Introduction

The family of carcinoembryonic antigen (CEA) constitutes glycoproteins that are involved in cell adhesion, thus referred to as carcinoembryonic antigen cell adhesion molecules (CEACAMS) and are produced during fetal development [1]. Many CEACAMS are overexpressed in a variety of carcinomas, can therefore be detected both in tissue and in blood in those patients used as a serum marker for the prognosis of colorectal cancer, especially CEACAM5, or CD66e [2-4]. Elevation of serum CEA has also been seen in symptom-free patients, where endoscopic examinations have verified the presence of tumors [5]. Blood levels of CEA can also be used as a preoperative prognostic marker for colorectal and breast cancers [6, 7] and may also be elevated in other cancer types, such as lung [8, 9], prostate [10], and adrenocortical cancer [11]. Its usefulness as a general marker is, however, limited in these diseases,
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Positron emission tomography (PET) is a sensitive tool for visualization of various diseases. This technique is based on the introduction of a positron emitting nuclide, such as $^{14}C$, $^{18}F$ or $^{68}Ga$, in molecules that bind to a target of interest. The most commonly used radiotracer is $[^{18}F]$fluoro-deoxy-glucose ($[^{18}F]$FDG), which accumulates in tissue with high glucose consumption such as in tumors. $[^{18}F]$FDG has been widely used in the diagnosis and in management of colorectal cancer [12]. However, one drawback of $[^{18}F]$FDG is that it is non-specific and may also accumulate in the inflamed tissues resulting in false-positive diagnosis as well as demonstrating negligible uptake in slowly-growing tumor cells resulting in false-negative diagnoses. A selective tool for a specific biomarker, like CEA, is therefore of interest; therefore, monoclonal antibodies against CEA have been radiolabeled with several different radio nuclides for use in radioisocintigraphy [13-18]. However, it is essential that the labeled radiotracer has a fast clearance and has low binding to other endogenous components, especially those in the proximity to the target of interest. Since most intact antibodies have a long biological half-life (days – weeks), this has hampered a wider use of radiolabeled antibodies in PET, where most radio nuclides have a very short half-life [19]. One way to overcome this problem is to use methods of pretargeting, i.e., to pretarget the tissue with a bispecific antibody followed by visualization of the bound antibody in a second step using smaller molecules with more appropriate kinetics some days later [20]. A recently developed pretargeting technique is to use complex antibodies or fragments of antibodies having multiple binding sites interacting with both antigen and reporter moiety carrying hapten molecules containing the radionuclide. In this report we have used a pretargeting system made by a trivalent, bispecific binding antibody TF2, consisting of two identical Fab fragments reacting against CEACAM5, is covalently linked to a different Fab fragment capable of reacting with a divalent hapten peptide containing histamine-succinyl-glycine (HSG) residues and a chelate to be used for attaching the appropriate radionuclide [21-23]. The specificity of TF2 to CEA has been demonstrated in previous studies [21, 24-26]. TF2 is pre-administered to allow the localization to the target and clearance from the blood within its pharmacokinetics time frame. The subsequent administration of the radiolabeled hapten peptide characterized with fast target (TF2) localization and blood clearance provides imaging of high contrast [21, 24].

Non-invasive visualization of the distribution and density of CEA not only in tissue fluids, but also in the patient in order to locate the spread of the CEA containing cancer by using the pretargeting technique in combination with PET could be advantageous for staging of the disease. This sensitive technique for visualization of various endogenous binding sites should be suitable for prognostic investigations of the presence of CEA, and due to the high sensitivity of PET this also allows for the detection of small metastases that may be occult by other imaging modalities.

A considerable number of radionuclide-labeled antibodies is investigated currently for cancer therapy with radionuclides such as $^{90}Y$, $^{125/131}I$, $^{213}Bi$, $^{188}Re$, $^{177}Lu$, $^{211}At$ where the radiation to healthy organs is high due to the slow pharmacokinetics and blood clearance, with high concentrations in the red marrow [27]. Thus, the introduction of the bispecific pretargeting concept contributes to the improvement of not only of diagnostic imaging but also possible therapy applications. The hapten molecule may contain various chelators (DTPA, DOTA, NOTA) for complexation with a variety of radionuclides, such as $^{111}In$, $^{99m}Tc$, $^{18}F$ [28, 29], $^{67}Ga$ or $^{68}Ga$ for the imaging in SPECT and PET studies of the distribution of CEA, or has been proven efficacious for therapy when labeled with $^{177}Lu$ or $^{90}Y$ [23].

The present in vitro study was undertaken to evaluate the binding of the trivalent bispecific antibody TF2 and the two haptons, Ga-IMP461 and Ga-IMP485 (Figure 1), in colorectal cancers removed from patients at surgery. Both haptons were labeled with $^{68}Ga$ for PET and with $^{67}Ga$ for SPECT for the in vitro evaluation.

Material and methods

Compounds and chemicals

The trivalent, bispecific binding antibody TF2 was prepared as described previously and stored at + 4°C until use [22]. Two haptons, IMP461(NOTA-D-Ala-D-Lys(HSG)-D-Tyr-D-Lys (HSG)-NH$_2$) and IMP485 (NOTA-MPAA-D-Lys…
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(HSG)-D-Tyr-D-Lys(HSG)-NH₂ (Figure 1) [20, 23], as well as TF2, were obtained from Immunomedics Inc., Morris Plains, NJ, USA. ⁶⁸Ga was obtained from a ⁶⁷Ge/⁶⁸Ga generator system (iThemba, IDB Holland bv, The Netherlands). HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, used as a powder), doubly distilled hydrochloric acid (Riedel de Haën), NaCl (38979), NaOH (306576, semiconductor grade) were obtained from Sigma-Aldrich Sweden (Stockholm, Sweden). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Water (18.2 MΩ), produced with a Purelab Ultrapure system (Elga Bucks, the UK), was used in all reactions. The purchased chemicals were used without further purification. ⁶⁷Ga (T½ = 3.2612 d, decay by electron capture (EC), gamma energy 93 keV 40%, 184 keV 20%, 300 keV 17%, 393 keV 5%) in 0.1M HCl solution was obtained from MDS Nordion Inc., Vancouver, BC, Canada and used without further purification. Strong anion exchange solid phase extraction cartridges (Chromafix, PS-HCO₃) were purchased from Macherey–Nagel GmbH, Düren, Germany.

Tissues

The colorectal cancer tissue samples were obtained from surgeries performed at Uppsala University Hospital, and were immediately frozen and stored at -80°C until sectioned. Tissues from cancerous colorectals as well as from neighboring tissue taken at a minimum of 10 cm from the site of the tumor were used. The sections were also stained with hematoxylin-eosin.

CEA-expressing LS174T human colorectal cancer xenografts from one nude rat and one nude mouse were sectioned and used as positive control tissues. All tissues were cryosectioned using a Leica cryocut to 20 µm, and put on Superfrost glass slides. The sections were stored at -25°C before use within a few months.

Analytical methods for radiolabeling

HPLC analysis was performed on a Beckman Nouveau HPLC system equipped with a Beckman 166 variable-wavelength UV detector and a flow-count radio detector (Bioscan, Inc., USA) connected in series. The reverse-phase separation was conducted using a Grace Vydac C18 Protein and Peptide HPLC column (Vydac, USA, 150 x 4.6 mm; 5µm). The gradient elution with 0.1% trifluoroacetic acid (TFA) aqua (A) and acetonitrile/water 70:30, containing 0.1% TFA (B), as mobile phase, was as follows: 5% B for 2 min, and then to 90% B in 8 min. The flow rate and UV wave length were 1 ml/min and 280 nm, respectively.

Pre-concentration and purification of ⁶⁸Ga

⁶⁸Ga (T½ = 68 min, β⁺ = 89% and EC = 11%) was available from a ⁶⁷Ge/⁶⁸Ga generator system (iThemba, IDB Holland bv, The Netherlands) in which ⁶⁸Ge (T½= 270 d) was attached to a column of an inorganic matrix based on stannous oxide. A pre-elution using 6 ml of 1 M HCl was performed four hours prior to the synthesis.
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68Ga was eluted with 6 ml of 1 M HCl into a vial containing 1.4 g NaCl and stirred until dissolved, giving a chloride concentration of 5 M. The solution was passed through the anion exchange cartridge (Chromafix, PS-HCO3), and afterwards the 68GaCl3 was eluted with 300 µl of water.


HEPES (0.048 g, 0.20 mmol) was dissolved in the 300 µl of preconcentrated 68Ga in a 2 ml Eppendorf tube. The pH was 4.2-4.6 (adjusted by the addition of 30% HCl (aq) or 10 M NaOH (aq) if necessary). Thereafter, an aqueous solution of IMP461 or IMP485 (1-6 nmol) was added. The reaction took place by using conventional heating at 90°C for 10-15 min. The purity of the labeled product was analyzed using HPLC and the pH of the reaction mixture was adjusted to ~7 by addition of 10 M NaOH (aq) prior to use in the biological experiments.


67Ga obtained as GaCl3 solution in 0.1M HCl (106 µL) was used without further purification. The amount of 67Ga (9 ± 1 MBq) used for the labeling was adjusted to the required specific radioactivity. The corresponding volume of the stock solution was added to the HEPES solution (0.3 M, 130 µl) in 0.1 HCl. The pH of the solution was adjusted to 3.5-4.5, if needed, with NaOH. Then 3 nmol of IMP461 or IMP485 were added, and the reaction mixture was incubated either at room temperature for 10 minutes or 90°C for 5 min. The radiochemical purity of [67Ga]Ga-IMP461 and [67Ga]Ga-IMP485 was assessed by UV-radio-HPLC, and the concentration of the conjugate and the tracer was determined from UV-HPLC standard plots.


NatGa of natural isotope composition (69Ga and 71Ga) was complexed with IMP461 and IMP485. Twenty mM aqua solution of NatGaCl3 (5 µl) were added to the HEPES solution (0.3 M) of 0.1 M HCl (200 µl) and the pH was adjusted with NaOH to 3.5-4.5. Then 10 nmol of either IMP461 or IMP485 were added and the reaction mixture was incubated at room temperature for 10 min or 90°C for 5 min. [NatGa]Ga-IMP461 and [NatGa]Ga-IMP485 were used as authentic references for the confirmation of the radio-HPLC chromatogram signals.

**Stability study**

The stability of formulated [67/68/NatGa]Ga-IMP461 and [67/68/NatGa]Ga-IMP485 counterparts in phosphate-buffered saline (PBS) at pH 7.4 was monitored at room temperature by UV-radio-HPLC during 4h, 17h and 3d, respectively, for 68Ga, 67Ga and NatGa. Appearance of radio-signals was followed for the compounds comprising 68Ga and 67Ga. The stability of the molecules containing NatGa was followed by UV-HPLC and determinations of the corresponding absorbance and concentration were made.

**Autoradiography**

The assay conditions were optimized by varying concentrations of the bispecific binding antibody and of the radioligand on rat and human tumors and control tissue. Both [67Ga]Ga-IMP461 and [68Ga]Ga-IMP461 were used in the optimization studies.

The optimal assay conditions for in vitro autoradiography were found to be as follows. The thawed sections were preincubated for 60 min at 37°C in Tris-HCl buffer (Tris-HCl 0.05 M, pH7.4, with BSA 0.2%, MgCl2 5 mM, bacitracin 50 mg/l) without and with bispecific antibody diluted 1:10000 (“pretargeting”). The sections were then washed in buffer twice for two min in ice-cold buffer to remove unbound antibody. The incubation was conducted for 60 min at 37°C with [67/68Ga]Ga-IMP461. Unbound radiotracer was then removed by washing twice for three min in ice-cold buffer. The sections were dried at 37°C for 7-10 min and exposed to phosphor imager plates (Molecular Dynamics, Amersham Biosciences, UK) over night before analyzing in a PhosphorImager SI (Molecular Dynamics, Amersham Biosciences, UK) and quantification using ImageQuant TL (GE Healthcare).

**Results**


The optimization of the 68Ga-labeling synthesis was conducted using IMP461. The lowest amount of the hapten molecule in the protocol
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providing radioactivity incorporation (RAI) of >95% was 3 nmol. The nearly quantitative RAI and biological compatibility of HEPES buffer allowed for the omission of the purification step. This amount was used throughout the study in order to provide highest possible specific radioactivity and resulting in relevant comparison of the biological results. SRA values varied, dependent on the age of the generator, from 41 to 117 MBq/nmol. The pre-concentration of the generator eluate was conducted using methods described earlier [30].

The amount of $^{67}$Ga used in the labeling reactions was defined by the product specific radioactivity determined by the biological experiment design. It was below (3-10 pmol) the amount (200 pmol) when the concentration of metal impurities starts to interfere with the labeling complexation [31]. The amount of the hapten molecules used for the synthesis was defined by the values optimized for the $^{68}$Ga-labeling in order to keep the same amount and ensure relevant comparison. The specific radioactivity was $3.1 \pm 0.1$ MBq/nmol.

The hapten counterparts were complexed with stable isotopes of gallium, $^{69,71}$Ga ($^{nat}$Ga), $^{nat}$Ga -IMP461 and $^{nat}$Ga -IMP485 were synthesized for the authentic reference purpose and in order to follow the stability of the compounds for longer time periods. The reaction mixture was spiked with the reference and monitored by UV-Radio-HPLC. The radiosignals of $^{67/68}$Ga-IMP461 (Rt = 5.12 ± 0.04) and $^{67/68}$Ga-IMP485 (Rt = 5.35 ± 0.14), and the UV-signals, respectively, from $^{nat}$Ga-IMP461 (Rt = 5.25 ± 0.04) or $^{nat}$Ga-IMP485 (Rt = 5.10 ± 0.05) co-eluted with slight delay due to in-line configuration of the detectors. The original compounds, IMP461 (Rt = 5.41 ± 0.01) and IMP485 (Rt = 5.68 ± 0.01), were well resolved from their respective complexes with stable gallium and allowed unambiguous determination during the stability tests. All compounds demonstrated high stability in the buffer at room temperature for at least 4, 17 and 72 h, respectively, containing $^{67}$Ga, $^{68}$Ga and $^{nat}$Ga. The detected signals corresponded only to the compounds of interest.

Optimization and validation for autoradiography

In the optimization studies using $^{67/68}$GaGa-IMP461, the concentration of the bispecific binding TF2 antibody varied between 1:1000 and 1:100000 (Figures 2 and 3). Maximum binding was obtained at a dilution of approximately 1:5000, but sufficient binding contrast was obtained with a dilution of 1:10000, which therefore was used in subsequent studies. Varying the concentrations of $^{68}$GaGa-IMP461, based on radioactivity (from 0.05 MBq/ml to 0.5 MBq/ml), showed that high intensity of binding was obtained already at the lowest concentration, although the maximum binding was increased with increasing hapten concentrations. In subsequent studies concentrations close to 0.25 MBq/ml (corresponding to 2-6 nM, depending upon specific radioactivity obtained) were used. A lower concentration of $^{67}$GaGa-IMP461 was found to be optimal at

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**Figure 2.** Autoradiography using $^{68}$GaGa-IMP461 and bispecific antibody TF2 on a mouse tumor. Effects of varying antibody concentration and hapten concentration, respectively.
lower concentrations, due to the longer nuclide half-life (0.01 MBq/ml, corresponding to approximately 3 nM) (Figure not shown). Similar conditions and concentrations were subsequently used for $^{67/68}$Ga-IMP485.

In the initial studies we used rat and mouse tumor xenografts and single human colorectal cancers from surgical specimens. All four ligands, regardless radionuclide or hapten, showed strong binding to pre-targeted tissue. The addition of $^{67/68}$Ga-IMP461 to sections without in vitro pretargeting, i.e., without prior pre-treatment with the bispecific antibody TF2, resulted in absence of, or very low binding of the radioligand to either xenografts (Figure 2) or primary human tumors. Using the optimal conditions as mentioned above very distinct images were seen in the murine xenografted tumor (Figure 2).

**Patient tissue study**

The bispecific antibody, TF2, and the haptens $^{67/68}$Ga-IMP461 and $^{67/68}$Ga-IMP485 were used for the study of specimens from colorectal cancers obtained at surgery. The tissue sections were subsequently analyzed using hematoxylin-eosin staining in order to verify the autoradiographic localization. In all tissue judged from the staining to be part of the tumor, extensive and strong labeling was found (Figure 4). Moreover, distinct labeling was also found in the epithelium of most samples of tissue ob-

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**Figure 3.** Effects of varying antibody concentration and hapten concentration on autoradiography using $^{68}$Ga-IMP461 and bispecific antibody TF2. Half maximum was reached at antibody concentrations of approximately 1:10000 and 1:50000 with hapten concentration of 0.5 and 0.05 nM, respectively.

**Figure 4.** Autoradiography using $^{67/68}$Ga-IMP461 and $^{67/68}$Ga-IMP485 and bispecific antibody TF2 on two samples from two colorectal cancer patients.
tained at least 10 cm from the cancer. However, in one of the samples, considered to be taken from the ileum, much lower labeling was found. No marked differences in the radioactivity uptake, dependent on the radionuclide (\(^{67}\text{Ga}\) vs. \(^{68}\text{Ga}\)) or hapten molecule (IMP461 vs. IMP485), were found. All four radioligands showed strong binding to the tumor tissue as well as to the epithelial tissue in the surgical specimen to a similar extent.

**Discussion**

These in vitro autoradiographical studies confirm previous in vivo studies on animal xenografts of human tumors and primary human tumor specimens that the TF2 bispecific antibody, labeled with \([^{67/68}\text{Ga}]\text{Ga-IMP461}\) or \([^{67/68}\text{Ga}]\text{Ga-IMP485}\), can be used for the visualization of CEA-containing cancers. Moreover, the in vitro imaging allows for higher resolution and thus more detailed information and more precise distribution of the target in the tissue providing valuable information on cancer cell extension. The signal-to-noise ratio in the colorectal cancer tissues was excellent, and could be considered specific as the radioligand did not bind to the tissue in the absence of pretargeting with the bispecific antibody. The labeling of CEA in the epithelial cells could be easily distinguished from the labeling of the tumor, because the localization of the epithelium resulted in a thin, more localized image than what was achieved in the tumor tissue. The good specificity versus binding to non-cancerous tissue thus makes this pretargeting in vitro autoradiography very reliable.

A pre-concentration procedure was performed for \(^{68}\text{Ga}\) by anion exchange using sodium chloride instead of hydrochloric acid [30]. To obtain >95% radioactivity incorporation in the \(^{68}\text{Ga}\)-labeling reaction it was necessary to use 3 nmol of the IMP461 precursor. Using this protocol for radioactivity incorporation no further purification was needed prior to the biological experiments. This is important because purification can lead to a decrease in specific radioactivity by means of losses by time and also by the procedure itself.

In the previous published studies a large number of variants of the hapten molecules have been used. The hapten can be labeled with several different radionuclides, e.g. \(^{18}\text{F}\) [20, 24, 29, 32], \(^{111}\text{In}\) [20, 33], \(^{99m}\text{Tc}\) [20, 34] for diagnosis, as well as with other radionuclides for radiotherapy (\(^{90}\text{Y}\) [20], \(^{177}\text{Lu}\) [35]). However, the use of \(^{68}\text{Ga}\) [32] is not common for these studies, in spite of obvious advantages such as availability and suitable half-life. In this study, we have used and compared two haptons, IMP485 and IMP461, both with NOTA as a chelator, labeled with \(^{67}\text{Ga}\) or \(^{68}\text{Ga}\). We have also labeled both haptons with the SPECT radionuclide, \(^{67}\text{Ga}\), for comparison. It is obvious from the figures that the two haptons result in identical in vitro images, and that any of the two radionuclides may be used.

In surgery of colorectal cancer patients, the surgeon resected the cancerous tissue, as well as neighboring tissue at a minimum of 10 cm from the site of the tumor. In this study the tissue obtained 10 cm from the tumor was used as control. However, previous immunohistological studies have shown that the epithelial cells of the normal colon also contain CEA, although to a lower extent than tissue with cancer cells [36-38]. Thus, this very sensitive pretargeting technique can also visualize CEA in putatively normal tissue adjacent to cancer. However, as the epithelial cells have a very distinct localization in comparison to the cancer cells, the difference between the two cell types is easily recognized in the autoradiograms. It must be appreciated, however, that normal-appearing epithelium adjacent to cancers of the colon and rectum may have elevated CEA expression, as compared to intestinal tissues from individuals free of cancer in this organ [36-38].

It can be concluded that pretargeting the CEA with the bispecific antibody TF2 followed by the addition of Ga-labeled hapten is extremely sensitive in visualizing CEA. This methodology can therefore be a very specific method that is complementary to other histochemical techniques in the diagnosis of colorectal cancer in biopsies or in samples taken from surgery. These tissue findings support the view that the pretargeting technique may also be a step forward in diagnosing patients with colorectal cancer, either using \(^{67}\text{Ga}\) and SPECT or \(^{68}\text{Ga}\) and PET for imaging. Furthermore the use of high specific radioactivity might be valuable if repeated PET studies using \(^{68}\text{Ga}\) can be undertaken in order to optimize the therapeutic protocol, as was discussed previously [23].
Competing interests

Dr David M Goldenberg and Dr William McBride have financial interests in Immunomedics, Inc. The other authors declare no competing interests.

Authors’ contributions

HH was responsible for the in vitro binding studies and was responsible for the writing of the manuscript. IV, EB, JU, WMB and BL adapted and performed all radiochemistry work. AM performed all animal in vivo work with the xenografted animals from which the animal tumors were taken. LP was responsible for surgery and for obtaining suitable tissue from the colorectal cancer. PM and AW prepared the samples for sectioning, were responsible for the sectioning and performed the pathological examinations of the human tissue samples. BL and DMG were the initiators of the project. All authors have contributed to the writing of the manuscript.

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