SYNTHESIS AND COMPARISON OF TARGETED T1-CONTRAST AGENTS BASED ON DOTA AND DTPA COMPLEXES

Tatiana Abakumova [a], Maxim Abakumov [b], Dmitry Bychkov [c], Alexander Kabanov [d,e], Natalia Nukolova [a,d*], Vladimir Chekhonin [a,b]

[a] *Department of Fundamental and Applied Neurobiology, Serbsky National Research Center for Social and Forensic Psychiatry, Kropotkinskiy 23, Moscow, 119991, Russia*

[b] *Pirogov Russian National Research Medical University, Ostrovityanova 1, Moscow, 117997, Russia*

[c] *Department of Geology, Lomonosov Moscow State University, Leninskie Gory 1, Moscow, 119991 Russia*

[d] *Department of Chemistry, Lomonosov Moscow State University, Leninskie Gory 1/3, Moscow, 119991, Russia*

[e] *Center for Nanotechnology in Drug Delivery and Division of Molecular Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7362*

**corresponding author: nukolova@serbsky.ru*

Abstract:

Permeability of blood brain barrier (BBB) plays a key role in drug delivery to the brain tissues. Many brain abnormalities such as tumors, demyelination, necrosis, inflammation are accompanied with enhanced penetration of BBB. Based on this fact we proposed the targeted polymer-based contrast agents for MRI visualization of brain tumors. The goal of our study was to synthesize macromolecular multi-chelate complexes of gadolinium with further conjugation with specific monoclonal antibodies. For this purpose poly-L-lysine (PLL) was modified with chelating agents - DTPA or DOTA - using carbodiimide chemistry and conjugated with monoclonal antibodies to connexin 43 (mAb Cx43). Afterwards these conjugates were loaded with Gd(III) ions. During synthesis the immunochemical activity of conjugated mAbs was preserved up to 85%. Maximal non-toxic concentration of contrast agents was 0.6 mg/ml. Important, that obtained contrast agents had higher T1-relaxivity values (6.5 and 8 $mM^{-1}s^{-1}$ for DTPA and DOTA chelating motif) in comparison with commercial available agent Magnevist (3.4 $mM^{-1}s^{-1}$). T1-contrast agent based on PLL and specific monoclonal antibody was successfully synthesized and characterized. These agents had a high relaxivity and high affinity to Cx43.

Keywords: T1 contrast agent, connexin 43, targeted delivery, DOTA and DTPA

1. INTRODUCTION

Recently a lot of studies have focused on targeting the central nervous system (CNS) abnormalities for therapeutic and diagnostic purposes [1]. One of the most used techniques for visualization of brain pathologies is magnetic resonance imaging (MRI). Injection of contrast agents (*e.g.* gadolinium (Gd) based agents) before examination provides enhancement of the contrast between various tissues and leads to more accurate diagnosis by MRI. Nowadays there are a lot of approaches which could improve the properties of contrast agents, such as relaxivity, stability, specificity to selected tissue, *etc.* [2]. For example, relaxivity (the ability to reduce the relaxation time of the coordinated water protons) could be increased by optimization of molecular parameters of contrast agent. It is attainable by increasing of water molecules in inner sphere of the complexes [3], conjugation chelates with macromolecules (polyaminoacids , dextrans , liposomes and *etc.*), linking multiple Gd-complexes together. [2]High specificity to the environment and tissues could be achieved by development of contrast agents with pH-responsive shell [4] or by conjugation with targeting moieties [5]. MRI visualization of CNS abnormalities might be possible *via* delivery of specific

contrast agents through compromised endothelium in pathological area, where blood-brain barrier (BBB) is disrupted and could be permeable for macromolecular agents and peptides [6]. Stability of contrast agents might be optimized by using chelating agents with high thermodynamic stability and kinetic constants [2], such as diethylene triamine pentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). In our work we combined listed approaches and developed the targeted macromolecular contrast agents based on the monoclonal antibodies to brain specific antigen (Cx43). This allowed increasing the relaxivity due to conjugation of multiple chelate complexes based on DOTA or DTPA with polymer backbone and enhancing the specificity due to conjugation with targeting group. Also we compared the parameters of obtained DOTA- and DTPA-derivatives for their further *in vivo* evaluation.

2. MATERIALS AND METHODS:

2.1. Materials:

Polylysine hydrobromide (PLL, Mw 15-30 kDa), diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), gadolinium(III) chloride hexahydrate ($GdCl_3$*6H2O), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). DMEM media, 10% fetal bovine serum (FBS), 0.25% trypsin/EDTA and Alexa 594 labeled goat anti-mouse IgG were from Invitrogen (Carlsbad, CA, USA). Triton X-100, Tween 20 were purchased from Applichem (Ottoweg, Germany). MTS reagent was from Promega (Madison, USA); Magnevist was from Bayer Shering Pharma AG (Germany).

2.2. Methods:

2.2.1 Synthesis of targeted contrast agent mAb-PLL-DTPA-Gd and mAb-PLL-DOTA-Gd

To synthesize targeted contrast agent we conjugated monoclonal antibody (mAb) with PLL-DTPA or PLL-DOTA complexes and loaded them with Gd(III). We used monoclonal antibodies to connexin 43 (Cx43) as a targeting group and immunoglobulin G (IgG) as a control. Briefly, carboxyl groups of DTPA or DOTA (16 mg of each) were activated by EDC:NHS (ratio 1:1, water, 15 min) and added dropwise to 0.5 ml of PLL solution (1 mg/ml, water, pH>8) followed by overnight incubation at room temperature (r.t.). Obtained polymer complexes were purified from unbound chelator (DTPA or DOTA) using PD-10 desalting columns and lyophilized. Free amino groups of PLL were measured by Fluram assay to estimate the amino groups modification (%). Then carboxyl groups of PLL-DTPA or PLL-DOTA were activated by EDC (pH 5.5) and then conjugated with amino groups of mAb (0.01 M HEPES, 0.9% NaCl) at molar ratio [PLL-DOTA or PLL-DTPA]:[EDC]:[mAb]=20:10:1. After incubation (12 h, 4°C) obtained contrast agents were purified from byproducts using size exclusion chromatography (Sepharose CL-6B, HEPES, pH 7.4) and loaded with 2-molar excess of $GdCl_3$ (HEPES, pH 6.5, overnight, 4°C). After that, obtained conjugates were purified by PD-10 desalting columns and concentrated on Amicon filters (3 kDa, Millipore).For fluorescent microscopy amino groups of PLL-DTPA or PLL-DOTA were labeled by fluorescein isotiocyonate (FITC), which was activated by EDC (water, 5 min) and stirred with polymer for 2 h at r.t. in dark. Then unbound dye was removed using PD-10 desalting columns and FITC-labeled conjugate was attached to mAb and loaded with Gd(III) as previously described. Obtained targeted contrast agents were sterilized using sterile filters (Fisherbrand, 0.22 μm) and stored at 4°C until further use. Stability of contrast agent was evaluated in HEPES buffer (pH 7.4, 7 days, 4°C) and plasma (10 % v/v, 1 day, 37°C).

2.2.2 X-ray fluorescent analysis and relaxivity measurement

Concentration of Gd(III) was analyzed by «dried drop» method using X-ray fluorescent (XRF) spectrometer «REspect» (Tolokonnikov, Russia) with addition of molybdenum as an inner standard. Gd(III) concentration was measured based on the concentration of molybdenum and obtained values of signal intensities. Percentage of loaded Gd(III) was calculated as the percent ratio of mass of incorporated Gd(III) to total mass

of contrast agent. For relaxivity measurements the targeted contrast agents and commercial analog Magnevist were diluted in HEPES buffer at Gd(III) concentrations from 0.1 to 0.5 mmol and analyzed using inversion-recovery consecution with following parameters: TR=16000, TE=7.1, TI=50, 100, 200, 400, 600, 800, 1000, 1500, 1980 (17°C, 7 T, Bruker BioSpin, ClinsScan, Germany) [7].

2.2.3 Enzyme-linked immunosorbent assay (ELISA) and immunofluorescent analysis

We used standard ELISA protocol [8] and immunofluorescent analysis to evaluate the targeting ability of the mAbs after conjugation to PLL-DTPA or PLL-DOTA. For ELISA assay Cx43 antigen were diluted in 0.1 M NaHCO3 (4 μg/ml, pH 9.6) and added to ELISA high binding plates (Sarstedt, Germany). Targeted contrast agents and controls (free mAb to Cx43, non-specific IgG) were added to the wells (37°C, 1 h) followed by incubation with anti-mouse IgG-peroxidase antibodies (A9917, 0.1 μl/ml, 1 h, 37°C). After incubation with ABC-kit, TMB solution and thorough washing, the reaction was stopped with 3 N HCl 100 μl/well and absorbance was measured using a microplate reader VICTOR X3 (Perkin Elmer, USA) at 450 nm.Immunofluorescent analysis of the obtained contrast agents was performed on glioma C6 cells, which over-express Cx43 antigen [9]. Glioma C6 cells were incubated with FITC-labeled contrast agents for 40 min and further fixed with 4% paraformaldehyde and double-stained with Alexa594-anti-mouse IgG antibodies. Images were collected using fluorescent microscopy (Leica DMB S100).

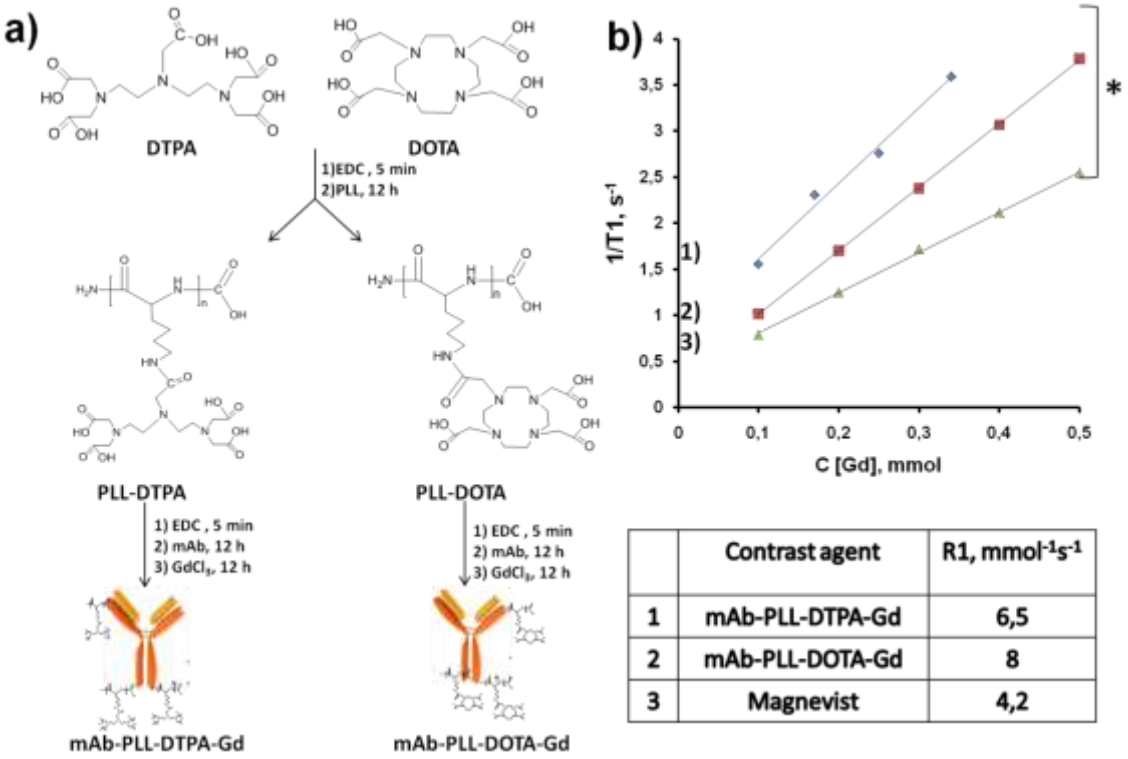
2.2.4 In vitro cytotoxicity

Cytotoxicity of obtained contrast agents was analyzed on human embryonic kidney cells (HEK 293) and rat glioma C6 cells. Cells were seeded in 96-well plates (5 000 cells/well) in DMEM media supplemented with 10 % FBS for 48 h before experiments (37°C, 5 % CO_2). Then cells were treated with targeted contrast agents and Magnevist at concentration up to 2 mg Gd/ml for 24 h, and then cultured for additional 24 h in fresh media at 37°C. Cytotoxicity was determined by colorimetric MTS assay [10].

3 RESULTS:

3.1 Synthesis of DTPA and DOTA-based targeted contrast agents

Targeted contrast agent with multimeric DTPA or DOTA chelate complexes were prepared by conjugation of carboxylic groups of PLL-DTPA (or DOTA) with amino groups of monoclonal antibodies and then followed by Gd(III) complexation (**Fig. 1, a**). Conjugates were purified by gel filtration chromatography (Sepharose CL-6B, HEPES buffer) and concentration of Gd(III) was measured by XRF. We found that loading of Gd(III) depended on the chelating agents (DTPA or DOTA). Thus efficacy of Gd(III) incorporation into contrast agent was about 40% and 80% for DTPA and DOTA derivatives (400 μg and 800 μg Gd(III) per 1 mg of mAb). Moreover, T1-relalxivity values of targeted DTPA and DOTA-based contrast agents were about 6.5 $mmol^{-1}s^{-1}$ and 8 $mmol^{-1}s^{-1}$, respectively, and were 2-fold higher than that of Magnevist (3.4 $mmol^{-1}s^{-1}$), **Fig. 1, b**. Analysis of immunochemical activity of obtained contrast agents did not revealed differences between DTPA and DOTA-derivatives. Both contrast agents had high preservation rate of antibody activities (up to 85% from initial,) and even after lyophilization they kept their immunochemical activity (more than 70%). Stability of contrast agent was studied in HEPES for 7 days at 4°C. All samples were active, stable and did not incline to aggregate. Moreover, the samples were stable in blood serum (10% v/v) and showed no precipitation during overnight incubation at 37ºC.



	Contrast agent	R1, $mmol^{-1}s^{-1}$
1	mAb-PLL-DTPA-Gd	6,5
2	mAb-PLL-DOTA-Gd	8
3	Magnevist	4,2

Fig. 1. Scheme of synthesis (**a**) and relaxivity measurements (**b**) of obtained contrast agents: 1) mAb-PLL-DOTA-Gd; 2) mAb-PLL-DTPA-Gd and 3) Magnevist.

3.2 Immunofluorescent analysis of targeted contrast agents

To analyze the cellular uptake of obtained contrast agents in Cx43-overexpressing cells, glioma C6 cells were treated with FITC-labeled specific and non-specific contrast agents (Figure 2). No specific binding of IgG-contrast agent to C6 cells was observed. Contrary, the relatively high accumulation of mAbCx43-PLL-DTPA-Gd in glioma C6 cells was detected by fluorescent microscopy (Figure 2, a). Double staining with Alexa594-antimouse IgG antibodies confirmed co-localization of PLL-DTPA-Gd (green color on the figure) with conjugated antibodies in the cell (red color on figures), which proved that conjugate did not dissociate inside of the cells (yellow color on the figure). The same data were obtained for DOTA-derivatives using glioma C6 cells.

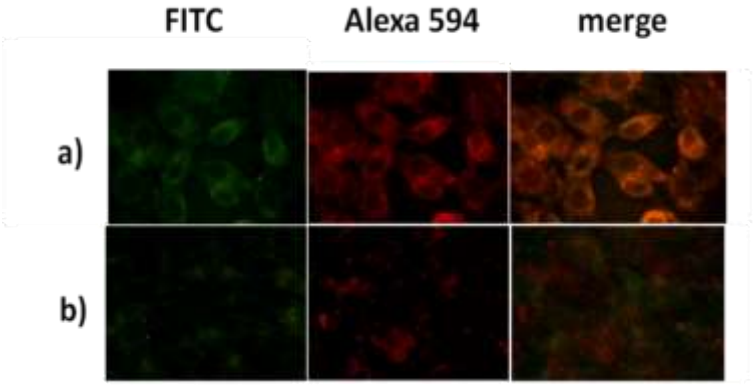


Fig. 2. Immunofluorescent analysis of contrast agents conjugated with **a**) specific mAbCx43 and **b**) non-specific IgG in live glioma C6 cells. The cells were pulsed for 40 min with the FITC-labeled contrast agents, followed by antibodies staining

3.3 In vitro cytotoxicity of targeted contrast agents

It is known that high amount of lysine residues in PLL-based contrast agents provides them with significant toxicity due to high positive charge and limits their application [11]. For that we prepared two types of contrast agent: with low amount of chelates (only 10% of Lys modified with DTPA) and high amount of chelates that further used in this study. Also, it is known that toxicity of DTPA derivatives might be higher than that for DOTA derivatives due to kinetic and thermodynamic stability of the Gd-complexes [12]. Therefore we analyzed the toxicity of obtained DTPA-based contrast agents conjugates with on glioma C6 and HEK 293 cell lines. Contrast agent possesed low amount of DTPA molecules had cytotoxic effect on both cell lines (IC_{50} were 1.58 μg Gd/ml and 1.88 μg Gd/ml for HEK293 and glioma C6, respectively). Contrast agents developed in this work did not reveal any toxicity up to concentration 0.6 mg Gd/ml that is much higher than that used in the clinic [13].

4 DISCUSSION

Contrast-enhanced MRI is an important tool in diagnostics of brain pathologies, such as cancer, neurodegeneration, inflammation and etc. Nowadays there are a lot of strategies to enhance properties of contrast agents thereby improve the quality of MRI image. Thus, conjugation of contrast agents with targeting moieties could enhance specificity to the tissue or organ *via* increasing of local concentration of Gd(III) in pathological area [2]. Visualization of CNS pathologies by macromolecule contrast agents might be possible due to diffusion of these agents across compromised endothelium and reduction of non-specific extravasation into healthy tissue [6]. The spectrum of application of DTPA and DOTA chelating agents is wide in modern medicine and research, since they can be used not only for MRI, but also for positron emission tomography (PET). Thus, Sabbah et al. successfully conjugated anti-ferritin antibodies with radiopharmaceutical ligands for PET imaging of pancreatic cancer [14]. Efficacy of bombesin-DOTA and DTPA- derivatives was demonstrated in A42J7 animal model; these agents were continued to the phase I as a potential PET agent for patients with prostate carcinoma [15]. In our study, we designed two types of targeted contrast agents based on DOTA and DTPA complexes of Gd(III) to improve visualization of intracranial glioma: mAbCx43-PLL-DTPA-Gd and mAbCx43-PLL-DOTA-Gd. All reactions involving mAb were carried out in HEPES buffer, since mAbs were inclined to lose their activity in acetate buffer and, for PBS buffer, even small amount of free Gd(III) caused the precipitation. Incorporation of Gd(III) into contrast agents with different chelating agents was analyzed using different methods. We believe that obtained DOTA-derivative has higher potential for further in vivo study, since this agent has better Gd-loading (about 80% w/w), higher relaxivity (about 8 $mmol^{-1}s^{-1}$) and good stability in comparison with mAbCx43-PLL-DTPA-Gd. Moreover, free Gd(III) ions is known to be very toxic and chelating complexes with high stability constant (25,4 vs. 22,1 for DOTA and DTPA, respectively [12]) might be more suitable for the development of diagnostic agents. ELISA demonstrated specific binding of the mAbCx43-PLL-DTPA-Gd and mAbCx43-PLL-DOTA-Gd to its antigen, suggesting that modification of mAb by macromolecules did not alter its functional properties. To the contrary, only a marginal binding was observed in the case of IgG-conjugates due to nonspecific hydrophobic interactions. All obtained contrast agents (mAbCx43-PLL-DTPA-Gd and mAbCx43-PLL-DOTA-Gd) were stable in blood serum, which is important for their further use. It should be noted that all obtained conjugates kept their immunochemical activity and were able to bind to their target after storage as detected by ELISA. It was shown that obtained targeted contrast agents has no significant toxicity in glioma C6 and HEK293 cell lines. Fluorescent analysis confirmed that the Cx43-specific targeted contrast agents were able to recognize its molecular site and selectively accumulate in glioma C6 cells.

CONCLUSIONS

We prepared and compared two types of Cx43-targeted macromolecular contrast agents (DTPA and DOTA derivatives) for MRI visualization of brain pathologies. Both of them were stable, non-toxic and had high specificity to glioma C6 cells. Contrast agent with DOTA chelates had higher Gd(III) incorporation and

relaxivity value in comparison with DTPA- derivative. Nevertheless, it is difficult to predict which one will be the leader in further in vivo studies, since there is no difference in efficacy and safety between Gd-DTPA and Gd-DOTA in randomized clinical trial [13].

ACKNOWLEDGEMENTS:

This work supported by grant №11G34.31.0004, RFBR grant №13-04-01383, program «UMNIC» of Foundation for Assistance to Small Innovative Enterprises, RSF grant №14-15-00698 (MRI protocols development) and financial support by agreement № 182-MRA between Lomonosov Moscow State University, Skolkovo Institute for science and technology and Massachusetts Institute of Technology

LITERATURE:

[1] E. Kanal, K. Maravilla, H.A. Rowley, Gadolinium Contrast Agents for CNS Imaging: Current Concepts and Clinical Evidence. AJNR Am J Neuroradiol (2014).
[2] P. Caravan, Strategies for increasing the sensitivity of gadolinium based MRI contrast agents. Chem Soc Rev 35 (2006) 512-523.
[3] J. Xu, B. O'Sullivan, K.N. Raymond, Hexadentate hydroxypyridonate iron chelators based on TREN-Me-3,2-HOPO: variation of cap size. Inorg Chem 41 (2002) 6731-6742.
[4] B. Yoo, M.D. Pagel, An overview of responsive MRI contrast agents for molecular imaging. Front Biosci 13 (2008) 1733-1752.
[5] P. Caravan, Protein-targeted gadolinium-based magnetic resonance imaging (MRI) contrast agents: design and mechanism of action. Acc Chem Res 42 (2009) 851-862.
[6] H. Maeda, The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzyme Regul 41 (2001) 189-207.
[7] H. Tamura, I. Yanagawa, T. Hikichi, K. Matsumoto, S. Takahashi, K. Sakamoto, T1 measurements with clinical MR units. Tohoku J Exp Med 175 (1995) 249-267.
[8] V.P. Chekhonin, O.I. Gurina, T.B. Dmitrieva, Shepeleva, II, I.A. Ryabukhin, G.N. Babich, Monoclonal anti-GFAP antibodies: extraction, characteristics, and immunoenzyme assay. Bull Exp Biol Med 132 (2001) 772-775.
[9] V.P. Baklaushev, G.M. Yusubalieva, E.B. Tsitrin, O.I. Gurina, N.P. Grinenko, I.V. Victorov, V.P. Chekhonin, Visualization of Connexin 43-positive cells of glioma and the periglioma zone by means of intravenously injected monoclonal antibodies. Drug Deliv 18 (2011) 331-337.
[10] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 65 (1983) 55-63.
[11] M. Meyer, A. Zintchenko, M. Ogris, E. Wagner, A dimethylmaleic acid-melittin-polylysine conjugate with reduced toxicity, pH-triggered endosomolytic activity and enhanced gene transfer potential. J Gene Med 9 (2007) 797-805.
[12] S. Laurent, L.V. Elst, R.N. Muller, Comparative study of the physicochemical properties of six clinical low molecular weight gadolinium contrast agents. Contrast Media Mol Imaging 1 (2006) 128-137.
[13] M. Oudkerk, P.E. Sijens, E.J. Van Beek, T.J. Kuijpers, Safety and efficacy of dotarem (Gd-DOTA) versus magnevist (Gd-DTPA) in magnetic resonance imaging of the central nervous system. Investigative radiology 30 (1995) 75-78.
[14] E.N. Sabbah, J. Kadouche, D. Ellison, C. Finucane, D. Decaudin, S.J. Mather, In vitro and in vivo comparison of DTPA- and DOTA-conjugated antiferritin monoclonal antibody for imaging and therapy of pancreatic cancer. Nucl Med Biol 34 (2007) 293-304.
[15] W.A. Breeman, M. de Jong, J.L. Erion, J.E. Bugaj, A. Srinivasan, B.F. Bernard, D.J. Kwekkeboom, T.J. Visser, E.P. Krenning, Preclinical comparison of (111)In-labeled DTPA- or DOTA-bombesin analogs for receptor-targeted scintigraphy and radionuclide therapy. J Nucl Med 43 (2002) 1650-1656.