

Multivalent Scaffold Proteins as Superagonists of TRAIL Receptor 2–Induced Apoptosis

Jeffery S. Swers¹, Luba Grinberg¹, Lin Wang¹, Hui Feng¹, Kristen Lekstrom¹, Rosa Carrasco², Zhan Xiao², Ivan Inigo², Ching Ching Leow², Herren Wu¹, David A. Tice², and Manuel Baca¹

Abstract

Activation of TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2) can induce apoptosis in a variety of human cancer cell lines and xenografts, while lacking toxicity in normal cells. The natural ligand and agonistic antibodies show antitumor activity in preclinical models of cancer, and this had led to significant excitement in the clinical potential of these agents. Unfortunately, this optimism has been tempered by trial data that, thus far, are not showing clear signs of efficacy in cancer patients. The reasons for discrepant preclinical and clinical observations are not understood, but one possibility is that the current TRAILR2 agonists lack sufficient potency to achieve a meaningful response in patients. Toward addressing that possibility, we have developed multivalent forms of a new binding scaffold (Tn3) that are superagonists of TRAILR2 and can induce apoptosis in tumor cell lines at subpicomolar concentrations. The monomer Tn3 unit was a fibronectin type III domain engineered for high-affinity TRAILR2 binding. Multivalent presentation of this basic unit induced cell death in TRAILR2-expressing cell lines. Optimization of binding affinity, molecular format, and valency contributed to cumulative enhancements of agonistic activity. An optimized multivalent agonist consisting of 8 tandem Tn3 repeats was highly potent in triggering cell death in TRAIL-sensitive cell lines and was 1 to 2 orders of magnitude more potent than TRAIL. Enhanced potency was also observed *in vivo* in a tumor xenograft setting. The TRAILR2 superagonists described here have the potential for superior clinical activity in settings insensitive to the current therapeutic agonists that target this pathway. *Mol Cancer Ther*; 12(7); 1235–44. ©2013 AACR.

Introduction

Cells can undergo apoptosis via 2 different pathways: the intrinsic and extrinsic. The intrinsic pathway is preferentially triggered by intracellular proteins such as p53 in response to many different damaging influences including DNA damage, a defective cell-cycle or loss of cell survival factors. Intrinsically triggered apoptosis is mainly regulated by proteins of the Bcl-2 family that control the release of proapoptotic factors from the mitochondrial intermembrane space. In contrast, the extrinsic pathway is triggered by activation of cell surface death receptors, which belong to the tumor necrosis factor (TNF) receptor superfamily. Ligand-induced crosslinking of these receptors initiates a p53-independent intracellular signal transduction cascade that leads to cellular apoptosis.

TNF-related apoptosis inducing ligand receptor 2 (TRAILR2) is a member of the TNF receptor superfamily that, when activated, induces apoptosis in a broad range of cancer cells, but not normal cells (1, 2). For this reason, agonists of TRAILR2 are being developed as anticancer therapeutics, including TRAIL, the natural ligand for this receptor, as well as agonistic monoclonal antibodies (3–6). As with other members of the TNF-family, TRAIL is a homotrimeric ligand that is initially produced as a membrane-bound protein, but can be released in soluble form following proteolytic processing. Relative to soluble TRAIL, agonistic antibodies against TRAILR2 are less potent in inducing cell death and require higher-order crosslinking to enhance their *in vitro* activity (7–9). The crosslinking requirement is also important to their *in vivo* activity, and Fcγ receptors on tumor-associated leukocytes are thought to provide a crosslinking scaffold that promotes antibody-dependent, TRAILR2-mediated apoptosis of cancer cells (10).

While the activity of soluble TRAIL does not seem to depend on higher-order crosslinking, a number of reports have shown that multimerization of the trimeric ligand does result in enhancement of *in vitro* activity (11, 12). Indeed, higher-order presentation of many TNF-family ligands is a requirement for effective triggering of biologic signals (13–16). In these cases, recruitment of 3 copies of receptor by a soluble TNF-family ligand is insufficient to

Authors' Affiliations: Departments of ¹Antibody Discovery and Protein Engineering, and ²Oncology, MedImmune, LLC, Gaithersburg, Maryland

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Corresponding Author: Manuel Baca, Department of Antibody Discovery and Protein Engineering, MedImmune, LLC, One MedImmune Way, Gaithersburg, MD 20878. Phone: 301-398-5980; Fax: 301-398-8980; E-mail: bacam@medimmune.com

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initiate an intracellular signaling cascade. Nature has devised various strategies for increasing the valency of trimeric TNF-family ligands including clustering of membrane-bound ligands (17), oligomerization of soluble trimers (18), or sequestration on proteoglycan surfaces (19). With this in mind, it is somewhat surprising that bivalent IgG antibodies can activate TRAILR2, though as previously described, the *in vivo* activity of agonist antibodies seems to be dependent on Fc-mediated effects. Taken together, these observations suggest that the ability to increase the valency of a TRAILR2 agonist could be a powerful means to enhance its potency.

In addition to agonistic antibodies, the development of other biologic mimetics of TRAIL has been reported, this including oligomeric forms of TRAIL receptor-binding peptides (20, 21) and scaffold proteins (22, 23). An ideal therapeutic that activates the TRAILR2 pathway would combine the potency of the natural ligand with the favorable manufacturing attributes of antibodies. To achieve this, the specificity and high-affinity binding of antibodies is needed, but also the flexibility to vary the valency of target binding, a key determinant that could affect the strength of TRAILR2 activation.

In this article, we describe the isolation of monomeric TRAILR2-binding scaffold proteins based on a fibronectin type III scaffold, and engineering of these into multivalent proteins that induced cell death in TRAIL-sensitive and TRAIL-resistant cell lines. Through manipulation of target-binding affinity, fusion format, and valency, we obtained highly potent TRAILR2 agonists that significantly outperform the natural TRAIL ligand in inducing tumor cell apoptosis *in vitro* and *in vivo*.

Materials and Methods

Cell culture and protein reagents

Human cancer cell lines H2122 and H460 (NSCLC), Colo205 (colorectal adenocarcinoma), Jurkat (T-cell leukemia), and hepatocellular carcinoma cell lines PLC/PRF/5, Hep3B, HepG2, SNU-387, SNU-398, and SNU-449 were all obtained from the American Type Culture Collection. These cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS and maintained in 5% CO₂ at 37°C. The HEK-293F cell line (Invitrogen) was grown in FreeStyle serum-free medium (Invitrogen). No authentication of any of these cell lines was done by the authors.

Recombinant TRAILR2-Fc fusion protein used in phage display selections and *in vitro* assays was from R&D Systems. Recombinant human TRAIL used for *in vitro* studies was commercially sourced from Chemicon/Millipore. Soluble human TRAIL used in xenograft studies was produced in-house by expression of an untagged fragment in *Escherichia coli* (*E. coli*; residues 114–281). Purification of this material was conducted by metal chelating and ion exchange chromatography as reported elsewhere (24). Purified material was formulated in 20 mmol/L Tris-HCl pH 7.0, 300 mmol/L arginine-HCl, and characterized

by size exclusion chromatography/multi-angle light scattering to ensure that this material had the correct trimeric structure, and was free of aggregated material. The bioactivity of in-house produced recombinant TRAIL was confirmed in a Colo205 cell viability assay, and shown to have equivalent potency to commercially sourced material (EC₅₀ values within 2-fold).

Generation of TRAILR2-binding Tn3 proteins

Generation of Tn3 libraries, selection of human TRAILR2-specific Tn3 proteins, and the construction, expression, and characterization of multivalent tandem Tn3 constructs are described in the Supplementary Materials and Methods.

TRAILR2-mediated apoptosis of cancer cell lines

Viability of cells in culture was measured by ATP quantitation, using a CellTiter-Glo kit (Promega). The cells were plated at a density of 10,000 cells/well in 75 μ L of complete medium (RPMI 1640 medium supplemented with 10% FBS). Following overnight incubation at 37°C, 25 μ L of test agent in complete medium was added and the cells were cultured 72 hours (20 hours for H460) before viability analysis with CellTiter-Glo according to the manufacturer's instructions. All treatments were carried out in duplicate wells. Relative cell viability was determined by dividing the luminescence values for treated cells by the average luminescence for untreated viable cells. Dose-response plots of cell viability versus compound concentration were generated, and cell killing potency (EC₅₀) was determined as the concentration of test agent that reduced cell viability by 50%. In addition to viability assessment by ATP quantitation, morphologic changes such as cell shrinkage, rounding, and membrane blebbing were also evaluated by microscopic inspection of cells. To measure TRAILR2-mediated caspase activation, cells were treated in the same manner as for cell viability assays, except caspase activity was assessed 6 hours after addition of test agent, using a Caspase-Glo 3/7 kit (Promega) according to the manufacturer's protocol. Fold induction of caspase 3/7 was defined as caspase activity for treated cells divided by the activity for untreated control cells.

In vivo Colo205 xenograft study

Colo205 cells were maintained as a semi-adhesive monolayer culture at 37°C under 5% CO₂ in RPMI 1640 medium that contained 10% FBS. The cells harvested by trypsinization were resuspended in Hank's Balanced Salt Solution, and athymic female nude mice (10 mice per treatment group) were each injected subcutaneously in the right flank with 3×10^6 cells. The treatment study was initiated when tumors reached an average size of approximately 180 mm³. TRAIL was diluted from stock solution with 20 mmol/L Tris-HCl and 300 mmol/L arginine-HCl pH 7 and administered intravenously at 30 mg/kg daily for a total of 5 doses according to body weight. TRAILR2-specific tandem Tn3 protein was diluted from a stock

solution with PBS and administered intravenously at 30 mg/kg (first study) or 3 and 0.3 mg/kg (second study), daily for a total of 5 doses. Tumor volumes and body weight measurements were recorded. Tumor measurements were made using an electronic caliper and tumor volume (mm^3) was calculated using the formula tumor volume = $[\text{length (mm)} \times \text{width (mm)}^2]/2$. Percent partial regression (PR) is defined as the percentage of mice in group where tumor volume was less than 50% of volume at time of staging for 2 successive measurements. Percent complete regression (CR) was defined as the percentage of mice in group where no palpable tumor was detectable for 2 successive measurements. All procedures were conducted in accordance with federal, state, and Institutional guidelines in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility and were approved by the MedImmune Institutional Animal Care and Use Committee.

Results

Isolation and reformatting of TRAILR2-specific Tn3 proteins

Tn3 is a monobody protein scaffold (25) based upon the third fibronectin type III domain of tenascin C (26). This monomeric domain, which we have termed Tn3, resembles an antibody variable region in that it has 3 CDR-like loops that can be randomized for selection of novel-binding proteins (Supplementary Fig. S1). To isolate Tn3 proteins that bind specifically to human TRAILR2, a phage-displayed Tn3 library was panned against recombinant TRAILR2-Fc fusion protein. From this, 1C12 was identified as a TRAILR2-specific Tn3, that when expressed as a soluble protein, could bind TRAILR2 with moderate affinity ($K_d = 4 \mu\text{mol/L}$).

Monomeric 1C12 did not show any agonistic activity on TRAILR2-expressing cells (data not shown). Multimeric bi- and tetraivalent forms of 1C12 were generated by fusing this Tn3 module to fragments of human IgG1 (Fig. 1A). Fusion to the Fc region was used to generate a bivalent construct, whereas co-expression of 1C12 fused to the constant regions of IgG1 heavy chain and kappa light chain was used to generate a 2 chain tetraivalent protein. Negative control proteins were also generated by fusing a nonbinding Tn3 (clone D1) to fragments of IgG1.

Analytical size exclusion chromatography (SEC) and SDS-PAGE characterization was used to confirm that recombinant 1C12-Fc and 1C12-Ig had the expected oligomeric state, and that the purified proteins were free of aggregated material. Binding of these constructs to TRAILR2 was confirmed by ELISA and we also found that soluble TRAIL could inhibit this interaction, suggesting that 1C12 and TRAIL share an overlapping epitope on TRAILR2 (data not shown). The ability of bi- and tetraivalent 1C12 to activate cellular TRAILR2 was then assessed in a cell viability assay using H2122 cells, a TRAIL-sensitive tumor cell line (5, 7). Both constructs had an inhibitory effect on cell viability, consistent with induc-

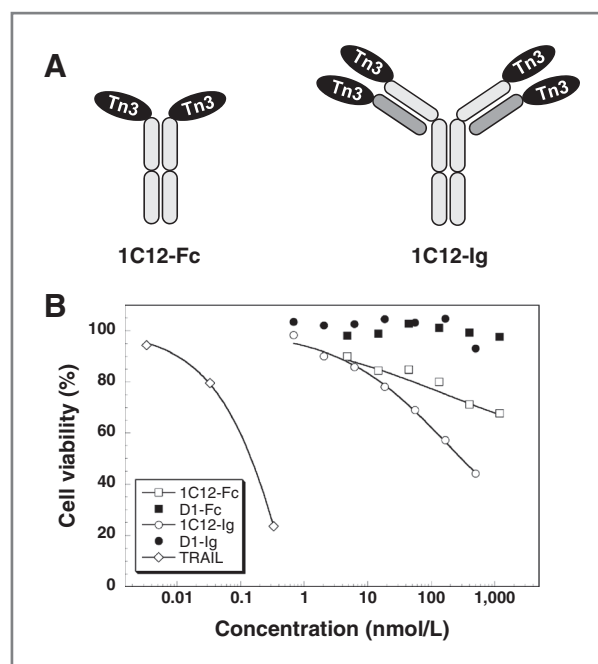


Figure 1. Generation and characterization of multivalent TRAILR2-binding Tn3. A, cartoon representations of bi- and tetraivalent forms of 1C12 based on fusion to antibody constant regions. B, inhibition of cell viability by multivalent TRAILR2-binding Tn3. Bivalent 1C12-Fc and tetraivalent 1C12-Ig showed modest inhibition of H2122 cell viability, whereas control proteins that do not bind TRAILR2 (D1-Fc and D1-Ig) had no effect.

tion of TRAILR2-dependent apoptosis (Fig. 1B). In this assay, 1C12-Ig exhibited greater activity, presumably due to its higher valency enabling greater clustering of cell surface TRAILR2. By contrast, neither of the control Tn3 fusion proteins, D1-Fc and D1-Ig, had any effect on H2122 cell viability. Relative to TRAIL, the activity of the 1C12 fusion constructs was modest and several orders of magnitude less potent.

Affinity maturation of a TRAILR2-specific Tn3

In an effort to improve the bioactivity of 1C12-Ig, we set out to enhance the TRAILR2 binding affinity of the 1C12 Tn3 module. A series of phage-displayed affinity maturation libraries were generated, in which each of the variable CDR-like loops in 1C12 was selectively mutagenized. This process was applied iteratively over several stages, with the newly optimized sequence after each stage providing the template for further rounds of library generation and binding selections. The affinity matured variants were expressed in *E. coli* as monomeric proteins, purified to homogeneity, and their TRAILR2-binding affinities were determined by surface plasmon resonance (Table 1). Sequence changes accumulated in each of the surface binding loops of 1C12 resulted in up to 100-fold affinity improvement. The highest affinity clone, G6, contained 15 substitutions relative to 1C12, and bound TRAILR2 with an equilibrium dissociation constant (K_d) of 43 nmol/L.

Table 1. Sequences and TRAILR2 binding affinities of Tn3 variants

Clone	Sequence			K_d (nmol/L)	K_d 1C12/ K_d mutant
	BC loop	DE loop	FG loop		
Wild-type	FKPLAEIDG	TEDENQ	RRGDMSSNPA		
1C12	AKPEKWDGSIYG	NSRHTA	FTPYGAKSNPA	4130 ± 281	
G3	AKPEKWD <u>PP</u> LWG	NSRHTA	FTPYGAKSNPA	422 ± 45	10
1E11	AKPW <u>VD</u> PPPLWG	<u>QQ</u> KHTA	<u>FD</u> PYGAKSNPA	103 ± 9	40
C4	AKPW <u>VD</u> PPPLWG	<u>QQ</u> KHTA	<u>FD</u> PYNKRNVPA	50 ± 2	83
G6	AKPW <u>VD</u> PPPLWG	<u>QQ</u> KHTA	<u>FD</u> PYGMRS <u>K</u> PA	43 ± 2	96
D1	SPGERIWMFTG	TEDENQ	PNYERISNPA	no binding	

NOTE: Loop sequences are shown for each Tn3 clone. 1C12 and its derivatives (G3, 1E11, C4, and G6) contained a Thr → Ala point mutation within the framework at position 29 (see Materials and Methods for Tn3 sequence numbering). Underlined sequence denotes changes relative to 1C12 for affinity optimized variants. Clone D1 is a control Tn3 that binds to the antibody palivizumab.

Improved binding affinity leads to greater agonistic activity

To investigate the effect of Tn3 affinity on TRAILR2 agonistic activity, the panel of clones in Table 1 was expressed in HEK293F cells and purified as 2 chain, tetravalent Tn3-Ig fusion proteins. H2122 cells were treated with each Tn3-Ig construct or soluble TRAIL, and cell viability was assayed after 72 hours. Increases in TRAILR2-binding affinity led to enhanced potency of Tn3-Ig proteins and increases to the maximum level of cell viability inhibition (Fig. 2). Inhibition of cell viability was associated with morphologic changes typical of TRAILR2-induced apoptosis, such as membrane blebbing, cell rounding, and detachment from the culture plate. By contrast, the nonbinding D1-Ig–negative control had no effect on cell viability or morphology. This showed that TRAILR2 binding was essential for cytotoxicity and that changes in affinity, for the most part, correlated with changes in bioactivity. Despite these improvements to bioactivity, the most potent sample (G6-Ig; EC_{50} = 3.2 nmol/L; max. inhibition 76%) was considerably less active than soluble TRAIL (EC_{50} = 0.080 nmol/L; max. inhibition 98%). This led us to consider additional strategies by which the agonistic activity might be improved.

Tandem Tn3 fusion proteins exhibit enhanced agonistic activity

We next considered alternative molecular formats that were not based on fusion to antibody fragments for generating multivalent constructs. Given the natural occurrence of proteins containing tandem repeats of Fn3 domains, we reasoned that linear fusion of Tn3 modules, like beads on a string, could provide a flexible platform for generating multivalent-binding proteins. This format was also attractive for the ease in which valency could be tuned by adjusting the number of Tn3 repeats. However, we were unsure if such proteins would express and fold correctly, or retain target-binding activity, as N-terminal fusion to Fn3-based scaffolds can hinder target binding in some cases (27).

A series of tandem 1E11 expression constructs were generated, coding for fusion of 2, 4, 6, or 8 modules of 1E11, each separated by Gly₄-Ser-containing linkers (1E11T2, 1E11T4, 1E11T6, and 1E11T8). When expressed in *E. coli*, the single 1E11 module was secreted at high level and accumulated in the culture media. To our surprise, tandem 1E11 fusion proteins were also expressed at high levels in *E. coli*, and could similarly leach into the culture

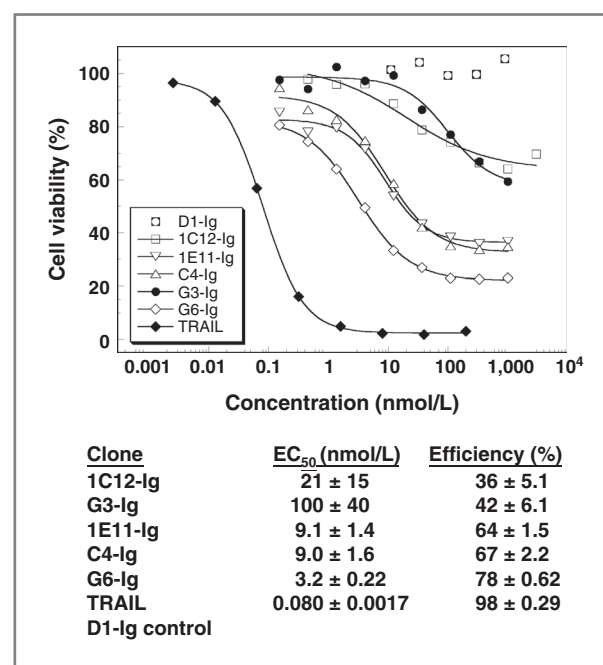


Figure 2. TRAILR2 affinity correlates with enhanced tumor cell line activity. Tetravalent Tn3-Ig fusion proteins containing 1C12, its affinity matured derivatives, or the non-TRAILR2-binding clone D1, were tested for their ability to inhibit the viability of TRAILR2-expressing H2122 cells. Soluble TRAIL was also tested. For each construct, the viability of treated cells was assessed after 72 hours by CellTiter-Glo. Increases in the affinity of Tn3 binding to TRAILR2 correlated with increases in potency, as defined by EC_{50} (molar concentration of agonist required to obtain half-maximal inhibition) or efficiency (maximal level of cell viability inhibition).

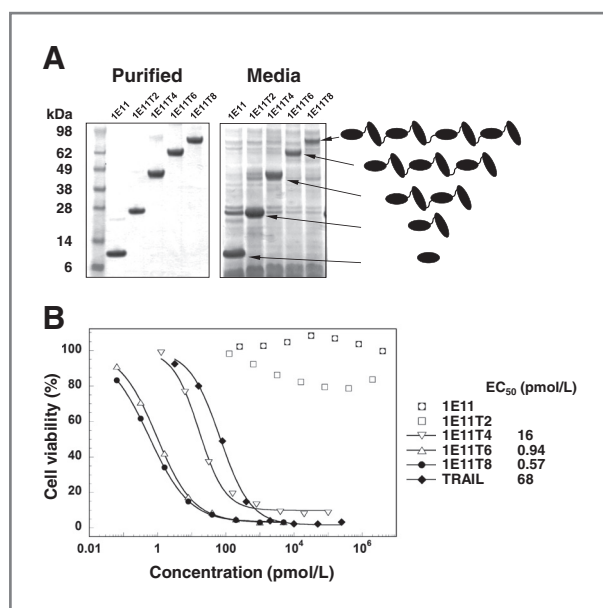


Figure 3. Expression and characterization of TRAILR2-specific tandem Tn3 constructs. **A**, recombinant expression in *E. coli*. Nonreducing SDS-PAGE analysis of crude bacterial media (right gel) and affinity purified samples (left gel) for tandem proteins containing repeats of the 1E11 Tn3 module. The cartoon schematic shows the number of Tn3 units, connected by flexible linkers, in each tandem construct. **B**, inhibition of H2122 cell viability by TRAILR2-specific tandem Tn3 proteins. 1E11, or tandem proteins containing 2, 4, 6, or 8 repeats of this module were incubated with H2122 cells for 72 hours, after which cell viability was assayed. Soluble TRAIL was also tested. Mono- and bivalent 1E11 had little or no effect on cell viability, but tetra-, hexa-, and octavalent proteins were highly cytotoxic and significantly more potent than soluble TRAIL. EC₅₀ values represent the concentration of test agent that reduced cell viability by 50%.

media (Fig. 3A). Following Ni-chelating chromatography, purified protein yields ranged from 400 mg/L for monovalent 1E11 down to 40 mg/L for 1E11T8. Intermediate yields were obtained for the remaining constructs. Analytical SEC revealed low to moderate levels of high molecular weight aggregate for each of the purified constructs (<20% of total protein), thus, aggregate-free samples were generated by preparative SEC of Ni-affinity purified material.

Tandem Tn3 fusion proteins are potent TRAILR2 agonists

We assessed the ability of tandem 1E11 proteins to activate cellular TRAILR2, by incubating H2122 cells with constructs containing 1, 2, 4, 6, and 8 copies of 1E11 (Fig. 3B). Before cellular analysis, analytical SEC characterization was carried out to confirm that all samples contained more than 98% monomeric content. As expected, monovalent 1E11, which cannot crosslink cellular TRAILR2, did not inhibit the growth of H2122 cells. Bivalent 1E11T2 had a marginal effect on cell viability, but further increases in valency resulted in a dramatic induction of cellular activity. Tetraivalent 1E11T4 potently inhibited cell viability, with half maximal inhibition (EC₅₀) at 13 pmol/L. This

represents a remarkable 1,000-fold increase in bioactivity over the corresponding antibody-like molecule 1E11-Ig, despite the equivalent valency for these 2 constructs. 1E11T4 also achieved a higher level of maximum cell viability inhibition in comparison with 1E11-Ig. Further increases in activity were observed for 1E11T6 and 1E11T8. Both of these constructs inhibited H2122 cell viability at subpicomolar concentrations, could achieve near 100% inhibition at saturating doses, and were considerably more potent than soluble TRAIL. An octavalent tandem Tn3 control protein that does not bind TRAILR2, D1T8, had no effect on H2122 cell viability at concentrations up to 2 μmol/L (data not shown).

TRAILR2-specific tandem Tn3 maintain superagonist activity across multiple tumor cell lines

To further characterize TRAILR2 agonistic activity, we evaluated tandem Tn3 proteins against a broader panel of cancer cell lines. Given the superior cellular activity of G6-Ig over 1E11-Ig, tandem proteins G6T4 and G6T8 (containing 4 and 8 repeats of G6) were prepared and tested for cytotoxicity across the TRAIL-sensitive cell lines H2122, Colo205, Jurkat, and H460. Across each of these cell lines, G6T8 exhibited low or subpicomolar activity and was 1 to 2 orders of magnitude more potent than TRAIL (Fig. 4A). G6T4 was less potent, but similar to TRAIL across the different cell lines. Apoptotic morphologic changes such as cell shrinkage, rounding, and membrane blebbing were observed for G6T8, G6T4, and TRAIL-treated cells, but not the negative control D1T8. These data have shown that G6T8 had ultra potent TRAILR2 agonistic activity that is well in excess of soluble TRAIL, regardless of the cell line.

To verify that cytotoxicity was dependent on binding to cellular TRAILR2, the ability of soluble TRAILR2-Fc fusion protein to block the cytotoxic effects of G6T8 was evaluated. When incubated with 100 pmol/L G6T8, H2122 cell death was inhibited in a dose-responsive manner by co-incubation with soluble TRAILR2-Fc (Fig. 4B). Inhibition of G6T8-induced cytotoxicity by soluble TRAILR2-Fc protein was also observed for other cell lines (Supplementary Fig. S2). We also evaluated whether G6T8 could bind to other members of the TRAIL receptor family by ELISA, but found that binding was restricted to TRAILR2 (Supplementary Fig. S3). Together, these data support that cytotoxicity is due to specific TRAILR2 binding on cells. To confirm that cytotoxicity was linked to the activation of apoptotic pathways, executioner caspase activity was assayed following treatment of H2122 cells with G6T8, TRAIL, or the negative control D1T8 (Fig. 4C). As expected for TRAILR2 signaling, robust activation of caspases 3 and 7 was observed for cells treated with G6T8 and TRAIL, but not D1T8. Consistent with results from cell viability assays, G6T8 was more potent in activating caspase activity than TRAIL. Similar results were seen with other TRAIL-sensitive cell lines (Supplementary Fig. S4). Together, these results support that cell death was initiated by TRAILR2 activation, and not an alternative pathway.

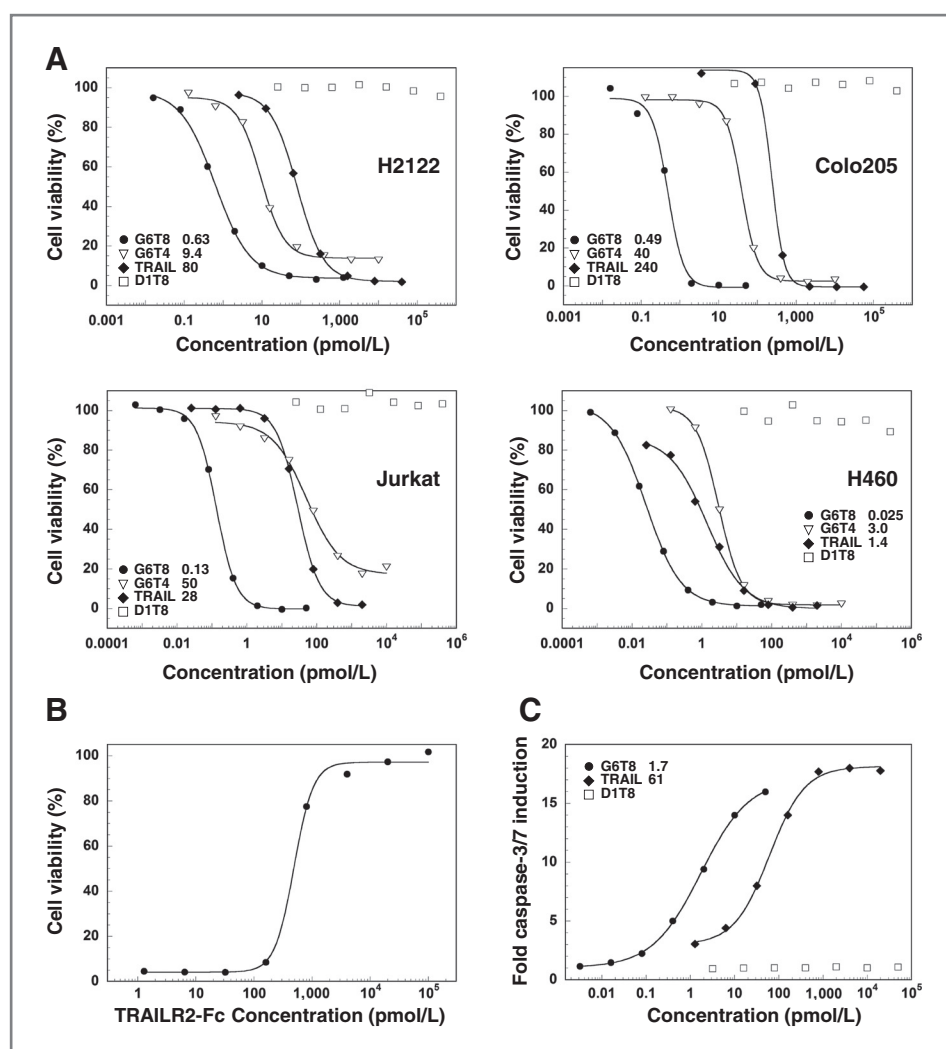


Figure 4. Characterization of cytotoxic activity of TRAILR2-specific multivalent tandem Tn3 proteins. A, killing of multiple TRAILR2-expressing tumor cell lines. TRAIL-sensitive tumor cell lines were treated with TRAILR2-specific tandem Tn3 proteins containing either 4- or 8-repeats of the G6 module (G6T4, G6T8), with soluble TRAIL protein or with a negative control protein containing 8-repeats of a nonbinding Tn3 (D1T8). EC₅₀ values (pmol/L) for inhibition of cell viability are shown within each graph. B, specificity of TRAILR2-induced killing. H2122 cells were incubated with 100 pmol/L G6T8 and different concentrations of soluble TRAILR2-Fc fusion protein. Inhibition of cell death by soluble TRAILR2-Fc is consistent with G6T8 cytotoxicity being dependent on binding to cellular TRAILR2. C, caspase activation by G6T8. H2122 cells were treated with G6T8, soluble TRAIL, or the negative control D1T8 for 6 hours, after which caspase 3 and 7 activity was measured in cell lysates. Data are reported as induction of caspase activity, that is, the ratio of activity in treated versus untreated cells. Concentrations of G6T8 and TRAIL (pmol/L) that resulted in half-maximal caspase induction are shown within the graph.

While G6T8 exhibited impressive *in vitro* activity against TRAIL-sensitive cell lines, many TRAILR2-expressing tumor cell lines are resistant to TRAIL-induced apoptosis. To evaluate whether such cell lines are sensitive to G6T8, we tested its effect on a set of 6 human hepatocellular carcinoma cell lines that express TRAILR2 but are resistant to TRAIL-mediated killing (28). Exposure to high concentrations of TRAIL for 3 days had no effect on the viability of Hep3B, SNU-398, and SNU-387 cells, and a modest effect on HepG2 cells (Fig. 5). By contrast, Hep3B and HepG2 cell viability was quite sensitive to G6T8 treatment, SNU-387 cells were partially sensitive, but SNU-398 cells were resistant. These data show that G6T8 is effective against some TRAIL-resistant tumor cell lines. The insensitivity of one cell line in this set also shows that G6T8 is not cytotoxic against all TRAILR2-expressing cells.

***In vivo* Colo205 xenograft study**

To determine the *in vivo* activity of a tandem Tn3 TRAILR2 agonist, we selected G6T6 containing 6 modules

of the G6 Tn3. This molecule was selected given its comparable *in vitro* activity with G6T8 (Supplementary Fig. S5), but with higher recombinant expression yield, which aided the scale up production to support animal studies. Importantly for studies in mice, G6T6 was specific for human TRAILR2 and no binding to mouse TRAILR was detected by ELISA (data not shown), consistent with the result for G6T8 (Supplementary Fig. S3). Accordingly, this molecule would not be expected to activate endogenous TRAILR2 in mice.

The *in vivo* half-life of G6T6 in mice was evaluated by assaying the concentration in serum collected from mice that received a single 5 mg/kg intravenous injection. Interestingly, despite the large size of this protein (62 kDa), G6T6 was cleared rapidly from circulation, with a serum half-life on the order of 0.6 hours (data not shown). This half-life is longer than the 4-minute half-life reported for human TRAIL in mice (29), but much shorter than the half-life of TRAILR2 agonistic antibodies.

The *in vivo* antitumor activity of G6T6 was then compared with TRAIL in a xenograft study using Colo205

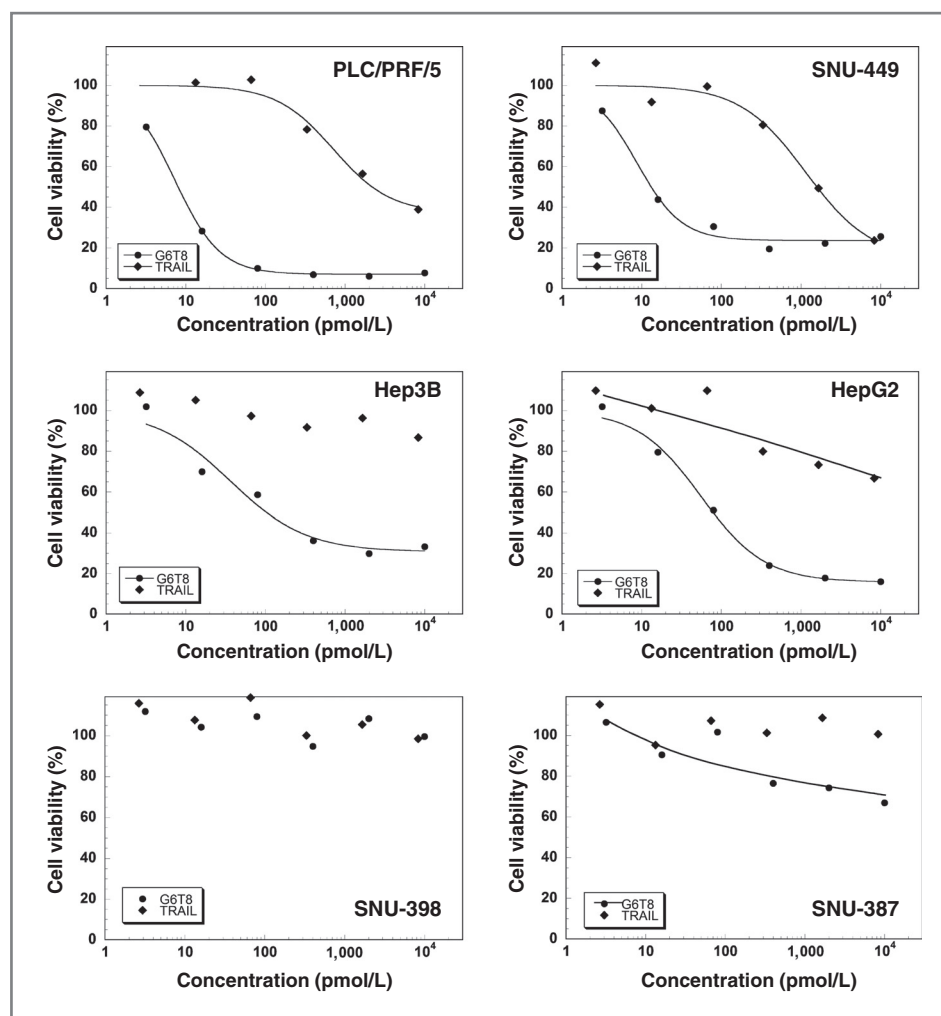


Figure 5. Activity of TRAILR2-specific tandem Tn3 protein on TRAIL-resistant HCC cell lines. Cell lines were treated with G6T8 or soluble TRAIL for 72 hours, before assessment of cell viability by CellTiter-Glo.

cells. In this study (Fig. 6A), athymic mice were administered 30 mg/kg of TRAIL or G6T6 intravenously, daily for a total of 5 doses. This dose level and frequency was selected to match that used in a previous study of TRAIL (29). While 30 mg/kg TRAIL resulted in modest tumor growth delay, the equivalent dose of 30 mg/kg G6T6 resulted in complete regression of tumors in all mice by day 45. G6T6 was well tolerated at this dose level and did not lead to decreases in body weight, though as noted previously, G6T6 would not be expected to activate endogenous mouse TRAIL receptor. In a second xenograft study, we further explored the potency of G6T6 by examining lower doses of 3 mg/kg and 0.3 mg/kg (Fig. 6B). At the dose of 3 mg/kg, tumors regressed completely in all mice by day 47 of the study. Even at the low dose of 0.3 mg/kg of G6T6, 90% of mice showed partial tumor regression. These impressive data showed that the superagonist activity observed *in vitro* translated into ultra potent *in vivo* proapoptotic activity that can eliminate TRAIL-sensitive tumors in a xenograft setting.

Discussion

Despite the preclinical promise of TRAILR2 agonists, clinical trial results in cancer patients has thus far been disappointing (30, 31). The reasons for this are not understood, though it is known that many TRAILR2-expressing tumor cell lines and primary tumor cells exhibit a TRAIL-resistant phenotype. Considerable efforts to identify synergistic agents has led to the identification of a myriad of anticancer drugs that can augment the antitumor effect of TRAIL agonists in preclinical studies (32–35). However, as with results from monotherapy trials, recent phase II trials of TRAILR-agonists combined with cytotoxic chemotherapy suggest that the addition of TRAILR-targeted drug does not improve outcomes compared with standard treatment alone (36). Clinical studies are ongoing, but based on results thus far, it may be that the biology of TRAILR2 is different in *in vitro* model systems compared with human cancers, or current agonists lack sufficient potency to achieve a positive therapeutic outcome. To address the second possibility, there is a need for new agents that can activate the TRAILR2

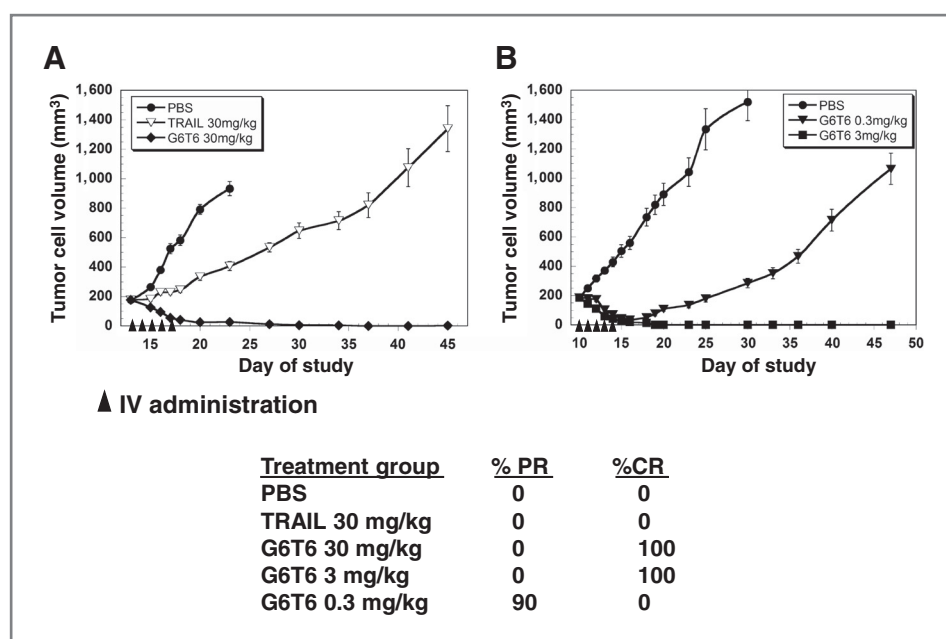


Figure 6. Antitumor activity of TRAILR2-specific tandem Tn3 protein in a Colo205 xenograft model. Tumors were allowed to grow to a mean tumor volume of approximately 180 mm³ before injection of test agent. Treatments were administered once per day for 5 days at the indicated doses, with 10 mice per treatment group. A, treatment with 30 mg/kg TRAIL slowed tumor growth relative to PBS-treated mice, but treatment with 30 mg/kg G6T6 resulted in complete tumor regression in all mice. B, low-dose treatment with G6T6. Colo205 tumor-bearing mice were treated with PBS or G6T6 at a dose of 3 or 0.3 mg/kg. Complete tumor regression was again observed for all mice treated at 3 mg/kg. Significant tumor shrinkage was observed in mice treated at 0.3 mg/kg, but tumors began to regrow after cessation of treatment.

pathway with greater potency than existing agonistic therapies.

In this study, we generated a new class of TRAILR2 agonists based on multivalent presentation of TRAILR2-binding Tn3 scaffold proteins. The effects of affinity, molecular format, and multivalency were evaluated for their contribution to agonistic activity. Each variable had considerable impact on activity. Affinity was evaluated in the context of tetravalent antibody-like Tn3-Ig fusion proteins. Here, improvements in binding affinity for the component Tn3 monomer correlated with enhanced agonism. Surprisingly, changes to molecular format exerted a more dramatic effect on agonistic activity than changes in Tn3-binding affinity. A change in the tetravalent multimerization platform, from an antibody-like construct to tandem fusion of 4 Tn3 modules, led to a 1,000-fold enhancement of TRAILR2 agonistic activity. This may indicate that linear fusion provides greater flexibility for individual Tn3 units to productively engage TRAILR2, in contrast to the antibody-like format where the Fc and/or Fab-like arms may impose steric constraints on how TRAILR2 is recruited at a cell surface.

Consistent with studies on multimerization of soluble TRAIL trimer (11, 12), we found that increasing the TRAILR2-binding valency had a dramatic effect on agonistic activity. The modular tandem Tn3 fusion format enabled us to explore the activity–valency relationship for a series of multivalent TRAILR2-binding proteins. Using H2122 cells as a model TRAIL-sensitive cell line and 1E11 as the TRAILR2-specific Tn3, we found that mono- and bivalent 1E11 constructs had little or no agonistic activity, but the tetravalent protein induced apoptosis at low picomolar concentrations. We did not produce trivalent 1E11 (to simplify the number of constructs generated), but these data show a rapid onset of agonist activity as the

valency increases from 2 to 4. Further enhancements to activity were observed for hexa- and octavalent tandem constructs, but the small difference between these 2 suggests that valency-driven effects become saturated at this level of receptor clustering. These observations are important for a number of reasons. First, it suggests there may be an upper limit beyond which increases in valency will have negligible impact on agonistic activity. Conversely, at low valency, an increase in the number of TRAILR2-binding units can have a very dramatic effect on activity. While these data are specific to TRAILR2, we believe that artificial ligands against other TNFR-family members could share similar activity–valency relationships, though onset and saturation of signaling as a function of valency may differ for each receptor, and be further modified according to other factors such as binding affinities and epitopes.

The anticancer potential of TRAILR2 agonists, as well as activators of other TNFR-family members, are currently being explored in a number of clinical trials (2, 37). Most of these agents are monoclonal antibodies in preference to the natural ligands, largely due to superior drug-like properties for antibodies such as pharmacokinetic behavior, stability, and manufacturability. However, IgG molecules are bivalent, and as shown in this study, higher valencies and nonantibody molecular formats can lead to vastly superior activation of TRAILR2-mediated apoptosis. This explains why some agonistic antibodies against TRAILR2 show low or no *in vitro* activity in the absence of higher-order cross-linking and are less active than soluble TRAIL trimer (7, 38, 39). Despite the fact that such antibodies have lower valency, they can exhibit antitumor activity in murine xenograft studies (7, 8, 39). A recent study with drozitumab, an agonistic TRAILR2 mAb, showed that binding to Fcγ receptors was essential to the

antitumor activity in mice (10). The *in vivo* activity of agonistic antibodies against a number of TNFR family members in certain animal models has now been shown to depend on binding to Fc γ receptors, particularly the inhibitory Fc γ RIIB receptor (10, 40–42). Although the mechanism of Fc γ R-dependence remains to be fully elucidated, it is possible that Fc γ receptor engagement facilitates higher-order presentation of agonistic antibodies on the surface of immune cells, and that this enables productive engagement of the target TNFR family member.

In contrast to agonistic antibodies, the highly potent *in vitro* and *in vivo* activity observed with multivalent Tn3-based TRAILR2 agonists was independent of any requirement for higher-order crosslinking or Fc receptor binding. This has advantages for therapeutic applications, as the *in vivo* activity would not be effected by polymorphisms of FcR (10, 43), nor require a contribution from immune cells, the function of which may be impaired in cancer patients (44). On the flip side, the short *in vivo* half-life of tandem Tn3 proteins is a limitation, however, this could be modified through further engineering, or may not be required for an agonistic therapy. For these reasons, we believe that tandem Tn3 proteins hold promise as an attractive alternative to antibodies for therapeutic activation of TRAILR2, and that this technology could be extended to the development of therapeutic agonists of other TNFR family members.

References

- Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997;277:815–8.
- Abdulghani J, El-Deiry WS. TRAIL receptor signaling and therapeutics. *Expert Opin Ther Targets* 2010;14:1091–108.
- Forero-Torres A, Shah J, Wood T, Posey J, Carlisle R, Copigneaux C, et al. Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). *Cancer Biother Radiopharm* 2010;25:13–9.
- Herbst RS, Eckhardt SG, Kurzrock R, Ebbinghaus S, O'Dwyer PJ, Gordon MS, et al. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol* 2010;28:2839–46.
- Kaplan-Lefko PJ, Graves JD, Zoog SJ, Pan Y, Wall J, Branstetter DG, et al. Conatumumab, a fully human agonist antibody to death receptor 5, induces apoptosis via caspase activation in multiple tumor types. *Cancer Biol Ther* 2010;9:618–31.
- Camidge DR, Herbst RS, Gordon MS, Eckhardt SG, Kurzrock R, Durbin B, et al. A phase I safety and pharmacokinetic study of the death receptor 5 agonistic antibody PRO95780 in patients with advanced malignancies. *Clin Cancer Res* 2010;16:1256–63.
- Yada A, Yazawa M, Ishida S, Yoshida H, Ichikawa K, Kurakata S, et al. A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. *Ann Oncol* 2008;19:1060–7.
- Adams C, Totpal K, Lawrence D, Marsters S, Pitti R, Yee S, et al. Structural and functional analysis of the interaction between the agonistic monoclonal antibody Apomab and the proapoptotic receptor DR5. *Cell Death Differ* 2008;15:751–61.
- Miller K, Meng G, Liu J, Hurst A, Hsei V, Wong WL, et al. Design, construction, and *in vitro* analyses of multivalent antibodies. *J Immunol* 2003;170:4854–61.
- Wilson NS, Yang B, Yang A, Loeser S, Marsters S, Lawrence D, et al. An Fc γ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* 2011;19:101–13.
- Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, Deforge L, et al. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. *J Biol Chem* 2005;280:2205–12.
- Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A, et al. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med* 1998;187:1205–13.
- Haswell LE, Glennie MJ, Al-Shamkhani A. Analysis of the oligomeric requirement for signaling by CD40 using soluble multimeric forms of its ligand, CD154. *Eur J Immunol* 2001;31:3094–100.
- Holler N, Tardivel A, Kovacsics-Bankowski M, Hertig S, Gaide O, Martinon F, et al. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol* 2003;23:1428–40.
- Mackay F, Schneider P. Cracking the BAFF code. *Nat Rev Immunol* 2009;9:491–502.
- Zhang G. Tumor necrosis factor family ligand-receptor binding. *Curr Opin Struct Biol* 2004;14:154–60.
- O'Reilly LA, Tai L, Lee L, Kruse EA, Grabow S, Fairlie WD, et al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 2009;461:659–63.
- Bossen C, Cachero TG, Tardivel A, Ingold K, Willen L, Dobles M, et al. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood* 2008;111:1004–12.
- Kimberley FC, van Bostelen L, Cameron K, Hardenberg G, Marquart JA, Hahne M, et al. The proteoglycan (heparan sulfate proteoglycan)

Disclosure of Potential Conflicts of Interest

Z. Xiao, D.A. Tice, and M. Baca have ownership interests (including patents) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.S. Swers, Z. Xiao, H. Wu, D.A. Tice, M. Baca
Development of methodology: J.S. Swers, L. Wang, M. Baca
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Swers, L. Grinberg, H. Feng, I. Inigo, C.C. Leow
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.S. Swers, L. Wang, C.C. Leow, D.A. Tice, M. Baca
Writing, review, and/or revision of the manuscript: J.S. Swers, D.A. Tice, M. Baca
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.S. Swers, K. Lekstrom, R. Carrasco
Study supervision: Z. Xiao, H. Wu, D.A. Tice, M. Baca

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- binding domain of APRIL serves as a platform for ligand multimerization and cross-linking. *FASEB J* 2009;23:1584–95.
20. Li B, Russell SJ, Compaan DM, Totpal K, Marsters SA, Ashkenazi A, et al. Activation of the proapoptotic death receptor DR5 by oligomeric peptide and antibody agonists. *J Mol Biol* 2006;361:522–36.
 21. Pavet V, Beyrath J, Pardin C, Morizot A, Lechner MC, Briand JP, et al. Multivalent DR5 peptides activate the TRAIL death pathway and exert tumoricidal activity. *Cancer Res* 2010;70:1101–10.
 22. Lee CH, Park KJ, Sung ES, Kim A, Choi JD, Kim JS, et al. Engineering of a human kringle domain into agonistic and antagonistic binding proteins functioning *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2010;107:9567–71.
 23. Allen JE, Ferrini R, Dicker DT, Batzer G, Chen E, Oltean DI, et al. Targeting TRAIL death receptor 4 with trivalent DR4 Atrimer complexes. *Mol Cancer Ther* 2012;11:2087–95.
 24. Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, et al. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* 2000;39:633–40.
 25. Koide A, Bailey CW, Huang X, Koide S. The fibronectin type III domain as a scaffold for novel binding proteins. *J Mol Biol* 1998;284:1141–51.
 26. Leahy DJ, Hendrickson WA, Aukhil I, Erickson HP. Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. *Science* 1992;258:987–91.
 27. Getmanova EV, Chen Y, Bloom L, Gokemeijer J, Shamah S, Warikoo V, et al. Antagonists to human and mouse vascular endothelial growth factor receptor 2 generated by directed protein evolution *in vitro*. *Chem Biol* 2006;13:549–56.
 28. Shin EC, Seong YR, Kim CH, Kim H, Ahn YS, Kim K, et al. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. *Exp Mol Med* 2002;34:114–22.
 29. Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of *in vivo* efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* 2001;299:31–8.
 30. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene* 2013;32:1341–50.
 31. den Hollander MW, Gietema JA, de Jong S, Walenkamp AM, Reyners AK, Oldenhuis CN, et al. Translating TRAIL-receptor targeting agents to the clinic. *Cancer Lett* 2013;332:194–201.
 32. Ashkenazi A, Herbst RS. To kill a tumor cell: the potential of proapoptotic receptor agonists. *J Clin Invest* 2008;118:1979–90.
 33. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* 2008;8:782–98.
 34. Shankar S, Srivastava RK. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. *Drug Resist Updat* 2004;7:139–56.
 35. Thorburn A, Behbakht K, Ford H. TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them. *Drug Resist Updat* 2008;11:17–24.
 36. Soria JC, Mark Z, Zatloukal P, Szima B, Albert I, Juhasz E, et al. Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer. *J Clin Oncol* 2011;29:4442–51.
 37. Grewal IS. Overview of TNF superfamily: a chest full of potential therapeutic targets. *Adv Exp Med Biol* 2009;647:1–7.
 38. Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, et al. Functional analysis of TRAIL receptors using monoclonal antibodies. *J Immunol* 1999;162:2597–605.
 39. Li J, Knee DA, Wang Y, Zhang Q, Johnson JA, Cheng J, et al. LB135, a novel anti-DR5 agonistic antibody induces tumor cell-specific cytotoxic activity in human colon tumor cell lines and xenografts. *Drug Dev Res* 2008;69:69–82.
 40. Li F, Ravetch JV. Inhibitory Fc γ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science* 2011;333:1030–4.
 41. Xu Y, Szalai AJ, Zhou T, Zinn KR, Chaudhuri TR, Li X, et al. Fc γ Rs modulate cytotoxicity of anti-Fas antibodies: implications for agonistic antibody-based therapeutics. *J Immunol* 2003;171:562–8.
 42. Haynes NM, Hawkins ED, Li M, McLaughlin NM, Hammerling GJ, Schwendener R, et al. CD11c+ dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors. *J Immunol* 2010;185:532–41.
 43. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003;21:3940–7.
 44. Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Semin Cancer Biol* 2006;16:3–15.