Classification: BIOLOGICAL SCIENCES

A tumor-targeted immune checkpoint blocker

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SUPPORTING INFORMATION

MATERIALS AND METHODS:

Growth inhibition activity assay on B16SIY cells. B16SIY cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated with various concentrations of conjugates or controls at 37°C with 5% CO_2. After 24 hour, 10 μL AlamarBlue Reagent (Thermo Fisher Scientific, IL) was added to each well, and cells were incubated for 4 hours at 37°C. After incubation, the cell number was evaluated by determination of fluorescence at excitation-560 nm / emission-590 nm on a microplate reader. Data were plotted in Graphpad Prizm.

Cytotoxicity assay on B16SIY cells. B16SIY cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated with various concentrations of conjugates or controls at 37°C with 5% CO_2. After 24 hours, 25 μL culture was transferred to a fresh 96-well flat clear bottom plate, and 25μl of the CytoTox 96 Reagent (Promaga, WI) was added to each sample aliquot. Absorbance intensities were then measured following manufacturer's instruction. Data were plotted and analyzed in Graphpad Prizm.

Thermal stability analysis. The thermal stability of antibodies was determined with Thermal Shift Dye Kit (Thermo Fisher Scientific, IL) according to manufacturer's instruction. Briefly, 12.5 μL 0.2 mg/mL protein in PBS was added to 5 μL thermal shift buffer, followed by 2.5 μL diluted thermal shift dye (8x). The mixture was

loaded onto a 384 well plate and the protein denaturation curve was determined with ViiATM 7 Real-Time PCR System according to the manufacturer's manual.

Stability of NDP-MSH-αPD-L1 conjugates in mouse serum. NDP-MSH-αPD-L1 conjugate or controls were added into 100 μL fresh mouse serum with a final concentration of 1 uM, and incubated at 37 °C for 0h, 6h, 24h, 48h, and 72h. After incubation, tubes were immediately frozen in liquid nitrogen, and stored at -80 °C until further processing. The amount of the antibody in each tube was determined by PD-L1 binding sandwich ELISA by the above procedures, and quantified by extrapolating the fluorescence signal into the linear range of a standard curve (signal vs concentration). The percentage was normalized against that at time zero.

Pharmacokinetics of NDP-MSH-αPD-L1 conjugates in mice. Protein in PBS (pH7.4) was administered intraperitoneally into C57BL/6 mice (Jackson Laboratory, n=3) at a single dose of 4 mg/kg. Blood samples were withdrawn from day 0 to day 14. The samples were stored in heparinized collection tubes, spun down and stored at -80 °C until further processing. After thawing and proper dilution of each plasma sample, the amount of antibody in each blood sample was quantified by PD-L1 binding sandwich ELISA as described above. The concentration of NDP-MSH-αPD-L1 conjugate was quantified by extrapolating the signal into a linear range (signal vs concentration) of a standard curve. Pharmacokinetic parameters were estimated using the modeling program WinNonlin (Pharsight).

SUPPLEMENTARY FIGURES:

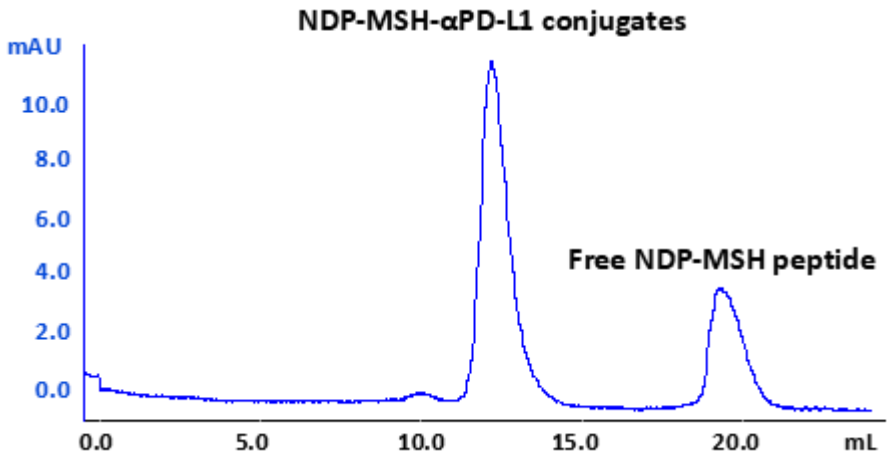

Figure S1. NDP-MSH-αPD-L1 conjugates were separated from free NDP-MSH peptide by size-exclusion gel chromatography. NDP-MSH/αPD-L1 was injected into Superdex 200 10/300 GL column, and followed by elution with PBS (pH7.4) buffer. UV absorbance at 280 nm was plotted versus the elution volume.

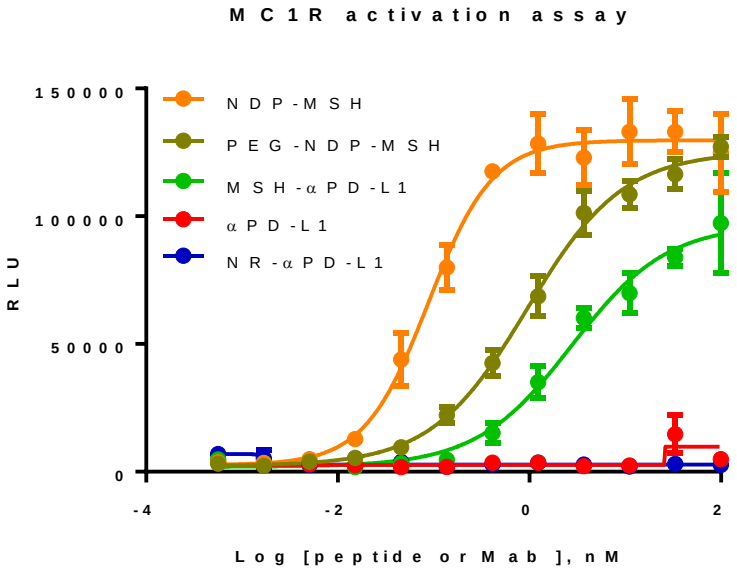

Figure S2. NDP-MSH-αPD-L1 conjugates activated MC1R in a dose-dependent manner using HEK 293 cells overexpressing MC1R and a CRE-Luc reporter. RLU=relative light units.

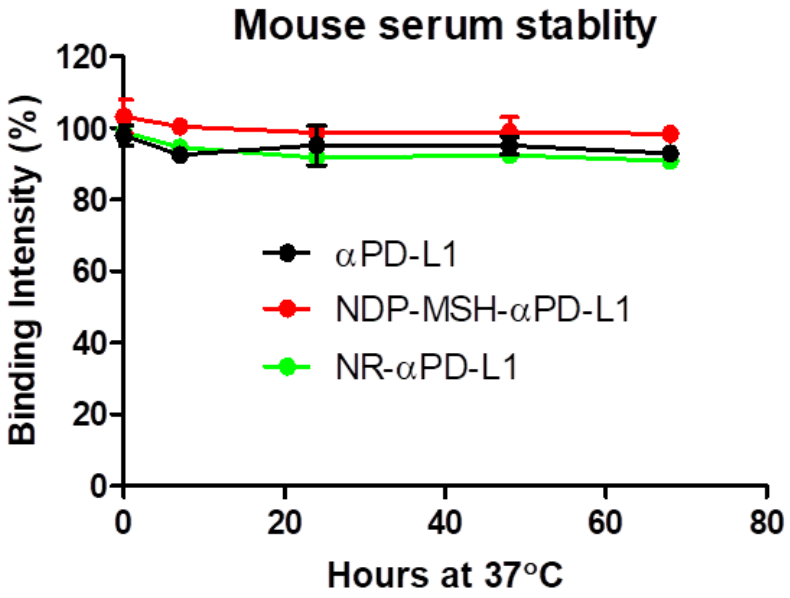

Figure S3. Mouse serum stability measurement of NDP-MSH-αPD-L1 and controls. The amount of NDP-MSH-αPD-L1 and controls was measured by sandwich ELISA binding with PD-L1 extracellular antigen.

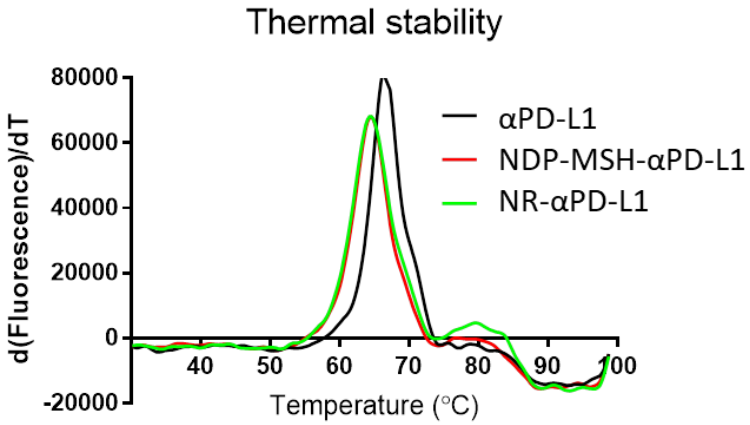

Figure S4. Thermal stability of NDP-MSH-αPD-L1 and controls. 0.2 mg/mL proteins in PBS mixed with the Thermal shift buffer and Thermal shift dye in the Thermal Shif Dye Kit (Thermo Fisher Scientific). The protein denaturation curve was determined with ViiATM 7 Real-Time PCR System.

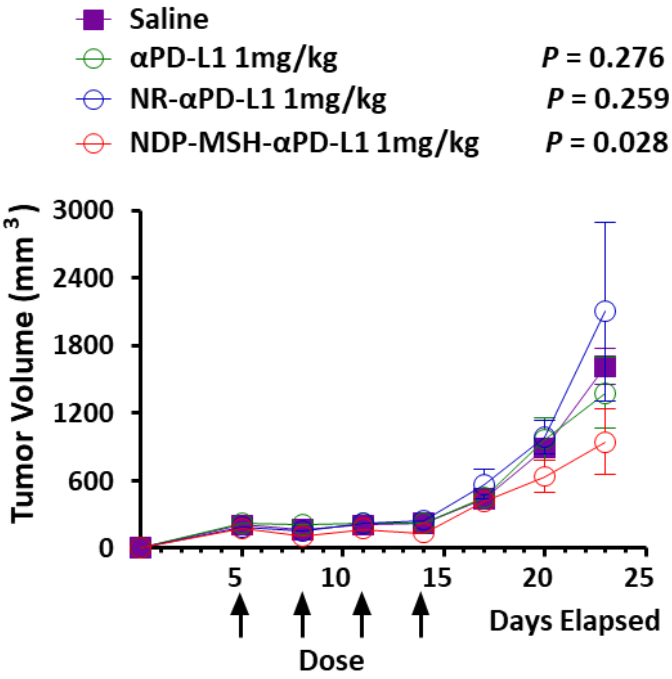

Figure S5. In vivo efficacy of NDP-MSH-αPD-L1 in mouse B16-SIY melanoma syngeneic models. C57BL/6 mice were s.c. inoculated with 1.5 million B16-SIY melanoma cells, and received NDP-MSH-αPD-L1 or controls every two days for a total of four times at 1 mg/kg. Tumor was measured three times a week with calipers and tumor volume was calculated. Each data point represents mean tumor volume of 10 mice in each group ± SD. Arrows indicate the time of drug injection. *p* value < 0.05 compared to the control groups (saline) were considered significant.

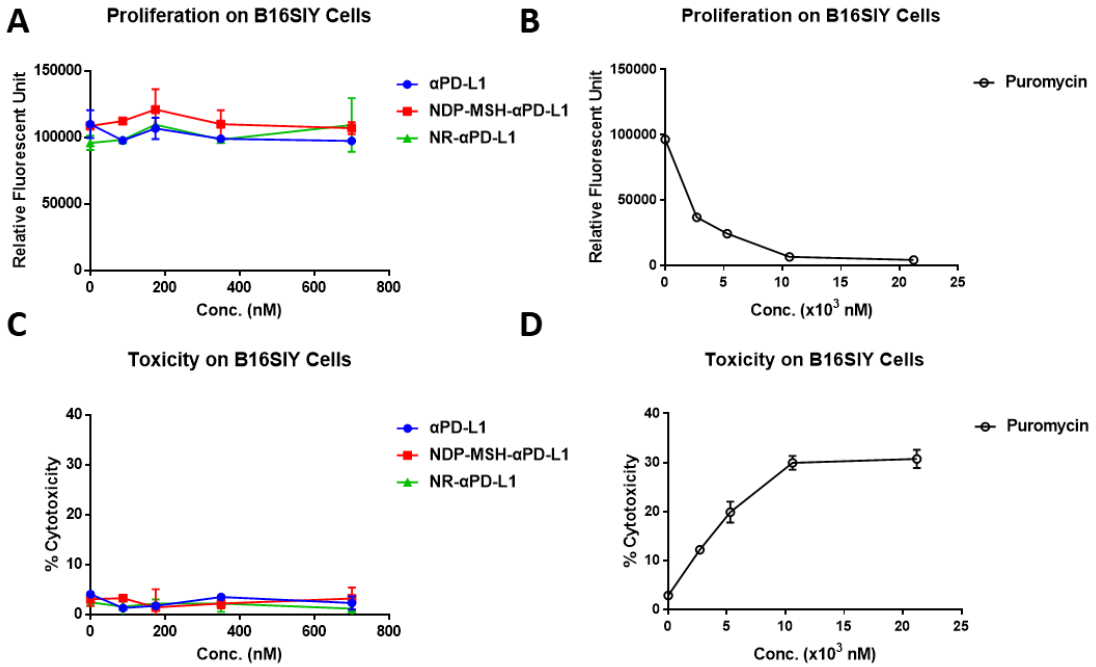

Figure S6. Growth inhibition activity and cytotoxicity assays on B16SIY cells with NDP-MSH-αPD-L1 conjugates and antibody controls (A). Puromycin acted as a positive control to inhibit cell growth in a dose-dependent manner (B). Direct cytotoxicity assay on B16SIY cells with NDP-MSH-αPD-L1 conjugates and antibody controls (C). Puromycin acted as a positive control to induce cell death in a dose-dependent manner (D). Error bars represent SD of duplicate samples.