*Figure S1: PRS-343 specificity for 4-1BB within TNF receptor superfamily. Seven recombinant human TNF receptor superfamily proteins were purchased from Sino Biological (4-1BB: 10041-H08H, RANK: 16078-H08H, GITR: 13643-H08H, Ox40: 10481-H08H) or R&D Systems (CD30: 6126-CD, TNF-RII: 1089-R2, TNF-RI: 636-R1) and used for determination of PRS-343 selectivity for 4-1BB. Proteins were coated to an ELISA plate, PRS-343 was added in a dilution series and detected via HRP-labeled anti-human IgG Fc antibody. Within the set of tested TNF receptor family proteins, PRS-343 binds exclusively to 4-1BB.*

*Figure S2: Characterization of multiple bispecific formats of a 4-1BB targeting Anticalin protein recombinantly fused to an anti-HER2 antibody (A). ELISA based binding properties of all formats were compared to parental building blocks (C) while the ability to activate T-cells (IL2 induction) was assessed in a co-culture assay.*

*Figure S3: Cytokine release assay with PRS-343. PBMC were isolated from the blood of twelve healthy donors and incubated for 72 hours with PRS-343 either air dried, in soluble form, or wet coated. Four concentrations of PRS-343 in a volume of 50 µl were tested in each setting as indicated in the figure. The anti-CD3 monoclonal antibody OKT3 at three different concentrations served as the positive control, and an IgG4 isotype antibody was the negative control. Supernatant levels of ten cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, GM-CSF, IFN-γ and TNF-α) were analyzed. The figure shows the average response for the ten donors that displayed a significant response to OKT3, and for a selection of the most relevant cytokines.*

***Figure S4****: Dose-dependent 4-1BB activation of a 4-1BB over-expressing Jurkat Nf-kB reporter cell line induced by PRS-343 with ON preincubation of the drug in the presence of NCI-N87 (HER2 high), MKN45 (HER2 low) and HepG2 (HER2 null) cell lines, or without tumor cells. Briefly, cancer cells were seeded onto tissue culture plates with PRS-343 (at 10, 1, 0.1 and 0,01 nM) and incubated ON. All plates were then washed twice with PBS. 4-1BB over-expressing Jurkat Nf-kB reporter (at 3:1 ratio) were added to each well. Following a 6 hours incubation, Bio-Glow luciferase reagent was added to each well and luminescence was measured.*

***Figure S5:*** *Fold increase changes of a panel of cytokines induced by human T-cells co-stimulated by PRS-343 in the presence of SKBR-3 (HER2 high) or MCF-7 (HER2 low).*

***Figure S6:*** *h-4-1BB expression in T-cells during co-culture assay. Using a similar set up as described in M&M, purified Pan T-cells (from healthy donors) and SK-BR3 high Her2 expressing cells were co-incubated in the presence of coated anti-CD3 antibody. After 24, 48 and 72 hours of co-incubation, Pan T-cells were collected and 4-1BB expression on CD3 positive cells was assessed by flow cytometry. (A) shows the % of 4-1BB positive T-cells for two donors and (B) shows histogram of h-4-1BB expression on CD3 positive T-cells from two donors (red 24 hours; blue 48 hours; brown 72 hours).*

*Table S 1: Relative HER2 cell surface expression on a panel of cell lines. Expression was experimentally determined using a specific anti-HER2-antibody binding capacity (sABC [HER2]) and quantitative indirect immunofluorescence in flow cytometry (QIFIKIT). HER2 surface levels are also provided relative to the level on SKBR3 cells which were chosen as a reference cell line.*