Novel DOTA-based prochelator for divalent peptide vectorization: synthesis of dimeric bombesin analogues for multimodality tumor imaging and therapy†

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Dimeric peptidic vectors, obtained by the divalent grafting of bombesin analogues on a newly synthesized DOTA-based prochelator, showed improved qualities as tumor targeted imaging probes in comparison to their monomeric analogues.

Several malignant human tumors overexpress various peptide hormone receptors on their cell surface.[1] These receptors have become increasingly important as targets for molecular imaging and targeted radionuclide therapy of tumors using receptor-avid small peptides.[2] The expression of somatostatin receptors in neuroendocrine tumors has been successfully exploited for the imaging and therapy of somatostatin receptor expressing tumors using radiolabeled somatostatin analogues in a clinical setting.[2,3] Several other peptide receptors such as cholecystokinin, neurotensin and bombesin receptors have also attracted considerable interest in recent years.[4] Bombesin receptors are of particular interest as they are overexpressed in several of the more prevalent cancers including prostate, breast and small cell lung cancer.[5]

A high and specific uptake of imaging probe/targeted drug in the tumor is crucial for effective imaging/therapy. To achieve this goal, design and synthesis of peptidyl ligands having high receptor affinity and selectivity is essential. Application of multivalent ligands showed markedly increased binding affinity with their respective receptors compared to monovalent ligands.[6] The multivalent concept has been used for the development of targeted radiotracers for tumor imaging.[7] Extensive studies on radiolabeled multimeric cyclic RGD peptides demonstrated that an increase in peptide multiplicity can significantly enhance the integrin $\alpha_v\beta_3$ binding affinity and improve the tumor targeting capability of the radiotracer.[8]

Because of its excellent chelating properties and the high *in vivo* and *in vitro* stabilities of the corresponding metal complexes, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is widely employed for the conjugation of reporter molecules with targeting ligands.[9] DOTA forms very stable complexes with a large number of metal ions, which are used in different fields of molecular imaging like Gd^{3+} for MRI,[10] ^{111}In and ^{67}Ga for SPECT,[2] ^{68}Ga and ^{64}Cu for PET[11] and Eu^{3+} and Tb^{3+} for optical imaging.[12] Thus, the synthesis of DOTA-based prochelators for the multivalent grafting of targeting ligands is of significant interest to develop improved targeting vectors that could find application in different imaging modalities. Here we report the synthesis of a new DOTA-based prochelator and its application for the divalent conjugation of bombesin analogues. The divalent conjugates were labeled with ^{177}Lu (as a Gd^{3+} surrogate) and *in vitro* assays were performed using PC-3 (human prostate adenocarcinoma) cells to compare the tumor cell uptake and cellular retention with their corresponding monovalent analogues.

In addition, relaxivity measurements of the gadolinium complexes of the monovalent and divalent conjugates were performed to assess their potency as targeted MRI contrast agents. The synthesis of prochelator (**04**) containing two free carboxylic acid groups for the divalent vectorization of targeting peptides started from commercially available DO2A-*tert*-butyl ester (**01**) (Scheme 1). *N*-alkylation of **01** with 2 equivalents of (*R*/*S*)-α-bromoglutaric acid 1-*tert*-butyl ester 5-benzyl ester (**02**)[13] yielded **03** in 63%. Subsequent hydrogenation of **03** over 10% Pd–C catalyst followed by chromatographic purification yielded the prochelator **04** in 86%, which was further characterized by using ESI-MS and NMR (1D-^{1}H, ^{1}H-^{1}H-COSY, 1D-^{13}C and ^{1}H-^{13}C-HSQC experiments). The new prochelator **04** exists in different isomeric forms (*RR*, *SS*, *RS* & *SR*) as it was synthesized by using racemic **02**.[14] The prochelator **05** containing one free carboxylic acid group was synthesized following the procedure described earlier.[13]

The application of prochelator **04** for the divalent vectorization with peptide ligands has been demonstrated by synthesizing divalent bombesin analogues (Scheme 1). In a typical reaction, two equivalents of bombesin peptide was added to prochelator **04**, dissolved in DMF and pre-activated with HATU and the pH was adjusted to 8 using DIPEA. After overnight coupling at room temperature, the DMF was evaporated and the crude mixture was directly deprotected by adding TFA/TA/H_2O/TIPS (95 : 3 : 1 : 1, *v*/*v*/*v*). The crude divalent peptide conjugates were purified by HPLC (yield 48 to 55%) and characterized by ESI-MS and MALDI. Analysis of HPLC fractions by MALDI-MS revealed the presence of considerable quantities of undesired modified divalent peptide conjugates produced by the HATU mediated nitrile formation *via* dehydration of the carboxamide side chain of asparagine and

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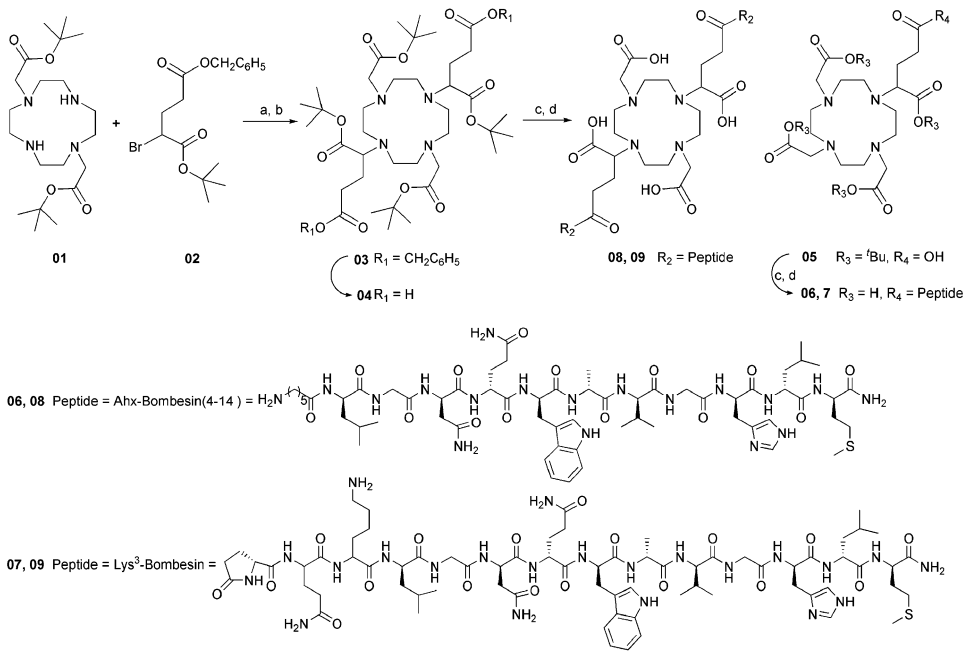

Scheme 1 Synthesis of DOTA-based prochelators and their conjugation to bombesin analogues. (a) K_2CO_3, AcCN, 18 h, RT; (b) 10% Pd–C/H_2, MeOH, 24 h, RT; (c) Peptide, HATU, DIPEA, 12 h; (d) TFA/TA/H_2O/TIPS (95 : 3 : 1 : 1, *v*/*v*/*v*), 8 h.

glutamine.[15] The divalent peptide conjugates showed excellent labeling properties with many radiometals of interest such as ^{177}Lu, ^{111}In, and ^{68}Ga affording a radiolabeling yield >99.5% at a specific activity of >50 GBq μmol^{-1}. Further, the facile complexation of these conjugates with Gd^{III} illustrates their potential application as targeted MRI contrast agents.

A key objective in MRI contrast agent research is to devise high relaxivity contrast agents based on gadolinium. To devise such high relaxivity systems, macromolecules have been explored as scaffolds for the presentation of Gd^{III} complexes. Every attempt to enhance the relaxivity by the covalently linked high molecular weight macromolecular conjugates is hindered by inefficient motional coupling or slow water exchange rate that quenches the theoretical relaxivity gain.[10] Multivalent functionalization of cyclen with a glutaric acid derivative has potential advantages as it: (a) facilitates multiple attachment of medium molecular weight macromolecules such as targeting peptides and (b) enhances the relaxivity due to the reduced tumbling rate of the conjugate and fast water exchange.[16] As a proof of the concept, we have determined the T_1 relaxivities, r_1, of the synthesized conjugates at proton Larmor frequencies of 30, 40 and 60 MHz (Table 1). All the conjugates have probably one inner-sphere water molecule and exhibited significant relaxivities at 60 MHz, ranging from 9.3 to 19.2 mM^{-1} s^{-1} (at 25 °C) and 6.9 to 14.7 mM^{-1} s^{-1} (at 37 °C). The values represent the 2- to 4-fold enhancement of relaxivity relative to Dotarem® (Gd-DOTA, the clinically used MRI contrast agent with a relaxivity of 4.1 mM^{-1} s^{-1} (at 25 °C) and 3.0 mM^{-1} s^{-1} (at 37 °C) at 60 MHz).[17] It is also worth noting that the divalent conjugates exhibit a 2-fold enhanced relaxivity compared to their monovalent analogues.

The initial evaluation of the conjugates as tumor targeting vectors has been conducted *in vitro* using the gadolinium surrogate radiometal ^{177}Lu. The specific tumor cell uptake and cellular retention of the monovalent and divalent bombesin conjugates were studied in GRP-receptor expressing PC-3 cells. All the four radiolabeled conjugates showed high and specific tumor cell uptake in PC-3 cells as shown in Fig. 1a. After 4 h, ^{177}Lu-06, ^{177}Lu-07, ^{177}Lu-08 and ^{177}Lu-09 showed specific internalization of 26.52 ± 0.83%, 18.26 ± 1.06%, 41.9 ± 2.14% and 35.91 ± 1.46%, respectively. Blocking studies performed using large excess of Tyr^4-bombesin demonstrate that the internalization was receptor mediated. The data revealed that the divalent presence of the targeting ligand results in approximately a 2-fold

Table 1 T_1 relaxivities of the gadolinium complexes of monovalent and divalent peptide conjugates at multiple proton Larmor frequencies[a]

	r_1/mM^{-1} s^{-1}					
	30 MHz		40 MHz		60 MHz	
Conjugate	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
Gd–06	9.5	7.2	9.4	7.0	9.3	6.9
Gd–07	11.9	8.9	11.8	8.7	11.7	8.6
Gd–08	16.5	12.5	16.4	12.4	15.8	12.0
Gd–09	20.2	15.3	20.1	15.2	19.2	14.7

[a] Longitudinal ^{1}H relaxation rate times T_1 were measured on Bruker Minispecs mq30 (30 MHz, 0.71 T), mq40 (40 MHz, 0.94 T) and mq60 (60 MHz, 1.41 T).

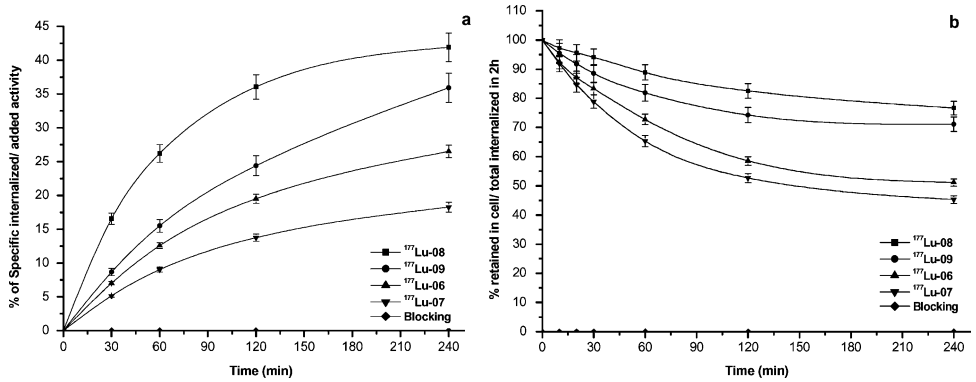

Fig. 1 Comparative *in vitro* evaluation of tumor targeting potential of ^{177}Lu labeled conjugates **06**, **07**, **08** and **09** with PC-3 cells. Values and standard deviations are the result of two independent experiments with triplicates in each experiment. (a) Rate of internalization; (b) cellular retention.

increase in the tumor cell uptake. This is either due to bivalent binding resulting in receptor dimers leading to an increased rate of internalization or it may also be explained assuming monovalent binding and increased probability of rebinding after dissociation. Both mechanisms may eventually result in increased internalization rates.

For cellular retention studies, the radiopeptides were allowed to internalize for 2 h; cells were then washed twice with PBS and the receptor-bound ligands were removed by washing with glycine buffer, pH 2.8. Cells were then incubated by adding fresh medium and the radioactivity retained in the cell after 10, 20, 30, 60, 120, and 240 min was measured. Fig. 1b illustrates the cellular retention of these compounds over time, expressed as the percentage of radioactivity left in the cell from the total amount internalized. Within 4 h, 49% of ^{177}Lu-06 and 56% of ^{177}Lu-07 were externalized from the cell. Comparatively, divalent conjugates exhibit better cellular retention (77% of ^{177}Lu-08 and 72% of ^{177}Lu-09 retained after 4 h).

In conclusion, the work demonstrates the synthesis of a new DOTA-based prochelator for the facile divalent conjugation of targeting biomolecules such as peptides. The improved tumor targeting capabilities and enhanced relaxivities exhibited by divalent bombesin analogues are highly promising for the development of these divalent conjugates as potential targeting probes, which could be employed as both targeted MRI contrast agents and radiopharmaceuticals.

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