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Supporting Information

For

Diminished Viability of Human Ovarian Cancer Cells by Antigen-specific Delivery of Carbon Monoxide with a Family of Photoactivatable Antibody-photoCORM Conjugates

Brian Kawahara,[†] Lucy Gao,[§] Whitaker Cohn,[§] Julian P. Whitelegge,[§] Suvajit Sen,[‡] Carla Janzen,[‡] Pradip K. Mascharak[†]*

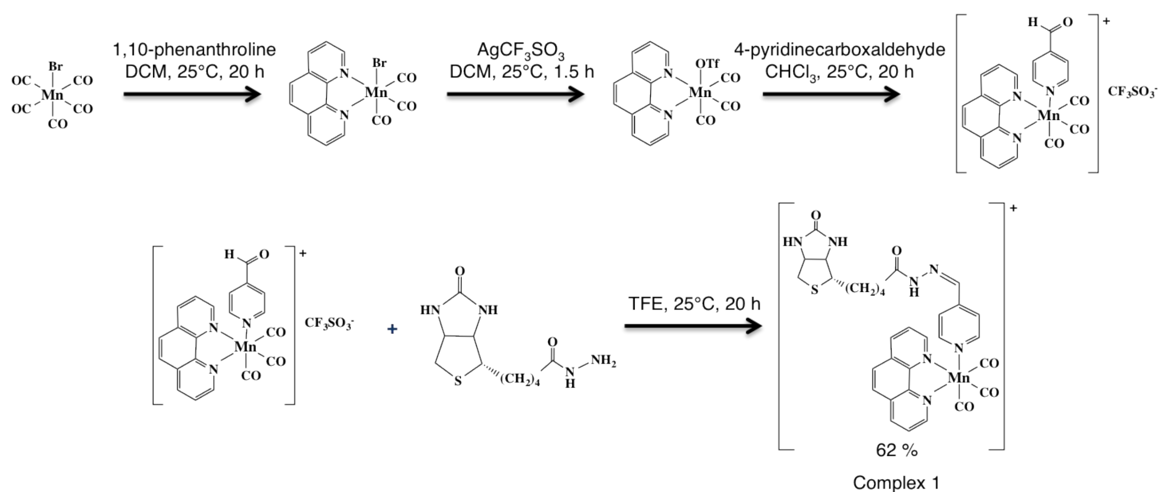
Contribution from

[†]Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064, USA

[§]Pasarow Mass Spectrometry Laboratory, Jane and Terry Semel Institute for Neuroscience and Human Behavior, University of California at Los Angeles, Los Angeles, CA 90095, USA

and

[‡]Department of Obstetrics and Gynecology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA



Scheme S1. Synthetic scheme for Complex **1**: biotinylated, photoactivatable CO-releasing molecule (photoCORM).

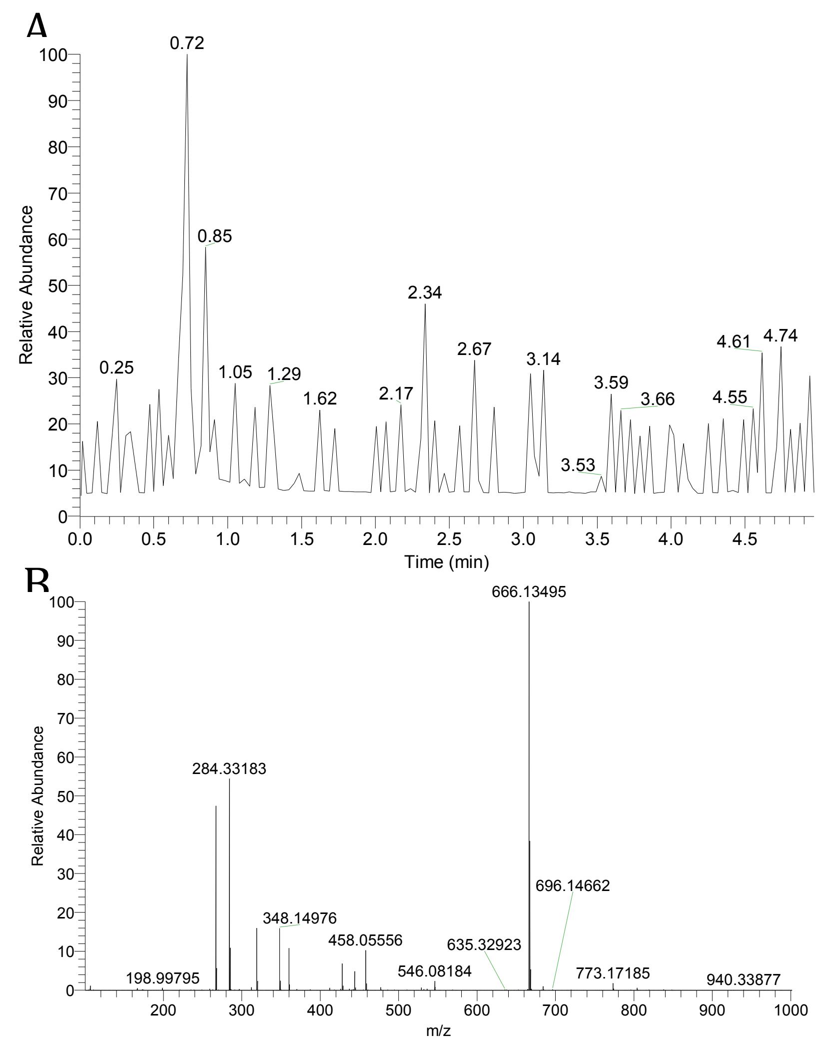


Figure S1. By electrospray ionization mass spectrometry (ESI-MS), (A) Total ion count (TIC) chromatogram of 0-5 min for a 5 min run of Complex **1** via flow injection analysis and (B) full mass spectrum (100-1000 m/z) for retention time=0.70-0.72. Found: 666.13495, calculated for $C_{31}H_{29}N_7O_5SMn$ 666.13314, Δ ppm = 3.4 ppm, Δ mDa = 2.2.

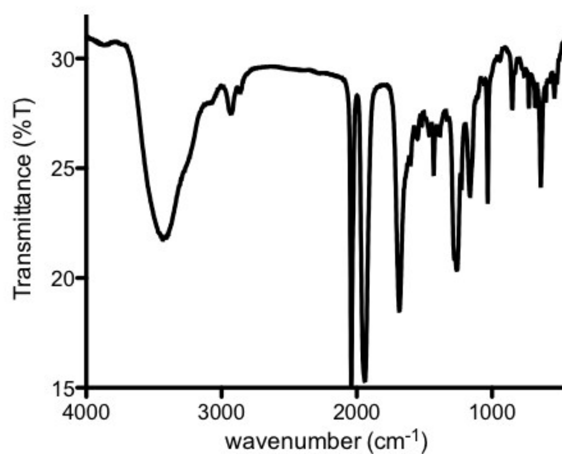


Figure S2. Infrared (IR) spectrum of Complex **1**. IR spectrum of solid Complex **1** was recorded in KBr matrix. $\nu_{C=O}$: (cm^{-1}): 2030, 1941, 1687.

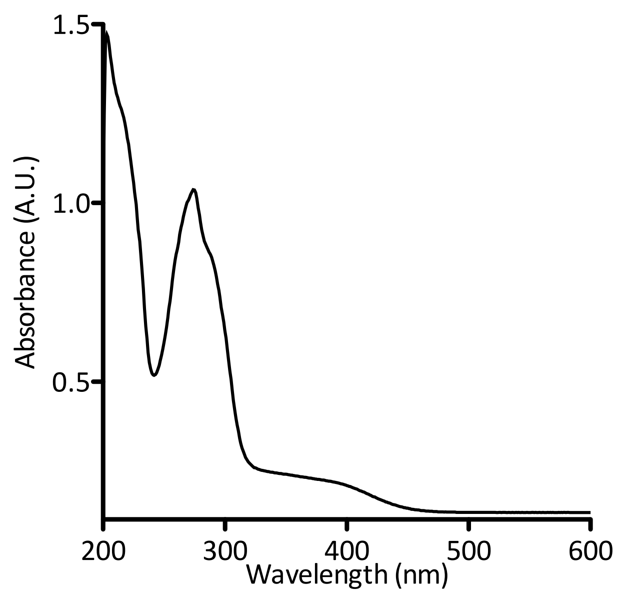


Figure S3. Electronic absorption spectrum of Complex **1** in 1x PBS, 25°C.

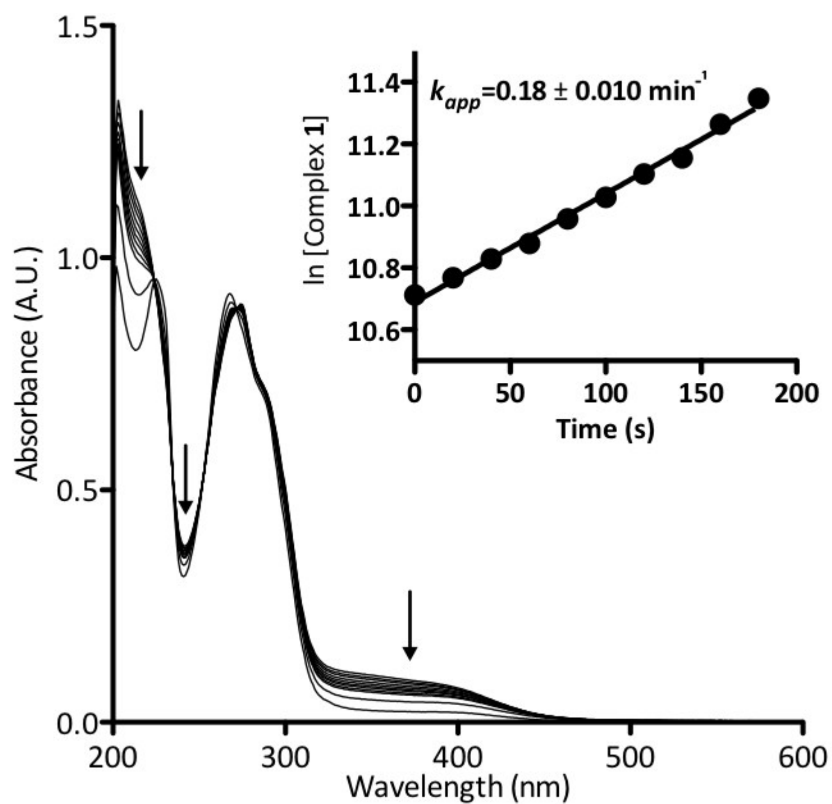


Figure S4. Electronic spectrum of Complex **1** upon illumination with low power (10 mW/cm^2), broadband visible light in 20-second intervals. Inset: Integration of the release rate for the photodegradation of Complex **1,** calculated at 390 nm, with low power (10 mW/cm^2), broadband visible light for indicated time.

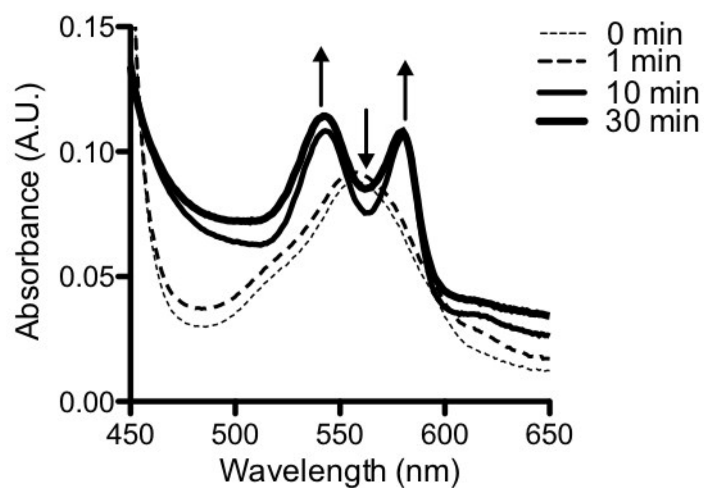


Figure S5. Myoglobin assay for CO release from Complex **1** dissolved and performed in 1X phosphate buffered saline (PBS), triggered by low power (10 mW/cm²), visible light for indicated time.

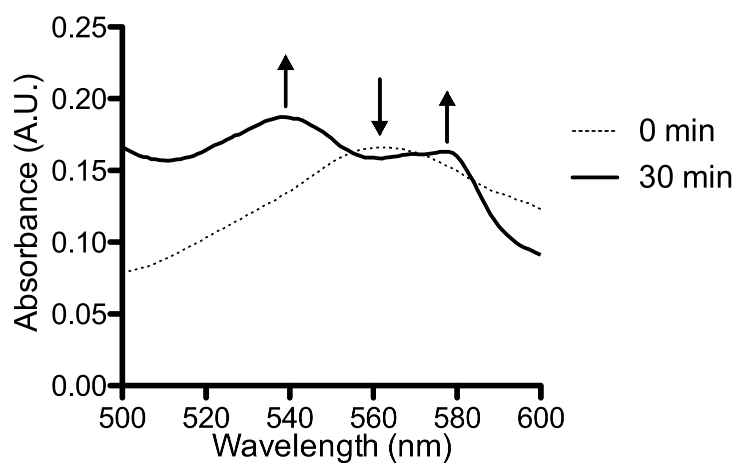


Figure S6. Myoglobin assay for CO release from Complex **1** dissolved in human serum and incubated for ≥1 h at 37°C, followed by exposure to low power (10 mW/cm²), broadband visible light for 30 min.

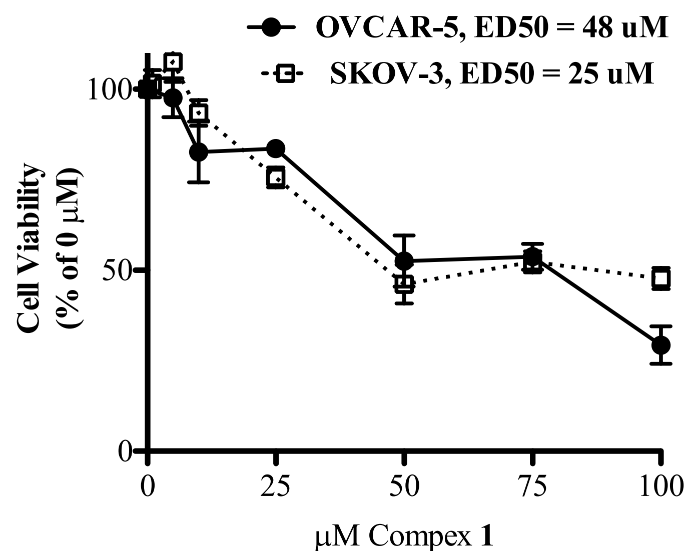


Figure S7. Effect of treatment of ovarian cancer cell lines with indicated concentrations of Complex **1** upon illumination with visible light on cell viability, measured 24 h post-treatment in ovarian cancer cell lines OVCAR-5 and SKOV-3. Data representative of n=3 independent experiments.

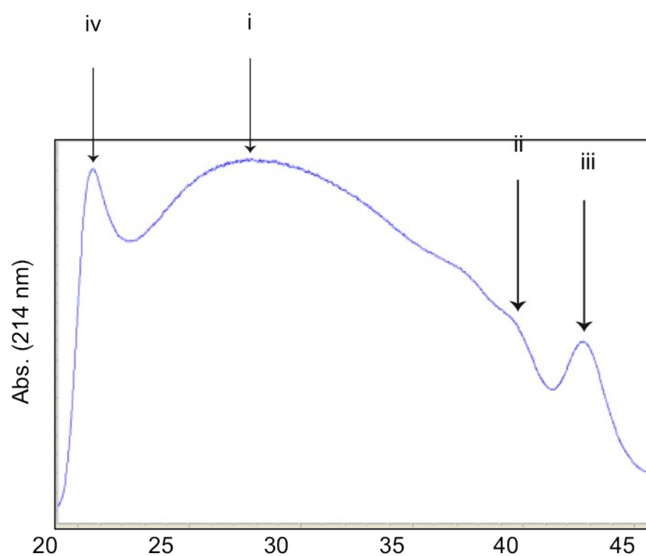


Figure S8. Chromatogram of Complex **2** following size exclusion chromatography. [retention time, ~molecular weight, identity] (i) [28.4 min, 366 kDa, IgG + 4 streptavidin]. (ii) [39.6 min, ~155 kDa, IgG + 0 streptavidin]. (iii) [42.7 min, ~121 kDa, IgG Fragments]. (iv) [20.6 min, ~659 kDa, void volume].

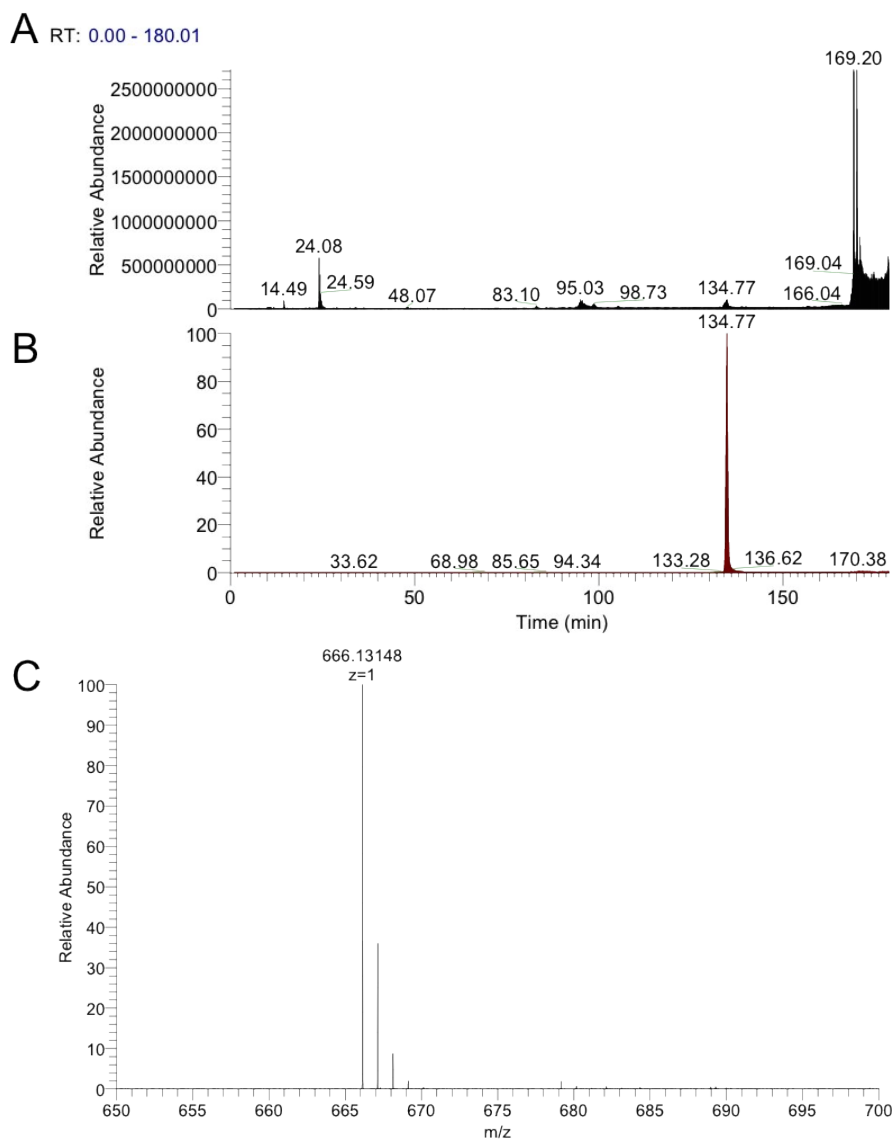


Figure S9. Detection of Complex **1** in tryptic digest of antibody-photoCORM conjugate (Ab-photoCORM). (A) Total ion count (TIC) of Ab-photoCORM sample. (B) Chromatogram of Ab-photoCORM, mass filter range m/z = 666.12593-666.13925. (C) Full mass spectrum at retention time 134.16-135.34 min.

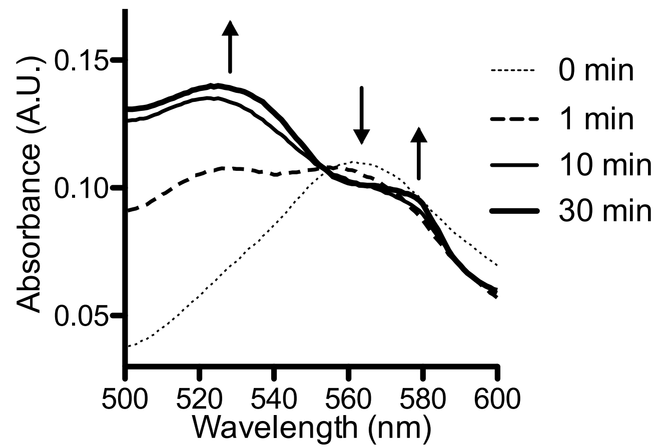


Figure S10. Myoglobin assay for CO release from antibody-photoCORM conjugate (Ab-photoCORM) dissolved in 1X phosphate buffered saline (PBS), triggered by low power (10 mW/cm^2), broadband visible light for indicated times.

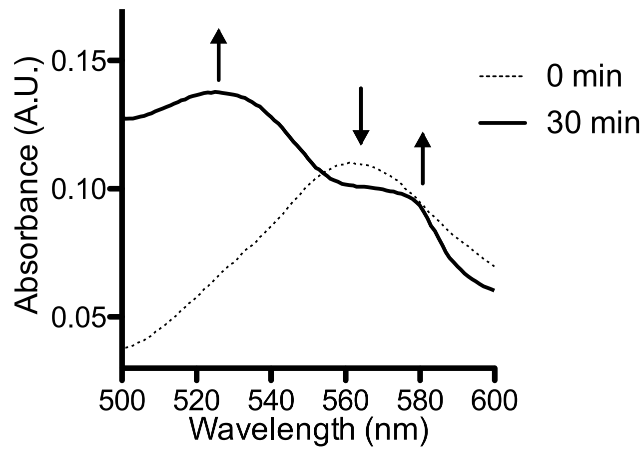


Figure S11. Myoglobin assay for CO release from the antibody-photoCORM conjugate (Ab-photoCORM) after ≥1 h incubation in human serum at 37°C, followed by exposure to low power (10 mW/cm^2), broadband visible light for 30 min.

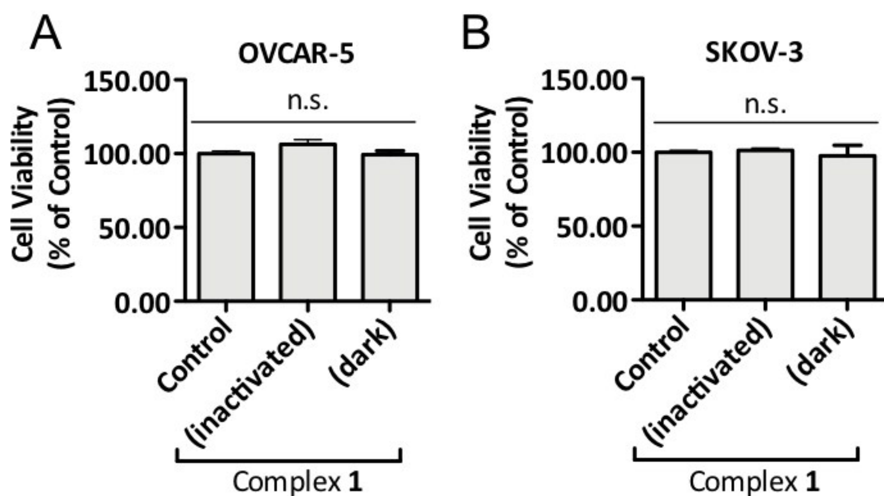


Figure S12. Cell viability of ovarian cancer cell lines OVCAR-5 and SKOV-3 treated with light-inactivated Complex **1** and Complex **1** in the dark to assess the cytotoxicity of non-CO components of Complex **1**. Data representative of n=3 independent experiments. (* $p<0.05$)

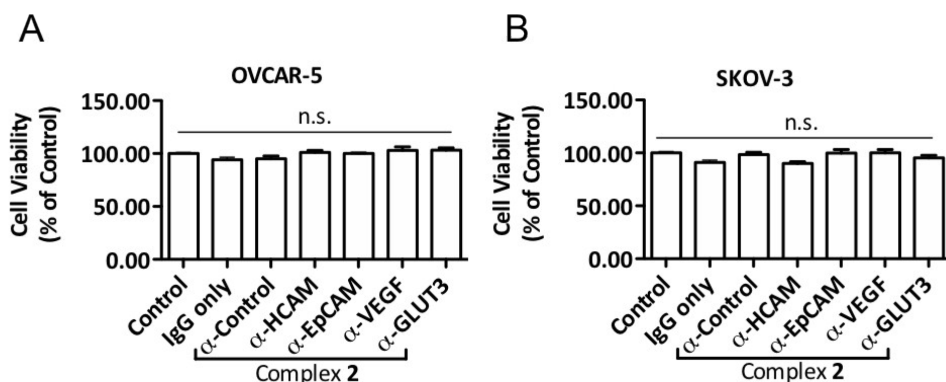


Figure S13. Cell viability, as measured by reduction of MTT 24 h post-treatment, of (A) OVCAR-5 and (B) SKOV-3 treated with 2 µg/mL Complex **2** and control antibodies. Family of Complex **2** constructed from mouse monoclonal antibodies raised against human HCAM (α-HCAM), EpCAM (α-EpCAM), GLUT3 (α-GLUT3) and VEGF-1 (α-VEGF). Contol treatments with vehicle control (Control), IgG without streptavidin (IgG) and Complex **2** synthesized from control mouse IgG (α-Control) included. Data representative of n=3 independent experiments. (* $p<0.05$)

$$\left(\frac{\text{2-10 µg Ab-photoCORM}}{\text{mL}}\right)\left(\text{0.2 mL cell culture media}\right)\left(\frac{\text{1-4 mol streptavidin}}{\text{1 mol Ab-photoCORM}}\right)\left(\frac{\text{4 mol Complex } \mathbf{1}}{\text{1 streptavidin}}\right)\left(\frac{\text{3 mol CO}}{\text{1 mol Complex } \mathbf{1}}\right) = \text{~23-262 pmol CO}$$

Scheme S2. Calculations for estimation of molar release of CO from antibody-photoCORM conjugates.

Experimental Section

Materials

Biotin-hydrazide (A8007-100mg) was procured from Apex Biotech, Ltd. (Xuzhuang, Shaanxi, PRC). Mouse monoclonal antibodies raised against HCAM (sc-7297), EpCAM (sc-53277), GLUT3 (sc-74399), VEGF-1 (365578) and normal mosue IgG (sc-2025) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Synthesis of biotin-photoCORM (Complex 1)

$[Mn(CO)_5(Br)]$ (100 mg, 0.36 mmol) and 1,10-phenanthroline (phen) (66 mg, 0.36 mmol) was dissolved in 25 mL dichloromethane (DCM) and allowed to stir in the dark for 20 h at 25°C. The solution, initially dark yellow, was dried down under vacuum to a yellow powder. Next, 1.5-fold excess $AgCF_3SO_3$ (140 mg, 0.54 mmol) was dissolved in 20 mL DCM and added to the yellow product, and allowed to stir for 1.5 h at 25°C in the dark. The cloudy green solution was subsequently filtered through a wet Celite pad, and the filtrate was evaporated to dryness. The resulting yellow powder was dissolved in 50 mL chloroform. To that stirring solution, 4-pyridinecarboxaldehyde (pyAl) (385 mg, 3.6 mmol) was added drop wise and allowed to stir for 20 h at 25°C in the dark. The next day, the solution was dried down under vacuum, revealing orange/yellow microcrystals of $[Mn(CO)_3(phen)(PyrAl]CF_3SO_3$ (155 mg, 0.27 mmol, 75%). To 223 mg (0.39 mmol) of $[Mn(CO)_3(phen)(PyrAl]CF_3SO_3$, biotin-hydrazide (100 mg, 0.39 mmol) dissolved in 20 mL of freshly distilled 2,2,2-trifluoroethanol was added, in a similar manner to a previous study.[1] The dark yellow solution was allowed to stir for 20h at 25°C in the dark. The yellow brown solution was concentrated under vacuum to ~2 mL, then chromatographed on a basic alumina column (50-200 µm particle diameter). The column was then washed with DCM (to remove unreacted $[Mn(CO)_3(phen)(PyrAl]CF_3SO_3$ and biotin-hydrazide and finally Complex **1** was eluted with DCM/methanol (3/2 v/v).

^{1}H NMR (400 MHz, $[D_4]$-methanol]): δ=1.37-1.84 (m, 6H), 2.30 (t, 2H, 7.4 Hz), 2.68 (m, 1H), 2.89 (m, 1H), 3.18 (m, 1H), 4,24 (m, 1H), 4.47 (m, 1H), 7.53 (m, 2H), 7.86 (s, 1H), 8.20 (m, 4H), 8.38 (m, 2H), 8.87 (m, 2H), 9.88 (m, 2H); IR (KBr): v = 2039, 1939, 1685 cm^{-1}(C=O); HRMS (ESI): *m*/*z* calcd for $C_{31}H_{29}N_7O_5SMn$: 666.13315 [*M*+]; found: 666.13539, Δ ppm = 3.4 ppm, Δ mDa = 2.2; elemental analysis calcd (%) for $C_{31}H_{29}N_7O_5SMn$: C 55.86, H 4.35, N 14.71,

O 12.01, S 4.80, Mn 8.26; found: C 55.84, H 4.39, N 14.71, O 12.01, S 4.80, Mn 8.25.

Physical Measurements

^{1}H NMR spectra of Complex **1** were collected at 298 K on a Varian Unity Inova 500 MHz instrument. FT-IR of Complex **1** was collected on a PerkinElmer Spectrum-One FT-IR. UV–vis data of Complex **1** were recorded on a Varian Cary 50 UV–vis spectrophotometer.

Myoglobin Assay

Horse heart myoglobin was dissolved in 1X PBS, pH=7.4 to a final concentration of 50 μM and reduced with 0.1 % sodium dithionite in quartz cuvette under aerobic conditions. In a second cuvette, Complex **1** was dissolved in 1X PBS to a final concentration of 50 μM. Antibody-photoCORM conjugates (Ab-photoCORM) were dissolved in 1X PBS to a final concentration of 10 μg/mL. For myoglobin assays performed in human serum, either Complex **1** or Ab-photoCORMs was dissolved to final concentrations of 50 μM or 10 μg/mL respectively in human serum, followed by ≥1h incubation at 37°C. Photogenerated CO, triggered by low power, broadband visible light (10mW/cm^2) from Complex **1** or Ab-photoCORM was released into the headspace and transferred to the reduced Mb solution via a cannula and positive pressure with N_2(g). The extent of the conversion of Mb to carboxymyoglobin (MbCO) was monitored by the change in absorbance at 540 nm, a reliable determination of CO-release from organometallic carbonyl complexes.[2] The source of low power, broadband visible light was an IL 410 Illumination System purchased from Electro Optical Components, Inc. (Santa Rosa, CA, USA). Visible light power was measured with a Field MaxII-TO laser power meter purchased from Coherent (Palo Alto, CA, USA).

Photolysis Experiments

The rate of CO release (k_{CO}) for Complex **1** at 25 °C in 1x PBS was assessed with in 1 cm x 1 cm quartz cuvettes. The k_{CO} of Complex **1** (concentration = 3.0 × 10^{-5} M, 390 nm, 25°C) was determined by recording the electronic absorption spectra, monitoring changes in the spectra following exposure to light at regular intervals. k_{CO} was then calculated from the ln[Complex **1**] versus time (*t*) plot.

Synthetic strategy of streptavidin-conjugated mouse IgG (Complex 2)

Conjugation of 1 mg mouse IgG, either control or antigen-specific IgG, with streptavidin was performed utilizing the Streptavidin Conjugation Kit (ab102921, Cambridge, MA, USA). Native gel electrophoresis and size exclusion chromatography were used to analyze and characterize streptavidin-conjugated antibodies. Complex **2** was quantified for use in subsequent cellular studies by measuring total protein using a Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific, Waltham, MA).

Native Gel Electrophoresis

2 μg of streptavidin-IgG conjugate was combined with native loading dye (62.5 mM Tris-HCl, pH=7.4, 40% glycerol and 0.01% bromophenol blue) and loaded onto a 4-12% Mini-PROTEAN TGX Precast Protein Gels (#4561095, Bio-Rad, Hercules, CA, USA) and separated under non-reducing, native conditions. Protein bands were visualized using Coomassie Brilliant Blue R-250 (#161-0436).

Size Exclusion Chromatography

Separation and simultaneous UV absorbance detection at 214 nm of streptavidin-conjugated antibodies and antibody-photoCORM conjugates was performed using a 7.5 D x 60 cm, 3 μm Tosoh TSK G4000SW (stainless steel) column. The column was preconditioned with molecular weight standards. The mobile phase was prepared with 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH = 6.8 and sterile filtered and degassed prior to use. Separation species based on size was performed at a flow rate of 0.5 mL/min, 25 °C. Fractions of streptavidin-conjugated antibodies and antibody-photoCORM conjugates were further characterized by bottom up proteomics.

Synthesis of antibody-photoCORM Conjugates (Ab-photoCORM)

Complex **2** (100 μg, ~273 pmol) pre-dissolved in 500 μL 1X PBS was reacted with excess Complex **1** (40.0 ng, 60 nmol) pre-dissolved in 500 μL 1X PBS for 1h at 25°C in the dark. The antibody-photoCORM conjugates (Ab-photoCORMs) were purified using size exclusion chromatography. Bottom up proteomics and HPLC-MS/MS analysis was utilized to characterize the composition of Ab-photoCORMs. Detection of Complex **1** in Ab-photoCORMs was observed in full MS scans in the bottom up proteomic assays.

Bottom Up Proteomics Analysis

10 μg of each antibody-photoCORM conjugate, as determined by BCA Protein Assay, were solubilized in 200 μL lysis buffer (12 mM sodium lauroyl sarcosine, 0.5% sodium deoxycholate, 50 mM triethylammonium bicarbonate (TEAB)) followed by 10 min bath sonication and heating at 95°C for 5 min. The samples were then diluted to 0.5 mg total protein/mL with lysis buffer, then a 100 μL aliquot was treated with 5 mM tris(2-carboxyethyl) phosphine (TCEP) prepared in 50 mM aqueous TEAB at 60°C for 30 min. Next, the samples were treated with 10mM chloroacetamide, prepared in 50 mM TEAB, for 30 min at 25°C in the dark. Samples were diluted 5-fold in 50 mM TEAB, then incubated overnight with Sequencing Grade Modified Trypsin (1:100, g trypsin: g total protein). The next day, an equal volume of ethyl acetate/trifluoroacetic acid(TFA, 100/1, v/v) was added to samples, followed by 5 min vigorous vortexing and centrifugation (13,000 x g, 5 min). Desalting of samples was performed similar to that previously described.[3] Dried samples were reconstituted in acetonitrile/water/TFA (2/98/0.1, v/v/v), loaded onto a C18-silica disk (3M, Maplewood, MN, USA) placed inside a 200 μL pipet tip. Prior to sample loading onto the disk, it was equilibrated with methanol (20 μL), acetonitrile/water/TFA (20 μ L, 80/20/0.1, v/v/v), then finally acetonitrile/water/TFA (2/98/0.1, v/v/v). The

samples loaded onto the disks were washed with acetonitrile/water/TFA (20 µL, 2/98/0.1, v/v/v) and eluted with acetonitrile/water/TFA (40 µL, 80/20/0.1, v/v/v). Eluents were concentrated under vacuum centrifugation and reconstituted in 10 µL water/acetonitrile/formic acid, 98/2/0.1, v/v/v). 5 µL aliquots were injected onto a reverse phase nanobore HPLC column (AcuTech Sceintiic, C18, 1.8 µm particle size, 360 µm x 20 cm, 150 µm ID), equilibrated in water/acetonitrile/formic acid (98/2/0.1, v/v/v: min/%; 0/0, 5/3, 18/7, 74/12, 144/24, 153/27, 162/40, 164/80, 174/80, 176/0, 180/0) using an Eksigent NanoLC-2D system (Sciex, Framingham, MA, USA). The flow from the column was directed towards nanospray ionization source connected to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific). Data-dependent mass spectra were acquired alternating between full scan (m/z 350-2000, automated gain control target 3 x 10^3, 50 ms maximum injection time, FWHM resolution 70,000 at m/z 200) and up to 10 MS/MS scans (quadrupole isolation of charge states ≥1, isolation width 1.2 Th) with optimized fragmentation conditions (normalized collision energy of 32, dynamic exclusion of 30 s, AGC target 1 x 10^6, 100 ms maximum injection time, FWHM resolution 35,000 at *m/z* 200). Analysis of raw data and peptide/protein identification of the antibody-photoCORM conjugates was performed using Mascot to search the UniProt-Mouse database. Common Contaminants database was also searched to identify streptavidin. Probability based scoring was used to determine significance of data, where reported scores = -10 x $Log_{10}(P)$, where P is the absolute probability that the observed match between the experimental data and the database sequence is a random event.[4,5] Scores >67 are considered significant ($p<0.05$).[4,5] Complex **1** associated with Ab-photoCORM was observed in the full MS scan data (Figure S6).

Cell culture

Ovarian cancer cell lines OVCAR-5 and SKOV-3 were obtained from American Type Culture Collection (Manassas, VA, USA). OVCAR-5 and SKOV-3 were grown in RPMI 1640 Medium (11875119, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, 16000) and 100 U/mL penicillin-streptomycin (15070063) were all purchased from ThermoFisher Scientific. Cells were passaged ≤ 10 times after acquisition from the manufacturer.

Cell Viability (MTT) Assay

Cell viability was assessed by the cellular reduction of tetrazolium dye MTT performed in 96-well tissue culture plates. 2 × 10^3 cells/well were allowed to seed overnight in a 37 °C incubator + 5% CO_2. The following day, cells were treated as indicated with Complex **1** or Complex **2**, then assessed for viability 24 h post-treatment. Following removal of cell culture media, 0.5 mg/mL MTT dissolved in fresh 1× DMEM was added and allowed to incubate for 2 h in a 37 °C incubator + 5% CO_2. Cell viability was quantified by measuring the relative amount of MTT reduced to insoluble formazan. Following solubalization of formazan in 10% SDS

+ 0.01 N HCl, formazan was measured by taking the absorbance at 570 mm, reference wavelength taken at 690 nm.

Western Analysis

Whole cell lysates were extracted using RIPA lysis buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS and 1× protease inhibitor cocktail. BCA Protein Assay assayed soluble fractions for total protein content. 20 µg of soluble cell lysates from samples were resolved on 10% SDS-PAGE gel and transferred to poly(vinylidene difluoride) (PVDF) membrane. All following blocking and antibody solutions were prepared in 1x PBS + 0.1% Tween 20. Membranes, following blocking in 5% nonfat dried milk for 18 h at 4°C, were probed with primary (1:1000 dilution) antibody overnight at 4°C and then horseradish peroxidase (HRP)-conjugated secondary (1:10,000 dilution) antibody for 1 h at 25°C. Immunofluorescent signals were amplified with Pierce ECL Plus Western blotting substrate (32132, ThermoFisher Scientific).

Live-cell Immunosorbent Assay

2 x 10^3 cells/well of 96-well tissue culture plates were allowed to seed overnight at 37°C + 5% CO_2. The next day, cells were treated as indicated with 0-10 µg/mL antibody-photoCORM conjugates (Ab-photoCORM), as measured by BCA Protein Assay. Immunosorbence of Ab-photoCORMs to the adherent live cells was allowed to occur for 60 min in the dark at 37°C + 5% CO_2. α-Control Ab-photoCORM, utilizing control mouse IgG (sc-2025, Santa Cruz Biotechnology), was utilized as control to assess the specificity of the other antibody-photoCORM conjugates. Following incubation, the media was gently aspirated, followed by three 250 µL washes with 1X PBS in the dark to remove any non-specific binding. 100 µL fresh cell culture media was added, followed by illumination with low power, visible light for 30 min to trigger release of CO from any Ab-photoCORM present after immunosorbence and washing. Cell viability, as measured by the reduction of MTT, was assayed 24 h post-illumination of light.

Statistical Analysis

Data are expressed as the mean ± standard error mean (range) or as percentage of control value where indicated. Comparisons between two groups were made using the Student's t-test. Comparisons between more than two groups were made using the One-way ANOVA/ Tukey's post hoc test. p-values < 0.05 were considered statistically significant. All calculations were performed using GraphPad Prism software package (GraphPad Software Inc., San Diego, USA).

Supporting References

[1] M. Salmain, N. Fischer-Durand, L. Cavalier, B. Rudolf, J. Zakrzewski, G. Jaouen, *Bioconjug. Chem*. **2002**, *13*, 693-698.

[2] A. J. Atkin, J. M. Lynam, B. E. Moulton, P. Sawle, R. Motterlini, N. M. Boyle, M. T. Pryce, I. J. S. Fairlamb, *Dalton Trans.* **2011**, *40*, 5755-5761.

[3] J. Rappsilber, M. Mann, Y. Ishihama, *Nat. Protoc.* **2007**, *2*, 1896-1906.

[4] T. Koenig, B. H. Menze, M. Kirchner, F. Monigatti, K. C. Parker, T. Patterson, J. J. Steen, F. A. Hamprecht, H. Steen, *J. Proteome Res.* **2008**, *7*, 3708-3717.

[5] D. J. C. Pappin, P. Hojrup, A. J. Bleasby, *Curr. Biol.* **1993**, *3*, 327-332.