Supporting information

Efficient drug delivery and induction of apoptosis in colorectal tumors using a Death Receptor 5-targeted nanomedicine

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Supplementary Figures:

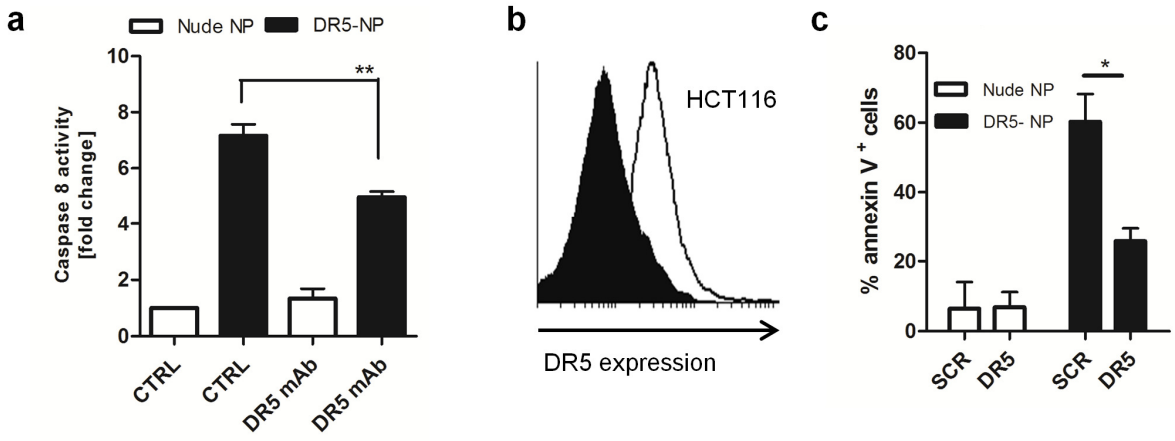

Figure S1. Pro-apoptotic effects of DR5-NPs are DR5 dependent. (a) Caspase 8 activation induced by treatment of HCT116 cells with 0.2 mg/ml DR5-NPs for 6 h with and without pre-treatment with 5x excess DR5-specific antibody (17 µg/ml) or PBS (CTRL), mean±SD (n=3). (b) Reduced DR5 cell surface expression on HCT116 cells after receptor silencing with DR5-specific siRNA (filled) or scramble control (clear). (c) Annexin V binding to HCT116 cells after transfection with DR5-specifc or scramble control (SCR) siRNA and treatment with 0.01 mg/mL NPs for 12 h, mean±SD.

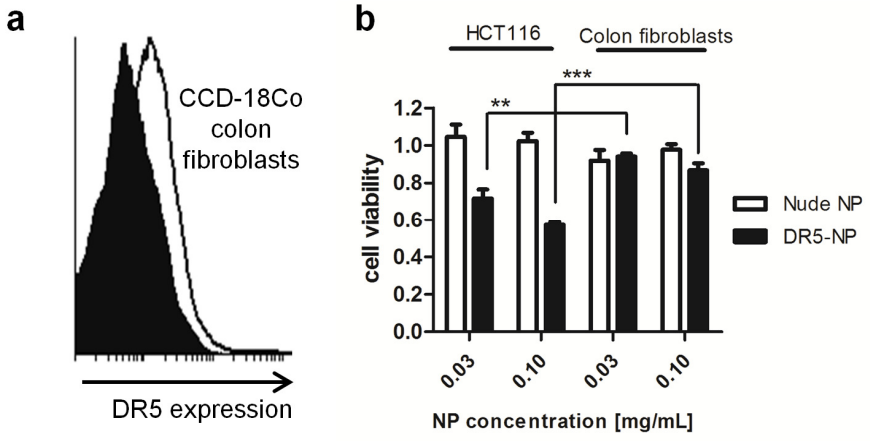

Figure S2. DR5-NPs exhibit reduced cytotoxic effects against normal colon fibroblasts. (a) DR5 cell surface expression on CCD-18Co human colon fibroblasts; isotype control antibody (filled) and DR5-specific antibody (clear). (b) Cell viability of HCT116 cells and human colon fibroblasts following treatment with nude or DR5-NPs for 24 h, mean±SD (n=5-6).

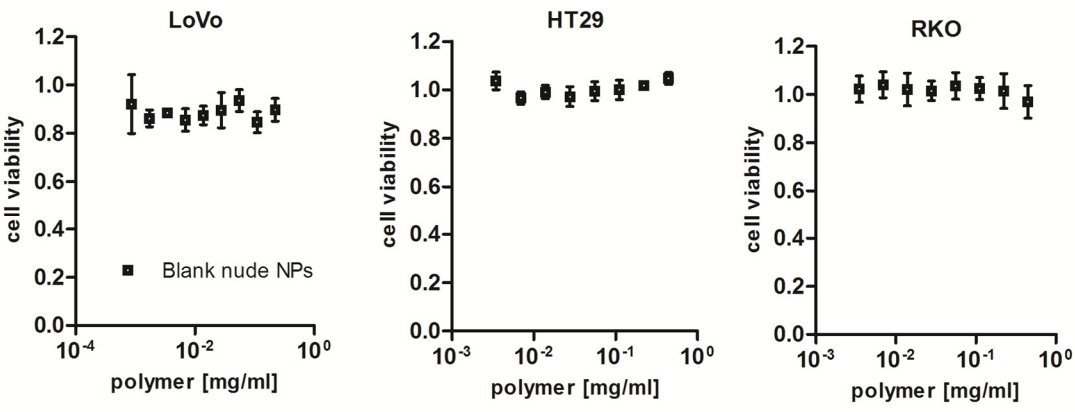

Figure S3. Effect of blank nude NPs on cell viability of colorectal cancer cell lines. Cell viability of LoVo, HT29 and RKO colorectal cancer cell lines after treatment with blank nude NPs for 24 h, mean±SD (n=3).

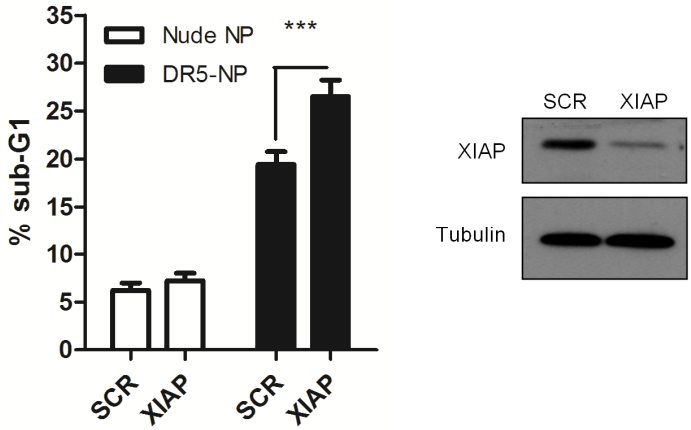

Figure S4. XIAP silencing enhances pro-apoptotic effect of DR5-NPs in resistant cells. Quantification of apoptotic (sub-G1) $BAX^{-/-}$ HCT116 cells analyzed by RNase A/PI staining and flow cytometry following transfection with XIAP or scramble control (SCR) siRNA and subsequent treatment with nude or DR5-NPs (0.05 mg/mL) for 6 h, mean±SD (n=3); Western blot confirming the down-regulation of XIAP in comparison to scramble control siRNA-transfected cells.

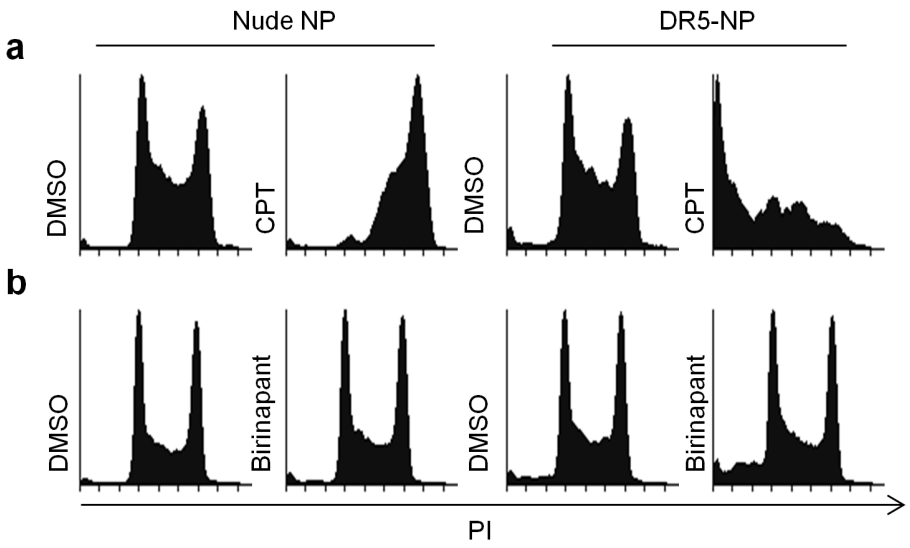

Figure S5. Combination therapies enhance cytotoxic effects of DR5-NPs against resistant HCT116 cells. RNase A/PI staining and flow cytometry analysis in $BAX^{-/-}$ HCT116 cells following pre-treatment with CPT (0.1 µM) for 16 h (a) or Birinapant (1 µM) for 1 h (b) and subsequent treatment with nude or DR5-NPs (0.05 mg/mL) for 6 h.

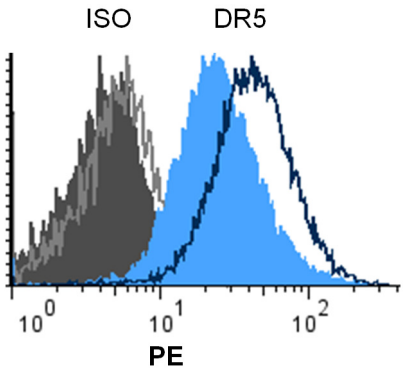

Figure S6. CPT exposure up-regulates DR5 cell surface expression. The cell surface of HCT116 cells was stained using isotype and DR5-specific antibodies (grey and blue peaks respectively) after cell exposure to 6 nM CPT for 24 h (clear peaks) or control (filled peaks).

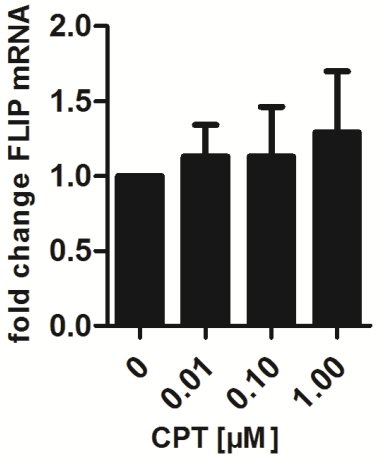

Figure S7. Assessment of FLIP mRNA levels by qPCR in HCT116 cells following treatment with free CPT for 24 h, mean±SD (n=3).

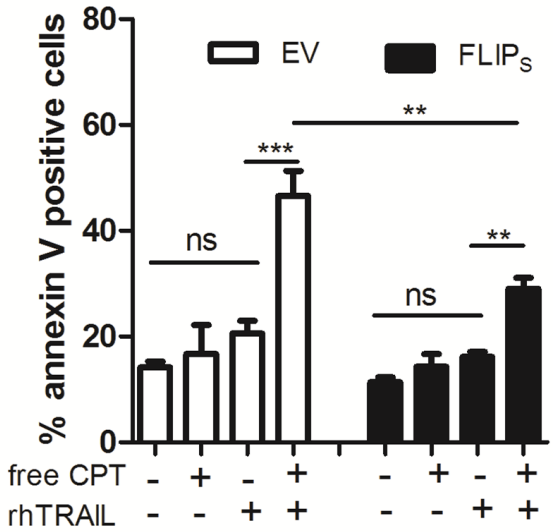

Figure S8. FLIP expression suppresses the pro-apoptotic activity of recombinant human (rhTRAIL). Annexin V binding to $FLIP_S$- and EV-transduced HCT116 cells following treatment with CPT (0.5 μM) and/or recombinant rhTRAIL (20 ng/mL) for 12 h, mean±SD (n=3).

Supplementary Material and Methods

PLGA-PEG$_{3400}$-COOH copolymer synthesis

The acid bearing PEG-PLGA copolymer was synthesized from PLGA RG502H (Evonik Industries, Germany) and NH_2-PEG_{3400}-COOH (Laysan Bio Inc, Arab, AL) using carbodiimide chemistry similar to a method described before [1]. Briefly, PLGA (0.042 mmol) was dissolved in 4 mL anhydrous dichloromethane (DCM) along with N-Hydroxysuccinimide (NHS, 0.130 mmol) and *N,N'*-Dicyclohexylcarbodiimide (DCC, 0.146 mmol) and allowed to react under nitrogen, stirring for 24 h. The activated polymer was precipitated with ice-cold diethyl ether, dried and 0.008 mmol of the material reacted with NH_2-PEG-COOH (0.017 mmol) in 1 mL of anhydrous DCM in presence of *N*,*N*-diisopropylethylamine (0.039 mmol) for 18 h under stirring. The copolymer was then precipitated with ice-cold diethyl ether, washed twice with a 75/25% (v/v) mixture of ice-cold methanol/diethyl ether and the structure verified by ^{1}H-NMR (Bruker Ultrashield 400 plus) with the anticipated peaks as observed by Cheng *et al.* [2]. (^{1}H-NMR (400 MHz, $CDCl_3$), δ 5.20 (m, –C(O)C*H*(CH_3)O–), 1.58 (m, –C(O)CH(C*H* $_3$)O–), 4.83 (m, –C(O)C*H*$_2$O–), 3.64 (m, –OC*H* $_2$C*H* $_2$–)).

NP characterization

Antibody attachment was measured on the NP surface by BCA protein assay (Thermo Scientific, UK) using conatumumab as protein standard. CPT loading per mg of polymer was assessed in the NP pellet by measurement of fluorescence at $330_{Ex}/460_{Em}$ and equal rhodamine 6G loading confirmed at $485_{Ex}/520_{Em.}$ NP characteristics were analyzed in PBS by dynamic light scattering with the Zetasizer Nano ZS (Malvern Instruments, UK) and SEM (Jeol 6500 FEGscanning electron microscope, Japan). *In vitro* drug release was assessed in

Slide-A-Lyzer dialysis cassettes with 7 kDa pore size (Thermo Scientific, UK). 30 mg of polymer in 1 mL were dialyzed against 29 mL of 50% FBS in PBS at 37°C under shaking. At the indicated time points, the supernatants were collected and analyzed for drug content by fluorescence at $330_{Ex}/460_{Em}$ and compared to a CPT standard, which was prepared in the release medium and incubated therein for the duration of the study. To assess the stability of the antibody-polymer conjugate, the NPs were incubated in 50% FBS in PBS and collected by centrifugation at 20 000 *g* for 20 min after 6, 24, 48 and 96 h. NPs were washed once in PBS and HCT116 cells treated to assess pro-apoptotic activity. Alternatively, the NPs were denatured and a Western blot performed using goat anti-human HRP-linked antibody (1:10000, Santa Cruz Biotechnology, Germany).

Cell viability of human colon fibroblasts

CCD-18Co human colon fibroblasts were purchased from ATCC and cultured in EMEM supplemented with 10% FBS. Colon fibroblast cells (5000 cells per well) were seeded in black 96 well plates with clear bottom and left overnight to adhere. The cells were treated with nude and DR5-NPs for 24 h and the cell viability measured using the cell-permeable GF-AFC Substrate from the ApoTox-Glo Triplex assay (Promega, UK).

Quantitative PCR (qPCR)

RNA was extracted from cell protein using RNeasy Plus Mini Kit (Qiagen, UK) and concentration measured using Nanodrop ND-1000 (Thermo Scientific, UK). The cDNA was received from 2 µg of RNA using the Transcriptor First Strand cDNA Synthesis Kit. Quantitative PCR was carried out using a Light Cycler 480 system and hydrolysis probes for *CFLAR* and *GAPDH* (Roche Diagnostics).

Supplementary References

1. Betancourt, T, Byrne, JD, Sunaryo, N, Crowder, SW, Kadapakkam, M, Patel, S *et al.* (2009). PEGylation strategies for active targeting of PLA/PLGA nanoparticles. *J Biomed Mater Res* A **91**:263-76.
2. Cheng, J, Teply, BA, Sherifi, I, Sung, J, Luther, G, Gu, FX *et al.* (2007). Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. *Biomaterials* **28**:869-76.