Treating Tissue Factor–Positive Cancers With Antibody-Drug Conjugates That Do Not Affect Blood Clotting

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Abstract:

The primary function of Tissue Factor (TF) resides in the vasculature as a cofactor of blood clotting; however, multiple solid tumors aberrantly express this transmembrane receptor on the cell surface. Here, we developed anti-TF antibody-drug conjugates (ADCs) that did not interfere with the coagulation cascade and benchmarked them against previously developed anti-TF ADCs. After screening an affinity-matured antibody panel of diverse paratopes and affinities, we identified one primary paratope family that did not inhibit conversion of Factor X (FX) to activated Factor X (FXa) and did not affect conversion of prothrombin to thrombin. The rest of the antibody panel and previously developed anti-TF antibodies were found to perturb coagulation to varying degrees. To compare the anti-cancer activity of coagulation-inert and inhibitory antibodies as ADCs, a selection of antibodies was conjugated to the prototypic cytotoxic agent monomethyl auristatin E (MMAE) through a protease-cleavable linker. The coagulation-inert and inhibitory anti-TF ADCs both killed cancer cells effectively. Importantly, the coagulation-inert ADCs were as efficacious as tisotumab vedotin, a clinical stage ADC that affected blood clotting, including in patient-derived xenografts from three solid tumor indications with a need for new therapeutic treatments—squamous cell carcinoma of the Head and Neck (SCCHN), ovarian and gastric adenocarcinoma. Furthermore, a subset of the anti-TF antibodies could also be considered for the treatment of other diseases associated with upregulation of membranous TF expression, such as macular degeneration.
Introduction

An emerging class of anticancer treatments consists of ADCs. These agents combine the specificity of an antibody against a membrane-associated protein with the cytotoxicity of a chemotherapeutic compound. With four ADCs—ado-trastuzumab emtansine, brentuximab vedotin, inotuzumab ozogamicin and gemtuzumab ozogamicin—approved for treatment (1-3), more than fifty ADCs are at various stages of clinical development (2,4). Emerging clinical data suggests that a subset of these ADCs have a relatively narrow therapeutic window (5,6). Novel linkers and payloads are anticipated to widen this window (4,7,8). Antibodies that do not impact the normal function of the target could also improve the safety and tolerability of the ADC (6).

For example, the anti-EGFR ADCs depatuximab mafodotin and ABBV-221 target a tumor-selective EGFR epitope, with low normal tissue binding (9). Furthermore, improvements in antibody technology enable isolation of candidate antibodies with the desired binding affinity, species cross-reactivity, cellular internalization, and biological activity or lack thereof (10).

Tissue Factor (TF; also known as coagulation factor III (F3), thromboplastin, CD142) is a promising ADC target. Under normal physiological conditions, membranous TF expression is restricted (11,12). Vascular injury results in exposure of cell surface TF to blood. Once Factor VII (FVII) binds TF, limited proteolysis by plasma proteases results in conversion of FVII into activated FVII (FVIIa). The TF:FVIIa complex subsequently binds and converts FX into its activated form, FXa. Cleavage of prothrombin into thrombin by FXa in association with FVa and divalent calcium is followed by platelet activation and thrombin-mediated cleavage of fibrinogen to fibrin (11).

In contrast to restricted surface expression in normal tissues, TF exhibits membranous TF expression on multiple solid tumor indications (13). Surface TF is expressed in gastrointestinal cancers, urogenital cancers, gliomas, melanomas, lung cancer and breast cancer (13). While TF has not been identified as an oncogene, its expression is regulated by hypoxia in the tumor microenvironment and various cancer mutations, such as oncogenic Ras and loss of PTEN (13,14). In cancer cells, co-expression of TF with its ligand, FVII, and its signaling receptors, protease activated receptors (PARs), can promote tumor growth by inducing immune-modulating and pro-angiogenic cytokines (14). Aside from its surface expression in cancer, TF undergoes efficient internalization and lysosomal targeting (15,16), enabling efficient delivery of ADCs (15).

TF-specific ADCs from three different preclinical studies have been reported to be efficacious in cancer xenografts (17-19). The ADC from the first preclinical study, tisotumab vedotin (17), has advanced into a Phase IIa trial, with a clinical response in one third of patients with cervical cancer (20). However, adverse events associated with perturbation of coagulation were detected (20,21). The antibody moiety of tisotumab vedotin competed directly with FVII for binding to TF and had a modest impact in the selected in vitro coagulation assays (17). In the second preclinical study, the TF-specific antibody SC1, conjugated to MMAE or the maytansinoid DM1, had anticancer activity, but also inhibited coagulation (19). The TF-specific antibody used in the third preclinical study of an ADC interfered with clotting by inhibiting binding of FVII to TF (18,22).

In this study, we set out to develop a TF-specific ADC with no impact on the coagulation cascade. An antibody panel with diverse paratopes and affinities was evaluated for perturbation of coagulation and anti-cancer activity. One paratope family of antibodies in particular did not...
affect blood clotting, and when conjugated to MMAE showed anticancer efficacy comparable to tisotumab vedotin.

**Materials and Methods**

**Proteins**

Human and cynomolgus monkey TF extracellular domain (ECD) fragments were expressed as C-terminal His or Fc-gamma fragment fusions using recommended procedures (ThermoFisher Scientific, Waltham, MA, USA). Details are provided in the Supplementary Materials and Methods section. The sequence identity between the extracellular domains of human and cynomolgus monkey TF is 94.5%.

**Antibodies**

Human antibodies against human TF were isolated from a full-length human IgG library using an *in vitro* yeast selection system and associated methods (23). A synthetic naïve human antibody library of ~10^{10} in diversity, was designed, generated in yeast, and propagated as described previously (23,24). TF-binding mAbs were enriched by incubating biotinylated TF-His monomers at different concentrations with antibody expressing yeast cells followed by magnetic bead selection (Miltenyi Biotec, Auburn CA) and fluorescence-activated cell sorting on a FACSIAria II cell sorter (BD Biosciences, San Jose CA) using streptavidin secondary reagents in several successive selection rounds. After the last round of enrichment, yeast cells were plated onto agar plates, clones were analyzed by DNA sequencing and used for IgG production. Heavy chains from the naïve outputs were used to prepare light chain diversification libraries, which were then used for additional selection rounds. In particular, heavy chains were extracted from the fourth naïve selection round outputs and transformed into a light chain library to create new libraries ~10^8 in diversity. Six human TF-specific antibodies (mAb 1, 25, 29, 39, 43 and 54) were prioritized for affinity maturation in two phases: additional diversification of the heavy chain followed by additional diversification of the light chain. Further diversification of the heavy chain was accomplished by diversification of CDR-H1 and CDR-H2, and diversification of CDR-H3 following CDR-H1 and CDR-H2 diversity pool optimization for improved binding affinity. CDR-H1 and CDR-H2 were diversified with a premade library with CDR-H1 and CDR-H2 variants of a diversity of ~10^8. CDR-H3 was diversified by constructing a library with oligonucleotide-based NNK degeneracy across the entire CDR-H3. Affinity-matured antibodies that underwent the first phase of affinity maturation contain a number and letter in their identifiers, and the number of V_{H} CDR amino acid changes in each antibody relative to the parent antibody is listed in Supplementary Table S1A. Further diversification of the light chain was accomplished by diversification of CDR-L3 within CDR-L1 and CDR-L2 diversity pools selected for improvements in binding affinity. CDR-L3 diversification was accomplished by constructing a library with oligonucleotide-based NNK degeneracy across the entire CDR-L3. The antibodies that underwent this second phase of affinity maturation contain an additional number at the end of their identifiers, and the number of V_{L} CDR amino acid changes in each antibody relative to the parent antibody is listed in Supplementary Table S1B. While the screening campaign was conducted with yeast-derived antibody material (Fig. 1), Expi293-derived antibody material was used for the remainder of this study. Also, a germ-line point mutation in framework region 3 of 43E was removed in 43Ea.
Alexa Fluor antibody and FVII-Fc conjugates were generated using Alexa Fluor 488 5-sulfo-dichlorophenol esters (A488) (ThermoFisher Scientific).

Cell lines
The A431, HCT-116, MDA-MB-231, HPAF-II and RF/6A cell lines were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were maintained as recommended. All cell lines from ATCC have been thoroughly authenticated using morphology, karyotyping and PCR-based approaches to rule out intra- and interspecies contamination. CHO cells were acquired from ThermoFisher Scientific. Assays were conducted with cells that did not exceed 15 passages in culture.

FX conversion assay
To evaluate the ability of TF:FVIIa to convert FX into FXa in the presence of human antibodies against TF, a cell-based FX conversion assay was conducted (25). Briefly, 5x10^4 MDA-MB-231 cells were plated into 96-well plates (Greiner Bio-One, Monroe, NC, USA) and cultured overnight. After removal of the cell culture media and addition of FX at a final concentration of 200 nM in a HEPES buffer with 1.5 mM CaCl₂, cells were incubated with 50 nM or a titration of the antibodies for 15 min at 37°C. Upon reconstitution of the binary TF:FVIIa complex with a final concentration of 20 nM of FVIIa, cells were incubated for 5 min at 37°C. After quenching the reaction with ethylenediaminetetraacetic acid (EDTA) in a black 96-well plate, generated FXa was measured with 50 μM of SN-7 6-amino-1-naphthalenesulfonamide-based fluorogenic substrate (Haematologic Technologies, Essex Junction, VT, USA). Percent FXa generation in the presence of anti-TF antibody relative to a no-antibody control was reported.

Cell viability assay
To evaluate internalization of the anti-TF antibodies, a secondary ADC cytotoxicity assay was conducted as previously described (26). Cells were plated in 384-well plates (Greiner Bio-One). Antibodies and an anti-human Fc Fab conjugated to the tubulin inhibitor mono-methyl auristatin F (MMAF) (Moradec, San Diego, CA, USA) were serially diluted as shown, starting at 5 and 30 nM, respectively. The anti-human Fc Fab conjugated to MMAF consisted of a polyclonal antibody specific to the Fc region of human IgGs with a DAR of 1.2 to 1.5. Plates were incubated for 3 to 5 days, followed by lysis in CellTiter-Glo (CTG) assay reagent (Promega, Madison, WI, USA). The mean and standard deviation of 4 replicates were graphed in Prism (GraphPad, La Jolla, CA, USA). For each ADC complex, the IC₅₀ and its associated 95% confidence interval were calculated in Prism using a 4-parameter binding model.

Binding assays
Cell-based antibody binding was assessed by flow cytometry as previously described (27). Details are provided in the Supplementary Materials and Methods. The median fluorescence intensities (MFIs) at each dilution were plotted and EC₅₀’s derived using a 4-parameter binding model in Prism.

Kinetic measurements for the anti-TF antibodies were conducted on an Octet QK384 (Pall ForteBio, Fremont, CA, USA) or a Biacore (GE Healthcare Bio-Sciences, Marlborough, MA, USA). ForteBio affinity measurements were performed during the screening campaign (Fig. 1 and Supplementary Table S1) as previously described (28). Details are provided in the Supplementary Materials and Methods. For the Biacore-based measurements (Supplementary
Table S2), the antibody was covalently coupled to a chip using an amine-coupling kit and a five-point titration of TF-His was analyzed using recommend procedures (GE Healthcare Bio-Sciences). Kinetic data was analyzed and fitted globally using a 1:1 binding model.

**Thrombin Generation Assay (TGA)**

The TGA was performed using the calibrated-automated-thrombogram (CAT) instrument (Diagnostica Stago SAS, Asnières sur Seine, France) (29). The test method design was equivalent to a standard CAT assay measurement, except that the plasma source was normal pooled plasma (NPP) collected in 11 mM citrate supplemented with 100 μg/mL of corn trypsin inhibitor. The anti-TF antibodies were diluted and mixed with NPP. Relipidated TF was added to a 96-well assay plate, followed by addition of the antibody/NPP mixture. Directly after combining the relipidated TF with the antibody/NPP, thrombin generation was initiated by the addition of calcium and the thrombin substrate. The STAGO software was used to report the following parameters: Peak IIa (highest thrombin concentration generated on the thrombin generation curve [nM]); Lag Time (time from assay start to the moment 10 nM of thrombin is formed [min]); ETP (endogenous thrombin potential, area under the curve [nM x min]); and ttPeak (time from assay start to Peak IIa [min]). Percent peak thrombin generation (% Peak IIa), percent endogenous thrombin potential (% ETP), and percent ttPeak (% ttPeak) in the presence of each antibody relative to a no-antibody plasma control on the same plate were also reported (Supplementary Table S3).

**FVII competition assay**

To evaluate competition between FVII and the human antibodies against TF, TF-positive A431 cells were first incubated for 1 h on ice with a titration of the human antibodies against TF or an IgG1 isotype control. Subsequently, FVII-Fc conjugated to A488 was added to the antibody-cell mixture at a final concentration of 20 nM. After another 1 h incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The A488 fluorescence data from viable cells was summarized using MFI. FVII-Fc binding was summarized with % FVII binding = \[\frac{MFI_{\text{anti-TF antibody labeled cells}} - MFI_{\text{unstained cells}}}{MFI_{\text{IgG1 control labeled cells}} - MFI_{\text{unstained cells}}}\].

**Generation of ADCs**

Antibodies were conjugated to MC-vc-PAB-MMAE (maleimidocaproyl-valine-citrulline-p-aminobenzoxycarbonylmonomethyl auristatin E) as previously described (30,31). Details are provided in the Supplementary Materials and Methods. Hydrophobic interaction chromatography and size exclusion chromatography were used to corroborate the absorbance-based DAR estimation and to ensure the ADC preparation was at least 95% monomeric, respectively.

**ADC cytotoxicity assays**

To evaluate ADC cytotoxicity, cells were plated in 384-well plates (Greiner Bio-One). Anti-TF antibodies conjugated to MC-vc-PAB-MMAE were serially diluted as shown. Plates were incubated for 3 days, followed by lysis in CTG assay reagent. CTG luminescence was measured and the mean and standard deviation of 4 replicates graphed in Prism. For each ADC, the IC_{50} and its associated 95% confidence interval (95% CI) were calculated in Prism using a 4-parameter binding model. When ranking the TF-specific ADCs in Supplementary Table S4, the
overall continuous primary ADC rank was calculated by equally weighing the rank obtained in A431, MDA-MB-231 and HPAF-II cultures.

**Immunofluorescence**

Immunofluorescence was conducted as described previously (32). Details are provided in the Supplementary Materials and Methods. The microtubule networks and nuclei were stained with anti-tubulin (11H10) rabbit mAb (A488 conjugate) (Cell Signaling Technology, Danvers, MA, USA) and DAPI, respectively.

**TF signaling assay**

IL-8 and GM-CSF protein levels were measured as described previously (33). Details are provided in the Supplementary Materials and Methods. A standard curve using recombinant GM-CSF or IL-8 (R&D Biosystems, Minneapolis, MN, USA) was used in Prism to calculate cytokine concentration in the cell culture supernatants. Percent IL-8 and GM-CSF (% IL-8 an % GM-CSF) at the reported antibody concentration were calculated relative to a no-antibody control.

**Antibody-dependent cellular cytotoxicity (ADCC)**

To evaluate ADCC activity, an ADCC Reporter Bioassay Core Kit (Promega) was used following the manufacturer’s protocol. Details are provided in the Supplementary Materials and Methods. The mean and standard deviation of 4 replicates were graphed in Prism. For each antibody and ADC, the EC$_{50}$ and its associated 95 % confidence interval were calculated in Prism using a 4-parameter binding model.

**Cell line–derived xenograft (CDX) models**

To evaluate the efficacy of the ADCs *in vivo*, xenograft studies in immune compromised mice were performed as described (26). Details are provided in the Supplementary Materials and Methods. The animals’ care was in accordance with institutional guidelines. Mean tumor volume (MTV) with the standard error of the mean (SEM) was plotted over time. Treatment efficacy was determined by calculating tumor growth inhibition (% TGI = 100 % x (final MTV – initial MTV of a treated group) / (final MTV – initial MTV of the control group))) before any of the animals in the vehicle arm were euthanized due to a tumor size ≥ 1200mm$^3$. Statistical comparisons between the MTVs were conducted using one-way ANOVA followed by Tukey’s multiple comparisons test comparing all groups. The *P*-values for each ADC compared to the vehicle control arm are reported. At the end of the study, efficacy was also determined in each treatment arm by counting the number of animals with a partial regression (PR) or a complete regression (CR) of the tumor. In a PR response, the tumor volume was 50 % or less of its day 1 volume for 3 consecutive measurements during the course of study, and equal to or greater than 14mm$^3$ for 1 or more of these measurements. In a CR response, the tumor volume was less than 14mm$^3$ for 3 consecutive measurements. When an animal exhibited a CR response at the end of the study, it was classified as a tumor-free survivor (TFS) instead of a CR. Throughout the ADC studies no significant body weight changes due to ADC treatment were observed.

**Patient-derived Xenograft (PDX) models**

TF-positive PDX models were performed in athymic nude mice (Envigo, Indianapolis, IN) to evaluate the efficacy of the ADCs *in vivo*. The animals’ care was in accordance with institutional
guidelines. Study animals were implanted unilaterally on the left flank with tumor fragments. Animals were randomized and treated as indicated in the figures. Animals were removed from study and euthanized once tumor size reached 1200mm$^3$ or skin ulceration was evident. In addition, the MTV curve for the treatment group in question was no longer shown once an animal was removed from study due to size. TGI and statistical analyses were conducted in the same manner as for the CDX studies. The CR and PR response definitions were as follows for the PDX studies: a PR responder had a MTV $\leq 30\%$ of MTV at day 1 for 2 consecutive measurements; a CR responder had an undetectable MTV for 2 consecutive measurements.

**Immunohistochemistry**
Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at 4-micron thickness and mounted onto positive-charged glass slides. The tissue sections were stained with the anti-TF antibody HTF-1 (ThermoFisher Scientific). Details are provided in the Supplementary Materials and Methods.

**Results**

**Screen to identify antibodies against tissue factor that do not affect FX conversion and exhibit cellular internalization**

In a discovery campaign with recombinant TF, six human TF–specific antibodies (1, 25, 29, 39, 43 and 54) were prioritized for antibody affinity maturation. Each antibody selected for optimization was from a different V$_\text{H}$ lineage, potentially resulting in greater epitope diversity, and thereby enabling selection of antibodies that do not impact coagulation and internalize efficiently. Affinity maturation of these six TF antibodies by diversification of the heavy chain complementary determining regions (CDR) resulted in a panel of fifty-four TF-specific antibodies with affinities ranging between 0.5 and 181 nM (Supplementary Table S1A). Within each V$_\text{H}$ lineage, affinities varied at least eight-fold, prompting bivariate analysis between affinity and activity for each lineage. To identify TF-specific antibodies that do not impact conversion of FX into FXa by the TF:FVIIa complex, we screened the parent and affinity-matured antibodies in a cell-based FX conversion assay. With a relatively small number of antibodies as a potential caveat for antibody group 39, a correlation analysis between affinity and inhibition of FX conversion within each V$_\text{H}$ lineage indicated that four of the six V$_\text{H}$ lineages exhibited moderate to strong correlation between the two parameters (Fig. 1A). In the four antibody groups with a substantial affinity-activity relationship—groups 1, 29, 39 and 54—antibodies with an affinity less than or equal to 5 nM exhibited more than 45 % inhibition (Figure. 1A, Supplementary Table S1A). In the two antibody groups without a substantial affinity-activity relationship—groups 25 and 43—the affinity ranges were 16 -181 nM and 1.7–14 nM, respectively. While the lack of an affinity-activity relationship for group 25 antibodies could be attributed to modest affinities, group 43 antibodies with an affinity less than or equal to 5 nM did not impact FX conversion by more than 10 % in the assays used (Figure. 1A, Supplementary Table S1A).

With the intent of developing a therapeutic ADC, internalization properties of the fifty-four TF-specific antibodies were evaluated in a cell-killing assay (Fig. 1B). Modest to strong correlation was observed between affinity and ADC IC$_{50}$ for groups 25, 29, 39, 43 and 54 (Fig. 1B), indicating that improved affinity correlated with more efficacious killing of cancer cells.
However, bivariate analysis of antibodies from group 1 revealed that improved affinity did not correlate substantially with improved ADC IC$_{50}$ for this group (Fig. 1B).

**Additional antibody affinity maturation to identify FX conversion–inert high-affinity antibodies with improved internalization characteristics**

Based on the lack of inhibition in the FX conversion assay for antibodies from groups 25 and 43, two members from each group were selected for additional affinity maturation to test whether improved binding affinity would affect activity in the FX assay. Affinity maturation by diversification of the light-chain CDRs resulted in a panel of fifty-two TF-specific antibodies with affinities ranging from 0.6 to 26 nM (Supplementary Table S1B).

None of the affinity-matured antibodies in this second panel affected FX conversion by more than 15 % (Fig. 1C). In addition, affinity maturation of group 25 led to a modest improvement in cell death (Fig. 1D). With some of the affinity-matured antibody clones from family 25 having binding affinities less than 1 nM, additional affinity maturation was not pursued.

A panel of ten TF-specific antibodies was selected for further characterization and a comparison against previously developed anti-TF antibodies. We prioritized antibodies that were inert or inhibitory in the FX conversion assay: six antibodies with varying affinities from the two groups that did not substantially affect FX conversion (i.e. 25A, 25A3, 25G1, 43B1, 43D7 and 43E) and the highest affinity antibody from each group that inhibited FX conversion by more than 50 % (i.e. 1F, 29E, 39A and 54E).

**Binding characteristics of anti-TF antibodies**

To ensure the difference in coagulation activity between the prioritized antibodies was not driven by different binding properties, the panel of antibodies was further evaluated in cell- and protein-based binding assays. The ten antibodies bound the TF-positive cancer cell line HCT-116 in a dose-dependent manner with a range of EC$_{50}$ values between 0.04 and 0.53 nM (Fig. 2A, Supplementary Table S2). The cell binding properties of the FX–inert and FX–inhibitory antibody categories were equivalent, with a similar range of EC$_{50}$ values for each category. Only a two-fold difference was observed between the lowest EC$_{50}$ in the FX–inert and –inhibitory antibody category, with 25A3 at 0.08 nM and 39A at 0.04 nM. A two-fold difference was also observed between the highest EC$_{50}$ antibody in the FX–inert and –inhibitory category, with 43Ea at 0.53 nM and 54E at 0.24 nM. Cell-based binding was confirmed in two additional cancer cell lines (Supplementary Fig. S1A and S1B). Biacore-based affinity measurements with a titration of human TF indicated that the TF-specific antibodies had affinities between 0.06 and 6.2 nM (Supplementary Table S2).

Throughout the TF-specific antibody selection campaign, affinity and cell binding were also evaluated on TF from cynomolgus monkey, the nonhuman primate to be used for toxicology. The Biacore-based affinity for human and cynomolgus TF differed less than 5-fold for all the TF-specific antibodies, with 29E as an exception (Supplementary Table S2). With the protein sequences of *Macaca fascicularis* and *mulatta* TF being identical, binding of the TF-specific antibody panel was also confirmed on the *Macaca mulatta* RF/6A cell line (Supplementary Table S2). Finally, while the anti-TF antibodies were selected against glycosylated TF from a human cell line, the antibodies also bound *E. coli*-derived TF (Supplementary Table S2).
In-depth characterization of anti-TF antibodies in coagulation assays

To corroborate the screening results, the FX conversion assay was performed with an eight-point antibody titration. As part of this effort, the TF-specific antibodies TF-011 (the antibody used in tisotumab vedotin), 5G9 and 10H10 were included as comparators (17,34). While 10H10 and the antibodies from lineage 25 and 43 were considered inert with less than 20 % inhibition at all concentrations tested, TF-011, 5G9 and the antibodies from lineage 1, 29, 39 and 54 exhibited higher inhibition (Fig. 2B and Supplementary Fig. S1C). Antibody 29E inhibited FX conversion by 32-37 % at the three highest concentrations (25, 50 and 100 nM), and 1F, 39A and 54E inhibited by 56-62 % at these concentrations (Fig. 2B). TF-011 and 5G9 inhibited FX conversion by 57-59 % and 67-70 % at these three concentrations (Supplementary Fig. S1C).

To understand the impact of diminished FXa generation by the TF-specific antibodies on the penultimate step in the coagulation cascade, a thrombin generation assay (TGA) was conducted (29). The TGA is considered a promising tool for monitoring patients on oral anticoagulants or replacement therapies and predicting thrombosis risk (35). Briefly, to mimic injury, we added recombinant TF to normal human plasma premixed with a four-point titration of each TF-specific antibody. Subsequent addition of Ca$^{2+}$ and a fluorescent thrombin substrate triggered TF-dependent coagulation and thrombin generation, respectively (Fig. 2C and 2D, Supplementary Table S3). At the 100 nM antibody concentration, 1F, 29E, 39A, 54E diminished the peak IIa concentration by 92, 76, 91 and 70 %, respectively (Fig. 2D). Similarly, 100 nM of 5G9 and TF-011 inhibited peak IIa concentration by 92 % and 91 %, respectively (Fig. 2D, Supplementary Table S3). Severely reduced thrombin generation in the presence of the two highest concentrations of 1F, 39A, 5G9 and TF-011 hampered endogenous thrombin generation (ETP) calculations and increased time to Peak IIa/thrombin generation (ttPeak) by at least 284 % at 50 nM (Supplementary Table S3). On the other hand, antibodies from group 25 did not impact the peak IIa concentration or ttPeak by more than single-digit percentage points (Fig. 2D and Supplementary Table S3). Group 43 antibodies and 10H10 exhibited mild interference with the peak IIa concentration: 100 nM of 43B1, 43D7, 43Ea and 10H10 reduced the peak IIa concentration by 33, 44, 13 and 34 %, respectively (Fig. 2D). In addition, 100 nM of 43B1, 43D7 and 10H10 showed at least a 29 % increase in ttPeak (Supplementary Table S3). However, the observed decline in peak IIa concentration and delayed ttPeak for group 43 antibodies and 10H10 did not result in more than a 10 % decline in the ETP (Supplementary Table S3). Overall, group 25 antibodies were completely inert in the penultimate step of the coagulation cascade when all three TGA parameters (ETP, Peak IIa concentration and ttPeak) were taken into consideration.

To establish whether the TF-specific antibodies interfered with binding of FVII to TF, competition between FVII and the TF-specific antibodies was evaluated. While the FX conversion–inert antibodies did not compete with FVII binding by more than 20 %, all the FX conversion–inhibitory antibodies (1F, 29E, 39A and 54E) competed with FVII binding (Fig. 2E). The previously described TF-specific antibodies TF-011, 5G9 and 10H10 (17,34) were also tested. TF-011 effectively competed with FVII, whereas 5G9 and 10H10 showed less than 25 % and 10 % competition at the highest concentration of antibody, respectively (Supplementary Fig. S1D). Thus, the FVII-competing antibodies 1F, 29E, 39A, 54E and TF-011 likely impaired FXa and thrombin generation by inhibiting the formation of an active TF:FVIIa complex. The results are also consistent with previous reports that indicated that 5G9 predominantly competes with substrate FX binding (36,37), resulting in the observed inhibition of FX conversion and thrombin
generation, while 10H10 inhibits TF-FVIIa mediated signaling without substantially affecting binding of FVIIa to TF (38).

The dichotomy in absence or presence of FVII competition suggested the TF-specific antibodies bound distinct epitopes on TF. Competition experiments using antibody pairs showed that antibodies from groups 25 and 43 bound overlapping epitopes, while the antibodies from groups 1, 29, 39 and 54 bound epitopes that did not overlap with the epitopes of groups 25 and 43 (Supplementary Fig. 2A–C). Furthermore, TF-011 did not compete with 25A3 and 43D7, but did compete with 39A (Supplementary Fig. 2A–C). However, possible overlap between 5G9’s epitope and those of group 25 and 43 antibodies illustrates the utility of high-resolution epitope mapping to further understand how antibodies from group 25 and 43 avoid interference with the coagulation cascade (Supplementary Fig. 2A-B).

**Anti-TF antibody internalization and toxin delivery**

To verify the TF-specific antibodies can mediate internalization and toxin delivery into cells, we conducted a cell-based assay in which the TF-specific antibodies were complexed with or without a Fab fragment conjugated to monomethyl auristatin F (Fab:MMAF). Because Fab:MMAF binds the Fc region of the TF-specific antibodies, cellular uptake of these complexes can trigger cell death, as previously shown in Fig. 1. While the TF-specific antibodies alone had no impact on cell viability in three-day cultures of TF-positive A431 cells (Supplementary Fig. S3A), the TF-specific antibodies in complex with Fab:MMAF showed dose-dependent cell killing with IC$_{50}$ values ranging between 0.07 and 0.14 nM (Supplementary Fig. S3B).

Cellular uptake was corroborated with fluorescently labeled TF-specific antibodies. In a quantitative assay based on internalized fluorescence and quenched surface-fluorescence, the TF-specific antibodies showed between 28 and 37 % internalization after a 4 h incubation (Supplementary Fig. S3C).

We then evaluated direct toxin delivery to cells by generating ADCs. MMAE was conjugated to cysteine residues on the TF-specific antibodies via a cleavable maleimide-containing linker with an average DAR of 3 to 4. The conjugation process did not alter the cell-binding properties of the TF-specific antibody moiety of the ADC (Supplementary Fig. S3D). Titrations of the TF-specific ADCs were added to A431 cells, with either a 72 h incubation or a 4 h incubation followed by removal of excess ADC and culture for another 68 h (Fig. 3A). Both treatments resulted in efficacious cell killing, with a 2.4 to 4.7-fold increase in IC$_{50}$ when excess ADC was removed from the culture after the 4 h incubation compared to the 72 h incubation (Fig. 3A). Removal of excess 25A3 and 39A ADC had the smallest impact on IC$_{50}$, with a 2.7 and 2.4-fold increase from 0.07 and 0.05 nM, respectively. The antibody internalization and short-term ADC exposure data were in agreement with previous reports in which TF and anti-TF antibodies undergo substantial cellular internalization (15,16).

Tumor angiogenesis, growth and metastasis have been reported to be regulated by FVIIa-dependent TF signaling (14). To understand whether FVIIa interfered with the activity of the TF-specific ADC, we treated A431 cells for 4 h with the TF-specific ADCs in the absence or presence of FVIIa and measured cell viability 68 h later. While the ADCs that competed with FVII (29E, 39A, 54E and TF-011) were negatively affected by the presence of FVIIa by at least 2.3-fold, the ADCs that did not compete with FVII (group 25 and 43 antibodies) were equally efficacious in the absence or presence of FVIIa (Supplementary Fig. S4A).

The activity of the TF-specific ADCs was confirmed on additional cancer cell lines with different levels of surface-exposed TF. The level of surface TF ranged from 1.9x10$^5$ to 5.7x10$^5$
copies in A431, MDA-MB-231 and HPAF-II cells (Supplementary Fig. S4B). HCT-116 cells expressed 2.2x10⁴ copies of surface TF, and CHO cells were used as a negative control for human TF (Supplementary Fig. S4B). While the TF-specific ADCs were also active on MDA-MB-231 and HPAF-II cells (Fig. 3B and 3C), the ADCs were less efficacious on HCT-116 cells, with some activity at the highest concentration and no reportable IC₅₀ value (Supplementary Fig. S4C). The TF-specific ADCs did not affect the viability of the CHO cultures (Supplementary Fig. S4D). When ranking the cell-killing efficacy of the ADCs on A431, HPAF-II and MDA-MB-231 cells, the top four ADCs in descending order were 39A, 29E, 25G1 and 25A3 (Supplementary Table S4A). When A431 cells were incubated for 4 h with the TF-specific ADCs followed by a washout, the top four ADCs in descending order were 39A, 25G1, 25A3 and 29E (Supplementary Table S4B). Thus, the top 2 ranking ADCs with no impact on coagulation were 25G1 and 25A3.

To illustrate the mechanism of action of the MMAE-based ADCs, we evaluated the disruption of the intracellular microtubule network in response to treatment. While the isotype control ADC did not affect the microtubule network, the 25A3 ADC disrupted the microtubule network effectively in A431 and HPAF-II cells (Fig. 3D).

In response to injury, inflammatory and angiogenic factors transiently increase expression of surface TF in the vasculature (39). We mimicked the transient upregulation of TF in cell culture by treating human umbilical vein endothelial cells (HUVECs) with a combination of inflammatory cytokines. Surface TF levels increased from 2.4x10³ copies in the absence of inflammatory cytokines to 1.2x10⁴ copies after 6 h of cytokine treatment (Supplementary Fig. S5A). In agreement with the transient nature of TF upregulation, surface TF was ~3-fold lower after 20 h of cytokine treatment relative to 6 h of treatment. Cytokine-treated endothelial cell cultures appeared resistant to anti-TF ADCs, as viability was unaffected in a 4-day viability assay (Supplementary Fig. S5B). To further understand the apparent resistance of endothelial cells to anti-TF ADCs, cell cycle progression was evaluated 24 h after addition of the cytokines and TF-specific ADCs. While the TF-specific ADCs induced an arrest at the G₂/M phase of the cell cycle in HCT-116 cells, the ADCs did not impact cell cycle progression in HUVECs treated with or without inflammatory cytokines (Supplementary Fig. S5C and S5D). An increase in phosphorylated histone H3 was observed in both HCT-116 cells and HUVECs upon treatment with unconjugated MMAE, indicating that the resistance in endothelial cells is specific for the MMAE-based ADC (Supplementary Fig. S5E).

Additional functional properties of TF-specific antibodies

Signaling by tumor cell–expressed TF:FVIIa complex induces proangiogenic and immune-modulating cytokines (14). To determine whether the TF-specific antibodies had an impact on TF signaling, cytokine secretion and Erk phosphorylation were evaluated in cell cultures treated with FVIIa. All the TF-specific antibodies inhibited IL-8 and GM-CSF protein levels with an IC₅₀ value ranging between 1 and 2.7 nM (Fig. 4A). Erk phosphorylation was ablated by pretreatment with 1F, 39A and 54E (fold induction between 0.8 and 1.2) and attenuated by 29E and the members of groups 25 and 43 (induction between 2.0 and 3.4) (Supplementary Fig. S6).

Both the TF-specific antibodies and ADCs can exert antibody-dependent cellular cytotoxicity (ADCC) via the IgG1 Fc domain of the antibody. In an ADCC assay with TF-positive A431 cells in which primary NK cells were replaced with a Jurkat cell line stably expressing human FcγRIIIa V158 and NFAT-induced luciferase, all the TF-specific antibodies
and ADCs exerted induction of luciferase-dependent luminescence with EC50 values ranging between 0.18 and 0.43 nM (Fig. 4B).

**ADCs against tissue factor exhibit antitumor activity in cell line– and patient-derived xenograft models**

The *in vivo* efficacy of the TF-specific ADCs was evaluated in multiple cell-line (CDX) and patient-derived xenografts (PDX). The target-specific ADCs were efficacious in the HPAF-II xenograft model using various dosing regimens (Fig. 5). In the first HPAF-II study, we compared TF-011 with two representative clones from the two groups that did not impact FX conversion (i.e. 25A and 43Ea). Tumor-bearing mice were treated with 5 mg/kg of ADC on day 1, 8 and 15 after randomization. Twenty-one days after randomization the efficacy of the 25A, 43Ea and TF-011 ADCs was equivalent, with tumor growth inhibition ranging between 131 and 136 % (Fig. 5A). In the second HPAF-II study, the highest affinity antibody that affected coagulation (i.e. 39A) and six antibodies with varying affinities from groups 25 and 43 (i.e. 25A, 25A3, 25G1, 43Ea, 43B1 and 43D7) were equally efficacious when dosed twice at 2 mg/kg (Fig. 5B). Tumor growth inhibition for the TF-specific ADCs ranged between 129 and 139 % on day 21, and 6 to 9 out of 10 animals per treatment arm were classified as tumor-free survivors at the end of the study (Fig. 5B).

In the MDA-MB-231 xenograft model, the ADCs were administered on day 1 and 8 post-randomization at 4 or 2 mg/kg (Fig. 5C and 5D). All the TF-specific ADCs were active at 4 mg/kg, with tumor growth inhibition ranging between 69 and 100 %, and a significant difference in mean tumor volume for each TF-specific ADC compared to the vehicle control arm (Fig. 5C). While a notable difference was observed in mean tumor volume between 25G1 and the other TF-specific ADCs, it was not statistically significant (*P > 0.05*, Fig. 5C). At 2 mg/kg of ADC, all the TF-specific antibodies showed suboptimal activity with varying degrees of significance in mean tumor volume compared to the vehicle control arm. 25A3, 39A and 43B1 showed the greatest degree of significance in mean tumor volume compared to the vehicle control arm (*P < 1x10^{-4}*) (Fig. 5D). The difference in mean tumor volume between 39A and the other antibodies was only significant for the comparison between 39A and 43Ea (*P < 0.05*, Fig. 5D).

Next, we benchmarked the activity of the 25A and 43Ea ADCs against tisotumab vedotin in three PDX models (Fig. 6). PDX tumor models reflect human tumor cell heterogeneity to a greater degree than cancer cell lines and CDX models (40). After profiling TF expression in PDX tumor models from multiple cancer indications—colorectal, esophageal, gastric, head and neck, lung, melanoma, ovarian, pancreatic, and sarcoma—we prioritized PDX models from three indications with varying levels of expression. While the squamous cell carcinoma of the head and neck (SCCHN) and ovarian adenocarcinoma PDX had H-scores of 250 and 220, respectively, the gastric adenocarcinoma PDX had an H-score of 155 (Fig. 6). Upon randomization of tumor-bearing mice, treatment occurred on a weekly basis either twice or three times with the dose ranging between 2.5 and 5 mg/kg (Fig. 6). In all the PDX models a significant reduction in mean tumor volume was observed for each TF-specific ADC compared to the isotype control arm (*P < 1x10^{-4}*) with no significant difference between the various TF-specific ADCs (*P > 0.05*, Fig. 6). In the head and neck and ovarian PDX model, the number of complete responders and tumor-free survivors did not exceed 2 out of 10 animals at the end of the study in any of the treatment groups (Fig. 6A and 6B). However, in the gastric PDX the 25A treatment arm had 2 partial responders, 2 complete responders and 3 tumor-free survivors, and
the TF-011 arm contained 1 complete responder and 5 tumor-free survivors at the end of the study (Fig. 6C).

To understand whether the activity of the ADC was solely attributable to the delivery of toxin by the anti-TF antibody to the xenograft, the activity of a subset of naked antibodies was evaluated in the HPAF-II xenograft model. Unlike the 25A, 25A3 and 43Ea ADCs, the naked antibody equivalents lacked substantial anticancer activity when dosed twice at 10 mg/kg (Fig. 6D).

Overall, the coagulation-inert and -inhibitory ADCs were equally efficacious in CDX and PDX models. A member of group 25 has been selected for further preclinical and clinical development as an ADC.

Discussion

Here, coagulation-inert TF-specific ADCs with potent anticancer activity were developed. First, a panel of anti-TF antibodies was evaluated for perturbation of coagulation and cellular internalization. To ensure lack of coagulation interference was not due to poor binding affinity, the antibody panel underwent affinity maturation and coagulation-inert antibodies were identified. Affinity maturation also improved the anticancer efficacy of the coagulation-inert antibodies as ADCs. The prioritized coagulation-inert ADCs from antibody family 25 induced tumor growth inhibition in TF-positive solid tumor xenografts at levels comparable to tisotumab vedotin, a clinical stage ADC (17,20,21).

Tisotumab vedotin validated TF as a promising clinical-stage target (20,21). In a phase I/II dose-escalation study of tisotumab vedotin, fourteen out of twenty-seventy patients achieved stable disease or presented with a better outcome (20,21). While tisotumab vedotin showed anticancer activity, some of the adverse events could be attributed to the biological activity of TF-011, the antibody used for the development of tisotumab vedotin. In the current study, TF-011 competed with FVII, partially inhibited the conversion of FX into FXa, and severely impacted thrombin generation at clinically relevant concentrations. In the phase I dose escalation cohorts from 0.3 to 2.2 mg/kg of tisotumab vedotin, the C\textsubscript{max} concentrations ranged from 32 up to 370 nM (21). One of the most common adverse events recorded in the trial was epistaxis, with an incidence of 48 % (20,21). We postulate that these nosebleeds were triggered by TF-011-induced perturbation of coagulation. In addition, one SCCHN patient died from tumor-related bleeding at 0.6 mg/kg of ADC. While this death could be related to the cancer itself, the frequency of epistaxis suggests that tisotumab vedotin may affect hemostasis.

Aside from mediating hemostasis, TF can also trigger thrombosis (11). Another TF-specific antibody, Sunol-cH36 underwent clinical development as an inhibitor of coronary thrombosis. In this naked antibody trial, dose-dependent spontaneous minor bleeding was observed (41). Sunol-cH36 had a putative mechanism of action similar to that of 5G9, with both antibodies inhibiting binding of FX to the TF:FVIIa complex (41,42). In the current study, 5G9 inhibited conversion of FX into FXa, and also interfered with thrombin generation. Therefore, the FX conversion and thrombin generation assays described herein might be predictive assays for clotting-related adverse events in the clinic. Indeed, the TGA is considered a bridge between \textit{in vivo} and \textit{ex vivo} coagulation, and an automated version can be used in the clinic on a large number of patients (35). We prioritized group 25 for further preclinical and clinical development, because none of the TGA parameters (i.e., ETP, Peak IIa concentration and ttPeak) were affected by group 25 antibodies. The automated TGA could be used during clinical development to...
understand whether the TGA parameters are also unaffected after dosing patients with a group 25 ADC.

In a population-based case-control study, the baseline risk of thrombosis in cancer was four-fold compared to a healthy population, with an additional seven-fold increase after treatment with chemotherapy (43). In cancer indications with a higher incidence of venous thromboembolism (VTE), such as pancreatic and brain cancer, a low dose of an antibody-based inhibitor of coagulation might attenuate thrombotic events (44). Indeed, treatment of human patients with tisotumab vedotin or Sunol-cH36 did not augment VTE. Furthermore, in light of the coagulation-inert properties of antibody family 25, we predict the clinical-stage ADC from this family not to elicit thrombosis.

The anticancer activity of the anti-TF ADCs was attributable to the delivery of the toxin to the TF-positive xenografts. While the TF-specific ADCs were efficacious in various xenograft models, the isotype control ADC and a high dose of naked anti-TF antibody showed little to no impact on tumor growth. ADC-dependent toxin delivery was consistent with the observation that cancer cells internalized about a third of fluorescently labeled anti-TF antibody over a 4 h time course. Because TF undergoes such rapid internalization, the anticancer activity of the anti-TF ADC described herein will likely not improve with a biparatopic anti-TF ADC or a bispecific ADC that bridges TF with another surface antigen that also undergoes rapid internalization, like the prolactin receptor or CD63 (45,46).

In the current study a high dose of naked antibody did not significantly impact tumor growth. However, co-injection of a cancer cell line with 10H10 resulted in tumor growth inhibition (34). In this prophylactic treatment setting, tumor growth inhibition was attributed to 10H10-dependent inhibition of TF:FVIIa signaling. Like 10H10, all the antibodies isolated from the screening campaign inhibited TF:FVIIa signaling by downmodulating Erk phosphorylation and cytokine production. Importantly, in the context of an ADC, the antibody’s ability to inhibit TF:FVIIa signaling could be masked by the anticancer activity of the internalized toxin.

The anti-signaling activity of the anti-TF antibody might be more relevant in cell populations resistant to MMAE-based ADCs. While the anti-TF ADCs affected cell cycle progression and viability of cancer cells, the ADCs did not alter cell cycle progression and viability of endothelial cells, consistent with previous reports in which endothelial cells were resistant to MMAE-based ADCs (47). This resistance was likely unrelated to TF copy number, as the difference in TF surface copy number between the cytokine-treated endothelial cells and the MMAE-based ADC–sensitive HCT-116 cancer cells was only two-fold. However, the lack of activity of the ADCs on endothelial cell cultures does not preclude a safe toxicity profile in normal tissue.

Stabilization of disease with tisotumab vedotin validated TF as an anti-cancer target for an ADC. To enable appropriate benchmarking against tisotumab vedotin, the prototypic linker-toxin vc-MMAE was used for all the in vivo efficacy studies. In the phase I/II study of tisotumab vedotin, the adverse events of peripheral neuropathy and neutropenia were consistent with MMAE-based ADCs, with a maximum tolerated dose (MTD) between 2–3 mg/kg (5). With the advent of safer linker-payloads such as XMT-1267 (48), we anticipate lack of MMAE-associated toxicities for the clinical-stage group 25 ADC.

Finally, the antibodies described herein could also be prioritized for the treatment of other diseases associated with upregulation of membranous TF expression, such as macular degeneration (49). A FVII-Fc fusion protein that binds TF reduces the size of choroid neovascular (CNV) lesions in various animal models (50,51). With the binding affinities of a
subset of the anti-TF antibodies below the affinity of FVII for TF (52), some of the antibodies could be more efficacious than the FVII-Fc fusion protein. A subset of the anti-TF antibodies will be evaluated in various CNV animal models.
Acknowledgments

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References


29. Samama MM, Mendell J, Guinet C, Le Flem L, Kunitada S. In vitro study of the anticoagulant effects of


Figure Legends

Fig. 1. Screen to identify anti-TF antibodies that do not impact coagulation and exhibit cellular internalization

(A) A diverse panel of anti-TF antibodies was evaluated in a binding and coagulation assay. The relationship between affinity and inhibition of coagulation, expressed as binding affinity of the anti-TF antibody to human TF (human K_D) versus inhibition of FX conversion by the anti-TF antibody (% FXa generation when cells were pretreated with 50 nM of antibody). Six antibody groups, each with a different symbol for the parent antibody and its associated affinity-matured antibody clones, are presented. In the legend, the parent antibody group identifier, the number of affinity-matured antibodies from each parent antibody, the affinity range within each group, and the bivariate equation and its associated correlation coefficient that capture the relationship between binding affinity and inhibition of FX conversion are shown (poor fit: R^2 < 0.40). Average % FXa generation was derived from three independent experiments, with each experiment conducted in duplicate.

(B) The panel of anti-TF antibodies was also evaluated in a cell killing–based internalization assay. The relationship between affinity and cell-killing activity in A431 cells, expressed as human K_D (as in panel A) versus the cell-killing IC_{50} value from a nine-point 3.5-fold titration starting at 10 nM of the anti-TF antibody complexed with 60 nM of an anti-human Fab conjugated to the auristatin MMAF. In the legend, the parent antibody group identifier, and the bivariate equation and its associated correlation coefficient that capture the relationship between binding affinity and cell-killing IC_{50} are listed.

(C) Additional affinity maturation of antibody clones 25A, 25G, 43B and 43D did not result in inhibition of coagulation. The relationship between antibody affinity and inhibition of coagulation is presented as in panel A. Average % FXa generation was derived from two independent experiments, with each experiment performed in duplicate.

(D) Additional affinity maturation of antibody clones 25A, 25G, 43B and 43D improved cell-killing activity of antibodies in group 25. The relationship between antibody affinity and cell-killing IC_{50} is presented as in panel B. The cell-killing IC_{50} values were derived from a nine-point 2-fold antibody titration starting at 4.5 nM of anti-TF antibody and 27 nM of Fab:MMAF in A431 cells.

Fig. 2. In-depth characterization of TF-specific antibodies in binding and coagulation assays

(A) Binding of anti-TF antibodies to human TF–positive HCT-116 cells. For each anti-TF antibody, the median fluorescence intensity (MFI) of antibody bound to cells is plotted against antibody concentration. Reportable cell EC_{50}’s are listed. The 95 % confidence intervals are reported in Supplementary Table S2. An IgG1 isotype control did not bind cells (no binding, nb). Representative data from one of three experiments are shown.

(B) TF:FVIIa–dependent conversion of FX into FXa on the cell surface of MDA-MB-231 cells in the absence or presence of an anti-TF antibody titration. Percent FX conversion to FXa (% FXa) is plotted against the anti-TF antibody concentration, with % FXa calculated relative to an antibody-free FX conversion reaction. The standard deviation of the mean % FXa is also graphed. Representative data from one of two experiments are shown.
(C) Thrombin generation in the presence of anti-TF antibody. Factor IIa/Thrombin generation in the absence or presence of 100 nM anti-TF antibody is plotted against time. The mean of a triplicate data set is shown. The standard deviation of the mean is listed in Supplementary Table S3. Representative data from one of two experiments are shown.

(D) The peak IIa/thrombin concentration associated with the time course in panel C is shown against a titration of anti-TF antibody. The mean of a triplicate data set is shown. Other parameters associated with the real-time IIa/thrombin generation measurement shown in panel C are summarized in Supplementary Table S3.

(E) FVII binding in the presence of anti-TF antibody. After pre-incubation of MDA-MB-231 cells with a titration of anti-TF antibody, the amount of FVII-Fc that bound cells is plotted as percent FVII binding (% FVII binding) relative to FVII binding in the presence of an IgG1 isotype control. An IC50 was not reported for antibodies that did not compete with FVII by more than 20% (no competition, nc). Representative data from one of two experiments are shown.

**Fig. 3. In vitro efficacy of anti-TF ADCs**

(A) Cell viability of A431 cell cultures after addition of a titration of anti-TF antibody conjugated directly to MMAE via a cleavable linker (MC-vc-PAB-MMAE). Prior to the cell viability measurement, the ADC titration was incubated continuously with the cells for 72 h, or the titration was washed out after a 4 h incubation and the cells were cultured for another 68 h. The isotype control lacked a reportable IC50 value (not applicable, na). The 95% confidence intervals for the IC50’s are reported in Supplementary Table S4.

(B, C) Cell viability of 5-day old cultures of MDA-MB-231 (B) or HPAF-II (C) cells incubated with a titration of anti-TF antibody conjugated to MC-vc-PAB-MMAE. Isotype control as in panel A. The 95% confidence intervals for the IC50’s are reported in Supplementary Table S4A.

(D) Staining of the microtubule network of A431 and HPAF-II cells treated for 20 h with MMAE-based ADCs. Representative images of tubulin from one of two experiments are shown in white or in green when overlaid with nuclei in blue. Scale bar, 10 microns.

**Fig. 4. Additional functional properties of anti-TF antibodies**

(A) FVIIa-dependent TF-signaling in MDA-MB-231 cells pretreated with an anti-TF antibody titration was evaluated by measuring the concentration of IL-8 and GM-CSF in cell culture supernatants. The IgG1 isotype control does not inhibit signaling and lacks a reportable IC50 (no inhibition, ni). Representative data from one of two experiments are shown.

(B) Antibody-dependent cellular cytotoxicity (ADCC) reporter luminescence was evaluated after a 6 h incubation of the reporter Jurkat cell line with TF-positive A431 cells and a titration of anti-TF antibody (left panel) or ADC (right panel). The ADCC reporter luminescence EC50 values for each anti-TF antibody or ADC are listed. An isotype control antibody and ADC lacked activity (EC50 value not applicable, na).

**Fig. 5. In vivo efficacy of anti-TF ADCs in cell line–derived xenograft models**

(A, B) Efficacy evaluation of anti-TF ADCs in HPAF-II xenograft model dosed weekly at 5 mg/kg for 3 weeks (A) or 2 mg/kg for 2 weeks (B). The treatment started on day 1 after animals with a tumor size of ~ 190 mm3 were randomized. The mean tumor volumes (Mean) and tumor growth inhibition (TGI) percentages on day 21 for all the animals on study are listed. The P-values for the mean tumor volume comparison between each ADC and the vehicle control arm...
are also listed. At the end of the study (day 59 for (A) and day 39 for (B)), the number of PR, CR and TFS animals were counted.

(C, D) Efficacy evaluation of anti-TF ADCs in MDA-MB-231 xenograft model dosed weekly at 4 mg/kg for 2 weeks (C) or 2 mg/kg for 2 weeks (D). The treatment started on day 1 after animals with a tumor size of ~160 mm$^3$ were randomized. The mean tumor volume and tumor growth inhibition on day 25 (C) and day 27 (D) for the animals on study are reported. The $P$-values for the mean tumor volume comparison between each ADC and the vehicle control arm are also listed. At the end of the study, the number of PR, CR and TFS animals were counted.

**Fig. 6.** *In vivo* efficacy of anti-TF ADCs in patient-derived xenograft (PDX) models and lack of anticancer activity of unconjugated anti-TF antibodies.

Efficacy evaluation of anti-TF ADCs dosed as indicated in PDX models of a head and neck carcinoma (A), an ovarian carcinoma (B), and a gastric adenocarcinoma (C). Immunohistochemistry (IHC) analysis indicated TF expression in each PDX, with an H-score of 250 for the head and neck carcinoma (A), 220 for the ovarian carcinoma (B) and 155 for the gastric adenocarcinoma (C). (D) The lack of anticancer activity of unconjugated anti-TF antibodies was evaluated in the HPAF-II xenograft model. ADC or naked antibody treatment started on day 1 after animals with a tumor size of ~210 mm$^3$ (A), ~190 mm$^3$ (B), ~140 mm$^3$ (C), and ~190 mm$^3$ (D) were randomized. Mean tumor volume, tumor growth inhibition and $P$-value calculations, and PR/CR/TFS enumerations were performed as in Fig. 5.
Figure 1

A

%FXa generation at 50 nM of antibody

0.1 1 10 100 1000

Human $K_\text{D}$ (nM)

B

ADC IC50 (nM)

0.1 1 10 100 1000

Human $K_\text{D}$ (nM)

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C

%FXa generation at 50 nM of antibody

0.1 1 10 100

Human $K_\text{D}$ (nM)

D

ADC IC50 (nM)

0.075 0.15 0.225

Human $K_\text{D}$ (nM)

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Figure 2

A

Median Fluorescence Intensity (MFI)

Antibody concentration (nM)

1E

0.05

0.17

0.08

0.09

0.15

0.04

0.28

0.04

0.28

0.53

0.24

IC50 (nM)

1.15

1.54

0.69

2.13

1F

25A

25A3

25G1

29E

39A

43B1

43D7

43Ea

54E

Isotype nb

B

% FXa generation

Antibody concentration (nM)

C

IIa/Thrombin (% relative to plasma)

Antibody concentration (nM)

D

Peak IIa/Thrombin (% relative to plasma)

Antibody concentration (nM)

E

% FVII binding

Antibody concentration (nM)

Antibody

1F

25A

25A3

25G1

29E

39A

43B1

43D7

43Ea

54E

Isotype

TF-011

5G9

10H10

Plasma
Figure 3

(A) Continuous 72 h incubation and culture

(B) 4 h incubation, followed by washout and 68 h culture

ADC IC50 (nM)

Cont. Washout

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<td>Isotype-vc-MMAE</td>
<td>na</td>
</tr>
<tr>
<td>TF-011-vc-MMAE</td>
<td>0.17</td>
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</table>

(B) ADC IC50 (nM)

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<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>25A</td>
<td>0.14</td>
</tr>
<tr>
<td>25A3</td>
<td>0.11</td>
</tr>
<tr>
<td>25G1</td>
<td>0.09</td>
</tr>
<tr>
<td>29E</td>
<td>0.07</td>
</tr>
<tr>
<td>39A</td>
<td>0.05</td>
</tr>
<tr>
<td>43B1</td>
<td>0.14</td>
</tr>
<tr>
<td>43D7</td>
<td>0.14</td>
</tr>
<tr>
<td>43Ea</td>
<td>0.15</td>
</tr>
<tr>
<td>Isotype na</td>
<td></td>
</tr>
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</table>

(D) A431 and HPAF-II

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<th>A431</th>
<th>HPAF-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype ctrl-vc-MMAE</td>
<td>25A3-vc-MMAE</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Tubulin</td>
</tr>
<tr>
<td>Tubulin + DAPI</td>
<td>Tubulin + DAPI</td>
</tr>
</tbody>
</table>

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Figure 5

A) Mean tumor volume, SEM (mm³)

- 25A-vc-MMAE
- 25A3-vc-MMAE
- 25G1-vc-MMAE
- 39A-vc-MMAE
- 43B1-vc-MMAE
- 43D7-vc-MMAE
- Isotype-vc-MMAE
- Vehicle

B) Mean tumor volume, SEM (mm³)

- 2 mg/kg, qwk x 2, IV

C) Mean tumor volume, SEM (mm³)

- 4 mg/kg, qwk x 2, IV

D) Mean tumor volume, SEM (mm³)

- 2 mg/kg, qwk x 2, IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 21 (n)</th>
<th>Mean ± [SEM] (mm³)</th>
<th>P</th>
<th>%TGI</th>
<th>Day 59 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>10</td>
<td>8 ± 2</td>
<td>&lt;0.0001</td>
<td>132</td>
<td>0/8/2</td>
</tr>
<tr>
<td>43Ea-vc-MMAE</td>
<td>10</td>
<td>8 ± 3</td>
<td>&lt;0.0001</td>
<td>131</td>
<td>0/6/4</td>
</tr>
<tr>
<td>Isotype-vc-MMAE</td>
<td>10</td>
<td>434 ± 76</td>
<td>NS</td>
<td>56</td>
<td>0/0/0</td>
</tr>
<tr>
<td>TF-011-vc-MMAE</td>
<td>10</td>
<td>7 ± 3</td>
<td>&lt;0.0001</td>
<td>132</td>
<td>0/5/5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>749 ± 68</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 25 (n)</th>
<th>Mean ± [SEM] (mm³)</th>
<th>P</th>
<th>%TGI</th>
<th>Day 49 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>10</td>
<td>174 ± 43</td>
<td>&lt;0.001</td>
<td>98</td>
<td>3/0/1</td>
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<tr>
<td>25A3-vc-MMAE</td>
<td>10</td>
<td>153 ± 43</td>
<td>&lt;0.0001</td>
<td>100</td>
<td>1/1/1</td>
</tr>
<tr>
<td>25G1-vc-MMAE</td>
<td>10</td>
<td>449 ± 102</td>
<td>&lt;0.0001</td>
<td>69</td>
<td>1/1/1</td>
</tr>
<tr>
<td>29E-vc-MMAE</td>
<td>10</td>
<td>180 ± 78</td>
<td>&lt;0.0001</td>
<td>97</td>
<td>1/0/3</td>
</tr>
<tr>
<td>39A-vc-MMAE</td>
<td>10</td>
<td>160 ± 45</td>
<td>&lt;0.0001</td>
<td>99</td>
<td>0/4/1</td>
</tr>
<tr>
<td>43B1-vc-MMAE</td>
<td>10</td>
<td>232 ± 66</td>
<td>&lt;0.0001</td>
<td>92</td>
<td>1/0/0</td>
</tr>
<tr>
<td>43D7-vc-MMAE</td>
<td>10</td>
<td>217 ± 49</td>
<td>&lt;0.0001</td>
<td>93</td>
<td>0/0/1</td>
</tr>
<tr>
<td>Isotype-vc-MMAE</td>
<td>10</td>
<td>873 ± 114</td>
<td>NS</td>
<td>24</td>
<td>0/0/0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>1098 ± 133</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 27 (n)</th>
<th>Mean ± [SEM] (mm³)</th>
<th>P</th>
<th>%TGI</th>
<th>Day 41 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>7</td>
<td>517 ± 52</td>
<td>&lt;0.001</td>
<td>67</td>
<td>0/0/0</td>
</tr>
<tr>
<td>25A3-vc-MMAE</td>
<td>7</td>
<td>495 ± 69</td>
<td>&lt;0.0001</td>
<td>68</td>
<td>0/0/0</td>
</tr>
<tr>
<td>25G1-vc-MMAE</td>
<td>7</td>
<td>550 ± 53</td>
<td>&lt;0.001</td>
<td>63</td>
<td>0/0/0</td>
</tr>
<tr>
<td>29E-vc-MMAE</td>
<td>7</td>
<td>223 ± 46</td>
<td>&lt;0.0001</td>
<td>92</td>
<td>2/0/0</td>
</tr>
<tr>
<td>39A-vc-MMAE</td>
<td>7</td>
<td>472 ± 58</td>
<td>&lt;0.0001</td>
<td>70</td>
<td>0/0/0</td>
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<tr>
<td>43B1-vc-MMAE</td>
<td>7</td>
<td>708 ± 104</td>
<td>&lt;0.01</td>
<td>49</td>
<td>0/0/0</td>
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<tr>
<td>43D7-vc-MMAE</td>
<td>7</td>
<td>728 ± 188</td>
<td>&lt;0.05</td>
<td>48</td>
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<tr>
<td>Isotype-vc-MMAE</td>
<td>7</td>
<td>1197 ± 88</td>
<td>NS</td>
<td>9</td>
<td>0/0/0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>1292 ± 180</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
</tr>
</tbody>
</table>
**Figure 6**

**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 44 (n)</th>
<th>Mean ±SEM [mm³]</th>
<th>P</th>
<th>%TGI</th>
<th>Day 60 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>10</td>
<td>74 [16]</td>
<td>&lt;0.001</td>
<td>124</td>
<td>5/0/1</td>
</tr>
<tr>
<td>43Ea-vc-MMAE</td>
<td>10</td>
<td>100 [18]</td>
<td>&lt;0.001</td>
<td>119</td>
<td>6/0/0</td>
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<tr>
<td>Isotype-vc-MMAE</td>
<td>10</td>
<td>780 [154]</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
</tr>
<tr>
<td>TF-011-vc-MMAE</td>
<td>10</td>
<td>49 [8]</td>
<td>&lt;0.001</td>
<td>128</td>
<td>6/0/2</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15 (n)</th>
<th>Mean ±SEM [mm³]</th>
<th>P</th>
<th>%TGI</th>
<th>Day 46 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>10</td>
<td>464 [35]</td>
<td>&lt;0.001</td>
<td>71</td>
<td>0/0/0</td>
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<tr>
<td>43Ea-vc-MMAE</td>
<td>10</td>
<td>439 [23]</td>
<td>&lt;0.001</td>
<td>74</td>
<td>0/0/0</td>
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<tr>
<td>Isotype-vc-MMAE</td>
<td>10</td>
<td>1131 [118]</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
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<tr>
<td>TF-011-vc-MMAE</td>
<td>10</td>
<td>452 [50]</td>
<td>&lt;0.001</td>
<td>72</td>
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**C**

<table>
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<tr>
<th>Treatment</th>
<th>Day 25 (n)</th>
<th>Mean ±SEM [mm³]</th>
<th>P</th>
<th>%TGI</th>
<th>Day 46 PR/CR/TFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25A-vc-MMAE</td>
<td>9</td>
<td>24 [7]</td>
<td>&lt;0.001</td>
<td>112</td>
<td>2/2/3</td>
</tr>
<tr>
<td>Isotype-vc-MMAE</td>
<td>9</td>
<td>1093 [143]</td>
<td>-</td>
<td>0</td>
<td>0/0/0</td>
</tr>
<tr>
<td>TF-011-vc-MMAE</td>
<td>9</td>
<td>46 [12]</td>
<td>&lt;0.001</td>
<td>112</td>
<td>0/1/5</td>
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</tbody>
</table>

**D**

<table>
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<th>Treatment</th>
<th>Day 15 (n)</th>
<th>Mean ±SEM [mm³]</th>
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<th>%TGI</th>
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</thead>
<tbody>
<tr>
<td>25A</td>
<td>9</td>
<td>938 [78]</td>
<td>NS</td>
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<td>25A3</td>
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<td>898 [73]</td>
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<td>43Ea</td>
<td>9</td>
<td>976 [114]</td>
<td>NS</td>
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<tr>
<td>Isotype</td>
<td>9</td>
<td>954 [86]</td>
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<tr>
<td>Vehicle</td>
<td>8</td>
<td>829 [67]</td>
<td>-</td>
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</tbody>
</table>
Molecular Cancer Therapeutics

Treating Tissue Factor-Positive Cancers With Antibody-Drug Conjugates That Do Not Affect Blood Clotting

Jan-Willem Theunissen, Allen G Cai, Maryam M Bhatti, et al.

Mol Cancer Ther  Published OnlineFirst August 20, 2018.

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