Electronic supplementary information

Assessment of the antitumor activity of a cyclopalladated ferrocene compound assisted by a dual-targeting drug delivery system

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1. Experiment Section

Materials

Ferrocenecarboxaldehyde, (S)-(+)-Tetrahydrofurfurylamine, $NaPdCl_4$, PPh_3 were from Titan (Shang Hai, China). Hyaluronan (HA) (MW 10 kDa), mono (6-amino-6-deoxy)-beta-cyclodextrin, N-hydroxysulfosuccinimide sodium salt (NHSS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were from Dalian Meilum Biotech Co., Ltd (Dalian Chian), phosphate buffer saline (PBS) were form beyotime (Shanghai, China).

Instruments

Melting points were measured on a Meltemp melting point apparatus. Optical rotation was measured with Perkin elmer model 341 polarimeter. ^1H NMR spectra were recorded on Bruker AM400 NMR spectrometer. Chemical shifts were reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$, δ = 7.26 ppm). Spectra were reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t =triplet, q = quartet, m = multiplet), coupling constants (Hz), integration and assignment. ^{13}C NMR spectra were collected on commercial instruments (100 MHz) with complete proton decoupling. Chemical shifts are reported in ppm from the tetramethylsilane with the solvent resonance as internal standard ($CDCl_3$, δ = 77.0 ppm). Ms spectra were recorded on UPLC-Xevo™ TQMS system equipped with an ESI source. C, H and N elemental determination were performed on a Euro EA 3000 elemental analyzer (Leeman, USA). Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Size distribution and zeta potential were measured using Mastersizer 3000; TEM were measured with Transmission Electron Microscope (Hitachi TM-1000), SEM was measured with scanning electron microscope (Hitachi H-7650). The concentrations of [Fc] were measured with Inductively Coupled Plasma Optical Emission (ICP-OES Optima 7000 DV), The concentrations of [Pd] were measured with ICP-MS (Agilent 7900 ICP-MS).

Compound **C1**: ferrocenecarboxaldehyde (2.14 g, 10 mmol) and (S)-(+)-Tetrahydrofurfurylamine (1.01 g, 10 mmol) were dissolved in dry toluene (100 mL). The flask containing the reaction mixture was connected to a condenser equipped with a Dean-Stark apparatus. The red solution was refluxed over an oil bath for about 6 h and then the carefully transferred into a Schlenk tube, into with 5 Å molecular sieve (3.0 g) were introduced. The mixture was further refluxed for 18 h and then washed with n-hexane. Characterization data for:

C1: yield: 1.88 g (66.4%); m.p. 63.7-64.2 °C, [α]20 D +25.2 (c 1.0 in CHCl3); ^1H NMR(400 MHz, $CDCl_3$, 25 °C, TMS): δ=8.13 (s, 1H), 4.64 (d, *J*=1.7 Hz, 2H), 4.40 – 4.29 (m, 2H), 4.19 (s, 5H), 4.17-4.09 (m, 1H) 3.89(dt, *J*=13.58, 6.8 Hz, 1H), 3.77 (dd, *J*=14.6, 7.4 Hz , 1H), 3.56 (d, *J* =5.3 Hz, 2H), 2.09-1.96 (m, 1H), 1.96-1.86 (m, 1H), 1.76-1.64 (m, 1H).

Compound **C2**: C1 (297 mg, 1.0 mmol) was added to a methanolic (30 mL) solution containing $NaPdCl_4$ (290 mg, 1 mmol) and $NaOAc{\cdot}3H_2O$ (140 mg, 1.0 mmol), and stirred at room temperature for 24 h, the result reaction mixture was dried

under high vacuum. The product was extracted into chloroform and passed through a SiO_2-column using $CHCl_3$/MeOH (9.5:0.5) as eluent. Concentration of the eluted solution produced **C2**. Characterization date for:

C2: yield: 0.19g (48%); m.p. 182-184 °C, [α] = -384.8 (**c** 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ=7.86 (s, 2H，N=CH), 4.78 (d, *J* = 14.2 Hz, 2H, NCH_2), 4.47-4.25 (m, 16H, C_5H_5+H^5,H^4 in C_5H_3+NCH_2), 4.00-3.60 (m, 6H, OCH_2+H^3 in C_5H_3), 3.19 (d, *J* = 8.7 Hz, 2H, OCH), 2.12 (d, *J* = 25.7 Hz, 2H, OCHCH2), 1.94 (d, *J* = 4.5 Hz, 4H, OCH_2CH_2), 1.65 (dd, *J* = 13.9, 5.9 Hz, 2H, $OCHCH_2$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ=174.3 (N=CH), 100.0 (C^1 in C_5H_3), 86.6 (C^2 in C_5H_3), 77.0 (C^5 in C_5H_3), 73.3 (C^3 in C_5H_3), 70.3 (C^4 in C_5H_3), 67.8 (C_5H_5), 65.8 (OCH_2), 63.2 (NCH_2), 62.0 (OCH), 28.7 (CH_2CH_2), 25.8 (CH_2CH_2) ppm; MS (ES+): calcd for $C_{32}H_{36}Cl_2Fe_2N_2O_2Pd_2$ $[M\text{-}Cl]^+$: 840.93, Found: 841.20; Anal. calcd for $C_{32}H_{36}Cl_2Fe_2N_2O_2Pd_2$: C, 43.87; H, 4.14; N, 3.20. Found: C, 43.85; H, 4.14; N, 3.20.

Compound **CP**: C2 (170 mg, 0.2 mmol) were added to an acetone (10 ml) solution containing PPh_3 (260 g 10 mmol), stirred at room temperature for 2 h. The result reaction mixture was dried under high vacuum. The product was extracted into dichloromethane and passed through a SiO_2-column with 4:1 petroleum ether/ethyl acetate. Concentration of the eluted solution of one successive red band produced, and compound **CP** which were recrystallized from dichloromethane/n-hexane (1:5) as reddish yellow plates. Characterization data for:

CP: yield: 0.13g (92%); m.p. 188-190 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ=8.14 (d, *J* = 8.3 Hz, 1H，N=CH), 7.76 (dd, *J* = 10.8, 7.4 Hz 6H, H^2, H^6 in Ph), 7.49 – 7.32 (m, 9H, H^3, H^4, H^5 in Ph), 4.41 – 4.33 (m, 3H, NCH_2+H^5 in C_5H_3), 3.98 (s, 1H, H^4 in C_5H_3), 3.93 (s, 5H, C_5H_5), 3.79 (dd, *J* = 15.1, 6.9 Hz, 1H, OCH_2), 3.71 (dd, *J* = 15.0, 7.1 Hz, 1H, OCH_2), 3.27 (s, 1H, H^3 in C_5H_3), 3.19 – 3.07 (m, 1H, OCH), 2.11-1.99 (s, 1H, $OCHCH_2$), 1.87 – 1.77 (m, 2H, OCH_2CH_2), 1.50 -1.38 (m, 1H, $OCHCH_2$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ=173.2 (N=CH), 134.9 (C_1 in Ph), 131.8 (C_2 and C_6 in Ph), 130.4 (C_3 and C_5 in Ph), 128.0 (C_4 in Ph), 103.3 (C_1 in C_5H_3), 87.5 (C_2 in C_5H_3), 77.8 (C_5 in C_5H_3), 76.6 (C_3 in C_5H_3) , 70.4 (C_5H_5), 69.1 (C_4 in C_5H_3), 67.6 (OCH_2), 66.4 (NCH_2), 61.8 (OCH), 28.6 ($OCHCH_2$), 25.6 (OCH_2CH_2) ppm. ^{31}P NMR (162 MHz, $CDCl_3$) δ 37.4. MS (ES+): calcd for $C_{34}H_{33}ClFeNOPPd$ $[M\text{-}Cl]^+$: 664.07, Found: 664.01. Anal. Calcd for $C_{34}H_{33}ClFeNOPPd$: C, 58.31; H, 4.75; N, 2.00. Found C, 58.32; H, 4.77; N, 1.98.

Synthesis of HACDs

Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (167.7 mg, 0.875 mmol) and N-hydroxysulfosuccinimide sodium salt (NHSS) (190 mg, 0.875 mmol) were added to a solution of HA (Mw = 10 000) (200 mg, 10 μmol, containing carboxyl 0.5mmol) in PBS (30 mL), and the mixture was stirred at room temperature for 30 min. Then, various amounts of mono-6-deoxy-6-ethylenediamino-β-CD (100-600 mg, 0.085-0.509 mmol) were added respectively, and the mixture was stirred for 24 h at room temperature. The resulting solution was dialyzed against an excess amount of deionized water for 5 days (change water per 4 h in day time). After being freeze-dried, HACDs with different degrees of substitution were obtained as white powder.

^{1}H NMR (400 MHz, D_2O, 25 ˚C): (1) HACD-1.97: δ 2.01 (s, 3H, H of methyl group of HA), 2.95-4.02 (m, 14.97H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.51 (dd, *J* = 32.4, 7.1 Hz, 2H, H of HA), 5.01-5.17 (m, 0.084H, H of C-1 of β-CD); (2) HACD-3.22: δ 2.01 (s, 3H, H of methyl group of HA), 3.01-4.07 (m, 15.94H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.51 (dd, J = 32.7, 7.5 Hz, 2H, H of HA), 4.99-5.19 (m, 0.96H, H of C-1 of β-CD); (3) HACD-4.55: δ 2.01 (s, 3H, H of methyl group of HA), 2.97-4.04 (m, 18.94H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.41-4.58 (m, 2H, H of HA), 5.00-5.20 (m, 1.36H, H of C-1 of β-CD); (4) HACD-5.94: δ 2.01 (s, 3H, H of methyl group of HA), 2.99-4.05 (m, 19.83H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.49 (d, *J* = 32.7 Hz , 2H, H of HA), 5.00-5.21 (m, 1.77H, H of C-1 of β-CD); (5) HACD-8.55: δ 2.01 (s, 3H, H of methyl group of HA), 2.98-4.09 (m, 20.27H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.39-4.58 (m, 2H, H of HA), 5.03-5.20 (m, 2.55H, H of C-1 of β-CD); (6) HACD-8.55: δ 2.01 (s, 3H, H of methyl group of HA), 2.98-4.09 (m, 20.27H, H of HA and C-3, C-5, C-6, C-2, C-4, and methylene on ethanediamine group of β-CD), 4.38-4.59 (m, 2H, H of HA), 5.00-5.16 (m, 2.55H, H of C-1 of β-CD).

Characterization of the HACD/CP and β-CD/CP Nano-micelles

The size and zeta potential in water of the nano-micelles HACD/CP and β-CD/CP were obtained using Mastersizer 3000 with a wavelength of 532 nm and a scattering angle of 90° at 25 °C. Each sample was tested for three times with the mean diameter shown for three replicate samples. The morphology was inspected by using TEM and SEM. Sample solution with a concentration of 20 mg/mL was dropped onto a copper grid for TEM or mica plate for SEM and dried at 37 °C, respectively. TEM inspections were made with an accelerating voltage of 80 kV.

Drug Releasing Behavior of HACD/CP and β-CD/CP

The drug release of the supramolecular micelles was investigated in phosphate buffer solution (PBS, pH 7.4, Beyotime, Haimen, China). In brief, 2 mL of β-CD/CP or HACD/CP micelles solution (50 mg/mL) was placed in a dialysis bag (MWCO = 3500) at 37 °C for the dialysis experiment in PBS solution containing various H_2O_2 (0, 0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM) concentrations for drug releasing. At certain time intervals, 4 mL of PBS medium was aspirated and another 4 ml of fresh PBS was added. The amount of Fe in the solution outside the dialysis bag was determined by ICP-AES, and the amount of CP was calculated the concentration of Fe. Besides, the dyndall effect was measured red laser pointer in dark place after incubated with H_2O_2 for 4 h at 37 ˚C.

HACD/CP (20 mg/ml) and hyaluronidase (HAase, Dalian Meilum Biotech Co., Ltd) were added to a penicillin bottle, after incubated at 37 ˚C for 4 h, the dyndall effect was measured with red laser pointer in dark place and the size distribution was measured with DLS.

Biological Studies

Human MDA-MB-468 cells and NIH3T3 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere.

CD44 Expression

About 100 µl of MDA-MB-231 or NIH3T3 cells (5x 10^4 cells) were incubated with 1 µl of anti-human CD44 antibody (Beyotime, Shanghai, China) for 30 minutes on ice. Then cells were rinsed twice using PBS to remove the unbound antibody. Next, the second antibody IgG(H+L) (FITC labeled, Beyotime) was added and incubated for 10 min and the cells were harvested and washed with ice-cold PBS. The fluorescence of the cells was analyzed using a Gallios flow cytometer (Beckman Coulter) in FL6 channel. Cells with no staining were set as negative control.

Cell Uptake Assay

5*10^5 MDA-MB-468 cells were seeded in 6-well plates with 2 mL of DMEM. After incubated for 24h, HACD/CP+HA (MDA-MB-468 cells were incubated with HA for 1 h, and then **CP** was added), HACD/CP, **CP** and DMSO were added. The final concentration of CP in HACD/CP+HA, HACD/CP and CP incubated with cells were 5 µM. After 6 h, the cells were washed with PBS for 3 times and harvest after counting. The cells were digested with nitric acid and the Pd concentration was measured by ICP-MS.

Cell Viability Assay

Cells were seeded in 96-well plates at a density of 5 × 10^3 cells/well with 100 µL medium. Cultured cells were treated with CP, HACD/CP, HACD/CP+HA, β-CD/CP, HACD or cisplatin at the indicated concentrations. After 72 h, 10 µL Alamar Blue reagent (Solarbio, Beijing, China) was added to the medium and the cells were incubated for 2–4 h until the color turned from blue to pink. The relative fluorescence intensity was measured using a Thermo Scientific Varioskan Flash multimode reader.

Cell Cycle and Apoptosis Assay

A total of 2×10^5 MDA-MB-468 cells were plated in a six-well plate for 24 h and then treated with 7.15 µM CP or HACD/CP for 24 h at 37°C. After incubation, the cells were harvested and washed with ice-cold PBS. The apoptosis ratio was performed with an annexin V-FITC Apoptosis Detection Kit (Beyotime).

Caspase-3 and 9 Activity Assays

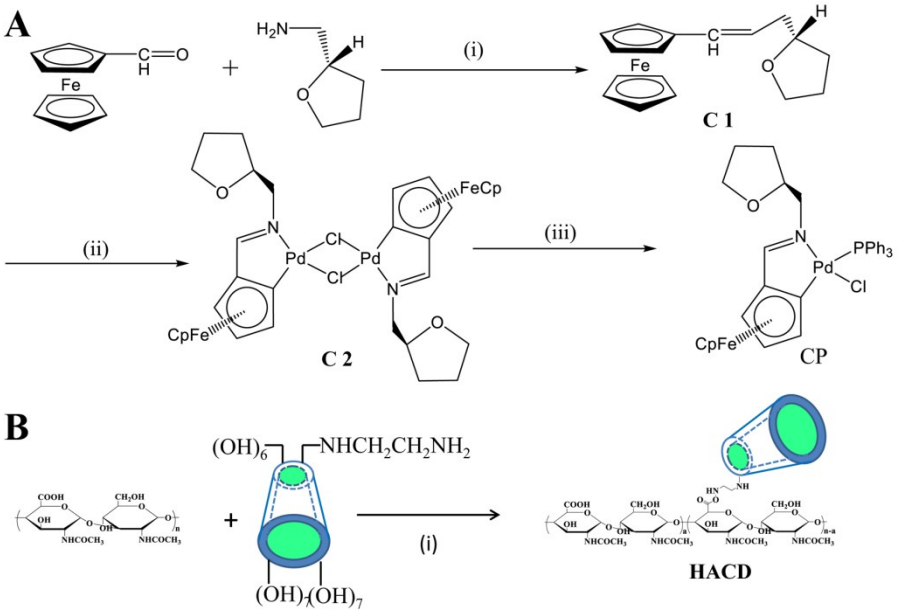
The activities of caspase-3 and -9 in MDA-MB-468 cells were assessed based on the specific protease-peptide substrate chromogenic reaction. In brief, MDA-MB-468 cells cultured in 6-well plates at 4×10^5 cell/well were treated for 12 h with PBS (as control) and CP or HACD/CP. After that, the cells were harvested, lysed and centrifugated. Then, aliquots of supernatants were collected and incubated with the peptide substrates of caspase-3 (Ac-DEVD-pNA) and-9 (Ac-LEHD-pNA) (Enzo Life Sciences, Inc., Farmingdale, NY, USA), respectively. The activities of caspase-3 and -9 were determined based on the absorbance at 405 nm by Thermo Scientific Varioskan Flash multimode reader. The total protein content of each sample was determined to normalize the obtained values by a BCA protein assay kit (Bestbio, Shanghai, China), and the activity ratio was calculated as compared to the blank

control.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA). All experiments were repeated at least thrice and representative results are presented. The data were compared by one-way ANOVA followed by Dunnett's post-hoc test. The differences were considered statistically significant when $p < 0.05$.

Scheme S1. Overview of Synthesized Compounds CP and HACD. A (i) $C_6H_5CH_3$, 110 °C, 24 h. (ii) $NaPdCl_4$, MeOH, r.t., 24 h. (iii) PPh3, CH_3COCH_3, r.t., 2 h. B (i) PBS, NHSS, EDC, r.t., 24h



2. NMR Spectra of C1, C2, CP and HACDs.

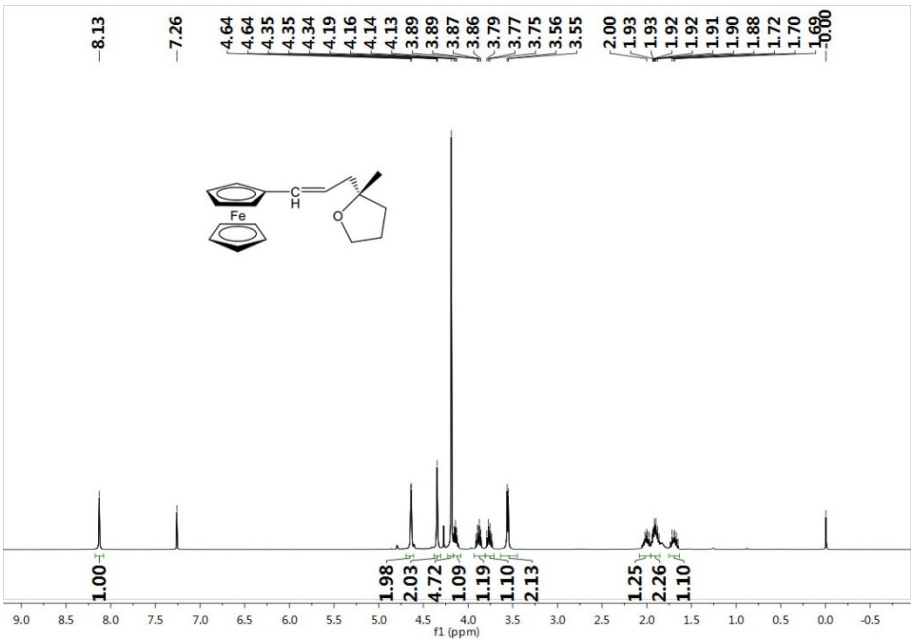


Figure S1. ^{1}H NMR Spectra of **C1** ($CDCl_3$, 400 MHz)

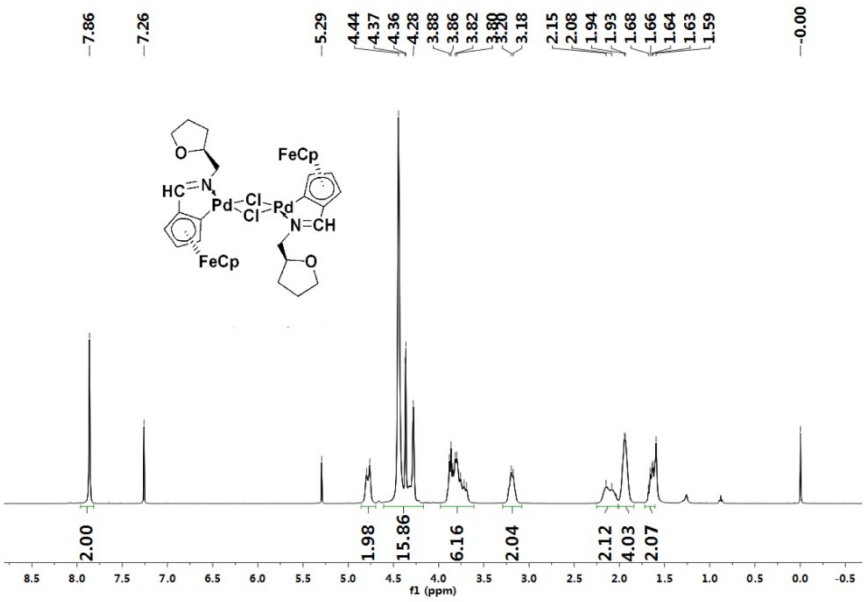


Figure S2. ^{1}H NMR Spectra of **C2** ($CDCl_3$, 400 MHz)

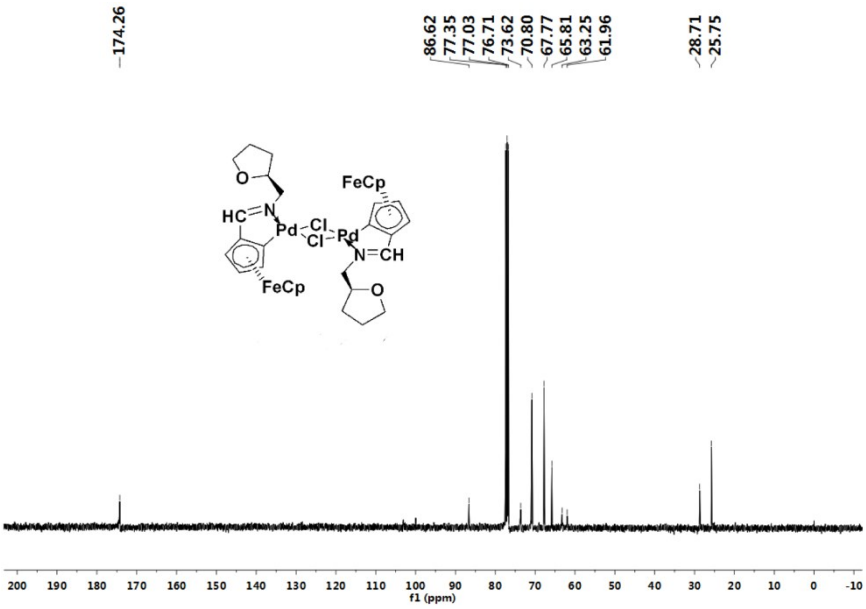


Figure S3. ^{13}C NMR Spectra of **C2** ($CDCl_3$, 100 MHz)

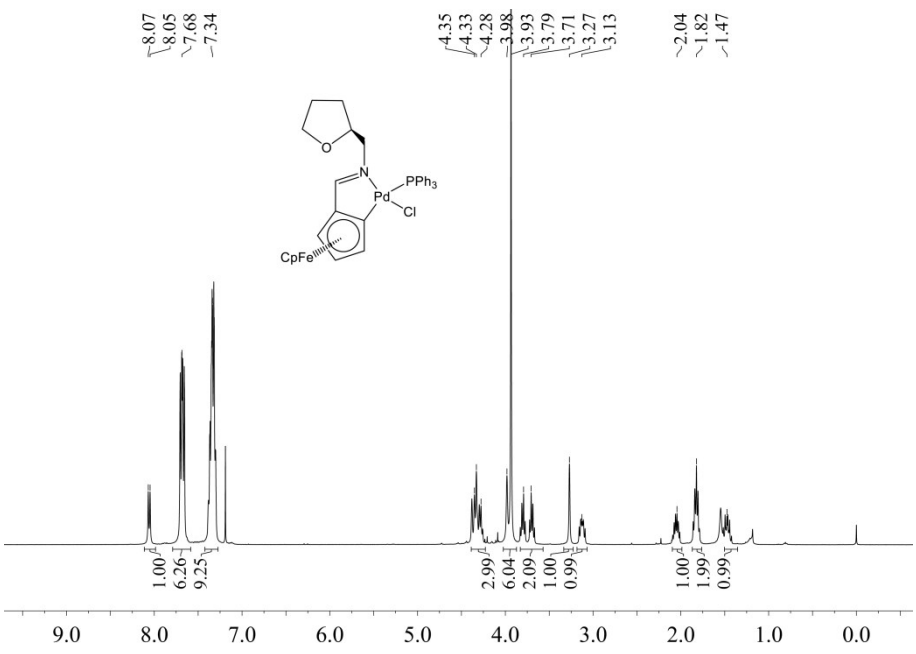


Figure S4. ^{1}H NMR Spectra of **CP** ($CDCl_3$, 400 MHz)

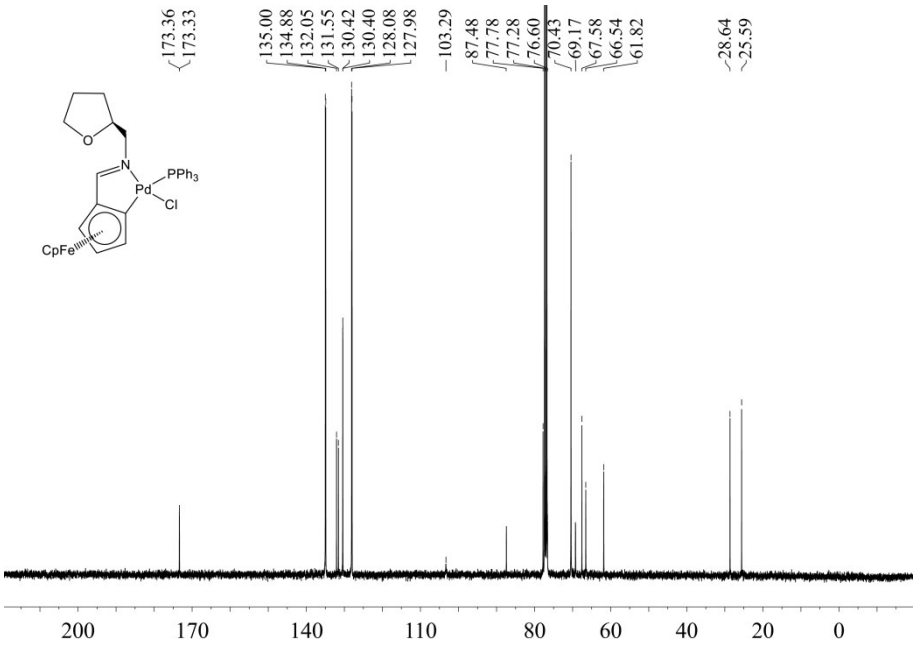


Figure S5. ^{13}C NMR Spectra of **CP** ($CDCl_3$, 100 MHz)

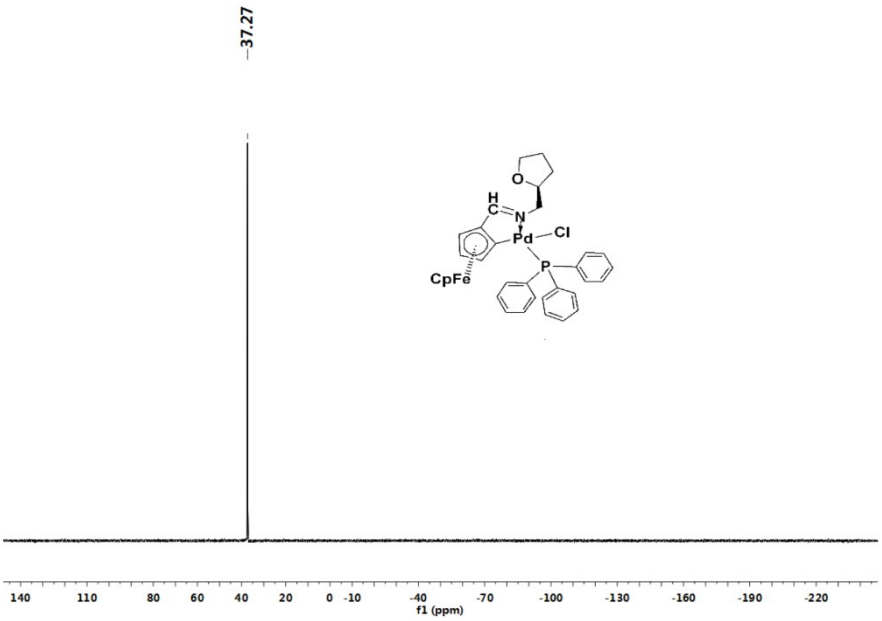


Figure S6. ^{31}P NMR Spectra of **CP** ($CDCl_3$, 400 MHz)

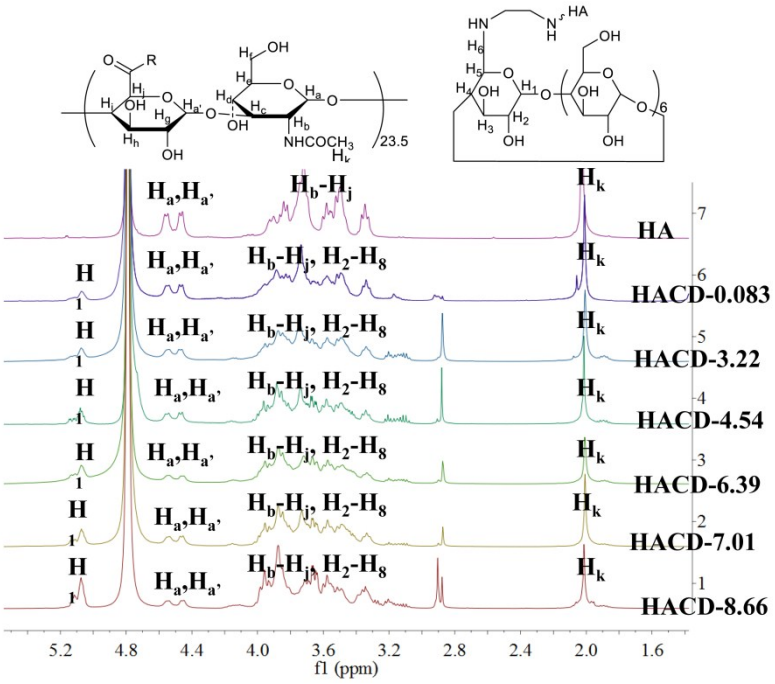


Figure S7. ^{1}H NMR (400 MHz) Spectra and Peaks Assignments of HA, HACD-0.08, HACD-3.22, HACD-4.54, HACD-6.39, HACD-7.01 and HACD-8.66 in D_2O at 25 °C.

3. H-H NOESY NMR Spectra of β-CD/CP and HACD/CP.

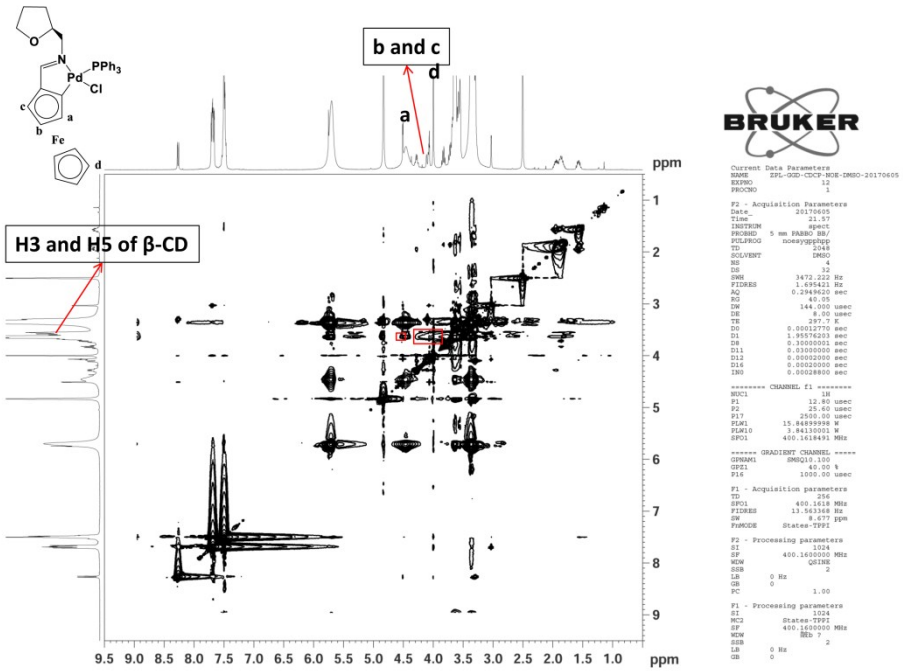
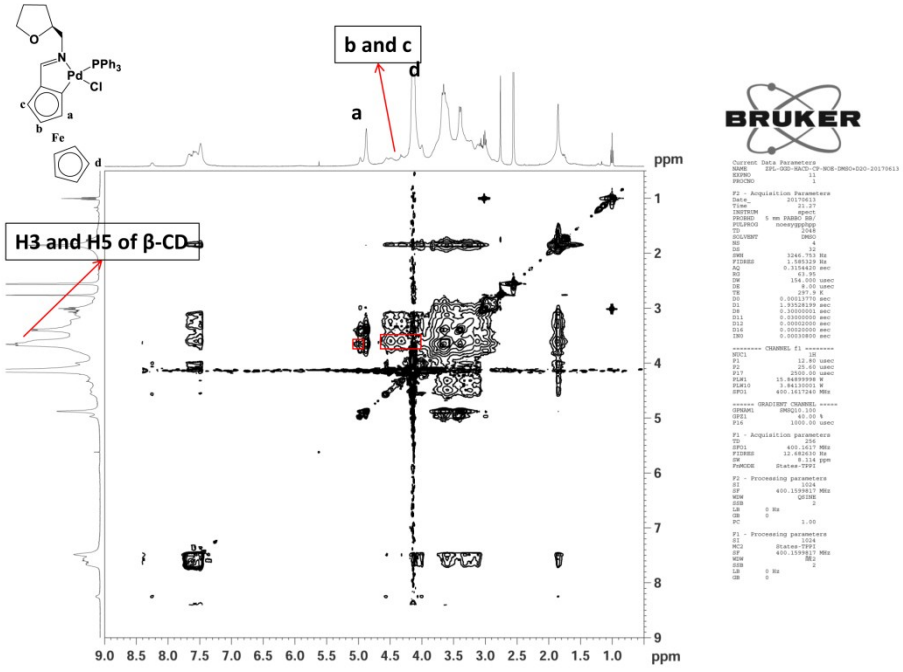


Figure S8. H-H NOESY NMR of β-CD/CP (DMSO-D6, 400 MHz, 25 ˚C)



Figrue S9. H-H NOESY NMR of HACD/CP (50% DMSO-D6 + 50% D_2O_2, 400 MHz, 25 ˚C)

4. Size Distribution.

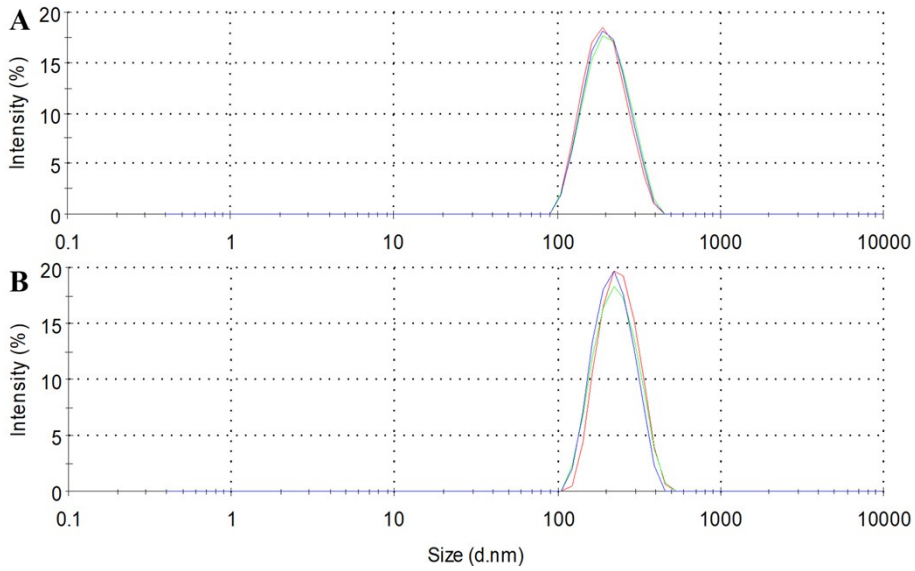


Figure S10. (A) Size Distribution of β-CD/CP; (B) Size Distribution of HACD/CP

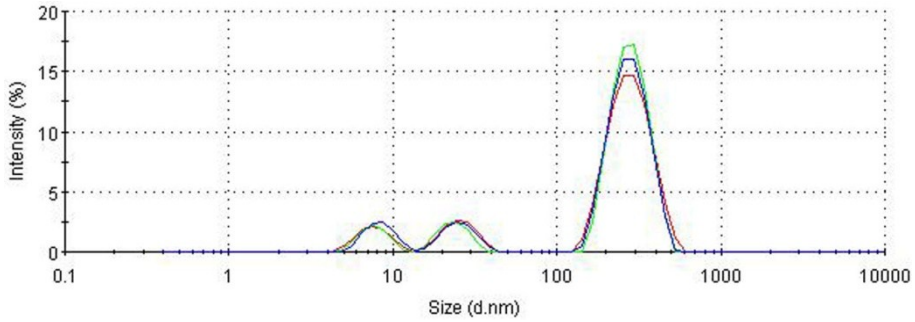


Figure S11. Size Distribution of HACD/CP in FBS (37 ˚C, 24h)

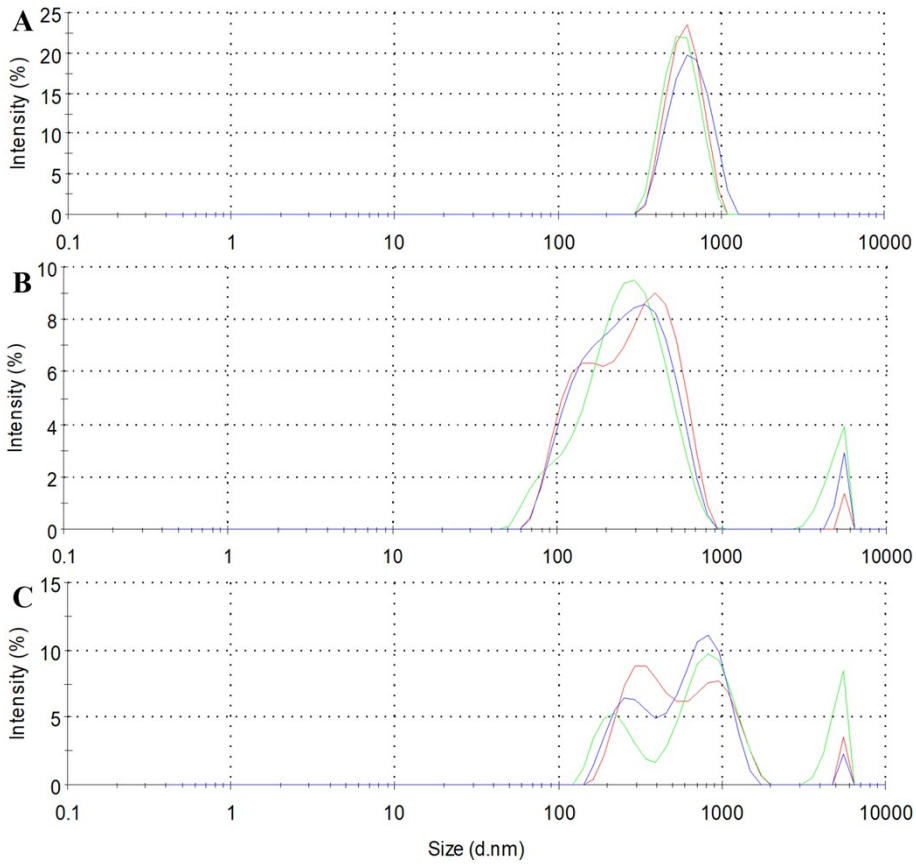


Figure S12. (A) Size Distribution of β-CD/CP in H_20_2 for 4 h; (B) Size Distribution of HACD/CP in H_20_2 for 4 h; (C) Size Distribution of HACD/CP in HAase for 4 h

5. Cell Viability of MDA-MB-468 and NIH 3T3.

Table S1. IC_{50} (μM) Values for Cisplatin, **CP**, HACD/**CP**, HACD/**CP**+HA and β-CD/**CP** Against the MDA-MB-468 and NIH 3T3 Cell Lines.[a]

	IC_{50} (μM)	
	MDA-MB-468	NIH 3T3
Cisplatin	10.38 ± 0.64	42.0 ± 2.1
CP	0.83 ± 0.08	4.49 ± 0.37
HACD/CP	0.96 ± 0.07	12.0 ± 0.76
HACD/CP+HA	2.63 ± 0.10	12.61 ± 0.29
β-CD/CP	2.76 ± 0.10	12.21 ± 0.99

[a].Cisplatin was dissolved in water. CP was dissolved in DMSO. The incubation time was 72 h. Dates are expressed as mean ± SD (N=3)

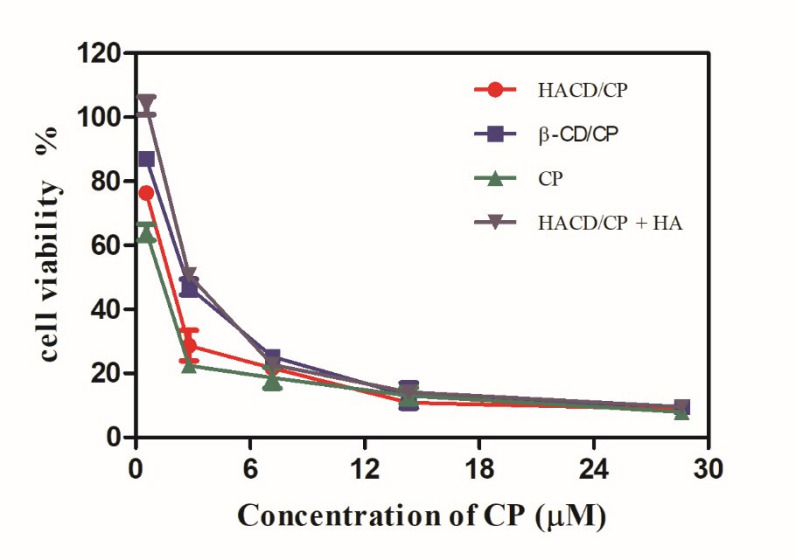


Figure S13. Cell Viability of MDA-MB-468 after 72 h of Treatment with CP, HACD/CP, HACD/CP+HA and β-CD/CP

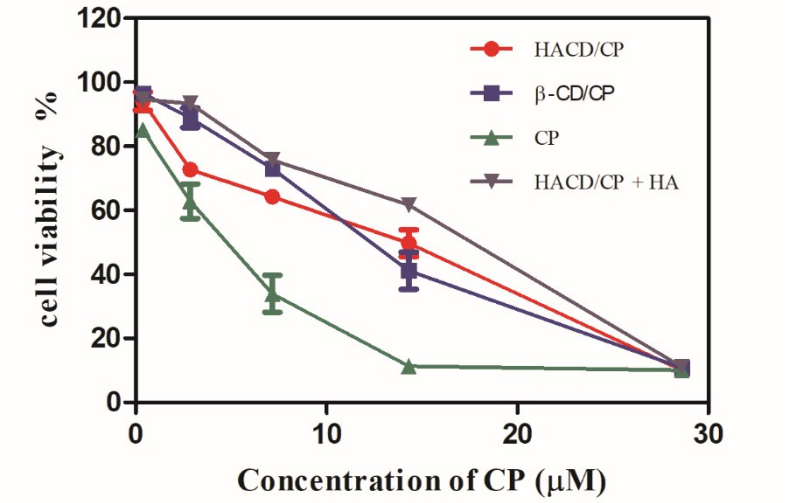


Figure S14. Cell Viability of NIH3T3 after 72 h of Treatment with CP, HACD/CP, HACD/CP+HA and β-CD/CP

6. CD44 Count.

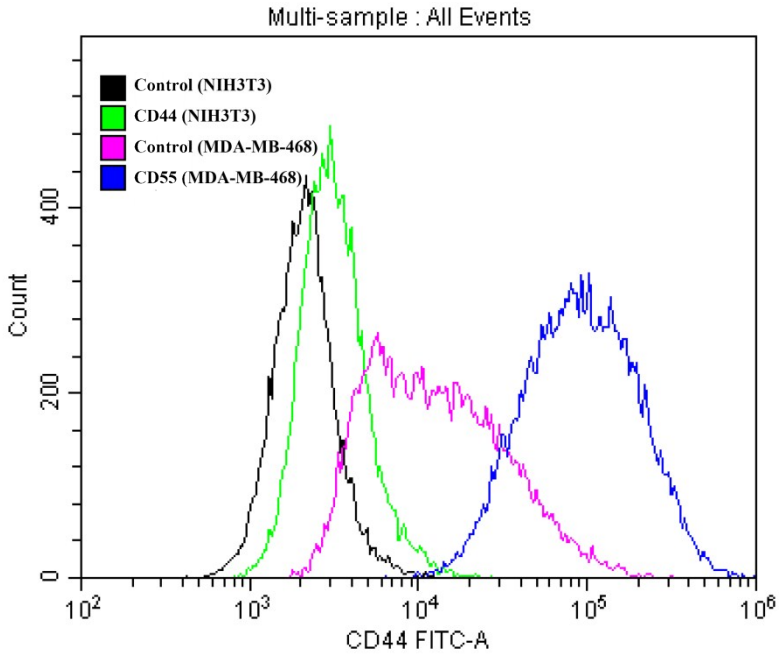


Figure S15 CD44 Expression of MDA-MB-468 and NIH3T3 Cells from Flow Cytometer analysis.

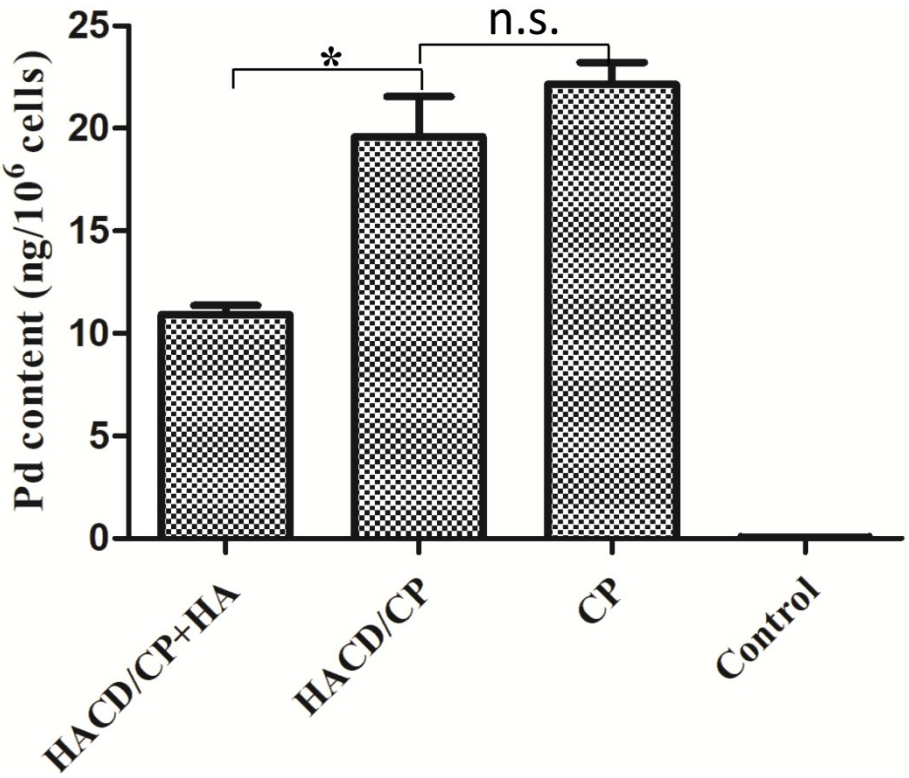


Figure S16. Palladium Uptake in MDA-MB-468 Cells (the concentration of CP in HACD/CP+HA, HACD/CP and CP were 5 μM, Dates are expressed as mean ± SD (N=3))

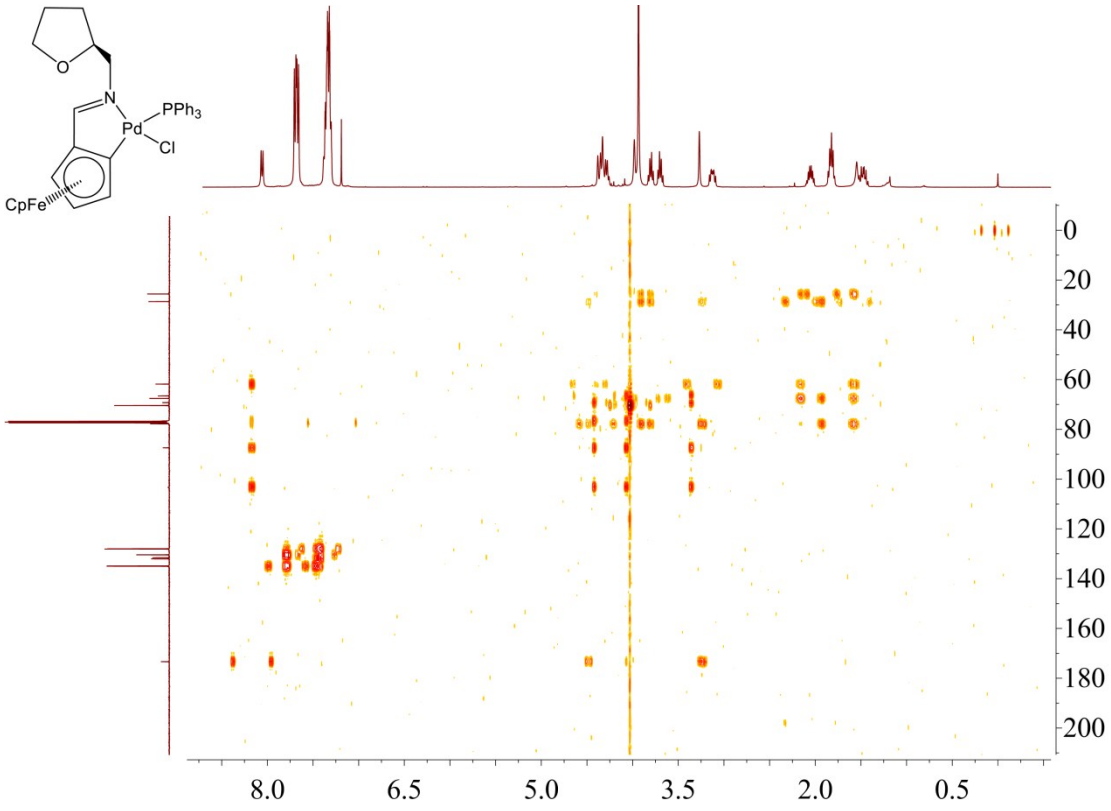


Figure S17. HMBC NMR Spectra of **CP** ($CDCl_3$, 400 MHz)