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Synthesis of trifunctional somatostatin based derivatives for improved cellular and subcellular uptake

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Abstract—It is now well established that the biological effects of Auger-emitting radionuclides are critically dependent on their subcellular location. Therefore, for their use in molecular imaging and targeted radionuclide therapy, attempts should be made to increase the nuclear specificity of the carriers. In the present paper the synthesis of novel trifunctional somatostatin derivatives containing a nuclear localization motif is described. These derivatives of [DOTA^0, Tyr^3]-octreotide (DOTATOC, DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) were obtained in high yields using Fmoc peptide synthesis in solid and in solution phase.

The molecular basis for the use of radiolabeled somatostatin (SS) analogs in peptide receptor mediated radionuclide therapy (PRRT) is provided by the overexpression of the five somatostatin receptors (sstr1–5) on a variety of human tumors, especially neuroendocrine tumors and their metastases.[1] The 'gold standard' is represented by DOTATOC (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, TOC = Tyr^3-octreotide), a radiopeptide which, labeled with ^{90}Y,[2] has almost a decade of clinical experience mainly in the targeted radiotherapy of neuroendocrine tumors.[3] ^{90}Y is a high energy β-emitter radionuclide, therefore more suitable for large tumors, its cytotoxicity being primarily due to the crossfire effect.[4] This is clinically significant, because it means that metastatic small cell clusters cannot be efficiently killed using β-emitters.

Auger-electron emitting radionuclides like ^{111}In, ^{67}Ga, ^{125}I, or ^{195m}Pt have potential for the therapy of small size cancers due to their high level of cytotoxicity, low energy and short-range biological effectiveness. Biological effects are critically dependent on the subcellular (and even subnuclear) localization of these nuclides.[5] [^{111}In-DTPA]-octreotide (DTPA = diethylenetriaminepentaacetic acid) has already been used for radionuclide therapy in patients with somatostatin receptor-positive tumors and its usefulness has been shown to be clearly dependent on the high accumulation of the radioligand in the tumor.[6] Therefore derivatives with a longer retention time in the cell and aiming at the nucleus would increase the potential of Auger-electron emitters in radiotherapy, but also improve other targeted therapy strategies.

Bearing this in mind we assumed that a new functional unit for nucleus targeting and prolonged cell retention could be added to the DOTATOC conjugate. For this we have chosen the nuclear targeting signal (NLS) of the SV40 large T antigen, the heptapeptide H-Pro-Lys-Lys-Lys-Arg-Lys-Val-OH.[7] This sequence serves as a tag to proteins, indicating their destination to the cell nucleus and assisting in the transport through the nuclear membrane. To function properly, the NLS conjugates must be located in the cytoplasm, but they are not readily incorporated into cells.[8] We report herein the design and synthesis of new trifunctional conjugates of a truncated analog of somatostatin bearing a function for receptor binding and internalization (TOC), one for the nucleus transfer (NLS) and one for the cytotoxic or reporter effect (^{111}In-DOTA).

The pharmacological profile of DOTATOC-like conjugates is greatly influenced by any structural alterations and care should be taken when designing new modifications. In order to test the best architecture for such a trifunctional derivative of somatostatin we incorporated

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the NLS in three different positions relative to the somatostatin analog sequence. The C-terminus modification (compound **I**) and the two N-terminus derivatives (compounds **II** and **III**) thus synthesized are shown in Figure 1. Compound **III** has a branched structure in which the NLS sequence is inverted (H-Val-Lys-Arg-Lys-Lys-Lys-Pro-OH).

Compounds **I** and **II** were fully synthesized on the solid phase using stepwise Fmoc peptide chemistry. Conjugate **I** was assembled on trityl-chloride resin (TCP). Coupling of Fmoc-Val-OH to the solid support was performed in dry dichloromethane in the presence of an excess of DIPEA (*N*-ethyldiisopropylamine). Conjugate **II** was anchored to 2-chlorotrityl chloride resin (CTCP) pre-loaded with Fmoc-Thr(*t*Bu)-ol. Additions of subsequent Fmoc-protected amino acids were carried out using DIC (*N*,*N*′-diisopropylcarbodiimide), HOBt (*N*-hydroxybenzotriazole), and DIPEA in DMF and 20% piperidine in DMF was used for Fmoc removal. 6-Aminohexanoic acid (Ahx) spacers were chosen between the NLS part and TOC for all three derivatives, and an additional β-Ala spacer between the chelator DOTA and the NLS sequence in compound **II** in order to circumvent the potential steric hindrance. The syntheses were straightforward despite the relatively long sequences; nevertheless capping with acetic acid anhydride was carried out after each coupling, starting from the fifth residue from the resin, as a safety measure. The DOTA-tris(*tert*-butyl) ester[9] was coupled on solid phase at the N-terminus of the peptides, using 2-(1-*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and DIPEA in NMP (*N*-methylpyrrolidone) for 2–4 h. The formation of the disulfide bridge in both conjugates was achieved in solution at room temperature, after cleavage of the fully protected linear conjugate from the resin, with I_2 in aqueous methanol. Total deprotection using TFA–water–triisopropylsilane (TIS)–thioanisole (95:2.5:1.25:1.25) for 4 h afforded the desired conjugates **I** and **II**, respectively, in good purities (75±5%), as assessed by multiwavelength detection HPLC.

Compound **III** was synthesized by either one of two strategies: solid phase peptide synthesis alone or by a convergent approach, involving the synthesis of protected fragments in solid phase and combination of these units in solution as illustrated in Scheme 1. The orthogonally-protected lysine derivative Fmoc-Lys-(ivDde)-OH[10] was chosen for the branching of this conjugate on the resin. The ivDde group is considerably more stable to piperidine than Dde, and is less prone to migrate from protected to unprotected lysine side chains.[11] After Fmoc cleavage the prochelator was coupled, thus allowing subsequently the ε-NH-ivDde removal with 3% hydrazine in DMF. Following cleavage from the resin, cyclization in solution and total deprotection, the crude product **III** obtained using method **A** (Scheme 1) had a purity of approximatively 85% as indicated by analytical HPLC. The overall yield of this synthesis was 21%.

We supposed that the synthesis of **III** would be more efficient and rapid via a convergent approach, involving synthesis and coupling of the component fragments (**1**), [Tyr^3, Lys^5(ivDde)]-octreotide, and (**2**) (Scheme 1, **B**). We opted for a minimal protection in this case to facilitate the succeeding conditions for coupling in solution. For the construction of the second fragment we utilized again the versatile Fmoc-Lys(ivDde)-OH derivative in a similar manner as in method **A**. As solid support we chose the TCP resin for its acid sensitivity, allowing cleavage with preservation of protecting groups Boc and *t*Bu. Following removal from the resin with 20% AcOH in DCM, fragment (**2**) was obtained with a purity of 80 ± 5% as revealed by HPLC and used without

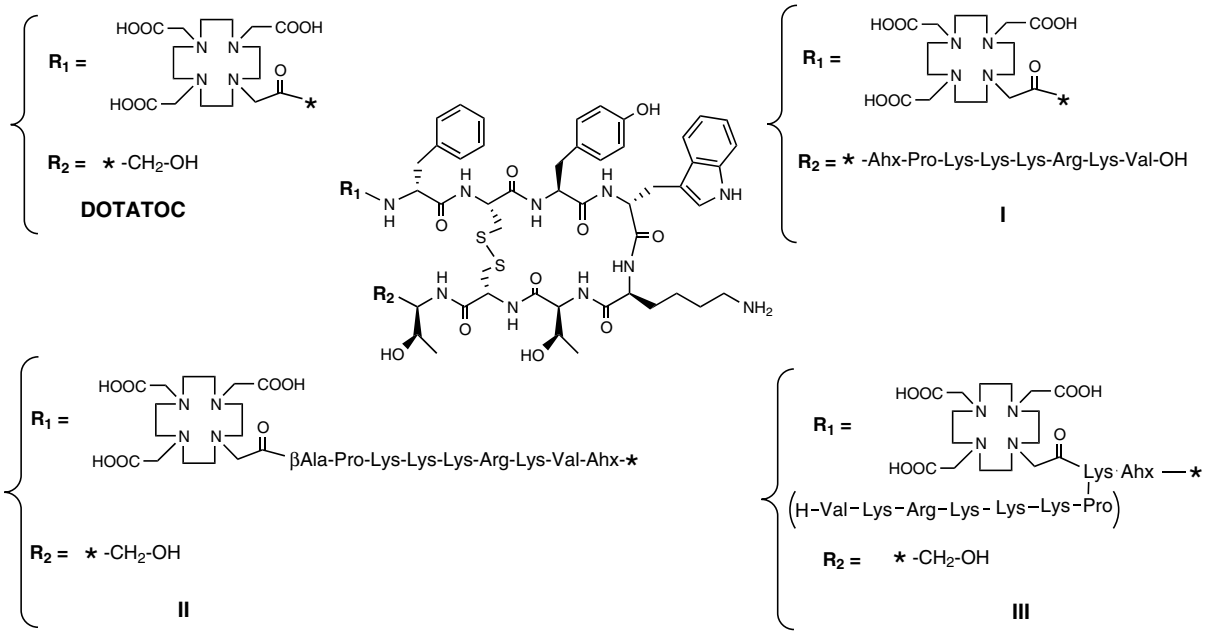

Figure 1. Chemical structures of the synthesized conjugates **I**–**III** and DOTATOC.