Supporting Information

Presentation and Delivery of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) *via* Elongated Plant Viral Nanoparticle Enhances Antitumor Efficacy

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Table of contents

1. Preparation of potato virus X (PVX) displaying nickel-coordinated nitrilotriacetic acid (PVX-Ni)

We developed a conjugation method to display nickel-coordinated nitrilotriacetic acid (Ni-NTA) as shown in Figure S1. PVX displaying Ni-NTA is denoted as PVX-Ni. PVX displays surface lysine side chains amenable for chemical modification;[1,2] the following protocols were established: a mixture of PVX and the heterobifunctional linker NHS-PEG4-azide (Thermo Fisher Scientific) were prepared at a 1:10 (PVX coat protein:linker) molar ratio in 10 mM potassium phosphate buffer (KP buffer, pH 7.0) (reaction **(i)**). The final concentration of PVX was adjusted to 2 mg mL^{-1}. The reaction was carried out at room temperature for four hours to obtain PVX displaying azido groups (PVX-N_3). The excess linkers were removed by dialysis in KP buffer for five to six hours. The concentration of the resulting PVX-Ni was determined from the absorbance at 260 nm, read on a Nanodrop 2000 UV/visible spectrometer (Thermo Fisher), and calculated using the Beer-Lambert law and PVX-specific extinction coefficient (2.97 mL mg^{-1} cm^{-1} at 260 nm).

Meanwhile, N_α,N_α-bis(carboxymethyl)-L-lysine powder (NTA-Lys, Sigma-Aldrich) was dissolved in KP buffer to yield a 10 mg mL^{-1} solution; the pH of the solution was adjusted to ~7.0 before the conjugation reaction, determined by pH paper. The linker propargyl-*N*-hydroxysuccinimidyl ester (100 mg mL^{-1} in DMSO, Sigma Aldrich) was added at a 1:1 molar ratio and allowed to react with NTA-Lys; the reaction was carried out on ice for five hours to obtain NTA with a terminal alkyne (propargyl-NTA) (reaction **(ii)**).

Prepared PVX-N_3 and propargyl-NTA were then mixed at a molar ratio of 1:10 to synthesize PVX with nitrilotriacetic groups (PVX-NTA). The required reagents needed for the Cu(I)-catalyzed azide-alkyne cycloaddition (CuCAAC, "click" chemistry) reaction were added in the following order: aminoguanidine (AMG, Sigma Aldrich) at 5 mM final concentration, mixture of copper (II) sulfate penta hydrate ($CuSO_4$, Acros Organics):Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, a kind gift from Prof. Jon Pokorski, Case Western Reserve University) at 0.25:1.25 mM final concentration, and sodium ascorbate (Na Asc, Sigma Aldrich) at 5 mM final concentration. The reaction was allowed to proceed for

45 min at room temperature and terminated by addition of EDTA 0.5 M (reaction **(iii)**). The reaction mixture was then dialyzed against PBS, pH 7.4 for five to six hours to remove excess NTA, the click chemistry reagents, and EDTA.

To introduce Ni^{2+} ions, PVX-NTA was dialyzed against PBS, pH 7.4 containing 1 mM Ni^{2+} overnight at room temperature (reaction **(iv)**). Excess Ni^{2+} was removed by additional dialysis against PBS, pH 7.4 for five to six hours or longer to obtain the purified PVX-Ni. The PVX-Ni particle concentration was determined by Nanodrop as described above. To confirm the presence of Ni^{2+} in the PVX-Ni formulation, PVX-Ni (2 mg mL^{-1}) was treated with 1,4-dithiothreitol (DTT, Gold Biotechnology) at a 100 mM final concentration; presence of Ni^{2+} is indicated by the solution turning into a yellow-brownish color, which is indicative of the reduction of Ni^{2+} ions (Figure S2).

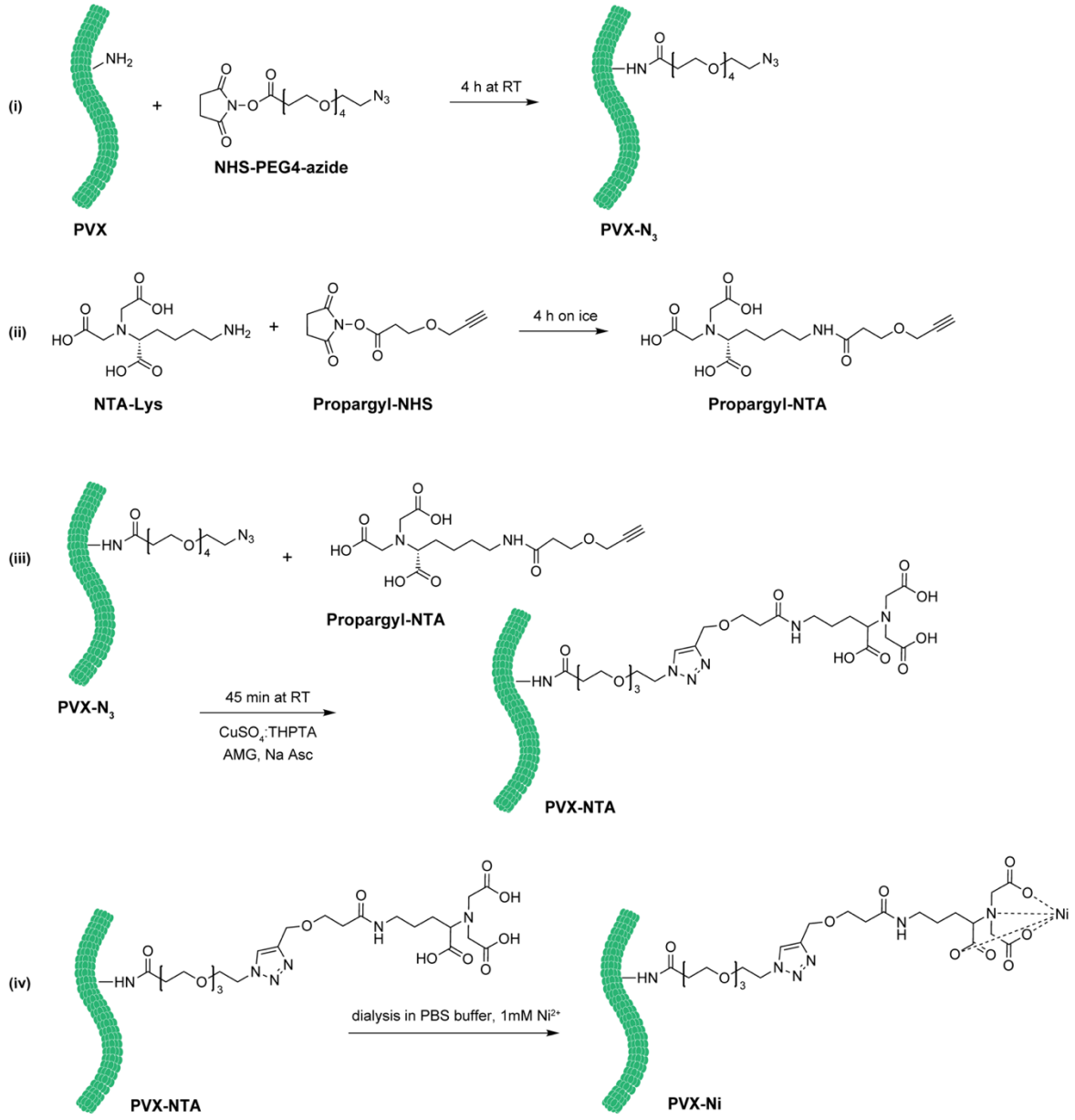

Figure S1. Reaction scheme for synthesis of PVX displaying nickel-coordinated nitrilotriacetic acid, denoted as PVX-Ni.

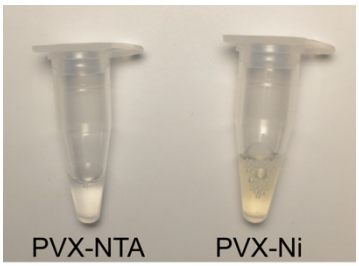

Figure S2. Treatment of PVX-NTA and PVX-Ni with 100 mM DTT. Reduction of Ni^{2+} ions results in yellow-brownish color of PVX-Ni, but not PVX-NTA – the latter does not contain any Ni^{2+} ions and thus remains clear .

2. Protein expression, purification, and characterization of TRAIL with an N-terminal His-tag (HisTRAIL)

The schematic of the constructed plasmid for expressing HisTRAIL is shown in Figure S3A. The expressed protein has an N-terminal His-tag, followed by a thrombin cleavage sequence allowing for removal of the His-tag if desired, and a C-terminal bioactive human TRAIL (aa 114-281).

Clear Coli® BL21DE3 carrying pET-28a(+)/HisTRAIL were inoculated in Luria-Bertani (LB) medium supplemented with kanamycin (Gold Biotechnology) at 50 μg mL^{-1} final concentration at 37°C; the culture was grown until an OD600 of 0.8 was reached. The culture was then induced with isopropyl β-D-thiogalactoside (IPTG, Gold Biotechnology) at 1 mM final concentration; culture growth was allowed to procced overnight at 22°C to induce protein expression. Cells were then harvested by centrifugation (5,000 × *g*, 10 min, 4°C); the pellet was resuspended in bacterial cell lysis buffer (Gold Biotechnology) containing lysozyme (Gold Biotechnology) at 1 mg mL^{-1} final concentration, and DNase I (NEB). Cell lysis was conducted at 37°C for 60 min. After centrifugation (13,000 × *g*, 30 min, 4°C) to remove cell debris, the supernatant containing HisTRAIL was collected. Protein purification was carried out using HisPurTM Ni-NTA resin (Thermo Fisher Scientific) for immobilized metal affinity chromatography; the protocol was according to the manufacturer's instructions. Eluted HisTRAIL in PBS, pH 7.4 containing 250 mM imidazole was treated with DTT at room temperature for 1 h (to reduce disulfide bonds), and then dialyzed against PBS, pH 7.4 overnight. The protein concentration was measured using a Nanodrop 2000 UV/visible spectrometer.

HisTRAIL expression and purification was confirmed by denaturing gel electrophoresis (SDS-PAGE) and western blot analysis (Figure S3B). A single band at 22 kDa was observed in SDS-PAGE gel under white light after Coomassie Blue (CB) staining, indicative of purified HisTRAIL in the monomeric state after being treated by DTT in the eluted solutions. In western blot analysis, HisTRAIL was detected by a rabbit polyclonal anti-TRAIL primary antibody (BioVision). Subsequently, the primary antibody was detected by an alkaline phosphatase-conjugated anti-rabbit antibodies (Invitrogen) and BCIP/NBT substrate (Invitrogen) was added for visualization. Multiple bands derived from HisTRAIL

oligomers were detected, reflecting the more sensitive detection of western blot *vs* SDS PAGE. After treated with DTT and dialysis, trimeric formation was confirmed by SDS-PAGE as shown in Figure S3C. A heterogeneous mixture of HisTRAIL monomers and trimers but not dimers were observed, indicating the reducing effects of DTT to remove di-sulfide bonds in dimers for trimeric formation.

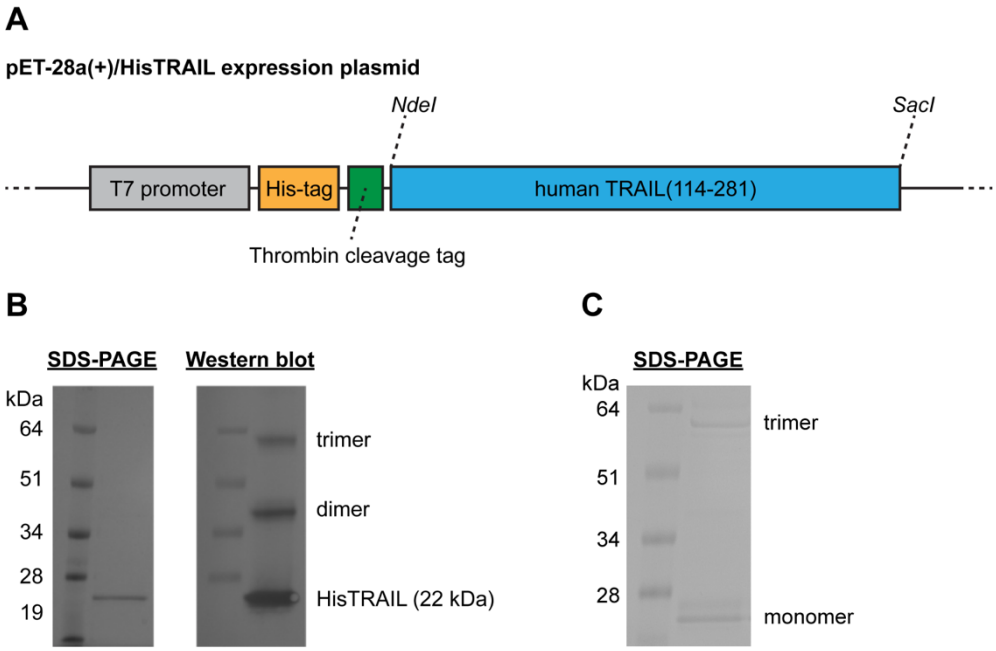

Figure S3. A) Schematic of the constructed pET-28a(+)/HisTRAIL plasmid encoding the bioactive domain of human TRAIL (aa 114-281) inserted at the corrresponding *NdeI* and *SacI* restriction enzyme sites, next to the N-terminal His-tag and thrombin cleavage tag from the orginial pET28a(+) plasmid. B) Confirmation of purified HisTRAIL by denaturing gel electrophoresis (SDS-PAGE) with DTT addition and western blot analysis. (C) Confirmation of trimeric HisTRAIL formation by SDS-PAGE without DTT addition.

3. Fluorescent labeling of HisTRAIL with Cy5

Water soluble sulfo-cyanine5 NHS ester (Cy5, Lumiprobe) was added to HisTRAIL (0.5 mg mL^{-1}) at a 1:1 molar excess and the reaction was allowed to proceed for two hours at room temperature; this reaction is designed to label TRAIL at lysine side chains. At higher ratios, HisTRAIL-Cy5 solutions tended to aggregate and thus significantly reduced the yield of fluorescently labeled HisTRAIL. Excess fluorophores were then removed using PD-10 columns (GE Healthcare) and additional dialysis against PBS, pH 7.4 overnight at 4°C. Fluorescently labeled HisTRAIL-Cy5 was analyzed by SDS-PAGE and visualized upon excitation at 632 nm to detect Cy5 and under white light after CB staining to detect HisTRAIL. Co-localization of the fluorescence and protein band indicates covalent modification of HisTRAIL with Cy5 (Figure S4A). The bands underneath the HisTRAIL or HisTRAIL-Cy5 monomer could be a fragment of HisTRAIL or internal cross-linked HisTRAIL. They can also be detected by western blot (data not shown). The number of Cy5 per HisTRAIL was calculated based on absorbance measurement using Nanodrop (Figure S4B) and the Beer-Lambert law with the HisTRAIL- and Cy5-specific extinction coefficients (27,390 M^{-1} cm^{-1} at 280 nm and 271,000 M^{-1} cm^{-1} at 647 nm, respectively). It was determined that in every 4.6 HisTRAIL molecules were modified by a Cy5 molecule.

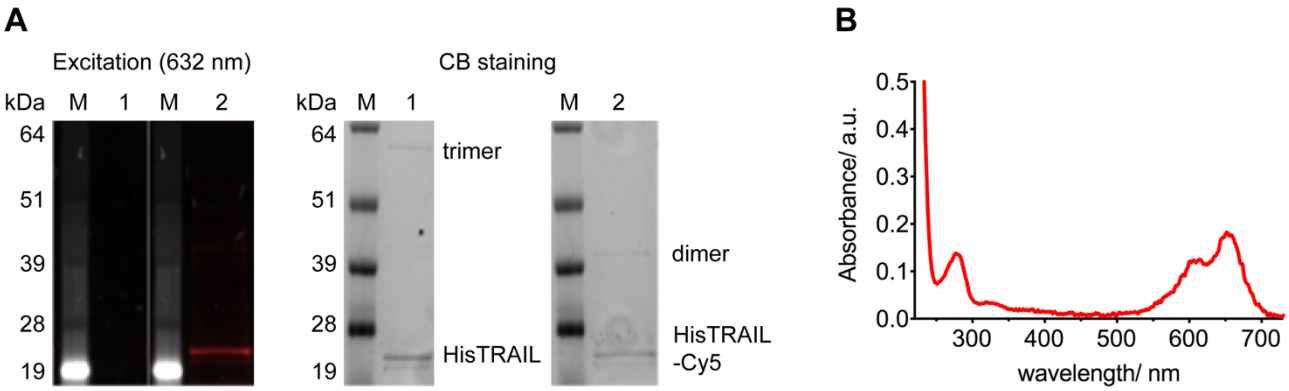

Figure S4. (A) SDS-PAGE gel analysis of (1) unlabeled HisTRAIL *vs* (2) fluorescently labeled HisTRAIL-Cy5, visualized upon excitation at 632 nm and under white light after CB staining. (B) UV/Visible spectrum of HisTRAIL-Cy5.

4. SEC profile of the native PVX particle

Figure S5 shows the SEC profile of native PVX particle eluting from the column at 14.5 mL, which is identical to the elution peaks of PVX-Ni and PVX-HisTRAIL-Cy5 in Figure 2B, confirming the intact filamentous structures of the PVX-based nanoplatforms after modification and purification.

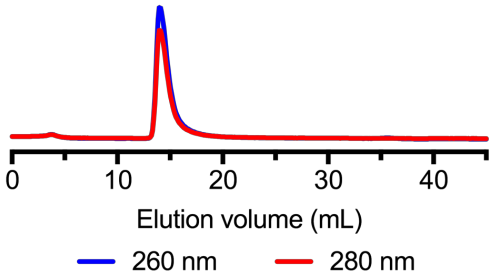

Figure S5. The SEC profile of native PVX particle eluting from the Superose-6 column. Native PVX elutes at 14.5 mL and the 260/280 nm ratio is 1.22.

5. Biotinylation of HisTRAIL

Similar to the method for labeling with Cy5 probe, a 1:1 molar ratio of biotin *N*-hydroxysuccinimide ester (MedChem Express) was added to HisTRAIL (0.5 mg mL^{-1}); the reaction was allowed to proceed for two hours in PBS, pH 7.4 at room temperature. Free biotin was removed using a 10-kDa cutoff spin filters (Millipore, 10 washes with PBS).

Biotin-conjugated HisTRAIL was analyzed by SDS-PAGE and western blot to assess the degree of biotinylation. Denatured HisTRAIL-biotin, after separation by SDS-PAGE, was transferred onto a nitrocellulose membrane (Thermo Scientific) and blocked for 60 min using 5% (w/v) skim milk powder dissolved in 0.1 M Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST). To detect biotinylated HisTRAIL, alkaline phosphatase-conjugated streptavidin (Sigma Aldrich) diluted in 5% (w/v) milk in TBST at a 1:500 ratio. BCIP/NBT substrate for alkaline phosphatase was added for visualization of biotinylated protein HisTRAIL-biotin (Figure S6).

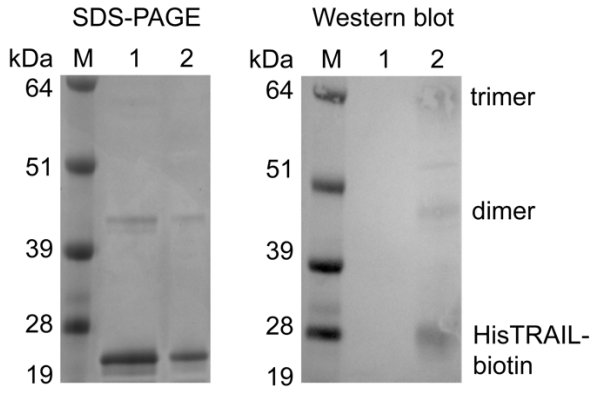

Figure S6. SDS-PAGE and western blot analysis of (1) unlabeled HisTRAIL *vs* (2) HisTRAIL-biotin.

6. SEC profile of the PVX-HisTRAIL conjugate particle

Figure S7 shows the SEC profile of PVX-HisTRAIL conjugate after ultracentrifugal purification; the elution profile indicates structural integrity of the particle as well as removal of free HisTRAIL (no observation at 25–39 mL) from the suspension. The PVX-HisTRAIL particle eluted at 14.5 mL; the elution profile matched the corresponding ones from wildtype PVX (Figure S5) and PVX-HisTRAIL-Cy5 (Figure 2B).

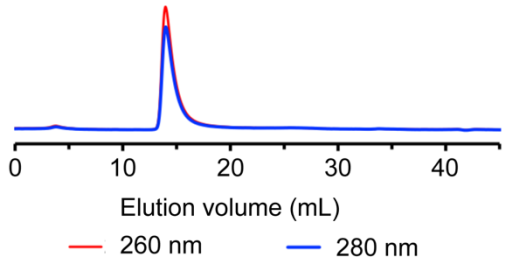

Figure S7. The SEC profile of purified PVX-HisTRAIL eluting from the Superose-6 column. PVX-HisTRAIL elutes at 14.5 mL, and the 260/280 nm ratio is 1.20, an indicator of the bound HisTRAIL.

7. *In vitro* treatment efficacy in SK-BR-3, a HER-2 expressing breast cancer model

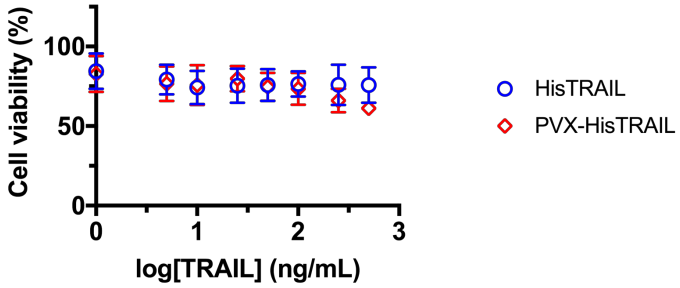

Figure S8. *In vitro* efficacy of HisTRAIL *vs* PVX-HisTRAIL in SK-BR-3, a HER2-amplified breast cancer cell line determined by MTT assay. Neither PVX-HisTRAIL or HisTRAIL did show cell killing in this cell line, consistent with previously reported data.[3] Data are expressed as means ± SD.

8. Body weights of treated mice

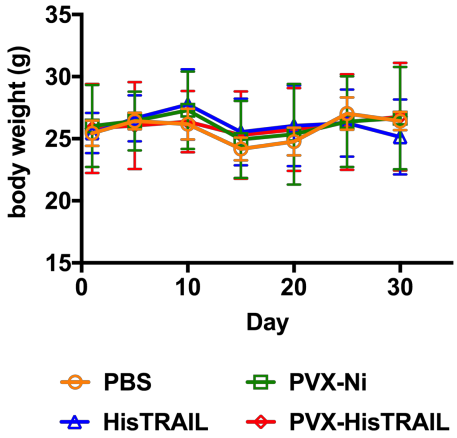

Figure S9. Body weights of mice treated by PBS, PVX-Ni, HisTRAIL, and PVX-HisTRAIL over 30 days of the study. No significant weight loss was observed.

9. References

(1) Steinmetz, N. F.; Mertens, M. E.; Taurog, R. E.; Johnson, J. E.; Commandeur, U.; Fischer, R.; Manchester, M. Potato Virus X as a Novel Platform for Potential Biomedical Applications. *Nano Lett.* **2010**, 10, 305–312.

(2) Le, D. H. T.; Hu, H.; Commandeur, U.; Steinmetz, N. F. Chemical Addressability of Potato Virus X for Its Applications in Bio/Nanotechnology. *J. Struct. Biol.* **2017**, 200, 360–368.

(3) Rahman, M.; Pumphrey, J. G.; Lipkowitz, S. The TRAIL to Targeted Therapy of Breast Cancer. *Adv. Cancer Res.* **2009**, 103, 43–73.