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Synthesis and preliminary in vitro evaluation of DOTA-Tenatumomab conjugates for theranostic applications in tenascin expressing tumors

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ABSTRACT

Tenatumomab is an anti-tenascin murine monoclonal antibody previously used in clinical trials for delivering radionuclides to tumors by both pre-targeting (biotinylated Tenatumomab within PAGRIT) and direct ^{131}Iodine labeling approaches. Here we present the synthesis and in vitro characterization of three Tenatumomab conjugates to bifunctional chelating agents (NHS-DOTA, NCS-DOTA and NCS-DTPA). Results indicate ST8198AA1 (Tenatumomab-DOTAMA, derived by conjugation of NHS-DOTA), as the most promising candidate in terms of conjugation rate and yield, stability, antigen immunoreactivity and affinity. Labeling efficiency of the different chelators was investigated with a panel of cold metals indicating DOTAMA as the best chelator. Labeling of Tenatumomab-DOTAMA was then optimized with several metals and stability performed confirms suitability of this conjugate for further development. ST8198AA1 represents an improvement of the previous antibody forms because the labeling with radionuclides like ^{177}Lu or ^{64}Cu would allow theranostic applications in patients bearing tenascin expressing tumors.

1. Introduction

Tenascin-C is a hexameric glycoprotein found in embryonic and adult extracellular matrices.[1] It can undergo alternative splicing resulting in large (monomer > 320 kDa) or small (monomer < 220 kDa) isoforms. The large Tenascin-C variant is preferentially expressed in malignant solid[2–7] and hematological tumors.[8] It is spatially and temporally related to tumor neovascularization and may exert anti-adhesive, and immunosuppressive activities. The presence of Tenascin-C within tumor-infiltrated tissues made it as an attractive candidate for antibody mediated therapy.

"Theranostic" or sometime named "theragnostic" is a neologism used in the last decade, which combines the words "therapeutic" and "diagnostic" for drugs or methods concerning the possibility of exploiting them both in therapeutic indications and for diagnostic applications. In this paper we have used the term theranostic because the results here reported could allow the use of this conjugate both in the therapeutic and diagnostic field. In detail, Tenatumomab (ST2146) is a murine monoclonal antibody recognizing the extra-cellular matrix protein Tenascin-C. It was originally imagined to deliver radionuclide therapy to tumors[9–11] and in its biotinylated version, proved to be useful for targeting radioactive biotin (^{90}Yttrium-BiotinDOTA) according to Pre-targeted Antibody Guided RadioImmunoTherapy (PAGRIT) clinical protocol.[12] Nevertheless, because such procedure requires the simultaneous production of multiple drugs, it was deemed too cumbersome to be pursued industrially and therefore the development of PAGRIT was discontinued in favor of simpler Tenatumomab versions. A clinical trial with ^{131}Iodine-Tenatumomab, in Tenascin-C positive cancer patients, was started but preliminary results suggested revising infusion modality (ClinicalTrials.gov NCT02602067) pointing out the need of an imaging guided approach to personalize treatment. In fact, despite fine specificity, monoclonal antibodies display inter-individual variability due to unpredictable factors like Fc receptor affinity, level of circulating antigen or presence of Anti-Drug Antibodies (ADA), thus requiring patient-specific information on the antibody distribution to personalize the radiotherapeutic treatment.[13,14]

Targeted radionuclide therapy (TRT) is a promising technique for cancer therapy but, so far, only ^{90}Y/^{111}In-Ibritumomab tiuxetan

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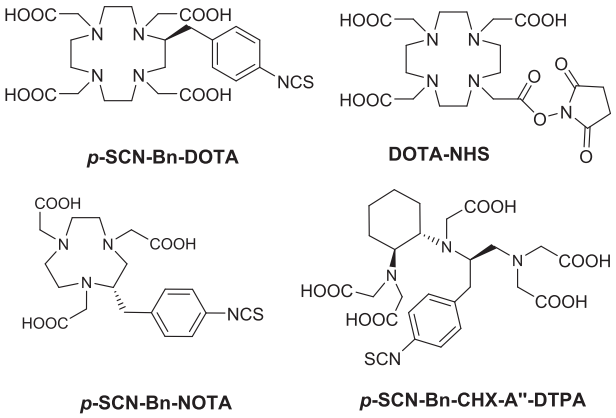


Scheme 1. Bifunctional chelating agents used in this work.

(Zevalin, Biogen IDEC) survived on the market for the therapy of Non-Hodgkin Lymphoma (NHL). The labeling of monoclonal antibodies with suitable radionuclides for SPECT and PET imaging and cancer therapy is an approach known as theranostic.[15] More than 20 antibodies or antibody-related therapeutics are currently investigated for theranostic purposes in patients.[16] In line with this trend, here we describe the chemical conjugation of three chelator moieties to Tenatumomab and the preliminary characterization of the related derivatives in a prospective theranostic use.

2. Results and discussion

2.1. Synthesis of cyclohexylamine-conjugated chelators

We started this study with the preparation of model compounds from four commercial bifunctional chelating agents (BFCAs), based on DOTA (1,4,7,10-tetraazacyclododecane tetraacetic acid), DTPA (diethylenetriamine pentaacetic acid) and NOTA (1,4,7-triazacyclononane triacetic acid) structures (Scheme 1).[17] These BFCAs were reacted with cyclohexylamine to mimic the bond formed in the conjugation of these ligands with antibody's external lysines (Scheme 1S). The reaction products thus formed were isolated in 55–75% yield, characterized by ^{1}H, ^{13}C NMR and HPLC-MS (see SI for details).

2.2. Complexation and preliminary stability of the model complexes

Each model chelator compound was complexed with 5 different metal ions: In(III), Y(III), Lu(III), Gd(III) and Cu(II). The Cu(II) complex with Cy-Bn-DOTA could not be further studied since a precipitate was formed immediately after addition of the metal ion to the ligand solution at three different pH (4–6). It is well established that the kinetic inertness of Ln(III) complexes is generally assessed either by measuring the rate of their dissociation under very acidic conditions or by determining the rate of transmetallation reaction, occurring in solution with competing Zn^{2+} or Cu^{2+} ions.[18] However, we focused here on testing the stability of the complexes under physiological conditions (PBS buffer at pH 7.4 and at 37 °C), by HPLC–MS, verifying that no further peaks were formed every day for seven days. In the case of diamagnetic metals, a further study was carried out via ^{1}H NMR, maintaining the solution in D_2O at 37 °C for one week. In the case of Gd (III), relaxivity was measured every day to determine its variation over time as an indication of the stability of the complex (if free Gd^{3+} ion was released, the relaxivity would increase over time).

Under these conditions, all the complexes resulted stable except LnNOTA-like complexes, as expected due to the presence of only six donor atoms in this chelator. In particular, a peak in the LC–MS chromatogram correspondent to the free ligand was clearly detectable after 24 h for the Y(III), Gd(III) and Lu(III) complexes of Cy-Bn-NOTA and after 72 h only 25% of the complex remained intact (see S.I., Figs. S1 and S2). Thus, after these preliminary tests, we decided to continue the study with the antibody conjugates with all BFCAs except *p*-SCN-Bn-NOTA.

2.3. Synthesis of the mAb-ligand conjugates

Between the different conjugates, NHS-DOTA showed higher reactivity compared to the other chelating agents used. In fact, it was sufficient a 10-fold molar excess and 45 min of reaction time to obtain a Chelator-Antibody-Ratio (CAR) in the desired range (CAR ~ 2). A 20-fold molar excess and two hours of reaction time were needed for *p*-SCN-Bn-DOTA and *p*-SCN-Bn-CHX-A″-DTPA to obtain similar ratios. No improvements were observed, in general, heating the reaction mixtures.

2.4. mAb-chelator conjugates analysis by MALDI

The comparison between the MALDI mass spectra of the naked Tenatumomab and each of the conjugated derivatives, NHS-DOTA, *p*-SCN-Bn-DOTA and *p*-SCN-Bn-CHX-A″-DTPA, showed an averaged CAR of 2.3, 1.8 and 2.3, respectively. The conjugation reactions were performed in triplicate and the molecular weight of the products averaged.

In order to get information on the localization of the chelating agent on the mAb, Tenatumomab and its derivatives were reduced with tris-(2-carboxyethyl)phosphine (TCEP) and analyzed by MALDI mass spectrometry. The results obtained showed unchanged molecular weight (at about 24 kDa) for the light chain of both the naked and the NHS-DOTA conjugated product (Tenatumomab-DOTAMA), while for the NCS derivatives also a molecular species with increased molecular weight was observed; the mass shift was attributed to the presence of one molecule of the chelating agent (Fig. S3).

For all the compounds, the heavy chain showed a difference in molecular weight attributable to the presence of one molecule of chelating agent conjugated except for Tenatumomab-DOTAMA. In fact, for the latter, the heavy chain of the unconjugated antibody was 51.9 kDa, while the derivative was 52.4 kDa. The difference was about 500 Da, corresponding to two DOTA residues (Fig. S4). From these data, it can be hypothesized that the conjugation reactions occurred preferentially on the heavy chain of the antibody, but, in the case of isothiocyanate, also conjugation to the light chain was possible.

2.5. Complexation of Tenatumomab-conjugates with metals ions, and stability

For the conjugates, antibody-chelator Tenatumomab-(DOTAMA), Tenatumomab-(Bn-DOTA) and Tenatumomab-(Bn-CHX-A″-DTPA), complexed with excess In^{3+}, Y^{3+}, Lu^{3+} ions (ratio metal:conjugate 5:1) in acetate buffer at pH 5.0, the results are summarized in Table 1.

Table 1
Yields of the complexation of Tenatumomab-chelator conjugates with In^{3+}, Y^{3+}, Cu^{2+} and Lu^{3+} (ratio MAb-chelator/metal ion 1:5) in 0.2 M acetate buffer, pH 5 and 37 °C and stability of the complexes over 7 days.

Entry	MAb-ML	Yield (%)	Stability after 7 day in PBS at 37 °C (%)
1	Tenatumomab-In(DOTAMA)	22	99.2
2	Tenatumomab-[In(Bn-CHX-A″-DTPA)]$^{2-}$	100	99.8
3	Tenatumomab-[In(Bn-DOTA)]$^{-}$	31	99.0
4	Tenatumomab-Y(DOTAMA)	37	99.3
5	Tenatumomab-[Y(Bn-CHX-A″-DTPA)]$^{2-}$	15	96.9
6	Tenatumomab-[Y(Bn-DOTA)]$^{-}$	50	99.2
7	Tenatumomab-Lu(DOTAMA)	100	98.0
8	Tenatumomab-[Lu(Bn-CHX-A″-DTPA)]$^{2-}$	25	99.6
9	Tenatumomab-[Lu(Bn-DOTA)]$^{-}$	48	88.7
10	Tenatumomab-Cu(DOTAMA)	100	95.5

Table 2
Yields of the complexation of Tenatumomab-chelator conjugates with In^{3+}, Y^{3+}, Cu^{2+} and Lu^{3+} (ratio MAb-chelator/metal ion 1:0.1) in two different buffers (0.2 M acetate buffer, pH 5 and 0.2 M Hepes buffer, pH 6.8).

Entry	Acetate buffer 0.2 M (pH 5.0)	Yield (%)	HEPES buffer 0.2 M (pH 6.8)	Yield (%)
1a	In(DOTAMA)	44.0 ± 2.7	In(DOTAMA)	46.5 ± 2.4
2a	$[In(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	61.4 ± 5.7	$[In(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	73.5 ± 6.8
3a	$[In(Bn\text{-}DOTA)]^{-}$	44.6 ± 7.9	$[In(Bn\text{-}DOTA)]^{-}$	65.2 ± 7.0
4a	Y(DOTAMA)	27.3 ± 0.7	Y(DOTAMA)	91.2 ± 8.7
5a	$[Y(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	77.5 ± 3.4	$[Y(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	86.2 ± 0.8
6a	$[Y(Bn\text{-}DOTA)]^{-}$	74.7 ± 7.7	$[Y(Bn\text{-}DOTA)]^{-}$	72.4 ± 7.1
7a	Lu(DOTAMA)	36.8 ± 0.1	Lu(DOTAMA)	16.9 ± 6.8
8a	$[Lu(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	56.6 ± 2.9	$[Lu(Bn\text{-}CHX\text{-}A''\text{-}DTPA)]^{2-}$	82.6 ± 7.0
9a	$[Lu(Bn\text{-}DOTA)]^{-}$	22.3 ± 1.9	$[Lu(Bn\text{-}DOTA)]^{-}$	0.2 ± 0.1
10a	Cu(DOTAMA)	53.1 ± 0.5	Cu(DOTAMA)	74.7 ± 7.3

In particular, unlike the model systems discussed earlier, the yields are not always quantitative. Indeed, for some complexes, the yield of complexation was found to be less than 50%, while for others the yield varies between 50 and 80%. Only three complexes were obtained with a quantitative yield. With regard to the stability measurements, a free metal release after one week was lower than 1% for most of the complexes.

Clearly, the model system cannot be coincident with the antibody-chelator system in which the concentrations involved are different and the buffer has a possible influence. Since the concentration of the radiometals during the labeling is supposedly much lower than that of the chelator (conjugate:metal ratio of 1:0.1), an assessment of complex stability was not considered necessary. The yields of complexation were carried out in triplicate. In the case of the Tenatumomab-DOTAMA conjugate, the Cu^{2+} complex was also prepared in the two selected buffers. The complexation yield was then determined via ICP-MS (Table 2).

The data reported in Table 2 shows that the Lu(Bn-DOTA) complex in Hepes buffer was virtually not formed (yield of 0.2%). It can be noted that for Lu(III) the synthesis in acetate buffer at pH 5 is preferable with respect to the Hepes buffer at pH 6.8 (as also reported in the literature),[19] whereas for the other metal ions the yields are typically higher in Hepes buffer at pH 6.8.

The stability of the conjugates of Tenatumomab with NHS-DOTA (ST8198AA1), NCS-DOTA (ST8199AA1) and NCS-DTPA (ST8200AA1) was investigated by SEC-HPLC analysis, incubating the conjugates in PBS at 37 or 56 °C up to 24 h. Molecular integrity was then evaluated by size exclusion chromatography in comparison with unconjugated Tenatumomab. Results show no significant change in chromatography profiles of all conjugates incubated at 37 °C while both Tenatumomab and conjugates were degraded at 56 °C (Fig. S5).

2.6. Surface plasmon resonance (SPR) results

Surface plasmon resonance (SPR) spectroscopy was applied to investigate kinetics parameter for interaction of Tenatumomab and its conjugates with Tenascin-C. Analysis of sensorgrams (see S.I. Figs. S6–S9) shows that best fitting is achieved by a bivalent model. All four antibodies (Tenatumomab and three conjugates to bifunctional chelating agents), however, show a dissociation from Tenascin-C almost insignificant (for kinetic data from fitting and fitted sensorgrams see S.I. Figs. S6–S9 and Table S1).

This makes it difficult to consider the dissociation data obtained as reliable, so we focused our attention on association region of sensorgrams. As it is possible to see in Fig. 1A, association constant k_{a1} of ST8198AA1 is quite similar to that of ST2146, while ST8199AA1 and ST8200AA1 k_{a1} constants are about seven time smaller than that of ST2146. Four sensorgrams (Fig. 1B) for relative Abs tested at the concentration of 15 nM are showed after normalization: the slope of curves for ST8199AA1 and ST8200AA1 clearly demonstrates that these two Abs associate more slowly to the antigen (dashed region).

2.7. Immunoreactivity

Tenascin-specific ELISA was used to test the immunoreactivity of conjugates compared to Tenatumomab. As shown in Fig. 2, ST8198AA1 resulted the most preserved derivative, although its residual immunoreactivity was reduced to about 50%. More in detail, residual immunoreactivity was 52% for Tenatumomab-DOTAMA (ST8198AA1), 26% for Tenatumomab-Bn-DOTA (ST8199AA1) and 22% for Tenatumomab-Bn-CHX-A″-DTPA (ST8200AA1) (mean of three experiments).

2.8. ^{111}In radiolabeling of Tenatumomab-DOTAMA

Radiolabeling efficiency by iTLC of ST8198AA1 conjugate was 77.0% and 47.2% for 1:0.12 and 1:0.7 (DOTAMA: ^{111}In atoms) respectively. The analysis repeated in the presence of a DTPA excess, which is challenging low affinity bonds, resulted of 58.5% and 23.5%, for 1:0.12 and 1:0.7 (DOTAMA: ^{111}In atoms), respectively. Based on both analyses, the best conjugate appeared to be the one with (DOTAMA: ^{111}In atoms) 1:0.12.

The reaction mixture was then purified on a PD-10 column and relevant fractions re-analyzed by iTLC, showing that most activity of the radiolabeled conjugate was eluted in the fractions 7–9 (Table 3).

2.9. Stability of radioconjugates

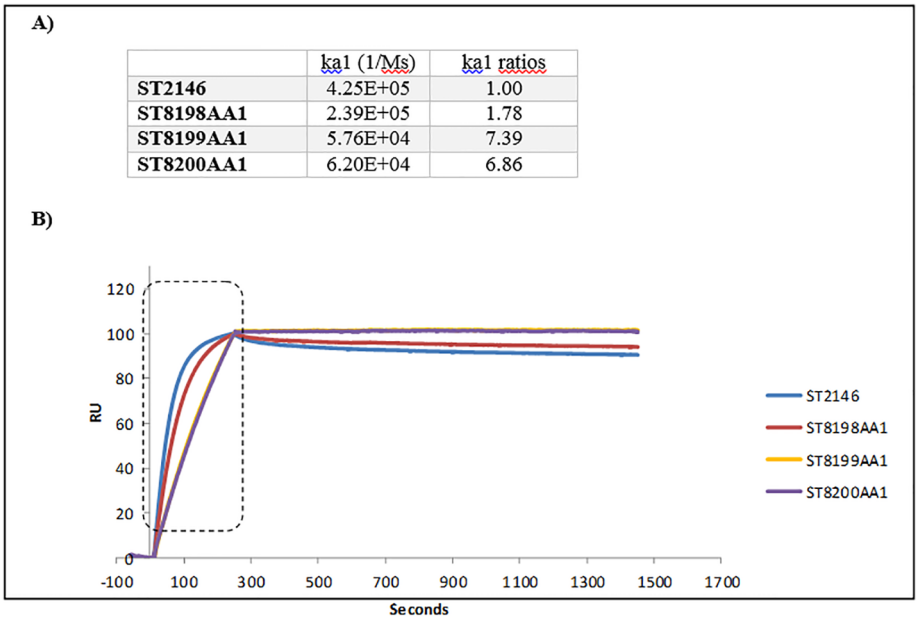
Preliminary stability of the radiolabeled material under the intended storage condition of investigational lots was confirmed by iTLC after 24 h at +4 °C (Table 4).

3. Conclusions

Tenascin-C, a hexameric glycoprotein preferentially expressed in malignant solid and hematological tumors and related to tumor neovascularization, anti-adhesive and immunosuppressive activities, represents an attractive candidate for antibody mediated therapy.

Tenatumomab (ST2146), a murine monoclonal antibody that recognizes the extra-cellular matrix protein Tenascin-C, was originally imagined for delivering radionuclide therapy to tumors. The ^{131}Iodine-Tenatumomab has been investigated in clinical trials in Tenascin-C positive cancer patients. Here we describe the chemical conjugation of chelator moieties to Tenatumomab and the preliminary characterization of the related derivatives as candidates for prospective theranostic uses.

The conjugation reaction of three different bifunctional chelators to Tenatumomab and then the complexation of the resulting antibody-chelator conjugates with different metal ions of interest for imaging or therapeutic applications, were screened. The results indicated that NHS-DOTA is the most efficient moiety that reacts preferentially on the heavy chain of the antibody. Moreover, the Tenatumomab-DOTAMA conjugate formed the complexes with Y(III), Lu(III) or Cu(II) in high yield both in the presence of excess of metal ion and in excess of



	ka1 (1/Ms)	ka1 ratios
ST2146	4.25E+05	1.00
ST8198AA1	2.39E+05	1.78
ST8199AA1	5.76E+04	7.39
ST8200AA1	6.20E+04	6.86

Fig. 1. A: k_{a1} values for Tenatumomab (ST2146), ST8198AA1, ST8199AA1 and ST8200AA1 (first column) and ratios of Tenatumomab k_{a1} value to derived antibodies k_{a1} values (second column). B: normalized sensorgrams for Tenatumomab, ST8198AA1, ST8199AA1 and ST8200AA1 tested at 15 nM; dashed region highlights differences in association kinetics of the four antibodies.

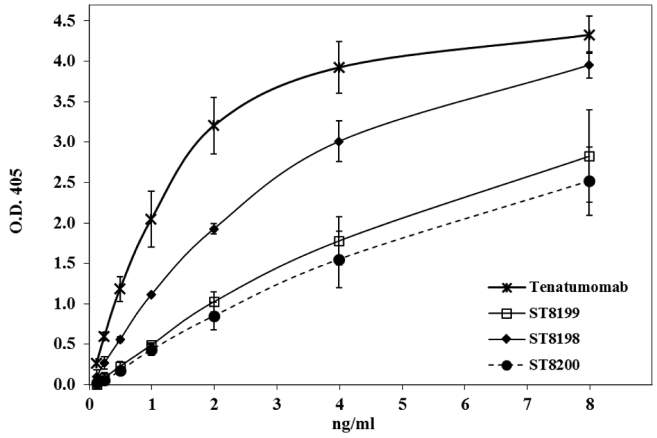


Fig. 2. Immunoreactivity of conjugates (ST8198AA1, ST8199AA1, ST8200AA1) as compared to Tenatumomab, tested by Tenascin-C specific ELISA. Detection through anti-mouse IgG-PA antibody and pNpp substrate addition. Optical density at 405 nm measured by spectrophotometer. Result are the mean (± SD) of three independent experiments.

Table 3
Percent of radiolabeled conjugate Tenatumomab-DOTAMA and free metal in PD-10 fractions by iTLC.

Fraction	Antibody coniugated metal	Free metal
6	99.72%	0.28%
7	99.59%	0.41%
8	98.43%	1.57%
9	83.96%	16.04%
10	39.50%	60.50%
11	25.01%	74.99%
12	21.46%	78.54%

antibody-chelator conjugates. A stability test in physiological conditions showed that the complexes are stable with negligible release of free metal from the chelator.

Monoclonal antibodies are entering clinical trials and being approved in record numbers with over 570 antibodies at various development stage.[20] Nevertheless, among radiolabeled antibodies only ibritumomab survived on the market and the percentage of

Table 4
Percent of radiolabeled conjugate Tenatumomab-DOTAMA and free metal in PD-10 fractions by iTLC, after 24 h at +4 °C.

Fraction	Antibody coniugated metal	Free metal
7	99.42%	0.58%
8	98.38%	1.62%
10	40.27%	59.73%

investigational radiolabeled antibodies is very low because of intrinsic logistic/technological difficulties in the development of such therapeutics. However, radionuclide therapy of tumors is particularly appealing because of the high direct therapeutic potential of irradiation and because of the emerging role of irradiation in unmasking tumor cells to the immune system thus adding efficacy to emerging immunotherapeutic approaches like those based on check point inhibitor antibodies.[21]

From the ibritumomab experience, it emerged the value of radioimmunotherapy for hematological tumors and the usefulness of dosimetry to model pharmacokinetic and optimize treatment.[22]

Unfortunately, radioimmunotherapy of solid tumors is much challenging because such tumors are much more radioresistant than hematological and less accessible to large molecules. Moreover, both solid and hematological tumors exhibit the problem of unpredictable level of circulating antigens that compete with tumor-associated and affect efficiency of tumor targeting as well as off-target accumulation of radioactivity due to sinking of immune-complexes in liver and spleen. Therefore, personalized treatments based on pre-therapeutic imaging is highly sought and made possible by theranostic approaches, which are based on the use of different radionuclides optimized for imaging or therapeutic purposes. In conclusion, we believe that Tenatumomab-DOTAMA is an improved version of previously clinically investigated forms of Tenatumomab because it could allow to perform pre-treatment imaging and dosimetry evaluations finally leading to personalized therapeutic treatments of tenascin expressing tumors.

4. Experimental part

4.1. Materials and methods

All chemicals were purchased from Sigma-Aldrich or Alfa Aesar unless

otherwise stated and were used without further purification. *p*-SCN-Bn-DOTA (*S*-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetra-acetic acid), NHS-DOTA, *p*-SCN-Bn-NOTA (2-*S*-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid) and *p*-SCN-Bn-CHX-A″-DTPA ([(*R*)-2-Amino-3-(4-isothiocyanatophenyl)propyl]-trans-(*S*,*S*)-cyclohexane-1,2-diamine-pentaacetic acid) were purchased from Macrocyclics Inc (US). Tenutomomab is a monoclonal anti-tenascin antibody developed by Alfasigma (former Sigma Tau; ST2146).

4.2. Complexation protocol for the model chelator compounds

To obtain information about their metal binding efficiency, the model ligands were dissolved in water (1 mL) and the pH was adjusted to 6.0. An excess of 1.1 M equivalents of the metals ions were added as the following salts (YCl_3; $Lu(NO_3)_3$; $GdCl_3$; $InCl_3$ and $CuCl_2$) and the pH was brought back to 6. The solutions were left stirring for 1 h at 37 °C and freeze-dried. HPLC–MS analyses showed the formation of the metal complexes except for the Cu(II) complex with Cy-Bn-DOTA as precipitation occurs as soon as the metal ion is added to the ligand (the product also precipitated at different pH values (4.0–6.0) (HPLC-MS analyses method: $AcONH_4$ pH 5.5 (A)/MeOH (B): 0–3 min 70% A; 3–18 min dal 30% al 100% B).

4.3. Stability study of the model complexes

The model cyclohexylamine-conjugated complexes obtained (Scheme 1S) were dissolved in 1 mL PBS (pH 7.4) and left at 37 °C for one week in a thermostatic water bath. Every 24 h HPLC analyses and ^1H proton spectra (except for Gd-complexes) were performed in order to investigate the stability of the complexes (HPLC–MS analyses method: $AcONH_4$ pH 5.5 (A)/MeOH (B): 0–3 min 70% A; 3–18 min dal 30% al 100% B).

4.4. Synthesis of the mAb-ligand conjugates

In order to optimize the conjugation conditions to bind in a range of 1.5–2.0 ligands for mAb, the effects of mAb/ligand molar conjugation ratio, reaction time, temperature, pH, and reaction buffer were studied.

First of all, the antibody was dialyzed overnight at 4 °C with 0.1 M $NaHCO_3$ buffer, pH 7.4, using a 14 M WCO membrane in order to change the buffer and to eliminate small interfering molecules. Each ligand was solubilized in DMSO just before starting the reaction at a concentration of 10 mM and added to the reaction mixture using 1:10 or 1:20 mAb/ligand molar ratios.

Depending on the ligand, the conjugation reaction was performed in 0.1 M $NaHCO_3$ buffer, pH 7.4, with 10-fold molar excess for NHS-DOTA, and 20-fold molar excess for *p*-SCN-Bn-DOTA, and *p*-SCN-Bn-CHX-A″-DTPA.

The reaction time was 45 min for NHS-DOTA and 2 h for the other two ligands, at room temperature under gentle shaking.

The conjugation mixture was dialyzed in a dialysis membrane with a molecular cut-off value of 14 kDa against 0.1 M $NaHCO_3$ buffer, pH 7.4 with two buffer changes to remove the unconjugated ligands. The concentration of each Tenatumomab derivative was determined spectrophotometrically at 280 nm (OD_{280nm}/1.35). Few microliters of the reaction products and of the starting naked antibody were desalted for MALDI mass spectrometry analysis.

4.5. mAb-chelator conjugates analysis by MALDI

As described above, in the Results and Discussion Section 2.3, native and reduced Tenatumomab as well as its conjugated derivatives were analyzed by MALDI mass spectrometry, in order to get information on the localization of the chelating agent. The results obtained showed unchanged molecular weight for the light chain of both the naked and the NHS-DOTA conjugated product, while for the NCS derivatives also a molecular species with increased molecular weight was observed (Fig. S3). While for the heavy chain of all the compounds analyzed, the mass spectra obtained showed a difference in molecular weight attributable to the presence of one molecule of chelating agent conjugated except for Tenatumomab-DOTAMA that showed a difference of about 500 Da (52.4 vs. 51.9 kDa), corresponding to two DOTA residues (Fig. S4). All these data suggest that conjugation reactions occurred preferentially on the heavy chain of the antibody with the sole exception of isothiocyanate where a conjugation also to the light chain was possible as shown by the peaks at about 48 kDa, attributable to the $[2\,M+H]^+$ of the light chain portion.

4.6. Complexation protocol of Tenatumomab-conjugates with metals ions

2 mg of each conjugates antibody-chelator (Tenatumomab-(DOTAMA), Tenatumomab-(Bn-DOTA) and Tenatumomab-(Bn-CHX-A″-DTPA)) dissolved in PBS pH 7.4, (+NaN_3 0.05%), were exchanged three times with the appropriate buffer (acetate buffer pH 5 or Hepes buffer pH 6.8) using Vivaspin concentrators (cutoff 10 kDa) and adjusting the final volume to 1 mL.

For preparations of the complexes two different molar ratios were used: 1:5 (Tenatumomab-conjugates:metal ions), to evaluate the maximum binding capacity, and 1:0.1 (Tenatumomab-conjugates:metal ions) to evaluate the binding efficiency in possible future conjugations with radiometals at low specific activity (for diagnostic use).

Both the metal-ions amounts (5 eq. or 0.1 eq.) were evaluated, with the following salts: YCl_3, $Lu(NO_3)_3$, $InCl_3$, and, only for DOTAMA $CuCl_2$, and the complexations were left stirring for 2 h at 37 °C. Moreover, for the molar ratio 1:5 the complexations was carried out in 0.2 M acetate buffer pH 5.0 (we have used acetate buffer as a suitable buffer, already reported on numerous literature articles, for the complexation of ligand-antibody conjugates,[17] while for the molar ratio 1:0.1 the complexation was performed also in 0.2 M hepes buffer pH 6.8.

The yield of the complexation reaction was determined by means of inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Optek X Series 2) after microwave mineralization of the samples. For this purpose, acid digestion of the conjugates in 800 µL of HNO_3 70% w/w and 200 µL of hydrogen peroxide 35% w/w in water were performed in quartz cuvettes. The cuvettes were then introduced into a closed vessel in the microwave oven, where a temperature-controlled microwave heating (200 °C) was applied for 45 min (microwaves power = 1200 W, Milestone Start D with internal temperature sensor).

4.7. Stability study of Tenatumomab-conjugates complexes

The stability was determined only for the Tenatumomab-conjugates:metal ions ratio 1:5.

For this purpose, the metal complexes were exchanged back to PBS buffer (+NaN_3 0.05%) (pH 7.4) through Vivaspin concentrators (cut-off 10 kDa) and left at 37 °C for one week in a thermostatic water bath. Every 24 h, the complexes were washed two times with PBS buffer (+NaN_3 0.05%) using Vivaspin concentrators (cut-off 10 kDa) and adjusting the final volume to 1 mL. The buffer collected during these washings were freeze dried and then mineralized by dissolving in nitric acid and heating at 120 °C for one night. Then, the solutions were diluted with water to obtain a final 1% (v:v) nitric acid concentration and finally analyzed by ICP-MS.

4.8. SEC-HPLC analysis

Stability of ST8199AA1, ST81998AA1 and ST8200AA1 was also investigated by SEC-HPLC analysis, after incubation of the conjugates in

PBS at 37 or 56 °C up to 24 h. Molecular integrity was then evaluated by size exclusion chromatography in comparison with unconjugated Tenatumomab.

50 μg of each sample were loaded on a TSKgel G3000SWX; phase diol, L × I.D. 30 cm × 7.8 mm, 5 μm particle size TOSOH BIOSCIENCE (SIGMA) #0008541. Mobile phase of 100 mM phosphate buffer solution (pH 7.0), 300 mM NaCl/Acetonitrile (90:10), at a flow rate of 1 ml/min. Detection by 280 nm absorbance.

4.9. Surface plasmon resonance study on Tenatumomab and Tenatumomab-conjugates complexes

All experiments were performed with a Biacore T200 (GE Healthcare). Tenascin-C was immobilized on CM5 sensor chips (GE Healthcare) according to standard amino coupling procedure: one flow cell surface was activated with EDC/NHS (GE Healthcare Amine Coupling Kit) and then, Tenascin (82 μg/mL in 10 mmol/L sodium acetate brought to pH 2.3 with HCl) was injected; capping of unreacted sites was achieved by flowing ethanolamine (GE Healthcare Amine Coupling Kit) to gain a final immobilization responses of about 790 RU. Another flow cell of the same chip was subjected to a blank immobilization to use it as reference cell (activation with EDC/NHS and capping of activated sites with ethanolamine without flowing Tenascin). Tenatumomab and derivatives ST8198AA1, ST8199AA1 and ST8200AA1 were flowed at 20 μL/min in both flow cells at seven concentration ranging from 0.45 nM to 30 nM in running buffer HBS-EP + (GE Healthcare) for a contact time of 240 s; after that only running buffer was flowed in both cells for a dissociation time of 1200 s and then the two surfaces were regenerated by flowing NaOH 50 mM at 20 μL/min for 30 s. Sensorgrams obtained by subtracting signal from the reference cell were analyzed by BiaEvaluation 1.0 software (Supporting information Figs. S6–S9 and Table S1).

4.10. Immunoreactivity (enzyme-linked immunosorbent assay) (ELISA)

MAXISORP 96-well plates (Nunc) were coated overnight at 4 °C with 100 μL/well of Tenascin-C (TG33) [0.5 μg/mL]. After blocking with 300 μL/well of PBS + 1%BSA for 1.30 h, plates were washed with PBS 0.1% Tween 20 and incubated at room temperature with serial dilutions of standard curve of samples starting from 8 ng/mL (8 serial dilutions in blocking solution).

After washings, anti-mouse IgG-PA antibody (Sigma A2429), diluted 1:1000 in PBS 0.1% Tween 20 1% BSA, was added at room temperature for 1 h and 30 min. Plates were washed again and incubated with pNpp for 30 min at room temperature, optical density at 405 nm was measured by ELISA reader (TECAN).

4.11. ^{111}In Radiolabeling of Tenatumomab-DOTAMA

Tenatumomab-DOTAMA (ST8198AA1) was radiolabeled with ^{111}Indium (^{111}In).

Briefly, 500 ng of ST8198AA1 in 0.5 mL of 0.2 M HEPES pH 6.8, were incubated with 1.5 MBq, or 8.9 MBq of radiometal (corresponding to about 1:0.12 and 1:0.70 chelator:radiometal atoms), 2 h at room temperature. Radiolabeling yield was tested by iTLC and then the antibody-radiometal conjugate purified from unreacted ^{111}In on PD-10 columns, collecting 0.5 mL fractions.

Interesting fractions were further tested by iTLC to monitor stability.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.05.047.

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