Abstract

Purpose: In the pretargeted antibody-guided radioimmunotherapy (PAGRIT) system, the combined use of two different antibodies directed against the same tumor antigen could represent a valid approach for improving tumor targeting and therapeutic efficacy. We developed a novel monoclonal antitenascin antibody, ST2485, and studied its biochemical and functional properties by in vitro and in vivo assays. We then investigated the first of the three-step therapy combining ST2485 with another antitenascin antibody, ST2146, previously described, to increase accumulation of biotinylated antibodies at the tumor site.

Experimental Design: Studies of immunoreactivity, affinity, immunohistochemistry, and biodistribution in xenograft model were carried out on ST2485. Analysis of the ST2485 and ST2146 combination was preliminary carried out by ELISA and BiaCore tests and then by in vivo distribution studies after administration of the radiolabeled biotinylated antibodies, followed by a chase with avidin as clearing agent.

Results: ST2485 was found to be a suitable antibody for therapeutic applications. Indeed, for its behavior in all tests, it was comparable with ST2146 and better than BC2, an antibody already used for clinical trials. The additivity of ST2146 and ST2485 in tenascin C binding, shown by in vitro tests, was confirmed by biodistribution studies in a xenograft model where tumor localization of the antibodies was near the sum of each antibody alone, with a tumor-to-blood ratio higher than 24.

Conclusion: The results reported in this study suggest that a monoclonal antitenascin antibody mixture can improve tumor targeting. This strategy could represent progress for therapeutic approaches such as PAGRIT.

Radioimmunotherapy using monoclonal antibodies (mAb) is a first-line strategy in cancer treatment because of its ability to specifically target cancer cells. The pretargeted antibody-guided radioimmunotherapy (PAGRIT) system allows the sequential administration of a biotinylated mAb, avidin/streptavidin, and a radiolabeled biotin molecule, leading to specific accumulation of radioactivity at the tumor site implemented by the multiple valence of avidin/streptavidin toward biotin.

Among the several tumor antigens identified as possible targets for antibody mediated therapy, tenasin C seems to be a good candidate for the treatment of several solid tumors (1, 2). Tenasin C is an extracellular hexameric glycoprotein whose monomer is composed of repeating epidermal growth factor (EGF)-like units followed by fibronectin-type repeats and a domain with homology to fibrinogen at the carboxy terminus. Alternative splicing in the A-D region of the fibronectin-type repeats gives rise to monomers of different sizes, whose longer form has been correlated with tumor phenotype (3). In the brain tumor, tenasin C is predominantly present in the extracellular matrix and the hyperplastic blood vessels, suggesting some role in the neovascularization of malignant gliomas (4). Both direct and pretargeted therapeutic approaches with labeled BC2, BC4, and 81C6 antitenascin antibodies have already shown promising results (5–11).

The pretargeting approach peculiarity is that it allows the injection of a high dose of antibody whose excess can be cleared by a chase procedure with a clearing agent preceding the administration of the effector drug. However, the limit to the drug amount administered is the saturating dose (i.e., the antibody amount above which the tumor/nontumor tissue ratios decrease and a toxic effect might be observed). The use of a mixture of tumor-saturating antibodies recognizing the same macromolecular antigen represents an interesting opportunity to increase tumor targeting. The administration of a cocktail of BC2 and BC4 antibodies benefited patients with grade III or IV gliomas (5, 6, 8) or with relapsed ovarian cancer (12). However, these antibodies were found unsuitable for further clinical trials owing to problems met during the development...
of their manufacturing process. We recently described (13) a novel antitenascin antibody, ST2146, which exhibits the highest tumor-targeting capacity in nude mice HT2 xenograft model compared with other monoclonal antitenascin antibodies, including BC2 and BC4. The HT2 model could be representative of those solid tumors where the pretargeting therapy with antitenascin C mAbs might be less effective compared with glioblastoma, which exhibits higher antigen density. In this context, where the tumor antigen is a limiting element, a combined approach would be useful to strengthen the targeting obtained by a single antibody.

81C6 and BC2 antibodies are known to bind epitopes located in the A-D splicing region of tenascin (9, 14). This region can, therefore, be considered a good target for the production of new mAbs.

We report herein the development and functional characterization of a novel antitenascin A-D fragment antibody, ST2485, and in vitro and in vivo studies aimed at demonstrating the potential use of biotinylated ST2146 and ST2485 combination in pretargeting.

**Materials and Methods**

**ST2485 development.** BALB/c mice were immunized with the recombinant A-D fragment of human tenascin (kindly provided by Dr. Luciano Zardi, Laboratory of Cell Biology, National Institute for Cancer Research, Genoa, Italy). Mice splenocytes were fused with nonproducing Sp2/0-Ag14 immunoglobulin myeloma cells (obtained from European Collection of Animal Cell Cultures, CBA, Genoa, Italy) by standard methods (15). The population of hybridomas obtained was screened by an ELISA test on tenascin purified from culture supernatant of SK-Mel 28 (human melanoma cell line obtained from Interlab Cell Line Collection, Genoa, Italy). The selected hybridomas secreting antitenascin antibodies were cloned twice through limiting dilution in a growth medium containing FCS and twice in a medium without animal proteins (Animal Derived Component Free Medium HyClone, HyQ Perbio). Two subsequent limited dilution subclonings led to the selection of the cST2485 hybridoma cell line used for the production of the Master Cell Bank and Working Cell Bank. Master Cell Bank samples were deposited at the Interlab Cell Line Collection (Genoa, Italy) for patent purposes (patent application RM2004A000105). The production of mAbs ST2485 was done by cultivation of ST2485 hybridoma cells in protein-free medium, in a 2 L perfusion bioreactor (MD2; B. Braun). The stability of cST2485 Postproduction Cell Bank was confirmed by limiting dilution. The ST2485 isotype was determined by the use of a commercial kit (mouse-hybridoma subtyping kit, Boehringer Mannheim, Monza, Italy).

**ST2485 purification and biotinylation.** Antibody purification was achieved by four chromatographic steps according to standard protocols using MEP HyperCell column (Biosepa, Fremont, CA), Q-Sepharose-XL column (Pharmacia, Milan, Italy; pH 6), SP-Sepharose-XL column and, finally, a Q-Sepharose-XL column (pH 9). The antibody in sodium bicarbonate buffer (pH 8.5) was biotinylated by adding a molar excess (10:1) of biotin- LC-NHS (Pierce, Rockford, IL) in DMSO to a 5 to 10 mg/mL mAb solution. After 1 hour at room temperature, the mixture was ultrafiltered on a 30,000 molecular weight cutoff membrane to separate the antibody from all reagents. The level of biotinylation (biotin-to-mAb molar ratio) was determined by the Green spectrophotometric method (16).

**Immunoreactivity.** Immuno-MAXISORP 96-well plates (Nunc, Rochester, NY) were coated at 4°C overnight with 100 μL/well of 0.5 μg/mL tenascin in PBS (pH 7.2) or recombiant A-D fragment 50 μg/mL. The plates were washed once with PBS-0.1% Tween 20 (washing buffer) and blocked with 300 μL/well of PBS, 0.1% Tween 20, 1% bovine serum albumin (blocking and diluting buffer) for 2 hours at room temperature. Plates were used immediately or dried and frozen at −20°C. The plates were incubated with serial dilutions of antitenascin mAbs (100 μL/well) for 1.5 hours at 37°C. Each dilution was assayed in duplicate. After three washings, the plates were incubated for 1.5 hours at 37°C with 100 μL/well of anti-mouse IgG (Fc-specific) alkaline phosphatase-conjugated (Sigma, St. Louis, MO) diluted 1:1,000 in blocking buffer. The plates were then washed four times, incubated with 200 μL/well of para-nitrophenylphosphate (Sigma) for 30 minutes at 37°C, stopped with 100 μL/well of 3 mol/L NaOH, and read at 405 nm with an ELISA spectrophotometer (Sirio S, SEAC, Florence, Italy).

**Competition ELISA.** The biotinylated mAb ST2485 alone or with increasing concentrations of unbiotinylated ST2485, BC2 (kindly provided by Dr. L. Zardi), or an unrelated IgG1 (Sigma) as competitor were dispensed (100 μL/well) on a tenascin-coated plate. Optimal concentrations of tenascin and biotinylated ST2485 were determined in preliminary experiments. After 2 hours at room temperature, the plate was washed thrice and incubated for 30 minutes at room temperature with 100 μL/well of horseradish peroxidase-streptavidin (Amersham, Uppsala, Sweden) diluted 1:1,500 in blocking buffer. After three washings, the plate was incubated for 30 minutes at room temperature with 200 μL/well of TMB substrate (Sigma); the reaction was stopped with 100 μL/well of 0.5 mol/L H2SO4 and the plate read at 405 nm with an ELISA spectrophotometer (Sirio S, SEAC).

**Epitope mapping.** Epitope mapping experiments were carried out by using seven peptides, 15 amino acids in length, with a short (three amino acids) overlapping sequence that were synthesized by the automatic solid phase method using a Model 431 A peptide synthesizer (Perkin-Elmer, Boston, MA). The peptides were N-terminally biotinylated during synthesis, purified by reversed-phase HPLC, analyzed by electrospray-mass spectrometry, and tested for binding to ST2485 by a solid phase assay as follows. Microtiter plates (Falcon, San Jose, CA) were coated with 100 μL/well mAb ST2485 at 50 μg/mL in PBS (pH 7.5) for 16 hours at 4°C. After washings with PBS, the wells were saturated with 200 μL/well of PBS containing 3% dried milk for 2 hours at room temperature. The plates were washed thrice, then incubated with 100 μL/well of biotinylated peptide solutions of different concentrations (200-100 to 50-10 μg/mL) in PBS containing 0.5% dried milk for 1 hour at 37°C. The washed plates were then added with 100 μL/well of a streptavidin-peroxidase conjugate (Sigma) diluted 1:1,000 in PBS-0.5% dried milk and, after 1 hour incubation, developed with a chromogenic substrate solution consisting of 0.2 mg/mL ABTS in 0.1 mol/L citrate buffer (pH 5.0) and 5 mmol/L hydrogen peroxide. The absorbance at 405 nm was measured with a Model 2250 ELA Reader (Bio-Rad, Hercules, CA).

For dot blot assay, 1 μL aliquots of an ST2485 solution (7 mg/mL) were spotted on small pieces of nitrocellulose membrane; after 15 minutes at room temperature, each membrane was saturated with 2 mL of blocking solution (PBS-5% dried milk) for 16 hours at 4°C, then incubated with each biotinylated peptide, 100 μg/mL in PBS-3% dried milk. After 1.5-hour incubation at room temperature, the membranes were washed five times with the same buffer and then incubated with 1 mL of a streptavidin-peroxidase conjugate (Sigma) diluted 1:25,000 in PBS-3% dried milk for 1 hour at room temperature. The membranes were washed five times with the same buffer, then incubated with the chemiluminescent peroxidase substrate luminol and the signal detected using the standard enhanced chemiluminescence kit (Sigma), according to the instructions of the manufacturer.

**BiaCore analysis.** BiaCoreX instrument, CM5 sensor chip, HBS buffer [10 mmol/L HEPES, 0.15 mol/L NaCl, 3.4 mmol/L EDTA, and 0.005% surfactant P20 (pH 7.4)], amine coupling kit (N-hydroxysuccinimide, N-ethyl-N'-dimethylaminopropylcarbodiimide), and ethanolamine were all obtained from Biosense (Milan, Italy).

A-D and the recombinant A-D fragment were immobilized on CM5 sensor chips according to standard amino coupling procedure. Briefly, the carboxymethylated dextran-coated surface was activated by a 7-minute injection of a solution containing 200 mmol/L...
Improved Tumor Targeting by Two Antitenascin mAbs

N-ethyl-N′-dimethylaminopropylcarbodiimide and 50 mmol/L N-hydroxysuccinimide; then, tenascin (82 μg/mL in 10 mmol/L sodium acetate brought to pH 2.3 with HCl) or A-D fragment (10.6 μg/mL in 10 mmol/L sodium acetate pH 3) was injected. A continuous flow of HBS at 5 μL/min was maintained and capping of unreacted sites was achieved by injecting 1 mol/L ethanolamine (pH 8.5). Two tenascin chips were generated giving final immobilization responses of 2.700 and 670 resonance units corresponding to 2.7 and 0.67 ng/mm², respectively. The A-D fragment chip gave a signal of 300 resonance units corresponding to 0.3 ng/mm² antigen density.

Sensorgrams for kinetic measurements were generated by the injection of mAbs (at five concentrations ranging from 3.9 to 500 nmol/L) in HBS at a flow rate of 30 μL/min. The binding was not affected by the flow rate (tested between 5 and 30 μL/min), suggesting no mass transport limitation for mAb binding. Kinetic data were collected in duplicate for each mAb concentration. The association and dissociation times were 120 and 180 seconds, respectively. The chip was regenerated by injection of repeated pulses of 100 mmol/L NaOH until the difference between the baselines before and after NaOH injection was <10 resonance units. Biosensor data were prepared, modeled, and fitted by means of the BIAevaluation 3.1 software. Evaluation of the data was done using a bivalent-analyte model with simultaneous determination of association and dissociation constants. The quality of the fitted data was evaluated by comparison calculated and experimental curves (residual values) and by the magnitude of the x² parameter.

The additive binding of biotinylated ST2146 and ST2485 to tenascin was tested by successively injecting each antibody at saturating concentration (3.7 μmol/L) and measuring the resonance units after dissociation occurred. The streptavidin-binding capacity was measured after injections of biotinylated antibodies followed by 30.7 μmol/L streptavidin (Biospa, Milan, Italy).

Immunohistochemistry. Immunohistochemistry studies on cryostatic sections of various human tumors and normal tissues were conducted in collaboration with Prof. Spagnoli (Tor Vergata University, Rome, Italy). Tissue slices were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. The tissue sections were dewaxed, incubated overnight at 4°C with the primary antibody, and further washed with the avidin-biotin-peroxidase complex for 30 minutes. After three washings with PBS, 3,3′-diaminobenzidine substrate was added and the reaction was stopped after 2 minutes by washing with tap water. Counterstaining was done with Mayer’s hematoxylin for 10 seconds. Negative controls included slides incubated with an isotype-matched, nonrelevant mAb or with the second antibody alone.

The ST2485 cross-reactivity toward murine tenascin was tested on paraffin-embedded sections of LMM3 murine breast tumor and normal murine small intestine tissue. After deparaffinization and hydration, the sections were processed as above using 10 μg/mL ST2485.

Radio labeling of monoclonal antibodies. The Greenwood et al. (17) chloramine T method was used for radiolabeling. Antibodies at 200 to 400 μg/mL in PBS buffer were mixed with chloramine T (Sigma) at a final concentration of 2 to 10 μg/mL and with 1 to 4 μCi (37-148 KBq) of Na¹²⁵I (New Life Science, Uppsala, Sweden) per microgram of antibody. After 5 to 10 minutes at room temperature, sodium metabisulfite, five times the concentration of chloramine T, was added to stop the reaction; free iodine and other reagents were removed by buffer exchanging thrice with sterile PBS on Vivaspin membrane, 30,000 molecular weight cutoff (Sartorius, Florence, Italy). Immunoreactivity of radiolabeled antibodies was checked by ELISA as described in the previous section with plates coated with tenasin at 1.0 μg/mL.

Xenograft model. BALB/c nu/nu mice (Charles River, Wilmington, MA) were transplanted s.c. with 5 × 10⁵ HT29 human colon carcinoma cells (obtained from DSMZ, Braunschweig, Germany) in 0.1 mL of PBS. After 15 days, mice were randomized into treatment groups (five animals per group) and i.v. injected with 125I-biotinylated ST2485 or 125I-biotinylated ST2146 (1-2 × 10⁶ cpm/animal) separately or together. Radiolabeled murine IgGs (Sigma) were used as a control. Twenty-four hours before the sacrifice, an avidin chase, 0.25 to 100 times the amount of the injected antibodies, was done. Five days after the antibody injection, mice were sacrificed by cervical dislocation and blood, spleen, kidney, liver, and tumor samples were collected. Each tissue was weighed, counted in a γ-counter (Packard, Camberra Schwadorf, Austria) and percentage of injected dose per gram of tissue (%d/g) was determined.

The care and husbandry of animals were in accordance with European Directive 86/609 and Italian legislation.

Results

ST2485 biochemical characterization

The ST2485 antibody was generated by immunizing BALB/c mice with the recombinant A-D fragment of tenasin C. After six rounds of cloning, the hybridoma cell line cST2485 was finally selected and the antibody purified from the protein-free culture broth.

<table>
<thead>
<tr>
<th>mAb</th>
<th>kₐ (mol/L⁻¹ s⁻¹) ± SE</th>
<th>kᵣ (s⁻¹) ± SE</th>
<th>Kₒ₁ (mol/L) ± SE</th>
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<tr>
<td>ST2485</td>
<td>6.02E+05</td>
<td>5.88E-04</td>
<td>9.77E-10</td>
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<tr>
<td>ST2485 biot</td>
<td>1.97E+03</td>
<td>8.23E-06</td>
<td></td>
</tr>
<tr>
<td>ST2146</td>
<td>2.80E+06</td>
<td>8.07E-04</td>
<td>2.88E-09</td>
</tr>
<tr>
<td>ST2146 biot</td>
<td>2.98E+03</td>
<td>1.72E-05</td>
<td></td>
</tr>
<tr>
<td>BC2</td>
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<tr>
<td>BC2 biot</td>
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<td>6.40E-05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00E+03</td>
<td>8.05E-05</td>
<td>5.40E-09</td>
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</tr>
<tr>
<td></td>
<td>8.91E+01</td>
<td>5.48E-05</td>
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Table 1. BiaCore kinetic values of ST2485, ST2146, and BC2, and their biotinylated counterparts
The supernatant was obtained through four chromatographic steps. The antibody isotype was IgG1/k as determined by ELISA (data not shown).

The immunoreactivity of ST2485 was evaluated on a coating of tenascin C and compared with BC2, an anti-A-D fragment antibody, and ST2146, an antibody against the EGF-like repeats of tenascin C. ST2485 and ST2146 showed similar reactivity toward the large tenascin C form, whereas the concentration of BC2 necessary to obtain 1.0 absorbance was >10 times the concentration of ST2485 (Fig. 1).

The affinity of ST2485 toward tenascin C or A-D fragment was estimated by surface plasmon resonance (BiaCore) following the bivalent model evaluation. On the tenascin C–coated chip (0.67 ng/mm²) $K_D$ of ST2485 was $9.77 \times 10^{-10}$ mol/L ($k_{a1} = 6.02 \times 10^5; k_{d1} = 5.88 \times 10^{-4}$); on the Tn(A-D)–coated chip (0.3 ng/mm²) $K_D$ of ST2485 was $8.95 \times 10^{-10}$ mol/L ($k_{a1} = 3.34 \times 10^5; k_{d1} = 2.99 \times 10^{-4}$). The effect of biotinylation (7-9 biotins/mAb) on immunoreactivity and affinity of the antibody was also evaluated. The immunoreactivity

![Fig. 2. ST2485 immunostaining of cryostatic sections derived from breast cancer and colon carcinoma tissues. The interstitial connective tissue is positively stained with ST2485, more homogeneously in breast carcinoma (A) or with a focal staining in colon carcinoma tissue (B) where blood vessels are also stained.](image)
of biotinylated ST2485 was found not significantly different from that of the unbiotinylated form by both ELISA (80% residual activity; data not shown) and BiaCore measurements that gave a value of $K_{D1} = 2.88 \times 10^{-9} \text{ mol/L}$ near to that of the unmodified ST2485. Table 1 shows the kinetic values of biotinylated and unbiotinylated ST2485, ST2146, and BC2 determined on tenascin C–coated chips. The affinity constants estimated for biotinylated and unbiotinylated ST2485 are comparable with those of ST2146, whereas both forms of BC2 have ~100 times less affinity to the antigen compared with the other mAbs. Immunohistochemical studies with ST2485 on cryostatic sections of different human tumors (breast, lung, and colon) have shown strong extracellular matrix staining for all the tissues (Fig. 2). Only a weak staining on the normal control tissues was observed (data not shown) as previously reported for other antitenascin C mAbs (2). Additionally, a study on the LMM3 murine tumor and normal murine tissue showed that ST2485 cross-reacted with murine tenascin (data not shown). This cross-reactivity was not surprising because within the tenascin C sequence containing the ST2485 epitope, human and mouse only differ in one amino acid (arginine versus lysine, both hydrophilic).

A competition ELISA was done between ST2485 and BC2 to verify any epitope sharing by the two antibodies. A 40% inhibition of BC2 binding to tenascin C or Tn(A-D) was shown by using increasing amounts of ST2485, which suggests a partial overlapping of the two epitopes (data not shown). Based on these results, the epitope mapping of ST2485 could be narrowed to the “pTN1” region where the BC2 epitope was already localized (14). Due to ST2485 cross-reactivity with murine tenascin, a sequence of 54 amino acids spanning A3 and A4 regions could also be excluded from the analysis because it is not shared by human and mouse. ST2485 epitope was therefore mapped at the edge of A4 and B repeats as evidenced by both dot blot and ELISA analyses done with the overlapping peptides described in Fig. 3. In both tests, the strongest signal was

![Diagram of tenascin structure and epitope location of some relevant antibodies with zooming on the region scanned for ST2485 binding. The biotinylated peptides were hybridized with ST2485 spotted onto nitrocellulose membrane or coated onto 96-well plates: Peptide number 6 gave the strongest signal in both tests.](Fig.3.
Tenascin structure and epitope location of some relevant antibodies with zooming on the region scanned for ST2485 binding. The biotinylated peptides were hybridized with ST2485 spotted onto nitrocellulose membrane or coated onto 96-well plates: Peptide number 6 gave the strongest signal in both tests.)

**Table 2.** Biodistribution study on HT29 xenograft of ST2485 (2 μg/mouse) with or without clearing step using avidin at different avidin-to-antibody ratios

<table>
<thead>
<tr>
<th>Organ</th>
<th>No Chase</th>
<th>Chase 1/4</th>
<th>Chase 1/1</th>
<th>Chase 5/1</th>
<th>Chase 25/1</th>
<th>Chase 100/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.80 ± 0.79</td>
<td>3.07 ± 0.23</td>
<td>2.67 ± 0.42</td>
<td>1.86 ± 0.42</td>
<td>0.83 ± 0.24</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.06 ± 0.19</td>
<td>1.26 ± 0.05</td>
<td>1.43 ± 0.24</td>
<td>1.23 ± 0.27</td>
<td>0.74 ± 0.17</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.94 ± 0.15</td>
<td>0.88 ± 0.07</td>
<td>0.83 ± 0.10</td>
<td>0.69 ± 0.13</td>
<td>0.42 ± 0.07</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>0.85 ± 0.14</td>
<td>0.99 ± 0.08</td>
<td>0.96 ± 0.15</td>
<td>0.74 ± 0.16</td>
<td>0.46 ± 0.09</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Tumor</td>
<td>8.20 ± 1.20</td>
<td>8.46 ± 0.33</td>
<td>10.66 ± 1.37</td>
<td>8.83 ± 1.26</td>
<td>7.31 ± 1.29</td>
<td>9.35 ± 1.02</td>
</tr>
</tbody>
</table>

*The percentage of injected dose per gram of tissue ± SD is reported for tumor and other organs.*
obtained with peptide 6, whereas weaker reactions were observed with peptides 2 and 7 (data not shown).

**ST2485 in vivo studies**

Biodistribution studies of biotinylated ST2485-labeled with $^{125}$I were carried out on nude mice implanted with the tenascin-expressing human tumor cell line HT29. In preliminary biodistribution experiments, we have investigated the influence of a clearing step with avidin at different ratios. As shown in Table 2, in the absence of chasing or using an avidin-to-antibody ratio ranging from 1:4 to 100:1, the percentage of injected antibody localized at the tumor site remained constant, independently of the avidin dose, whereas for all the other organs a significant decrease of nonspecific binding, proportional to the avidin dose, could be observed. Based on these results, an avidin chase, 100 times the amount of the injected antibodies, was done in later experiments.

Four doses ranging between 0.5 and 5 $\mu$g of ST2485 and control mouse IgGs were injected. Like ST2146 (13), the optimal dose for ST2485 was 2 $\mu$g/mouse, at which high tumor accumulation combined with lower background. The results reported in Fig. 4A show that, after 5 days, ST2485 is specifically localized in the tumor with a plateau reached at 2 $\mu$g/mouse. This represents the saturating dose in this model because it showed the most favorable tumor/nontumor ratios among those reported in Fig. 4B with values of 15.59, 2.25, 10.73, and 18.78 for blood, spleen, kidney, and liver, respectively.

**Additivity of ST2485 and ST2146**

*In vitro studies.* To evaluate a possible combined use of ST2146 and ST2485 in PAGRIT, the absence of interference between ST2485 and ST2146 and the additivity of their binding was preliminary shown by ELISA (data not shown).

The *in vitro* additivity of the two antibodies was also tested by surface plasmon resonance, consecutively injecting the two antibodies at saturating concentrations. As shown in Fig. 5, ST2485 and ST2146 are capable of binding additively to tenascin C with a resonance signal equal to the sum of the signals of the single antibodies (Fig. 5A). Using the same experimental system, the pretargeting capacity of the two biotinylated antibodies was then evaluated by streptavidin binding. The experiment was carried out on chips at high (2.7 ng/mm$^2$; Fig. 5B and C) and low (0.67 ng/mm$^2$) antigen density (not shown) to evaluate any interference from the simultaneous binding of the two antibodies. In both cases, the amount of streptavidin captured by the two antibodies bound to the tenascin C–coated chip was twice that bound to a single reagent, suggesting absence of steric hindrance.

*In vivo studies.* An *in vivo* study of ST2485 and ST2146 additivity was also carried out using the previously described animal model. The antibodies were administered at the single or combined tumor-saturating dose of 2 $\mu$g per antibody per mouse; 24 hours before the sacrifice, an antibody clearing chase with 100 times the excess of avidin was done and, at day 5, the animals were sacrificed and radioactivity in tumor, blood, spleen, liver, and kidney was measured. The results are shown in Fig. 6A, expressed as nanogram of antibody per gram of tumor. The combination of the two radiolabeled biotinylated antibodies shows a tumor localization that almost equals the sum of the single antibody values confirming the additivity of the *in vitro* results. The percentage of injected combined dose per gram of tumor was 14.18. No interference from mouse IgGs on the localization of ST2485 or ST2146 was observed. The tumor/nontumor ratios for the radiolabeled antibodies combination reported in Fig. 6B were 24.5, 11.47, 30.36, and 38.49 for blood, spleen, kidney, and liver, respectively.

**Discussion**

In the present study, we describe the development and characterization of a novel antitenascin antibody, ST2485, and its use either as single agent or in combination with another antitenascin mAb, ST2146, for pretargeting applications. Like BC2 and 81C6, two antitenascin antibodies already used in clinical trials, ST2485 does not recognize the short form of the tenascin C molecule lacking the entire A-D region, whereas binding to the large tenascin C form preferentially expressed in malignant tissues. ST2485 is a highly affine antibody with immunoreactivity toward tenascin C far higher than that of BC2 antibody and similar to that observed for ST2146. Its affinity at picomolar level with minimal reduction after biotinylation renders this antibody a valid candidate for pretargeting purposes. Furthermore, its biodistribution in the xenograft model evidenced a good tumor/nontumor ratio (Fig. 4) despite the cross-reactivity of ST2485 versus murine tenascin expressed in normal tissues.
It has been reported that the BC2 epitope maps in a region comprising the entire A4 domain as well as a portion of the surrounding A3 and B domains (14). Our competition ELISA results showed that ST2485 binds human tenascin at an epitope partially shared with BC2. We also showed that ST2485 cross-reacts with murine tenascin, which lacks the large portion of the domain interested in BC2 binding, a fact that narrowed the search for the ST2485 epitope. By using overlapping peptides, we mapped the antibody-binding site into a sequence of 15 amino acids at the conjunction of A4 and B domains.

The three-step PAGRIT strategy is based on biotinylated antibody accumulation at the tumor site and subsequent clearance from normal tissues by a chase step with a clearing agent, before streptavidin and then a biotin-conjugated radionuclide are administered to the patient. In this context, achievement of optimal tumor saturation is an important issue and the use of combined antibodies directed to one or more tumor-associated antigens could represent a valid mean to enhance tumor localization of the effector molecule (18).

We investigated the first of the three-step therapy combining ST2146 and ST2485 to maximize accumulation of biotinylated antibodies at the tumor site. Indeed, the large size of tenascin C molecule and its high density in tumor environment might represent favorable conditions for using several antibodies...
directed against different epitopes. A cocktail of BC2 and BC4 as pretargeting antibodies has already been used in clinical trials involving patients with grade 3 or 4 gliomas (5, 6, 8) or with relapsed ovarian cancer (12) with encouraging results.

Absence of mutual interference of the ST2146 and ST2485 mAbs in tenasin C binding has been verified here and their additivity assessed by both ELISA and surface plasmon resonance tests. We also evaluated, through the use of biotinylated antibodies, the streptavidin-binding capacity of the mAb-saturated tenasin immobilized at different densities on the sensor chips. In all cases, the amount of streptavidin that bound to the mAb mixture was doubled compared with individual mAbs, suggesting the accessibility of biotin moieties. These in vitro results are confirmed by in vivo studies on nude mice implanted with the human colon carcinoma cell line, HT29. This xenografted tumor expresses tenasin at lower level than glioma xenografts as based on our immunohistochemistry analysis (data not shown). The limiting amount of antigen available for antibody binding was reflected in the low amount of antibody (2 μg) sufficient to saturate the tumor compared with other reports where antibody treatments of glioma xenografts required higher saturating doses (19, 20). In our model, the mixture of ST2146 and ST2485 radiolabeled biotinylated antibodies, each at its saturating dose, generated tumor accumulation of radioactivity, reaching 93% of the sum of the single antibody values. The percentage of 14.18 of injected dose per gram of tumor, 5 days after treatment, reflects the data in the literature (4.97-30% i.d./g; refs. 21–24). In this study, the use of a proper tumor-saturating dose of antibody and of a high avidin/antibody ratio in the chase step gave values of the tumor-to-normal tissue ratios far above those reported in the cited papers. Preliminary results obtained in the absence of avidin clearing step or using an avidin-to-antibody ratio ranging from 1:4 to 100:1 showed that the percentage of injected antibody localized at the tumor site remains constant independently from the avidin chase, whereas for all the other organs a significant decrease of unspecific binding proportional to avidin concentration can be obtained.

The results described in the present paper are stimulating to the application of the two murine antibodies mixture in the PAGRIT system where an increase in the tumor uptake of radiolabeled biotin may reasonably be expected as a consequence of the higher concentration of biotinylated antibodies saturating two independent antigenic epitopes. There are other examples in the literature where the combined use of therapeutic agents brought improvement in clinical outcome. Immunotoxins designed for human B-cell lymphoma treatment have been used in severe combined immunodeficient (SCID) mouse model and, when used as mixture, anti–CD19-CD22 and anti–CD38-saporin fusion proteins were more effective than each component alone (25). Similar results were reported by Spiridon et al. (26) on the antigrowth activity of three antibodies generated against different epitopes of HER-2 tyrosine kinase receptor. They found that the mixture is more effective than individual mAbs in treating tumors in SCID mice, whereas in vitro activities, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, also did better. More recently, Nahta et al. (27) reported the combined use of two therapeutic antibodies (trastuzumab and pertuzumab) directed against the same target. In vitro assays on breast cancer cell lines have shown a synergistic effect of the antibody cocktail in inhibiting survival by increasing apoptosis. Finally, a recent report on a randomized phase II clinical trial evaluating the safety and efficacy of the concurrent administration of cetuximab and bevacizumab, two therapeutic antibodies against EGF receptor and vascular endothelial growth factor, respectively, indicated that the clinical response of patients treated with the mAbs combination was higher than that of the patients treated with a single antibody (28).

Optimization of tumor pretargeting is fundamental to maximize tumoridal activity while minimizing side effects in PAGRIT applications. In fact, our preclinical experience indicates that once the tumor antigen is saturated, the administration of additional antibody only produces a reduction in tumor-to-nontumor ratios and the clearing step will require higher or repetitive doses without any benefit for the efficacy of the therapy. Therefore, especially in those tumors where the target antigen is expressed at low level, the use of mAb combination targeting different epitopes of the limiting antigen or different antigens, each mAb at its saturating dose, could be advantageous.

![Image](http://example.com/image.png)

**Fig. 6.** In vivo additivity study of the two mAbs. A, amount of radiolabeled (*) antibodies in the tumor used singly or in mixture, expressed as nanogram per gram of tumor, 5 days after administration. B, tumor-to-nontumor ratio of the ST2146 and ST2485 combination.

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