An Antibody–Drug Conjugate That Targets Tissue Factor Exhibits Potent Therapeutic Activity against a Broad Range of Solid Tumors

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Abstract

Tissue factor (TF) is aberrantly expressed in solid cancers and is thought to contribute to disease progression through its procoagulant activity and its capacity to induce intracellular signaling in complex with factor VIIa (FVIIa). To explore the possibility of using tissue factor as a target for an antibody-drug conjugate (ADC), a panel of human tissue factor–specific antibodies (TF HuMab) was generated. Three tissue factor HuMab, that induced efficient inhibition of TF:FVIIa-dependent intracellular signaling, antibody-dependent cell-mediated cytotoxicity, and rapid target internalization, but had minimal impact on tissue factor procoagulant activity in vitro, were conjugated with the cytotoxic agents monomethyl auristatin E (MMAE) or monomethyl auristatin F (MMAF). Tissue factor–specific ADCs showed potent cytotoxicity in vitro and in vivo, which was dependent on tissue factor expression. TF-011-MMAE (HuMax-TF-ADC) was the most potent ADC, and the dominant mechanism of action in vivo was auristatin-mediated tumor cell killing. Importantly, TF-011-MMAE showed excellent antitumor activity in patient-derived xenograft (PDX) models with variable levels of tissue factor expression, derived from seven different solid cancers. Complete tumor regression was observed in all PDX models, including models that showed tissue factor expression in only 25% to 50% of the tumor cells. In conclusion, TF-011-MMAE is a promising novel antitumor agent with potent activity in xenograft models that represent the heterogeneity of human tumors, including heterogeneous target expression. Cancer Res; 74(4); 1–13. ©2013 AACR.

Introduction

Antibody-drug conjugates (ADC), which combine the tumor-targeting capacity of monoclonal antibodies with the antitumor activity of cytotoxic agents, received renewed attention in recent years. Trastuzumab emtansine (T-DM1), an ADC composed of the HER2-specific antibody trastuzumab and the cytotoxic agent DM1, increased progression-free survival in patients that had received prior treatment with unconjugated trastuzumab (1), demonstrating the added value of toxin conjugation to a monoclonal antibody. In addition, brentuximab vedotin, a CD30-specific antibody coupled to the microtubule disrupting agent monomethyl auristatin E (MMAE), was approved for the treatment of relapsed Hodgkin lymphoma and relapsed systemic anaplastic large cell lymphoma (2). With at least thirty products in clinical development, ADCs represent an exciting new class of anticancer drugs.

Tissue factor (TF), also called thromboplastin, factor III, or CD142, is aberrantly expressed in many solid cancers, including pancreatic, lung, cervical, prostate, bladder, ovarian, breast, and colon cancer. Expression has been described on tumor cells and the tumor vasculature, and has been associated with poor disease prognosis and increased metastatic properties (reviewed in ref. 3). This, in combination with the known internalizing capacity of tissue factor (4), led us to explore the possibility of using tissue factor as a novel target for an ADC.

Tissue factor is the main physiologic initiator of the extrinsic coagulation pathway. Proteolytic cleavage of factor VII (FVII), the physiologic ligand of tissue factor, generates activated FVII (FVIIa), which associates with tissue factor to form the TF:FVIIa complex. This complex proteolytically activates coagulation factor X (FX) to generate FXa, eventually leading to thrombin generation and clot formation (5). Tissue factor is expressed in a wide range of organs, including brain, heart, intestine, kidney, lung, placenta, uterus, and testes (6). Under physiologic conditions, tissue factor expression is mostly restricted to the cells of the subendothelial vessel wall, such as smooth muscle cells, pericytes, and...
fibroblasts, that are not in direct contact with the blood (6).
In healthy individuals, blood leukocytes do not express tissue factor on the cell surface, although tissue factor expression has been described on 1% to 2% of monocytes (7, 8). Activation of the coagulation cascade occurs when membrane-bound tissue factor is exposed to circulating FVII (a), for example, after disruption of the vessel wall by injury or after upregulation of tissue factor on monocytes under inflammatory conditions (9).

In addition to initiation of coagulation, formation of the TF:FVIIa complex on the cell membrane induces an intracellular signaling cascade by activation of protease-activated receptor 2 (PAR-2), resulting in the production of proangiogenic factors, cytokines and adhesion molecules (10). This signaling cascade is further amplified by coagulation factors generated downstream of the TF:FVIIa complex, such as FXa and thrombin, all of which recognize one or more receptors of the PAR family (10).

Tissue factor-expressing tumor cells are thought to exploit both tissue factor procoagulant activity and TF:FVIIa-mediated intracellular signaling. Experimental tumor models showed that interference with tissue factor using siRNA or monoclonal antibodies reduced tumor outgrowth, tumor-associated angiogenesis, and metastatic potential in vivo (11–13). Previous studies demonstrated that it is possible to generate tissue factor-specific antibodies that have minimal impact on tissue factor procoagulant capacity (13, 14), potentially allowing specific targeting of tissue factor-positive tumors without a major impact on hemostasis.

Here, we report the development of TF-011-MMAE, an ADC composed of a human tissue factor-specific monoclonal antibody, a protease-cleavable linker, and the potent cytotoxic agent MMAE. By carefully selecting tissue factor-specific antibodies that interfere with TF:FVIIa-dependent intracellular signaling, but not with tissue factor procoagulant activity, and that show efficient internalization and lysosomal targeting, we developed an ADC that efficiently kills tumor cells in vivo with only minimal effect on parameters of coagulation. TF-011-MMAE was extensively tested in preclinical efficacy studies, including studies in patient-derived xenograft (PDX) models that showed heterogeneous target expression.

Materials and Methods

Cells
Human tumor cell lines AsPC-1 (pancreas adenocarcinoma; 100,000–300,000 tissue factor molecules/cell), BxPC-3 (pancreas adenocarcinoma; >350,000 tissue factor molecules/cell), HCT-116 (colorectal carcinoma; <15,000 tissue factor molecules/cell), HPAF-II (pancreas adenocarcinoma; >350,000 tissue factor molecules/cell), MDA-MB-231 (breast adenocarcinoma; >350,000 tissue factor molecules/cell), SK-OV-3 (ovarian adenocarcinoma; 50,000–175,000 tissue factor molecules/cell), and TOV-21G (ovarian adenocarcinoma; <7,000 tissue factor molecules/cell) were obtained from the American Type Culture Collection. The epidermoid adenocarcinoma cell line A431 (>300,000 tissue factor molecules/cell) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, and HaCaT human keratinocytes (150,000–200,000 tissue factor molecules/cell) were a kind gift from Dr. Wiiger (Biotechnology Center of Oslo, Norway). To guarantee cell line authenticity, cell lines were aliquoted and banked, and cultures were grown and used for a limited number of passages before starting a new culture from stock. Cell lines were routinely tested for mycoplasma contamination. Tissue factor cell surface expression was quantified by QIFIKIT analysis (DAKO) according to the manufacturer's guidelines, using a mouse anti-human tissue factor antibody (R&D Systems).

Recombinant expression of full-length tissue factor or the tissue factor extracellular domain
A codon-optimized construct was generated for the expression of full-length tissue factor (Genbank accession no. NP001984), cloned into the mammalian expression vector pEE13.4 (Lonza Biologics), and transfected into Freestyle 293-F cells (HEK-293F, Invitrogen) or NSO cells as described (15). To generate recombinant His-tagged soluble tissue factor, PCR was used to amplify the part encoding the extracellular domain (aa 1-251) of tissue factor from the construct, adding a C-terminal His tag containing six His residues (TF-ECDHis). The construct was cloned in pEE13.4 and expressed in HEK-293F cells. TF-ECDHis was purified from cell supernatant using immobilized metal affinity chromatography.

Generation of human tissue factor–specific antibodies and ADCs
Human immunoglobulin G (IgG)-1κ tissue factor–specific antibodies (tissue factor HuMab) were generated by immunization of HuMab mice (Medarex; ref. 16) with TF-ECDHis and/or tissue factor-expressing NSO cells. Hybridomas were generated from mice that showed tissue factor–specific antibodies in serum, as assessed by binding to tissue factor-transfected HEK293F or A431 cells, or to bead-coupled TF-ECDHis using Fluorimetric Microvolume Assay Technology (Applied Biosystems). Tissue factor–specific hybridomas were identified by screening supernatants for tissue factor–specific antibodies as described above. To determine the antibody variable region sequences of tissue factor–specific hybridomas, mRNA was extracted and the immunoglobulin variable heavy and light chain regions were amplified, cloned, and sequenced. Recombinant antibodies were generated as described (17), and the recombinant IgG1κ was used for further characterization of the tissue factor HuMab. Fab fragments were generated as described (17). The IgG1κ antibodies IgG1-b12 (18) and HuMab-KLH (19) were included as isotype control antibodies.

Antibodies TF-011, -098, and -111, as well as IgG1-b12, were conjugated with MMAE through a protease-cleavable valine-citrulline (vc) dipeptide and a maleimidocaproyl-containing (mc) linker, or with monomethyl auristatin F (MMAF) through an mc linker as described (20, 21). The average drug-antibody ratio was 4:1.

Flow cytometry
Binding of tissue factor HuMab and tissue factor–specific ADCs (TF-ADC) to membrane-bound tissue factor was
analyzed by flow cytometry as described (22), using phycoerythrin-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories) to detect binding of tissue factor HuMab or ADCs.

**Biacore analysis**

The affinity of tissue factor HuMab for tissue factor was measured by surface plasmon resonance in a Biacore 3000 (GE Healthcare). Tissue factor HuMab was immobilized on a CM-5 sensor chip (GE Healthcare), according to the manufacturer’s guidelines, and a concentration series of TF-ECDHis was injected over the HuMab (30 µL/minute; 180 seconds). The HuMab surface was regenerated using 10 mmol/L glycine-HCl, pH 2.0. Kinetic analysis was performed using double reference subtraction and model 1:1 (Langmuir) binding analysis.

**FVIIa ELISA**

TF-ECDHis (0.5 µg/mL) was immobilized and incubated with recombinant FVIIa (100 nmol/L, Novo Nordisk) in the presence of tissue factor HuMab (1 hour, room temperature). Plates were washed and incubated with rabbit-anti-FVIIa (2.5 µg/mL; Abcam), followed by incubation with swine-anti-rabbit IgG-HRP (1:2,500; DAKO). Binding was visualized as described (17).

**Phosphorylation inhibition assay—Western blot analysis**

BxPC-3 or HaCaT cells were cultured in serum-free medium for 1.5 hours before preincubation with tissue factor HuMab (30 minutes, 37°C). Next, cells were stimulated with 10 mmol/L FVIIa (10 minutes, 37°C) and lysed. Phosphorylated extracellular signal-regulated kinase (p-ERK)-1/2 and total ERK1/2 were detected in cell lysates by Western blot analysis using standard procedures, using rabbit anti-p-ERK1/2 and rabbit anti-ERK1/2 (Cell Signaling technology) as primary antibodies, and donkey-anti-rabbit-IgG-HRP (Jackson Immunoresearch Laboratories) as detection antibody.

**Interleukin-8 release assay**

MDA-MB-231 cells were cultured in serum-free medium for 105 minutes before incubation with tissue factor HuMab (15 minutes). FVIIa (10 nmol/L) was added and after 5 hours (37°C), interleukin (IL)-8 production was measured in culture supernatant by ELISA (Sanquin) according to the manufacturer’s protocol.

**FXa generation assay**

Recombinant lipidated full-length tissue factor (Innovin; Dade Behring) was incubated with tissue factor HuMab in HEPES buffer containing 3 mmol/L CaCl2 (30 minutes, room temperature). FXa generation was initiated by adding 1 nmol/L recombinant FVIIa and 200 nmol/L FX (Enzyme Research Laboratories). After 30 minutes (37°C), the reaction was stopped by adding 5 mmol/L EDTA in HEPES buffer, and FXa was detected by measuring conversion of the FXa substrate Chromogenix-2765 (Instrumation Laboratory Company) according to the manufacturer’s guidelines.

**Thromboelastography**

Citrated human whole blood was obtained from healthy volunteers with the donor’s consent and approval from the Ethical Committee of the Florida Hospital Center. Whole blood was incubated with 10 µg/mL lipopolysaccharide (LPS) or PBS without Ca2+ and Mg2+ (4 hours, 37°C), followed by incubation with tissue factor HuMab (10 minutes, room temperature). Thromboelastography (TEG) was performed as described (23). In this system, the LPS-induced decrease in clotting lag time (R) represents a measure for tissue factor activity. Antibody-mediated inhibition of tissue factor activity was calculated as follows: % inhibition of tissue factor activity = 100 - ([RNo-LPS - Rout antibody + LPS]/RNo-LPS + Rout type-mAb + LPS) × 100.

**Immunofluorescent confocal microscopy**

SK-OV-3 and A431 cells were grown on glass coverslips (Thermo Fisher Scientific) at 37°C for 16 hours. Cells were incubated with 50 µg/mL leupeptin (Sigma) for 1 hour to block lysosomal activity, followed by incubation with 1 µg/mL tissue factor HuMab (1, 3, or 24 hours, 37°C). Cells were fixed with 4% formaldehyde (30 minutes, room temperature) and stained with fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG (Jackson Immunoresearch Laboratories) to identify tissue factor HuMab, and mouse anti-human CD107a (LAMP-1)-allophycocyanin (BD Pharmingen) to identify lysosomes. Staining was analyzed with a Leica SPE-II confocal microscope and LAS-AF software.

**Fab-TAMRA/QSY7 internalization and degradation assay**

Goat anti-human IgG Fab-fragments (Jackson Immunoresearch Laboratories) were conjugated with the fluorophore and quencher pair TAMRA/QSY7 (Fab-TAMRA/QSY7) as described (24). Tissue factor HuMab (1 µg/mL) was pre-incubated with Fab-TAMRA/QSY7 (2 µg/mL; 30 minutes, 4°C), and the complex was added to SK-OV-3 or A431 cells while shaking (200 rpm, 37°C). After 24 hours, TAMRA-fluorescence was measured on a FACS Canto-II (BD Biosciences).

**Cytotoxicity assay in vitro**

Cells were seeded in 96-well plates (2,500–5,000 cells/well) and incubated for 6 hours (37°C), before adding ADCs. After 3 to 5 days (37°C), the viability of the culture was assessed using Alamar Blue (Biosource International) according to the manufacturer’s guidelines. Staurosporine (Sigma, 10 µg/mL) was used a positive control (100% cell death) and untreated cells were used as a negative control. The percentage of viable cells was calculated as follows: % viable cells = ([fluorescence test sample – fluorescence staurosporine]/fluorescence untreated cells – fluorescence staurosporine] × 100.

**Antibody-dependent cell-mediated cytotoxicity assay**

Lysis of tumor cells by antibody-dependent cell-mediated cytotoxicity (ADCC) was measured in a 51Cr release assay as...
described (25), using A431, BxPC-3, and MDA-MB-231 cells as target cells and human peripheral blood mononuclear cells (PBMC), isolated from healthy donors (Sanquin), as effector cells.

Immunohistochemical analysis of tissue factor expression in PDX models
A tissue microarray containing formalin-fixed, paraffin-embedded (FFPE) PDX tissue (Oncotest GmbH) was incubated with FITC-labeled TF-011 or mouse anti-human cytokeratin antibody (Cell Marque; 1 hour, room temperature), after antigen retrieval (citrate/EDTA buffer, pH8, in a pressure cooker for 5 minutes for TF-011-FITC and citrate buffer, pH6, for mouse anti-cytokeratin). Endogenous peroxidase (PO) activity was exhausted by incubation with H2O2, and nonspecific antibody binding was blocked using chicken serum or normal human serum. Binding of TF-011-FITC was detected using rabbit anti-FITC (Zymed) and Powervision (anti-rabbit IgG1)-PO (Leica Biosystems). Mouse-anticytokeratin binding was detected using Ultravision-PO (Thermo Scientific). Peroxidase was visualized with amino-ethyl-carbazole, resulting in a red color. Nuclei were visualized using hematoxylin. Immunostaining was scored manually, by estimating the tissue factor–positive tumor area in relation to the total tumor area as identified by human cytokeratin staining. The tissue factor–positive tumor area was scored according to the following intervals: 0 (no tissue factor–positive cells), 0% to 25%, 25% to 50%, 50% to 75%, or >75% tissue factor–positive cells.

Xenograft models
Cell line-derived xenograft models were established in female SCID mice by subcutaneous injection of 2 to 10 × 10^5 (A431, AsPC-1, and BxPC-3), or 0.5 × 10^6 (HCT-116) tumor cells as described (22). Tissue factor HuMab were injected intraperitoneally 1 hour after tumor injection (prophylactic treatment) or when tumors had reached a size of 100 to 400 mm³ (therapeutic treatment, starting between day 8–13). All experiments were approved by the Utrecht University (Utrecht, the Netherlands) Animal Ethics Committee.

PDX models were initiated by subcutaneous implantation of human tumor fragments in the flanks of NMRI nu/nu mice at Oncotest GmbH. When tumors had reached a size of 80 to 200 mm³, mice were treated intravenously with 4 mg/kg ADC or 20 mg/kg paclitaxel (Teva-Gry Pharma). Tumor volume was determined as described above. All experiments were conducted according to the guidelines of the German Animal Welfare Act (Tierschutzgesetz).

Statistical analysis
Data were analyzed using GraphPad Prism 5 software. For mouse xenograft studies, differences in tumor size between treatment groups were analyzed by one-way ANOVA, using mean tumor sizes from the last day that all groups were complete (i.e., before mice in isotype control groups had to be sacrificed because of large tumor burden).

Results

Target-binding characteristics of tissue factor HuMab
From a large panel of human tissue factor–specific IgG1K antibodies (tissue factor HuMab), eight clones were selected for extensive functional characterization in vitro and in vivo. All tissue factor HuMab showed dose-dependent binding to tissue factor–positive MDA-MB-231 breast cancer cells (Fig. 1A). EC50 values ranged from 0.07 μg/mL for TF-011 to 0.49 μg/mL for TF-109 (subnanomolar to nanomolar range; Supplementary Table S1). Similar results were obtained using BxPC-3 pancreas adenocarcinoma and A431 epidermoid carcinoma cells (data not shown). Biacore analysis demonstrated that tissue factor HuMab bound tissue factor with affinities ranging from 1.8 nmol/L for TF-025 to 307 nmol/L for TF-098 (Supplementary Table S1).

Tissue factor–specific antibodies interfere with TF:FVIIa-mediated intracellular signaling
Tissue factor–specific antibodies may interfere with the interaction between tissue factor and FVIIa, possibly resulting in inhibition of TF:FVIIa-dependent intracellular signaling. To measure competition between tissue factor HuMab and FVIIa for tissue factor binding, FVIIa was incubated with TF-ECDHIs in the presence of tissue factor HuMab, and binding of FVIIa was detected by ELISA. Except for TF-044 and TF-013, tissue factor HuMab efficiently inhibited binding of FVIIa to tissue factor, with only 9% to 21% of FVIIa binding remaining at the highest antibody concentration tested (30 μg/mL; Fig. 1B; Table 1).

Next, the capacity of tissue factor HuMab to interfere with TF:FVIIa—dependent PAR-2 signaling was assessed by measuring antibody-mediated inhibition of ERK1/2 phosphorylation and IL-8 production, both of which have been implicated in tumor cell proliferation, migration, and metastatic potential (26, 27). When preincubated with BxPC-3 or HaCaT cells, all tissue factor HuMab, except TF-013, inhibited TF:FVIIa-induced ERK phosphorylation, as shown by Western blot analysis (Fig. 1C and data not shown). Inhibition of TF:FVIIa-induced ERK phosphorylation was confirmed in A431 cells using Alphascreen, which allowed more quantitative detection of p-ERK1/2 (Table 1 and Supplementary Fig. S1). The tissue factor HuMab that showed efficient inhibition of ERK1/2 phosphorylation, also inhibited TF:FVIIa-dependent production of IL-8 by MDA-MB-231 cells when antibodies were allowed to bind the cells before stimulation with FVIIa (Fig. 1D). In the reverse experiment, where the tumor cells were incubated with FVIIa before adding tissue factor HuMab, inhibition of TF:FVIIa-induced IL-8 production was clearly less efficient, confirming competition between FVIIa and tissue factor HuMab for tissue factor binding (Supplementary Fig. S2). In agreement with poor inhibition of FVIIa binding, TF-044 only moderately inhibited TF:FVIIa-dependent intracellular signaling, whereas TF-013 showed almost no inhibition.

These results suggest that tissue factor HuMab recognize distinct functional epitopes in the tissue factor extracellular
This was confirmed in a cross-competition study, which indicated that TF-011, -025, -098, -111, -109, and -114 bind different, but overlapping, epitopes, whereas TF-013 and TF-044 recognize a nonoverlapping epitope (Supplementary Table S2).

Tissue factor HuMab show minor interference with FXa generation and coagulation in vitro

Proteolytic activation of FX by the TF:FVIIa complex, generating FXa, is an important step in the extrinsic coagulation pathway. Depending on the binding domain, tissue

Figure 1. Functional characteristics of tissue factor HuMab in vitro. A, binding of tissue factor HuMab to MDA-MB-231 cells as assessed by flow cytometry. Results from a representative experiment are shown (n = 3). B, competition between tissue factor HuMab and FVIIa for tissue factor binding. FVIIa was incubated with TF-ECDHis in the presence of tissue factor HuMab, and binding of FVIIa was measured by ELISA. Results from a representative experiment are shown (n = 3); error bars, SEM. C, TF:FVIIa-induced ERK phosphorylation in the presence of tissue factor HuMab. BxPC-3 cells were incubated with FVIIa after preincubation with tissue factor HuMab, and p-ERK1/2 and total ERK1/2 were detected in cell lysates by Western blot analysis. Full-length blots are presented in Supplementary Fig. S7. D, TF:FVIIa-induced IL-8 production in MDA-MB-231 cells in the presence of tissue factor HuMab. Cells were incubated with tissue factor HuMab before stimulation with FVIIa. IL-8 production was measured in cell culture supernatants using ELISA. Results from a representative experiment are shown (n = 3); error bars, SEM. E and F, effect of tissue factor HuMab on whole-blood coagulation as assessed by TEG. Citrated whole-blood was incubated with LPS to induce tissue factor expression, followed by incubation with tissue factor HuMab. Coagulation was initiated by recalcification. E, inhibition of tissue factor activity in the presence of tissue factor HuMab. Tissue factor activity was defined as the difference in clotting lag time (R) between unstimulated and LPS-stimulated whole blood. Tissue factor HuMab-mediated inhibition of tissue factor activity was expressed as the percentage change in tissue factor activity. Data represent the average of three donors; error bars, SEM. F, TEG trace overlays of coagulation in the presence of 20 µg/mL TF-011, TF-013, TF-098, or an isotype control IgG. Results from a representative donor are shown (n = 3).
factor-specific antibodies may interfere with binding of FX to the catalytic domain of TF:FVIIa, thereby impairing FXa generation and coagulation (14). None of the tissue factor HuMab in our panel substantially inhibited FXa generation as shown in a chromogenic FXa generation assay (Table 1). TF-013 induced the highest inhibition of FXa generation, but even for this antibody, the reduction in FXa activity was maximally 22%. The impact of tissue factor HuMab (TF-011, TF-013, and TF-098) on whole-blood coagulation was assessed by TEG. Citrated whole blood, obtained from healthy donors, was incubated with LPS to induce tissue factor expression on monocytes and release of monocyte-derived tissue factor into the plasma. Incubation with TF-013 induced a small delay in LPS-induced clotting. At a concentration of 50 μg/mL, the average inhibition of tissue factor activity was 22% for TF-011 and 31% for TF-098. TF-013 showed a somewhat stronger inhibition of tissue factor procoagulant activity (66%). Results obtained at 10, 20, and 50 μg/mL tissue factor HuMab were comparable (Fig. 1E). Tissue factor HuMab did not have a major impact on other parameters of clot formation such as the clot kinetics (K value and α-angle) or clot strength (maximal amplitude), as shown by the similar shape of the TEG trace in the presence or absence of tissue factor HuMab (Fig. 1F and Supplementary Table S3). This was as expected, as tissue factor is thought to be important for the initiation but not the amplification or propagation of coagulation (9).

**Tissue factor HuMab are rapidly internalized after target binding**

Because ADCs generally rely on internalization for release of the payload, we characterized the internalization characteristics of tissue factor HuMab in the TAMRA/QSY7 assay. This assay uses a fluorophore (TAMRA) and quencher (QSY7) pair. In close proximity, for example, upon conjugation to the same protein, TAMRA fluorescence is quenched by QSY7. Tissue factor HuMab were complexed with TAMRA/QSY7-conjugated anti-human IgG Fab fragments (Fab-TAMRA/QSY7), and the complex was incubated with A431 cells. After 6 hours, TAMRA fluorescence was detected in cells that had been incubated with TF-011, TF-098, or TF-111 (Fig. 2A and B), indicating internalization of the HuMab-Fab-TAMRA/QSY7 complex and degradation in the reducing environment of the endosomes and lysosomes. Internalization was most efficient for TF-011 and TF-098. Interestingly, internalization of TF-011 was reduced when Fab fragments were used instead of the intact antibody (Fig. 2B), suggesting that internalization of TF-011 is stymied by bivalent target binding.

Efficient internalization of TF-011 was confirmed by confocal microscopy. One hour after incubation with SK-OV-3 cells, TF-011 was already detectable in intracellular vesicles, some of which colocalized with the lysosomal marker LAMP-1, indicating internalization and lysosomal targeting. Colocalization of TF-011 and LAMP-1 was enhanced after 3 hours, and after 24 hours, most TF-011 colocalized with LAMP-1, indicating efficient accumulation in the lysosomal compartment (Fig. 2C). Similar results were obtained with A431 cells (data not shown).

Our results demonstrate that tissue factor HuMab are rapidly internalized and degraded upon target binding, indicating that tissue factor may be a very suitable ADC target.

**Generation of tissue factor-specific ADCs and cytotoxicity in vitro**

Tissue factor-specific ADCs were generated by conjugation of TF-011,-098, and -111 with the dolastatin analogs MMAE or MAAF. Auristatins are potent cytotoxic agents that induce cell

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**Table 1. Tissue factor HuMab: functional characteristics in vitro**

<table>
<thead>
<tr>
<th>HuMab</th>
<th>IC_{50} (SD) μg/mL</th>
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<th>IC_{50} (SD) μg/mL</th>
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<tr>
<td>TF-011</td>
<td>0.19 (0.07)</td>
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<td>0.17 (0.07)</td>
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<td>TF-013</td>
<td>2.9 (4.2)</td>
<td>1.37 (0.31)</td>
<td>1.13 (0.27)</td>
<td>0.90 (0.21)</td>
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<tr>
<td>TF-025</td>
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<td>0.28 (0.06)</td>
<td>0.20 (0.05)</td>
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</tr>
<tr>
<td>TF-044</td>
<td>0.21 (0.04)</td>
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<td>0.28 (0.06)</td>
<td>0.17 (0.05)</td>
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<tr>
<td>TF-098</td>
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<td>TF-109</td>
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<tr>
<td>TF-111</td>
<td>0.33 (0.14)</td>
<td>&gt;1,000</td>
<td>52 (1)</td>
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<tr>
<td>TF-114</td>
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<td>0.16 (0.05)</td>
<td>0.16 (0.05)</td>
<td>0.14 (0.05)</td>
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*aFVIIa ELISA, average of three experiments.
*bAlphascreen Surefire ERK assay, A431 cells, average of two experiments.
*cMDA-MB-231 cells, average of three experiments.
*dInhibition measured at plateau of dose response curve at 30 μg/mL (1), 10 μg/mL (2), or 120 μg/mL (3).
*eInhibition (percentage) measured at plateau of dose–response curve (at 0.9 μg/mL IgG); average of two experiments.
death by disrupting microtubules (20, 21). MMAE was conjugated through the protease-cleavable vc linker, and can therefore be released from the antibody by lysosomal proteases, such as cathepsin B (21). MMAF was conjugated through a noncleavable linker, and relies on intracellular degradation of the ADC for release (20). Tissue factor HuMab were conjugated with an average of four auristatins per antibody, a ratio that was shown to provide the optimal therapeutic index for brentuximab vedotin (29). Direct comparison of tissue factor HuMab and TF-ADCs in vitro confirmed that target binding and internalization characteristics were preserved in the ADCs (Fig. 2A and Supplementary Fig. S3).

TF-ADCs showed excellent cytotoxicity in vitro. TF-ADCs efficiently and dose dependently killed A431 and HPAF-II cells (Fig. 3A and B), which express high levels of tissue factor on the plasma membrane (>300,000 molecules/cell). The EC50 for TF-ADC-mediated tumor cell killing in A431 and HPAF-II cells ranged from 4 to 10 ng/mL, for TF-011-MMAE and TF-098-MMAE, to 5 to 80 ng/mL, for TF-111-MMAF. In cell lines that showed low (HCT-116; <15,000 molecules/cell) or very low (TOV-21G; <7,000 molecules/cell) tissue factor expression, TF-ADCs showed very limited or no cytotoxic activity (Fig. 3C and D). Similarly, TF-ADCs did not show cytotoxic activity in tissue factor-negative tumor cells (data not shown). This, in addition to the lack of cytotoxic activity of the nonbinding control ADCs IgG1-b12-MMAE and IgG1-b12-MMAF, indicates that the efficacy of TF-ADCs is dependent on target expression.

Unconjugated tissue factor HuMab did not induce direct cytotoxicity in vitro (data not shown), indicating that the cytotoxicity of TF-ADCs was auristatin dependent.
Tissue factor HuMab and TF-ADCs efficiently induce ADCC in vitro

Monoclonal antibodies of the IgG1 isotype may exert cytotoxicity through Fc-mediated effector functions such as ADCC, and these effector mechanisms may be preserved upon conjugation with cytotoxic agents (30). Unconjugated TF-011, TF-098, and TF-111 potently induced killing of A431 cells by ADCC (Fig. 3E), with median EC50 values of 15 ng/mL (range 0.5–19 ng/mL), 18 ng/mL (range 5.0–57 ng/mL), and 76 ng/mL (range 15–102 ng/mL), respectively. Similar results were observed with BsPC-3 and MDA-MB-231 cells (data not shown). Importantly, the capacity to induce ADCC was preserved after conjugation with MMAE (Fig. 3E).

Antitumor activity of tissue factor–specific ADCs in cell line-derived xenograft models

All TF-ADCs potently inhibited tumor growth in established xenografts derived from HPAF-II and A431 cells, at a dose of 3 mg/kg (four injections in 2 weeks; Fig. 4A and Supplementary Fig. S4A). MMAE conjugates showed significantly better efficacy than their MMAF-conjugated counterparts (Fig. 4B and Supplementary Fig. S4B). Complete tumor regression, that is no measurable tumor remaining at 20 to 30 days after the last treatment, was observed for most mice in the TF-098-MMAE and TF-111-MMAE treatment groups, and for all mice in the TF-011-MMAE group. Strikingly, 3 out of 7 mice that had been treated with TF-011-MMAE remained tumor free until the end of the study (139 days after discontinuation of treatment). In mice that did show tumor recurrence after treatment with TF-011-MMAE (4 out of 7 mice in the HPAF-II model), measurable tumors were not detected until 56 to 70 days after discontinuation of treatment. Recurring tumors could successfully be retreated with TF-011-MMAE, TF-098-MMAE, or TF-111-MMAE (Fig. 4C and Supplementary Fig. S4C), indicating that tissue factor expression was maintained in tumor cells that
showed outgrowth after completion of the first treatment cycle. The isotype control ADCs IgG1-b12-MMAE and IgG1-b12-MMAF did not inhibit tumor growth, indicating that the efficacy of TF-ADCs was dependent on target binding. This was supported by the lack of activity of TF-ADCs in the low tissue factor expressing HCT-116 xenograft model (Supplementary Fig. S4D).
To study the potential contribution of ADCC and inhibition of TF:FVIIa-dependent intracellular signaling to the antitumor activity of TF-ADCs in vivo, xenograft studies were performed using unconjugated TF-011, TF-098, or TF-111. Prophylactic treatment with TF-011, TF-098, or TF-111 significantly reduced outgrowth of BxPC-3, HPAF-II, AsPC-1, and A431 xenografts (Supplementary Fig. S5A–S5D and data not shown). When treatment was initiated after the tumors had established (therapeutic treatment), TF-098 and TF-111 induced a small, albeit significant, reduction of tumor growth in the BxPC-3 model (Supplementary Fig. S5E and S5F). However, therapeutic treatment with unconjugated tissue factor HuMab was unable to inhibit tumor growth in the A431, AsPC-1, and HPAF-II xenograft models (Supplementary Fig. S5G and S5H and data not shown). This suggests that the antitumor activity of TF-ADCs in the established xenografts tested here is to a large extent mediated by the cytotoxic activity of MMAE or MMAF.

The antitumor efficacy of the most potent ADC, TF-011-MMAE, was assessed at different dose levels and dosing schedules. At weekly dosing (four doses), treatment with 1 mg/kg TF-011-MMAE was sufficient to induce tumor regression (Fig. 4D). When administered as a single dose, 2 and 4 mg/kg TF-011-MMAE induced tumor regression, whereas inhibition of tumor growth was observed after treatment with 0.5 or 1 mg/kg (Fig. 4E). Interestingly, comparison of tumor volumes on day 24, when mice had received either one or two doses of TF-011-MMAE, demonstrated that a single dose of 1 mg/kg TF-011-MMAE was more effective than two doses of 0.5 mg/kg, although the cumulative dose was the same in both treatment groups. Similarly, treatment with a single dose of 2 mg/kg was more effective than two doses of 1 mg/kg (Fig. 4F). This suggests that, at the same cumulative dose (exposure), dosing schedules giving a higher peak plasma level (C_{max}) are more effective. For treatment with a cumulative dose of 4 mg/kg, no difference in efficacy was observed between a single dose of 4 mg/kg and two doses of 2 mg/kg, because tumor regression was complete in both groups.

In summary, TF-ADCs showed potent antitumor activity in vivo, which was dependent on both tissue factor targeting and conjugation with auristatins. TF-011-MMAE was selected for further preclinical studies and clinical development (designated as HuMax-TF-ADC).

**TF-011-MMAE induces efficient tumor cell killing in PDX models with heterogeneous tissue factor expression**

Like most solid tumor targets, expression of tissue factor in cancer is heterogeneous between patients, within patients, and even within tumors (3). We addressed the capacity of TF-011-MMAE to inhibit the growth of tumors with heterogeneous target expression using PDX models, which are thought to represent the heterogeneity that exists between human tumors (31-33). Immunohistochemical (IHC) analysis of xenografted...
primary human tumor biopsies confirmed heterogeneity of tissue factor expression, and seven PDX models were selected on the basis of variable levels of tissue factor expression. Tissue factor expression was observed in >75% of tumor cells in the PDX models for lung and pancreas adenocarcinoma (Fig. 5A and B), and in 50% to 75% of tumor cells in the models for bladder carcinoma, prostate carcinoma, and lung squamous cell carcinoma (Fig. 5C–E). In PDX models for cervix squamous cell carcinoma and ovarian adenocarcinoma, only 25% to 50% of tumor cells were tissue factor positive (Fig. 5F and G).

PDX tumors were implanted subcutaneously in nude mice, and when tumors had established, mice were treated with TF-011-MMAE (4 mg/kg, two doses). In models where the sensitivity to microtubule-targeting agents was not known, a paclitaxel treatment group (20 mg/kg, 3–4 doses) was included. In the models for pancreas and lung adenocarcinoma, which showed >75% tissue factor–positive tumor cells, TF-011-MMAE induced complete tumor regression in all mice (Fig. 6A). Similarly, TF-011-MMAE induced efficient tumor regression in PDX models that expressed tissue factor in 50% to 75% of tumor cells (bladder cancer, prostate cancer, and lung squamous cell carcinoma; Fig. 6B). Importantly, tumor regression was also observed in models that showed tissue factor expression in only 25% to 50% of the tumor cells (ovarian and cervical cancer; Fig. 6C).

Treatment with two doses of HuMax-TF-ADC was at least as efficient as treatment with three or four doses of paclitaxel (Fig. 6). Moreover, TF-011-MMAE induced tumor regression in mice that showed tumor recurrence after paclitaxel treatment, even in mice with relatively large tumors (Supplementary Fig. S6). This demonstrates that prior treatment with paclitaxel did not

![Figure 6. Antitumor activity of TF-011-MMAE in PDX models with heterogeneous target expression. PDX models were established by subcutaneous implantation of tumor fragments in mice. When tumors had reached a size of 80 to 200 mm³, mice were randomized and treatment was initiated. Mice were treated with TF-011-MMAE or paclitaxel at the indicated doses and time points. IgG1-b12-MMAE was included as an isotype control ADC, IgG1-b12 was included as an isotype control IgG. A, tumor growth in PDX models showing >75% tissue factor–positive tumor cells (lung adenocarcinoma and pancreatic adenocarcinoma). tumor growth in PDX models showing 50% to 75% tissue factor–positive tumor cells (bladder adenocarcinoma, prostate adenocarcinoma, and lung squamous cell carcinoma). C, tumor growth in PDX tumors with 25% to 50% tissue factor–positive cells (cervical squamous cell carcinoma and ovarian adenocarcinoma). Datapoints, average tumor size per group (8 mice per group). Error bars, SEM.](https://www.aacrjournals.org/can/doi/10.1158/0008-5472.CAN-13-2440)
affect the sensitivity of the tumors to treatment with TF-011-MMAE, indicating that tissue factor expression and sensitivity to MMAE-mediated tumor cell killing were retained in tumors that showed outgrowth after paclitaxel treatment.

Discussion

TF-011-MMAE was selected from a panel of six tissue factor–specific ADCs, consisting of three different tissue factor HuMab conjugated with vcMMAE or mcMMAF. TF-011-MMAE showed excellent antitumor activity in vivo, with auristatin-mediated tumor cell killing as the dominant mechanism of action. Furthermore, TF-011-MMAE and unconjugated TF-011 induced inhibition of TF:FVIIa-mediated intracellular signaling and ADCC in vitro, although it is unclear to what extent these mechanisms may contribute to the inhibition of tumor growth in patients with cancer. To our knowledge, TF-011-MMAE is the first ADC that uses a tissue factor–specific antibody to deliver a cytotoxic agent to tumor cells.

Importantly, TF-011-MMAE induced complete tumor regression in PDX models, even if only a subpopulation of the tumor cells expressed tissue factor. PDX models are thought to represent the genetic and histologic heterogeneity in human tumors, and efficacy of treatment in such models was shown to have predictive value for the clinic (31–33). The high potency of TF-011-MMAE in tumors with nonhomogeneous target expression may be related to the capacity of MMAE to cause a bystander effect by diffusion across cell membranes after intracellular release (34). Especially in solid tumors, where antibody penetration may be limited (35, 36), this may be a major advantage. As opposed to uncharged MMAE, the negative charge of MMAF is thought to prevent diffusion across membranes (20). This difference in membrane permeability probably underlies the difference in efficacy observed between MMAE and MAF conjugates.

Although tissue factor plays a crucial role in coagulation and hemostasis, TF-011 showed minimal impact on coagulation in vitro. Previous studies suggested that tissue factor–specific antibodies can roughly be divided into two categories: those that inhibit FVIIa binding and/or TF:FVIIa-induced intracellular signaling with minor impact on tissue factor procoagulant activity, and those that interfere with FXa activation and coagulation without impacting on TF:FVIIa-induced intracellular signaling (14, 37). Our in vitro studies suggest that TF-011 belongs to the first category. This notion is supported by nonclinical toxicity studies in cynomolgus monkeys. TF-011 and TF-011-MMAE, which show comparable binding to cynomolgus monkey and human tissue factor, did not significantly impact on functional bleeding time or systemic parameters of coagulation in cynomolgus monkeys at doses up to 100 mg/kg or 5 to 6 mg/kg, respectively (Gemnab, data on file). It may seem counterintuitive that TF:FVIIa binding, the first step in the coagulation cascade, can be inhibited without impacting on hemostasis or clotting. This apparent paradox is most likely explained by the many amplification steps in the coagulation cascade downstream of TF:FVIIa (38). As a result, only little TF:FVIIa binding is required to maintain hemostasis. The work of Parry and colleagues, who demonstrated that transgenic mice expressing only 1% of normal tissue factor activity were viable and had relatively normal hemostasis (39), supports this.

In summary, TF-011-MMAE is a promising new ADC that is being developed for the treatment of solid tumors. Tissue factor is thought to be an excellent ADC target due to its broad expression profile across solid cancer types and rapid internalization and degradation after antibody binding. TF-011-MMAE induced complete tumor regression in PDX models derived from a broad range of solid tumors, demonstrating the high potency of TF-011-MMAE for treatment of cancer.

Disclosure of Potential Conflicts of Interest

W.K. Bleeker has ownership interest (including patents) in Gemnab. P.W.H.L. Parren has ownership interest (including patents) in Gemnab. No potential conflicts of interest were disclosed.

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Potent Antitumor Activity of an ADC Targeting Tissue Factor


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An Antibody–Drug Conjugate That Targets Tissue Factor Exhibits Potent Therapeutic Activity against a Broad Range of Solid Tumors

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