DOI: 10.1002/anie.201303108

Peptidomimetics

1,2,3-Triazoles as Amide Bond Mimics: Triazole Scan Yields Protease-Resistant Peptidomimetics for Tumor Targeting**

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The targeted delivery of diagnostic and therapeutic agents to tumors and metastases has emerged as a promising strategy for the management of cancer.[1] The functionalization of cytotoxic agents and imaging probes with targeting moieties (vectors) enables their specific delivery to tumors with an efficiency higher than in nonconjugated form and may thus reduce side effects and improve imaging, respectively.[2] Ideally, a vector should be readily synthesized, offer the possibility for conjugations, exhibit an appropriate stability, have a well-defined structure, and display nanomolar affinity towards a cell surface recognition element (e.g., receptors), which is overexpressed by tumors but not in nontargeted tissue.[3] Some naturally occurring, synthetically accessible small molecules fulfill these criteria and have found application in the clinic. For example, cyclic peptides (octreotide,[4] RGD[5]) labeled with diagnostic or therapeutic radionuclides are currently used in nuclear medicine. On the other hand, the employment of linear peptides (e.g., bombesin, neurotensin) in this context has been hampered despite their high potential in part due to their low stability in vivo.[6] Considerable research efforts have been made in the past in order to stabilize such peptides while not impacting their favorable biological properties, however, with varying degree of success.[6a] Therefore, new approaches for the metabolic stabilization of tumor-targeting peptides are needed.

It has previously been shown that 1,4-disubstituted 1,2,3-triazoles can effectively mimic *trans*-amide bonds because of their similar size, planarity, H-bonding capabilities, and dipole moment.[7] It has also been suggested that the replacement of an amide bond by a 1,2,3-triazole isostere could afford protease-resistant peptidomimetics.[7a,h,8] However, to the best of our knowledge, no study has yet reported the influence of such backbone modifications on the stability of the peptide. With the goal to develop a tumor-targeting peptidic vector with retained high receptor affinity but improved stability, we set out to replace systematically amide bonds of linear peptides with 1,4-disubstituted 1,2,3-triazoles by the Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC)[7a,9] and study the effect of the structural changes. For proof of concept of the new methodology (termed a triazole scan), we chose the minimal binding sequence of the peptide bombesin (BBN(7-14)). This short octapeptide (H-QWAVGHLM-NH_2) is a high-affinity agonist of the gastrin-releasing peptide receptor (GRPr), which is overexpressed in a variety of clinically relevant tumors including, prostate and breast cancer.[10] BBN(7-14) undergoes cell internalization by endocytosis upon activation of the GRPr and thus is widely studied for applications in drug delivery and nuclear oncology.[11] BBN(7-14) represents not only a good model peptide to illustrate the potential of this novel stabilization technique but also a challenge as other backbone-modification strategies (e.g., cyclization, carbonyl reduction, N-methylation) have had only moderate success in providing bombesin analogues with maintained affinity towards GRPr.[12]

We are particularly interested in BBN(7-14) as a tumor-targeting vector for the development of novel radiopharmaceuticals using radioactive metals (radiometals) in a theranostic approach.[13] For this purpose, the peptide can be functionalized N-terminally through a spacer unit with an appropriate chelator for complexation of a radiometal.In this work, we used the universal macrocyclic chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), a short hydrophilic tetraethylene glycol (PEG_4) spacer, and lutetium-177 (^{177}Lu) as a clinically established therapeutic radionuclide with a concomitant γ-emission for imaging.[14] In addition, the methionine residue in position 14 of the amino acid sequence was replaced by norleucine to avoid the formation of oxidation side products during radiolabeling.[15] These modifications have all been reported to be tolerated by the peptide.[16]

The peptide analogues described herein were synthesized by a solid-phase approach, which includes the CuAAC reaction. Amino alkyne and azido acid building blocks were prepared following reported procedures (Scheme 1). In brief, chiral Fmoc-protected amino alkynes were obtained by reduction of amino acid derived Weinreb amides followed by a Seyferth–Gilbert homologation of the in situ generated α-amino aldehydes using the Bestmann–Ohira reagent.[17] The enantiomeric purity of the alkyne building blocks was verified in each case.[18] Azido acids were synthesized from amino acids with retention of chirality by diazo-transfer

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[**] This work was supported by the Swiss National Science Foundation (grant no. 132280) and the Nora van Meeuwen-Häfliger Foundation. We thank A. Mascarin and E. Huxol for technical assistance and Dr. I. Muckenschnabel (Novartis, Basel, Switzerland) for assistance with MS analysis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201303108.

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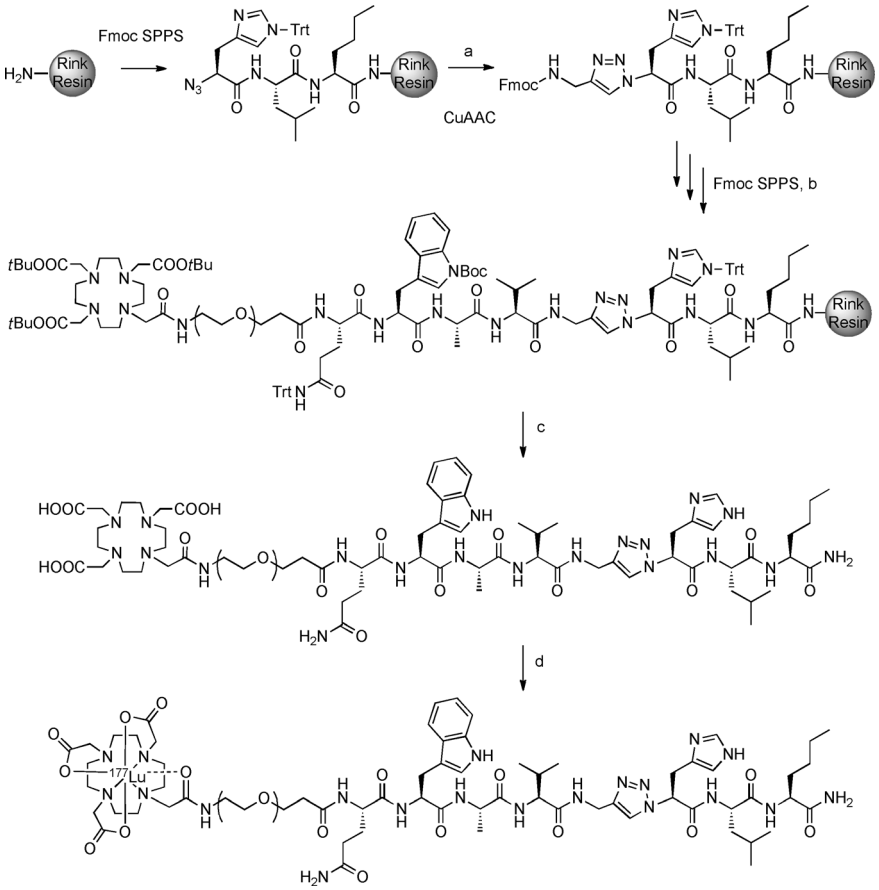

Scheme 1. Synthesis of radiolabeled peptidomimetics illustrated by the synthesis of **5**. Fmoc-SPPS: 1) 20% piperidine/DMF; 2) Fmoc-amino acid or azido acid, HATU, iPr$_2$NEt; DMF, a) (9*H*-fluoren-9-yl)methyl prop-2-yn-1-ylcarbamate, [Cu(CH$_3$CN)$_4$]PF$_6$, iPr$_2$NEt, DMF; b) DOTA(*tris*-*t*Bu), HATU, iPr$_2$NEt; DMF, c) trifluoroacetic acid/H$_2$O/PhOH/iPr$_3$SiH; d) ^{177}LuCl$_3$, 95 °C, ammonium acetate buffer (pH 5.0). Fmoc-SPPS: Fmoc-based solid-phase peptide synthesis, Fmoc: 9-fluorenylmethoxycarbonyl, *t*Bu: *tert*-butyl, Trt: trityl, DMF: *N*,*N*-dimethylformamide, HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium-3-oxide.

reactions employing the reagent imidazole-1-sulfonyl azide[19] either in solution or on the solid support.[20] The introduction of the 1,2,3-triazole amide bond surrogate into the amino acid sequence was achieved by CuAAC with the azide on the solid support and the corresponding amino alkynes using Cu-[CH$_3$CN]$_4$PF$_6$.[21] After completion of the amino acid sequence, the peptides were elongated N-terminally with a PEG$_4$ spacer and conjugated to the chelator DOTA. Cleavage from the support, subsequent deprotection, and purification by HPLC afforded the desired conjugates. Labeling of the bombesin analogues with ^{177}LuCl$_3$ using standard reaction conditions yielded radiolabeled compounds **1**–**10** in radiochemical yields and purities of $>95\,\%$.[16] The synthesis of compound **5** is shown as an example in Scheme 1.[18]

With radiotracers **1**–**10** in hand, we first studied their proteolytic stability by incubation in blood serum (Table 1). With the exception of compounds **2** and **10**, serum half-lives of backbone-modified compounds were significantly improved (by up to 20-fold) in comparison to the reference conjugate **1**. In general, we observed that the introduction of a 1,2,3-triazole in the C-terminal region of the peptide had the most pronounced effect on serum stability. Next, we investigated the influence of the backbone modifications on the biological properties of the targeting vector [Nle^{14}]BBN(7-14) in vitro using GRPr-overexpressing PC3 cells. Receptor-binding and cell-internalization characteristics of radiolabeled peptidomimetics **2**–**10** were assessed and compared with those of unmodified conjugate **1**.[18] In the case of compounds **3**, **4**, and **8**–**10**, receptor-specific cell binding and internalization was abolished, whereas conjugates **2**, **5**, and **7** displayed properties similar

Table 1: Structures and biological properties of radiolabeled compounds **1**–**10**.

Compound	Structure[a]	Half-life [h][b]	Uptake after 4 h[c,e]	K_D [nM][e,f]
1 (reference)	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-Val-Gly-His-Leu-Nle-NH$_2$	5	27.7	2.0 ± 0.6
2	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-Val-Gly-His-Leu-**Nleψ[Tz]-H**	6	29.1	3.0 ± 0.5
3	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-Val-Gly-His-**Leuψ[Tz]Nle**-NH$_2$	60	0.2	n.d.
4	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-Val-Gly-**Hisψ[Tz]Leu**-Nle-NH$_2$	>100	n.o.[d]	n.d.
5	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-Val-**Glyψ[Tz]His**-Leu-Nle-NH$_2$	17	28.3	3.1 ± 1.0
6	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-Ala-**Valψ[Tz]Gly**-His-Leu-Nle-NH$_2$	25	8.4	48.6 ± 11.5
7	[^{177}Lu]DOTA-PEG$_4$-Gln-Trp-**Alaψ[Tz]Val**-Gly-His-Leu-Nle-NH$_2$	16	24.5	5.9 ± 1.8
8	[^{177}Lu]DOTA-PEG$_4$-Gln-**Trpψ[Tz]Ala**-Val-Gly-His-Leu-Nle-NH$_2$	8	n.o.[d]	n.d.
9	[^{177}Lu]DOTA-PEG$_4$-**Glnψ[Tz]Trp**-Ala-Val-Gly-His-Leu-Nle-NH$_2$	14	n.o.[d]	n.d.
10	[^{177}Lu]DOTA-**PEG$_4\psi$[Tz]Gln**-Trp-Ala-Val-Gly-His-Leu-Nle-NH$_2$	5	0.5	n.d.

[a] ψ[Tz] represents the replacement of an amide bond by a 1,4-disubstituted [1,2,3]-triazole. [b] Determined in blood serum at 37 °C. [c] Ratio of specific receptor-bound and cell-internalized compound expressed in % of administered dose normalized to 10^6 cells. [d] n.o.: not observed; no specific binding or internalization was detected at a peptide concentration of 2.5 pmol/well. [e] All the values are means of at least two experiments performed in triplicate. [f] Determined by receptor saturation binding assay; n.d.: not determined.

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to those of the reference compound **1.** Finally, compound **6** exhibited slower binding and internalization kinetics, indicating a decreased affinity for the GRPr. Receptor affinities (K_D) towards GRPr of conjugates **1**, **2**, and **5–7** were determined by receptor saturation binding assays. As expected, the compounds selected based on the receptor-binding and cell-internalization experiments all showed excellent affinities. Compounds **2**, **5**, and **7** exhibited single-digit nanomolar K_D values and a receptor affinity comparable to that of reference **1** and other reported bombesin-based radiotracers.[15–16,22] Notably, the triazole scan of [Nle[14]]BBN-(7-14) provided four novel peptidomimetic analogues with conserved biological activity, a screening result which is superior to that of other reported peptide bond substitution strategies.[12a]

Compound **5** with the most promising properties (retained GRPr affinity and 3.5-fold improved serum stability) was selected for evaluation in vivo and a side-by-side comparison with the parent compound **1** (Figure 1). Biodistribution studies were performed in athymic nude mice bearing PC3

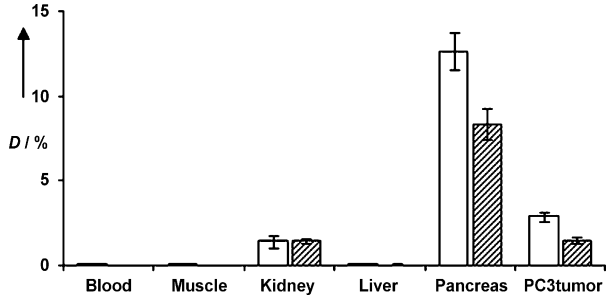

Figure 1. Biodistribution studies of compound **5** and reference compound **1** in PC3-xenografted athymic nude mice (only selected organs and tissue shown). Mice were sacrificed 4 h after intravenous injection of compound **5** (white bars) or reference **1** (dashed bars). Radioactivity in the organs is expressed as the percentage of injected dose per gram of tissue (*D*, mean ± standard deviation; $n = 3$ for each group). Receptor-specific uptake in GRPr-positive organs (e.g., pancreas) and tumors was verified by blocking experiments in the presence of excess natural BBS(1-14).

xenografts 4 h after injection of the radiotracers.[18] In general, both conjugates showed a similar biodistribution pattern. Accumulation in nontargeted tissue (e.g., muscle) was negligible for both compounds. Low levels of radioactivity were found in blood and in the liver, indicating fast blood clearance and the absence of hepatobiliary elimination, respectively. Unspecific uptake was observed in the kidneys as the result of renal excretion, a common feature of radiolabeled peptides. Strikingly, receptor-specific accumulation of radioactivity in receptor-positive organs (e.g., pancreas) was significantly enhanced for compound **5** and tumor uptake was doubled. Blocking experiments carried out by co-injection of natural bombesin suppressed GRPr-specific uptake, demonstrating that the triazole-modified peptide **5** retained its full in vivo specificity.[18] Thus, the introduction of a single 1,2,3-triazole heterocycle between Gly[11] and His[12] of [Nle[14]]BBN(7-14) increased the peptide's proteolytic stability and led to an improved tumor uptake in vivo.[23]

In summary, we report for the first time a triazole scan of a biologically relevant peptide and its utility for the identification of novel peptidomimetics with improved properties. Application of the new strategy to the bombesin fragment [Nle[14]]BBN(7-14) led to the identification of a series of peptide-based radiotracers with retained nanomolar receptor affinity and an up-to-fivefold improved serum stability. In vivo evaluation of a backbone-modified peptide analogue demonstrated the enhanced stability of the vector and its improved tumor-targeting capability. Further optimization of our lead compounds and application of triazole scans to other peptides of medicinal interest are currently in progress. We expect that this new methodology for the stabilization of peptides will find broad application in the field of tumor targeting with small peptides for drug delivery, imaging, and peptide receptor radiotherapy.

Received: April 13, 2013
Revised: May 24, 2013
Published online: July 5, 2013

Keywords: click chemistry · drug delivery · metabolic stabilization · peptidomimetics · radiopharmaceuticals

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