Supplementary information

**Supplementary Figure 1.** Simulation of the effect of injection time on the determination of pre-steady state affinity value. For the simulation the rate constants of the trimer:monomer complex of rhTRAIL WT and DR5-Fc were used: \( k_a = 11.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) and \( k_d = 0.36 \times 10^{-4} \text{ s}^{-1} \), thus \( K_D = 30 \text{ pM} \) (cp. Table 1). Binding of 18 concentrations of rhTRAIL (2 pM-262 nM; bottom to top) was simulated with program CLAMP99 v3.3 (24) using a 1:1 binding model and 4 different injection times: 150 s (A); 500 s (B); 2500 s (C); and 180,000 s (D). For each injection time, responses at the end of each injection were plotted against the concentration to create the binding isotherms (e). Equilibrium can be reached at high rhTRAIL concentrations in all time frames. However, only when using the very long injection time of 180,000 s (which is nearly 10 times the half life of the complex of 19254 s), a true equilibrium value can be reached at all concentrations and the binding curve results in the \( K_D \) value of 30 pM. Evidently, shorter injection times can result in apparent \( K_D \)’s values that are two orders of magnitude too high.

**Supplementary Figure 2.** Formation of the trimer:trimer complex by binding of rhTRAIL\(^{D269H/E195R}\) to captured DR4-Fc or DR5-Fc. A CM5 sensor chip was prepared by chemical coupling of protein A. A series of cycles was programmed comprising capturing 1205 RU DR4-Fc (A) or 1850 RU DR5-Fc (B), followed by an injection of rhTRAIL\(^{D269H/E195R}\) protein and finally regeneration. Eight concentrations of rhTRAIL\(^{D269H/E195R}\) were used, ranging from 1.95-250 nM (lower to upper curves). Although the data were double corrected for responses measured on the empty flow cell and for injection of buffer (7), a initial fast drop in SPR response was measured on the flow cell with captured DR4-Fc. This was not observed on the flow cell with captured DR5-Fc (indicating normal behavior of rhTRAIL\(^{D269H/E195R}\)), nor with rhTRAIL WT with captured DR4-Fc (indicating normal behavior of the captured DR4-Fc), strongly suggesting this to be a non-specific effect. Using a CM5 chip with an intermediate density of 240 RU DR4-Fc (C), this effect is significantly smaller, indicating the non-specific effect to be related to high densities. As a comparison, the sensorgrams of rhTRAIL WT (grey)
binding to 240 RU DR4-Fc (C) is also shown. In addition, the sensorgrams of binding rhTRAIL WT (grey) and rhTRAIL \textsuperscript{D269H/E195R} (black) to 300 RU DR5-Fc are added (D). The complexes formed at these intermediate receptor densities are no longer trimer:trimer complexes but most likely heterogeneous with a mean stoichiometry that is in the range of a trimer:dimer complex.

**Supplementary Figure 3.** The three receptor binding sites in rhTRAIL are not identical. The crystal structures of the trimer:trimer complexes of rhTRAIL and DR5 (A: PDB file 1D0G; B: PDB file 1DU3) demonstrate that the conformations of the three TRAIL subunits in the trimeric molecule are not fully identical as was also indicated especially for loops 190’s and 150’s (Hymowitz et al., 1999). An overlay of the three TRAIL subunits (respectively in red, green and purple) in both crystal models is presented with the 190’s loop on top. Preliminary calculations using Discovery Studio 2.5 (Accelrys, San Diego, USA) of the interaction energy suggest differences in interaction energies up to 5 kcal/mol when only considering interactions of residues 195-202 in the 190’s loop of each TRAIL subunit with its bound receptor molecule in structure 1D0G. A comparison of the interaction energies of the TRAIL-receptor pairs using all residues was not possible due to non-defined regions in the TRAIL or receptor structures. Nevertheless, these comparisons point out that the three receptor binding sites are not identical.
Supplementary Figure 1
Simulation of the effect of injection time on the determination of pre-steady state affinity value.
Supplementary Figure 2

Formation of the trimer:trimer complex by binding of rhTRAIL^{D269HE195R} to captured DR4-Fc or DR5-Fc.
Supplementary Figure 3

The three receptor binding sites in rhTRAIL are not identical.