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# Radiolabeled antagonistic bombesin peptidomimetics for tumor targeting†‡

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**The replacement of amide bonds in the backbone of peptides by proteolytically stable 1,2,3-triazole isosteres can provide novel peptidomimetics with promising properties for the development of tumor-targeting radiopeptides. On the basis of our previous work with radiolabeled agonistic bombesin (BBN) derivatives of the sequence [Nle$^{14}$]BBN(7–14), we substituted selected amide bonds of the structurally closely related antagonistic peptide analog JMV594. With the exception of the C-terminal modification, amide-to-triazole substitutions tolerated by [Nle$^{14}$]BBN(7–14) without loss of biological function led to abolished receptor affinity in the case of JMV594. These findings provide an additional piece of evidence for the currently disputed differences in the modes of action of agonistic and antagonistic gastrin-releasing peptide receptor (GRPR)-targeting radiopeptides.**

**Keywords:** 1,2,3-triazoles; amide bond mimics; bombesin; antagonists; radiopeptides; peptidomimetics; click chemistry

## Introduction

The development of radiopharmaceuticals based on peptides as tumor-targeting moieties (vectors) has been a focus of cancer research in radiopharmaceutical and nuclear medicinal research for about the past two decades. As a result, the first radiolabeled peptides (somatostatin analogues) have found routine applications in the clinic and are used in nuclear medicine as diagnostic probes and radiotherapeutics for the management of neuroendocrine tumors. In addition, a number of peptidic radiotracers are currently in preclinical and clinical development.[1]

Bombesin (BBN) is an amphibian analog of the mammalian gastrin-releasing peptide (GRP), a member of the family of regulatory peptides, which target G-protein coupled receptors. Specifically, BBN has a high affinity and specificity towards the GRP receptor (GRPR), a cell membrane receptor over-expressed in a variety of clinically relevant tumors including prostate, breast, colon, and small-cell lung carcinomas.[2] As a consequence, the 14 amino acid sequence of BBN (and fragments thereof) has been widely studied for the development of diagnostic and therapeutic radiopeptides for application in nuclear oncology.[3] Currently, two different classes of radiolabeled BBN derivatives are under investigation. 1) Agonistic versions that internalize into tumor cells by endocytosis upon activation of the receptor and trigger an intracellular response (e.g., release of $Ca^{2+}$). Historically, radiolabeled GRPR agonists have been studied almost exclusively based on the premise that radioactivity is accumulated with higher efficiency in tumor cells (enhanced tumor-to-background ratio) and that the proximity to the cell nucleus results in an improved efficacy where therapeutic radionuclides are employed.[4] 2) Radiolabeled GRPR antagonists are not internalized into cells but exhibit a persistent attachment to tumors resulting in an overall increased accumulation of radioactivity at tumors and metastases *in vivo*.[5] Even though the exact mode of action of the GRPR-targeting peptide antagonists has not yet been fully elucidated, they represent an interesting new class of tumor-targeting peptidic vectors for the development of radiotracers.[6]

We have recently shown that the replacement of amide bonds in the backbone of linear peptides by 1,2,3-triazole amide bond isosteres, readily available by the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC, click chemistry),[7,8] yields novel peptidomimetics with promising properties for the development of tumor-targeting radiotracers.[9] We termed the systematic replacement of amide bonds by 1,2,3-triazoles as *trans*-amide bond isosters a triazole scan. A triazole scan of a variant of the agonistic BBN binding sequence, [Nle$^{14}$]BBN(7–14),[10] yielded several novel peptidomimetics with retained nanomolar affinity ($K_D$) towards GRPR, an up to fivefold enhanced blood serum stability *in vitro* and improved tumor targeting capabilities *in vivo*. In particular, amide-to-triazole substitution between the amino acid residues Ala$^9$Val$^{10}$, Gly$^{11}$His$^{12}$ and the introduction of the heterocycle at the C-terminal Nle$^{14}$ of [Nle$^{14}$]BBN(7–14), provided the most promising peptidomimetic BBN agonists (Figure 1). In an effort to identify novel radiolabeled BBN antagonists, we set out to apply our methodology to the

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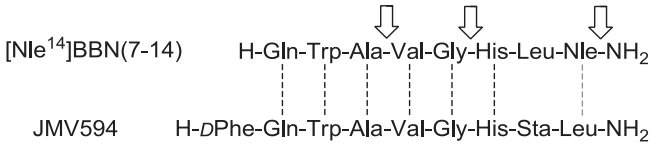



**Figure 1.** Comparison of the structures of agonistic bombesin (BBN) derivative [Nle$^{14}$]BBN(7–14) and antagonist JMV594. Dashed lines illustrate the structural similarity of the amino acid sequences, and arrows indicate positions where amide bonds can be replaced by 1,2,3-triazoles in the agonist [Nle$^{14}$]BBN(7–14) without loss of the biological properties of the vector. Nle, norleucine; Sta, statine: (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid.

structurally closely related BBN receptor antagonist JMV594,[5,11] a peptide with high-sequence homology differing only in the last two amino acids of the C-terminus and the additional DPhe at the N-terminus. On the basis of our previous investigations with [Nle$^{14}$]BBN(7–14), we reasoned that a comparison with JMV594 would be most appropriate when the same linker, chelator, and radionuclide are used. Thus, we employed a short, tetraethylene glycol (15-amino-4,7,10,13-tetraoxapentadecanoic acid, $PEG_4$-OH) spacer for the N-terminal conjugation of the macrocyclic chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA; Table 1) and lutetium-177 ($^{177}$Lu) as a therapeutic $\beta^-$ emitter with a concomitant $\gamma$ emission for imaging by single photon emission computed tomography.

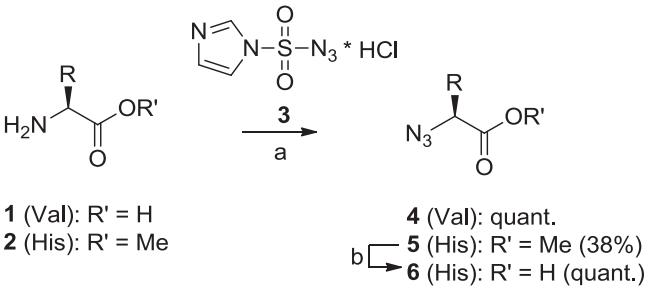
## Experimental

General methods, synthetic procedures, radiolabeling protocols, details of *in vitro* experiments, and analytical data of intermediates and final products can be found in the Supporting Information.

## Results and discussion

Building blocks required for the synthesis of 1,2,3-triazole-containing peptidomimetics as indicated in Figure 1 were prepared according to published procedures. Azide derivatives of amino acids **4** and **6** were synthesized from the corresponding amino acids **1** and **2** by a diazotransfer reaction employing the reagent imidazole-1-sulfonyl azide (**3**; Scheme 1).[12] In the case of histidine (His) derivative **5**, an additional saponification step furnished desired $\alpha$-azido acid **6**. For the insertion of a mono-substituted 1,2,3-triazole at the C-terminus of peptidomimetic **21** (Table 1), the azide functionality was introduced directly into the linker of the resin on solid support as described in the literature (Supporting Information).[13]

$\alpha$-Amino alkyne building blocks were prepared by multistep synthesis involving the reduction of amino acid-derived Weinreb amides **8** and **10** with DIBAL-H followed by a Seyferth–Gilbert homologation of the *in situ* generated $\alpha$-amino aldehydes using the Bestmann–Ohira reagent **11** (Scheme 2). Exchange of the




**Scheme 1.** Synthesis of $\alpha$-azido acids; a) **3**, $K_2CO_3$, $CuSO_4$, MeOH, RT; b) LiOH, MeOH/$H_2O$ (2:1), RT. For Val, R = $CH(CH_3)_2$; for His, R = $CH_2$(1-trityl-1H-imidazole).

Boc-protecting groups of intermediates **12** and **14** with Fmoc yielded $\alpha$-amino alkynes **13** and **15** for solid phase synthesis. Glycine analog **17** was prepared by Fmoc protection of commercial propargyl amine (**16**).

The synthesis of reference compound **18** and 1,2,3-triazole containing peptidomimetics **19–21** (Figure 2, Table 1) was accomplished by standard Fmoc solid phase peptide synthesis (Fmoc-SPPS) and CuAAC on solid support. In some cases, fragments of the peptides were synthesized using an automated synthesizer, whereas in other examples, amide couplings were performed manually (Experimental). CuAAC on solid support was performed according to published procedures employing Cu$[CH_3CN]_4PF_6$ as catalyst.[14,15] After completion of the amino acid sequence, the peptides were elongated N-terminally with a $PEG_4$ spacer and functionalized with the universal macrocyclic chelator DOTA. Following cleavage from the resin and deprotection, the peptide conjugates **18–21** were obtained in high purity (>95%) after preparative HPLC purification and structural identity was verified by mass spectrometric analysis (Table 1).

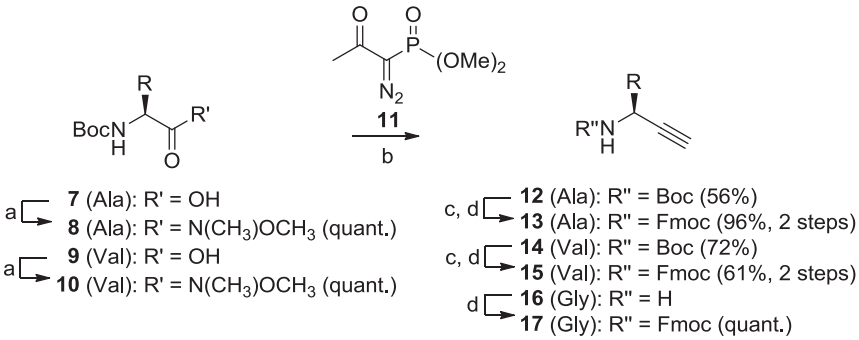
Radiolabeling of DOTA conjugates **18–21** with $^{177}LuCl_3$ was performed using standard labeling procedures at 95°C in $NH_4OAc$ (pH 5) as described by us and others.[9,16] Radiolabeled compounds [$^{177}$Lu(**18–21**)] were obtained in excellent radiochemical yield and purity (>95%) with a specific activity of 0.05–0.3 mCi/nmol (not optimized).

With radiolabeled reference peptide [$^{177}$Lu(**18**)] and peptidomimetics [$^{177}$Lu(**19–21**)] in hands, we next evaluated their receptor binding and cell internalization properties as well as their receptor affinity ($K_D$) *in vitro* using GRPR overexpressing PC3 cells (Figures 3 and 4). Receptor specificity was confirmed for all *in vitro* studies described henceforth by blocking experiments in the presence of an excess of natural BBN (Supporting Information). Radiopeptide [$^{177}$Lu(**18**)], used as a reference compound in this study, represents a novel antagonistic BBN radioconjugate. Despite structural differences to published radiolabeled JMV594 derivatives,[5,17] [$^{177}$Lu(**18**)] displayed receptor binding (approximately 30%) and cell internalization properties (approximately 8%) similar to

**Table 1.** Reference peptide **18** and 1,2,3-triazole containing peptidomimetics **19–21** obtained by Fmoc-SPPS and CuAAC on solid support

| Nr. | Structure | Yield (%) | $[M+H]^+$ (calc.) |
|---|---|---|---|
| **18** | DOTA-$PEG_4$-*D*Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-$NH_2$ | 28 | 1746.932 (1746.943) |
| **19** | DOTA-$PEG_4$-*D*Phe-Gln-Trp-Ala$\psi$**[Tz]**Val-Gly-His-Sta-Leu-$NH_2$ | 27 | 1770.957 (1770.954) |
| **20** | DOTA-$PEG_4$-*D*Phe-Gln-Trp-Ala-Val-Gly$\psi$**[Tz]**His-Sta-Leu-$NH_2$ | 13 | 1770.944 (1770.954) |
| **21** | DOTA-$PEG_4$-*D*Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu$\psi$**[Tz]**-H | 14 | 1770.993 (1770.954) |

$\psi$[Tz] indicates the position of the amide-to-triazole substitution.




**Scheme 2.** Synthesis of $\alpha$-amino alkynes; a) MeONMe HCl, BOP, *i*-$Pr_2NEt$, $CH_2Cl_2$, RT; b) i) DIBAL-H, $CH_2Cl_2$, −78°C; ii) dimethyl-1-diazo-2-oxopropylphosphonate, $K_2CO_3$, MeOH, RT; c) trifluoroacetic acid/$CH_2Cl_2$ (2:1), RT; d) Fmoc-OSu, *i*-$Pr_2NEt$, $CH_2Cl_2$, RT. For Ala, $R = CH_3$; for Val, $R = CH_2CH(CH_3)_2$; for Gly, $R = H$.

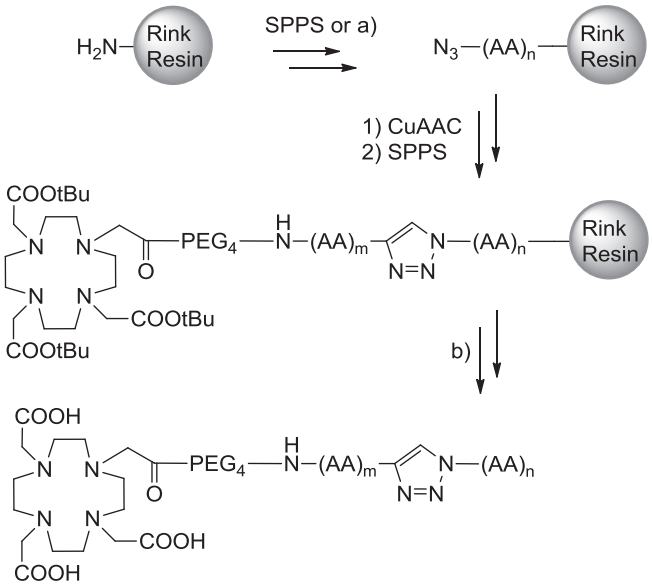



**Figure 2.** Synthesis of 1,2,3-triazole containing bombesin (BBN) antagonistic radiopeptidomimetics. SPPS: i) 20% piperidine/*N,N,*-Dimethylformadide (DMF); ii) Fmoc-amino acid, azido acid, Fmoc-$PEG_4$-OH, or DOTA($t$Bu)$_3$, HATU, *i*-$Pr_2NEt$; DMF; a) For compound **21** ($n = 0$): **3**, DMF; CuAAC: $\alpha$-amino alkyne, $[Cu(CH_3CN)_4]PF_6$, *i*-$Pr_2NEt$, DMF; b) trifluoroacetic acid/$H_2O$/PhOH/*i*-$Pr_3SiH$. SPPS, solid phase peptide synthesis; AA, amino acid.

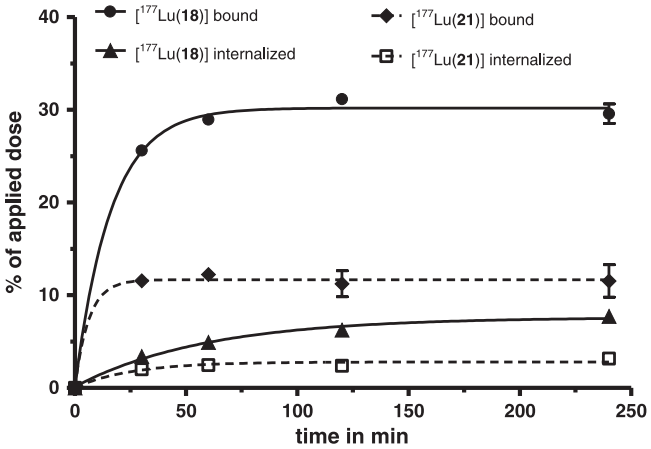



**Figure 3.** Specific receptor binding and cell internalization of antagonistic $^{177}$Lu-labeled peptide conjugates. Data are presented as mean ± standard deviation ($n = 3$ in triplicates) and fitted by nonlinear regression with GraphPad Prism 5.0.

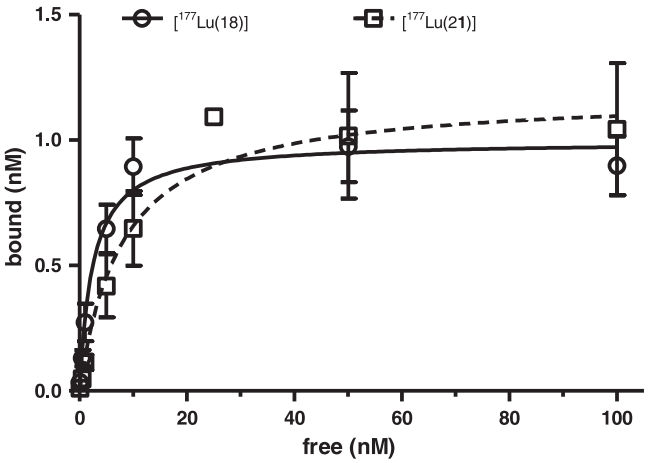



**Figure 4.** Receptor saturation assays of antagonistic $^{177}$Lu-labeled conjugates. Data are presented as mean ± standard deviation ($n = 2$–3 in triplicates) and fitted by nonlinear regression with GraphPad Prism 5.0.

that of reported derivatives. Interestingly, JMV594-derived [$^{177}$Lu(**18**)] exhibited to some extent cell internalization properties typically observed for agonists even though the vector has been reported as a full antagonist.[11] Disappointingly, [$^{177}$Lu(**19–20**)] did not bind to GRPR or internalize into PC3 cells, which indicates abolished receptor affinity. On the other hand, compound [$^{177}$Lu(**21**)] showed a similar but less pronounced receptor binding (13%) and cell internalization (3%) profile in comparison with reference compound [$^{177}$Lu(**18**)]. Receptor saturation experiments revealed a receptor affinity for [$^{177}$Lu(**18**)] and [$^{177}$Lu(**21**)] of $K_D = 2.7$ nM and $K_D = 8.1$ nM, respectively. The lower GRPR affinity of [$^{177}$Lu(**21**)] compared with reference [$^{177}$Lu(**18**)] correlates with the observed decreased accumulation of the 1,2,3-triazole-modified compound [$^{177}$Lu(**21**)] in PC3 cells.

The metabolic stability of [$^{177}$Lu(**18**)] and [$^{177}$Lu(**21**)] was investigated by incubation of the compounds in human blood serum at 37°C. Samples were taken within 1–48 h and analyzed by $\gamma$ HPLC after precipitation of proteins. Remarkably, both antagonistic radiopeptides were still intact to a large extent (65% and 75%, respectively) even after 48 h (Supporting Information). This resistance to proteolytic degradation by serum proteases is outstanding in comparison with radiolabeled BBN agonists, which usually exhibit a serum half-life in the range of a few hours.[9,18] We hypothesize that the exceptional *in vitro* stability of JMV594-derived antagonistic radioconjugates is at least in part responsible for their observed favorable characteristics as tumor-targeting radiopharmaceuticals.

## Conclusions

In summary, we have applied the amide-to-triazole substitution strategy of previously investigated agonistic GRPR-targeting peptide [Nle$^{14}$]BBN(7–14) to the structurally closely related antagonistic peptide JMV594.

Despite the high-sequence homology of the two peptides investigated, we observed that the majority of backbone modifications tolerated by [Nle$^{14}$]BBN(7–14) cannot be applied to JMV594. Our results are in accordance with published data reporting backbone modifications (N-methylation) of GRPR agonists and antagonists based on the described amino acid sequences.[19]

The data obtained in this study provide an additional piece of evidence that the currently disputed mode of action of agonistic versus antagonistic peptides (e.g., binding conformation of the peptides or receptor binding site, respectively) may be indeed different. In addition, we report here the first radiolabeled 1,2,3-triazole-containing GRPR-specific antagonistic radiopeptidomimetic. Investigations of other antagonistic GRPR-targeting radiopeptides with 1,2,3-triazole backbone modifications are currently ongoing.

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## Conflict of Interest

The authors have no conflicts of interest.

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## Supporting information

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