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ORIGINAL ARTICLE

Imaging of human glioma cells by means of a Syndecan-4 directed DOTA-conjugate

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Abstract The extracellular glycoprotein Tenascin-C (TN-C) is highly upregulated in gliomas. Therefore, many chemotherapies with radiolabeled antibodies against TN-C have been performed. However, TN-Cs binding partner Syndecan-4 did not play any role as a therapeutic or imaging target in gliomas. We constructed an imaging compound containing the magnetic resonance imaging (MRI) contrast agent gadolinium (Gd)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), the fluorescence dye sulforhodamine and a synthetic Syndecan-4-specific 21 amino acid peptide derived from TN-C. Magnetic resonance relaxometry, confocal laser scanning microscopy, and flow cytometry showed that the Syndecan-4-DOTA-Rhodamine conjugate was taken up into the cytoplasm of human U373 glioma cells without any cytotoxic effects. Competition experiments indicate that this uptake was receptor-mediated. This conjugate might be used for future MRI studies of brain tumors after systemic or intraoperative local application.

Keywords Syndecan-4 · U373 glioma cells · Tenascin-C · DOTA

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Introduction

Tenascin-C (TN-C) is an extracellular matrix protein. It is highly expressed during embryogenesis, wound healing and neoplastic processes (Saito et al. 2007).

Tenascin-C expression correlates with malignancy grade in astrocytic tumors and is associated with a poor prognosis. The expression of TN-C is absent or only low in adult healthy fully developed tissue. TN-C promotes glioma invasiveness by inducing matrix-metalloproteinase (MMP)-12-activation (Sarkar et al. 2006) and therefore the degradation of collagen 5 within the surrounding healthy tissue. TN-C also induces ß1 integrin through Syndecan-4 (Saito et al. 2007). Therefore, TN-C represents a promising target for glioma radioimmunotherapy. Radiolabeled Tenascin antibodies have been systemically or intratumorously administered (Reardon et al. 2007, 2008; Zalutsky et al. 2008).

However, the cell surface binding partner of TN-C, the Syndecan-4, has not found use in therapeutic or imaging studies concerning gliomas.

It was previously shown that Syndecan-4 is highly expressed on the surface of glioma cells (Watanabe et al. 2006).

It has been shown that TNIIIA2, a 21 amino acid peptide derived from TN-C, specifically binds to Syndecan-4 leading to activation of $\beta 1$ integrin (Saito et al. 2007). Syndecan-4 knockout led to reduced $\beta 1$ integrin activation by TNIIIA2 (Saito et al. 2007). Alanine scanning-mutagenesis showed that while some point mutations of TNIIIA2 led to complete loss of $\beta 1$ integrin activation, others like for example sequence T15A still induced strong $\beta 1$ integrin activation (Saito et al. 2007).

With the aim to construct a glioma imaging agent we coupled the 21 amino acid Syndecan-4 directed peptide

T15A (RSTQLPGLKAATHYAITIRGV; Saito et al. 2007) to the commonly clinically used extracellular magnetic resonance imaging (MRI) contrast agent gadolinium (Gd)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and also to the fluorescence dye rhodamine. These two imaging agents were bound at the peptide's C-terminus via the ε-amino groups of two lysines separated by two arginines as a spacer. The gadolinium-DOTA complex was chosen because of its high stability with a half-life of over 4000 hours (Lukes et al. 2001).

By means of magnetic resonance (MR) relaxometry, confocal laser scanning microscopy (CLSM), and flow cytometry the binding of our Syndecan-4-DOTA-rhodamine conjugate to U373 glioma cells was investigated. The U373 human glioma cells were chosen because Syndecan-4 expression has already been shown for them (Su et al. 2006).

The specificity for Syndecan-4 was tested by the competition with an unmarked peptide.

Materials and methods

Synthesis of the dual-labeled Syndecan-4 directed conjugate: Arg-Ser-Thr-Asp-Leu-Pro-Gly-Leu-Lys-Ala-Ala-Thr-His-Tyr-Ala-Ile-Thr-Ile-Arg-Gly-Val-Lys(RITC)-Arg-Arg-Lys(Gd–DOTA)

Conjugate synthesis was performed on 0.1 mmol scale by solid phase peptide synthesis on a Eppendorf ECOSYN P peptide synthesizer (Eppendorf-Biotronik, Hamburg, Germany) using the fmoc strategy. Tentagel S rink amid resin (Rapp-Polymere, Tübingen, Germany) was used as carrier. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) was used as coupling agent and diisopropyl-ethylamine (DIPEA) as adjuvant base for amino acid coupling in sequential peptide elongation. The fmoc protective group was cleaved off with 25% piperidine in dimethylformamide. The lysine side chain designated for 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was protected with Mmt (4-methoxytrityl). The lysine side chain carrying rhodamine was protected with Dde [1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl]. For introduction of DOTA the Mmt protective group was cleaved off by repeated treatment with a solution of 1% TFA (trifluoroacetic acid) and 1% TIPS (triisopropylsilane) in DCM during 1 h. After several washing steps with DMF and neutralizing of the resulting TFA-salt with DIPEA the deprotected side chain was accessible for DOTA coupling. Coupling was carried out with three equivalents of 1,4,7,10-tetraacacyclododecane-1,4,7-tris(tert.butylester)-10-acetic acid (Macrocyclics, Dallas, USA), three equivalents of TBTU and six equivalents of DIPEA during 1.5 h at room temperature. Then the Dde side chain protective group was cleaved off by repeatedly treating the resin with a solution of 2.5% hydrazinhydrate in DMF over the course of 1 h. After several washing steps with DMF, rhodamine was coupled to the peptide using 0.5 mM rhodamine-isothiocyanate with equal amount of DIPEA in dimethylsulfoxide (DMSO) at room temperature over night. For competition experiments the same peptide was produced with free side chains.

The peptide sequences synthesized were analyzed and separated by RP-HPLC (Merck Hitachi, L-4000 A UV detector) using a C8 column (150 × 10 mm; Reprosil 100; Dr Maisch GmbH, Germany) with the following solvent systems: (A) 0.055% v/v trifluoroacetic acid in water. (B) 0.05% v/v trifluoroacetic acid in 80% v/v acetonitrile in water. Evaluation was performed using a linear gradient from 20 to 100% B within 40 min at the flow rate of 2.5 ml/min and at 214 nm absorbance. The conjugate was purified from initially ca. 40 to >97% purity and then lyophilized.

The resulting substance was stirred for 5 h at 50°C with one equivalent of Gd(III)chloride hexahydrate in water at pH 5.6 (adjusted with NaOH from pH 3–4). After acidification with diluted acetic acid (pH 4.5–5; as a precaution because the Gd–DOTA complex is not stable in alkaline conditions) the substance was relyophilized. The loading with gadolinium was found to be quantitative.

The unmarked peptide and the rhodamine-DOTA-conjugate with and without gadolinium were verified by MALDI-TOF (matrix assisted laser desorption/ionization–time of flight) mass spectrometry on a Bruker Daltonics reflex IV (Bruker Daltonics Inc., Billerica, MA, USA) and by electrospray ionization triple quadrupole mass spectrometry on a Micromass VG Quattro II triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) (theoretical masses: 2808.3, 3695.0, 3847.6; measured masses: 2806.9, 3694.5, 3845.8) (Fig. 1).

Confocal laser scanning microscopy

U373 human glioma cells were grown to 70% confluency at 37°C, 5% CO_2 (vol/vol) in RPMI-1640 Ready Mix Medium containing L-glutamine and 10% fetal bovine serum (FBS)-Gold (PAA Laboratories, Pasching, Austria), in 4-well plates (NUNC, Wiesbaden, Germany) with about 250,000 cells per well.

The conjugate was dissolved at 130 µM in medium. Cells were incubated in the wells at 37°C in an atmosphere of 5% CO_2 for 60 min with 300 µl of the conjugate solution. After this, the cells were rinsed once with medium and then incubated with 300 µl of medium containing Annexin-Fluos (Roche Diagnostics, Mannheim, Germany). Annexin-Fluos binds to phosphatidylserine which is only accessible in the outer membrane leaflet of apoptotic or necrotic cells but not in intact cells where phosphatidylserine is exclusively found in the inner membrane leaflet.

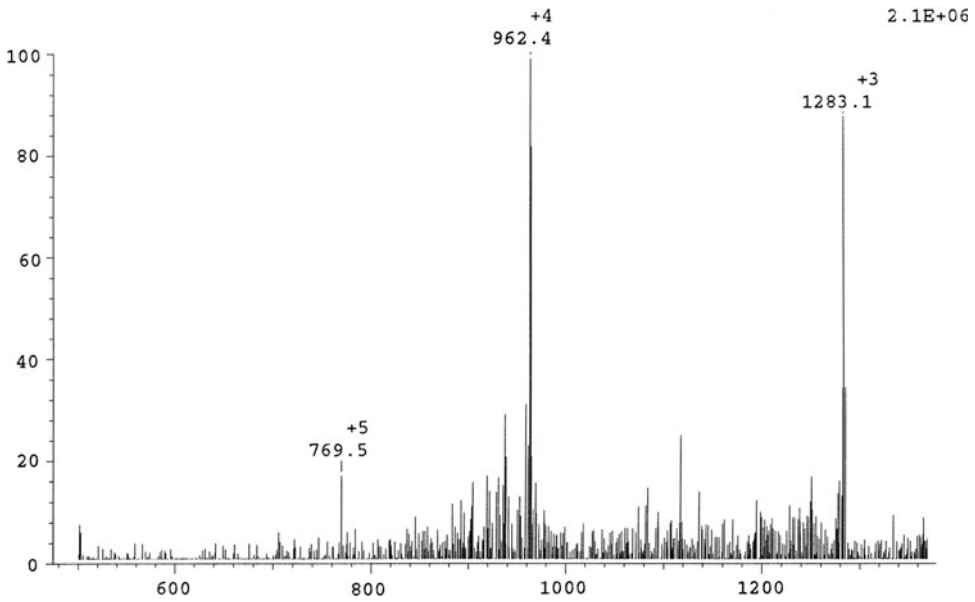

Fig. 1 Mass spectrometry. Electrospray ionization quadrupole mass spectrum of the Tenascin-C-derived conjugate containing rhodamine and gadolinium-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid. The mass peaks for the $[M + 3H^+]$, $[M + 4H^+]$ and $[M + 5H^+]$ ions are found at 1,283.1, 962.4 and 769.5 *m/z* resulting in a mass of 3,845.8 for the measured conjugate

Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Jena, Germany) (objective: LD Achroplan 40 × 0.6). For Annexin-Fluos and rhodamine fluorescence excitation, the 488 nm line of an Argon laser and the 543 nm line of a helium–neon laser with appropriate beam splitters and barrier filters were used. All measurements were performed at least three times on living, non-fixed cells.

Cell growth analysis

For cell growth analysis U373 cells were detached and seeded into eight 4-well plates (32 wells total for experiments in triplicate at 5 different growth times with and without the conjugate) at 20,000 cells per well (300 µl medium). Cells were left to attach for 12 h and then incubated with 300 µl of either fresh RPMI Ready Mix medium or 130 µM conjugate solution in RPMI Ready Mix medium. The conjugate solution was replaced with medium after 1 h.

After different cell growth times, beginning at 6 h after incubation until 72 h after incubation, cells were washed once with 300 µl of D-PBS buffer and then detached with 300 µl of Accutase™ (PAA Laboratories, Pasching, Austria). Cell density in these 300 µl was counted using a hemocytometer and the mean cell density of the sample was determined (three aliquots from one sample were counted). The logarithm of the cell density at each measurement time point was plotted in a graph. Linear regression of the plotted values yielded a value for the slope of the growth curve which was linear to the cell growth rate.

Magnetic resonance relaxometry

For MR relaxometry, U373 human glioma cells were grown in 75 cm^2 culture flasks (Corning Costar, Bodenheim, Germany) (70% confluency). Accutase™ (PAA Laboratories, Pasching, Austria) was added to achieve detachment of the cells, which were harvested and subsequently aliquoted into Eppendorf tubes (6×10^6 cells per tube). The cells in the tubes were incubated with 100 µl of the conjugate (130 µM). After a 60-min incubation period at 37°C in an atmosphere of 5% CO_2, the cells were washed three times with 300 µl of PBS and centrifuged at 800 rpm for 5 min.

For the competition experiments, cells were preincubated with 100 µl of 20-fold excess of unmarked peptide (2.6 mM) for 10 min. Then the unmarked peptide solution was replaced with 100 µl of a solution of 2.6 mM unmarked peptide and 130 µM conjugate and incubated for 60 min. Control samples were only incubated with 100 µl of medium.

Washing and centrifugation were performed in line with the other MRI samples.

In vitro relaxometry was performed with a 3 T whole body MRI-system (Trio, circular polarized wrist coil, Siemens, Erlangen, Germany).

Sagittal T1-weighted MR images were obtained using the following spin echo sequence:

Repetition time (TR): 200 ms, TE (echo time): 7.4 ms, flip angle 90°, averages: 1, concatenations: 2, measurements: 1, number of slices: 19, distance factor: 30%, slice thickness: 3 mm, field of view read: 180 mm, field of view phase: 100%, base resolution: 256, phase resolution: 100%, voxel size: 0.7 × 0.7 × 3.0 mm, scan time: 1:48 min.

T1 relaxation times were evaluated from signal intensities obtained by multiple spin echo measurements:

TR: 20–8,000 ms (50 different TR values), TE: 6.4 ms, flip angle 90°, averages: 1, measurements: 1, number of slices: 1, slice thickness: 1 mm, field of view read:

120 mm, field of view phase: 87.5, base resolution 128, phase resolution: 100%, voxel size: 0.9 × 0.9 × 1 mm.

Analyses and calculations were performed using a Matlab program (Math Works, Natick, MA, USA). T1 values were approximated by a three-parameter fit procedure.

All signal curves were examined and found to be monoexponential.

The investigations were performed in triplicate.

Flow cytometry

For fluorescence activated cell sorting (FACS), cells were grown in 75 cm^2 culture flasks (Corning Costar, Bodenheim Germany) (80% confluency) as described in the CLSM section. Accutase™ (PAA Laboratories, Pasching, Austria) was added to achieve detachment of the cells, which were harvested and subsequently aliquoted into Eppendorf tubes (Eppendorf, Hamburg, Germany) (1×10^6 cells per tube). The cells were incubated for 60 min with 100 µl of the conjugate (130 µM). As described before, competition samples were first preincubated with unmarked peptide for 10 min and then incubated for another 60 min with a mix of 2.6 mM unmarked peptide and 130 µM conjugate.

Afterwards, the cells were washed three times in 300 µl of PBS and centrifuged at 800 rpm for 5 min. Then 300 µl FACS buffer (D-PBS containing 1% paraformaldehyde) was added.

The samples were measured immediately after the end of the incubation. At least 20,000 events were recorded per sample. Fluorescence excitation was achieved by an argon laser (488 nm). Rhodamine fluorescence was detected using a 580–610 nm bandpass filter. All investigations were performed in triplicate.

Mean rhodamine fluorescence values of the samples were acquired using the WinMDI software (Joseph Trotter, Scripps Research Institute, San Diego, CA, USA) and then statistically evaluated.

Results

Confocal laser scanning microscopy

Many if not all cells were stained. Staining was cytoplasmic and the cell nuclei appeared to be free of conjugate. The cell cytoplasm showed dot-like staining pattern (Fig. 2a).

Lack of green fluorescence from the Annexin-Fluos vitality test (Roche Diagnostics, Mannheim, Germany) indicated that the cells remained viable.

Cell growth analysis

The slopes of the linear regression of the graphs for the cell density development after incubation with conjugate or fresh medium were similar only differing at the third digit (Fig. 2b).

Flow cytometry

Incubation of U373 glioma with 130 µM conjugate and 20-fold excess of unmarked peptide showed reduced mean fluorescence intensity compared to the samples without competition with unmarked peptide (Fig. 3a). Mean fluorescence intensity of the competition samples was reduced to just over 50% of the values for the incubation without competition.

Magnetic resonance relaxometry

T1 relaxation time values of samples where 20-fold excess of unmarked peptide was added to the conjugate solution showed lower uptake of Gd–DOTA–rhodamine conjugate at 130 µM conjugate concentration (mean T1 = 1,009 ms) compared to the sample without competition (mean T1 = 527 ms) (Fig. 3b, c). All T1 relaxation time values for the samples were shorter than those of the native control (mean T1 = 2,076 ms).

Discussion

Tenascin-C, an extracellular matrix protein, is highly expressed in gliomas and plays a key role in glioma growth.

Therefore, it has found use as a target for many radioimmunotherapy procedures (Reardon et al. 2007, 2008; Zalutsky et al. 2008).

Magnetic resonance imaging is a common method in brain tumor imaging due to its high anatomical resolution compared to other methods like positron emission tomography. Gadolinium-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is a commonly used MRI contrast agent in clinical routine examinations and also a very stable complex with a half-life of over 4000 hours (Lukes et al. 2001).

It was previously shown that various different Tenascin-derived 21 amino acid peptides (TNIIIA2, T15A and G20A) bind to the cell surface heparin sulfate proteoglycan, including Syndecan-4 (Saito et al. 2007, 2008). This binding results in the activation of ß1 integrin and stimulates tumor cell proliferation (Huang et al. 2001). Until now no imaging or anticancer therapeutic compounds targeting Syndecan-4 have found use in glioma research.

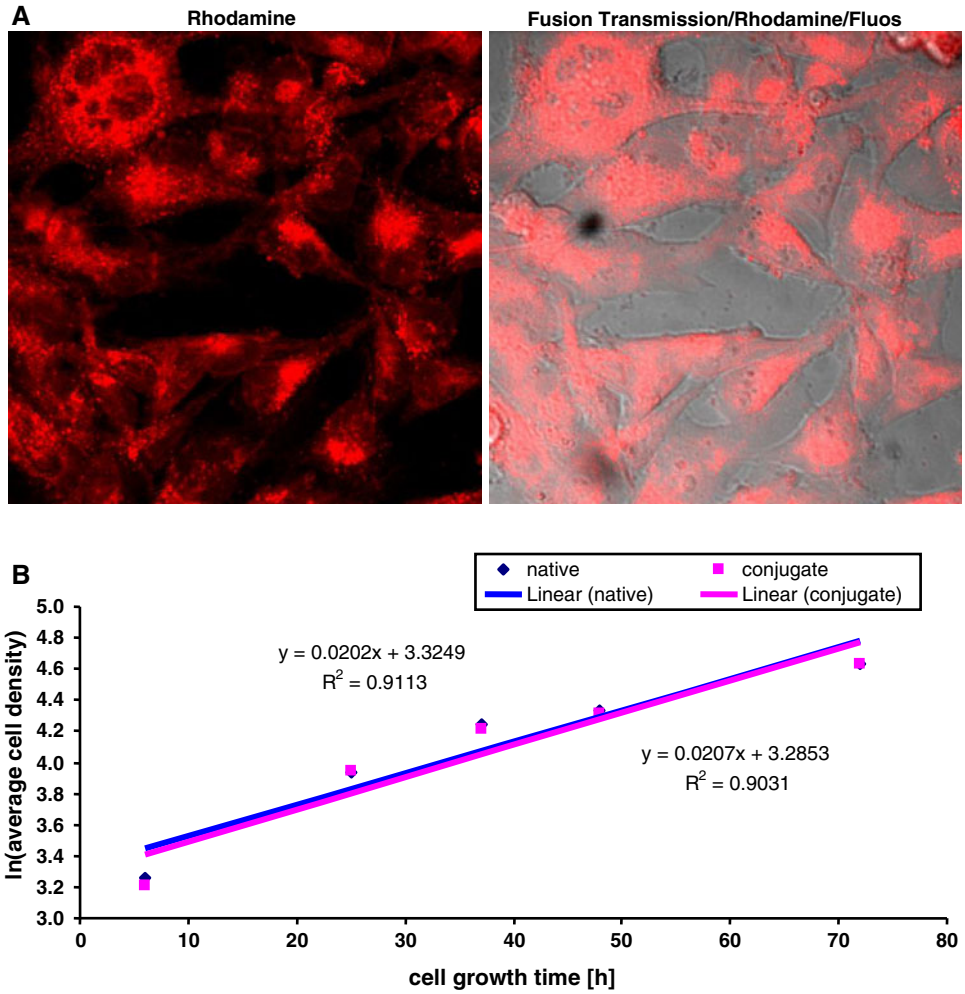

Fig. 2 **a** Confocal laser scanning microscopy. Images of living adherent U373 glioma cells incubated with the conjugate at 130 μM concentration show dot-like cytoplasmic staining and omitted cell nuclei. The majority if not all cells are stained. The Annexin-Fluos vitality test shows no green fluorescence, i.e. no cell death. **b** Cell growth analysis. The logarithm of human U373 glioma cell density in cell samples (native and after 1 h of incubation with the conjugate) after different cell growth times (6–72 h) is shown. Linear regression of the values yields the slope values of the cell growth curves which are directly proportional to cell growth. The slopes of the growth curves for native cells and cells incubated with the conjugate are very similar (native: 0.0202, conjugate: 0.0207)

We designed a Syndecan-4 directed conjugate containing the Syndecan-4-specific peptide T15A elongated by four amino acids (lysine-arginine-arginine-lysine) at the C-terminal end which served as a carrier for rhodamine and DOTA.

Gadolinium-DOTA can be detected by MRI. Rhodamine enables subcellular localization of the conjugate by CLSM.

With respect to future in vivo experiments where the conjugate concentration should be as high as possible to achieve good signal intensities, the concentration for the experiments was chosen at 130 μM. While this is very high compared to natural receptor ligands, the cells showed no signs of cell death and conjugate solubility still was not an issue.

The dual-labeled conjugate was taken up by the cytoplasm of U373 glioma cells. The cell nuclei remained conjugate free. The staining pattern was granular making a receptor-dependent uptake with subsequent internalization presumable (Fig. 1).

Such a Syndecan-4 associated endocytotic uptake was previously described by Tkachenko et al. (2004) using endothelial cells.

Binding of the conjugate to Syndecan-4 could be inhibited with excess of unmarked T15A peptide. This inhibitory effect was substantial at 20-fold excess of unmarked peptide. This has been observed in MRI as well as flow cytometry experiments and is a clear indicator for receptor-mediated conjugate uptake.

Vitality testing with Annexin-Fluos in CLSM as well as cell growth analysis showed that targeting Syndecan-4 with our conjugate did not result in cell death. This makes it of potential value for the use as a contrast agent.

Using rhodamine as label in addition to Gd–DOTA is important during the stage of in vitro experiments for qualitative and quantitative evaluation of the conjugate uptake by fluorescence detection. For in vivo experiments on animals the fluorescence labeling is still desirable for easy localization of the conjugate in frozen sections of the different tissues. For application on patients the

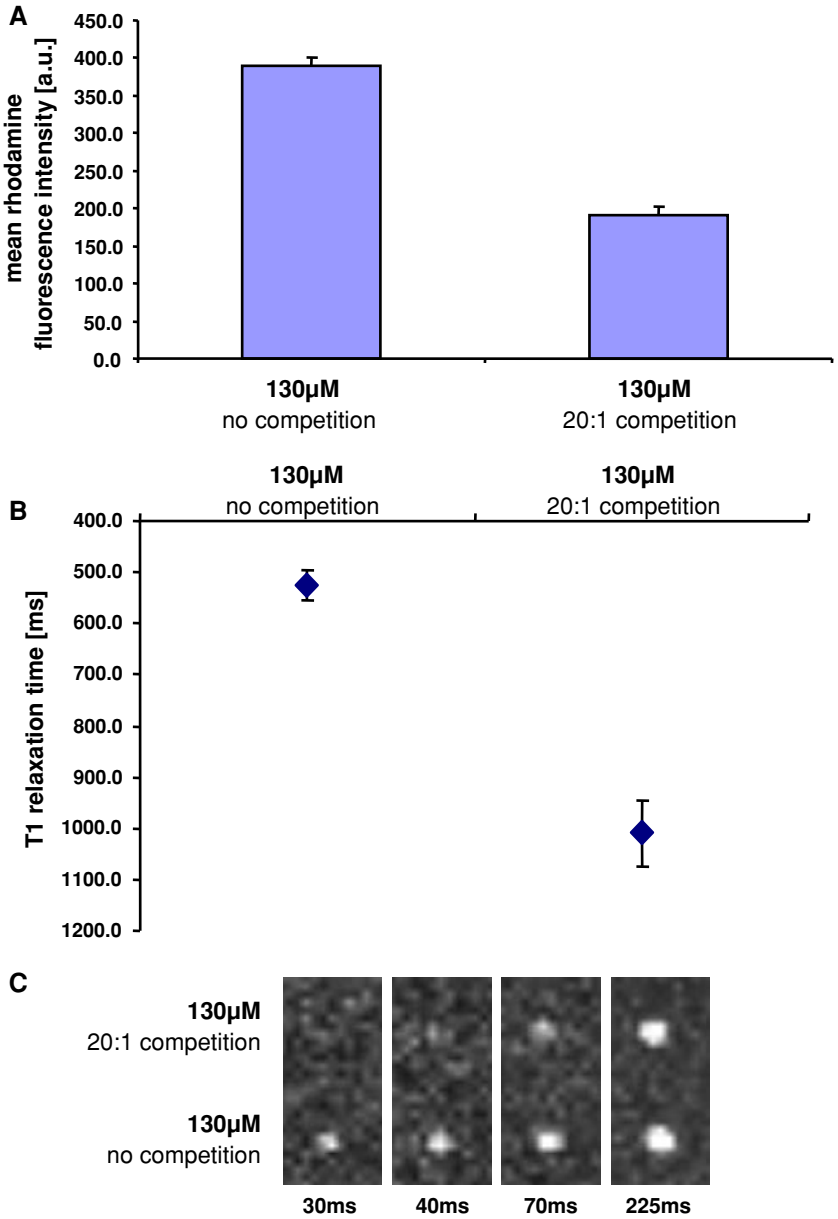

Fig. 3 **a** Flow cytometry. The 130 µM sample of human U373 cells with 20-fold excess of unmarked peptide (2.6 mM) show <50% mean rhodamine fluorescence intensity (191 arbitrary units, deviation: 11.8) compared to the sample incubated with 130 µM conjugate alone (390 arbitrary units, deviation: 11.0). **b** Magnetic resonance relaxometry. T1 relaxation time values of samples in which 20-fold excess of unmarked peptide (2.6 mM) was added to the 130 µM conjugate solution are higher (1,009 ms, deviation: 64.2 ms; weaker signal) than those for the incubation with 130 µM conjugate only (525 ms, deviation: 29.3 ms; stronger signal). Mean values and standard deviation from three investigations are depicted. The *y*-axis is inverted to reflect that shorter relaxation times correspond to stronger conjugate uptake. Mean T1 relaxation time value of the native control is 2,076 ms (deviation: 81.6 ms). **c** Axial T1 MRI slices. Axial T1-weighted magnetic resonance images of human U373 glioma cell pellets (0.5 ml cups) at four different repetition times (TR = 30, 40, 70, 225 ms). The upper sample was incubated with 130 µM conjugate and 20-fold excess of unmarked peptide, the lower sample with 130 µM conjugate alone. At TR = 30 ms the competition sample is not visible while the sample without competition already shows an MRI signal. At TR = 40 ms a slight signal of the competition sample can be seen while the non-competition sample is stronger than at TR = 30 ms. At TR = 70 ms the signal of the competition sample is clearly visible, but the non-competition sample's MRI signal is clearly stronger. At the highest TR value depicted (TR = 225 ms) both samples show similar high MRI signal

fluorescence dye could be replaced with either another Gd–DOTA or for example a compound like transferrin which can translocate the conjugate through the blood–brain barrier or even a therapeutic compound.

Possibilities for MRI of brain tumors have been very limited so far. The commonly used contrast agents remain in the extracellular space and cannot bind to the cells themselves. Therefore, in the brain, tumors cannot be

differentiated from inflammations like multiple sclerosis or fibrotic lesions. In all of these cases the blood–brain barrier is disrupted and therefore the contrast agent accumulates within the interstitial space resulting in signal enhancement. To certify the diagnosis of a brain tumor a biopsy is often necessary.

A similar problem occurs during brain tumor surgery controlled by intraoperative MRI. When the tumor tissue is dissected the extracellular contrast agent flows out along the interstitial space into the healthy brain tissue.

An intracellular, tissue-specific, receptor-directed contrast agent might help to circumvent these problems.

Since the Syndecan-4 receptor is also present in normal astrocytes (Watanabe et al. 2006), it is not clear to which extent the conjugate can differentiate between malignant and healthy astrocytes. However, in a first step we could demonstrate that our dual-labeled conjugate is taken up into the cytoplasm of human U373 glioma cells. Therefore, it shows promise for the use in intraoperative MRI either administered systemically or locally. Due to its cytoplasmic but not extracellular localization it may lead to more defined tumor margins in intraoperative MRI.

In its current state the conjugate would be suitable for WHO grade II–III and above brain tumors where the blood–brain barrier is disrupted. For lower grade brain tumors a ligand for Syndecan-1 would be preferable as this has not been found in healthy brain tissue (Watanabe et al. 2006). However, such a conjugate must be further modified to be able to pass the blood–brain barrier for example by coupling it with transferrin.

In summary the novel Syndecan-4-DOTA-rhodamine conjugate was taken up into the cytoplasm of human U373 glioma cells without any cytotoxic effects. Competition experiments indicate that this uptake was receptor-mediated.

The conjugate might be used for future MRI studies of brain tumors by systemic or intraoperative local application.

Further experiments will show whether this conjugate can distinguish between healthy and tumorous astrocytes based upon their different Syndecan-4 expression.

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