

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
9 September 2005 (09.09.2005)

PCT

(10) International Publication Number
WO 2005/082938 A2

(51) International Patent Classification⁷: **C07K 16/00**

(21) International Application Number:
PCT/IT2005/000078

(22) International Filing Date: 16 February 2005 (16.02.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
RM2004A000105 27 February 2004 (27.02.2004) IT

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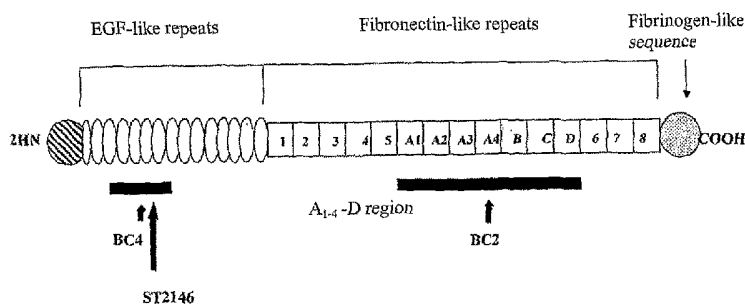
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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ,

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(54) Title: ANTI-HUMAN TENASCIN MONOCLONAL ANTIBODY

Schematic representation of human tenascin-C and of the strategy
followed for the generation of BC2-like antibodies



(57) Abstract: An anti-human tenascin monoclonal antibody is described, whose light and heavy chain variable region sequences are SEQ ID NO: 1 and SEQ ID NO: 2, respectively, its proteolytic fragments capable of binding to an antigenic epitope within the region A₍₁₋₄₎-D of human tenascin, its recombinant derivatives, its conjugates and its similar functional analogues capable of binding to an antigenic epitope within the A₍₁₋₄₎-D region of human tenascin.

Immunisation of Balb/c mice with Tn
(A₁₋₄-D) fragment

Fusion of spleen cells of immunised mice with
Sp2/0 Ag14 myeloma cells

Screening of culture supernatants of hybridomas
obtained on purified tenascin



TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Anti-human tenascin monoclonal antibody

The present invention relates to anti-human tenascin monoclonal antibodies, methods and materials for obtaining them, the use of said antibodies in diagnostic and treatment procedures for tumours expressing tenascin and materials containing said antibodies suitable for use in the medical field.

Background to the invention

The specificity of tumour therapy often constitutes a limiting factor in determining the success of the treatment. In fact, the onset of toxic effects and the reduced tolerability of a number of anticancer agents limit their use and the quality of life of the patients.

The reduction of toxicity is directly linked to the selectivity of the treatment for cancer cells alone. Monoclonal antibodies are the ideal instrument for specifically localising the tumour and, when combined with the avidin/biotin amplification system, constitute an extremely potent method for directing active molecules to the tumour site.

Tenascin is a molecule of the extracellular matrix, expressed during embryogenesis and in adult tissues during the processes of scar formation and tumour development, as well as in newly formed blood vessels. Tenascin is virtually absent in normal adult tissues, whereas it is expressed in the stroma of many solid tumours such as gliomas (*Burdon, et al., Cancer Res., 43:2796-2805, 1983*), carcinomas of the breast (*Chiquet-Ehrismann, et al., 1986*), lung (*Natali, et al., Intl. J. Cancer, 54:56-68, 1989*), fibrosarcomas and squamous-cell carcinomas (*Ramos, D.M. et al., Intl. J. Cancer, 75:680-687, 1998*). Tenascin is expressed in glioma, but not in the corresponding normal brain tissue. For an in-depth discussion of tenascin, the reader is referred to WO 92/04464, Wistar and related references.

On the basis of EP 0 496 074, G. Paganelli et al. have developed a method called "three-step pre-targeting" for the systemic and locoregional treatment of tumours, the results of which are reported in: *Cremonesi, M., et al., Eur. J. Nucl. Med., 26(2):110-120, 1999; Paganelli, G., et al., Eur. J. Nucl. Med., 26(4):348-357, 1999; Paganelli, G., et al., Cancer Biother. & Radiopharm., 16(3):227-235, 2001; Grana, C., et al., Br. J. Cancer, 86:207-212, 2001.*

Other references for this type of cancer treatment are: WO 94/04702, and US 5.578.287.

Three-step pre-targeting treatment, also known sotto by the trademark PAGRIT[®], is based on the sequential intravenous administration of a biotinylated anti-tenascin monoclonal antibody, streptavidin and ⁹⁰Y-biotin, with administrations of avidin and biotinylated albumin prior to the streptavidin and ⁹⁰Y-biotin, respectively ("chase" step), to reduce the circulating levels of the antibody and of streptavidin. The selectivity of the 3-step pre-targeting method is due to the use of the anti-tenascin monoclonal antibody. The targeting of extracellular matrix molecules presents the advantage, compared to targeting aimed at cell surface antigens, of not being affected by modulation of antigen expression by the tumour cell itself. The doses, administration times and "chases" of the pre-targeting treatment have been established for the purposes of obtaining optimal tumour to non-tumour biodistribution ratios.

The results obtained in 48 patients suffering from glioblastoma (GBM) or anaplastic astrocytoma (AA), entered into a study by Paganelli (*Paganelli, G., et al., Eur. J. Nucl. Med., 26(4):348-357, 1999*), have demonstrated a substantial lack of toxicity, except for a number of cases of allergic reaction to streptavidin, and preliminary efficacy of the treatment. In fact, 2 months after the end of the treatment, 25% of the patients showed a reduction of the tumour mass (Complete Response = 6%, Partial Response = 11%, Minor Response = 8%), and 52% of patients remained stable, with an overall response rate of 77%.

In a number of these patients, whose life expectancies were below six months, the response to treatment persisted for more than one year.

The role of the biotinylated anti-tenascin antibody is to localise in the tumour and mediate, via the biotinylated molecule, the accumulation of avidin and subsequently of ^{90}Y -biotin, directing the radioisotopes directly into the interior of the tumour. Anti-tenascin antibodies are already the object of patents and patent applications: US 5,624,659, Duke University; JP 2219590, Rikagaku; WO 92/04464, Wistar; and WO 03/072608, Sigma-Tau.

One particular anti-tenascin antibody is described in: *Siri, A., et al, Nucl. Acid Res., 19(3):525-531, 1991; Balza, E., et al, FEBS 332:39-43, 1993*; and its use for therapeutic purposes is described in: *Riva, P., et al., Acta Oncol. 38(3): 351-9, 1999; Riva, P., et al, Cancer, 80 (12): 2733-42; 1997; Riva, P., et al., Int. J. Cancer, 5: 7-13, 1992.*

The clone used for the generation of said antibody in the above-cited studies is called BC-2. The applicant has demonstrated that the clone BC-2 is not suitable for industrial development, in that it produces an additional non-functional light chain (probably deriving from the parent myeloma line) whose level of expression increases during development of the production process on a large scale, preventing industrial-scale purification of the antibody.

There is therefore a perceived need for an anti-tenascin monoclonal antibody that can be produced on an industrial scale at the purity levels required for pharmaceutical use.

The previous patent application WO 03/072608, filed in the name of Sigma Tau Industrie Farmaceutiche Riunite S.p.A., describes the generation of the anti-tenascin antibody ST2146, without the additional non-functional light chain, capable of recognising an antigenic epitope shared with antibody BC-4, also contaminated by a non-functional light chain.

Antibody ST2485, the object of the present invention, recognises an epitope partly shared with that of BC-2, and therefore located in the same protein region. This region is greatly expressed in various tumour tissues. It is therefore important to have a homogeneous monoclonal antibody which is specific for this region, to be used for cancer diagnosis or targeting.

Moreover, the present invention demonstrates that antibody ST2485 presents the advantage of binding to tenascin in addition to antibody ST2146, thus proving useful in pre-targeting methods involving the combined use of the two antibodies.

Summary of the invention

An anti-human tenascin monoclonal antibody has now been found which solves the above-mentioned problems, since it does not possess a non-functional light chain and presents the advantage of additivity when used in combination with another anti-tenascin antibody. This antibody is the object of the present invention, together with the method for obtaining it and its use in therapy, in particular for the preparation of a product useful for the treatment of diseases characterised by the expression of tenascin, such as, for instance, tumours.

The present invention relates to the antibody and to antibody fragments, optionally containing additional markers and diagnostic agents, to processes for obtaining said antibody and antibody fragments, to pharmaceutical compositions containing said antibody and its fragments and to diagnostic and therapeutic methods making use of them, as well as kits useful for implementing said methods.

The present invention also relates to the DNA encoding said antibody or fragments thereof, to vectors containing such DNA, to host cells containing such vectors, to the protein coded for by the nucleotide

sequences SEQ ID NO: 1 and SEQ ID NO: 2, to the DNA encoding the protein and the fragments, to the specific complementary determining regions (CDRs) and proteins containing such CDRs. In particular, the object of the present invention is also the hybridoma that produces said monoclonal antibody.

The antibody which is the object of the present invention is characterised by the sequences of the variable region of the light and heavy chains SEQ ID NO: 1 and SEQ ID NO: 2, respectively, shown in Figures 17 and 18. For the sake of brevity, the antibody which is the object of the present invention will be identified by the name ST2485. Also fragments of ST2485 or its chimeric or recombinant derivatives can be produced and used within the scope of the present invention. Also objects of the present invention are the proteolytic fragments of said antibody capable of binding to an antigenic epitope within the A1-4-D region of human tenascin. In the course of the description of the present invention what is meant by antibody fragments are those fragments capable of binding to an antigenic epitope within the A1-4-D region of human tenascin C.

According to the present invention, said antibody or proteolytic fragments are preferably biotinylated.

Another object of the present invention consists in the hybridoma cell line, called cST2485, producing the antibody ST2485.

The hybridoma cell line was deposited at the Centro di Biotecnologie Avanzate, Largo Rossana Benzi 10, Genoa, Italy, on 12 November 2003 in accordance with the provisions of the Budapest Treaty, under deposit number PD03003.

Also objects of the present invention are the recombinant derivatives of antibody ST2485, which may optionally be biotinylated. In particular, the recombinant derivatives preferred are those in which the murine constant region is replaced by its human counterpart (*Ferrer, C., et al.*,

J. Biotechnol., 52: 51-60, 1996), or those in which the murine constant region is replaced by a biologically active component, such as, for example, a member of the avidin family (*Penichet ML., Manuel, L., et al., J. Immunol.*, 163: 4421-4426, 1999), a growth factor useful for stimulating tumour-directed immunological effectors (for example, G-CSF, GM-CSF), or those in which the murine constant region is replaced by a pharmacologically active component, such as, for example, a superantigen, a toxin, a cytokine or any other protein useful for enhancing the anticancer therapeutic efficacy (*Di Massimo, AM, et al., British J. Cancer*, 75(6):822-828, 1997; *Parente D., et al., Anticancer Research*, 17(6A):4073-4074, 1997). The methods for obtaining such derivatives are well known to experts in the field.

Another object of the present invention consists in conjugated derivatives of antibody ST2485, optionally biotinylated.

In particular, the conjugated derivatives preferred are those in which the biologically active component is bound to the antibody by means of conventional systems. Examples of biologically active compounds are the members of the avidin family, a growth factor useful stimulating tumour-directed immunological effectors (for example, G-CSF, GM-CSF), or those in which the murine constant region is replaced by a pharmacologically active component, such as, for example, a superantigen, a toxin, a cytokine or any other protein useful for enhancing the anticancer therapeutic efficacy, anticancer drugs, and radioisotopes.

A further object of the present invention consists in the use of the antibody or its derivatives for the preparation of a pharmaceutical product useful in a method for cancer radioimmunotherapy carried out preferably according to the method called three-step pre-targeting, also known by the trade-mark PAGRIT®, including the administration to a subject suffering from a cancer expressing the tenascin antibody ST2485 or proteolytic fragments thereof, preferably biotinylated.

Recombinant derivatives of the antibody which is the object of the present invention and its conjugates can be used to advantage in cancer therapy. Therefore, the use of the antibody and its fragments or derivatives in the preparation of a medicine for the treatment of tumours expressing tenascin constitutes a further object of the present invention.

For the purposes of implementing radioimmunotherapy two therapeutic kits, one systemic and the other locoregional, are also described. These kits are also known by the trade-mark PAGRIT®. The systemic kit consists of 5 vials, including vial 1 containing the biotinylated antibody or fragments or derivatives thereof according to the present invention; vial 2 containing avidin; vial 3 containing streptavidin; vial 4 containing biotinylated human albumin; and vial 5 containing biotin DOTA (or derivatives of biotin described in WO 02/066075). The locoregional kits consists of 3 vials, corresponding to vials 1, 2 and 5 of the systemic kit. The vials are prepared so as to be suitable for injection in the subject to be treated, preferably a human subject. For the purposes of implementing radioimmunotherapy, a method called IART (Intraoperative Avidination for Radionuclide Treatment) is described, based on the intraoperative treatment of patients undergoing surgical removal of tumour masses with the reagents which are the object of the present invention, either alone or in combination with other components of the PAGRIT® kit.

The specific container, preferably in the form of a vial suitable for injection containing the antibody or its fragments in biotinylated form, constitutes a further object of the present invention.

According to one embodiment of the present invention, in the therapeutic kits the biotinylated antibody is combined with other anti-tenascin antibodies, preferably directed at the EGF-like region of the protein. Alternatively the biotinylated antibody can be coupled to other antibodies specific to the tumour. A general description of this type of

method is to be found in EP 0 496 074, *European Journal of Nuclear Medicine Vol.26, No4; April, 1999;348-357*, US 5,968,405.

Another object of the present invention is the use of the monoclonal antibody ST2485 for obtaining images for subsequent diagnostic purposes ("imaging") by means of *in-vivo* immunolocalisation in the tumour.

Another object of the present invention is the use of the monoclonal antibody ST2485 in combination with a second anti-tenascin antibody in a test for the production of a diagnostic kit for determining circulating levels of tenascin.

These and other objects of the present invention will be described in detail here below, also with the aid of examples and figures.

Brief description of the figures

Figure 1: schematic representation of human tenascin C and the strategy adopted to generate the BC-2-like antibody.

Figure 2: SDS-PAGE (a, b) and Western blot (c, d) analysis of antibody ST2485 in reducing and non-reducing conditions. In reducing conditions, a double band is observed at the level of the light chain of the antibody (b, d).

Figure 3: SDS-PAGE analysis of antibody ST 2485 subjected to deglycosylation by digestion with the enzyme PNGaseF (Peptide-N-Glycosidase from *Flavobacterium*): disappearance of the high molecular weight light chain band is observed.

Figure 4: hydroxyapatite chromatography profiles of antibodies ST2485 and BC-2.

Figure 5: binding specificity of ST2485 for human tenascin and for the Tn (A₍₁₋₄₎-D) fragment by means of Western blot analysis.

Figure 6: Competitive ELISA between ST2485 and BC-2 for binding to tenascin C (a) or to the Tn (A₍₁₋₄₎-D) fragment (b).

Figure 7: immunoreactivity of ST2485 compared to BC-2 on tenascin C (a) and on the Tn (A₍₁₋₄₎-D) fragment (b).

Figure 8: immunoreactivity of biotinylated ST2485 and BC-2 on tenascin C (a) and on the Tn (A₍₁₋₄₎-D) fragment (b).

Figure 9: cross-reactivity of antibody ST2485 with the murine tenascin expressed in LMM3 tumour (murine breast cancer) and in normal murine intestine.

Figure 10: protocol adopted for the ST2485 biodistribution study in nude mice.

Figures 11 and 11a: biodistribution of ST2485 at various administration doses compared with BC-2. The accumulation of ST2485 in the tumour, at all doses administered, is at least twice as much as that of BC-2.

Figures 12 and 12a: tumour to non-tumour ratios of ST2485 compared with BC-2. These ratios are higher for antibody ST2485, at all doses administered.

Figure 13: *in-vitro* interference (a) and additivity testing (b) of anti-tenascin antibodies ST2485 and ST2146 by means of ELISA test.

Figures 14 and 14a: *in-vitro* additivity test of antibodies ST2485 and ST2146 by means of BiaCore analysis.

Figure 15: scheme adopted in the *in-vivo* additivity study of antibodies ST2485 and ST2146.

Figure 16: *in-vivo* additivity of antibodies ST2485 and ST2146, expressed in ng of antibody localised per gram of tumour.

Figure 17: SEQ ID NO: 1 sequence of the variable region of the light chain of ST2485 (VL). Encoded amino acid sequence shown in SEQ ID NO: 3.

Figure 18: SEQ ID NO: 2 sequence of the variable region of the heavy chain of ST2485 (VH). Encoded amino acid sequence shown in SEQ ID NO: 4.

Detailed description of the invention

The inventors have prepared a new anti-human tenascin antibody called ST2485, whose light and heavy chain variable region sequences are SEQ ID NO: 1 and SEQ ID NO: 2, respectively, produced from the hybridoma cell line cST2485. The antibody is capable of binding to an antigenic epitope within the A₍₁₋₄₎-D region of human tenascin.

The proteolytic fragments of the antibody according to the present invention are capable of binding to an antigenic epitope within the A₍₁₋₄₎-D region of human tenascin.

Recombinant derivatives of the antibody according to the present invention are obtained according to conventional methods which are well known to experts in the field. The recombinant derivatives preferred are those in which the murine constant region is replaced by its human counterpart, or by a biologically or pharmacologically active component, or by a member of the avidin family.

According to the present invention, the conjugated derivatives are obtained by means of conventional methods well known in this field.

The preferred conjugated derivatives are those in which a biologically active portion is bound to the antibody. Examples of biologically active portions are the members of the avidin family, a growth factor useful for stimulating tumour-directed immunological effectors (such as G-CSF, GM-CSF), a pharmacologically active portion, such as, for example, a toxin, superantigen, cytokine or any other protein useful for enhancing the therapeutic anticancer effect, anticancer drugs, and radioisotopes.

For further information regarding the preparation of the recombinant and conjugated derivatives, the reader is referred to WO 03/072608.

According to the present invention, recombinant or conjugated derivatives of the monoclonal antibody are also referred to as "derivatives".

In one preferred embodiment of the present invention, the antibody, its fragments and derivatives can be biotinylated.

The antibody according to the present invention, its fragments and derivatives can also advantageously contain additional markers and diagnostic agents.

The present invention also includes the DNA encoding the monoclonal antibody or its fragments as defined above. The invention also comprises a vector containing said DNA, and a host cell containing said vector.

Vectors and host cells fall within the sphere of the common knowledge pertaining this field.

The present invention also comprises the protein coded for by the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 2 or its fragments, and the DNA encoding it.

The present invention also comprises specific CDRs (Complementary Determining Regions) of the above-mentioned antibody and proteins containing said CDRs.

The process for preparing the antibody according to the present invention consists of the following stages:

- a) immunisation of an animal, preferably a mouse, with the A₁-₄-D fragment of human tenascin;
- b) fusion of the somatic spleen cells of said animal with myeloma cells not producing immunoglobulins;
- c) selection of the monoclonal antibody.

The monoclonal antibody ST2485 is produced from the hybridoma cell line cST2485, identified above.

According to the present invention, the antibody or its fragments or its derivatives, optionally biotinylated, are used for the preparation of a pharmaceutical product useful for the treatment and diagnosis of diseases characterised by expression of tenascin. In particular, said diseases are tumours, and more particularly include glioma, breast cancer, lung cancer, fibrosarcoma and squamous cell carcinoma.

In one preferred aspect, the above-mentioned pharmaceutical product is used in the two-stage perioperative therapy of solid tumours, described in the International application WO 03/07268. In this type of therapy, the biotinylated antibody is administered, followed by avidin, thus constructing the "artificial receptor" for the actual subsequent anticancer agent. In this case, the anticancer agent will be delivered by biotin, which will be contained in a chemical compound suitable for forming a complex with the anticancer agent and hereinafter referred to as the biotin compound, and will be administered systemically in the postoperative phase. The biotin, in fact, will localise only where avidin is present and in this case we can be sure that avidin is present in the area we are interested in treating, in that it is introduced by the surgeon a few hours earlier (e.g. 4 to 72 hours) during the operation.

This is an advantage in that it drastically reduces the time elapsing between removal of the primary tumour and adjuvant therapy.

As regards the industrial aspects of the present invention, the antibody described herein will be suitably formulated in pharmaceutical compositions or therapeutic or diagnostic kits, according to normal practice in the pharmaceutical field.

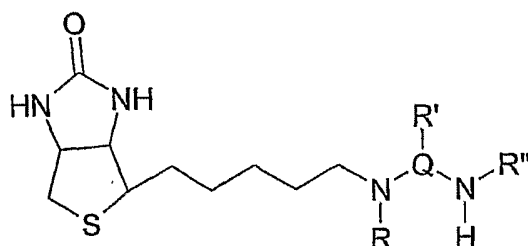
Pharmaceutical compositions and kits are entirely of the type which is conventional in this field and can be prepared by experts in the field even only on the basis of common knowledge. Examples of pharmaceutical compositions are provided in the references cited in the present invention. The same is true of the kits. Particularly preferred are the kits for tumour radioimmunotherapy, as described in the above-mentioned studies by Paganelli et al., and in EP 0 496 074, WO 02/066075, WO 03/072608, and WO 03/075960. A particular use of the radioimmunotherapy kit can be implemented with the device described in WO 03/069632.

Pharmaceutical compositions containing the antibody or its derivatives or fragments, optionally biotinylated in mixtures with at least one pharmaceutically acceptable excipient or vehicle are included in the scope of the present invention.

In a particular embodiment of the present invention, kits are supplied for systemic radioimmunotherapy, particularly three-step pre-targeting radioimmunotherapy, containing 5 vials, vial 1 containing an antibody or its fragments or recombinant derivatives or its conjugates or analogues, optionally biotinylated; vial 2 containing avidin; vial 3 containing streptavidin; vial 4 containing biotinylated human albumin; and vial 5 containing biotin DOTA.

In another embodiment of the present invention, the kits are for locoregional radioimmunotherapy and contain 3 vials which are the same as vials 1, 2 and 5 of the systemic kit.

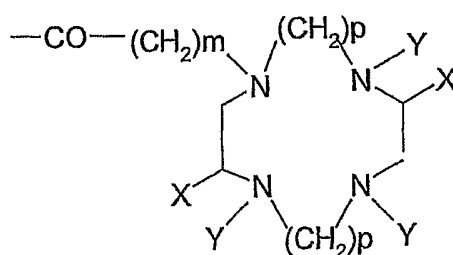
In a particularly preferred embodiment, in the vial containing biotin



DOTA, said biotin DOTA is the formula (I) compound

(I)

in which Q is a $-(CH_2)_n-$ group, where n is a whole number from 4 to 12, in which case R' is not present, or Q is selected from the group consisting of $-(CH_2)_a-CH(R')_b-(CH_2)_b-$, where a and b are independently whole numbers from 0 to n, R' is as defined here below, or Q is cyclohexyl, phenyl, in which case R' is a substituent on the cyclohexyl or phenyl ring;



R is hydrogen or $-\Lambda$ where $-\Lambda$ is a formula (II) macrocycle,

(II)

where the various Y's, which may be the same or different, are selected from the group consisting of hydrogen, straight or branched C_1 - C_4 alkyl, $-(CH_2)_m-COOH$, where m is a whole number from 1 to 3, X is

hydrogen, or the group $-\text{CH}_2\text{-U}$, where U is selected from the group consisting of methyl, ethyl, and p-aminophenyl, or X is the group $-(\text{CHW})_o\text{-Z}$, where o is a whole number from 1 to 5, W is hydrogen, methyl or ethyl, Z is a 5- or 6-member heterocyclic group containing one or more heteroatoms selected from O, N- R_1 , where R_1 is hydrogen or straight or branched $\text{C}_1\text{-C}_4$ alkyl, and S; or Z is selected from the group consisting of $-\text{NH}_2$, $-\text{NH-C(=NH)-NH}_2$, or $-\text{S-R}_2$, where R_2 is straight or branched $\text{C}_1\text{-C}_4$ alkyl;

p is the number 2 or 3;

R' is selected from the group consisting of hydrogen, straight or branched $\text{C}_1\text{-C}_4$ alkyl, $-(\text{CH}_2)_q\text{-T}$, in which T is selected from the group consisting of $-\text{S-CH}_3$, $-\text{OH}$, $-\text{COOH}$, and q is the number 1 or 2;

R'' has the same meanings as R' , upon the following conditions: if R is $-\Lambda$, R'' is hydrogen; if R is hydrogen, R'' is $-\Lambda$, or R and R'' are, respectively, $-(\text{CH}_2)_r\text{-}\Lambda$ (for R), where r is a whole number from 4 to 12, and $-\Lambda$ (for R''), Q being a $-(\text{CH}_2)_n\text{-}$ group, where n is a whole number from 4 to 12.

These compounds are described in WO 02/066075.

In a particularly preferred embodiment, in the vial containing avidin, the latter is an avidin dimer in which two molecules of avidin are bound via $-\text{NH}_2$ groups by means of suberate, or an avidin dimer in which two molecules of avidin are bound via $-\text{COOH}$ groups by means of polyethylene glycol with a molecular weight of 3,400, as described in WO 03/075960.

In the kits according to the invention, the antibody, its proteolytic fragments, its derivatives, optionally biotinylated, can be in combination with other anti-tenascin antibodies, preferably targeting the EGF-like region of the protein, or in combination with other tumour-specific antibodies.

All the aspects of this invention, in addition to applying to the therapy of tumours expressing tenascin, also apply to the diagnosis of tumours and particularly tumour immunolocalisation procedures, e.g. imaging. Another object of the present invention is a container, preferably in the form of a vial suitable for injection, containing an antibody or its proteolytic fragments or its derivatives, optionally biotinylated, and/or radiolabelled.

In another aspect of the invention, the antibody or its fragments, recombinant derivatives, or analogues are used in combination with a second tenascin-specific antibody, in a sandwich-type *in-vitro* ELISA assay, in conditions in which said second antibody binds to a second antigenic epitope of tenascin, for the purposes of determining circulating tenascin levels, and particularly the isoforms containing the A₍₁₋₄₎-D region.

The following example further illustrates the invention.

Example 1

For the purposes of generating a new hybridoma with BC-2 specificity but not producing a non-functional light chain, Balb/c mice were immunised with the A₍₁₋₄₎-D fragment of human tenascin, containing the epitope recognised by BC-2 (Siri, A., *et al.*, *Nucl. Acid Res.*, 19(3):525-531, 1991; Balza, E., *et al.*, *FEBS*, 332:39-43, 1993). The schematic representation of human tenascin C and the strategy adopted to generate the BC-2-like antibody are illustrated in Figure 1. Spleen cells of mice immunised with the Tn (A₍₁₋₄₎-D) fragment were fused with myeloma cells not producing immunoglobulins Sp2/0-Ag14 according to standard methods (Cianfriglia, M., *et al.*, *Methods Enzymol.*, 121:193-210, 1986), and the hybridoma population obtained was subjected to screening by means of the ELISA test on purified tenascin from SK-Mel 28 human melanoma cells (ICLC HTL99010). The hybridomas secreting anti-tenascin antibodies were cloned twice by means of limiting dilutions in growth medium containing FCS and

twice in protein-free medium of animal origin (Animal Derived Component Free Medium HyClone, HyQR Perbio).

Production of the ST2485 reference material was achieved by culturing the cST2485 hybridoma cells in a 2-litre bioreactor; two successive limiting dilution subclonings of the cST2485 Post Production Cell Bank (PPCB) led to the selection of the subclone cST2485/A3e/A12f, used for the production of the Master Cell Bank (MCB) and the Working Cell Bank (WCB).

ST2485 is a mouse immunoglobulin of IgG1/k isotype.

Figure 2 shows the SDS-PAGE (a, b) and Western blot (c, d) analysis of the antibody ST2485 in reducing and non-reducing conditions. In reducing conditions (b, d), a double band is observed at the level of the light chain of the antibody; Western blot analysis demonstrates that the two bands are immunoreactive as light chains of immunoglobulin. Hydroxyapatite chromatographic analysis of antibody ST2485, shown in Figure 3, confirms a mild heterogeneity of the antibody through the slightly asymmetrical peak. In the same analysis, on the other hand, the antibody BC-2 presents a distinctly heterogeneous profile, in which three peaks can be distinguished, of which only peak 1 corresponds to the totally functional homogeneous antibody.

To define the nature of the heterogeneity of antibody ST 2485, a sample of the latter was subjected to digestion of the N-glycoside residues with the enzyme PNGaseF (Peptide-N-glycosidase from *Flavobacterium*). The enzyme digestion was done using the Prozyme deglycosylation kit (cat. N°GE51001), in the conditions indicated by the manufacturer: approximately 8 µg of ST2485 and 5 mU of enzyme were reacted overnight at 37°C in 10 µl of reaction mixture. Antibody ST2485, digested and undigested, was run on 12% polyacrylamide gel in reducing conditions. The gel was stained with Coomassie Brilliant Blue.

The digestion caused the disappearance of the high molecular weight light chain band, as demonstrated by the SDS-PAGE analysis in reducing conditions illustrated in Figure 4. This finding shows that the two light chain bands of antibody ST2485 correspond to the glycosylated (high molecular weight) and non-glycosylated (low molecular weight) variants of the same. The existence of a potential N-glycosylation site was also confirmed by the immunoglobulin cDNA sequence data (Figure 17).

The variable region of the kappa light chain was amplified by the circularised cDNA using a pair of primers (5' GGGAAGATGGATACAGTTGGTG (SEQ ID NO: 5), 5' CAAGAGCTTCAACAGGAATGAG) (SEQ ID NO: 6) that recognise the constant region of the antibody as described by *M. Sassano et al. Nucl. Ac. Res. (1994) 22, 1768-1769*.

ST2485 binding to the A₍₁₋₄₎-D region of tenascin is specific, as demonstrated by the Western blot analysis presented in Figure 5. The antibody binds to human tenascin and to the A₍₁₋₄₎-D fragment, but not to the EGF-like fragment of the same protein, containing the epitope recognised by the BC-4 antibody.

Antibody ST2485 binds human tenascin in a distinct epitope or in one partly shared with BC-2, as demonstrated by the competitive ELISA assay between the two antibodies illustrated in Figure 6. The biotinylated antibody BC-2, at a concentration established in preliminary experiments, is dispensed with non-biotinylated BC-2 (curve 1) or ST2485 (curve 2) in increasing concentrations on plates sensitised with the antigen tenascin C (a), or Tn A₍₁₋₄₎-D fragment (b). The binding of the biotinylated antibody is measured after addition of HRP-streptavidin and the related chromogenic substrate TMB. Antibody ST2485 causes 40% inhibition of BC-2 binding to tenascin C or the Tn A₍₁₋₄₎-D fragment.

The immunoreactivity of ST2485 was evaluated in comparison with BC-2 by ELISA assay on tenascin C and on the Tn A₍₁₋₄₎-D fragment, as

shown in Figure 7. The antibodies ST2485 and BC-2 and the normal mouse immunoglobulins (nMIgG) were dispensed at increasing concentrations on plates sensitised with tenascin C (a) or with the Tn A₍₁₋₄₎-D fragment (b); the addition of the secondary antimouse antibody labelled with alkaline phosphatase and the related chromogenic substrate (pNPP) made it possible to determine the dose-response curve of the antibodies. The amount of ST2485 necessary to obtain 1.0 OD is approximately 13 times less than the amount of BC-2 on whole tenascin (a), and approximately 10 times less than the amount of BC-2 on the Tn (A₁₋₄-D) fragment (b).

The affinity of antibody ST2485 was evaluated both on tenascin C and on the Tn (A₁₋₄-D) fragment by means of BIAcore analysis. On tenascin C the KD₁ of ST2485 is 9.77×10^{-10} M ($ka_1 = 6.02 \times 10^5$; $kd_1 = 5.88 \times 10^{-4}$), whereas the KD₁ of BC-2 is 2.54×10^{-7} M ($ka_1 = 9.85 \times 10^3$; $kd_1 = 2.5 \times 10^{-3}$). On the Tn (A₁₋₄-D) fragment the KD₁ of ST2485 is 9.72×10^{-10} M ($ka_1 = 3.28 \times 10^5$; $kd_1 = 3.19 \times 10^{-4}$) whereas the KD₁ of BC-2 is 7.39×10^{-8} M ($ka_1 = 2.68 \times 10^4$; $kd_1 = 1.93 \times 10^{-3}$).

Maintaining the immunoreactivity after biotinylation constitutes a fundamental requisite of the antibody used in pre-targeting, and therefore the behaviour of biotinylated ST2485 (8.3 biotins/molecule) was evaluated by ELISA and BIAcore assays in comparison with biotinylated BC-2 (7.6 biotins/molecule). With reference to Figure 8, the antibodies ST2485 and BC-2, biotinylated and non-biotinylated, and the normal mouse immunoglobulins (nMIgG) were dispensed at increasing concentrations on plates sensitised with tenascin C (a) or with the Tn A₍₁₋₄₎-D fragment (b); the addition of the secondary antimouse antibody labelled with alkaline phosphatase and the related chromogenic substrate (pNPP) made it possible to determine the dose-response curve of each biotinylated antibody compared to the non-biotinylated one (residual immunoreactivity). The residual immunoreactivity of biotinylated ST2485 and BC-2 on tenascin C is equal to 76% and 88%, respectively. On the Tn A₍₁₋₄₎-D fragment, the

residual immunoreactivity for ST2485 and BC-2 is 73% and 83%, respectively.

The BIAcore analysis of the biotinylated antibodies on whole tenascin shows a KD_1 affinity = 2.88×10^{-9} M for biotinylated ST2485 ($ka_1 = 2.8 \times 10^5$; $kd_1 = 8.07 \times 10^{-4}$) and a KD_1 affinity = 3.71×10^{-7} M for biotinylated BC-2 ($ka_1 = 6.0 \times 10^3$; $kd_1 = 2.23 \times 10^{-3}$). Biotinylated ST2485 thus maintains good immunoreactivity and affinity characteristics, conserving an affinity for tenascin approximately 100 times greater than that of biotinylated BC-2.

Immunohistochemical studies on various human tumours (breast, lung, stomach, colon) have demonstrated localisation of ST2485 at the level of the extracellular matrix, similar to that reported for BC-2 (*Natali, PG, et al., Int. J. Cancer, 47: 811-16, 1991*). These studies have also demonstrated the ability of ST2485 to produce cross-reaction with murine tenascin, including that expressed in normal mouse tissues, as can be seen in Figure 9. Sections of LMM3 tumour (murine mammary carcinoma) and normal murine intestine fixed in formalin were incubated with 10 μ g/ml of ST2485 or of normal murine IgG1 antibody (control). After incubation with the biotinylated secondary anti-mouse antibody and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) binding to tenascin was detected with the addition of the colorimetric substrate DAB (Vector). The contrast was done with Mayer's haematoxylin. The LMM3 and murine intestine sections are positive with ST2485 but not with the control antibody. It was not possible to perform these studies in comparison with BC-2 since that antibody does not recognise fixed tissue sections embedded in paraffin. The ability of ST2485 to recognise murine tenascin, including that expressed by normal tissues, enhances the significance of the results obtained in the antibody biodistribution studies in the murine model described here below.

For the purposes of evaluating the ability of the antibody to localise in the tumour mass, biodistribution studies of biotinylated 125 I-labelled

ST2485 and BC-2 were carried out. These studies were conducted in nude mice which had been implanted with human tumours expressing tenascin, according to the protocol represented schematically in Figure 10.

The animals were subcutaneously inoculated with 4×10^6 human colon carcinoma HT-29 cells in 0.1 ml of sterile saline solution. After 15 days, when the tumour had reached a mass of approximately 200 mg, groups of five animals received intravenous administrations of ^{125}I -BC2, ^{125}I -ST2485, or normal mouse ^{125}I -immunoglobulins (nMIg), all biotinylated (7-9 biotins/mol) and at five different doses: 0.2-0.5-1-2-5 $\mu\text{g}/\text{mouse}$ in 100 μl of sterile PBS. Five days after administration of the antibodies the animals were sacrificed and samples of blood, spleen, kidney, liver and tumour were taken for determining the radioactivity present. Twenty-four hours prior to the sacrifice the animals received an intravenous administration of avidin in 100 μl of sterile PBS, at doses 100 times greater than that of the antibody (chase).

The results, presented in Figures 11 and 11a, show that both BC-2 and ST2485 localise specifically in the tumour, regardless of the dose (the amounts of antibody are expressed as percentage of the dose injected per gram of tissue: %ID/gr). Moreover, ST2485 shows a greater localisation in the tumour compared with BC-2 at all doses assayed, always maintaining a higher tumour to non-tumour ratio (Figures 12 and 12a). In particular, at the dose of 1 $\mu\text{g}/\text{mouse}$ the accumulation of ST2485 in the tumour is equal to 16% of the injected dose, whereas the accumulation of BC-2 does not exceed 5% at any of the doses assayed. At the dose of 1 $\mu\text{g}/\text{mouse}$ the tumour:blood ratio for ST2485 is greater than 20 (optimal dose), whereas for the antibody BC-2 this ratio never exceeds 6 at any of the doses assayed. In the other organs, the tumour to non-tumour ratios for ST2485 at the dose of 1 $\mu\text{g}/\text{mouse}$ are 9, 26, and 43 in relation to the spleen, kidney and liver, respectively. For BC-2, on the other hand, these ratios are always lower than 7, at all the doses assayed. The tumour to non-tumour ratios are shown in Figures 12 and 12a.

For the purposes of evaluating the possibility of the combined use of the anti-tenascin antibodies ST2485 and ST2146 in the PAGRIT® and IART methods, ST2416 being directed at an epitope localised in the EGF-like region of the protein (*De Santis, R., et al., Br. J. Cancer, 88: 996-1003, 2003, WO 03/072608*), *in-vitro* and *in-vivo* additivity experiments were conducted with the two antibodies. Figure 13, in two ELISA tests performed on plates sensitised with tenascin C, shows the absence of epitope interference between the antibodies ST2485 and ST2146 (13a) and the additive nature of their binding to the antigen (13b). For the evaluation of the interference (a) biotinylated antibody ST2485, at increasing concentrations, was dispensed on microplates sensitised with tenascin C in the absence (1) and in the presence (2) of ST2146 at saturating concentration: the superimposed curves 1 and 2 indicate the absence of epitope interference between the two antibodies, in that the binding of biotinylated ST2485 to tenascin is not affected by the presence of ST2146 (and vice versa; data not shown). Curve 3 shows the signal obtained when seeding biotinylated ST2485 in the presence of non-biotinylated saturating ST2485 (control).

Figure 13b shows the additivity test, in which the biotinylated antibodies ST2485 and ST2146 were dispensed, in saturating doses, separately or together, on microplates sensitised with tenascin C: the mixture of the two antibodies produces a signal equal to the sum of the signals, thus demonstrating that the binding of the two antibodies is additive.

The *in-vitro* additivity of the two antibodies was also assayed using BIACore analysis, as shown in Figures 14 and 14a. The tenascin was immobilised on the chip (CM5 sensor chip, Biosense) according to the method described (*De Santis R.; et al.; Br. J. Cancer, 88: 996-1003, 2003*). Two consecutive injections of the first antibody were administered (ST2485 in a, ST2146 in c) at saturating concentrations (10 μ M), followed by injection of the second anti-tenascin antibody (ST2146 in a; ST2485 in c). Sensorgrams a) and c) show that the two

antibodies are capable of binding to the respective epitopes in an additive manner, as shown by the increase in the resonance signal produced by each single antibody. Sensorgrams b) and d) represent the injections of ST2485 and ST2146 respectively, each followed by the respective isotypical control antibodies, mIgG2b and mIgG1.

The additivity study of the two antibodies ST2485 and ST2146 was also conducted *in-vivo* in the previously described animal model. The study scheme is illustrated in Figure 15, whereas the results obtained are presented in Figure 16, expressed in ng of antibody per gram of tumour. The data confirm the additivity of the two antibodies also in the animal model. The mixture of the two labelled antibodies, in fact, presents an accumulation in the tumour amounting to 93% of the theoretical value (mathematical sum of the two individual labelled antibodies). Groups of control animals were treated with mixtures of radiolabelled antibody and second "cold" antibody to avoid any interference.

The variable region of the kappa light chain was amplified starting from the circularised cDNA using a pair of primers (5' GGGAAGATGG-ATACAGTTGGTG 3' (SEQ ID NO: 5), 5' CAAGAGCTTCAACAGGAAT-GAG 3') (SEQ ID NO: 6) that recognise the constant region of the light chain of the antibody, as described by *M. Sassano, et al., Nucl. Ac. Res., (1994) 22, 1768-1769.*

The variable region of the gamma heavy chain was amplified starting from the circularised cDNA using a pair of primers (5' ATGGAGTTAG-TTTGGGCAGCAG 3' (SEQ ID NO: 7), 5' GCACAACCACCATACTGAG-AAG 3') (SEQ ID NO: 8) that recognise the constant region of the heavy chain of the antibody, as described by *M. Sassano, et al., Nucl. Ac. Res., (1994) 22, 1768-1769.*

Figure 17 shows the SEQ ID NO: 1 sequence of the variable region of the light chain (VL) of ST2485.

Figure 18 shows the SEQ ID NO: 2 sequence of the variable region of the heavy chain (VH) of ST2485.

The comparative characterisation of ST2485 compared to BC-2 demonstrates that the antibody ST2485 possesses the following characteristics:

- it recognises an epitope within the A₍₁₋₄₎-D region of human tenascin, close to or partly overlapping that of BC-2;
- it is a homogeneous antibody as regards the composition of the light and heavy chain, in that the heterogeneity observed is due to light chain glycosylation variants;
- it is more immunoreactive than BC-2;
- it has greater affinity for the antigen than BC-2;
- it is comparable to BC-2 as regards the maintenance of immunoreactivity after biotinylation;
- it is superior to BC-2 as regards tumour localisation in the animal model;
- it binds in an additive manner with the anti-tenascin monoclonal antibody ST2146 to tenascin C, both *in-vitro* and *in-vivo* in the animal model.

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CLAIMS

1. Anti-human tenascin monoclonal antibody, preferably murine, whose light and heavy chain variable region sequences are SEQ ID NO: 1 and SEQ ID NO: 2, respectively, its proteolytic fragments capable of binding to an antigenic epitope within the A₍₁₋₄₎-D region of human tenascin, its recombinant derivatives, its conjugates and similar functional analogues capable of binding to an antigenic epitope within the A₍₁₋₄₎-D region of human tenascin.
2. Fragments of the antibody according to claim 1, optionally containing additional markers and diagnostic agents.
3. Recombinant derivative of the antibody according to claim 1, in which the murine constant region is replaced by its human counterpart.
4. Recombinant derivative of the antibody according to claim 1, in which the murine constant region is replaced by a biologically active component.
5. Recombinant derivative of the antibody according to claim 1, in which the murine constant region is replaced by a pharmacologically active component.
6. Recombinant derivative of the antibody according to claim 1, in which the murine constant region is replaced by a member of the avidin family.
7. Derivatives of the antibody according to claim 1 conjugated with biologically active substances.

8. Biotinylated antibody according to claim 1 or biotinylated fragments according to claim 2, or biotinylated derivatives according to claims 4-7.
9. DNA encoding the antibody according to claim 1 or fragments according to claim 2.
10. A vector containing the DNA according to claim 9.
11. Host cell containing the vector according to claim 10.
12. Protein coded for by the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 2 or its fragments.
13. DNA encoding the protein or its fragments according to claim 12.
14. Specific CDRs (Complementary Determining Regions) of the antibody according to claim 1 and proteins containing said CDRs.
15. Hybridoma producing the antibody according to claim 1, deposited at the Centro di Biotecnologie Avanzate, Largo Rossana Benzi 10, Genoa – Italy on 12 November 2003 in accordance with the provisions of the Budapest Treaty, under deposit number PD03003.
16. Process for the preparation of the antibody according to claim 1 comprising
 - a) immunisation of an animal with the A₍₁₋₄₎-D fragment of human tenascin;
 - b) fusion of somatic spleen cells of said animal with myeloma cells not producing immunoglobulins;
 - c) selection of the monoclonal antibody.
17. Use of the antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or of its fragments according to claim

2, optionally biotinylated, or of its biotinylated derivatives according to claims 4-7 for the preparation of a pharmaceutical product useful for the treatment or diagnosis of a disease characterised by expression of tenascin.

18. Use according to claim 17, in which said disease is a tumour.

19. Use according to claim 18, in which said tumour is selected from the group consisting of glioma, cancer of the breast, carcinoma of the lung, fibrosarcoma and squamous-cell carcinoma.

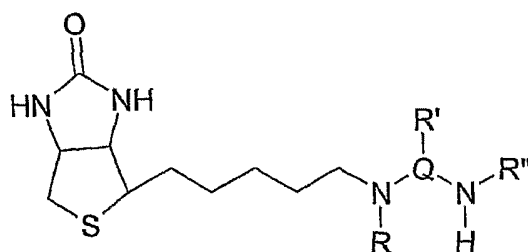
20. Use of the antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or of its fragments according to claim 2, optionally biotinylated, or of its biotinylated derivatives according to claims 4-7 for the preparation of a pharmaceutical product useful for the two-stage perioperative therapy of solid tumours.

21. Pharmaceutical or diagnostic compositions containing an antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or its biotinylated derivatives according to claims 4-7 in mixtures with at least one pharmaceutically acceptable vehicle and/or excipient.

22. Kit for systemic radioimmunotherapy, particularly three-step pre-targeting radioimmunotherapy, consisting of 5 vials: vial 1 containing an antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or fragments according to claim 2, optionally biotinylated, or biotinylated derivatives according to claims 4-7; vial 2 containing avidin; vial 3 containing streptavidin; vial 4 containing biotinylated human albumin; and vial 5 containing biotin DOTA.

23. Kit for locoregional radioimmunotherapy consisting of 3 vials; vial 1 containing an antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or biotinylated derivatives according to claims 4-7; vial 2 containing avidin; and vial 3 containing biotin DOTA.

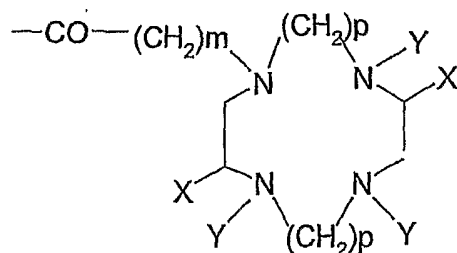
24. Kit according to claim 22 or 23, in which, said biotin DOTA in vial 5 or vial 3, respectively, is the formula (I) compound



(I)

in which Q is a $-(CH_2)_n-$ group, where n is a whole number from 4 to 12, in which case R' is not present, or Q is selected from the group consisting of $-(CH_2)_a-CH(R')-(CH_2)_b-$, where a and b are independently whole numbers from 0 to n, R' is as defined here below, or Q is cyclohexyl, phenyl, in which case R' is a substituent on the cyclohexyl or phenyl ring;

R is hydrogen or -A where -A is a formula (II) macrocycle,



(II)

where the various Y's, which may be the same or different, are selected from the group consisting of hydrogen, straight or branched C₁-C₄ alkyl, -(CH₂)_m-COOH, where m is a whole number from 1 to 3, X is hydrogen, or the group -CH₂-U, where U is selected from the group consisting of methyl, ethyl, and p-aminophenyl, or X is the group -(CHW)_o-Z, where o is a whole number from 1 to 5, W is hydrogen, methyl or ethyl, Z is a 5- or 6-member heterocyclic group containing one or more heteroatoms selected from O, N-R₁, where R₁ is hydrogen or straight or branched C₁-C₄ alkyl, and S; or Z is selected from the group consisting of -NH₂, -NH-C(=NH)-NH₂, or -S-R₂, where R₂ is straight or branched C₁-C₄ alkyl;

p is the number 2 or 3;

R' is selected from the group consisting of hydrogen, straight or branched C₁-C₄ alkyl, -(CH₂)_q-T, in which T is selected from the group consisting of -S-CH₃, -OH, -COOH, and q is the number 1 or 2;

R" has the same meanings as R', upon the following conditions: if R is -Λ, R" is hydrogen; if R is hydrogen, R" is -Λ, or R and R" are, respectively, -(CH₂)_r-Λ (for R), where r is a whole number from 4 to 12, and -Λ (for R'), Q being a -(CH₂)_n- group, where n is a whole number from 4 to 12.

25. Kit according to any one of claims 22-24, in which vial 3 contains an avidin dimer in which two avidin molecules are bound via the -NH₂ groups by means of suberate.

26. Kit according to any one of claims 22-24, in which said vial 3 contains an avidin dimer in which two avidin molecules are bound via the -COOH groups by means of polyethylene glycol with a molecular weight of 3,400.

27. Kit according to claim 22 or 23, in which the antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or its

biotinylated derivatives according to claims 4-7, are combined with other anti-tenascin antibodies, preferably targeting the EGF-like region of the protein.

28. Kit according to claim 22 or 23, in which the antibody or its proteolytic fragments, or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or its biotinylated derivatives according to claims 4-7, are combined with other tumour-specific antibodies.

29. Use of the antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or its biotinylated derivatives according to claims 4-7, in the preparation of compositions useful in the tumour immunolocalisation procedure.

30. Container, preferably in the form of a vial, suitable for injection, containing an antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, and/or radiolabelled or its fragments according to claim 2, optionally biotinylated, or its biotinylated derivatives according to claims 4-7.

31. Tumour imaging method including the administration of an antibody or its proteolytic fragments or its recombinant derivatives or its conjugates or analogues according to claim 1, optionally biotinylated, or its fragments according to claim 2, optionally biotinylated, or its biotinylated derivatives according to claims 4-7 to a person suffering from or suspected of suffering from a tumour, and the detection of said tumour.

32. Method according to claim 31, in which the antibody or its proteolytic fragments or recombinant derivatives or conjugates or analogues are radiolabelled.

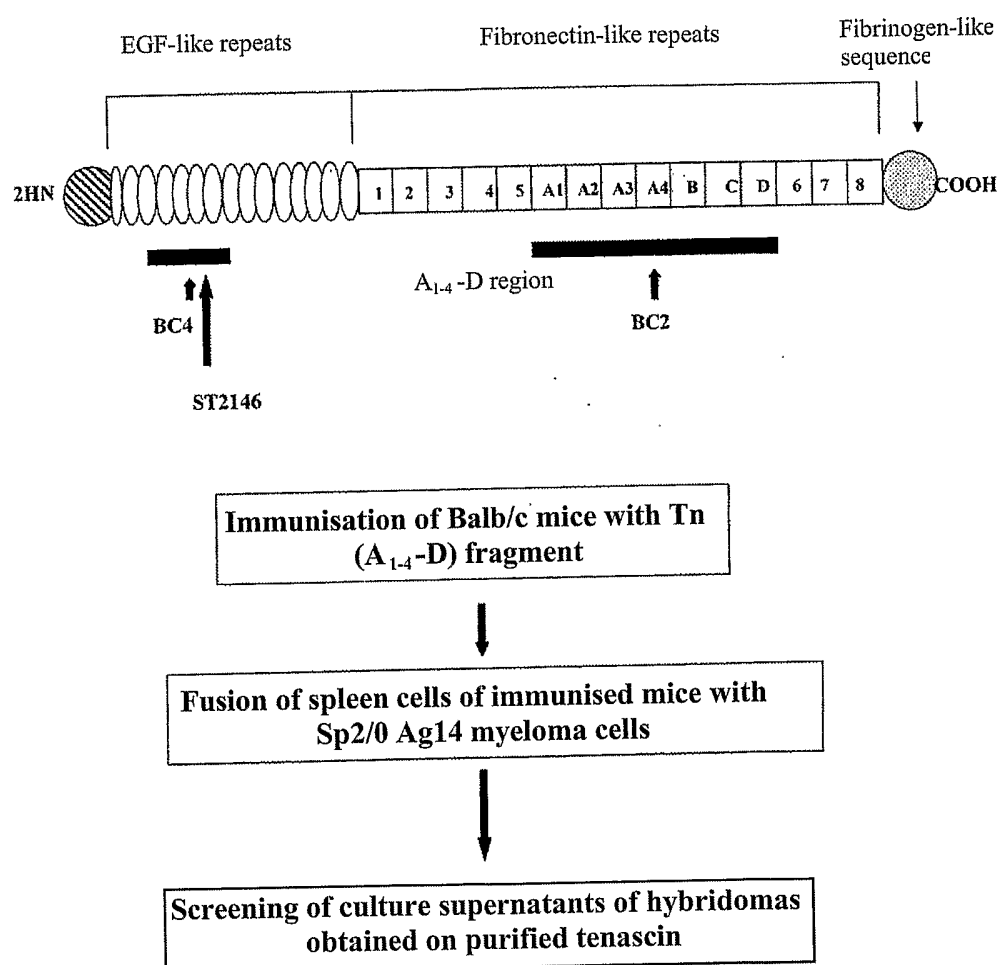
33. Combination containing the antibody or its proteolytic fragments or recombinant derivatives or conjugates or analogues according to claim 1, or fragments according to claim 2, or derivatives according to claims 4-7 and a second tenascin-specific antibody.

34. Use of the combination according to claim 33 in a sandwich-type *in-vitro* ELISA assay, in conditions in which said second antibody binds to a second antigenic epitope, for the purposes of determining circulating tenascin levels, particularly levels of the isoforms containing the A₍₁₋₄₎-D region.

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Figure 1

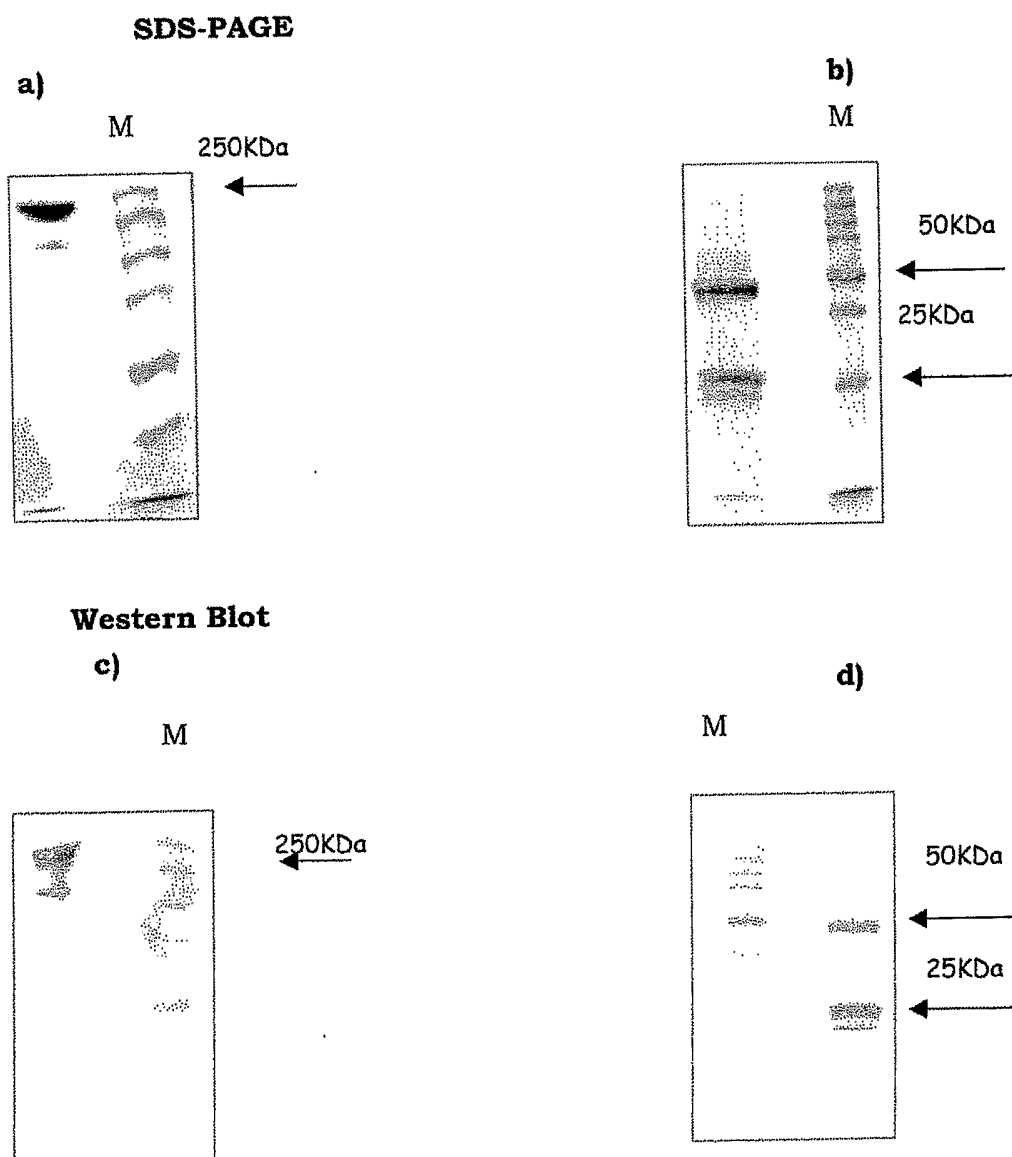
Schematic representation of human tenascin-C and of the strategy followed for the generation of BC2-like antibodies



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Figure 2

SDS-PAGE and Western Blot analysis of ST2485 antibody, in reducing (b, d) and non-reducing (a, c) conditions.

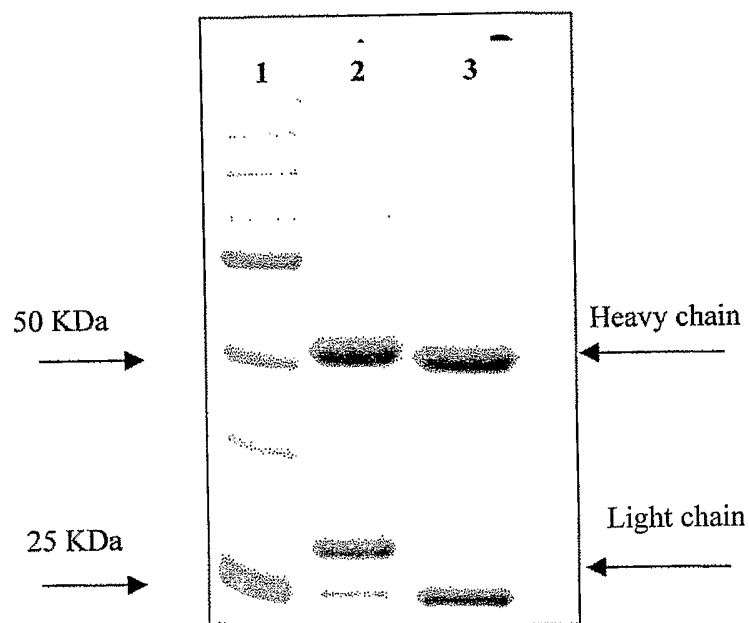


M: molecular weight standards

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Figure 3

ST2485 antibody digestion with *Flavobacterium* Peptide-N-glycosidase enzyme (PNGase F).

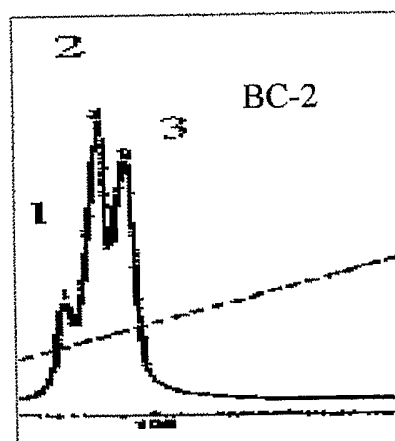
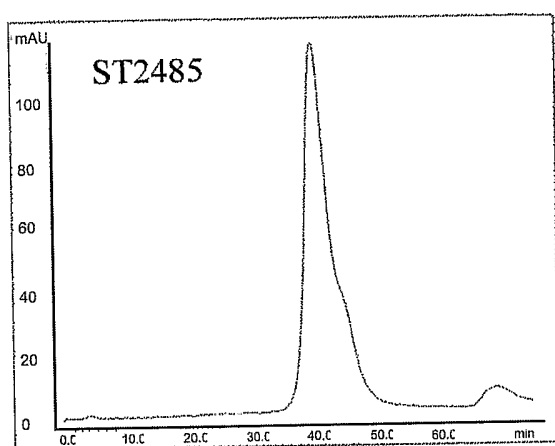


- 1: Molecular weight marker
- 2: Non-digested ST2485
- 3: PNGaseF-digested ST2485

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Figure 4

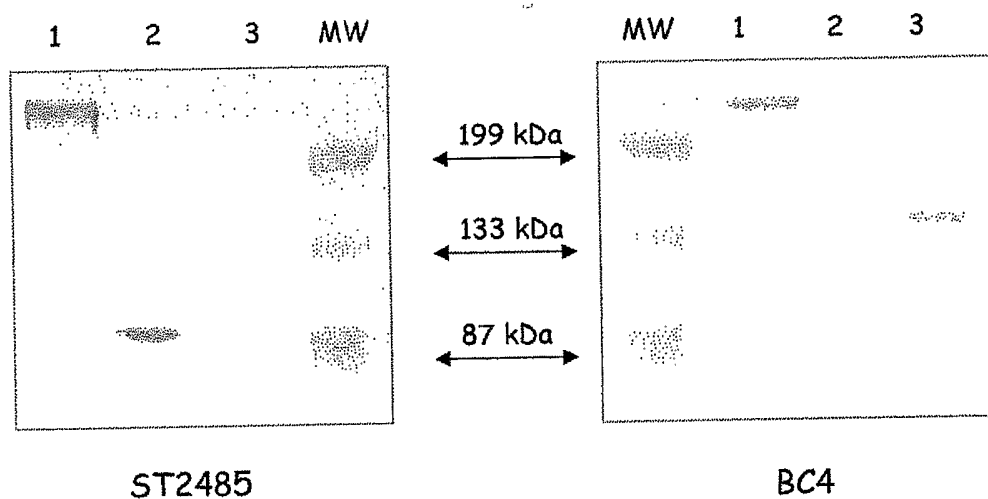
BC-2 and ST2485 antitenascin antibodies hydroxyapatite chromatography.



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Figure 5

ST2485 antitenascin antibody Western Blot Analysis



1: Tenascin-C

2: Tn A₍₁₋₄₎-D Fragment

3: Tenascin EGF-like region recombinant fragment, containing the epitope recognized by BC-4 antibody.

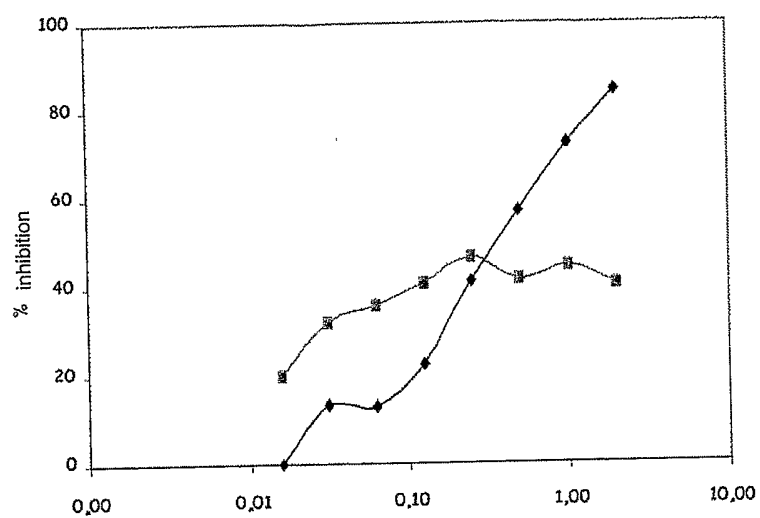
MW: molecular weight standards

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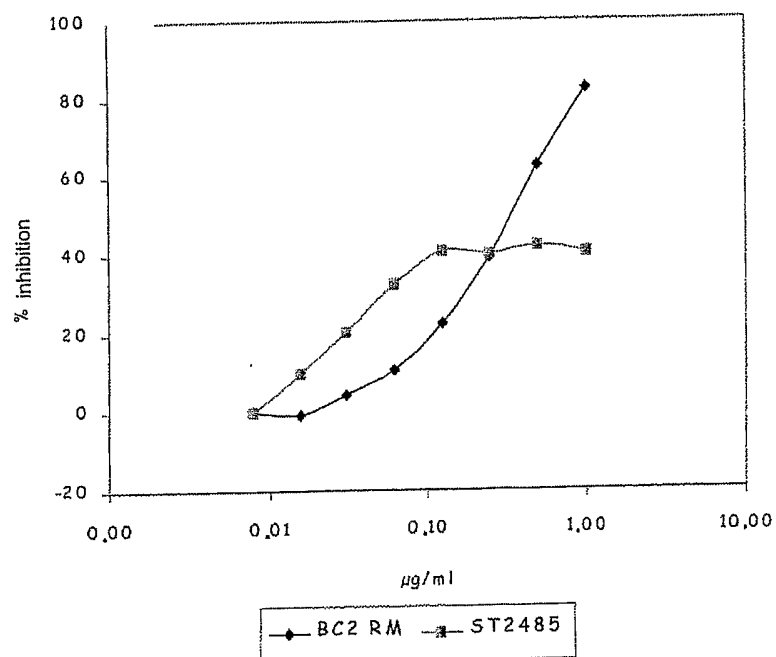
Figure 6

Competitive ELISA test between ST2485 and BC-2 for antigen binding.

a)



b)

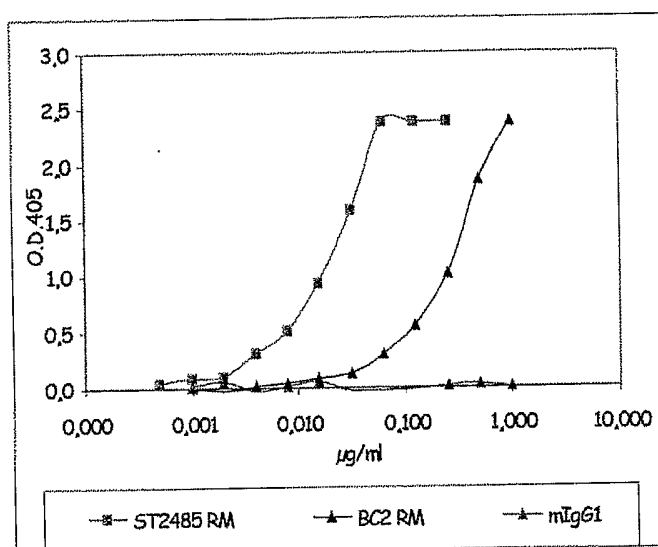


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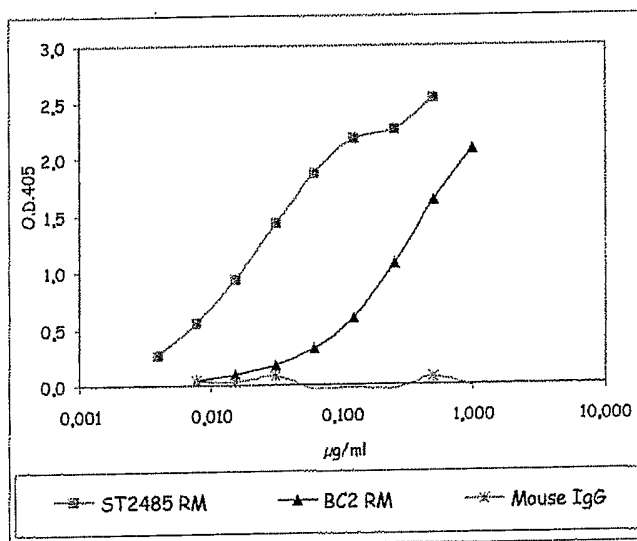
Figure 7

Immunoreactivity of ST2485 antibody in comparison with BC-2, on tenascin C (a) and on Tn A₍₁₋₄₎-D fragment (b).

a)



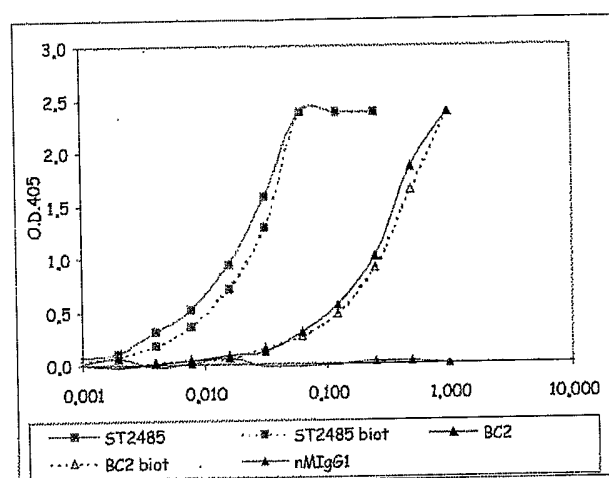
b)



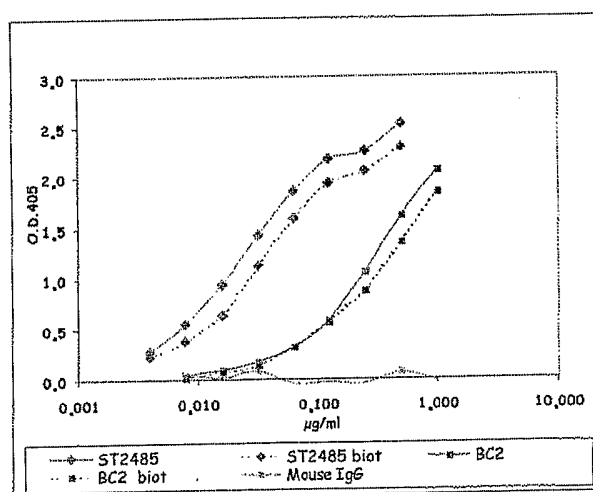
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Figure 8

Immunoreactivity of ST2485 and BC-2 biotinylated and non-biotinylated antibodies, on tenascin-C (a) and on Tn A₍₁₋₄₎-D fragment (b).



a)

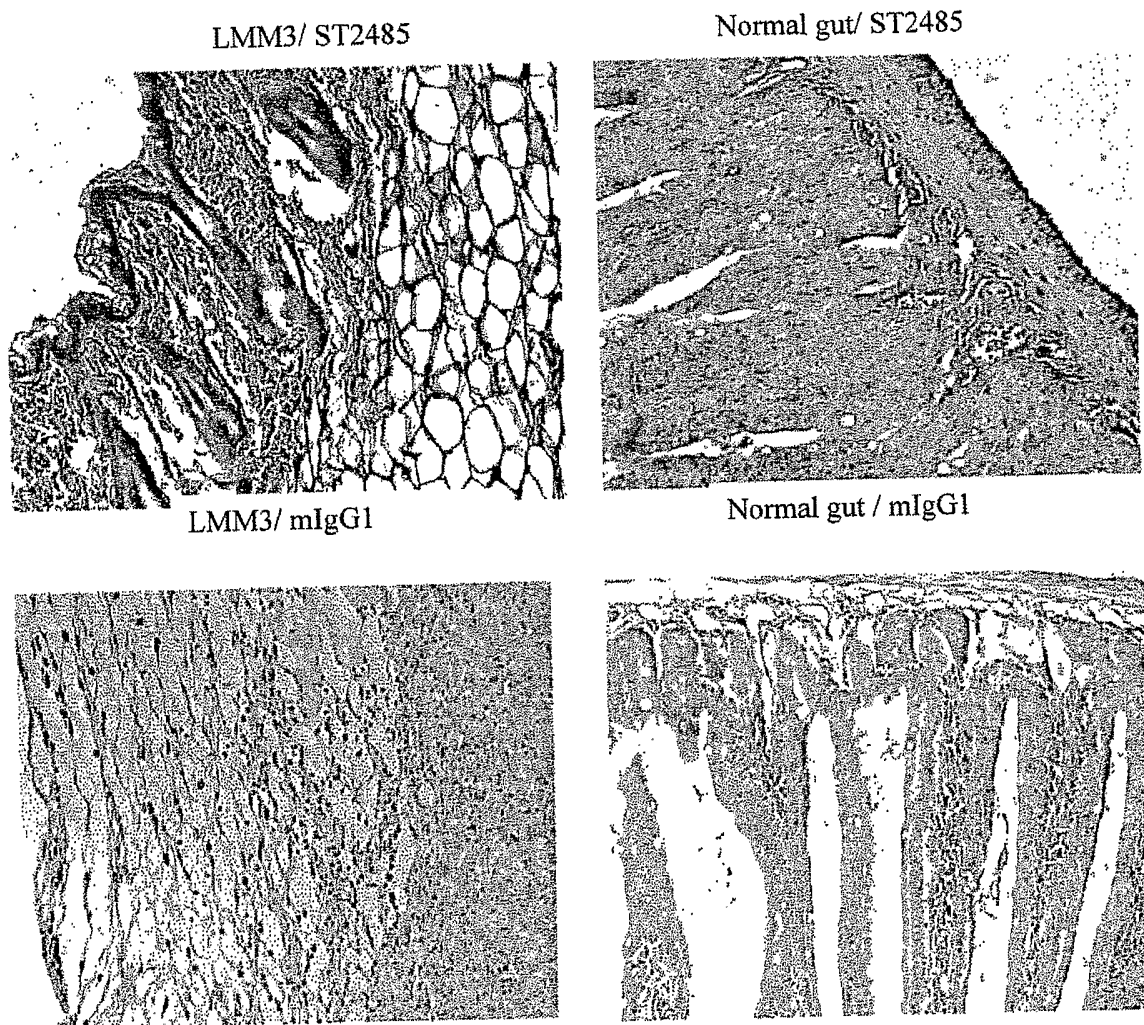


b)

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Figure 9

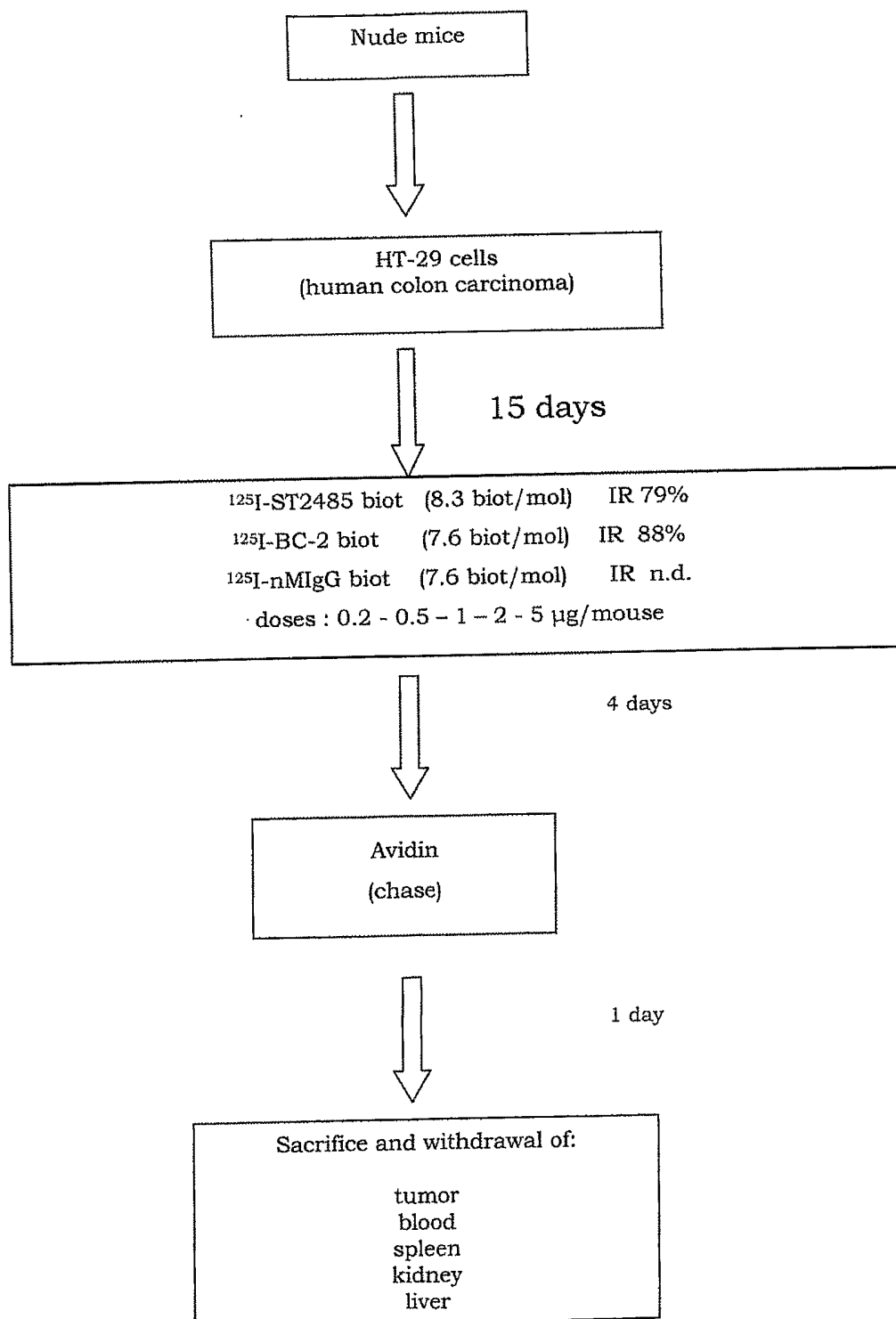
Cross-reactivity of ST2485 antibody with murine tenascin.



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Figure 10

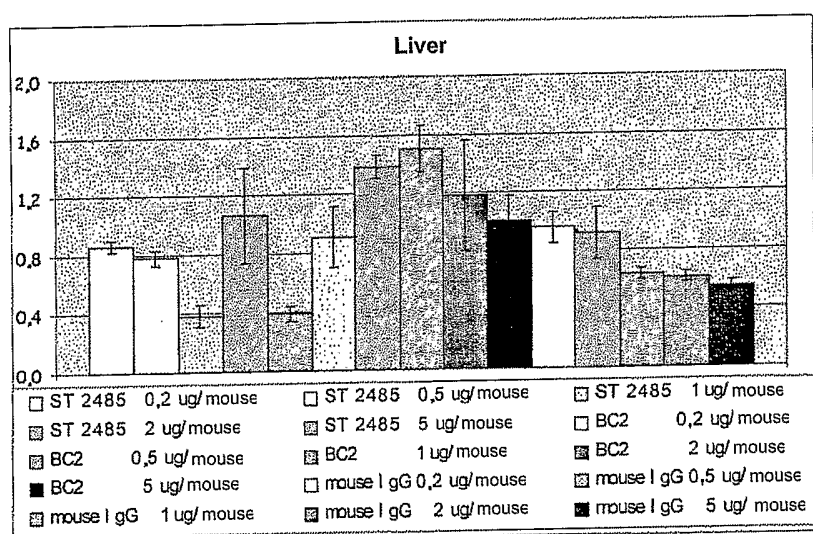
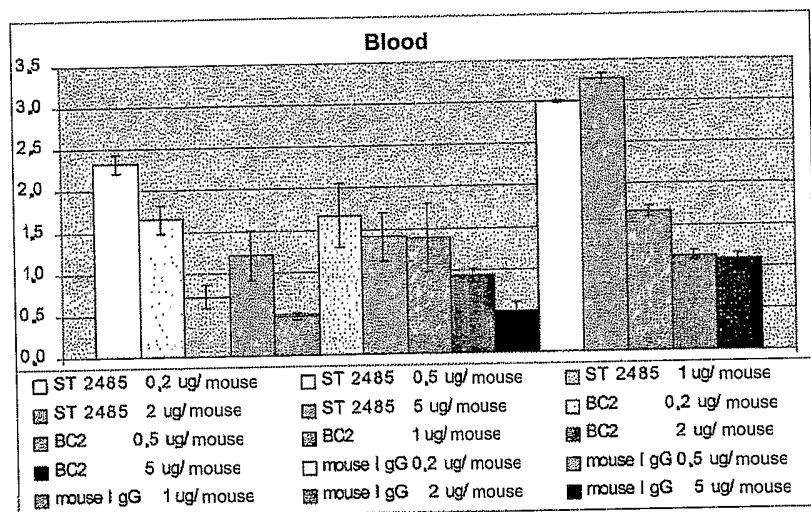
Biotinylated ST2485 and BC-2 antibodies biodistribution study protocol in human tenascin-expressing tumor-implanted nude mice.



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Figure 11

Biotinylated ST2485 and BC-2 biodistribution in human tenascin-expressing tumor-transplanted nude mice. The antibody amount is expressed as percent of the injected dose per tissue gram (% I.D./gr).



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Figure 11a

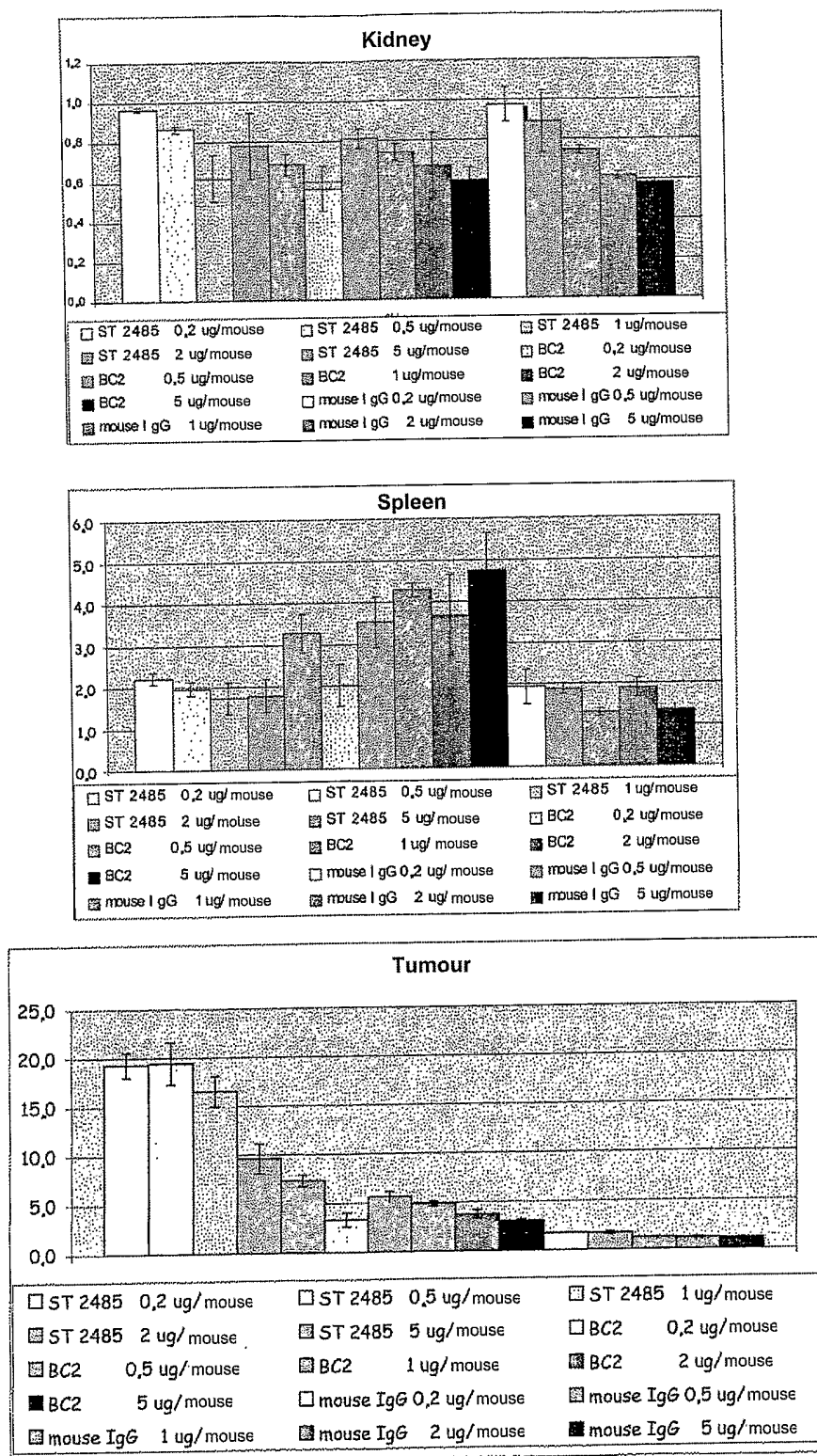


Figure 12

Biotinylated ST2485 and BC-2 biodistribution in nude mice: tumor/non tumor ratio.

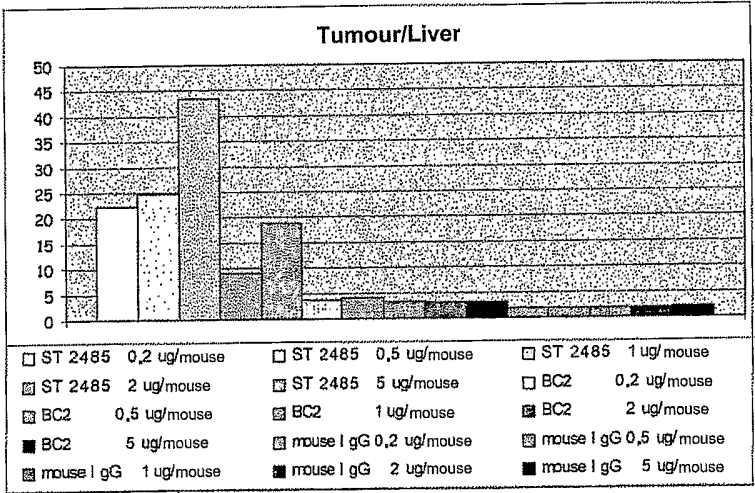
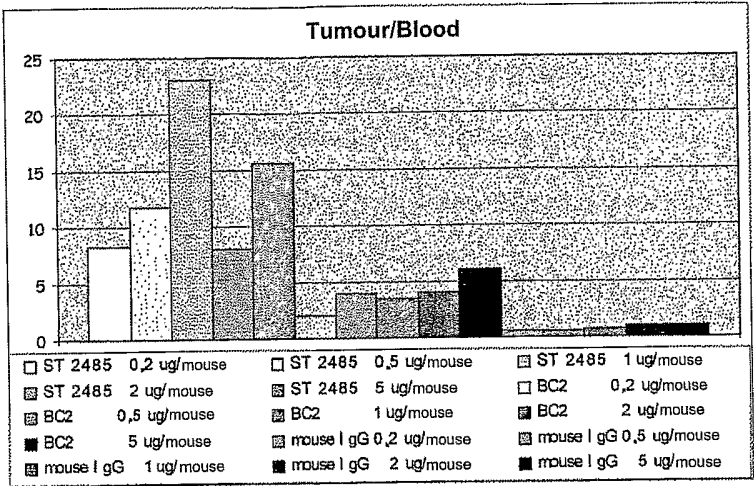
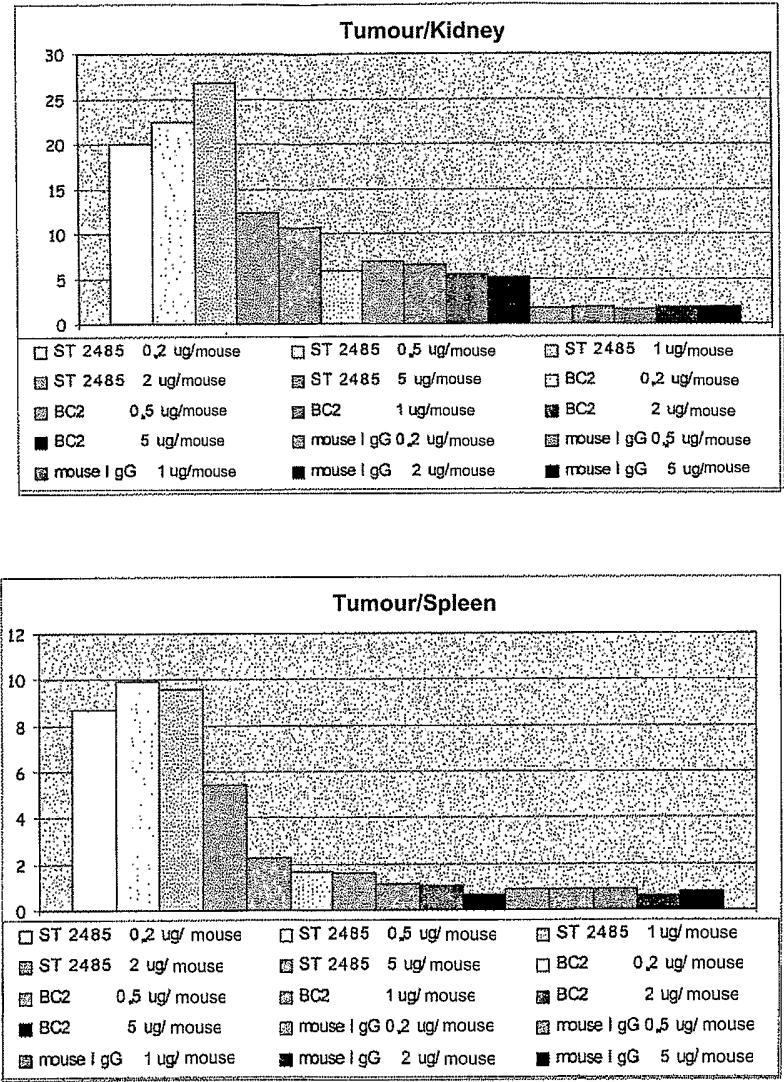


Figure 12a

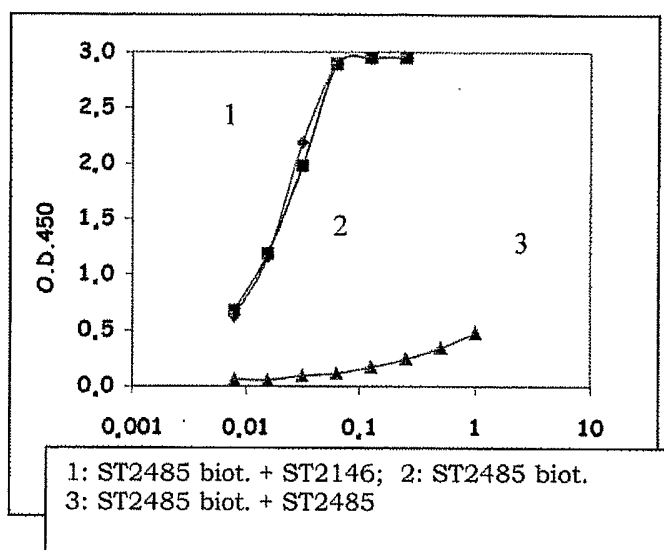


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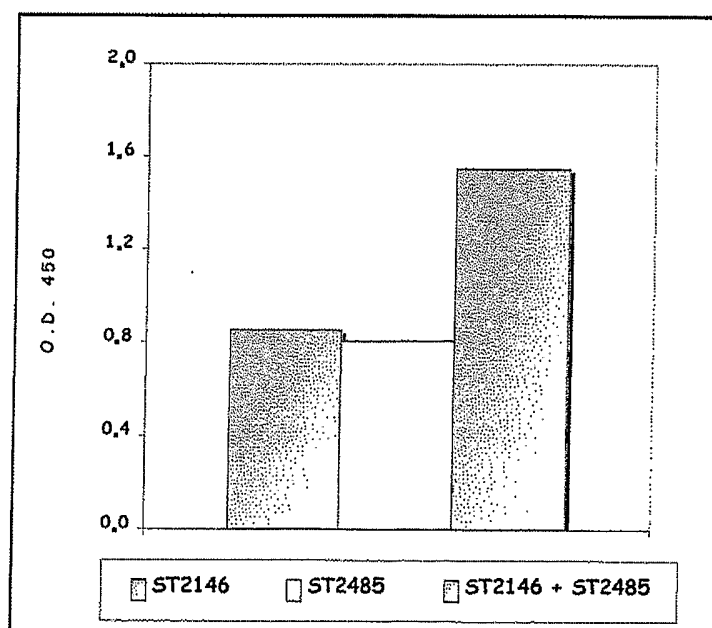
Figure 13

Interference (a) and additivity (b) ST2485 and ST2146 antitenascin antibodies *in vitro* evaluation by ELISA test.

a) Interference



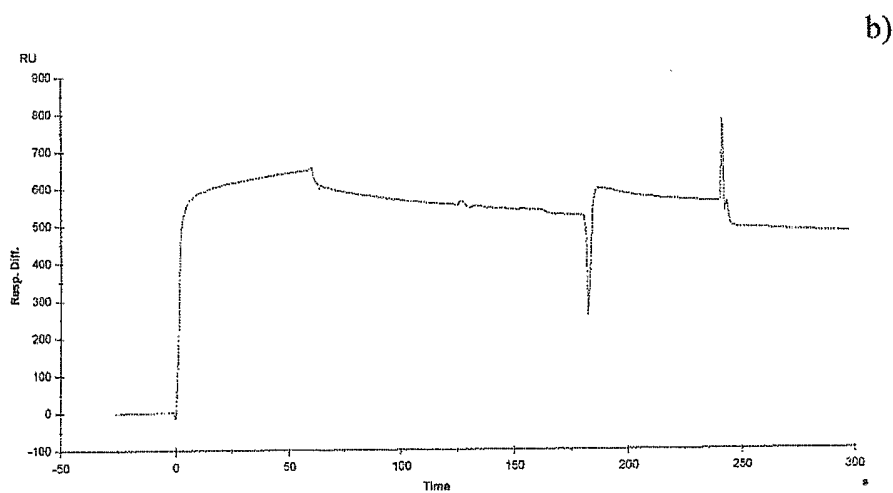
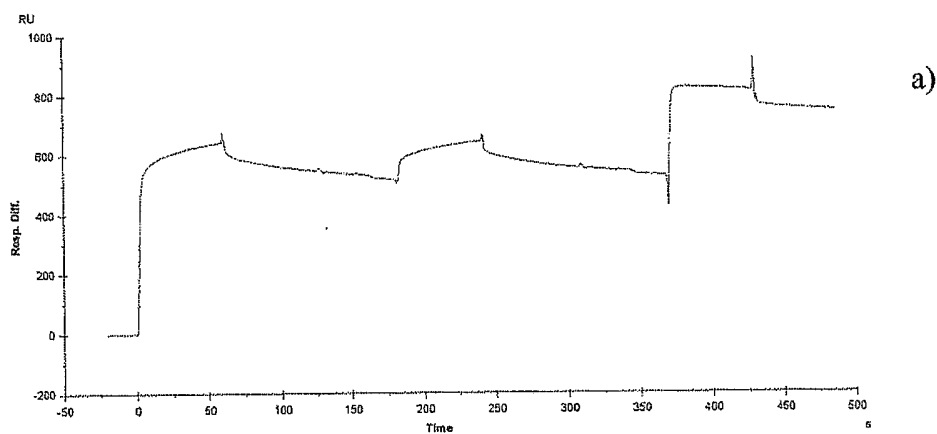
b) Additivity



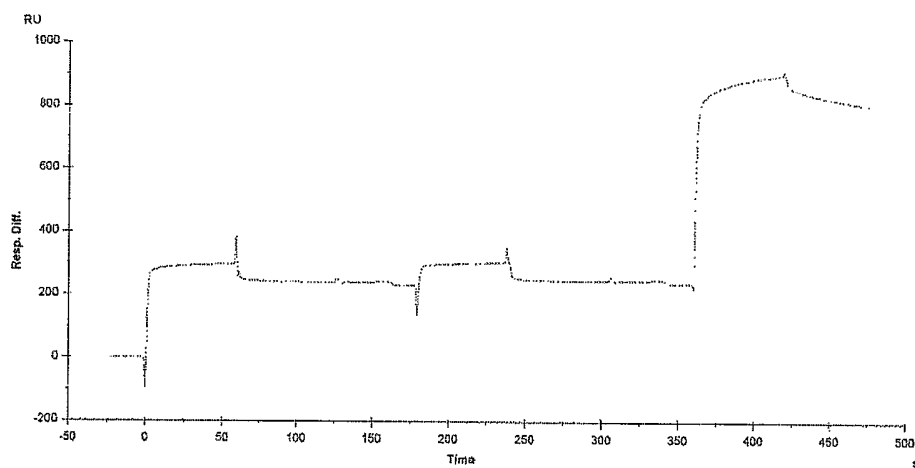
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Figure 14

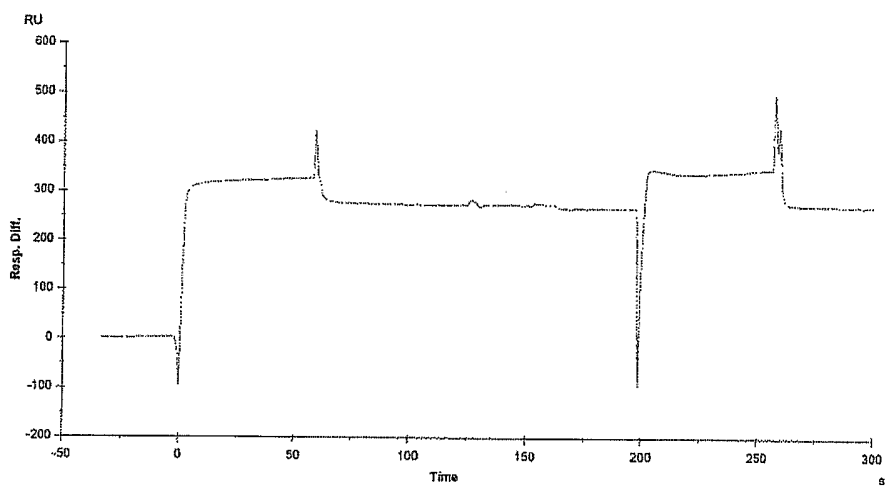
Antibodies ST2485 and ST2146 tenascin binding *in vitro* additivity by
BLACore



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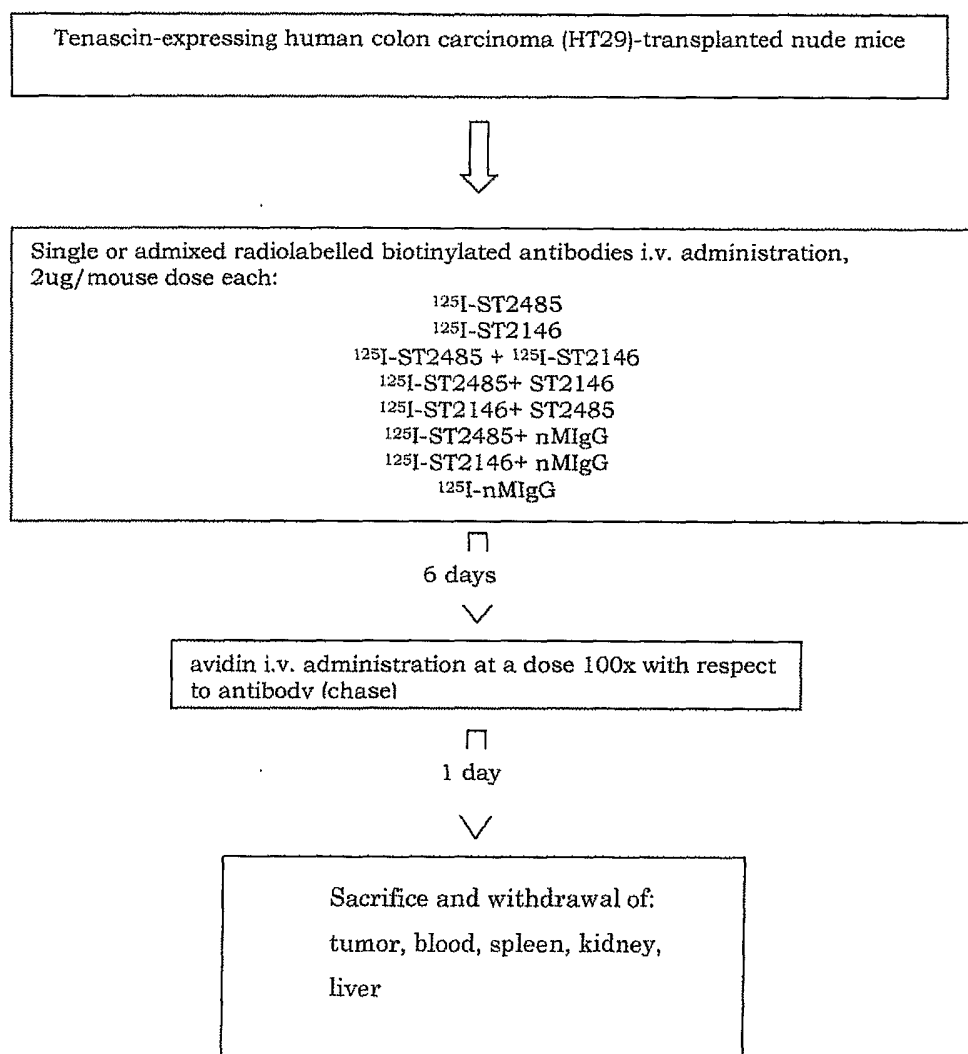
Figure 14a

c)



d)

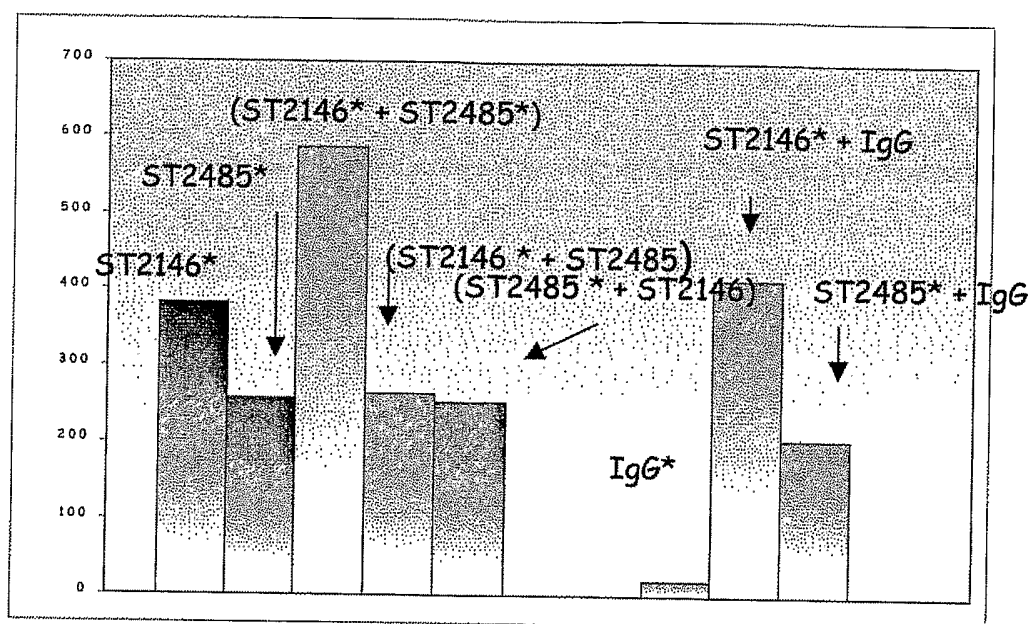
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Figure 15Schematic representation of *in vivo* additivity study in animal model

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Figure 16

ST2485 e ST2146 antibodies additivity in animal model; tumor seat localization.



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Figure 17

SEQID 1 sequence of ST2485 kappa light chain variable region (VL).

Signal peptide

ATGGATTTCAGTGCAGATTTCAGCTTCCTGCTAATCAGTGCTTCAGTCATAATGTCCAGAGGACAAA
 Met Asp Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser Val Ile Met Ser Arg Gly Gln

TTGTTCTCTCCAGTCTCCAGCAATCCTGTCTGCATCTCCAGGGGAGAAAGTCACAATGACTTGC
 Ile Val Leu Ser Gln Ser Pro Ala Ile Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys

↓ N-glycosylation

CDR1
AGGGCCAACTCAAGTGTACGTTTCATGCACTGGTACCAGCAGAAGCCAGGATCCTCCCCCAAACC
 Arg Ala Asn Ser Ser Val Arg Phe Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys

CDR2
CTGGATTATGTCATCCAACTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGG
 Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly

CDR3
GACCTCTTATTCTGTACAATCAGCAGAGTGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGC
 Ser Gly Thr Ser Tyr Ser Val Thr Ile Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln

ACTGGAGTAGTAATTCACCCAGGACGTTGGGTGGAGGCCACCAAGGTGGAAATCAGACGGGCT
 Gln Trp Ser Ser Asn Ser Pro Arg Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Arg Arg Ala

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Figure 18

SEQID 2 sequence of ST2485 gamma heavy chain variable region (VH)

Signal peptide

ATGGGATGGAGCTGGATCTTTCTCTCCTCCTGTCAGGAAGTGCAGGTGTCCACTCTGAGGTCCAGCTG
 Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly Val His Ser Glu Val Gln Leu

CAACAGTCTGGACCTGAGCTGGTGAAGCCTGGAGCTTCAATGAAGATTTCCTGCAAGGCTTCTGG
 Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser

CDR1

TTACTCATTCACTGGCTACACCATGAACCTGGGTGAAGCAGAGCCATGGAAAGAACCCTGAATGGA
 Gly Tyr Ser Phe Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu Glu Trp

CDR2

TTGGACTTATTAATGCTCACAATGGTGGTACTACCTACAACCAGAGTTCAAAGGGCAAGGCCACA
 Ile Gly Leu Ile Asn Pro His Asn Gly Gly Thr Thr Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr

TTAACTGTAGACAAGTCATCCAACACAGCCTACATGGAGCTCCTCAGTCTGACATCTGAGGACTC
 Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp

CDR3

TGCAGTCTATTACTGTACAAGACCEGGGGGTTACTACTGGTTCTTCGATGTCTGGGGCGCAGGGA
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SEQUENCE LISTING

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 DE SANTIS, Rita
 PELLICCIA, Angela
 PALOMBO, Giovanna
 CARMINATI, Paolo

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Val Ile Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Ile	
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ctg tct gca tct cca ggg gag aag gtc aca atg act tgc agg gcc aac	144
Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Asn	
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Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro	
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Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Val Thr Ile	
85 90 95	
agc aga gtg gag gct gaa gat gct gcc act tat tac tgc cag cag tgg	336
Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp	
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 Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
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 Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe
 35 40 45
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 Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
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 Glu Trp Ile Gly Leu Ile Asn Pro His Asn Gly Gly Thr Thr Tyr Asn
 65 70 75 80
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 Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Asn
 85 90 95
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 Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val
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 Tyr Tyr Cys Thr Arg Pro Gly Gly Tyr Tyr Trp Phe Phe Asp Val Trp
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 35 40 45

3/4

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Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro
 65 70 75 80

Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Val Thr Ile
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Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp
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Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Asn
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Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val
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