxycoumarin derivatives can induce cancer cell apoptosis through ERK/MAPK signaling pathway [23].

Based on the above, we herein report a novel dual-targeting platinum(IV) anticancer prodrug named Cou-platin, *cis*,*cis*,*trans*-{Pt$(NH_3)_2Cl_2(OH)$[2-((2-oxo-2H-chromen-7-yl)oxy)acetate]}, by introducing 7-hydroxycoumarin to the axial position of *cis*,*cis*,*trans*-[Pt$(NH_3)_2Cl_2(OH)_2$] derived from cisplatin, whose anticancer activities and underlying mechanisms were studied.

2. Results and discussion

2.1. Synthesis and characterization of Cou-platin

The preparation of Cou-platin is shown in Scheme 1. 7-Hydroxycoumarin was first treated with a linker, bromoacetic acid to produce

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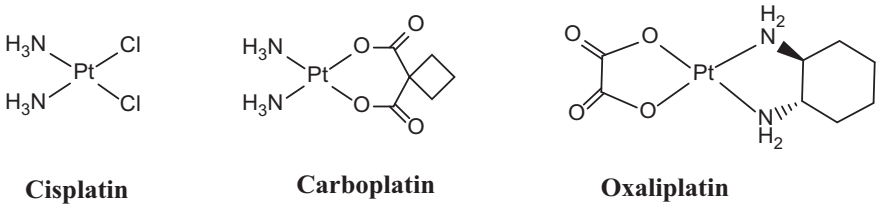
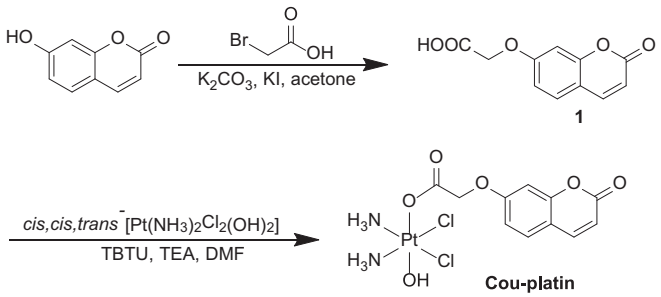


Fig. 1. FDA approved platinum(II) anticancer agents.



Scheme 1. Synthesis of the target compound, Cou-platin.

intermediate **1**. By taking the carboxylic acid of the linker, Cou-platin was then prepared by treatment of *cis,cis,trans*-$[Pt(NH_3)_2Cl_2(OH)_2]$ with equivalent molar amount of intermediate **1** in the presence of O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and triethylamine (TEA) in dried DMF. The final product of Cou-platin was characterized by microanalysis, ^{1}H NMR and ^{13}C NMR spectra together with ESI-MS mass spectrometry. The purity of Cou-platin was determined to be > 97% by reverse-phase HPLC (Fig. S1).

2.2. Reduction and stability study of Cou-platin

Ascorbic acid and glutathione are overexpressed in cancer cells [24], which gives platinum(IV) based anticancer drugs a target point so that they could be reduced by ascorbic acid or glutathione and release active free drugs. As Cou-platin was expected to be reduced by biomolecule such as ascorbic acid or glutathione, the reduction of Cou-platin was investigated in the presence of ascorbic acid by HPLC. As shown in Fig. 2, the reduction reaction of Cou-platin by ascorbic acid proceeded gradually. It was noted that the compound released from Cou-plaitn was 7-hydroxycoumarin instead of intermediate **1** as referring to the HPLC diagram of 7-hydroxycoumarin (Fig. S2), this might arise from the instability of phenolic ether bond in the presence of nucleophilic agents. Actually, the solution behavior of platinum(IV) complexes is very complicated because not only the reduction occurs at the axial position, but also the hydrolysis at the equatorial position also happens in the aqueous solution [16]. Despite cisplatin was not found due to its weak chromophore under the ultraviolet detecting condition in Fig. 2, those results indicated that Cou-platin as a platinum(IV) based anticancer prodrug could be reduced by ascorbic acid to release cytotoxic agents, which was consistent with our design. However, the specific reduction mechanism of Cou-platin needs further investigation in future.

The stability of Cou-platin was also studied by HPLC under both phosphate buffered saline (PBS, pH = 7.4) and biological conditions. As shown in Fig. S3, Cou-platin was stable in PBS solution for 12 h, and no disassociation was observed. However, no useful information was obtained in the solution of cell culture medium, because the peak of Cou-platin was overlapped by the constituent components of the medium, and we could not find a suitable condition to separate them for further analysis (Fig. S4).

2.3. In vitro cytotoxicity

The in vitro cytotoxicity of Cou-platin was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against six human cancer cell lines: HepG2 (hepatoma), HCT116 (colon), A549 (lung), MCF7 (breast), SGC7901 (stomach), SGC7901/cis (cisplatin resistant) and HUVEC (human umbilical vein endothelial cells) with cisplatin and 7-hydroxycoumarin as positive controls. The corresponding half maximal inhibitory concentration (IC_{50}) values are given in Table 1. As predicted, the cytotoxicity of 7-hydroxycoumarin was negligible. It is noted that Cou-platin showed superior cytotoxicity to cisplatin against all tested cancer cell lines. Particularly, Cou-platin showed significantly enhanced inhibitory effect against HCT116 cells with a 30-fold higher cytotoxicity than cisplatin. Besides, Cou-platin also exhibited increased toxicity against HepG2, SGC7901 and SGC7901/cis cancer cells with 29-fold, 23-fold and 19-fold lower IC_{50} values than cisplatin, respectively. Despite Cou-platin showed considerable anti-proliferation activity against cisplatin resistant SGC7901/cis cells but it did not overcome the drug tolerance. The resistance index of Cou-platin was calculated as 5.37, which was higher than that of cisplatin (resistance index = 4.38). However, the IC_{50} value against HUVEC cells of Cou-platin was 4-fold higher than that of cisplatin, suggesting that, in contrast to cisplatin, Cou-platin may be selectively cytotoxic for human cancer cells. These in vitro results encouraged us to further explore the mechanism of inhibitory effect of Cou-platin upon cancer cells.

2.4. Cellular accumulation

To figure out the underlying mechanism of the considerable cytotoxicity of Cou-platin, inductively coupled plasma mass spectrum (ICP-MS) was used to evaluate the cellular accumulation of Cou-platin in cancer cells. As shown in Fig. 3 and Table 2, platinum amount in HCT116 cells treated with Cou-platin was 1232 ± 65 ng/(10^6 cells), which was 37-fold higher than that of cisplatin. In other two cancer

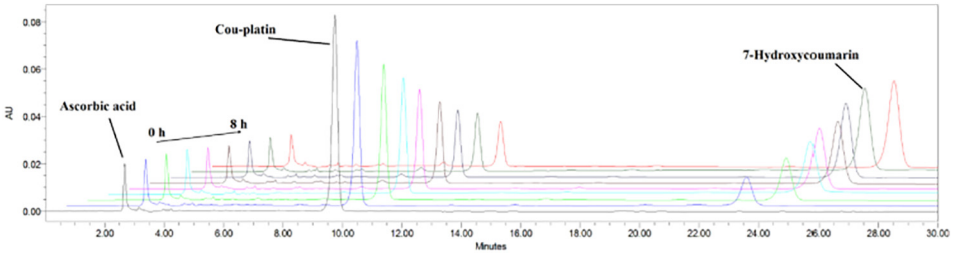


Fig. 2. HPLC analysis of Cou-platin incubated with ascorbic acid.

Table 1
IC_{50} values of Cou-platin and cisplatin against six human cancer cells.[a]

Cell types	Compounds, IC_{50} (μM)		
	Cisplatin	7-Hydroxycoumarin	Cou-platin
HepG2	6.94 ± 0.48	> 100	0.24 ± 0.05
HCT116	9.34 ± 0.85	> 100	0.31 ± 0.02
A549	4.51 ± 0.35	> 100	4.02 ± 0.26
MCF7	6.40 ± 0.48	> 100	2.46 ± 0.18
SGC7901	1.86 ± 0.15	> 100	0.08 ± 0.01
SGC7901/cis	8.15 ± 0.63	> 100	0.43 ± 0.02
HUVEC	7.86 ± 0.34	> 100	29.79 ± 1.58
Resistance index[b]	4.38	–	5.38

[a] Cancer cells were treated with a series dose of drugs for 72 h and measured with IC_{50} values. The data were presented as the mean ± SD for three independent experiments.

[b] Resistance index = (IC_{50} value of SGC7901/cis)/(IC_{50} value of SGC7901).

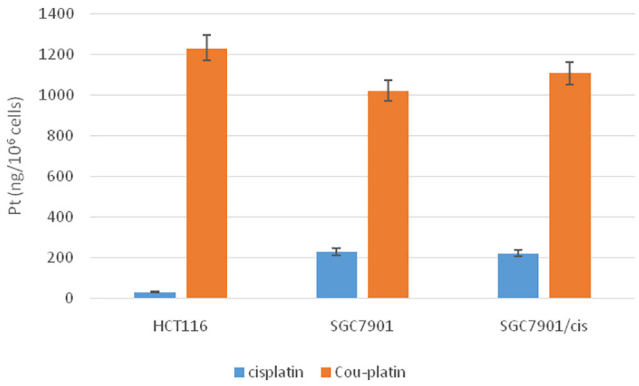


Fig. 3. Cellular accumulation of Cou-platin and cisplatin in three cancer cells. All tested cancer cells were incubated with cisplatin and Cou-platin (15 μM respectively) for 24 h at 37 °C. The Data were expressed as the mean ± SD for three independent experiments.

Table 2
Cellular platinum amount of Cou-platin and cisplatin in three cancer cell lines.[a]

Compounds	Pt (ng/10^6 cells) in cells		
	HCT116	SGC7901	SGC7901/cis
Cisplatin	33 ± 3	230 ± 17	222 ± 10
Cou-plaitn	1232 ± 65	1023 ± 49	1109 ± 57

[a] All tested cancer cells were incubated with cisplatin and Cou-platin (15 μM respectively) for 24 h at 37 °C. Results are expressed as the mean ± SD for three independent experiments.

cells, Cou-platin also possessed dramatically increased platinum amount compared with cisplatin, 1023 ± 49 ng/(10^6 cells) in SGC7901 and 1109 ± 57 ng/(10^6 cells) in SGC7901/cis. Just like other platinum(IV) based anticancer drugs reported before, Cou-platin also has a lipophilic group so that it can enter cancer cells more efficiently by passive diffusion and has such enhanced cellular accumulation pattern [25–29]. These results demonstrated that Cou-platin can enter cancer cells more efficiently than cisplatin after a treatment for 24 h and exhibits high cellular persistence [30]. Compared with the in vitro cytotoxicity results (Table 1), Cou-platin showed high anti-proliferation activity against both wild and resistant SGC7901 cells. This is in tune with the results of cellular accumulation. But it did not overcome the drug resistance. This implies that chemoresistance does not come from different drug accumulation.

2.5. Cell image

Due to the existence of a coumarin moiety, Cou-platin can be used as a track agent to show the drug uptake in cells under the laser scanning confocal microscopy (LSCM). HCT116 cells were incubated with Cou-platin (30 μM) for 3 h. Then the free drug was washed off and the treated cells were analyzed by LSCM. As shown in Fig. 4, with excitation by using 405 nm light source, green fluorescence was observed in HCT116 cells, indicating that Cou-platin could be uptaken by HCT116 cells. Besides, the morphology of HCT116 cells became round from spindle-shaped (Fig. 4A), demonstrating that the cells were undergoing apoptosis or death. The result was consistent with the corresponding cytotoxicity by MTT assay.

2.6. Cell cycle

Flow cytometry was used to investigate cell cycle block pattern of Cou-platin with cisplatin at equitoxic concentration (at concentration of IC_{50} value). Cou-platin arrested HCT116 cells in G2 phase as cisplatin did, which is a common phenomenon caused by anticancer drugs [31,32]. Unlike cisplatin reducing the portion of G1 phase of HCT116 cells dramatically, Cou-platin showed almost no effect on this phase (Fig. 5). Besides, after treatment of cisplatin, the percentage of S phase of cells was reduced to 54.44% compared with the negative control (58.92%). Interestingly, Cou-platin reduced the portion of S phase of HCT116 cells by 14.80%. This result was in line with an anticancer drug that contained a 7-hydroxycoumarin moiety reported previously [33]. Hence, both cisplatin and 7-hydroxycoumarin may exert their effect in the cell cycle of HCT116 cells.

2.7. Apoptosis studies

To further study the underlying anticancer mechanism of Cou-platin, the analysis of apoptosis of our compound and cisplatin toward

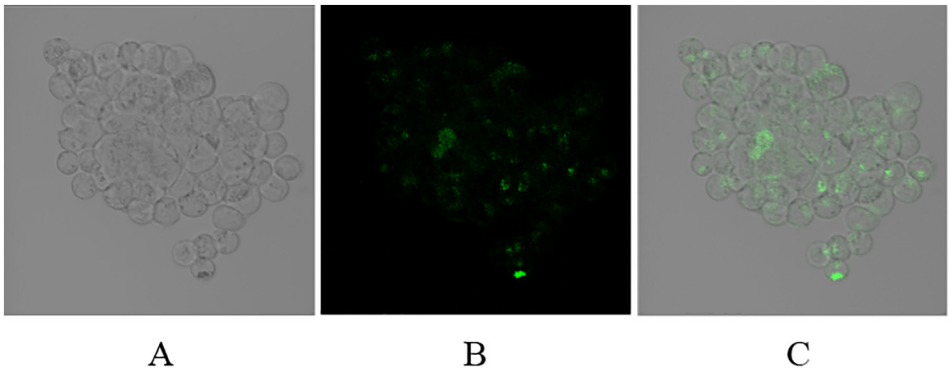


Fig. 4. Cell image of HCT116 cells treated with Cou-platin. (A) Bright field; (B) Fluorescence image; (C) Overlay of the merged images of (A) and (B). HCT116 cells were treated with 30 μM Cou-platin for 3 h. The excitation wavelength was 405 nm and emission wavelength was 488 nm.

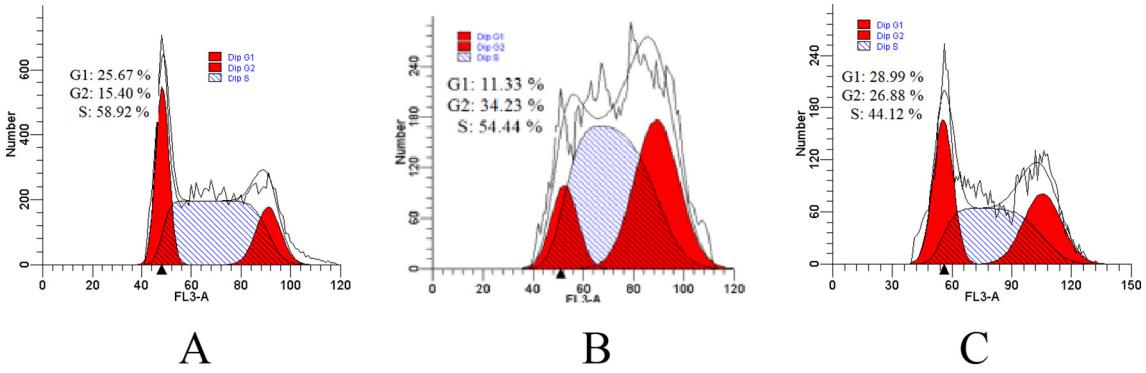
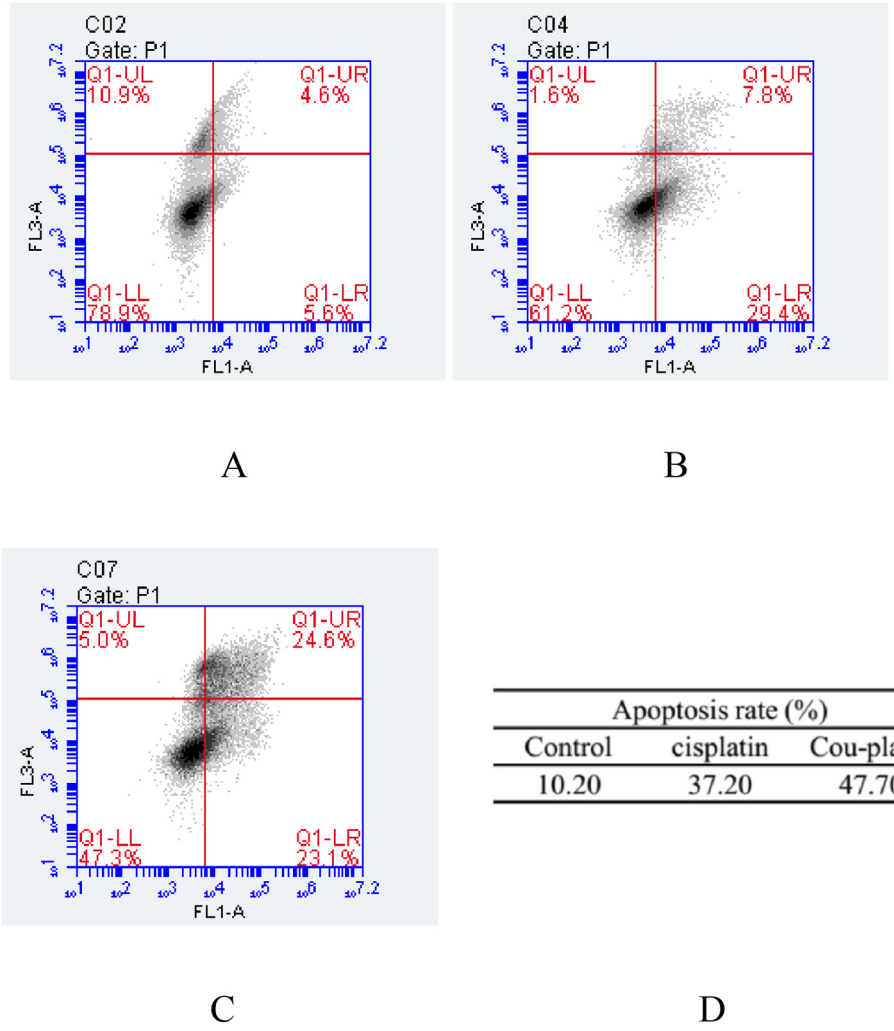


Fig. 5. Cell cycle distribution of HCT116 cells treated with (A) DMF; (B) cisplatin and (C) Cou-platin. HCT116 cells were incubated with 9.3 μM cisplatin and 0.3 μM Cou-platin for 24 h at 37 °C.



Apoptosis rate (%)		
Control	cisplatin	Cou-platin
10.20	37.20	47.70

Fig. 6. Cell apoptosis results of HCT116 cells treated with Cou-platin and cisplatin. (A) DMF; (B) Cisplatin and (C) Cou-platin are graphical representations; (D) Digital representation of apoptosis rate. HCT116 cell line was incubated with 9.3 μM cisplatin and 0.3 μM Cou-platin for 24 h at 37 °C.

HCT116 cells was studied at equitoxic concentration (at concentration of IC_{50} value) by Annexin V-FITC/Propidium Iodide (PI) assay. Cells treated with DMF were used as negative control. As shown in Fig. 6, Cou-platin caused 47.70% cells apoptosis, which was in accordance with MTT results, indicating that Cou-platin can inhibit HCT116 cells via apoptosis pathway more effectively than cisplatin.

2.8. Western blot assay

Based on the cell apoptosis and cycle results, Western blot technique was applied to investigate the influence of Cou-platin exerted on the related protein expression. HCT116 cells were incubated with cisplatin and Cou-platin for 24 h, respectively. The expression of apoptosis related protein in HCT116 cells was shown in Fig. 7A and B. It was noticed that Cou-platin upregulated the expression of Bax, cleaved

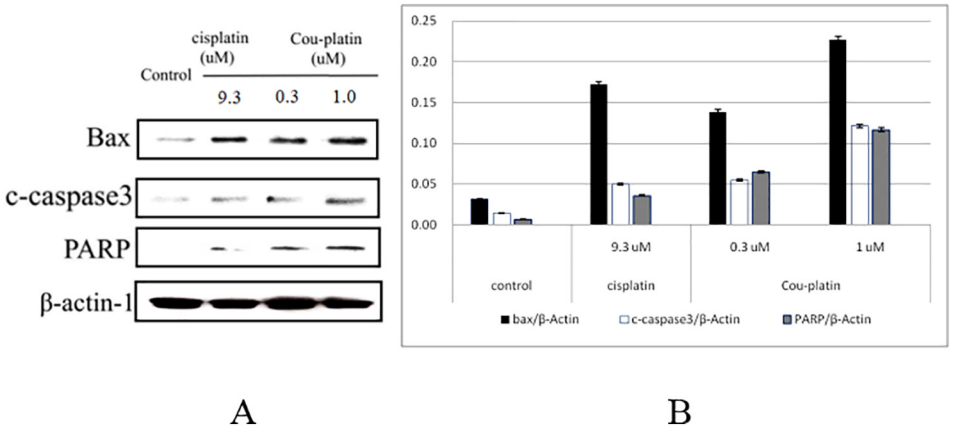


Fig. 7. Cell apoptosis pathway Western blot analysis of HCT116 cells incubated with cisplatin and Cou-platin. (A) Blots; (B) Relative gray intensity analysis. HCT116 cells were incubated with 9.3 μM cisplatin and 0.3 and 1.0 μM Cou-platin for 24 h at 37 °C. Relative gray intensity = (gray intensity of indicated protein)/(gray intensity of β-actin-1). The data are representative of three independent experiments and shown as the mean ± S.D.

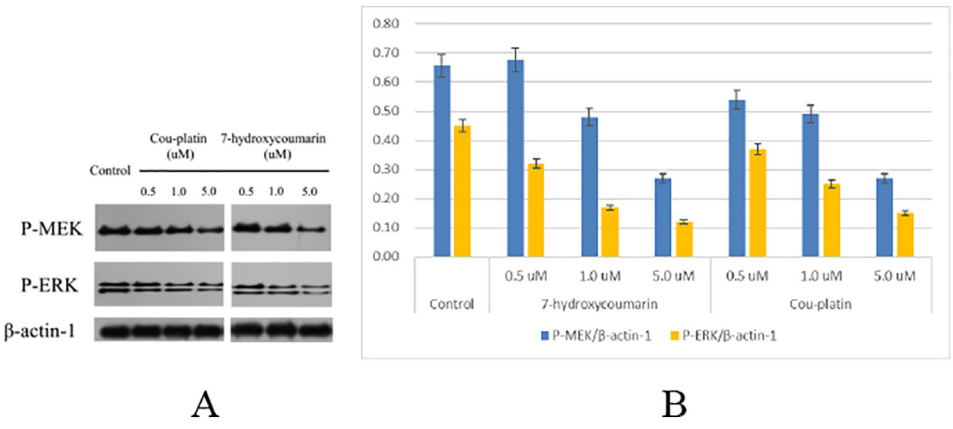


Fig. 8. ERK/MAPK pathway Western blot analysis of HCT116 cells incubated with 7-hydroxycoumarin and Cou-platin. (A) Blots; (B) Relative gray intensity analysis. HCT116 cells were incubated with 7-hydroxycoumarin and Cou-platin (0.5, 1.0, 5.0 μM) for 24 h at 37 °C. Relative gray intensity = (gray intensity of indicated protein)/(gray intensity of β-actin-1). The data are representative of three independent experiments and shown as the mean ± S.D.

caspase3, cleaved PARP and cyto-c (Fig. S5) in a concentration dependent manner, which indicated that Cou-platin could activate the apoptosis pathway and finally inhibited the proliferation of HCT116 cells. Taken the cellular accumulation results, this may come from the enhanced drug accumulation of Cou-platin [34,35]. Moreover, we further investigated the inhibitory effect of Cou-platin on phosphorylation of mitogen-activated extracellular signal-regulated kinase 1 (MEK1, key node of ERK/MAPK pathway) and its unique downstream target ERK1 [18]. As shown in Fig. 8A and B, Cou-platin indeed downregulated the phosphorylation of MEK1 and ERK1 in a concentration dependent manner and shared the same trend and degree as the positive control. It should be noted that cisplatin also possess the ERK/MAPK pathway modulation ability [36]. As a derivative of 7-hydroxycoumarin, Cou-platin could inhibited ERK/MAPK pathway efficiently.

2.9. In vivo antitumor activity

Encouraged by the in vitro results, we further investigated the in vivo tumor inhibitory effect of Cou-platin in nude mouse HCT116 tumor xenograft models. Mice with tumors were divided into five groups including (1) control, (2) cisplatin (5 mg/kg), (3) oxaliplatin (5 mg/kg), (4) Cou-plaitn (5 mg/kg, equal mass dose to cisplatin), and (5) Cou-plaitn (9 mg/kg, equimolar dose to cisplatin), which were treated by tail vein injection once a week for 21 days. Tumor volumes and weights are illustrated in Fig. 9A and B. In the equal mass dose, unfortunately, Cou-plaitn exhibited a little weaker inhibitory activity than cisplatin and oxaliplatin. While in the equimolar dose, the in vivo antitumor activity of Cou-plaitn was promoted, which was nearly comparable to that of cisplatin. However, it was of interest to note that Cou-platin was less toxic than cisplatin and oxaliplatin upon the change of body weight of mice treated with Cou-platin in contrast to that with the positive drugs (Fig. 9C).

3. Conclusions

In summary, a novel compound, named Cou-platin, comprising 7-hydroxycoumarin and a platinum(IV) moiety derived from cisplatin was designed and obtained via a linker. HPLC assay showed that the compound could release cisplain and 7-hydroxycoumarin upon reduction by ascorbic acid as expected. But the specific reduction mechanism of Cou-platin needs a further studied. In vitro test indicated that Cou-platin had stronger anticancer activity against all the tested cancer cell lines than cisplatin. Related experiment exhibited that introduction of 7-hydroxycoumarin led to the increased cellular accumulation of Cou-platin in several cancer cells in contrast to cisplatin. Both cell cycle and cell apoptosis analysis indicated that Cou-platin arrested HCT116 cell cycle in a mix manner of cisplatin and 7-hydroxycoumarin and could induce cell apoptosis more efficiently than cisplatin. Western blot analysis revealed that Cou-platin inhibited HCT116 cells via cell apoptosis pathway and the ERK/MAPK pathway. In vivo tests showed that Cou-platin, at equimolar dose to cisplatin, could effectively inhibit tumor growth in nude mouse HCT116 tumor xenograft models almost the same as cisplatin and oxaliplatin, but with less toxicity. However, we are aware that the limitation of using the equimolar dose in the in vivo test. Consequently, our work demonstrated that 7-hydroxycoumarin and cisplatin have a jointed effect on the antitumor activity of the resulting platinum(IV) complex, which can be useful for developing new anticancer agents.

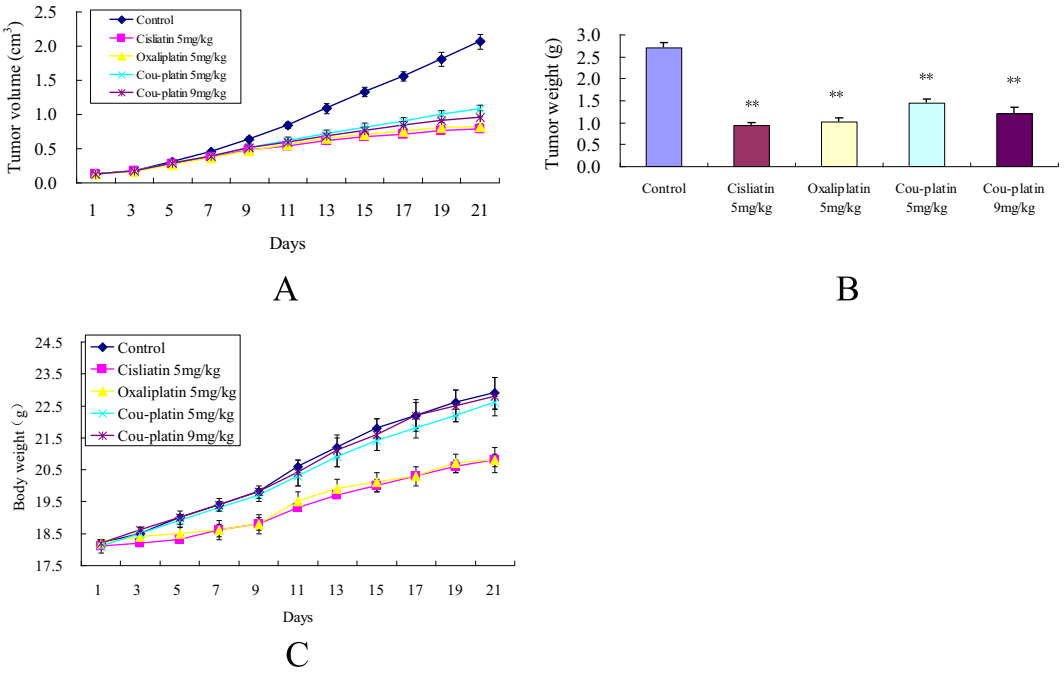


Fig. 9. In vivo antitumor activity of Cou-platin in nude mouse HCT116 tumor xenograft models. (A) Tumor volume of mice during the administration period; (B) Tumor weight of mice; (C) Curve of mice body weight recorded during administration period. Mice were sacrificed and tumors were weighed after 21-day treated with drugs. The data were presented as the mean ± SEM. *P < 0.05.

4. Materials and methods

4.1. HPLC experiments

For reduction experiment, 0.5 ml of Cou-platin (0.3 mM) in PBS (pH = 7.4, contains 10% DMF) and 0.5 ml of ascorbic acid (3.0 mM) in PBS (pH = 7.4) were mixed and incubated at 37 °C in dark for 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h and 8 h respectively and then studied by HPLC with an eluent comprised of MeOH and H_2O (1:10, v:v, containing 1% trifluoroacetic acid) at r.t. Flow rate was 1.0 ml/min. Reversed-phase HPLC was carried out on a ODS column (250 × 4.5 mm). The wavelength of UV detector was 280 nm. For stability experiment, 0.5 ml of Cou-platin (3.0 mM) in PBS (pH = 7.4, contains 10% DMF) was incubated at 37 °C in dark and studied by HPLC every 3 h with the same condition as reduction experiment.

4.2. Cell culture

Human cancer cell lines HepG2, HCT-116, A549, MCF7, SGC-7901, SGC-7901/cis and HUVEC were purchased from Jiangsu KeyGEN BioTECH company (China). HepG2, A549, MCF7 and HUVEC cell lines were cultured in 5% CO_2 with DMEM added by 1% penicillin/streptomycin (Beyotime, Nantong, China) and 10% fetal bovine serum (FBS) (Hyclone, Lifescience, USA) at 37 °C. HCT116 and SGC-7901 cell lines were cultured in 5% CO_2 with RPMI-1640 added by 1% penicillin/streptomycin and 10% FBS. SGC-7901/cis cell line was cultured and screened in RPMI-1640 added by 1% penicillin/streptomycin, 10% FBS and 800 ng/ml cisplatin before use. Cancer cell lines were passed every 2 days and recovered from frozen stocks upon reaching pass number 20.

4.3. MTT assay

In vitro anticancer activity of cisplatin and Cou-platin against HepG2, HCT116, A549, MCF7, SGC7901 and SGC7901/cis cancer cell lines were carried out by the MTT assay. Cancer cells were plated in 96-well plates at density of 10^5/ml per well. After an overnight culturing, cells were exposed to culture medium containing cisplatin and Cou-platin separately at a series of concentrations and incubated for 72 h at 37 °C. Then the cell viability was evaluated by the MTT method according to literature method [37].

4.4. Cellular accumulation

Drug uptake of HCT116, SGC7901 and SGC7901/cis cells against cisplatin and Cou-platin was evaluated by ICP-MS. After an overnight culturing in 6-well plates, cancer cells were incubated with cisplatin and Cou-platin (15 μM respectively) for 24 hat 37 °C. Then cancer cells were washed by PBS buffer (pH 7.4) for three times, and harvested after trypsinization. The cells were concentrated and digested by addition of HNO_3 to obtain the samples. Finally, the samples were examined by ICP-MS.

4.5. Cell image

HCT116 cells were cultured in a six well plate containing a coverslip. Then the cells were incubated with 30 μM Cou-platin for 3 h. After that, the cells were washed with PBS for three times to wipe out the free drug. Then the coverslip was taken out and put on a glass slid. The glass slid was detected by LSCM operating at a 405 nm excitation wavelength and at 488 nm detecting emission wavelength.

4.6. Cell cycle

Cell cycle was carried out by flow cytometry (FCM) according to a method report previously [38]. Data were collected and analyzed by FlowJo software (TreeStar, Inc.).

4.7. Cell apoptosis

According to manufacturer's protocol (Shanghai Yisheng Biotechnology Co. Ltd., 40302ES50) of Annexin V-FITC assay, HCT116 cells were treated with 9.3 μM cisplatin and 0.3 μM Cou-platin at 37 °C for 24 h, respectively. Then the cells were collected and resuspended in binding buffer (pH 7.5, 10 mM HEPES, 2.5 mM $CaCl_2$ and 140 mM

NaCl). After that, cells were incubated with Annexin V-FITC and PI in dark for 10 min at r.t. Finally, cells were analyzed with flow cytometry (FACSCalibur, Becton Dickinson, USA) and computer station running Cell-Quest software (BD Biosciences, Franklin Lakes, NJ, USA).

4.8. Western blot

HCT116 cells were cultured until the cell density reached 80%. Then positive control (cisplatin or 7-hydroxycoumarin) and Cou-platin were added, and the cells were cultured for 24 h at 37 °C. Proteins were extracted by lysis buffer. The concentration of protein was measured using the BCA (bicinchoninic acid) assay with a Varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA). Then protein (20 mg/lane) in equal concentration were separated by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Bio-Rad) in transblot apparatus (Bio-Rad). The blots were blocked with 5% defatted milk powder in TBST (Tris-buffered saline plus 0.1% Tween 20) for 1 h, and then incubated with a series of primary antibodies against Bax, PARP, caspase3, cytochrome *c*, p-MEK and p-ERK overnight at 4 °C. After that, the membrane was washed with PBST three times and incubated with IRDye 800 conjugated secondary antibody for 1 h at 37 °C. Detection was performed by an Odyssey scanning system (Li-COR, Lincoln, Nebraska). GAPDH or β-actin was used as loading control.

4.9. In vivo evaluation

25 nude mice (BALB/c; purchased from Shanghai Ling Chang biotechnology company, China; body weight range from 15 to 18 g) were divided into five groups randomly (control, cisplatin 5 mg/kg, oxaliplatin 5 mg/kg, Cou-platin 5 mg/kg and Cou-platin 9 mg/kg). HCT116 cells in PBS (10^7 cells in 0.1 ml) were injected subcutaneously into the right axilla of nude mice. When the volume of volume reached 100–150 mm^3 on day 14, equal volume of drugs was administrated at indicated concentration via tail vein injection. 5% dextrose injection was used as a vehicle and negative control. Cisplatin and oxaliplatin were dissolved in vehicle while Cou-platin was dissolved in a small amount of DMF and then diluted with Tween 80 and 5% dextrose injection (DMF: Tween 80: 5% dextrose injection = 10: 2: 88). Tumor volume and body weight were recorded every other day after drug administration. All mice were sacrificed after 3 weeks of administration and the tumor volumes were measured with electronic digital calipers and determined by measuring length (a) and width (b) to calculate volume ($V = ab^2/2$).

Acknowledgments

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Appendix A. Supplementary data

The synthesis procedure of Cou-platin in detail, HPLC analysis of Cou-platin, standard sample of 7-hydroxycoumarin, stability study of Cou-platin and cyto-C Western blot analysis. This material is available free of charge via the Internet. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2018.05.015.

References

[1] N.P. Farrell, Drugs Future 37 (2012) 795–806.
[2] R.C. Todd, S.J. Lippard, Metallomics 1 (2009) 280–291.
[3] G.J. Bosl, R.J. Motzer, N. Engl. J. Med. 337 (1997) 242–254.
[4] P.J. O'Dwyer, J.P. Stevenson, S.W. Johnson, B. Lippert (Ed.), Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug, Verlag Helvetica Chimica Acta, Zürich, 1999, p. 31.
[5] X. Wang, Z. Guo, Chem. Soc. Rev. 42 (2013) 202–224.
[6] S. Dhar, S.J. Lippard, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 22199–22204.
[7] Y. Yuan, R.T.K. Kwok, B.Z. Tang, B. Liu, J. Am. Chem. Soc. 136 (2014) 2546–2554.
[8] Q. Cheng, H. Shi, H. Wang, Y. Min, J. Wang, Y. Liu, Chem. Commun. 50 (2014) 7427–7430.
[9] R.K. Pathak, S. Marrache, J.H. Choi, T.B. Berding, S. Dhar, Angew. Chem. Int. Ed. 53 (2014) 1963–1967.
[10] M.D. Hall, T.W. Hambley, Chem. Rev. 232 (2002) 49–67.
[11] L.R. Kelland, G. Abel, M.J. McKeage, M. Jones, P.M. Goddard, M. Valenti, B.A. Murrer, K.R. Harrap, Cancer Res. 53 (1993) 2581–2586.
[12] K.R. Harrap, L.R. Kelland, M. Jones, P.M. Goddard, R.M. Orr, S.E. Morgan, B.A. Murrer, M.J. Abrams, C.M. Giandomenico, T. Cobbleigh, Adv. Enzym. Regul. 31 (1991) 31–43.
[13] V. Pichler, J. Mayr, P. Heffeter, O. Dömötör, É.A. Enyedy, G. Hermann, D. Groza, G. Köllensperger, M. Galanksi, W. Berger, B.K. Keppler, C.R. Kowol, Chem. Commun. 49 (2013) 2249–2251.
[14] L. Ma, R. Ma, Y. Wang, X. Zhu, J. Zhang, H.C. Chan, W. Zhang, X. Chen, S.–.K. Chiu, G. Zhu, Chem. Commun. 51 (2015) 6301–6304.
[15] Z. Xu, J. Zhao, S. Gou, G. Xu, Chem. Commun. 53 (2017) 3749–3752.
[16] J. Zhao, Z. Xu, J. Lin, S. Gou, Inorg. Chem. 56 (2017) 9851–9859.
[17] J. Zhao, W. Hua, G. Xu, S. Gou, J. Inorg. Biochem. 176 (2017) 175–180.
[18] P.J. Roberts, C.J. Der, Oncogene 26 (2007) 3291–3310.
[19] R. Hoshino, Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-i, H. Wada, J. Fujimoto, M. Kohno, Oncogene 18 (1999) 813 – 822.
[20] M. Curini, F. Epifano, F. Maltese, M.C. Marcotullio, S.P. Gonzales, J.C. Rodriguez, Aust. J. Chem. 56 (2003) 59–60.
[21] K.C. Fylaktakidou, D.J. Hadjipavlou-Litina, K.E. Litinas, D.N. Nicolaides, Curr. Pharm. Des. 10 (2004) 3813–3833.
[22] D. Conley, E.M. Marshall, Proc. Am. Assoc. Cancer Res. 28 (1987) 63.
[23] C. Wang, F. Xu, Y. Niu, Y. Wu, J. Sun, Y. Peng, L. Liang, P. Xu, Lett. Drug Des. Discov. 10 (2013) 727–732.
[24] A.I. Fiaschi, A. Cozzolino, G. Ruggiero, G. Giorgi, Eur. Rev. Med. Pharmacol. Sci. 9 (2005) 361–367.
[25] L.R. Kelland, B.A. Murrer, G. Abel, C.M. Giandomenico, P. Mistry, K.R. Harrap, Cancer Res. 52 (1992) 822–828.
[26] L.R. Kelland, P. Mistry, G. Abel, S.Y. Loh, C.F. O'Neill, B.A. Murrer, K.R. Harrap, Cancer Res. 52 (1992) 3857–3864.
[27] K.J. Mellish, L.R. Kelland, Cancer Res. 54 (1994) 6194–6200.
[28] M.G. Ormerod, R.M. Orr, C.F. O'Neill, T. Chwalinski, J.C. Titley, L.R. Kelland, K.R. Harrap, Brit. J. Cancer. 74 (1996) 1935–1943.
[29] M.J. Mckeage, G. Abel, L.R. Kelland, K.R. Harrap, Brit. J. Cancer 69 (1994) 1–7.
[30] M. Ravera, E. Gabano, I. Zanellato, I. Bonarrigo, M. Alessio, F. Arnesano, A. Galliani, G. Natile, D. Osella, J. Inorg. Biochem. 150 (2015) 1–8.
[31] F. Gümüş, G. Eren, L. Açik, A. Celebi, F. Öztürk, S. Yılmaz, R.I. Sağkan, S. Gür, A. Özkul, A. Elmalı, Y. Elerman, J. Med. Chem. 52 (2009) 1345–1357.
[32] J. Zhao, D. Wang, G. Xu, S. Gou, J. Inorg. Biochem. 175 (2017) 20–28.
[33] M.M. Liu, X.-Y. Chen, Y.-Q. Huang, P. Feng, Y.–.L. Guo, G. Yang, Y. Chen, J. Med. Chem. 57 (2014) 9343– – 9356.
[34] E. Gabano, M. Ravera, D. Osella, Dalton. T. 43 (2018) 9813–9820.
[35] M. Ravera, E. Gabano, I. Zanellato, A. Gallina, E. Perin, A. Arrais, S. Cantamessa, D. Osella, Dalton. T. 46 (2017) 1559–1566.
[36] A. Gozdz, A. Vashishta, K. Kalita, E. Szatmari, J.J. Zheng, S. Tamiya, N.A. Delamere, M. Hetman, J. Neurochem. 106 (2008) 2056–2067.
[37] F.H. Chen, N. Lu, H.W. Zhang, L. Zhao, L.C. He, H.P. Sun, Q.D. You, Z.Y. Li, Q.L. Guo, Mol. Pharmacol. 82 (2012) 958–971.
[38] A. Siddiqui-Jain, D. Drygin, N. Streiner, P. Chua, F. Pierre, S.E. O'Brien, J. Bliesath, M. Omori, N. Huser, C. Ho, C. Proffitt, M.K. Schwaebe, D.M. Ryckman, W.G. Rice, K. Anderes, Cancer Res. 70 (2010) 10288–10298.