Supplementary Table 1: Small compounds for synergy screening

A total of eleven small compounds were selected for synergy screening with D4-LR in T3M4 cells. The basic information and reasons for selection are summarized in the table below.

ID	Name	NCGC ID	Top Conc (nM)	Reason for Selection	Class
1	Daporinad	NCGC00182868-02	1000	consistent antagonism with T19 (positive control)	Assay Ctrl
2	Panobinostat	NCGC00263117-02	4000	top synergistic MoA and confirmed	HDAC
3	Quisinostat	NCGC00346487-01	4000	top synergistic MoA and confirmed	HDAC
4	Trametinib	NCGC00263180-01	4000	T3M4 - KRAS mutation & top synergistic MoA	KRAS
5	SN38	NCGC00167831-01	1000	std care & confirmed	TOP1
6	Gemcitabine	NCGC00168784-08	10000	std care but the weak synergy	RRM
7	Actinomycin D	NCGC00025059-04	200	top synergistic MoA and confirmed	Translation
8	Navitoclax (ABT-263)	NCGC00188344-05	4000	top synergistic MoA and confirmed	BCL
9	LY-2874455	NCGC00346459-01	4000	top synergy	FGFR
10	Aclarubicin	NCGC00167503-02	1000	std care & confirmed	TOP2
11	Palbociclib	NCGC00263129-01	10000	std care & no activity & synergy in Hep3B	CDK4

T19, anti-GPC3 immunotoxin(1)

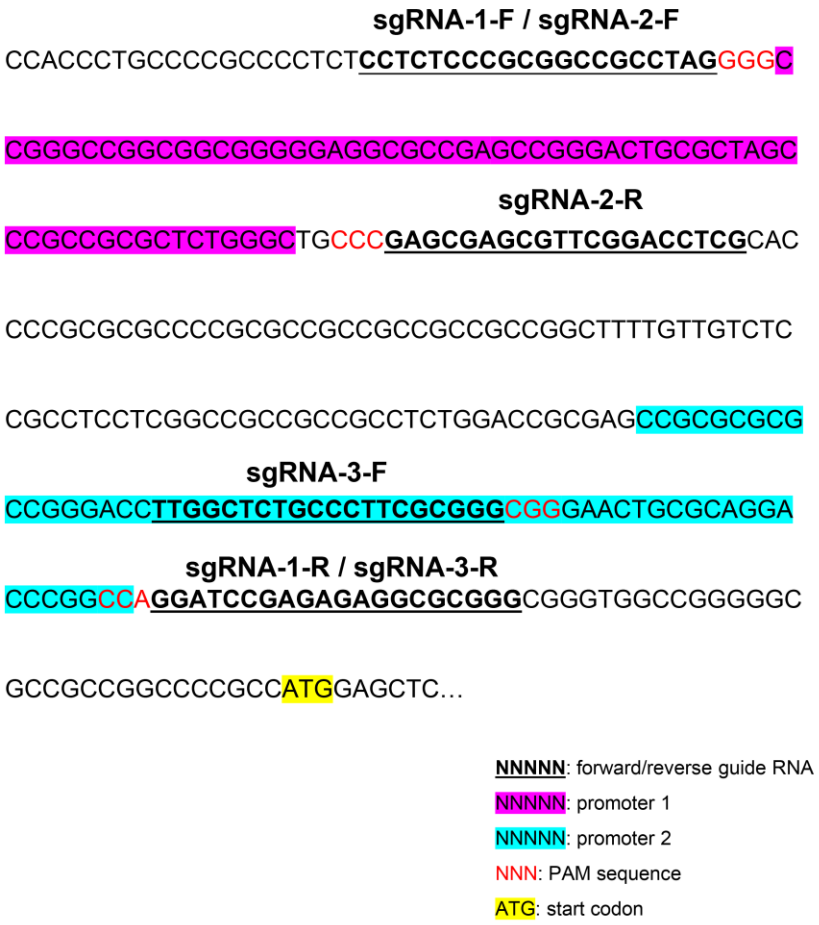
Std, standard.

Supplementary Table 2: Blood Analysis and Necropsy Results

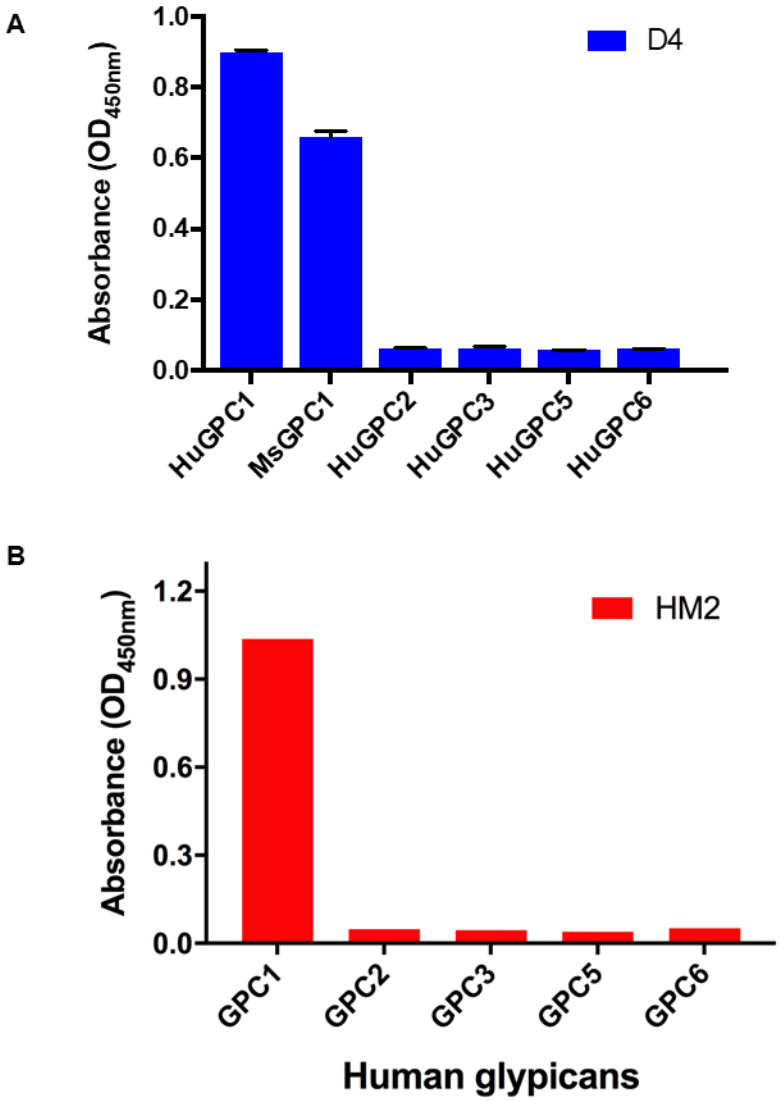
Blood analysis and necropsy. Athymic nude mice were intraperitoneally injected with 2×10^6 T3M4 cells. Mice were treated by *i.p.* injection every other day for a total of 14 injections. N=3/group. Values represent the mean ± SD. All the treating groups were compared to the PBS control group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. An increase in white blood cell levels was observed in mice from the PBS group. Elevated levels of red blood cells in mice from groups treated with PBS, D4-LR, or irinotecan were also detected. Serum chemistry analysis revealed a mild upregulation in alanine aminotransferase following D4-ABD-LR+irinotecan treatment relative to other treatments, suggesting slightly increased liver damage for that combinational therapy. All treatment strategies resulted in liver and spleen shrinkage when compared with the PBS group.

	PBS	D4-LR	D4-ABD-LR	Irinotecan	D4-LR+Iri	D4-ABD-LR+Iri	Normal Range
White blood cells (K μL^{-1})	19.87 ± 3.09	9.07 ± 2.57***	7.06 ± 0.22***	7.45 ± 2.32***	5.33 ± 1.87***	8.12 ± 1.51***	1.8-10.7
Neutrophils (K μL^{-1})	14.03 ± 3.85	3.91 ± 1.60***	3.78 ± 0.79***	3.82 ± 2.26***	2.14 ± 0.34***	3.56 ± 0.83***	0.10 - 2.40
Lymphocytes (K μL^{-1})	3.46 ± 0.56	3.75 ± 0.76	2.43 ± 0.44	3.02 ± 0.46	2.50 ± 1.06	3.47 ± 0.51	0.90 - 9.30
Monocytes (K μL^{-1})	1.77 ± 0.75	0.68 ± 0.13**	0.54 ± 0.30**	0.36 ± 0.02**	0.32 ± 0.19**	0.69 ± 0.06**	0.00 - 0.40
Eosinophils (K μL^{-1})	0.55 ± 0.06	0.66 ± 0.23	0.22 ± 0.10	0.22 ± 0.06	0.34 ± 0.27	0.31 ± 0.37	0.00 - 0.20
Basophils (K μL^{-1})	0.06 ± 0.03	0.07 ± 0.08	0.08 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.08 ± 0.06	0.00 - 0.20
Red blood cells (M μL^{-1})	37.47 ± 2.32	47.37 ± 6.05**	9.39 ± 0.63***	41.27 ± 0.81	9.31 ± 0.33***	9.34 ± 0.73***	6.36-9.42
Albumin (g dL^{-1})	2.17 ± 0.06	3.50 ± 0.00**	3.63 ± 0.15**	3.77 ± 0.85***	3.63 ± 0.06**	4.03 ± 0.31***	2.50-4.80
Alkaline phosphatase	47.67 ± 4.04	81 ± 14.11**	60.33 ± 10.26	62.00 ± 8	75.00 ± 2.65*	58.33 ± 12.21	62.00-209.00
Alanine aminotransferase	25.67 ± 0.58	42.00 ± 5	74.67 ± 20.21***	35.67 ± 7.64	59.33 ± 5.51**	131.67 ± 11.02***	28.00-132.00
Total bilirubin (mg dL^{-1})	0.20 ± 0.00	0.30 ± 0.00	0.27 ± 0.06	0.33 ± 0.06*	0.23 ± 0.06	0.27 ± 0.06	0.10-0.90
Blood urea nitrogen (mg dL^{-1})	15.00 ± 3.61	20.00 ± 1.00	25.33 ± 2.31***	19.67 ± 0.58	25.00 ± 1***	28.00 ± 2.65***	18.00-29.00
Creatinine (mg dL^{-1})	0.20 ± 0.00	0.23 ± 0.06	0.33 ± 0.06	0.30 ± 0.10	0.20 ± 0.00	0.23 ± 0.06	0.20-0.80
Total protein (g dL^{-1})	5.73 ± 0.15	5.70 ± 0.30	5.60 ± 0.17	5.57 ± 0.25	5.63 ± 0.35	5.67 ± 0.15	3.60-6.60
Globulin (g dL^{-1})	3.57 ± 0.21	2.13 ± 0.25**	2.00 ± 0.1**	1.80 ± 0.79***	2.00 ± 0.36**	1.63 ± 0.4***	0.00-0.60
Organ Weight (g)							
Brain	0.44 ± 0.02	0.47 ± 0.00	0.43 ± 0.03	0.42 ± 0.04	0.43 ± 0.02	0.42 ± 0.01	
Heart	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.13 ± 0.00	0.11 ± 0.00*	0.12 ± 0.01	
Kidney	0.35 ± 0.03	0.35 ± 0.01	0.30 ± 0.04	0.32 ± 0.01	0.30 ± 0.02	0.28 ± 0.01*	
Liver	1.54 ± 0.18	1.25 ± 0.03*	1.07 ± 0.03***	1.06 ± 0.07***	1.01 ± 0.08***	1.05 ± 0.12***	

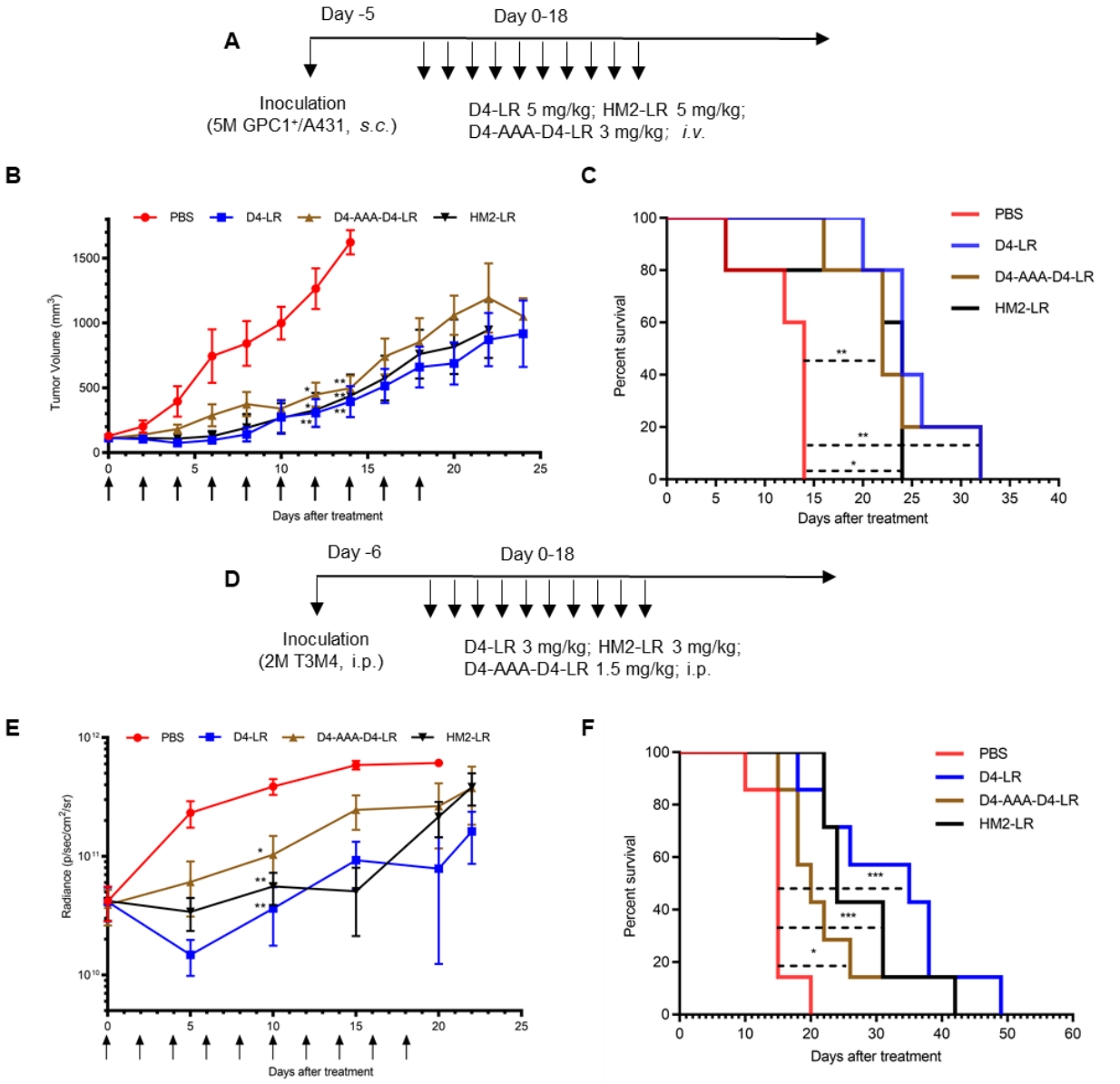
Lung	0.17 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.01
Pancreas	0.11 ± 0.02	0.16 ± 0.02	0.15 ± 0.03	0.15 ± 0.04	0.14 ± 0.03	0.13 ± 0.01
Spleen	0.24 ± 0.06	0.14 ± 0.04*	0.12 ± 0.02*	0.14 ± 0.07*	0.13 ± 0.01*	0.12 ± 0.02*



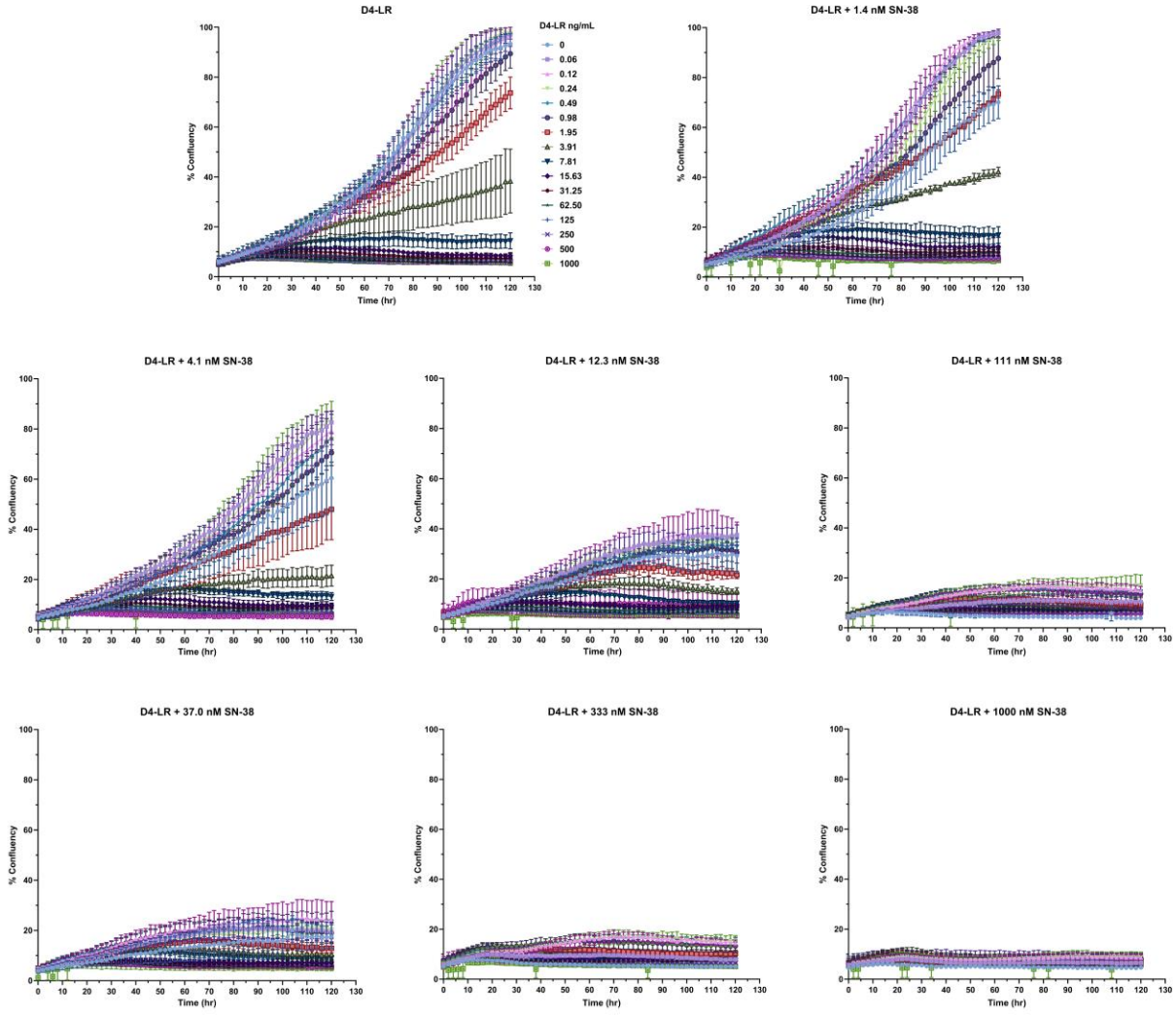
Supplementary Figure 1. Sequences of the predicted GPC1 promoters and three pairs of sgRNAs. The underlined parts are forward or reverse sgRNAs. The bases in magenta or cyan indicate the sequences of predicted GPC1 promoter 1 or 2, respectively. PAM sequences are in red. ATG highlighted in yellow represents the start codon.



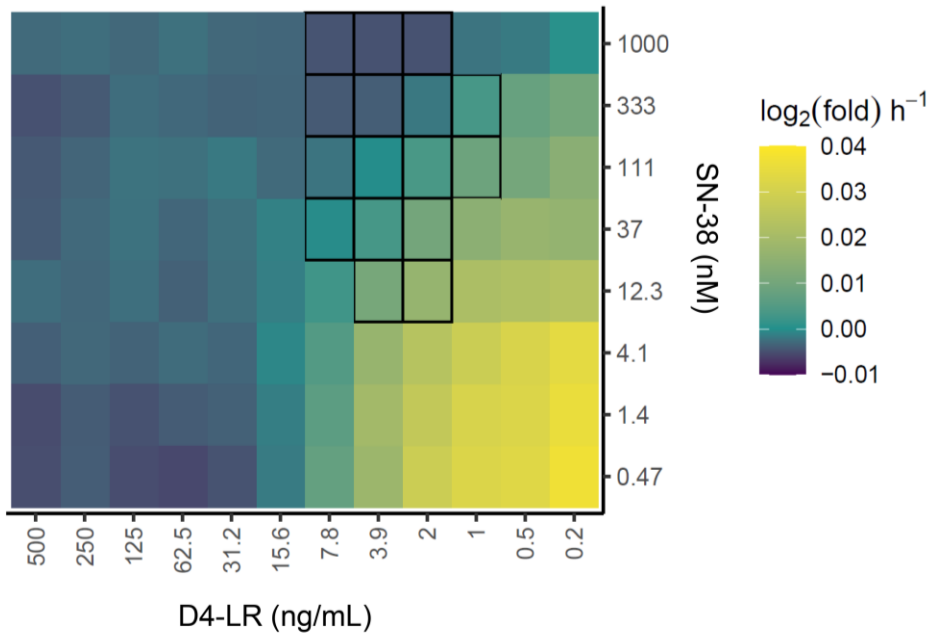
Supplementary Figure 2. Binding of D4 or HM2 antibodies to human or mouse glypicans. A, Binding activity of D4 antibody against human glypicans and mouse GPC1 analyzed by ELISA. **B,** Binding ability of HM2 antibody against human glypicans analyzed by ELISA.



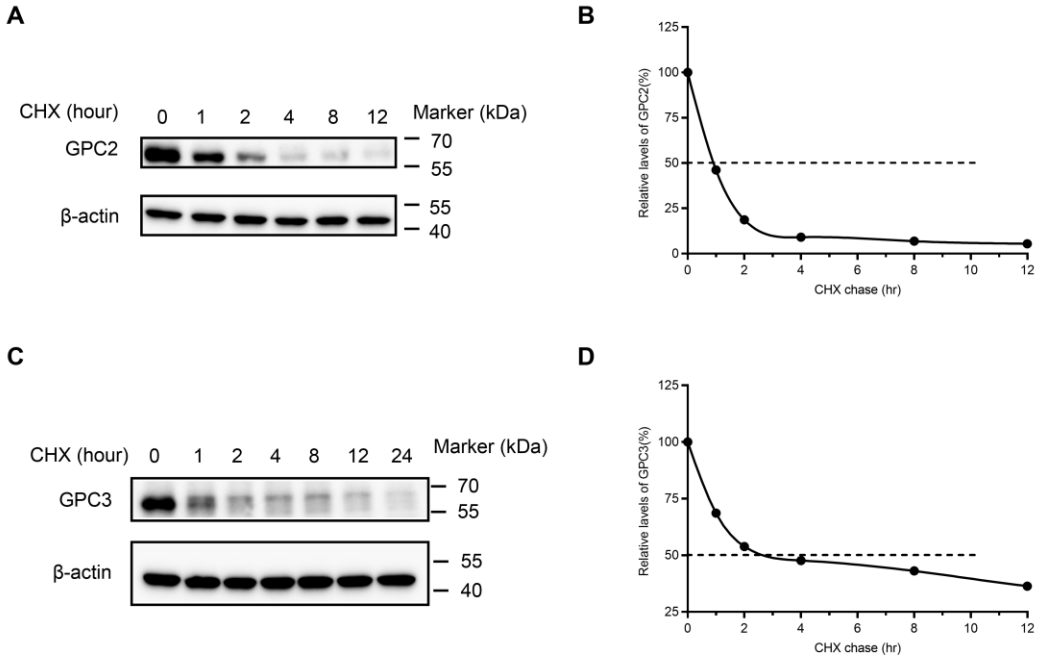
Supplementary Figure 3. Anti-GPC1 immunotoxins inhibited tumor growth *in vivo*. A, Five-week-old female athymic nude mice were injected with 5 x 10^6 GPC1$^+$/A431 cells in the right dorsal flank. Mice (n=5 per group) were treated 10 times with D4-LR (5 mg/kg), D4-AAA-D4-LR (3 mg/kg), or HM2-LR (5 mg/kg) by tail vein injection on the days indicated with a black arrow. **B,** Average tumor volume ± SEM for each experimental group in the GPC1$^+$/A431 subcutaneous mouse model. **C,** Kaplan-Meier survival curve for each group in GPC1$^+$/A431 subcutaneous mouse model. **D,** Five-week-old female athymic nude mice were inoculated with 2 x 10^6 T3M4 cells in the right abdominal cavity. Mice (n=5 per group) were treated with D4-LR (3 mg/kg), D4-AAA-D4-LR (1.5 mg/kg), or HM2-LR (3 mg/kg) by *i.p.* injection on the days indicated with a black arrow. **E,** Average tumor volume ± SEM for each experimental group in the T3M4 xenograft mouse model. **F,** Kaplan-Meier survival curve in T3M4 xenograft mouse model.



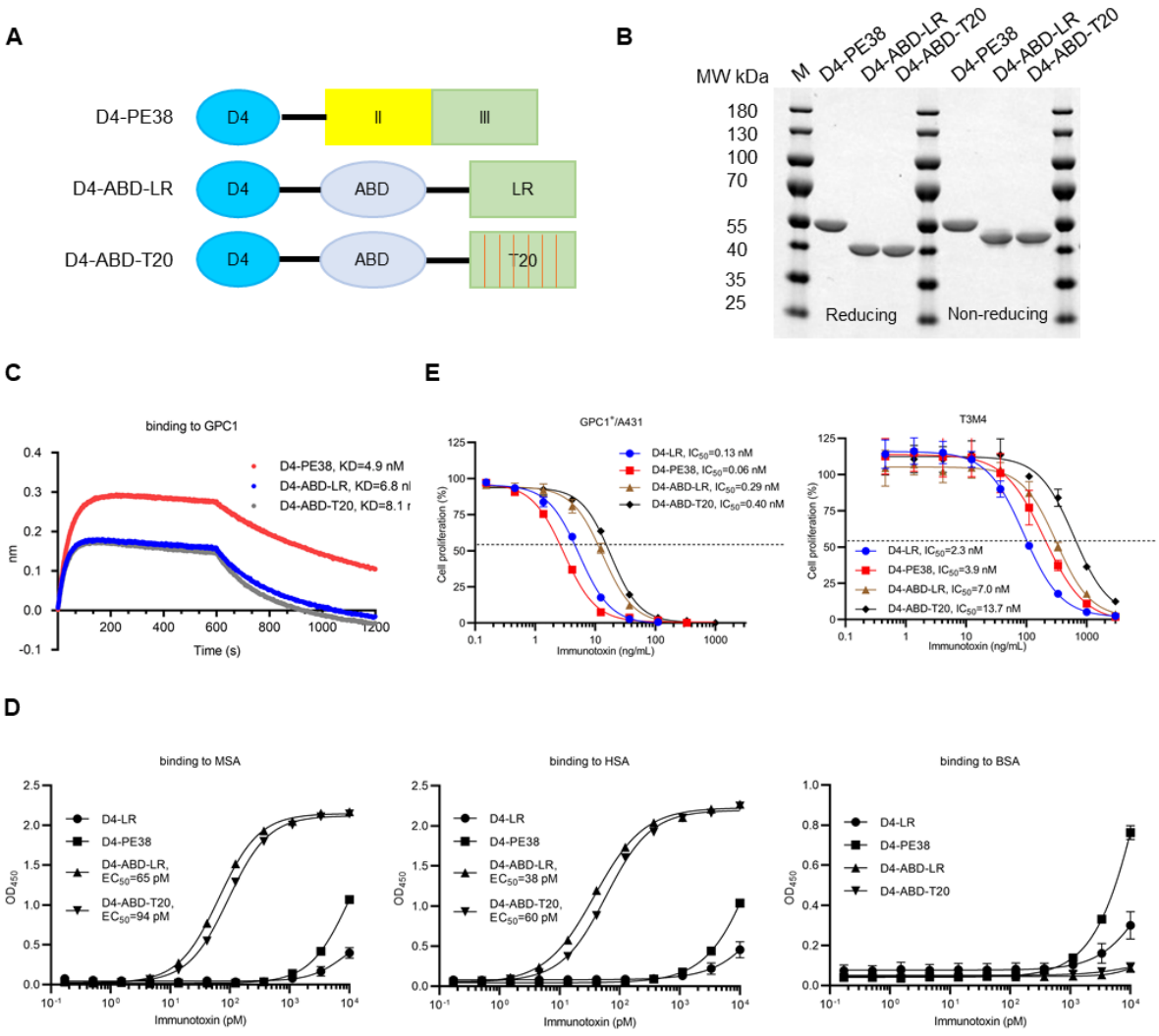
Supplementary Figure 4. IncuCyte assay indicating the confluency of T3M4 cells treated with D4-LR alone or in combination with SN-38 at different concentration points. Cell confluency was continuously monitored every two hours over 5 days.



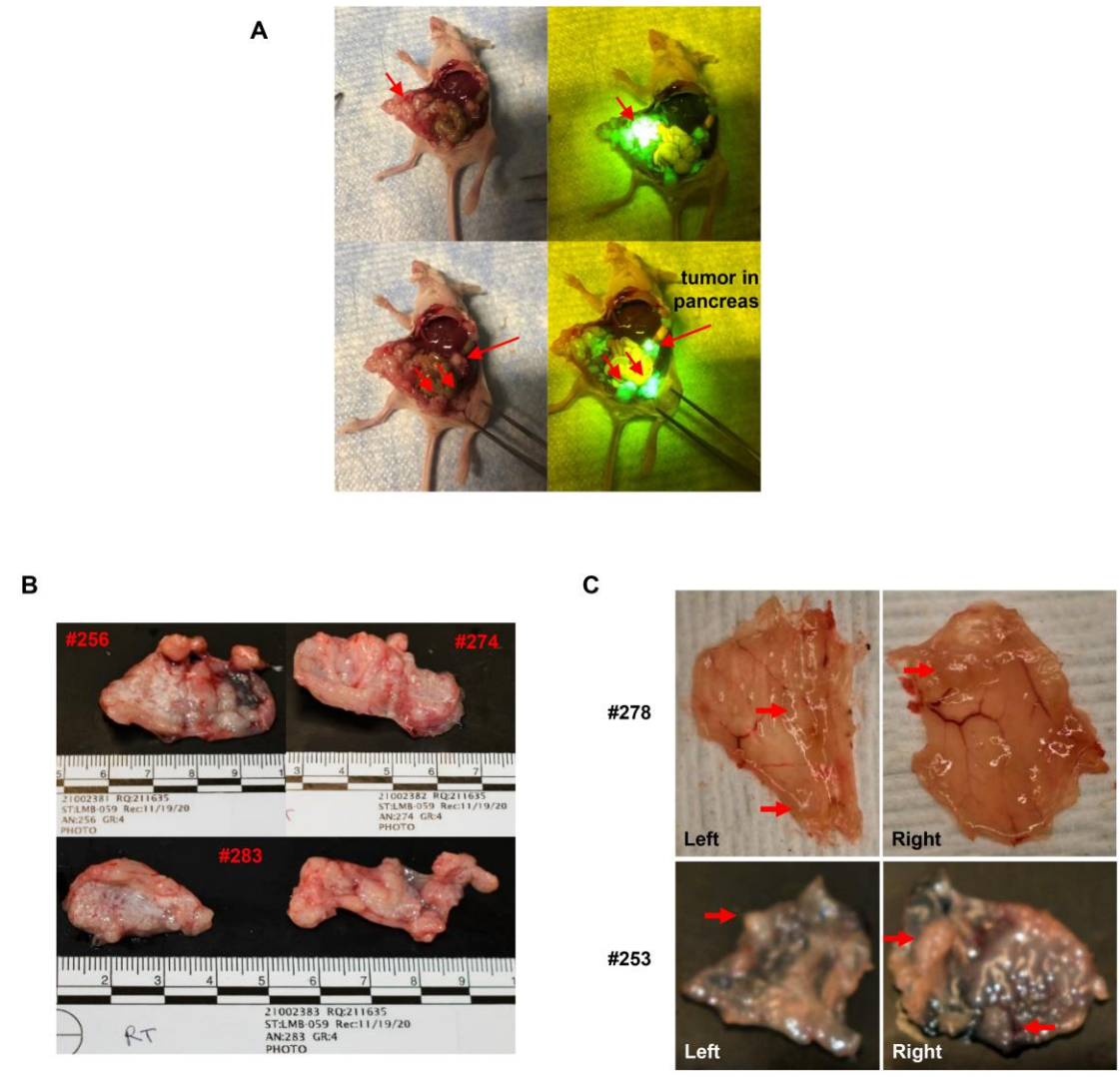
Supplementary Figure 5. Synergy analysis of D4-LR and SN-38. Black rectangles in the block heatmap highlight the dose combinations which reduced the growth rate by >0.005 $\log_2$fold per hour (using the HSA model).



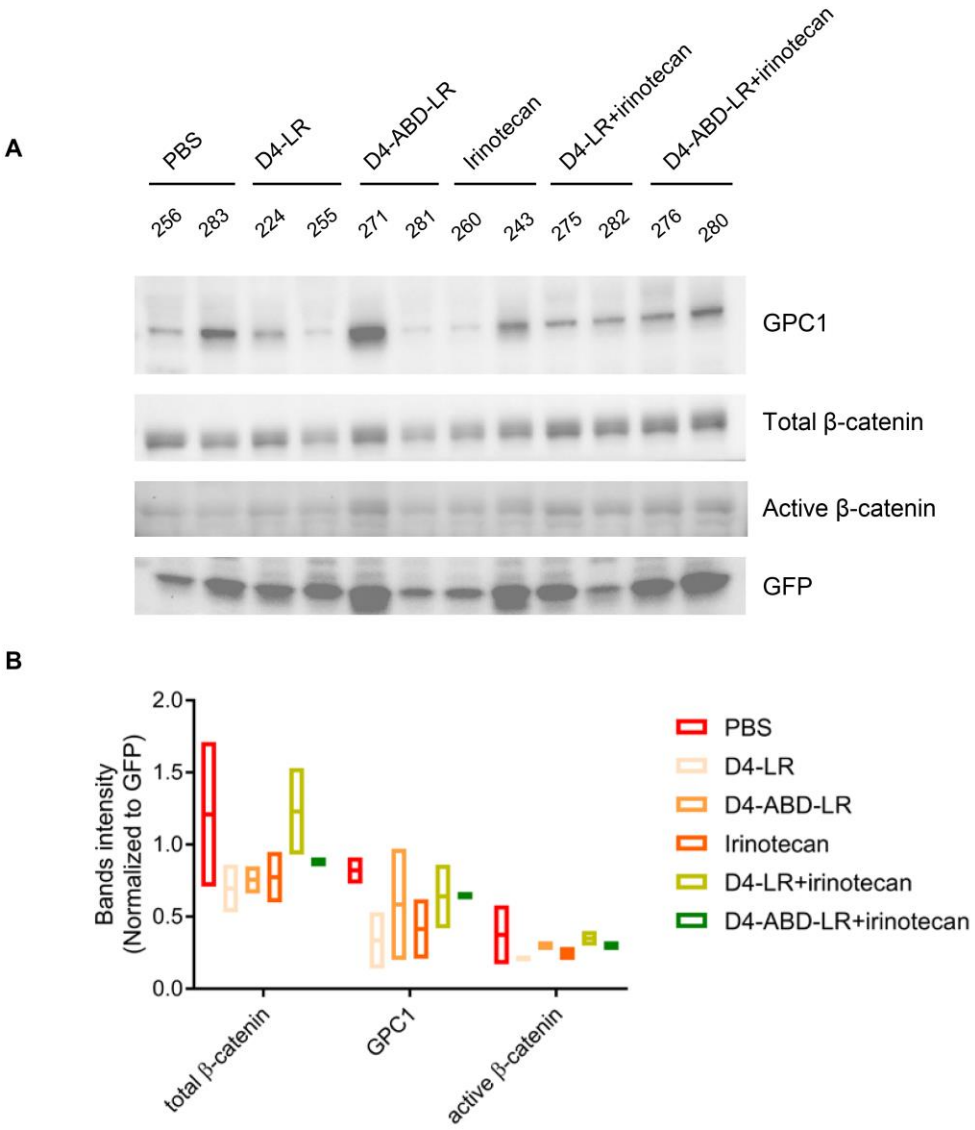
Supplementary Figure 6. Turnover rate analysis for GPC2 and GPC3. A, Western blot analysis, detecting GPC2 expression levels in IMR5 cells (GPC2-positive) after cycloheximide treatment. **B,** Signals of GPC2 in A were quantified with Image Lab software (Bio-Rad). The GPC2 band intensity was normalized to β-actin and then normalized to the t=0 controls and plotted. **C,** Levels of GPC3 in Hep3B cells (GPC3-positive) treated with cycloheximide. **D,** The GPC3 band intensity was normalized to β-actin, and then normalized to the t=0 controls and plotted.



Supplementary Figure 7. D4-ABD-LR showed great binding to human and mouse serum albumin. A, Schematic of three new D4-based immunotoxins, D4-PE38, D4-ABD-LR, and D4-ABD-T20. PE38 contains both domains II and III of *Pseudomonas* exotoxin A. T20 is an LR toxin fragment containing 6-point mutations targeting T cell epitope. **B,** SDS-PAGE gel analysis. **C,** Binding affinity to GPC1 analyzed by Octet. **D,** Binding ability of D4-based immunotoxins to bovine serum albumin (BSA), human serum albumin (HSA), and mouse serum albumin (MSA). Values represent mean ± SD. **E,** Cytotoxicity of D4-based immunotoxins on GPC1^{+}/A431 and T3M4 cells. Values represent mean ± SD.



Supplementary Figure 8. Tumors from the mice treated with PBS, compounds, or immunotoxins. A, Representative pictures of a dissected mouse from PBS group. Red arrows indicate tumors tagged with GFP grow in the abdominal cavity or on the peritoneal wall under bright light (left) and blue light (right). **B,** Representative images of isolated tumors from mice in PBS group (#256, #274, #283). **C,** Representative images of small nodules on the peritoneal wall from mice treated with D4-LR (#278) or irinotecan (#253).



Supplementary Figure 9. Expression levels of GPC1, β-catenin, active β-catenin in harvested tumors. A, Harvested tumors (from two individual mice per group) were lysed and investigated for the expression levels of β-catenin, active β-catenin, and GPC1, by western blot analysis. **B,** Quantified data of WB bands intensity. The intensity of all bands in A was quantified by normalizing to GFP that indicates tumor cells (T3M4 tumor cells tagged with GFP and luciferase).

Reference

1. Fleming BD, Urban DJ, Hall MD, Longerich T, Greten TF, Pastan I, *et al*. Engineered Anti-GPC3 Immunotoxin, HN3-ABD-T20, Produces Regression in Mouse Liver Cancer Xenografts Through Prolonged Serum Retention. Hepatology **2020**;71(5):1696-711 doi 10.1002/hep.30949.