The serum protein transthyretin as a platform for dimerization and tetramerization of antibodies and Fab fragments to enable target clustering

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Transthyretin (TTR) is an abundant homotetrameric serum protein and was selected here for engineering higher-valency molecules because of its compact size, simple structure, and natural propensity to tetramerize. To demonstrate this utility, we fused TTR to the C terminus of conatumumab, an antibody that targets tumor necrosis factor–related apoptosis-inducing ligand receptor 2, as heavy chains to form antibody dimers and Fab heavy chains to form Fab tetramers. Moreover, we used constant heavy domain 3 heterodimerization substitutions to create TTR-mediated conatumumab tetramers. The conatumumab–TTR fusions displayed substantially enhanced potency in cell-based assays, as well as in murine tumor xenograft models. We conclude that antibody–TTR fusions may provide a powerful platform for multimerizing antibody and Fab fragments to enhance the capabilities of human therapeutics that benefit from target clustering and higher-order antigen-binding valency.

Transthyretin (TTR) is a compact (14 kDa), abundant serum protein that carries the thyroid hormone thyroxine (1, 2) and is responsible for extending the serum T₁/₂ of retinol-binding protein (3); however, it is not essential because TTR knockout mice are viable (4, 5). TTR is also a major component of cerebrospinal fluid, and most if not all brain TTR is produced by the choroid plexus, unlike serum TTR, which is predominantly produced by the liver (2, 6–9). Certain familial mutations in TTR can induce amyloidogenesis (10, 11), which is believed to be primarily driven by mutation-induced loss in thermal stability of the tetramer, and this is currently a major area of study (12, 13). TTR is a noncovalently associated homotetrameric protein that, unlike most serum proteins, does not contain any disulfide bonds or glycosylation. The tetramer of TTR is strongly associated and does not undergo thermal unfolding at less than 80 °C, which can be further enhanced by the addition of a single mutation to eliminate the mutually repulsive lysines at position 15 (14). TTR has a single cysteine, which is often found as a mixed disulfide species with cysteine or GSH (15), but this cysteine is not required for tetramer formation.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a TNF superfamily member that induces cellular apoptosis by activating death receptors DR4 and DR5/TRAIR2 (16, 17). Antibodies against TRAIL-R2, such as conatumumab (AMG 655, or Ab1), induce tumor cell apoptosis in multiple tumor types via caspase activation (18); however, AMG 655 failed to demonstrate significant clinical benefit in phase 2 trials (19–21). Receptor clustering is often required for activation of TNF family members (22), and many anti-death receptor antibodies require cross-linking for maximal activity (18, 23–25). Subsequently, it has been demonstrated that enhanced target clustering significantly enhances the anti-tumor activity of Ab1 in cell-based assays (26), and antibody-mediated target clustering has been shown to improve the potency for other targets in this superfamily (27–31).

Other engineering strategies to multimerize DR5 and induce apoptosis without the requirement for cross-linking have been reported. TAS266 is an agonistic tetravalent construct containing four high-affinity single-variable domains that cluster DR5 molecules (32); however, this molecule was discontinued in clinical development. Tetrameric anti-DR5 antibody constructs have also been generated by fusing single-chain variable fragments targeting DR5 with the tetramerization domain of p53 and human serum albumin (33). A comparison of antibody valency and epitope targeting demonstrated tetravalent constructs that bind two distinct DR5 epitopes showed more potent activity than bivalent antibodies or antibodies that target a single DR5 epitope (34). Nonantibody scaffolds for DR5 multimerization have also been explored. A DR5-targeting peptide was covalently coupled to a dextran scaffold fused to an Fc to generate a flexible multimeric construct that effectively induced apoptosis (35). Together, these different DR5-targeting molecules demonstrate formats that multimerize DR5 binding, especially in a tetravalent format, and can drive DR5-mediated apoptosis without the requirement for additional cross-linking.

Both the N and C termini of TTR are exposed on the surface of the tetramer, making them amenable to fusion with proteins (Fig. 1). Here we used the natural tetramer forming ability of TTR to generate antibody dimers and Fab fragment tetramers with only modest modifications to TTR and no modifications to the antibody other than fusion of TTR to the C terminus of the antibody or Fab fragment. In addition, we were able to form antibody tetramers using TTR with the addition of two charged mutations in each Fc CH3 dimerization interface. These higher-order antibody and Fab molecules significantly increase the potency of Ab1 both in vitro and in vivo.
**Results**

**TTR fusion construct design, production, and analytics**

The 2X-Ab1-TTR molecule was constructed by fusing the C terminus of the human anti-TRAILR2 antibody (Ab1) heavy chain directly to the N terminus of human TTR without linkers. Because there are two copies of the heavy chain in the full Ab1, this results in the formation of a TTR dimer at the C terminus of the Ab1, which subsequently forms a noncovalent TTR tetramer resulting in tail-to-tail dimerization of the Ab1, leaving the antigen-binding domains unobstructed (Fig. 1). The 4X-Ab1-TTR molecule was assembled in a similar manner; however, charge pair mutations in the CH3 domain that discourage heavy chain homodimerization and thus favor heterodimerization of the CH3 domain enabled fusion of only one TTR monomer per full Ab1. This results in formation of an Ab1 tetramer complex (Fig. 1). The 4X-Fab1-TTR molecule was produced by fusing the C terminus of the Fab1 heavy chain to the N terminus of the TTR with the addition of two intervening glycines to form a linker (Fig. 1). To aid in purification of the fusion of Fab1, which lacks an Fc, a polyhistidine tag was added to the C terminus of the TTR.

The constructs were stably expressed in CHO-K1 cells at 2–10-liter scale, and the resultant molecules were purified by affinity capture chromatography followed by cation-exchange chromatography yielding 72 mg/liter (4X-Fab1-TTR), 41 mg/liter (2X-Ab1-TTR), and 26 mg/liter (4X-Ab1-TTR) product determined to be 96.2, 94.9, and 83.1% pure.

![Diagram of TTR molecules](image-url)
respectively, as determined by analytical size-exclusion chromatography (SEC).

The oligomerization state of the fusion proteins was determined by analytical SEC. Each molecule showed a shift to shorter SEC peak retention time as would be expected for the increasing size of the molecules (Ab1, 148 kDa; 4X-Fab1-TTR, 248 kDa; 2X-Ab1-TTR, 345 kDa; and 4X-Ab1-TTR, 635 kDa), indicating that the molecules were primarily assembled as expected (Fig. 2A and Fig. S1). The prepeaks may be dimers of the assemblies. Mass spectral analysis further confirmed the TTR fusion assemblies by detecting intact molecular masses of 248,423, 345,622, and 635,962 Da for the 4X-Fab1-TTR, 2X-Ab1-TTR, and 4X-Ab1-TTR, respectively.

The propensity of the fusion assemblies to exchange with unfused TTR was determined by incubating the fusion molecules with 24-fold molar excess unfused tetramer at 37 °C for 28 days. After the 28-day incubation period, the SEC profiles of the fusion molecules (between 4 and 5.5 min) and the unfused TTR tetramers (between 6.5 and 7.0 min) did not significantly change (Fig. 2B). If TTR subunit exchange was occurring, an intermediate size species eluting between the fusion molecules and the unfused molecules would be expected.

**TTR fusion constructs potency in cell-based assays**

The cytotoxic activity of the TTR fusion constructs was evaluated in vitro against the colorectal cancer cell lines Colo205-Luc and SW403. In these cell lines, the parental antibody, Ab1, demonstrates little to no cell killing. Cross-linking of the antibody with protein G enables cell killing in a dose-dependent manner (Figs. 3 and 4), consistent with the requirement of antibody clustering for activity (36). Interestingly, the cytotoxic activity of the three TTR fusion constructs was more potent than that induced by the cross-linked Ab1 (Figs. 3 and 4). The most potent activity was observed with the 4X-Ab1-TTR construct. Similar results were observed in both cell lines.

**Pharmacokinetic profiles of TTR fusion constructs**

The pharmacokinetic (PK) profiles of the antibodies and TTR fusion molecules were assessed in mice after IV administration. The benchmark antibodies conatumumab (Ab1) and a variant of conatumumab harboring two complementarity-determining regions mutations that render the molecule unable to bind TRAILR2 (Ab2) both demonstrated a PK profile typical for an antibody. Because both the target-binding Ab1 and non-
target-binding Ab2 have nearly identical PK properties (AUC₀⁻⁻ of 38,482 and 39,483 nM/h respectively), target-mediated clearance is unlikely a factor in the PK of these molecules (Fig. 5 and Table 1). The 2X-Ab1-TTR fusion displayed slightly more favorable coverage (AUC₀⁻⁻ of 57,167 nM/h) than either benchmark antibody. The 4X-Ab1-TTR fusion showed initial PK properties similar to those of the benchmark antibodies and the 2X-Ab1-TTR fusion, but after 336 h the PK appears to be slightly less favorable than the benchmark antibodies (AUC₀⁻⁻ of 26,457 nM/h). As expected, the 4X-Fab1-TTR fusion, lacking an Fc for neonatal Fc receptor-mediated recycling, showed substantially inferior PK properties compared with any other molecules in the study (AUC₀⁻⁻ of 3,025 nM/h). Together, these data support the idea that the 2X-Ab1-TTR and 4X-Ab1-TTR have PK properties similar to that of Ab1 with the potential for increased efficacy.

**Murine xenograft models**

The antitumor activity of the TTR fusion molecules was evaluated in colorectal cancer xenograft models in vivo. SW403 cells were implanted subcutaneously into immunocompromised mice, and the tumors were allowed to reach 58–66 mm³. The mice were then treated with TTR fusion constructs, antibody, or vehicle only, twice a week for 3 weeks. As expected, treatment of mice with the control antibody Ab2 did not impact tumor growth (Fig. 6). Treatment with either Ab1 or the 2X-Ab1-TTR construct modestly inhibited tumor growth, but this effect was not statistically significant (Fig. 6). In contrast, both the 4X-Fab1-TTR and 4X-Ab1-TTR fusions were very effective in inhibiting tumor growth in this model, with tumor stasis observed after only two doses (\( p < 0.05 \)). The body weight of the animals was unaffected by treatment, consistent with the lack of cross-activity of Ab1 or the TTR fusion constructs for mouse TRAILR2 (Fig. 6).

The TTR fusion molecules were also evaluated in the Colo205-Luc xenograft model. Immunocompromised mice were implanted subcutaneously with Colo205-Luc cells, and the tumors were allowed to grow to 40–48 mm³. The mice were then randomized and treated with TTR fusion molecules, Ab1, control antibody, or vehicle only. As in the SW403 model, the non–target-binding Ab2 did not impact tumor growth (Fig. 7). Unexpectedly, the 2X-Ab1-TTR fusion construct also did not have antitumor activity in the Colo205-Luc model (Fig. 7). In contrast, Ab1 significantly inhibited tumor growth, with similar efficacy as the 4X-Fab1-TTR and 4X-Ab1-
Transthyretin-mediated multimerization of antibodies

**Figure 7.** TTR fusion proteins inhibit Colo205-Luc tumor xenografts. At day 8, the mice (n = 10) were treated (red arrows) with b.i.w. IV injections with binding-site normalized doses (1.38 nmol binding sites/dose) of the test articles for 3 weeks (Ab1 (100 μg), Ab2 (100 μg), 2X-Ab1-TTR (126 μg), 4X-Ab1-TTR (109 μg), and 4X-Fab1-TTR (43 μg)). Tumor volume measurement was determined by measuring the length and width of tumors using a caliper. One or two animals from groups 4X-Fab1-TTR and Ab2 respectively were euthanized prior to the end of the study because tumor size exceeded 1,500 mm³.

TTR constructs (Fig. 7). Both Ab1 and the 4X-Fab1-TTR construct were able to induce tumor stasis in the Colo205-Luc model after just two treatments. The 4X-Ab1-TTR construct promotes tumor regression after two treatments, suggesting that this construct is most effective in promoting antibody clustering and tumor cell killing.

**Discussion**

For some therapeutic applications, the ability of an antibody to cluster the target can be advantageous (27–31). The use of monoclonal sIgA to produce dimeric antibodies and IgM to make pentameric or hexameric antibodies as therapeutics has been attempted (37–48); however, these platforms tend to produce heterogeneous products (49, 50), and they rapidly clear from the blood because of receptor-mediated transport to mucosal layers (51, 52). Although there are technologies other than sIgA and IgM that enable antibody clustering, none of these use natural human serum proteins; therefore, these alternatives have an increased risk of inducing undesired anti-drug antibody responses (53). Technologies to chemically multimerize synthetic TRAILR2-binding peptides have also been demonstrated to potently activate cell death (35); however, this approach is also at increased risk for inducing anti-drug antibodies. TTR is uniquely suited to enable higher-order antibody structures using an abundant natural human serum protein that is compact, highly stable, and simple, lacking glycosylation and disulfide bonds. In addition, both the N and C termini of TTR are exposed on the surface and can be used to fuse proteins, enabling the formation of up to an octovalent molecule. Two modifications to WT TTR are advantageous, C10A to eliminate the one cysteine in the molecule that can increase complexity by forming unwanted mixed disulfide species, and K15A, which can further enhance tetramer stability by eliminating the self-repulsion this lysine creates at the tetramerization interface (14).

We demonstrated that the TTR platform can be utilized to form antibody dimers, antibody tetrabmers, and Fab1 tetrabmers, which form a single molecule with four to eight antigen-binding sites. We also demonstrated that our fusion constructs do not have a propensity to exchange with unfused TTR tetrabmers in vitro, indicating a low risk of exchange with endogenous TTR in vivo. When the TTR multimerization technology was applied to an anti-TRAILR2 antibody, the potency in target cell killing assays was dramatically increased. The rank ordering of molecule potency in vitro translated to xenograft models in vivo, where the 4X-Fab-TTR and 4X-Ab1-TTR had significant antitumor efficacy as compared with the 2X-Ab1-TTR construct. The increased potency of Ab1 in vivo compared with in vitro may be due to the presence of normal IgG1 effector function in Ab1, which was likely substantially attenuated in the aglyco 2X-Ab1-TTR molecules. Interestingly, the tetramerized Fab1 was much more efficacious than the dimerized Ab1 with the same number of binding sites. This indicates that the geometry of the Fab domains may play an important role promoting efficacy, because the dimerized antibody has two rigid Fc domains separating the Fab1 pairs, whereas the Fab1 tetramer may be more flexible. Because the Fc domains in the antibody dimer were not glycosylated and therefore have substantially reduced effector function, and the antibody tetramer with four Fc domains was highly effective, Fc functionality is not likely to explain this difference. The nearly equivalent potency of the tetramerized Fab1 and tetramerized Ab1 in the SW403 xenograft model was unexpected, because the tetramerized Fab1 showed very rapid clearance in the PK study, despite similar activity in the in vitro cell killing assays. This suggests that in vivo, short exposure to the clustered anti-TRAILR2 activity is all that is required to start the tumor cell death cascade. Alternatively, it could be that the smaller Fab1 tetramer had better tumor penetration.

In the human adenocarcinoma Colo205-Luc murine tumor xenograft model, Ab1 performed as well as the tetramerized Fab1 and tetramerized Ab1 during the treatment phase of the study. However, after treatment was withdrawn, the tetramerized Ab1 showed superior tumor growth suppression for the remainder of the study. This result suggests that the tetramerized antibody produced a more durable antitumor response. This may be related to the octovalency of the Ab1 tetramer providing maximum target clustering and killing most if not all target cells. This is consistent with the finding that oligomerization of Ab1 for TRAILR2 clustering is critical for activity and likely explains why the monomeric Ab1 antibody failed to demonstrate efficacy in the clinic (26). Although the Colo205-Luc
xenograft model may be more sensitive to anti-TRAILR2 antibodies and fusion constructs than the SW403 xenograft model, together the data support the ideas that forced clustering of the antibody drives potent cytotoxic activity and that the 4X-Ab1-TTR configuration is the preferred geometry for target engagement.

The TTR multimerization platform presents a powerful and versatile tool that enables high order multivalent presentation of proteins using all human components. When used for antibody and Fab oligomerization, the tail-to-tail assembly ensures that all antigen-binding domains are correctly oriented with the N terminus exposed, which avoids unwanted steric hindrance and subsequent loss of affinity observed with head to tail fusion systems. This technology can be leveraged when target clustering is advantageous, such as with oncology or infectious disease treatment, possibly enabling a new class of human therapeutics.

### Materials and methods

#### Cloning

All TTR fusion constructs were based on the anti-TRAILR2 antibody conatumumab, also called AMG 655, referred to here as Ab1. The TTR used in these studies are cysteine 10 to alanine and lysine 15 to alanine (C10A/K15A) variants. The TTR antibody homodimer fusion (2X-Ab1-TTR), TTR antibody homotetramer fusion (4X-Ab1-TTR), and TTR Fab1 homotrimer fusion (4X-Fab1-TTR) constructs were assembled using standard PCR-based cloning methods into the mammalian pSLX240 vector (Selekis). The 2X-Ab1-TTR construct was assembled by directly fusing the C terminus of the antibody heavy chain (HC), with an N297G mutation to prevent glycosylation, to the N terminus of TTR. The 4X-Fab1-TTR construct was assembled by fusing the C terminus of the Fab1 heavy chain starting at the cysteine that forms the intermolecular disulfide bond with the light chain (LC) by the addition of two linking glycines between the Fab1 and the N terminus of the TTR. In addition, a polyhistidine tag with two linking glycines was added to the C terminus of the TTR. The 4X-Ab1-TTR construct was assembled by fusing the C terminus of one of the two antibody heavy chains, with an N297G mutation to prevent glycosylation, directly to the N terminus of TTR. Heterodimerization of the antibody heavy chains was accomplished by using charge pair mutations in the CH3 domains (54), with one heavy chain fused to TTR and the other heavy chain not fused. Ab1 was produced as previously described (18). The inactive control antibody, Ab2, contains two mutations in the complementarity-determining regions of Ab1 that prevent target binding and was produced as previously described (55).

#### Expression

The fusion proteins were stably expressed in suspension adapted CHO-K1 cells. Transfections were performed using Lipofectamine LTX (Invitrogen™) according to the manufacturer’s protocol. A total of 30–36 μg of the mammalian expression plasmid DNA was used at a 1:1 ratio (HC-TTR:LC) for the 2X-Ab1-TTR and 4X-Fab1-TTR fusions, and a 1:1:1 ratio (HC:HC-TTR:LC) was used for the 4X-Ab1-TTR molecule. The flasks were incubated at 36 °C with 5% CO2 shaking at 150 rpm for 6 h, at which point 9–12 ml of growth medium was added to each flask and incubated for an additional 48–72 h. For selection, the medium was replaced with 23–25 ml of growth medium supplemented with selective antibiotics 72 h post-transfection. The selection medium was changed two or three times/week, diluting cultures when needed to ensure cultures did not overgrow (<5–6 × 10^6 viable cells/ml), until cell viability and density recovered. Large-scale productions (2.3–2.5 liters) were carried out in shake flasks at 36 °C. Production runs were seeded at 2 × 10^6 viable cells/ml in production medium, and the conditioned medium was harvested on day 5 by centrifugation followed by filtration.

#### Purification

The 2X-Ab1-TTR and 4X-Ab1-TTR fusion proteins were purified using an AKTA Purifier (GE Healthcare Life Sciences) liquid chromatography system with two coupled 5-ml protein A Fast Flow (ProA) HiTrap (GE Healthcare Life Science) columns. The conditioned medium was loaded directly onto the ProA columns, then washed with 5 column volumes (CV) of Dulbecco’s PBS (DPBS) (Thermo Fisher Scientific) and eluted with 8 CV of 50 mM acetic acid, pH 3.2. The ProA elution pools were titrated to pH 5.0 using 2 M Tris-HCl, pH 9.2, and then diluted with nine volumes of sterile water. The conditioned elution pools were dialyzed against 2 liters of 20 mM HEPES, 150 mM sodium chloride, pH 7.0 twice using 10-kDa molecular mass cutoff Slide-a-lyzers (Thermo Fisher Scientific). The buffer-exchanged pools were purified on an 18-ml SP Sepharose high-performance (SP HP) (GE Healthcare Life Science) column employing a 5-CV wash with 20 mM monobasic sodium phosphate, pH 7.0, and then diluted with nine volumes of sterile water. The conditioned elution pools were dialyzed against 2 liters of 20 mM HEPES, 150 mM sodium chloride, pH 7.0 twice using 10-kDa molecular mass cutoff Slide-a-lyzers (Thermo Fisher Scientific). The conditioned medium was harvested on day 5 by centrifugation followed by filtration.

The 4X-Fab1-TTR fusion protein was purified using an AKTA Purifier liquid chromatography system with a 50-ml nickel-Sepharose Excel (GE Healthcare Life Sciences) column. The conditioned medium was loaded directly onto the nickel-Sepharose Excel column; then washed with 10 CV of 20 mM monobasic sodium phosphate, 0.5 mM sodium chloride, 10 mM imidazole, pH 7.4; and eluted with 8 CV of 10–500 mM imidazole gradient. The nickel-Sepharose Excel pool was dialyzed against 2 liters of 20 mM HEPES, 150 mM sodium chloride, pH 7.0, twice using 10-kDa molecular mass cutoff Slide-a-lyzers. The purified samples were then concentrated using VivaSpin 10-kDa molecular mass cutoff centrifugal filtration units (Sartorius) and purified on a 320-ml Superdex 200 column (GE Healthcare Life Science) using a 1.4 CV isocratic mobile phase (20 mM HEPES, 300 mM sodium chloride, pH 7.0). Fractions were selected for pooling based on their SDS-PAGE and analytical SEC profiles. The SP HP pools were concentrated using VivaSpin 10-kDa molecular mass cutoff centrifugal filtration units (Sartorius) followed by sterile filtration through 0.2 μm Supor syringe filters ( Pall).
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employing a 5-CV wash with 20 mM monobasic sodium phosphate, pH 7.0, and eluted with a 20 CV 0 mM to 500 mM sodium chloride gradient. The fractions were selected for pooling based on their SDS-PAGE and analytical SEC profiles. The SP HP pool was dialyzed against 2 liters of 10 mM MES, 150 mM sodium chloride, pH 7.0, twice using a 10-kDa molecular mass cutoff Slide-a-lyzer. The dialyzed sample was concentrated using a VivaSpin 10-kDa molecular mass cutoff centrifugal filtration unit and then sterile-filtered through a 0.2-μm Supor syringe filter.

Protein analysis

Protein concentrations were determined by UV absorbance at 280 nm (A280) using a NanoDrop 2000 (Thermo Fisher Scientific). The samples were analyzed by denaturing, nonreducing 4–12% Bis-Tris NuPAGE gels using MES running buffer (Thermo Fisher Scientific), per the manufacturer’s instructions. The samples were analyzed on a Phenomenex SEC 3000 column, 7.8 × 300 mm (Phenomenex) using a 50 mM sodium phosphate, 250 mM sodium chloride, pH 6.9, isocratic mobile phase at 1 ml/min observing the absorbance at 280 nm. Mass spectral analysis of the fusion proteins was performed by buffer-exchanging the samples into 200 mM ammonium acetate and then brought to 5 μM as a final working concentration for native MS studies.

Subunit exchange study

TTR fusions were mixed with 24-fold molar excess unfused TTR tetramer in DPBS and incubated at 37 °C. Immediately after mixing and 28 days later, the samples were analyzed on a BEH 200 Å, 1.7-μm, 4.6 × 300-mm column (Waters) using an isocratic mobile phase with 100 mM sodium phosphate, 50 mM sodium chloride, 7.5% ethanol, pH 6.9, at 0.4 ml/min observing the absorbance at 280 nm. Mass spectral analysis of the fusion proteins was performed by buffer-exchanging the samples into 200 mM ammonium acetate and then brought to 5 μM as a final working concentration for native MS studies.

Pharmacokinetics

The PK profiles of the fusion proteins, Ab1 and Ab2 were determined after intravenous (IV) injection in male CD-1 mice (n = 3/group) at normalized molar equivalent levels: 6.5 mg/kg of 4X-Ab1-TTR, 3.5 mg/kg of 2X-Ab1-TTR, 2.5 mg/kg of 4X-Fab1-TTR, and 1.5 mg/kg of Ab1 and Ab2. Serum samples were collected from 75 μl of blood collected at 0.5, 2, 8, 24, 48, 72, 96, 192, 336, 504, 672, and 840 h after the dose. Each sample was maintained at room temperature after collection, and following a clotting period of 30–40 min, the samples were centrifuged at 22–8 °C at 11,500 rpm for 10 min using a calibrated Eppendorf 5417R centrifuge system (Brinkmann Instruments, Inc.). The collected serum was then stored at −60 to −80 °C until analysis.

To measure the total amount of test article in mouse serum samples, a regular binding 96-well MSD plate (Meso Scale Discovery) was coated with 2 μg/ml of rabbit anti-Ab1 polyclonal antibody (Amgen Inc.) in PBS and then incubated overnight at 4 °C. The plate was then washed and blocked with 1-Block™ (Thermo Fisher Scientific) overnight at 4 °C. The standards and quality controls (QCs) were prepared in mouse serum, and the samples were diluted in naïve CD-1 mouse serum if dilution was required. The standards, QCs, and samples were then diluted 1:20 in a buffer containing PBS, 1 mM NaCl, 0.5% Tween 20, and 1% BSA. The plate was washed three times with ~200 μl of 1× KPL buffer (KPL Inc.), and subsequently 50 μl of the diluted standards, QCs, and samples were transferred into the anti-Ab1 antibody coated plate and incubated for 1.5 h at room temperature (~25 °C). The plate was washed three times with ~200 μl of 1× KPL wash buffer, and then 50 μl of 100 ng/ml of mouse anti-hu Fc antibody, clone 135.1 (Amgen Inc.), conjugated to SULFO-TAG (Meso Scale Discovery) in 1-Block™ containing 5% BSA was added and incubated for 1.5 h at room temperature. For the 4X-Fab1-TTR construct, 50 μl of 250 ng/ml of mouse anti-κ LC, clone KCF-9 (Amgen Inc.) conjugated to biotin was added and incubated for 1.5 h; then, after washing the plate with 1× KPL wash buffer, 50 μl of 100 ng/ml of streptavidin conjugated to SULFO-TAG was added and incubated for 15 min. The plate was then washed six times with ~200 μl of 1× KPL wash buffer, followed by addition of 150 μl of 1× Read Buffer T (Meso Scale Discovery), and the electrochemiluminescent signal was measured using an MSD 6000 plate reader (Meso Scale Discovery). The serum concentration data were analyzed using noncompartmental methods with Phoenix® (Phoenix 64, Build 6.4.0.768, Pharsight® Corp.).

Cell-based assays

Human colon carcinoma cell lines Colo205 and SW403 were obtained from the American Type Culture Collection and maintained at 37 °C at 5% CO₂ in RPMI 1640 GlutaMAX culture medium (Thermo Fisher Scientific) supplemented with 10% FBS (Sigma, F9423-500mL) and 1% antibiotic–antimycotic (Thermo Fisher Scientific). The Colo205 cells were stably transfected with luciferase to generate Colo205–Luc cells, using standard procedures (56). For in vitro evaluation of TTR fusion protein activity, Colo205–Luc cells were plated at 10⁴ cells/well, and SW403 cells were plated at 4,000 cells/well of a 384-well microplate (Perkin Elmer) the day of the assay. The cells were incubated with the TTR fusion proteins or with Ab1 in the absence or presence of 1 μg/ml protein G (Sigma P4689) for 24 h. The protein G was used to promote cross-linking of the antibodies (18). Triplicate samples were analyzed. After 24 h of incubation at 37 °C with 5% CO₂, cell viability was assessed using the CellTiter-Glo cell viability assay (Promega). The CellTiter-Glo reaction was carried out according to the manufacturer’s instructions, and luminescence was measured using a BioTek Synergy Neo2 multimode reader (BioTek). The data were analyzed using Prism (Graph Pad) using nonlinear regression with a four-parameter variable slope.

Efficacy studies in xenograft models

For xenograft studies, mice were purchased from Charles River Laboratories and housed in groups of five in sterilized clear polycarbonate cages with microisolator tops. The cages were changed twice weekly. Harlan Teklad feed (8656) and water chlorinated with sodium hypochlorite to 3 mg/liter chloride were supplied ad libitum. Housing temperature was maintained between 18 and 22 °C, and relative humidity was maintained between 50 and 70%. The animal housing provided a...
The activity of the antibodies and fusion proteins was evaluated in a Colo205 human colorectal adenocarcinoma xenograft model as follows. Colo205-Luc cells were maintained at 37 °C at 5% CO₂ in RPMI 1640 culture medium supplemented with 10% FBS (Sigma, 2442-500mL), 4 mM t-glutamine (Hyclone, SH30034.01), 1 mM HEPES (Hyclone, SH30237.01), 1 mM sodium pyruvate (Sigma, S8636-100mL), and 2.5 g/liter glucose (Sigma, G8769). Female NU/NU Nude mice (Charles River Laboratories: NU-Foxn1nu) were injected with 1 × 10⁶ cells (in 100-μl volume) subcutaneously in the right flank. On day 7 post-tumor implantation, the mice were distributed into five treatment groups with 10 mice each, such that each group had similar mean tumor volumes. The mice received twice a week (b.i.w.) intraperitoneal (IP) injection, with the doses normalized to the number of target-binding sites in each molecule to 1.38-nmol binding sites per dose: Ab1 (100 μg), Ab2 (100 μg), 2X-Ab1-TTR (126 μg), 4X-Ab1-TTR (109 μg), and 4X-Fab1-TTR (43 μg). All treatments started on day 8 and ended on day 25 post-tumor implantation. All molecules were freshly prepared in diluent (DPBS) on the treatment date just prior to injection.

Tumor volume was determined by measuring tumor length and width with an ABS Digimatic solar caliper and calculated as 0.5 × L × W², where W was the smaller of the two measurements and expressed in mm³. Body weight measurements were determined using a PB602-S balance and reported as body weight change, which is calculated as 100 × (Wc/Wi), where Wc is the current body weight, and Wi is the body weight at day 4. The animals were euthanized prior to the end of the study if the tumor size exceeded 1,500 mm³. Statistical analysis

Data from days 7–39 are expressed as means plus or minus standard errors and plotted as a function of time. Statistical significance of observed differences between growth curves was evaluated by repeated measures analysis of variance for the transformed tumor volume data with Dunnett adjusted multiple comparisons. The analysis was done using SAS PROC MIXED procedure with model effects of transformed baseline tumor volume, day, treatment, and day-by-treatment interaction; a REPEATED statement where day was a repeated value, animal was the subject, and a Toeplitz covariance structure; and an LSMEANS statement to do a Dunnett analysis comparing the control group with the other treatment groups. The data were log or square root transformed according to Hortz’s method and transformed baseline tumor volume was included as a covariate in the model to account for possible pre-treatment tumor volume differences. If the log or square root transformation failed to achieve a proper residual distribution, a nonparametric repeated measure analysis of variance model was used with the same model effects on rank of the tumor volume. P values of <0.05 were considered statistically significant.

**SW403 xenograft model**

SW403 human colorectal adenocarcinoma cells were maintained at 37 °C in 5% CO₂ in RPMI 1640 culture medium (Sigma R0883) supplemented with 10% FBS (Sigma, 2442-500mL) and 4 mM t-glutamine (Hyclone, SH30034.01). Female NU/NU Nude mice (Charles River Laboratories: NU-Foxn1nu) were injected with 5 × 10⁶ cells (in 100-μl volume) subcutaneously in the right flank on day 0. On day 7 post-tumor implantation, the mice were distributed into six treatment groups with 10 mice each, such that each group had similar mean tumor volumes. The mice were administered b.i.w. IP injections of antibody or TTR fusion protein, with the doses of treatments normalized to the number of target-binding sites in each molecule to 2.76 nmol binding sites/dose: Ab1 (200 μg), Ab2 (200 μg), 2X-Ab1-TTR (252 μg), 4X-Ab1-TTR (218 μg), and 4X-Fab1-TTR (86 μg). The vehicle treatment was DPBS. All treatments started on day 15 and ended on day 32 post-tumor implantation. All molecules were freshly prepared in diluent (DPBS) on the treatment date just prior to injection.

Tumor volume and body weight measurements were determined by the methods described above. The animals were euthanized prior to the end of the study if the tumor size exceeded 1,500 mm³. Statistical analysis was done as described above.

**Data availability**

All of the data are contained within the article and the supporting information.

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**Abbreviations**—The abbreviations used are: TTR, transthyretin; PK, pharmacokinetic(s); TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; Ab1, antibody 1 (conatumumab, or AMG 655); Ab2, antibody 2 (TRAILR2); CH3, constant heavy domain 3; HC, heavy chain; LC, light chain; DR, death receptor; SEC, size-exclusion chromatography; CV, column volume(s); TNF, tumor necrosis factor; DPBS, Dulbecco’s PBS; SP HP, SP Sepharose high-performance; IV,
intravenous; QC, quality control; FBS, fetal bovine serum; b.i.w., twice a week; IP, intraperitoneal.

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