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# Utility of epirubicin-incorporating micelles tagged with anti-tissue factor antibody clone with no anticoagulant effect

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## Key words

Drug delivery system (DDS), epirubicin, NC-6300, polymeric micelles, tissue factor (TF)

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Tissue factor (TF), an initiator of the extrinsic blood coagulation cascade, is over-expressed in different types of cancer. Tissue factor overexpression is also known as a poor prognostic factor in pancreatic cancer. We recently developed anti-TF antibody (clone1849)-conjugated epirubicin-incorporating micelles (NC-6300), and reported that this anti-TF1849-NC-6300 showed enhanced antitumor activity against TF-high expressed human pancreatic cancer cells, when compared with NC-6300 alone. However, clone 1849 antibody inhibited TF-associated blood coagulation activity. We studied another anti-TF antibody, clone 1859, which had no effect on blood coagulation and prepared anti-TF1859-NC-6300. In addition, to determine the optimum size of the antibody fragment to conjugate with NC-6300, three forms of the 1859 antibody (whole IgG, F[ab']<sub>2</sub>, and Fab') were conjugated to NC-6300. The antitumor effect of each anti-TF1859-NC-6300 was studied *in vitro* and *in vivo*, using two human pancreatic cancer cell lines, BxPC3 with high-expressed TF, and SUIT2 with low levels of TF. *In vitro*, all forms of anti-TF1859-NC-6300 showed higher cytotoxic effects than NC-6300 in BxPC3, whereas this enhanced effect was not observed in SUIT2. Likewise, all forms of anti-TF1859-NC-6300 significantly suppressed tumor growth when compared to NC-6300 in the BxPC3, but not in the SUIT2, xenograft model. Among the three forms of conjugates, anti-TF1859-IgG-NC-6300 had a higher antitumor tendency in TF-high expressed cells. Thus, we have confirmed an enhanced antitumor effect of anti-TF1859-NC-6300 in a TF-high expressing tumor; anti-TF1859-IgG-NC-6300 could be used to simplify the manufacturing process of the antibody–micelle conjugation for future clinical studies.

Drug delivery systems using nanoparticles are comprised of both passive and active targeting. Passive targeting can be achieved by an EPR effect.<sup>(1,2)</sup> In active targeting, mAbs or ligand(s) to tumor-related receptors are conjugated on the surface of nanoparticles to use the specific binding ability between an antibody and antigen/ligand and its receptor. NC-6300 is an epirubicin-incorporating polymeric micelle system and exerts an enhanced antitumor effect through the EPR effect.<sup>(3–6)</sup> We have recently developed an antibody-conjugated NC-6300 as a second generation micelle system; it is a hybrid of active and passive targeting.

Tissue factor, the trigger protein of extrinsic blood coagulation, is overexpressed in various human tumor cells and is involved in tumor proliferation, invasion, and metastasis.<sup>(7,8)</sup> In addition, overexpression of TF on tumor cells is closely related to poor patient prognosis in various cancers.<sup>(8–13)</sup> We previously developed an anti-TF antibody (clone 1849)<sup>(14)</sup> conjugated with NC-6300 (anti-TF1849-NC-6300) and showed that

it had superior antitumor activity when compared with NC-6300 alone.<sup>(15)</sup>

However, we know that the anti-TF1849 antibody inhibits the blood coagulating activity of TF and may cause a coagulation abnormality *in vivo*. Therefore, we obtained a new anti-TF-antibody, clone 1859, which has no anticoagulant activity. Using this clone, we developed anti-TF1859-NC-6300. In addition, to determine the optimum size of the antibody fragment to conjugate with NC-6300, we prepared three different forms of anti-TF1859-NC-6300: (i) anti-TF1859-IgG-; (ii) anti-TF1859-F(ab')<sub>2</sub>-; and (iii) anti-TF1859-Fab'-NC-6300. We used the three forms to study antitumor activities of these micelles in human pancreatic cancer cells.

## Materials and Methods

**Selection of anti-TF mAb based on the anticoagulant activity and affinity to human TF antigen.** To select a suitable anti-human TF mAb with less anticoagulant activity and strong

specific binding affinity, we screened three types of rat anti-human TF antibodies, which were produced by the hybridoma clones established previously.<sup>(14)</sup> To evaluate the anticoagulant activity of the newly developed mAbs, each mAb (1 nM) and recombinant human TF (62.5 pM) were reacted for 1 h at 37°C in a 96-well plate. Human coagulation factor VII and X were then applied to wells and reacted for 30 min at 37°C. At the termination of the reaction, a substrate specific to activated human coagulation factor X was applied, and the Xa production was estimated by measuring absorbance at 405 nm.

To evaluate the affinities, the antigen–antibody complex reactions of several anti-TF mAbs were assessed by surface plasmon resonance using Biacore T200 (GE Healthcare, Uppsala, Sweden). Recombinant human TF (1 µg/mL) in 10 mM sodium acetate was immobilized onto a Series S Sensor chip (CM5) to yield a binding signal of 20 resonance units, using an amine coupling kit. Various doses of human TF antibody (10–80 nM) diluted in running buffer (HBS-EP+ buffer; GE Healthcare) were then injected at a flow rate of 30 µL/min for 1 min of contact time through the sensor chip. The BIA evaluation software (GE Healthcare) that was obtained using the Langmuir 1:1 binding model was used to analyze the binding data. The affinity (KD) was calculated using the equation  $KD = \text{dissociation rate constant (Kd)}/\text{association rate constant (Ka)}$ .

**Test compounds.** NC-6300 was prepared by NanoCarrier Co., Ltd. (Kashiwa, Japan). Epirubicin was purchased from Pfizer Japan Inc. (Tokyo, Japan).

**Preparation of anti-TF1859-NC-6300.** Anti-TF1859-IgG (1 mg/mL) was prepared in 10 mM sodium acetate (pH 2.4) and 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After preheating at 37°C for 15 min, pepsin was added at protein : pepsin ratio of 200:1, the reaction mixture was incubated for 80 min, and the reaction was then stopped by adding 3 M Tris-HCl (pH 8.5). Gel filtration chromatography was carried out using Bio Logic Duo Flow (Bio-Rad, Hercules, CA, USA), and F(ab')<sub>2</sub> was isolated. Next, 1 mg/mL F(ab')<sub>2</sub> in 5 mM EDTA in PBS was reduced by

adding DTT at a final concentration of 5 mM. The mixture was then heated at 37°C for 30 min, dialyzed against 5 mM EDTA in PBS, and the final product, Fab' of the 1859 antibody, was produced. The final products were verified using SDS-PAGE.

The IgG, F(ab')<sub>2</sub>, and Fab' of the 1859 antibody were conjugated with NC-6300 based on our antibody/drug-conjugated micelle technology described previously<sup>(15)</sup> (anti-TF1859-IgG-NC-6300, anti-TF1859-F[ab']<sub>2</sub>-NC-6300, and anti-TF1859-Fab'-NC-6300, respectively). Diameters were measured with a Zetasizer Nano-ZS (Malvern, Malvern, UK).

**Clotting assay.** A total of 20 µL human recombinant TF 50 ng, anti-TF1859-IgG 5 µg, or anti-TF1859-(IgG, F[ab']<sub>2</sub> or Fab')-NC-6300 (equivalent to 5 µg anti-TF mAb), and ultrapure water were incubated at 37°C for 15 min using 96-well plates; 100 µL citrated normal human plasma and 100 µL CaCl<sub>2</sub> (25 mM) was then added, and the time to fibrin clot formation for each combination was measured. In the control group, PBS was added. Fibrin precipitation could be quantified by determining the absorbance at 350 nm, and the time to fibrin clot formation was determined when measured absorbance increased by 25% from baseline. The absorbance was measured every 45 s at 37°C using a SpectraMax plate reader (Molecular Devices Japan, Tokyo, Japan). The experiments were carried out in triplicate.

**Cell cultures and cell selection based on tissue factor expression.** The human pancreatic cancer cell lines BxPC3 and SUIT2 were purchased from ATCC (Rockville, MD, USA) and from the JCRB Cell Bank (Osaka, Japan) respectively. Both the cell lines were maintained in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and 100 units/mL penicillin, and 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B suspension (Wako) in a 5% CO<sub>2</sub> incubator at 37°C.

**Cellular uptake and affinity analysis.** Flow cytometry was carried out to examine the cellular affinity of antibodies and anti-TF1859-NC-6300 for BxPC3 and SUIT2. BxPC3 and SUIT2 were washed with PBS and detached by incubation with a non-enzymatic cell dissociation solution (×1) for 15 min at 37°C. Suspensions of cells with B.E. PBS were put into flow cytometry-compatible tubes (BD Biosciences, Franklin Lakes, NJ, USA) at 2 × 10<sup>5</sup> cells/tube. Suspensions were then centrifuged at 500 g for 5 min, and the supernatant was removed. The cells were incubated for 30 min on ice with primary antibodies and anti-TF1859-NC-6300 (equivalent to 0.5 µg mAb for 1 × 10<sup>6</sup> cells) diluted in B.E. PBS. Then, the cells were washed with B.E. PBS and incubated for 30 min on ice with goat anti-rat IgG-Alexa647 (Invitrogen, Grand Island, NY, USA) as a secondary antibody diluted in B.E. PBS. Controls contained only secondary antibody without primary antibody. After washing and staining with propidium iodide, the cells were applied to a flow cytometer (Guava EasyCyte; GE Healthcare). Multi-parametric analyses were carried out using FlowJo 7.5.5 software (Ashland, OR, USA).

**In vitro growth inhibition assay.** The growth-inhibitory effects were evaluated by a yellow-color formazan dye produced by

**Table 1. Human tissue factor (TF) activity assay of three mAbs**

Clone no.	Human TF activity, %
1849	5.0 ± 1.8
1006	86.9 ± 0.8
1859	98.5 ± 2.6
Control	100.0 ± 1.8

**Table 2. Affinity of three mAbs to human tissue factor antigen assessed by surface plasmon resonance**

Clone no.	Ka (1/Ms)	Kd (1/s)	KD (M)
1849	4.2.E+05	3.8.E–05	9.1.E–11
1006	1.5.E+05	1.2.E–04	8.3.E–10
1859	1.9.E+06	2.4.E–04	1.2.E–10

The affinity (KD) was calculated using the equation:  $KD = \text{dissociation rate constant (Kd)}/\text{association rate constant (Ka)}$ .

**Table 3. Particle size of NC-6300 and anti-TF1859-IgG-, anti-TF1859-F(ab')<sub>2</sub>-, and anti-TF1859-Fab'-NC-6300**

	NC-6300	Anti-TF1859-IgG-NC-6300	Anti-TF1859-F(ab') <sub>2</sub> -NC-6300	Anti-TF1859-Fab'-NC-6300
Mean particle diameter, nm	54.3 ± 0.24	58.3 ± 0.44	54.2 ± 0.76	48.4 ± 0.35

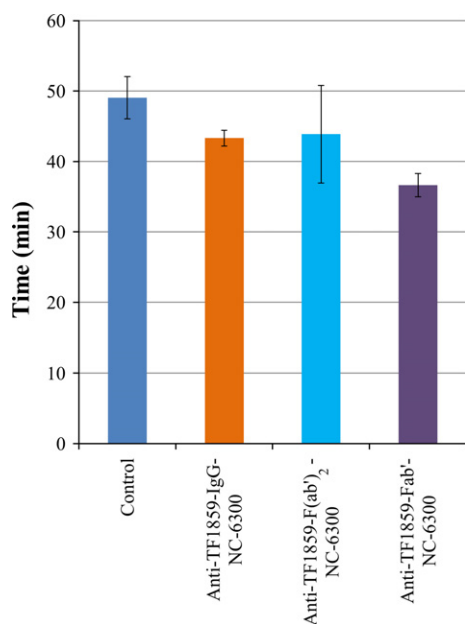
TF, tissue factor.

dehydrogenase activities in cells of tetrazolium salt (WST-8). BxPC3 and SUI2 were plated at 2000 cells in 100  $\mu$ L medium/well in 96-well plates and were incubated for 24 h at 37°C. The medium was removed, and cells with each compound (epirubicin, NC-6300, anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, or anti-TF1859-Fab'-NC6300) with medium at an epirubicin equivalent of 0.1, 1, 10, and 100 nM and 1, 10, and 100  $\mu$ M were then incubated for 96 h. At the termination of the incubation, the medium was removed, and 100  $\mu$ L of a 10% WST-8 solution was added to each well and incubated for 2 h at 37°C. Absorbance value at 450 nm was measured using the SpectraMax plate reader. As a result of these measurements, a 50% inhibitory concentration (IC<sub>50</sub>) was obtained.

**In vivo antitumor activity.** Female BALB/c nu/nu mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions in cages, provided with standard food, and given free access to sterilized water. Five-week-old mice were s.c. inoculated with  $1 \times 10^7$  BxPC3 cells or  $2 \times 10^6$  SUI2 cells in the flank region. Seven or 14 days after inoculation with BxPC3 or

SUI2, respectively, when the tumor size had reached approximately 180 mm<sup>3</sup>, the mice were randomly divided into six test groups consisting of five mice each (day 0). Mice were injected i.v. with the test compound through the lateral tail vein on days 0, 7, and 14. The test groups received anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, anti-TF1859-Fab'-NC-6300, NC-6300, or free epirubicin in saline. The concentration of each compound was 10 mg/kg epirubicin equivalents. The normal control group received saline. The length (*a*) and width (*b*) of the tumor masses and body weights were measured biweekly, and the tumor volume (TV) was calculated as follows:  $TV = (a \times b^2)/2$ . From an ethical point of view, animals in which the tumor volume exceeded 2000 mm<sup>3</sup> were euthanized. All animal experiments were carried out in compliance with the Guidelines for the Care and Use of Experimental Animals approved by the Committee for Animal Experimentation of the National Cancer Center, Japan.

**Statistics.** Clotting assay data were reported as mean  $\pm$  SD, using multiple comparison to a control (Dunnett's one-sided test). The IC<sub>50</sub> data were reported as mean  $\pm$  SD, using multiple comparison (Tukey's HSD, two-sided test). *In vivo* data was evaluated by *post-hoc* comparison, which is a repeatedly measured ANOVA followed by multiple comparison (Tukey's HSD, two-sided test). The level of significance for all the tests was set at  $P < 0.05$ . These analyses were carried out using spss version 18.0 (SPSS, Chicago, IL, USA).



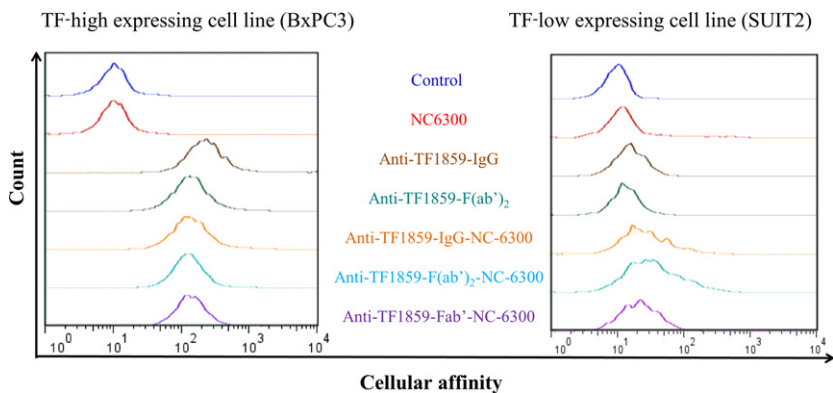
**Fig. 1.** Time to fibrin clot formation with anti-TF1859 antibody and anti-TF1859-IgG-NC-6300 was measured using a clotting assay. The time was determined when the absorbance at 350 nm increased by 25% from baseline. Data are expressed as the mean  $\pm$  SD;  $n = 4$ . TF, tissue factor.

## Results

**Selection of anti-TF mAb based on anticoagulant activity and affinity to human TF antigen.** Compared with controls, the human TF activity influenced by clones 1849, 1006, and 1859 were 5%, 87%, and 98%, respectively. The data indicated that anti-TF1859 possessed the lowest anticoagulant activity among mAbs tested (Table 1).

The affinities of the three clones were evaluated by surface plasmon resonance sensing. Clones 1849, 1006, and 1859 had the KD value of approximately  $9.1 \times 10^{-11}$  M,  $8.2 \times 10^{-10}$  M, and  $1.2 \times 10^{-10}$  M, respectively (Table 2). Clone 1849 had the highest and 1859 had the second highest affinities. The association constant (Ka) of 1859 was slightly higher than 1849, whereas its dissociation constant (Kd) was lower than 1849. Consequently, clone 1859 was selected because it had the least anticoagulant activity and the second highest affinity to the human TF antigen.

**Characteristics of micelles.** The mean particle diameters of anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, and anti-TF1859-Fab'-NC-6300 were  $58.3 \pm 0.44$ ,  $54.2 \pm 0.76$ , and  $48.4 \pm 0.35$  nm, respectively (Table 3). Those particular



**Fig. 2.** Comparative binding activities were determined with flow cytometry analysis among anti-TF1859-NC-6300 using BxPC3 (tissue factor [TF]-high expressing) and SUI2 (TF-low expressing) pancreatic cancer cells.



micelle formulations contained the same amount of epirubicin per micelle, and the numbers of antibodies tagged to micelles were two to four molecules per micelle in all three formulations.

**Clotting assay.** The time to fibrin clot formation in the incubation with anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, anti-TF1859-Fab'-NC-6300, and control were 43 ± 1, 44 ± 7, 37 ± 2, and 49 ± 3 min (*P* = 0.997, *P* = 0.995, and *P* = 1.000, compared to control), respectively (*n* = 4). The

three forms of anti-TF1859-NC-6300 did not prolong the fibrin clot formation time when compared with the control (Fig. 1).

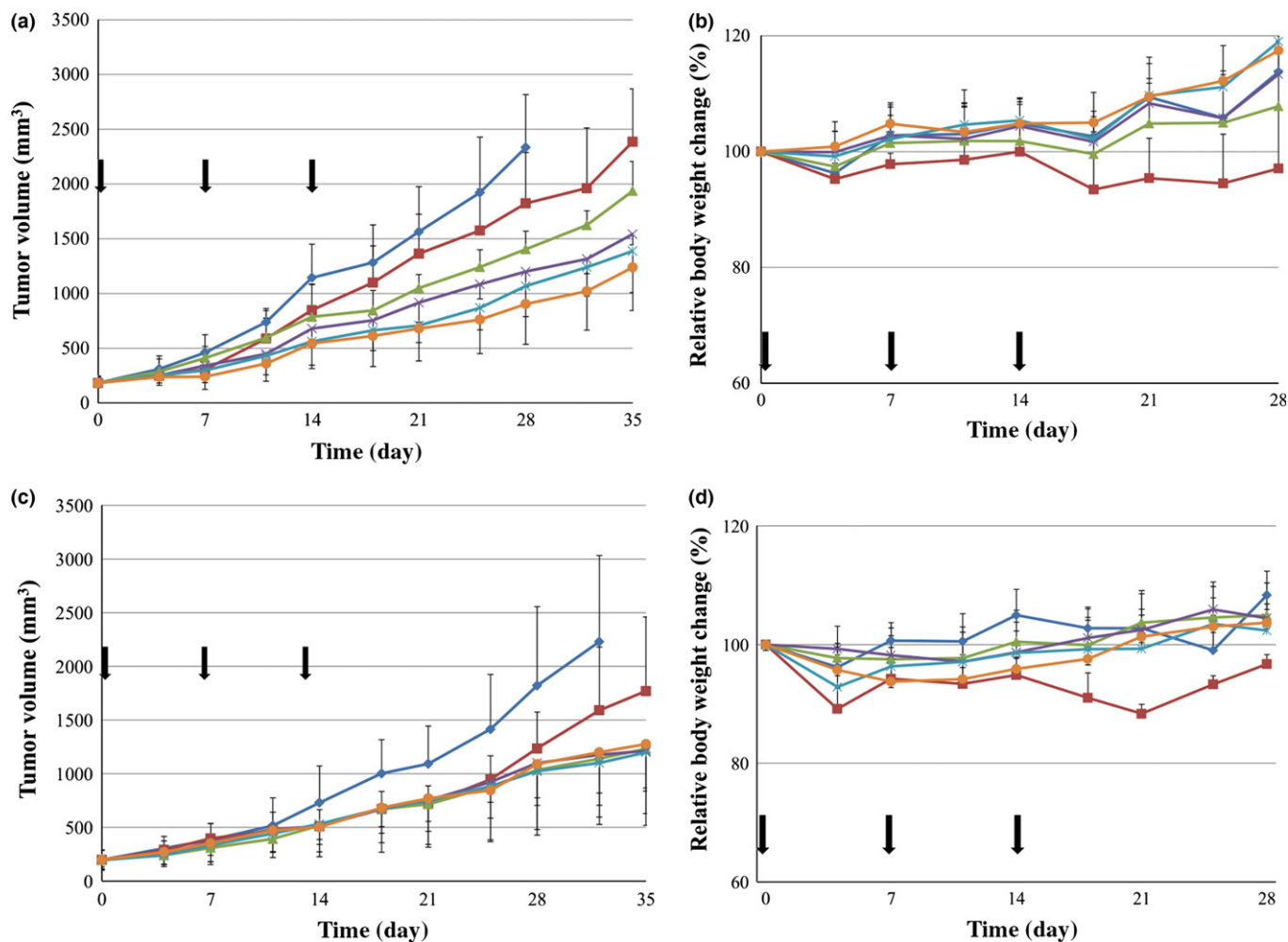
**Flow cytometry analysis of anti-TF1859-NC-6300.** Binding affinities of three formulations of anti-TF-NC-6300 conjugated with 1859 IgG, F(ab')<sub>2</sub>, and Fab' were almost the same as that of anti-TF mAb in BxPC3. The binding activities of the three formulations of anti-TF1859-NC-6300 and anti-TF1859 mAb to BxPC3 were approximately 10-fold higher than that to SUIT2. Otherwise, no significant differences were observed in terms of the binding activities of IgG, F(ab')<sub>2</sub>, and Fab' to BxPC3, whereas the affinities of anti-TF (IgG, F[ab']<sub>2</sub>, and Fab')-NC-6300 and NC-6300 were almost the same in the SUIT2 cell line (Fig. 2).

**In vitro cytotoxicity.** The IC<sub>50</sub> values of NC-6300, anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, and anti-TF1859-Fab'-NC-6300 for the BxPC3 cells were 329 ± 23 nM, 224 ± 60 nM, 233 ± 49 nM, and 183 ± 39 nM, respectively (Table 4). The three forms of anti-TF1859 (IgG, F[ab']<sub>2</sub>, and Fab')-NC-6300 were all superior to NC-6300 in the BxPC3 cell line in terms of cell killing effectiveness *in vitro* (*P* = 0.026, 0.043, 0.003, respectively). No significant differences were observed among the three forms of anti-TF1859-NC-6300 in the BxPC3 cell line. In contrast, the IC<sub>50</sub> values of NC-6300, anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-

**Table 4.** Inhibitory concentration values of various drugs in pancreatic cancer cell lines using WST-8 assay

Drug	BxPC3		SUIT2	
	IC <sub>50</sub> , nM	<i>P</i> -value	IC <sub>50</sub> , nM	<i>P</i> -value
Epirubicin	65 ± 2		145 ± 40	
NC-6300	329 ± 23		636 ± 13	
Anti-TF1859-IgG-NC-6300	224 ± 60	0.026*	662 ± 8	0.014*
Anti-TF1859-F(ab') <sub>2</sub> -NC-6300	233 ± 49	0.043*	642 ± 3	0.839*
Anti-TF1859-Fab'-NC-6300	183 ± 39	0.003*	634 ± 13	0.996*
Anti-TF1859-IgG	N/A		N/A	

\**P*-value versus NC-6300. N/A, not applicable; TF, tissue factor.



**Fig. 3.** *In vivo* tumor growth inhibition assay. Antitumor activities and changes in body weight in xenograft mice models in BxPC3 (a,b) and SUIT2 (c,d) pancreatic cancer cell lines. The treatment was given on days 0, 7, and 14 (*n* = 5). ♦, Control; ■, epirubicin; ▲, NC-6300; ×, anti-TF1859-Fab'-NC-6300; \*, anti-TF1859-F(ab')<sub>2</sub>-NC-6300; ●, anti-TF1859-IgG-NC-6300. Arrows, compound injections; bars, SD; points, mean.

NC-6300, and anti-TF1859-NC-6300 for the SUIT2 cells were  $636 \pm 13$  nM,  $662 \pm 8$  nM,  $642 \pm 3$  nM, and  $634 \pm 13$  nM, respectively (Table 4). The three forms of anti-TF1859 (IgG, F(ab')<sub>2</sub>, and Fab')-NC-6300 were not superior to NC-6300 in the SUIT2 cell line ( $P = 0.014, 0.839, 0.996$ , respectively).

**In vivo tumor growth inhibition assay.** The antitumor activities of anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, and anti-TF1859-Fab'-NC-6300 exceeded that of NC-6300 in the BxPC3 xenograft models ( $P = 0.021, P < 0.001, P < 0.001$ , respectively; Fig. 3a). Among anti-TF1859-NC-6300, anti-TF1859-IgG-NC-6300 versus anti-TF1859-F(ab')<sub>2</sub>-NC-6300 and anti-TF1859-F(ab')<sub>2</sub>-NC-6300 versus anti-TF1859-Fab'-NC-6300 were not significant. Anti-TF1859-IgG-NC-6300 was superior to anti-TF1859-Fab'-NC-6300 ( $P = 0.002$ ). In contrast, in SUIT2 xenografts, anti-TF1859-IgG-NC-6300, anti-TF1859-F(ab')<sub>2</sub>-NC-6300, and anti-TF1859-Fab'-NC-6300 were all equivalent to NC-6300 (Fig. 3c). There were no treatment-related deaths. No significant body weight loss was observed among any of the anti-TF1859-NC-6300 groups, NC-6300 groups, or control groups. However, the free epirubicin administration groups showed significant body weight loss compared with all the other groups in both BxPC3 and SUIT2 models (Fig. 3b,d).

## Discussion

In the present study, anti-TF1859-NC-6300 showed superior antitumor activity to NC-6300 in the TF-high expressing human pancreatic cancer model. This is similar to our previous report concerning anti-TF1849-NC-6300.<sup>(15)</sup> Anti-TF1849-NC-6300 was distributed throughout all of the tumor tissues and was efficiently internalized into tumor cells; it showed potent antitumor activity in TF-high expressing cancer cells, indicating that the conjugation of anti-TF mAb to micelles offered that advantage. The present study showed that no differences in tumor growth inhibition among three forms of anti-TF1859-NC-6300 and NC-6300 were observed in the xenograft SUIT2. The sizes of the three forms of anti-TF1859-NC-6300 ranged from 50 to 60 nm, which was not very different from the 50 nm size of non-modified NC-6300. This result indicates that the slightly larger size of anti-TF1859-NC-6300 did not affect the EPR effects within these size ranges. In the TF-high expressing BxPC3 xenograft, anti-TF1859-IgG-NC-6300 tended to have a superior effect when compared to the Fab' fragment forms, although there were no significant differences among the three types of the antibody-micelle conjugates. To simplify the manufacturing process of the mAb-micelle conjugation, it is reasonable to select the whole IgG of 1859 mAb for further studies. In addition, as previously reported,<sup>(15)</sup> we showed that a higher tumor accumulation of anti-TF-NC-6300 compared to NC-6300 was seen, regardless of the TF expression levels. In order to examine the immunological and pharmacokinetic effects by the formulation of various immunoconjugates, we will have to analyze pharmacokinetic parameters of total epirubicin (micelle-encapsulated epirubicin and free epirubicin) and free epirubicin in plasma for future clinical pharmacology. Although we are developing a chimeric

human mAb of the clone used in this study, difference in antigenicity of antibodies (IgG, F(ab')<sub>2</sub>, and Fab') on micelles should also be evaluated before the clinical trial using a non human primate model.

As a possible adverse effect of anti-TF1849-NC-6300, a tendency towards bleeding is of concern, as anti-TF1849 has anticoagulant activity. However, we are not sure whether it actually causes a bleeding tendency in clinical use. Our present data indicated that anti-TF1859 mAb and its conjugated micelles may not cause a bleeding tendency in humans because they have no anticoagulant activity in human blood. Unfortunately, both anti-human TF antibodies, clones 1849 and 1859, used in this study, have no cross-reactivity with mouse TF; thus, we could not assess their influence on mouse blood coagulation *in vivo*. Therefore, this issue should be evaluated in clinical studies.

In contrast with results in oncology patients, hematuria was observed in 44% patients who were treated with anti-TF antibody for acute respiratory distress syndrome in a phase 1 clinical trial.<sup>(16)</sup> With regard to solid tumors, anticancer drugs conjugated with human TF antibody were developed in pre-clinical studies.<sup>(17,18)</sup> Safety and toxicity concerns are currently under evaluation in the first in-human clinical trial.<sup>(17)</sup>

In conclusion, anti-TF1859-NC-6300 has a low risk of bleeding because of its low anticoagulant activity, and it has a potent antitumor effect in a human pancreatic cancer model, especially TF-high expressing tumors. Results from the present study warrant a comparative clinical evaluation of efficacy and safety between anti-TF1859-IgG-NC-6300 and anti-TF1849-IgG-NC-6300.

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## Disclosure Statement

The authors have no conflict of interest.

## Abbreviations

B.E. PBS	PBS containing 2 mM EDTA and 0.1% BSA
EPR	enhanced permeability and retention
HSD	honest significant difference
Ka	association constant
Kd	dissociation constant
KD	affinity
TF	tissue factor
TV	tumor volume

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