Supporting Information for

A photoresponsive antibody-siRNA conjugate for activatable immunogene therapy of cancer

Xingxing Wang,[#] Xiao Xiao,[#] Yi Feng, Jinbo Li* and Yan Zhang*

State Key Laboratory of Analytical Chemistry for Life Sciences, Jiangsu Key Laboratory of Advanced Organic Materials, School of Chemistry and Chemical Engineering, Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, Nanjing 210023, China.

[#]These authors contributed equally.

*Emails: jinboli@nju.edu.cn (Jinbo Li); njuzy@nju.edu.cn (Yan Zhang)

Contents

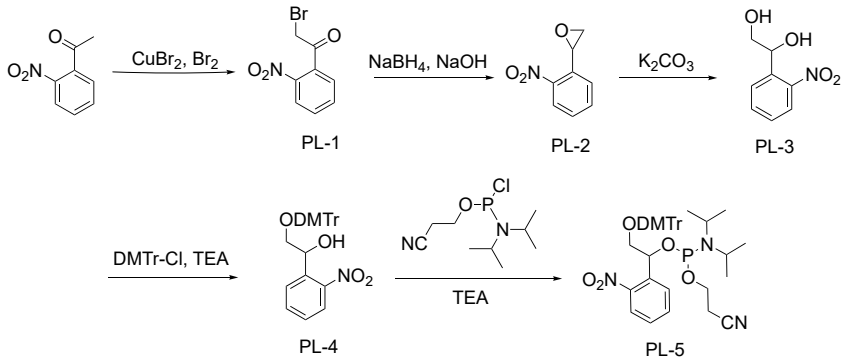
Materials and Methods

1. General materials and methods

All chemicals and solvents were purchased from J&K chemicals (Shanghai, China), TCI chemicals (Shanghai, China), Sinopharm Chemicals (Shanghai, China) or Tansoole (Shanghai, China). Traut's Reagent and Lipofectamine 2000 were purchased from ThermoFisher (Shanghai, China). High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, Lysotracker, DAPI, and ECL reagent were ordered from Life Technologies (Shanghai, China). Antibodies and protease inhibitor cocktail were purchased from Cell Signaling Technology (Shanghai, China). RIPA buffer and 5*SDS loading buffer were purchased from Solarbio (Beijing, China). αPD-L1 was purchased from MedChemExpress (Shanghai, China). IL-2 and ELISA kits were purchased from Sino Biological lnc. (Beijing, China). LDH cytotoxicity assay kit was purchased from Beyotime (Shanghai, China). All aqueous solutions were treated by diethy pyrocarbonate (DEPC) before use.

^{1}H NMR and ^{31}P NMR spectra were obtained on a 400 MHz Bruker AVANCE III–400 spectrometer. Chemical shifts are reported in δ (ppm) relative to the solvent residual peak. Coupling constants are reported in Hz with multiplicities denoted as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). HRMS was done on Agilent 6550 iFunnel Q-TOF LC/MS. HPLC was carried out on Thermo UltiMate 3000 with CH_3CN/H_2O (0.1 M CH_3COONH^+) as eluents. Ion exchange chromatography (IEC) was performed on an Akta system equipped with a HiTrap Capto S column.

2. Synthesis and characterization of the photo-cleavable linker



The photo-cleavable linker PL-5 was synthesized according to the method reported by us before.[1]

Synthesis of PL-1

2-nitroacetophenone (5.04 g, 30 mmol) and $CuBr_2$ (13.41 g, 60 mmol) were dissolved in a mixture of ethyl acetate-chloroform (v/v, 1/1, 70 mL). Then, several drops of liquid bromine were slowly added and the reaction mixture was stirred at 80 ^oC for 8 h before cooling down to room temperature. The precipitate was filtered out, and the supernatant was washed with saturated brine and dried over anhydrous sodium sulfate. After the solvent was removed by vacuum, the crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, v/v, 3/1) to give the product PL-1 (4.72 g, 65%). ^1H NMR (400 MHz, DMSO) δ 8.24 (d, J = 8.2 Hz, 1 H), 7.93 (t, J = 7.6 Hz, 1 H), 7.83 - 7.78 (m, 1 H), 7.77 (dd, J = 7.6, 1.4 Hz, 1 H), 4.85 (s, 2 H).

Synthesis of PL-2

PL-1 (4.39 g, 18 mmol) was dissolved in 1,2-dioxane (30 mL). Then, $NaBH_4$ (2.05 g, 54 mmol in 20 mL methanol) was slowly added through cooling by ice bath. The reaction mixture was stirred at room temperature until PL-1 completely disappeared as monitored by TLC. Then, aqueous NaOH solution (10%) was added to the reaction mixture through cooling by ice bath, and the mixture was stirred for 0.5 h at room temperature before the mixture was extracted with ethyl ether and dried over anhydrous sodium sulfate. After the mixture was filtered and concentrated, the crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, v/v, 5/1) to give the product PL-2 (2.17 g, 72.9%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, J = 8.2 Hz, 1 H), 7.66 (t, J = 7.5 Hz,1 H), 7.60 (d, J = 7.8 Hz, 1 H), 7.47 (t, J = 7.5 Hz ,1 H), 4.48 (dd, J = 4.3, 2.7 Hz, 1 H), 3.29 (dd, J = 5.5, 4.5 Hz, 1 H), 2.67 (dd, J = 5.6,

2.6 Hz, 1 H).

Synthesis of PL-3

For the synthesis of PL-3, aqueous K_2CO_3 solution (10%, 50 mL) was added to a solution of PL-2 (1.98 g, 12 mmol in 16 mL 1,2-dioxane). The resulted mixture was refluxed for 18 h before cooling down to room temperature. Then, hydrochloric acid was added to the mixture to adjust the pH value to 5. After the mixture was extracted with ethyl ether and dried over anhydrous sodium sulfate, the solution was filtered and concentrated. The crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, v/v, 1/1) to give the product PL-3 (1.8 g, 82%). ^{1}H NMR (400 MHz, DMSO) δ 7.85 (d, J = 8.1 Hz, 1 H), 7.78 (d, J = 7.8 Hz, 1 H), 7.70 (t, J = 7.6 Hz, 1 H), 7.49 (t, J = 7.0 Hz, 1 H), 5.63(d, J = 4.6 Hz, 1 H), 5.09 - 5.05 (m, 1 H), 4.92 - 4.89 (m, 1 H), 3.48 - 3.43 (m, 2 H).

Synthesis of PL-4

For the synthesis of PL-4, PL-3 (0.549 g, 3.0 mmol) was dissolved in a mixture of dichloromethane and triethylamine (v/v, 4/1, 6.25 mL). 4, 4′-dimethoxytriphenylmethyl chloride (1.016 g, 3.0 mmol in 5 mL dichloromethane) was added dropwise to the solution. The mixture was stirred for 2 h at room temperature. After the mixture was concentrated, the crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, v/v, 5/1) to give the product PL-4 (0.94 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, J = 8.1 Hz, 1 H), 7.78 (dd, J = 7.9 Hz, 1 H), 7.60 (t, J = 7.5 Hz, 1 H), 7.42 - 7.38 (m, 3 H), 7.30 - 7.26 (m, 7 H), 7.23 - 7.21 (m, 1 H), 6.83 - 6.80 (m, 4 H), 5.53 (dd, J = 7.3, 3.7 Hz, 1 H), 3.79 (s, 6 H), 3.63 (dd, J = 9.4, 3.4 Hz, 1 H), 3.16 (dd, J = 9.5, 7.4 Hz, 1 H).

Synthesis of PL-5

PL-4 (0.37 g, 1 mmol) and triethylamine (250 μL) were dissolved in anhydrous CH_2Cl_2 (3 mL) in a dry flask, followed by injection of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.35 g, 0.7 mmol in 1 mL anhydrous CH_2Cl_2) under dry nitrogen. The resulted solution was stirred at room temperature for 1 h. After the mixture was concentrated, the crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate, v/v, 2/1) to give

the compound PL-5 (226 mg, 33%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.87 (d, J = 8.2 Hz, 1 H), 7.80 (d, J = 8.9 Hz, 1 H), 7.57 (t, J = 7.6 Hz, 1 H), 7.41 - 7.37 (m, 3 H), 7.29 - 7.24 (m, 6 H), 7.20 - 7.18 (m, 1 H), 6.81 - 6.78 (m, 4 H), 5.80 - 5.77 (m, 1 H), 3.89 - 3.85 (m, 1 H), 3.78 (m, 6 H), 3.49 - 3.40 (m, 2 H), 3.28 - 3.24 (m, 1 H), 2.61 - 2.46 (m, 2 H), 2.28 - 2.22 (m, 2 H), 1.16 - 1.14 (d, J = 6.8 Hz, 6 H), 0.85 - 0.82 (d, J = 6.8 Hz, 6 H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.

3. Synthesis and characterization of siPD-L1, Cy3-labeled siPD-L1 and si-ctrl

All the oligonucleotides including siPD-L1 and si-ctrl were synthesized on a K&A H-8 DNA/RNA synthesizer. siPD-L1: 5'-GUGGCAUCCAAGAUACAAAdTdT-3' (guide strand), 5'-UUUGUAUCUUGGAUGCCACdTdT-3' (passenger strand). si-ctrl: 5'-UUCUCCGAAC-GUGUCACGUUUdTdT-3' (guide strand), 5'-AAACGUGACACGUUCGGAGAAdTdT-3' (passenger strand). 3'-amino-modifier-CPG was used for solid phase synthesis. PL-5 and Cy3 phosphonamidite were directly used as monomers on DNA/RNA synthesizer for preparation of siPD-L1, which contains 3'-NH_2. After cleavage from resin and further deprotection, these oligonucleotides were purified with HPLC. After lyophilization, the siPD-L1 was dissolved in DMSO and NHS-PEG-MAL stock solution was added. The reaction mixture was shaken at 25 ^{o}C for 12 h. The crude product was purified by HPLC with CH_3CN/H_2O (0.1 M CH_3COONH^{+}) as eluents (CH_3CN from 2% to 40% in 30 min). The collected products were desalted and lyophilized. Then, the oligonucleotides were dissolved in DEPC-treated H_2O and their concentrations were determined using NanoDrop 2000 (Thermo Scientific). These siRNAs were characterized by LC-MS. Guide strand of siPD-L1 with 3'-NH_2: *m/z*: $[M+H]^+$ calcd. 7171, found: 7171; guide strand of Cy3-labeled siPD-L1: *m/z*: $[M+H]^+$ calcd. 7782, found: 7782; passenger strand of siPD-L1: *m/z*: $[M+H]^+$ calcd. 6585, found: 6584; guide strand of si-ctrl with 3'-NH_2: *m/z*: $[M+H]^+$ calcd. 7666, found: 7666; passenger strand of si-ctrl: *m/z*: $[M+H]^+$ calcd. 7391; found: 7390. Double-stranded siPD-L1 and si-ctrl were prepared by annealing the guide strand with the passenger strand.

Reference

[1] Chen, L.; Sun, Y.; Li, J.; Zhang, Y., *Chem. Commun.* **2020,** *56*, 627-630.

Supporting Figures

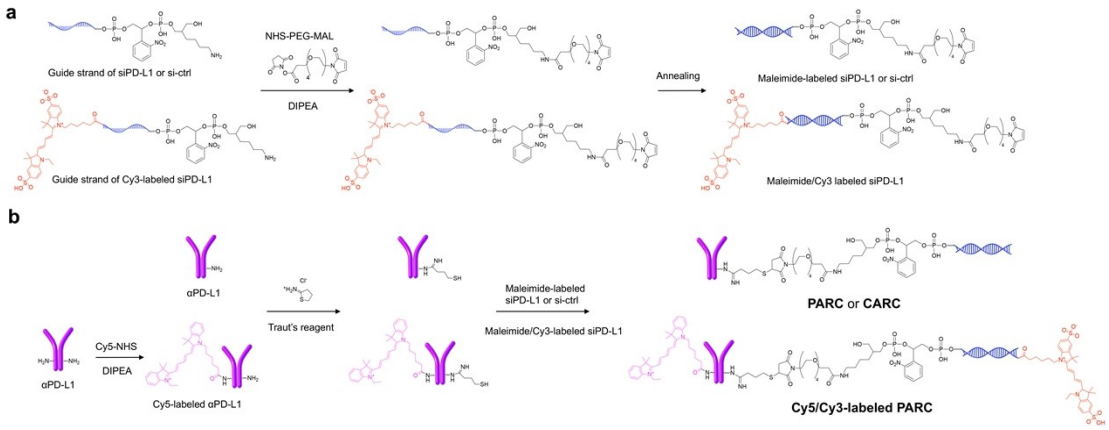


Figure S1. Chemical routes for (**a**) synthesis of labeled siPD-L1 or si-ctrl and (**b**) preparation of **PARC**, **CARC**, or Cy5/Cy3-labeled **PARC**.

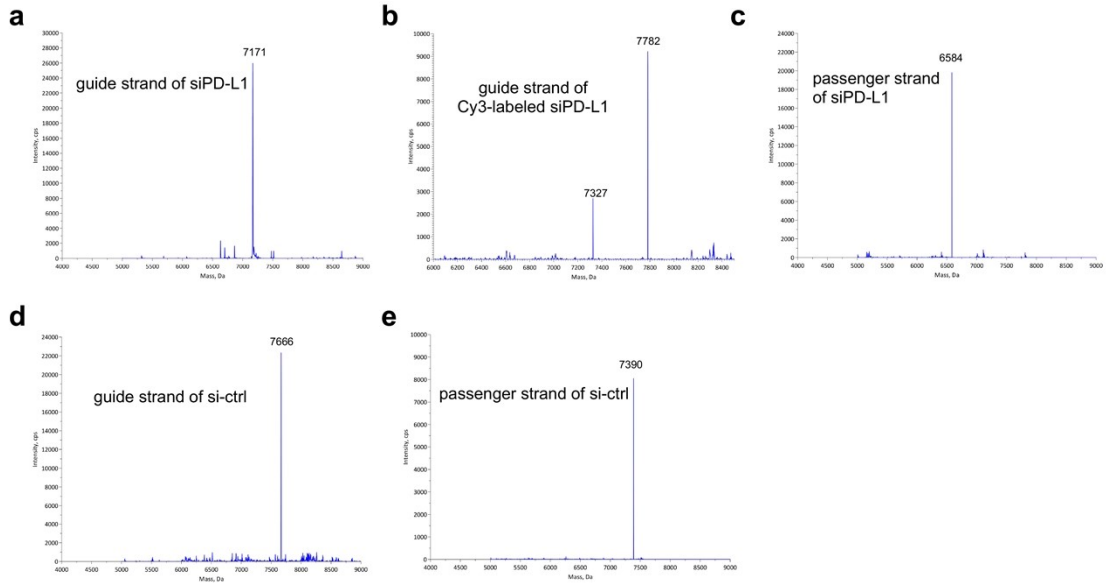


Figure S2. MS spectra of (**a**) guide strand of siPD-L1, (**b**) guide strand of Cy3-labeled siPD-L1, (**c**) passenger strand of siPD-L1, (**d**) guide strand of si-ctrl, and (**e**) passenger strand of si-ctrl.

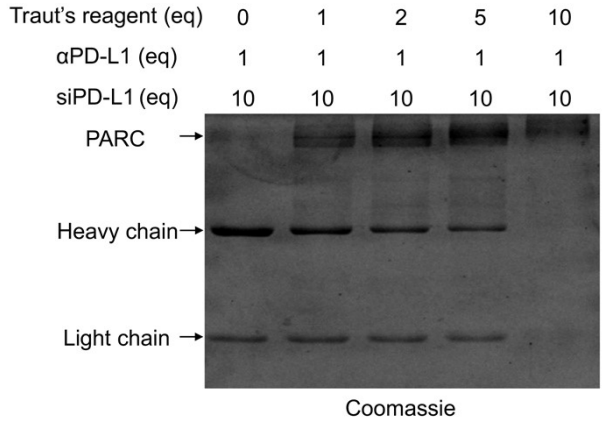


Figure S3. SDS-PAGE analysis of reaction mixtures of αPD-L1 (1 eq.) treated with Traut's reagent (0-10 eq.) and maleimide-labeled siPD-L1 (10 eq.). αPD-L1 was fully converted into **PARC** at a Traut's reagent/αPD-L1/siPD-L1 ratio of 10/1/10.

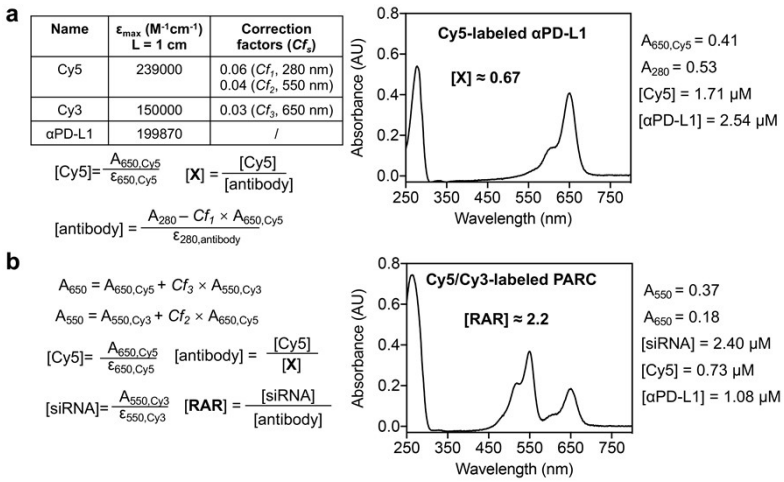


Figure S4. To measure siRNA-to-antibody ratio (RAR), Cy5 and Cy3 were used to respectively tag αPD-L1 and siPD-L1, whose amounts were further quantified by measuring Cy5/Cy3 according to the Lambert-Beer law. UV-vis absorbance spectra of (**a**) Cy5-labeled αPD-L1 and (**b**) Cy5/Cy3-labeled **PARC**. The (**a**) Cy5-to-antibody ratio (X) and (**b**) RAR were calculated using formula described in the figure. (**a**) X was measured after αPD-L1 was labeled with Cy5 and (**b**) further used to determine αPD-L1 concentration.

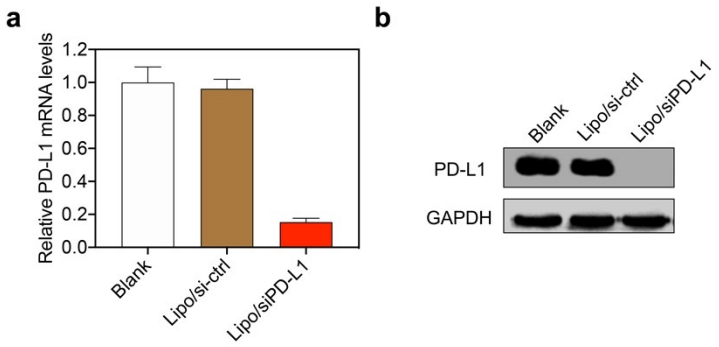


Figure S5. (**a**) RT-qPCR analysis of PD-L1 mRNA levels and (**b**) western blotting analysis of PD-L1 protein levels in HCT116 cells transfected with si-ctrl (200 nM) or siPD-L1 (200 nM). Data are shown as mean ± SD (n=3). GAPDH served as the internal control.

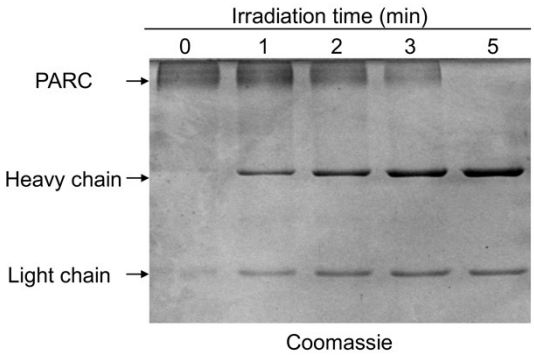


Figure S6. SDS-PAGE analysis of **PARC** irradiated with 365 nm light (10 mW/cm^2) for 0, 1, 2, 3, or 5 min.

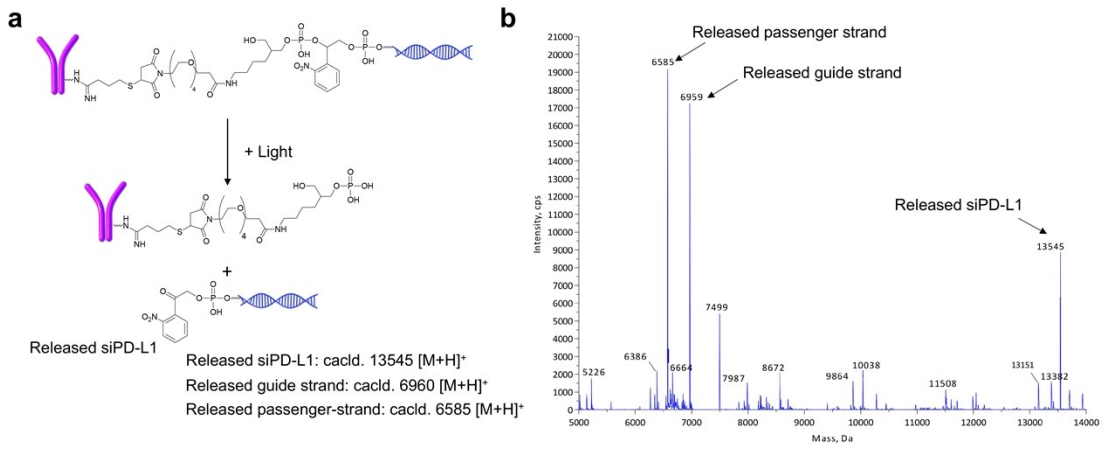


Figure S7. (**a**) Photolysis chemistry of **PARC** and calculated molecular weight of released siPD-L1. (**b**) MS spectrum of released siPD-L1.

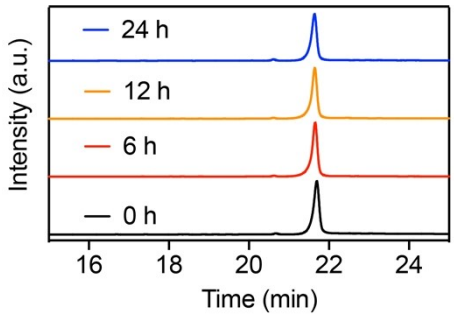


Figure S8. IEC analysis of **PARC** incubated in PBS at 37 °C for 0, 6, 12, or 24 h.

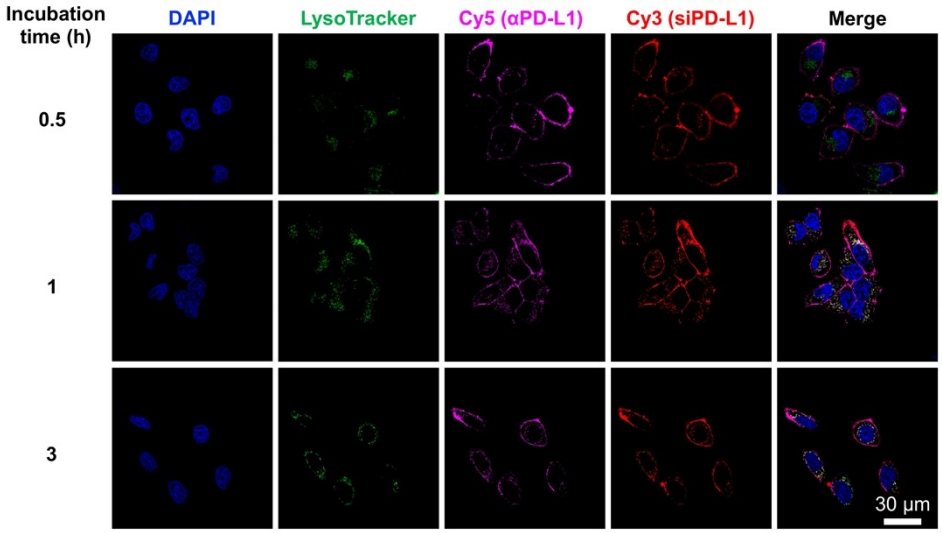


Figure S9. Confocal fluorescence images of HCT116 cells treated with Cy5/Cy3-labeled **PARC** (150 nM) for 0.5, 1, or 3 h. The nucleus and lysosome were stained with DAPI and LysoTracker, respectively.

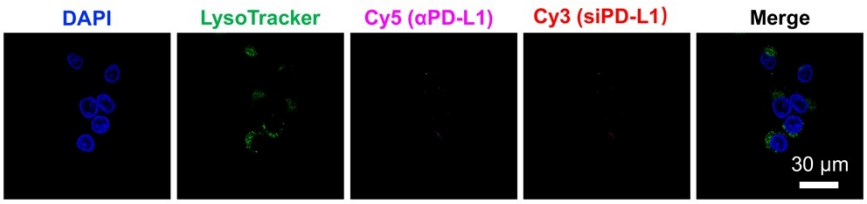


Figure S10. Confocal fluorescence images of PD-L1-negative HCT116 cells treated with Cy5/Cy3-labeled **PARC** (150 nM) for 24 h. The nucleus and lysosome were stained with DAPI and LysoTracker, respectively. PD-L1-negative HCT116 cells were prepared by transfection with siPD-L1 (200 nM).

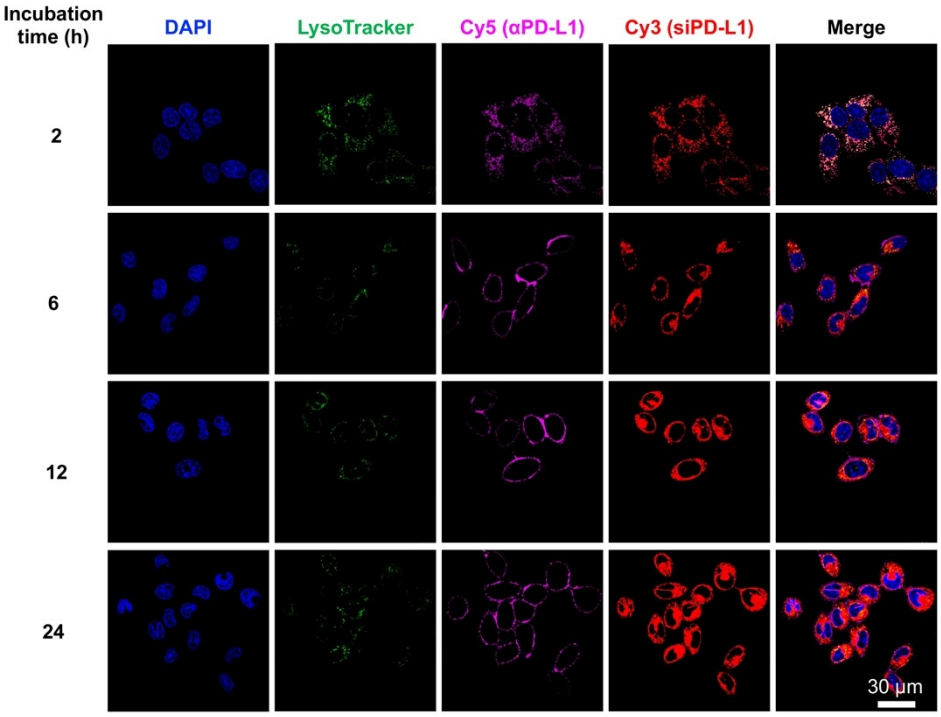


Figure S11. Confocal fluorescence images of HCT116 cells treated with Cy5/Cy3-labeled **PARC** (150 nM) for 2, 6, 12, or 24 h. Cells were irradiated with 365 nm light (10 mW/cm^2) for 5 min at 1 h. The nucleus and lysosome were stained with DAPI and LysoTracker, respectively.

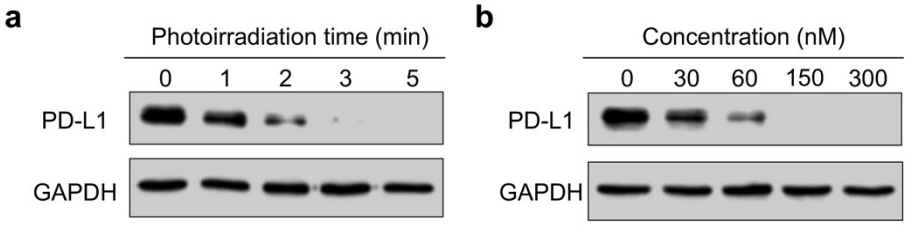


Figure S12. Western blotting analysis of PD-L1 protein levels in HCT116 cells treated with **PARC** for 48 h. Cells were irradiated with 365 nm light (10 mW/cm^2) after incubation for 1 h. (**a**) **PARC**, 150 nM; irradiation time, 0-5 min. (**b**) **PARC**, 0-300 nM; irradiation time, 5 min. GAPDH served as the internal control.

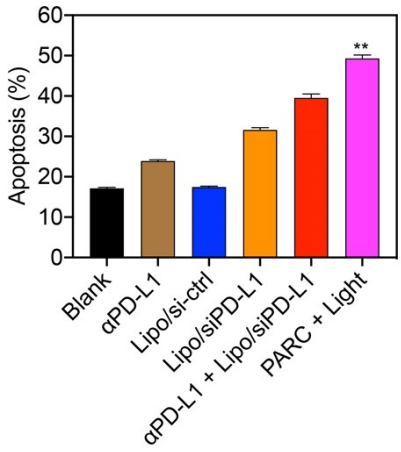


Figure S13. Apoptosis rate of HCT116 cells determined by lactate dehydrogenase release assay. HCT116 cells were treated with αPD-L1 (150 nM), si-ctrl (330 nM), siPD-L1 (330 nM), αPD-L1 (150 nM) + siPD-L1 (330 nM), or **PARC** (150 nM) for 48 h. si-ctrl or siPD-L1 were transfected by Lipofectamine 2000 (Lipo) according to the manufacture's protocol. Cells were irradiated with 365 nm light (10 mW/cm^2) for 5 min after incubation for 1 h. For co-culture assay, PBMCs were added to HCT116 cells at an effector-to-target ratio of 5:1 and incubated for another 24 h. Data are shown as mean ± SD (n=3). $^{**}P < 0.01$, relative to other groups.

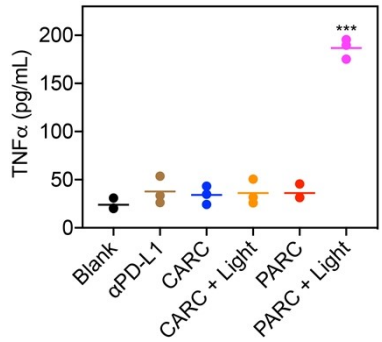


Figure S14. TNFα levels in the culture media. HCT116 cells were treated with αPD-L1 (150 nM), **CARC** (150 nM), or **PARC** (150 nM) for 48 h. Cells were irradiated with 365 nm light (10 mW/cm^2) for 5 min after incubation for 1 h. For co-culture assay, PBMCs were added to HCT116 cells at an effector-to-target ratio of 5:1 and incubated for another 24 h. Data are shown as mean ± SD (n=3). $^{***}P < 0.001$, relative to other groups.

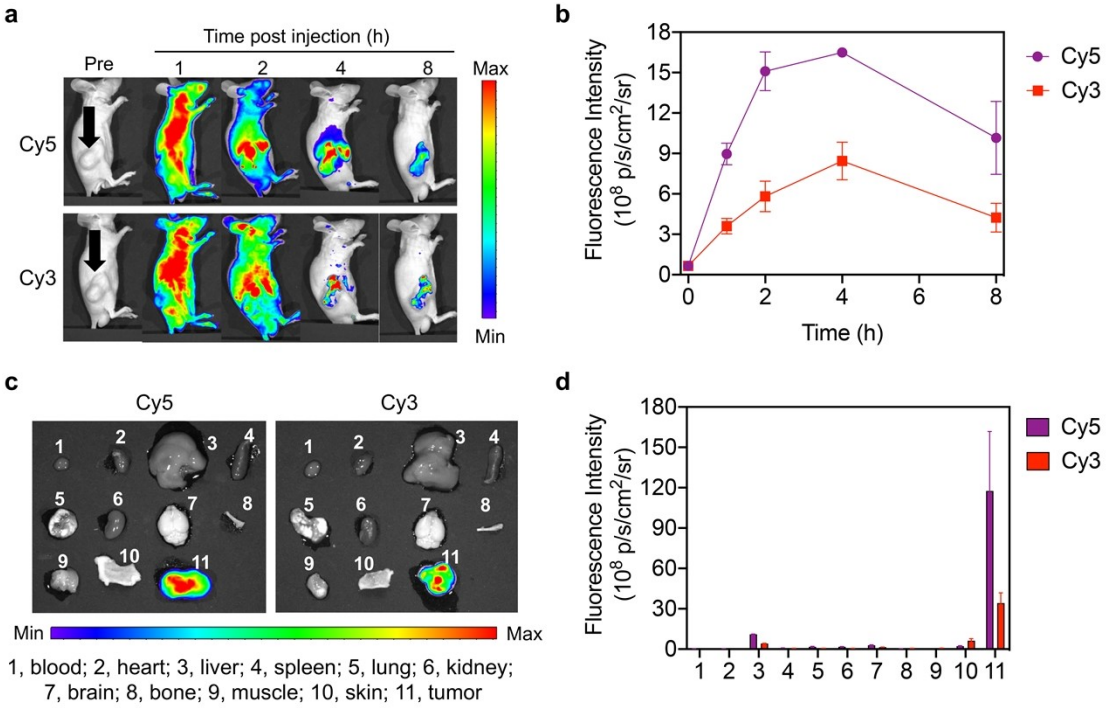


Figure S15. (**a**) Representative Cy5 and Cy3 fluorescence images of HCT116-tumor bearing mice and (**b**) fluorescence intensities in tumors at different time points after tail vein injection of Cy5/Cy3-labeled **PARC** (0.7 nmol). Dark arrows indicate tumor locations. (**c**) Representative Cy5 and Cy3 fluorescence images of major organs resected from mice at 8 h post injection and (**d**) fluorescence intensities. Data are shown as mean ± SD (n=3).

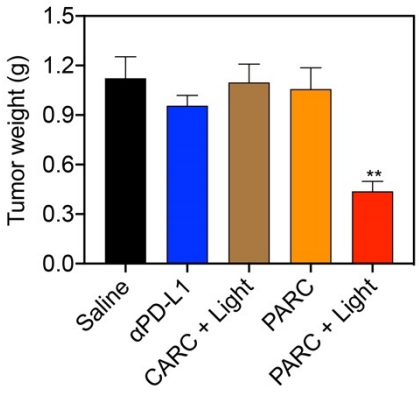


Figure S16. Weights of tumor tissues dissected from NCG mice that were intravenously injected with saline, αPD-L1 (0.7 nmol), **CARC** (0.7 nmol), or **PARC** (0.7 nmol). Tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each injection. Data are shown as mean ± SD (n=5). $^{**}P < 0.01$, relative to other groups.

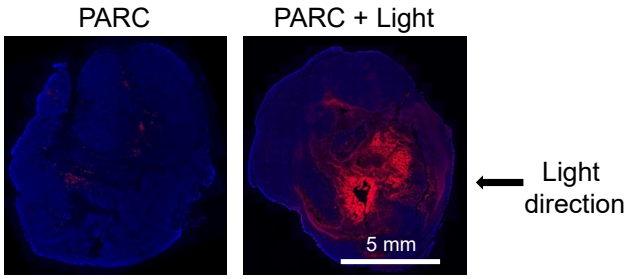


Figure S17. TUNEL staining of tumor tissues dissected from NCG mice that were treated with **PARC**. For + Light group, tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each injection. The apoptotic cells (red) in the tissues were labeled with Tunnelyte Red, and the nuclei (blue) were stained with Hoechst.

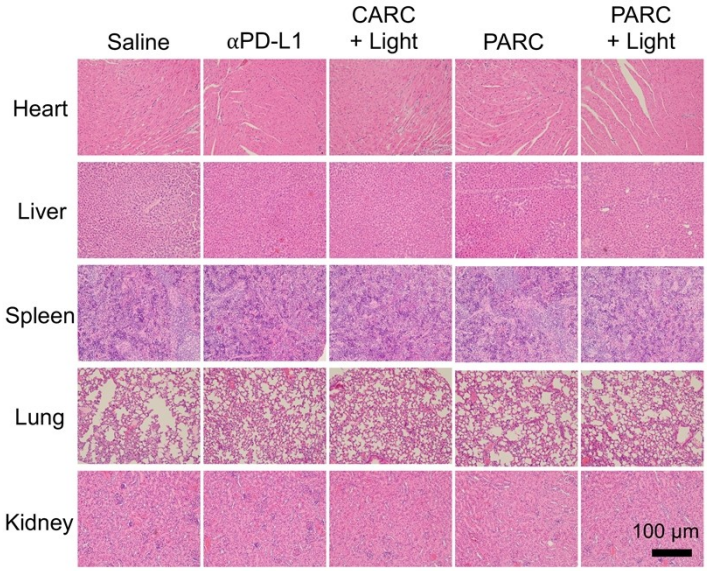


Figure S18. H&E staining of heart, liver, spleen, lung, kidney tissues dissected from NCG mice that were intravenously injected with saline, αPD-L1 (0.7 nmol), **CARC** (0.7 nmol), or **PARC** (0.7 nmol). Tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each injection.

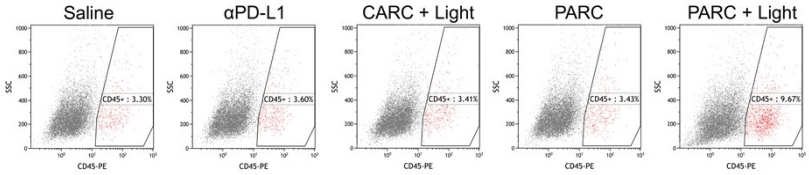


Figure S19. Flow cytometry analysis of CD45$^+$ lymphocytes in tumor tissues dissected from NCG mice that were intravenously injected with saline, αPD-L1 (0.7 nmol), **CARC** (0.7 nmol), or **PARC** (0.7 nmol). Tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each

injection.

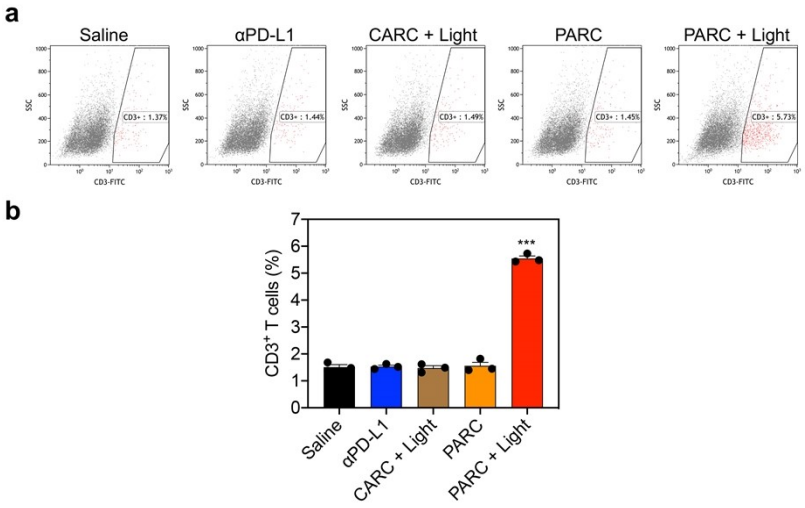


Figure S20. (**a**) Flow cytometry analysis of $CD3^+$ T cells in tumor tissues dissected from NCG mice that were intravenously injected with saline, αPD-L1 (0.7 nmol), **CARC** (0.7 nmol), or **PARC** (0.7 nmol). Tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each injection. (**b**) Quantitative analysis. Data are shown as mean ± SD (n=3). ***$P < 0.001$, relative to other groups.

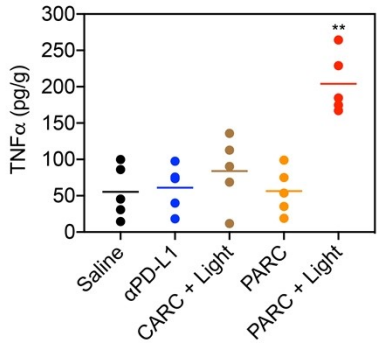
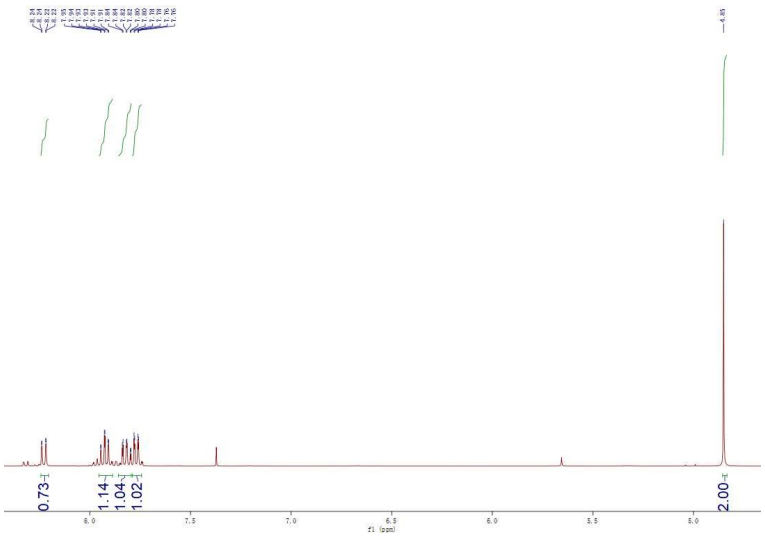


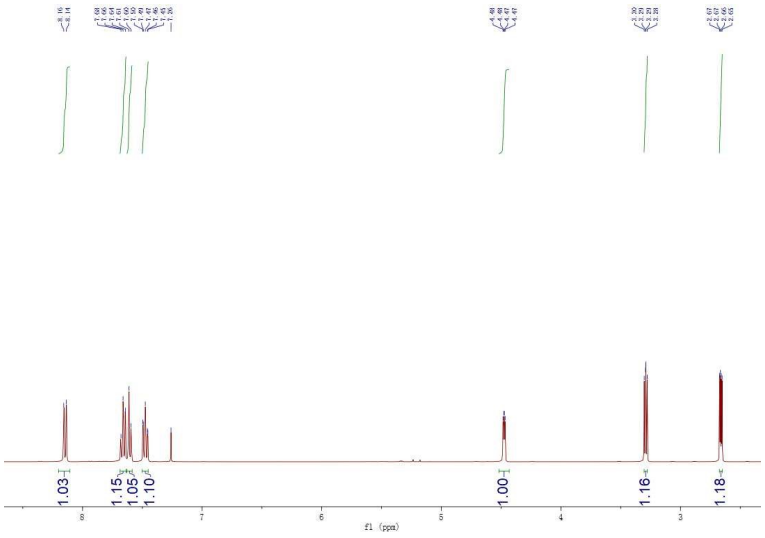
Figure S21. TNFα levels in tumor tissues dissected from NCG mice that were intravenously injected with saline, αPD-L1 (0.7 nmol), **CARC** (0.7 nmol), or **PARC** (0.7 nmol). Tumors were irradiated with 365 nm light (50 mW/cm^2) for 5 min at 4 h post each injection. Data are shown as mean ± SD (n=5). **$P < 0.01$, relative to other groups.

NMR Spectra

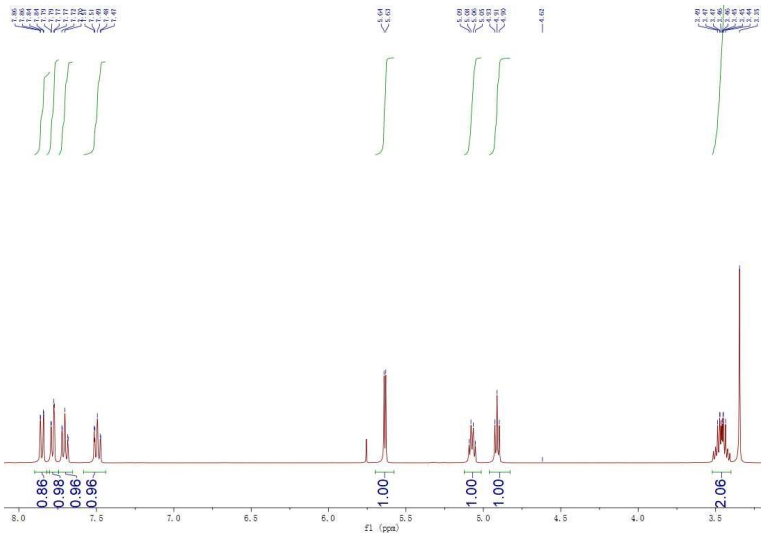
^{1}H NMR of PL-1



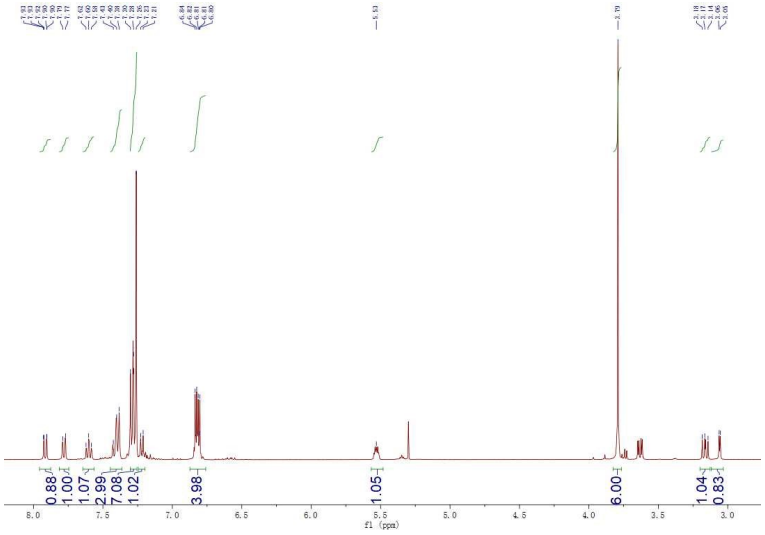
^{1}H NMR of PL-2



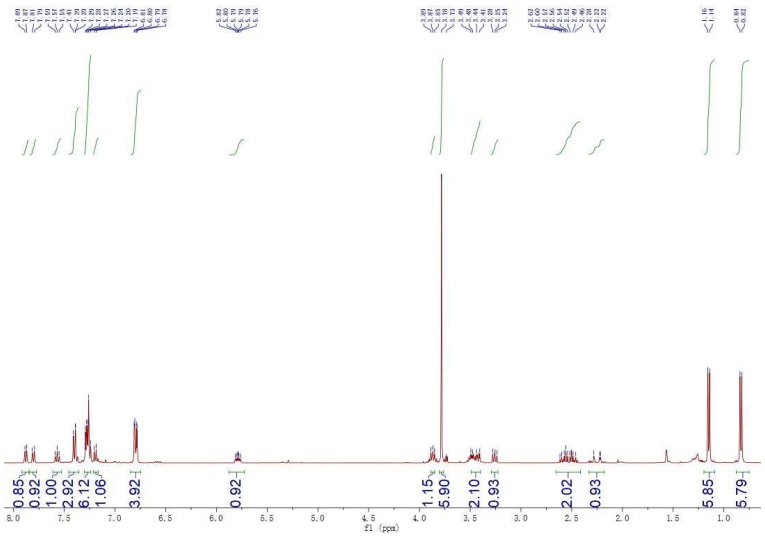
^{1}H NMR of PL-3



^{1}H NMR of PL-4



^{1}H NMR of PL-5



^{31}P NMR of PL-5