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Luminescent cyclometalated platinum(II) complexes with amino acid ligands for protein binding

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Part I. Experimental Section

Materials. Human serum albumin (HSA), Cohn IV-4 protein [HSA (21%), α-HG (39%), β-HG (40%)], γ-HG, calf thymus DNA (ct DNA) were purchased from Sigma Chemical Co. Ltd. and purified by the literature method.[1] The DNA concentration per base pair and protein concentation were determined by UV-vis absorption spectroscopy using the following molar extinction coefficients at the indicated wavelengths: calf thymus DNA, ε_{260} = 13200 bp cm^{-1} M^{-1};[2] HSA, ε_{279} = 35300 cm^{-1} M^{-1}. A plasmid DNA, pDR2 (10.7 kb), was purchased from Clontech Laboratories Inc. (Palo Alto, USA). Unless otherwise stated, protein binding experiments were performed in phosphate buffered saline (PBS) solutions (10 mM PBS, 10 mM Na_2HPO_4-NaH_2PO_4, 0.9% NaCl, pH 7.4) at 20.0 ^oC. phenylalanine (phe), tryptophan (try) and glycine (gly) were obtained from Aldrich. Pt(C^N)(Hthpy)Cl was prepared according to literature methods. The stock solutions (10 mM) of Pt^{II} complexes for titration studies were prepared in either DMSO or MeCN, and further dilution to designated concentrations were made using deionized water. All the stock solutions were kept at –20 ^oC in the dark between experiments.

HepG2 (hepatocellular cancer),[3] HeLa (human cervix epitheloid carcinoma), NCI-H460 (lung cancer), MCF-7 (breast cancer) and CCD-19Lu (normal lung fibroblast) cells were obtained from American Type Culture Collection. SF-268 (brain cancer) was obtained from National Cancer Institute of NIH, USA. Cell proliferation Kit I (MTT) from Roche was used for cytotoxicity evaluation.

Physical Measurement. Absorption spectra were recorded on a Perkin-Elmer Lambda 19 UV-visible spectrophotometer. Emission spectra were recorded on a SPEX Fluorolog-2 Model fluorescence spectrophotometer. Emission lifetime measurements were performed with a Quanta Ray DCR-3 pulsed Nd:YAG laser system (pulse output 355 nm, 8 ns). Error limits were estimated: λ (±1 nm); τ (±10%); ϕ(±10%). ^{1}H NMR spectra were recorded on a Bruker DPX-300 NMR spectrometer. Positive ion FAB mass spectra were recorded on a Finnigan MAT95 mass spectrometer. FPLC analysis was performed using Amazon FPLC system, Unicon 4.1 software and fraction collector.[4] UV melting studies were performed using a Perkin-Elmer Lambda 900 UV-visible spectrophotometer equipped with a Peltier temperature programmer PTP-6. Flow cytometry measurements were performed with an EPICS XL cytometer (Coulter Corporation, Miami, FL) equipped with an argon laser.

Spectroscopic Titration. Solutions of the platinum(II) complexes (37.5 μM) were prepared in PBS buffer. Aliquots of a millimolar stock HSA solution (0–250 μM) were added. Absorption spectra were recorded in the 200–600 nm range, after equilibration at 20.0 ° C for 10 min. Emission spectra were recorded in the 400–800 nm range, after equilibration at 20.0 ° C for 10 min. The concentration of the un-bounded metal complex, c_F, is obtained by equation (1):

$$c_F = c_T - c_B \quad (1)$$

where c_T is the total concentration of the free and bound forms of the metal complex, c_B is the concentration of the bound complex. A plot of r/c_F vs r is constructed according to equation (2):[5]

$$r/c_F = K(1 - nr)[(1 - nr)/(1 - (n - 1)r)]^{n-1} \quad (2)$$

where r is c_B/[HSA], K is the binding constant and n is the binding stoichiometry.

Competitive Studies by Emission measurement. Complexes **1**, **2** or **3** (at one molar equivalent to HSA) were added to a PBS solution of HSA in the absence and presence of plasma protein competitors. The competitors used were α-HG, β-HG and γ-HG. The concentrations of the competitors used were calculated using the plasma protein ratio of average healthy individuals (HSA:α-HG:β-HG:γ-HG ≈ 52:20:11:11 w/w) and of individuals with extreme hypoalbuminemia (HSA:α-HG:β-HG:γ-HG ≈ 20:20:11:11 w/w).[6] Emission spectra were recorded in the 400–800 nm range, after equilibration at 20.0 ° C for 10 min.

Native Polyacrylamide Gel Electrophoresis (*nu*-PAGE). Binding of **1**–**3** toward plasma proteins were studied by using *nu*-PAGE. Complexes **1**, **2** or **3** (at one molar equivalent to HSA) were added to PBS solution of HSA (15 μM), plasma proteins at normal ratio (HSA:α-HG:β-HG:γ-HG ≈ 52:20:11:11 w/w) or plasma proteins at hypoalbuminemia ratio (HSA:α-HG:β-HG:γ-HG ≈ 20:20:11:11 w/w).[6] The solution was then incubated at 20.0 ° C for 10 min.

nu-PAGE analysis was conducted using 4% stacking gel and 10% resolving gel. The gel was prepared according to Bio-Rad Mini-PROTEAN® 3 Cell Instruction Manual. Briefly, 10% resolving gel was prepared by mixing 4.2 mL H_2O, 3.3 mL acrylamide/bis solution (acrylamide 29.2 g, N'N'-bis-methylene-acrylamide 0.8 g per 100 mL H_2O) and 2.5 mL gel buffer (1.5 M Tris-HCl, pH 8.8). Immediately prior to pouring the gel into the cast, 50 μL of ammonium persulfate (10%) and 5 μL of N,N,N',N'-tetramethylethylenediamine (TEMED) were added and mixed with gentle swirling. After the resolving gel had set, 4% stacking gel was prepared in a similar way with 6.2 mL H_2O, 1.3 mL acrylamide/bis solution, 2.5 mL gel buffer (0.5 M Tris-HCL, pH 6.8), 50 μL of ammonium persulfate and 10 μL TEMED. The stacking gel was then poured on top of the resolving gel and a 10-well comb was inserted. After the gel had set, the comb was cautiously removed. The gel was then placed into the inner chamber assembly of the Mini-PROTEAN® 3 Cell system and put into the running tank. Running buffer (3.03 g Tris and 14.4 g glycine per 1L) was then poured into the tank to cover the gel.

Sample was prepared by dissolving in sample buffer [5.55 mL H_2O, 1.25 mL 0.5 M Tris_HCl (pH 6.8), glycerol and 0.2 mL 0.5% (w/v) bromophenol blue]. The sample solution (10 μL) was then loading on to the well with a 10 μL auto-pipettes. The sample was separated by a constant current of 70–90 mA.

In this method, denaturant sodium dodecyl sulfate (SDS) and reducing reagent such as β-mercaptoethano were omitted in gel preparation and buffer system compared to the denaturing SDS-PAGE so that the protein can preserve the native conformation during electrophoresis.

Anion-exchange Fast Protein Liquid Chromatography Analysis. Binding of **1**–**3** toward plasma proteins were studied by using anion-exchange fast protein liquid chromatography (FPLC). Complexes **1**, **2** or **3** (at one molar equivalent) were added to PBS solution of HSA, plasma proteins at normal ratio (HSA:α-HG:β-HG:γ-HG ≈ 52:20:11:11 w/w) or plasma proteins at hypoalbuminemia ratio (HSA:α-HG:β-HG:γ-HG ≈ 20:20:11:11 w/w).[6] The solution was then incubated at 20.0 ° C for 10 min.

The FPLC operating conditions were:

Column	Mono Q HR5/5	
Injection loop	100 μL	
Flow rate	1 mL/min	
Eluents	(A) 0.05 M Tris-HCl, pH 7.4	
	(B) A + 0.25 M ammonium acetate	
Gradient Elution	Time (min)	% B
	0	0
	9	60
	9	100
	28	100
Detection	Absorbance at 280 nm	

Restriction Endonuclease Fragmentation Assay. Digestion of a plasmid pDR2 DNA (10.7 kb) with a restriction enzyme, ApaI (Boehringer Mannheim), was performed by mixing the DNA (21 nM bp^{-1}) in 1× SuRE/Cut Buffer A with ApaI (1 unit/μL), followed by incubation at 37 °C for 1 h.[7] A mixture of ethidium bromide (4 μM), Hoechst 33342 (200 μM), cisplatin (*cis*-$Pt(NH_3)_2Cl_2$) (200 μM), **1**–**3** (200 μM) and pDR2 (10.7 kbp, 21 nM bp^{-1}) in digestion buffer was first incubated at room temperature for 5 min followed by addition of restriction enzyme (1 unit/μL). Two controls of pDR2 in the absence and presence of restriction enzyme in digestion buffer were prepared. All the solutions were incubated at 37 °C for 1 h; after restriction enzyme digestion the samples were analyzed by agarose gel electrophoresis.

UV Melting Study. Solutions of DNA (20 μM bp^{-1}) in the absence and presence of the Pt^{II} complex [DNA base pair:Pt = 1:1] were prepared in a Tris buffer solution (5 mM Tris, 50 mM NaCl, pH 7.2). The temperature of solution was increased at a rate of 1 °C min^{-1}, and the absorbance at 260 nm was monitored. The T_m values were determined graphically from the absorbance vs temperature plot.

Cytotoxicity Test (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) Assay). Cells were seeded in a 96-well flat-bottomed microplate at 20000 cells/well in 150 μL of growth medium solution [10% fetal calf serum (FCS, Gibco), 1% Sigma A-7292 Antibiotic and Antimycotic Solution in minimal essential medium (MEM-Eagle, Sigma)]. Complexes **1**–**3** and cisplatin (positive control) were dissolved in DMSO (dimethyl sulfoxide) and mixed with the growth medium (final concentration ≤4% DMSO). Serial dilution of each complex was added to each well. The microplate was incubated at 37 °C, 5% CO_2, 95% air in a humidified incubator for 48 h. After incubation, 10 μL MTT reagent (5 mg/mL) was added to each well. The microplate was re-incubated at 37 °C in 5% CO_2 for 4 h. Solubilization solution (10% SDS in 0.01 M HCl); 100 μL was added to each well. The microplate was left in an incubator for 24 h. Absorbances at 550 nm were measured by a microplate reader. The IC_{50} values of **1**–**3** (concentration required to reduce the absorbance by 50% compared to the controls) were determined by the dose-dependence of surviving cells after exposure to the metal complex for 48 h.

Flow Cytometric Analysis. The sheath fluid was an isotonic solution (Isoflow Coulter 8547008, Coulter Corporation). An excitation wavelength of 488 nm at 15 mW was used. About 10,000 cells were analyzed in each sample. Cancer cells (NCI-H460 cell line) were cultured at a concentration of approximately 2×10^5 cells/mL. Complex **1** (2 μM) was added to the cultures. Staurosporin Streptomyces was used as positive control. After treatment, the cultures were incubated in 5% CO_2 at 37 °C. Cells were collected at 6 h interval. The genomic DNA was extracted according to the literature method,[1] and analyzed by Annexin V plus PI staining.

Pt(C^N)(phe) (1). A suspension of Pt(C^N)(Hthpy)Cl (0.20 g, 0.36 mmol), phenylalanine (0.07 g, 0.40 mmol) and NaOH (0.02 g, 0.40 mmol) in methanol (20 mL) was refluxed for 16 h to give a clear solution. The solution was filtered and the volume was reduced to 5 mL. The shinny orange solid was washed with H_2O and diethyl ether. Recrystallization by diffusion of diethyl ether into an acetonitrile solution afforded an orange crystalline solid. Yield: 42%. FAB-MS: *m/z* 520 [M^+]. ^{1}H NMR (300 MHz, CD_2Cl_2): 8.53 (d, 1H, J = 5.7 Hz), 7.60 (t, 1H, J = 7.2 Hz), 7.43–7.36 (m, 5H), 7.31 (d, 1H, J = 4.8 Hz), 7.11 (d,1H, J = 8.0 Hz), 6.72 (t, 1H, J = 6.4 Hz), 6.60 (d, 1H, J = 4.8 Hz), 5.06 (s, 1H), 3.99–3.91 (m, 2H), 3.41 (dd, 1H, J = 14.2, 3.7 Hz), 3.24 (dd, 1H, J = 14.1, 10.9 Hz). Anal. Calcd. for $C_{18}H_{16}N_2O_2PtS$: C, 41.62; H, 3.10; N, 5.39. Found: C, 41.97; H, 2.95; N, 5.12. UV-vis (MeOH): λ/nm (ε/mol^{-1}dm^{3}cm^{-1}) 254 (11200), 297 (12700), 311 (sh, 11500), 327 (sh, 9800), 406 (3200).

Pt(C^N)(trp) (2). The procedure was similar to that of **(1)**, except tryptophan (0.08 g, 0.40 mmol) was used. Yield: 40%. FAB-MS: *m/z* 559 [M^+]. ^{1}H NMR (300 MHz, dmso-d_6): 10.87 (s, 1H), 8.56 (d, 1H, J = 5.1 Hz), 7.90 (t, 1H, J = 7.8 Hz), 7.56–7.47 (m, 3H), 7.33 (d, 1H, J = 8.1 Hz), 7.28 (s, 1H), 7.13 (t, 1H, J = 6.5 Hz), 7.05 (t, 1H, J = 7.3 Hz), 7.00 (t, 2H, J = 6.2 Hz), 6.45 (s, 1H), 5.24 (s, 1H), 3.64 (dd, 1H, J = 4.0, 8.7 Hz), 3.41 (dd, 1H, J = 3.9, 15.1 Hz), 3.09 (dd, 1H, J = 8.8, 15.1 Hz). Anal. Calcd. for $C_{20}H_{17}N_3O_2PtS$: C, 43.01; H, 3.07; N, 7.52. Found: C, 42.8, H, 3.36; N, 7.73. UV-vis (MeOH): λ/nm (ε/mol^{-1}dm^{3}cm^{-1}) 254 (12200), 284 (sh, 13300), 291 (15000), 328 (sh, 9200), 406 (3600).

Pt(C^N)(gly) (3). The procedure was similar to that of **(1)**, except glycine (0.04 g, 0.50 mmol) was used. Yield: 45%. FAB-MS: *m/z* 430 [M^+]. ^{1}H NMR (300 MHz, dmso-d_6): 8.59 (d, 1H, J = 5.3 Hz), 7.95 (t, 1H, J = 7.5 Hz), 7.65 (d, 1H, J = 4.5 Hz), 7.53 (d, 1H, J = 7.9 Hz), 7.18 (t, 1H, J = 6.0 Hz), 6.98 (d, 1H, J = 4.6 Hz), 6.21 (s, 2H), 3.40 (t, 2H, J = 8.0 Hz). Anal. Calcd. for $C_{11}H_{10}N_2O_2PtS$: C, 30.77; H, 2.35, N, 6.52. Found: C, 30.35; H, 2.66; N, 6.36. UV-vis (MeOH): λ/nm (ε/mol^{-1}dm^{3}cm^{-1}) 254 (12200), 284 (sh, 13300), 291(14700), 328 (sh, 9200), 406 (3600).

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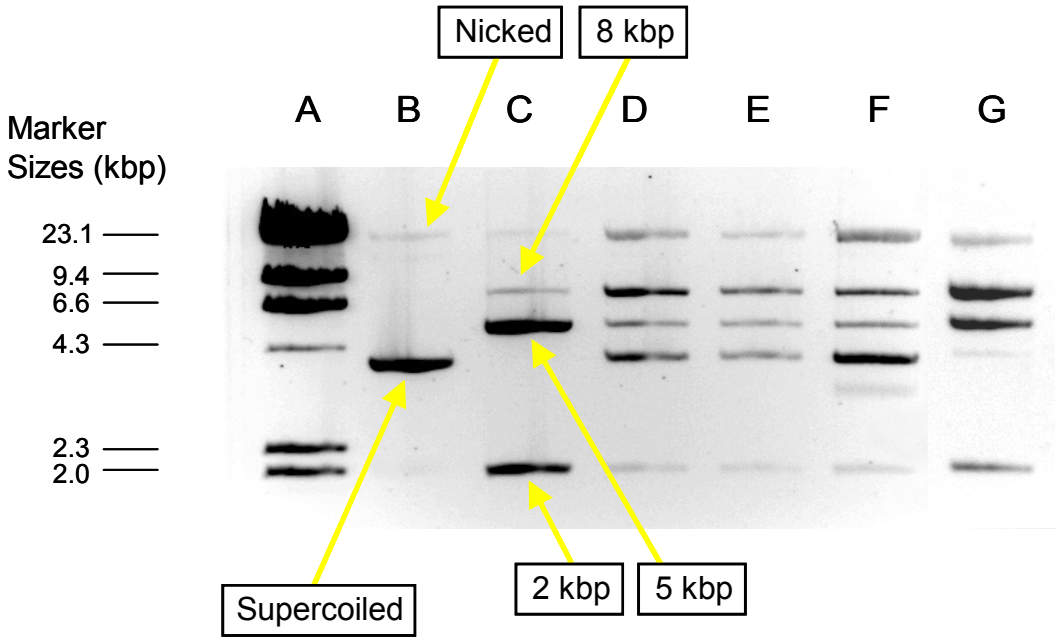
Part II. Figures

Fig. S1. Inhibition of restriction endonuclease ApaI cutting sites by various small molecules. Lane A is size marker. Lanes B and C are undigested and ApaI (1 unit/μl) digestion products of pDR2 DNA (10.7 kbp, 21 nM bp^{-1}), respectively.[a] Lanes D–F are the digestion products of pDR2 DNA in the presence of DNA interacting molecules: ethidium bromide (4 μM) (Lane D), Hoechst 33342 (200 μM) (Lane E), cisplatin (200 μM) (Lane F).[b] Lane G is the digestion products of pDR2 DNA in the presence of **1** at 200 μM (Lane G).[c]

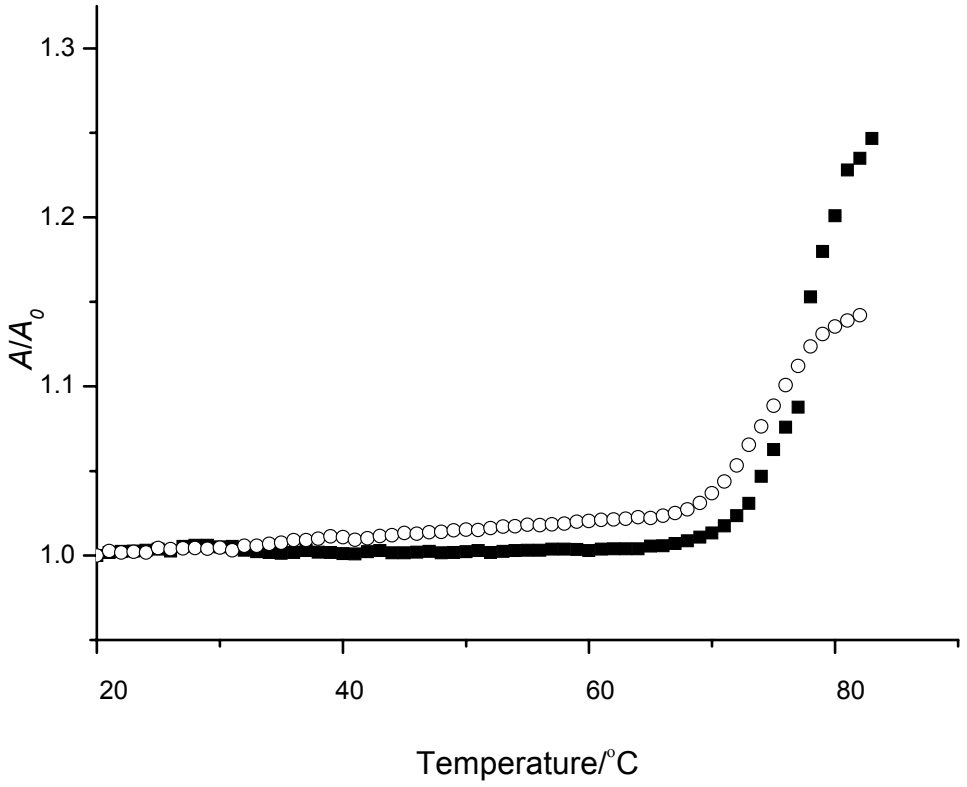


[a] Two bands corresponding to the supercoiled and nicked DNA were observed for the undigested DNA (Lane B). After ApaI digestion of pDR2, three bands corresponding to DNA fragments with 8, 5, and 2 kbp were obtained and resolved by agarose gel electrophorsis (Lane C).

[b] In the presence of the classical intercalator – ethidium bromide (4 μM), the minor groove binder – Hoechst 33342 (200 μM), or the intrastrand crosslinker – cisplatin (200 μM), DNA digestion was incomplete and bands attributed to the whole plasmid plus fragments were observed (Lanes D–F).

[c] Because complex **1** do not bind to DNA, treatment of pDR2 and ApaI with **1** at concentration 200 μM in 1× SuRE/Cut Buffer A failed to inhibit the ApaI digestion and the three bands of DNA fragments with 8, 5, and 2 kbp were obtained (Lane G).

Fig. S2. Plots of A/A_0 vs temperature of ct DNA (20 μM) (■) and ct DNA in the presence of **1** (○) with a 1:1 ratio of DNA base pair to **1** in Tris buffer solution.[a]



[a] The melting temperature (T_m) of the untreated ct DNA was found to be 77 °C, and no increase in T_m upon treatment of ct DNA (50 μM) with **1** ([ct DNA]:[Pt] = 1:1).

Fig. S3. UV-visible spectra of **1** (37.5 μM) in PBS buffer with increasing ratio of [HSA]/[Pt] = 0–1.36 at 20.0 ° C. Inset: Plot of r/C_F vs r.

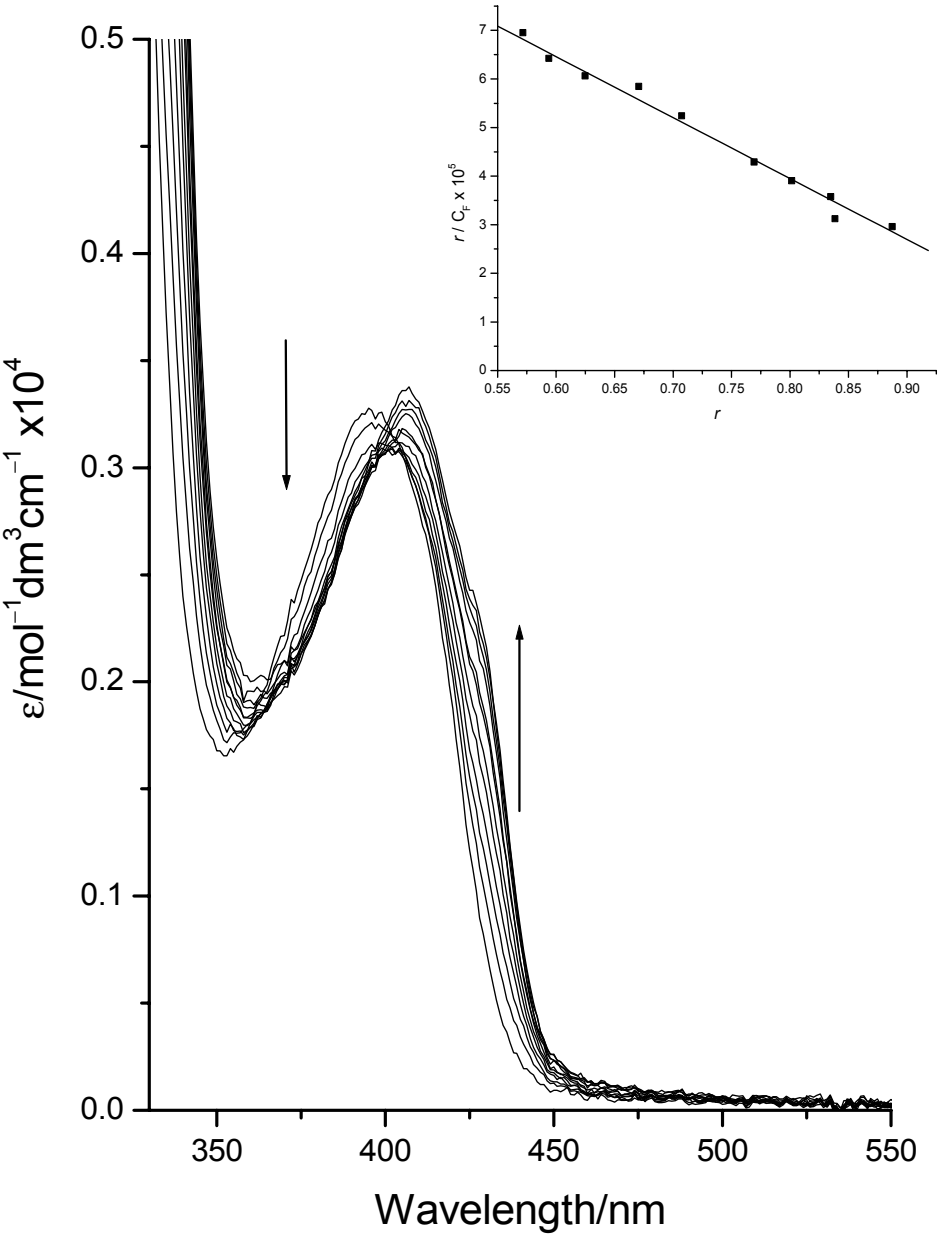


Fig. S4. Plot of I/I_o vs [HSA]/[Pt] for the emission titration study of the **1**–HSA interaction.

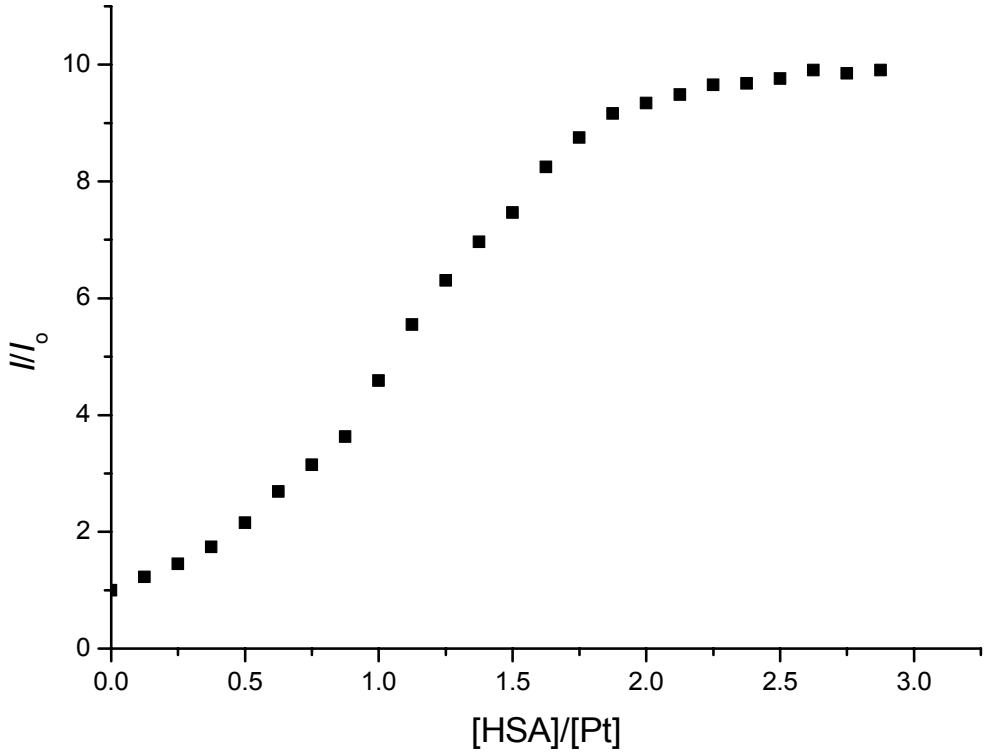


Fig. S5. Emission spectral traces of **2** (4.5 μM) in PBS buffer with increasing ratio of [HSA]/[Pt] = 0–16.0 at 20.0 ° C.

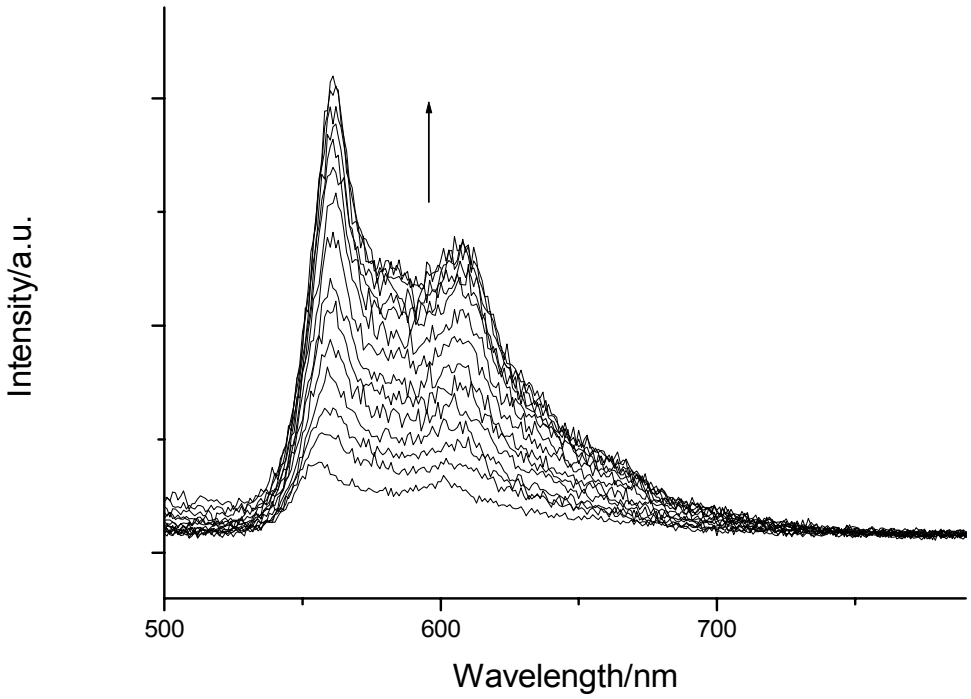


Fig. S6. Emission spectral traces of **3** (4.5 μM) in PBS buffer with increasing ratio of [HSA]/[Pt] = 0–9.0 at 20.0 ° C.

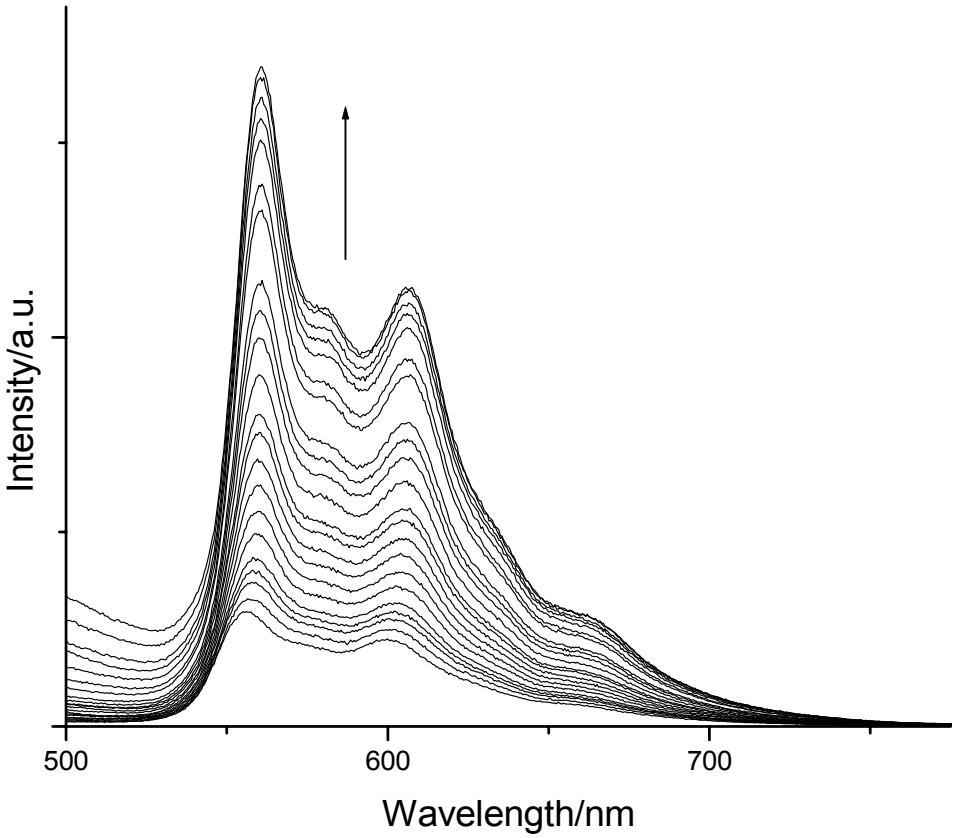


Fig. S7. Chromatograms of a mixture of plasma proteins at normal ratio (HSA:*α*-HG:*β*-HG:*γ*-HG ≈ 52:20:11:11 w/w) in the presence of **1**. (—) indicates the absorbance of the eluent. (⬚) indicates emission intensity of the eluent in 1 mL fractions with excitation at 400 nm.

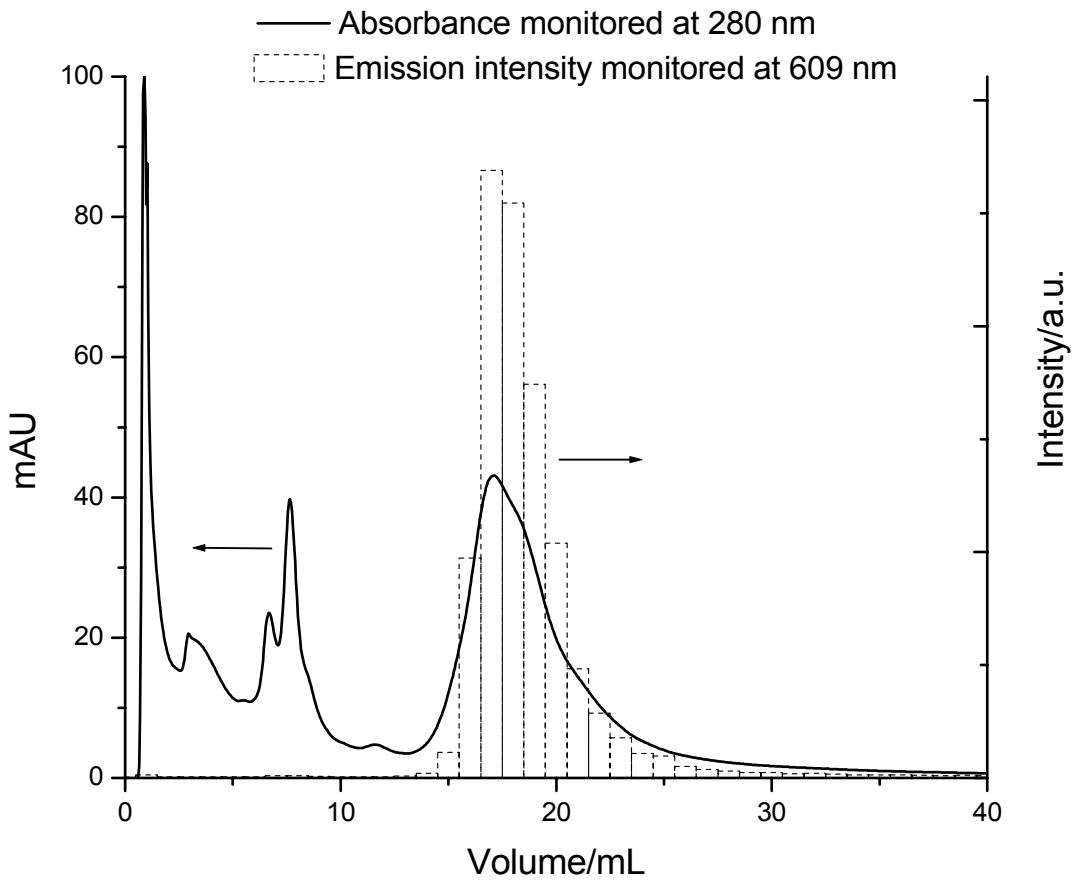


Fig. S8. FACS analysis of apoptotic NCI-H460 cells by Annexin-V-FLUOS and propidium iodide. a) single parameter Annexin-V-Fluos (cultivation for 8 h in the presence of **1**), b) dual parameter (cultivation for 8 h in the absence of **1**) and c) dual parameter (cultivation for 8 h in the presence of **1**); cluster R1 = living cells, R2 = apoptotic cells, R3 = necrotic cells and R4 = late apoptotic cells and necrotic cells.

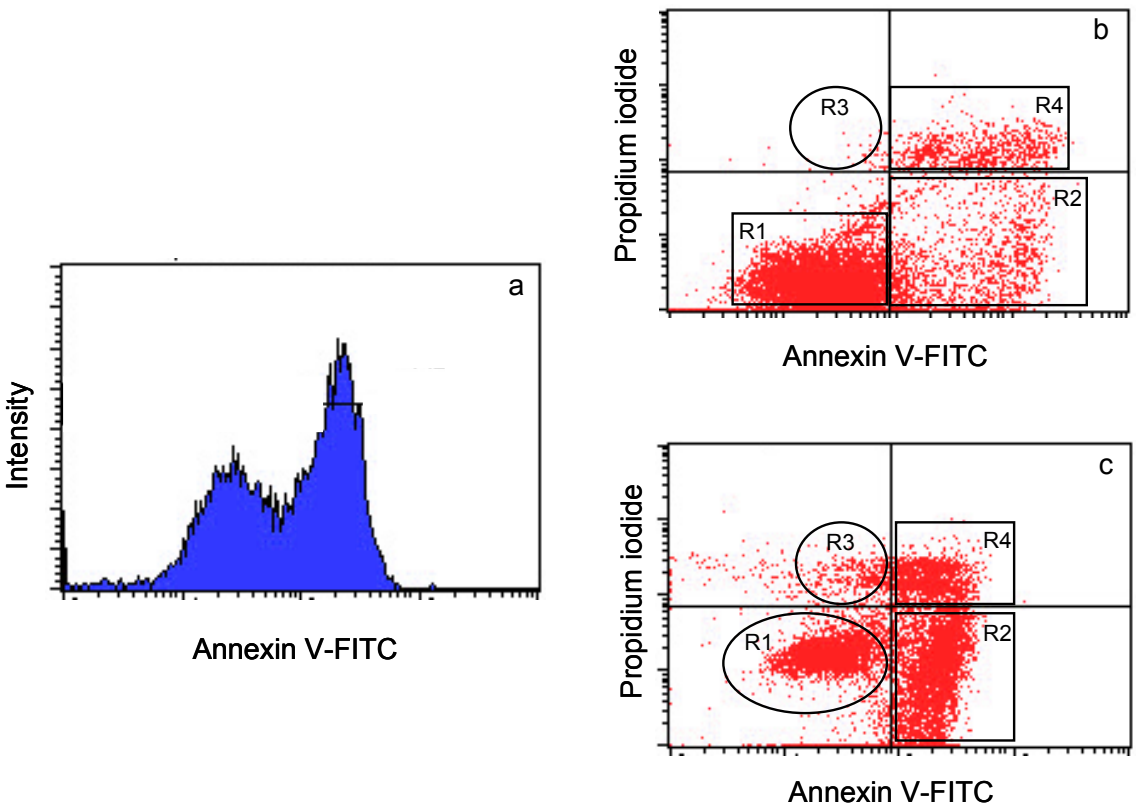


Table S1. Summary of competitive emission studies for **1**

Complex	Protein	I/I_0	Deviation of I from I_0 [I_0 refers to emission intensity of **1** in the presence of pure HSA]
1	Pure HSA	5.34	0.0 %
	HSA + *γ*-HG at normal ratio	5.34	0.0 %
	HSA + *γ*-HG at hypoalbuminemia ratio	5.40	1.1 %
	HSA + *α*, *β*, *γ*-HG at normal ratio	5.91	10.6 %
	HSA + *α*, *β*, *γ*-HG at hypoalbuminemia ratio	6.21	16.4 %

Table S2. Summary of relative emission intensities of the bound HSA–Pt for **1**

Complex	Spot area	IDV [a.u.][a]	%[b]
1	M1	108570	80.9
	M2	102306	75.5
	M3	109980	77.0
	D1	25600	19.1
	D2	23040	17.0
	D3	25916	18.1
	P1	0	0
	P2	7628	5.6
	P3	6935	4.9

[a] IDV= Integrated Density Value

[b] Emission Intensity