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1,5-Disubstituted 1,2,3-Triazoles as Amide Bond Isosteres Yield Novel Tumor-Targeting Minigastrin Analogs

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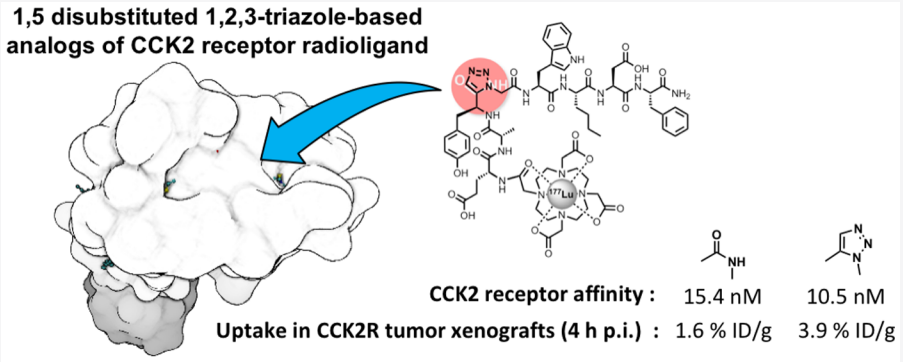

ABSTRACT: 1,5-Disubstituted 1,2,3-triazoles (1,5-Tz) are considered bioisosteres of *cis*-amide bonds. However, their use for enhancing the pharmacological properties of peptides or proteins is not yet well established. Aiming to illustrate their utility, we chose the peptide conjugate [Nle^{15}]MG11 (DOTA-DGlu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2) as a model compound since it is known that the cholecystokinin-2 receptor (CCK2R) is able to accommodate turn conformations. Analogs of [Nle^{15}]MG11 incorporating 1,5-Tz in the backbone were synthesized and radiolabeled with lutetium-177, and their pharmacological properties (cell internalization, receptor binding affinity and specificity, plasma stability, and biodistribution) were evaluated and compared with [Nle^{15}]MG11 as well as their previously reported analogs bearing 1,4-disubstituted 1,2,3-triazoles. Our investigations led to the discovery of novel triazole-modified analogs of [Nle^{15}]MG11 with nanomolar CCK2R-binding affinity and 2-fold increased tumor uptake. This study illustrates that substitution of amides by 1,5-disubstituted 1,2,3-triazoles is an effective strategy to enhance the pharmacological properties of biologically active peptides.

KEYWORDS: *1,2,3-Triazoles, peptidomimetics, structure−activity relationships, radiopharmaceuticals, tumor targeting, cancer*

Five-membered N-heterocycles have attracted attention as amide bond mimics for the past 20 years. Due to the similar size, polarity, planarity, and their ability to create hydrogen bonds, imidazoles, oxa(dia)zoles, tetrazoles, and triazoles, among others, have been used to replace amide bonds in order to improve the pharmacological characteristics of biologically active compounds.[1] These replacements are performed to overcome the inherently low proteolytic stability of amides or to probe binding conformations since heterocycles can mimic conformationally locked amide bonds.[2−6] Despite the large number of examples illustrating the potential of replacing amide bonds with small heterocycles in drug-like compounds with low molecular weight,[5,6] the use of this approach in peptides is not very common yet. Disubstituted tetrazoles as well as 1,2,4- and 1,2,3-triazoles are among the heterocycles that allow a precise replacement of an amide bond (Figure 1).[7−9] Triazole synthesis can be performed in solution or on solid support, the latter making their use particularly appealing for peptide chemists.[10−12] In this context, 1,2,3-triazoles (Tz) are heterocycles that, while having a similar dipolar moment, are capable of mimicking both *trans*- and *cis*-amide bonds, depending on their 1,4- or 1,5-substitution pattern, respectively, while being synthetically accessible (Figure 2).[13]

The practicality of 1,2,3-triazoles as peptidomimetics has been greatly enhanced by the discovery of the copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC), which selectively gives access to 1,4-disubstituted 1,2,3-triazoles (1,4-Tz).[14,15] Since then, 1,4-Tz have been quickly adopted as bioisosteres of amide bonds in peptidomimetics to modify the pharmacological properties of peptides or proteins.[16−20] The systematic replacement of amide bonds by 1,4-Tz (termed

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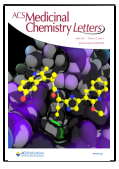

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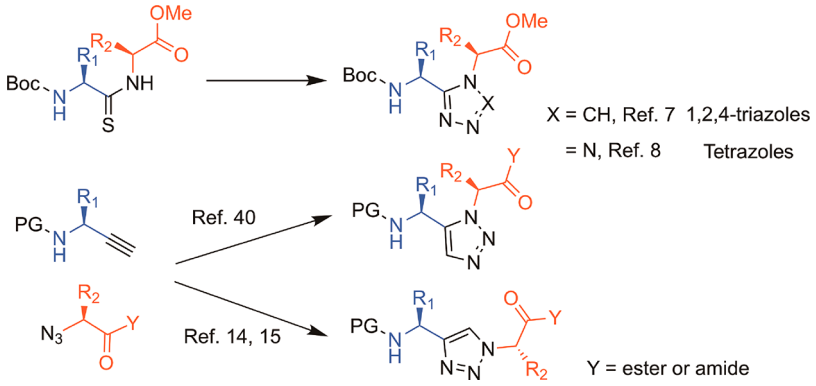

Figure 1. Mimetics of amide bonds based on N-heterocycles.

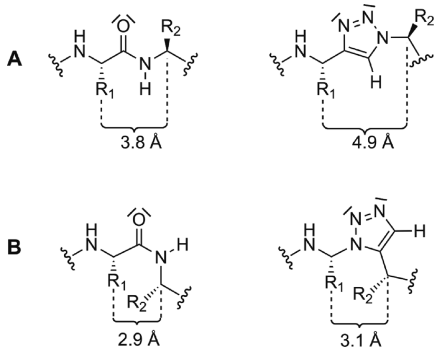

Figure 2. Comparison between *trans*-amide and *cis*-amide bonds and 1,4- (A) and 1,5-disubstituted 1,2,3-triazoles (B).

a triazole scan) has been used as a tool to probe the contribution of the backbone to the stability and the activity of biologically relevant peptides similar to an *N*-methylamide scan.[21] So far, this approach has been applied to enkephalin,[22] angiotensin,[23] bombesin,[24−26] neurotensin,[27,28] and minigastrin.[29,30] Examples of peptidomimetics based on 1,5-disubstituted 1,2,3-triazoles (1,5-Tz) are scarce. 1,5-Tz, obtained by the ruthenium-catalyzed azide−alkyne cycloaddition (RuAAC), are useful to probe the bioactive conformation of peptides, since they can mimic constrained structures such as β-turns or hairpins,[17,31,32] and their use as valuable amide bioisosteres in peptide backbones has been demonstrated both theoretically[33,34] and experimentally.[11,17,35]

We have recently reported the triazole scan of [Nle^{15}]MG11 (compound **1**, Figure 3) labeled with lutetium-177 (^{177}Lu).[29,30] This conjugate consists of a peptide with high affinity for the CCK2R coupled to the macrocyclic chelator DOTA to enable labeling with radiometals and shows potential in nuclear medicine for targeting a variety of tumors such as medullary thyroid carcinoma and small cell lung cancer. We have previously shown that single or multiple 1,4-Tz can be incorporated between residues DGlu^{10} to Trp^{14} of compound **1**. In the majority of the examples, the nanomolar affinity of the resulting peptidomimetics toward the target receptor is preserved and an increased proteolytic stability was observed. In addition, several linear and cyclic peptides and peptidomimetics mimicking β-turns have shown high affinities toward the CCK2R.[36−38] On the basis of these reports, we set out to study the influence of amide-to-1,5-Tz switches on the CCK2R-binding properties of compound **1** in positions likely to be involved in β-turn conformation. Triazole-containing analogs of compound **1** were synthesized, radiolabeled with [^{177}Lu]-Lu^{3+}, and evaluated *in vitro* (cell internalization, receptor affinity and specificity, metabolic stability) and *in vivo* (biodistribution in mice bearing CCK2R-positive xenografts).

With the aim of identifying 1,5-Tz-modified derivatives of compound **1** with retained tumor-targeting properties, bonds between Gly^{13}-Trp^{14} (**5**), Tyr^{12}-Gly^{13} (**6**), Ala^{11}-Tyr^{12} (**7**) were substituted with a 1,5-Tz moiety. Modifications between Trp^{14}-Nle^{15} and DGlu^{10}-Ala^{11} were discarded since these bonds are distant from the suspected location of the turn.[29,30] The heterocycles were inserted in the peptide sequence following the strategy exemplified for conjugate **7** in Scheme 1. Azido benzyl esters of amino acids were synthesized via diazo transfer using 1*H*-imidazole-1-sulfonyl azide.[39] Amino alkynes were synthesized from commercially available L-amino acids as previously described.[24,27] Despite our efforts, we were not able to obtain the excellent optical purities that we observed in the past.[24,27] As a result, amino alkyne mimics of Fmoc-Ala-OH and Fmoc-Tyr(*t*Bu)-OH were isolated in a L:D ratio of 66:34 and 90:10, respectively (see Supporting Information).[24,27] Partial racemization during the

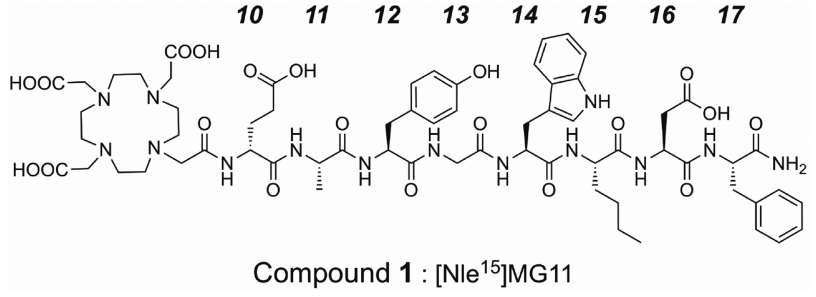

Figure 3. Structure of [Nle^{15}]MG11 with numbering of the amino acid residues.

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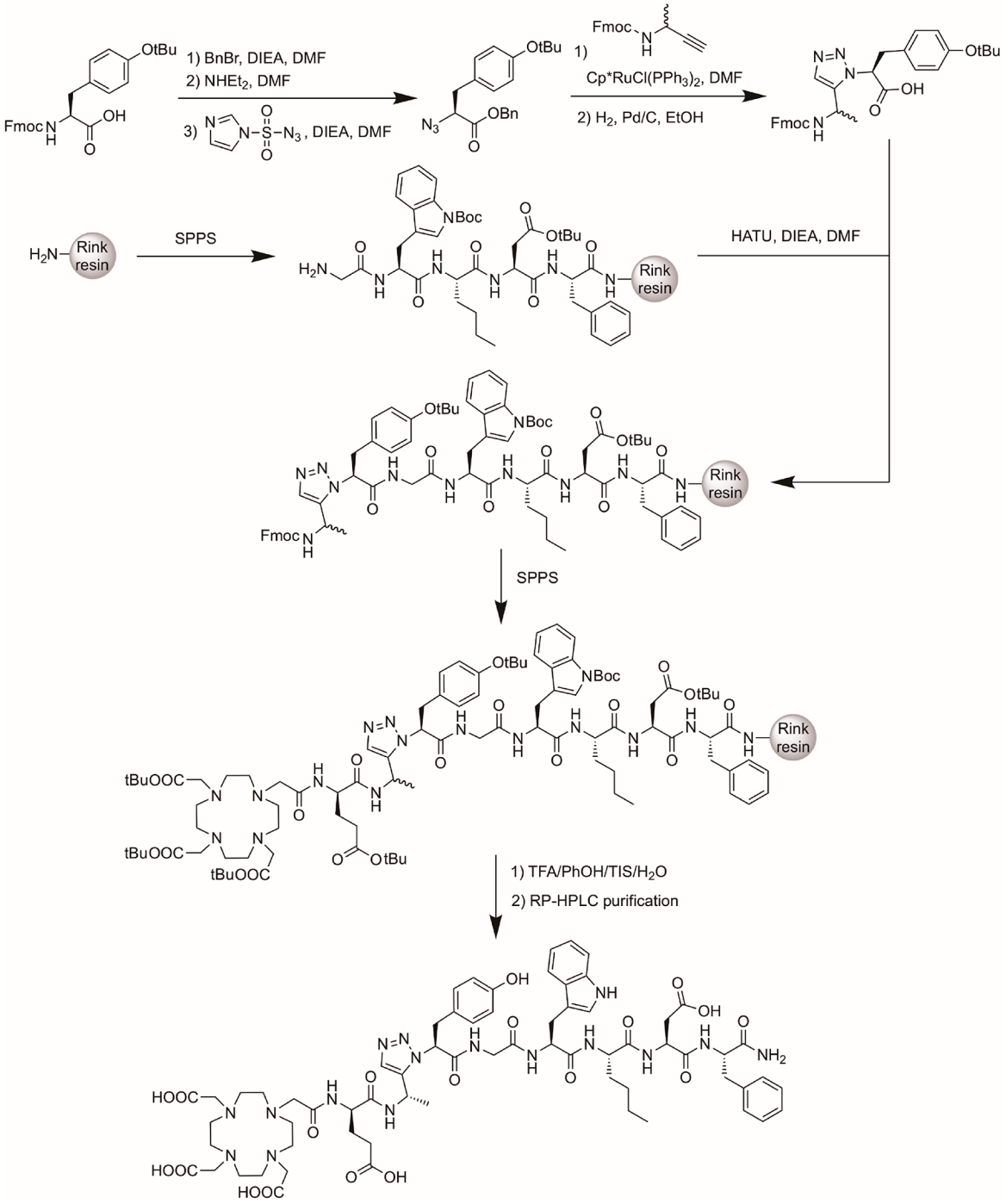
Scheme 1. Synthesis of 1,5-Tz-containing peptidomimetic DOTA conjugate 7.



Seyferth−Gilbert homologation has been observed in the past,[29] and alternative procedures have since been proposed for the synthesis of optically pure amino alkynes.[34] Despite the inconvenience, amino alkynes were used in the peptidomimetic syntheses as a mixture of enantiomers. Cp*RuCl(PPh$_3$)$_2$ and reaction temperatures of 60 °C [40,41] afforded the cycloaddition products in solution within 2−8 h (see Supporting Information). RuAAC was followed by deprotection of the carboxylic acid by hydrogenation using Pd/C as catalyst. The obtained pseudodipeptide building blocks were coupled to the growing peptide sequence on solid support using HATU as a coupling reagent. The remaining amino acids were coupled using standard Fmoc/*t*Bu solid-phase peptide synthesis (SPPS) until completion of the DOTA-peptide conjugates (Scheme 1). After cleavage from solid support, deprotection by TFA, and purification by RP-HPLC, the three DOTA-substituted peptidomimetics **5**−**7** were obtained in satisfactory yields (30−50%) and characterized by analytical RP-HPLC and HR-MS (see Supporting Information). In the case of the syntheses of **6** and **7**, as was expected based on the enantiomeric purity of the α-substituted amino alkynes, two diastereoisomers were found in the same ratios as the L:D ratios of the aminoalkynes (see Supporting Information). The two diastereoisomers were separated by RP-HPLC, and only the

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Table 1. Summary of *in Vitro* Results of Compounds [^{177}Lu]Lu-1−7

compd	sequence	internalization at 4 h (% a. d.)	IC_{50} (mean), (95% confidence interval) (nM)[b]	half-life (h)
[^{177}Lu]Lu-1[a]	[^{177}Lu]Lu-DOTA-DGlu-Ala-Tyr- Gly-Trp-Nle-Asp-Phe-NH_2	32.2 ± 3.2	15.4 (11.0−21.1)	3.9
[^{177}Lu]Lu-2[a]	[^{177}Lu]Lu-DOTA-DGlu-Ala-Tyr-Glyψ[1,4-Tz]Trp-Nle-Asp-Phe-NH_2	41.7 ± 3.9	15.6 (12.3−19.7)	3.8
[^{177}Lu]Lu-3[a]	[^{177}Lu]Lu-DOTA-DGlu-Ala-Tyrψ[1,4-Tz]Gly-Trp-Nle-Asp-Phe-NH_2	54.3 ± 5.1	1.7 (1.3−2.3)	2.6
[^{177}Lu]Lu-4[a]	[^{177}Lu]Lu-DOTA-DGlu-Alaψ[1,4-Tz]Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	29.6 ± 2.7	20.9 (17.0−25.7)	51.4
[^{177}Lu]Lu-5	[^{177}Lu]Lu-DOTA-DGlu-Ala-Tyr-Glyψ[1,5-Tz]Trp-Nle-Asp-Phe-NH_2	1.5 ± 0.9	447.7 (352.8−568.1)	91.1
[^{177}Lu]Lu-6	[^{177}Lu]Lu-DOTA-DGlu-Ala-Tyrψ[1,5-Tz]Gly-Trp-Nle-Asp-Phe-NH_2	50.9 ± 4.1	10.5 (8.1−13.7)	2.2
[^{177}Lu]Lu-7	[^{177}Lu]Lu-DOTA-DGlu-Alaψ[1,5-Tz]Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	30.3 ± 1.4	64.8 (51.7−81.3)	6.7

[a]Data of reference compound [^{177}Lu]Lu-**1** and 1,4-Tz analogs [^{177}Lu]Lu-**2**−**4** are reproduced for comparison.[29] [b]Competition experiments were performed with nonradioactive analogs of the compounds **1**−**7** labeled with ^{175}Lu.

major compounds, which were assumed to have the desired stereochemistry, were later examined in *in vitro* and *in vivo* assays (Scheme 1).

Compounds **5**−**7** were radiolabeled with [^{177}Lu]$LuCl_3$ to obtain radioconjugates [^{177}Lu]Lu-**5**−**7** (Table 1) in high radiochemical yields and purities of ≥95%, with specific molar activities between 25 and 50 MBq/nmol (not optimized).[29,30] Representative chromatograms of quality controls by γ-HPLC can be found in the Supporting Information.

Receptor-mediated internalization of the radioconjugates was evaluated using A431 cells stably transfected with the CCK2R (A431-CCK2R cells)[42] and is expressed as the percent of added radioactivity per 0.85 million cells (Table 1 and Figure 4). Blocking experiments (performed by addition of a

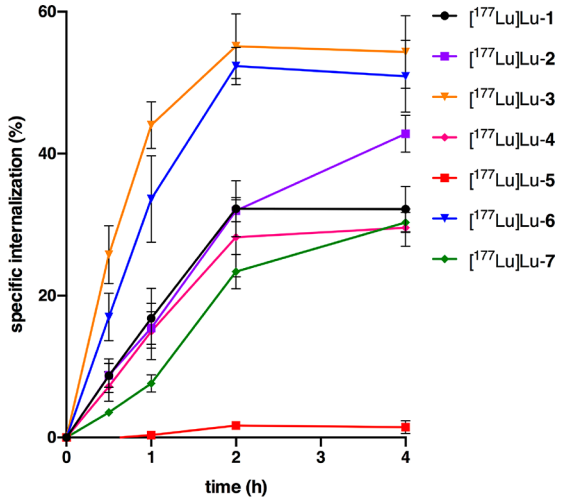

Figure 4. Specific internalization kinetics of conjugates [^{177}Lu]Lu-**1** to **7** in A431-CCK2R cells at 37 °C. Data of previously reported compounds [^{177}Lu]Lu-**1**−**4**[29] are included for comparison (n = 3 in triplicates).

5000-fold excess of minigastrin) decreased the internalization of radiolabeled compounds to less than 1% in all cases, hence demonstrating specific receptor-mediated interaction (see Supporting Information). With the exception of compound [^{177}Lu]Lu-**5**, the other 1,5-Tz analogs of compound **1** were able to bind and internalize into receptor-positive A431-CCK2R cells. Conjugates [^{177}Lu]Lu-**6** and [^{177}Lu]Lu-**7** reached similar or higher cell internalizations than reference compound **1** (51−30% vs 32%, respectively), and the internalization rates and kinetics resembled closely the ones of their 1,4-substituted counterparts [^{177}Lu]Lu-**3** and -**4**, respectively (54% and 30%, respectively, Table 1, Figure 4).

The affinity (IC_{50}, Table 1) of the novel triazolopeptides toward the CCK2R was determined by a competition-binding assay. Briefly, the established CCK2R-ligand [^{177}Lu]Lu-PP-F11N (DOTA-(DGlu)$_6$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2)[43] was added to A431-CCK2R cells and displaced by increasing concentrations of nonradioactive ^{175}Lu-labeled peptidomimetics **5**−**7** for 1 h. The cells were washed and lysed to determine the bound fraction of [^{177}Lu]Lu-PP-F11N as a function of the concentration of added competitors (see Table 1 and the Supporting Information). As anticipated from the internalization assays, compound [^{175}Lu]Lu-**5** with the lowest cell internalization had the lowest affinity for its target (IC_{50} ∼ 450 nM). Compounds [^{175}Lu]Lu-**6** and [^{175}Lu]Lu-**7** showed IC_{50} values of 11 nM and 65 nM, respectively, with only conjugate [^{175}Lu]Lu-**6** approaching the affinity of the reference compound [^{175}Lu]Lu-**1** (15 nM). Unlike compound [^{177}Lu]Lu-**2**, with a 1,4-Tz between Gly^{13}-Trp^{14} mimicking a *trans*-amide, compound [^{175}Lu]Lu-**5**, with a 1,5-Tz that mimics a *cis*-amide bond, did not show high nanomolar affinity toward the receptor. This result could indicate that the backbone of the peptide requires a linear (all-*trans*) conformation from the C-terminus until Gly^{13}. This observation confirms our previous hypothesis that Gly^{13} separates the C-terminal part of [Nle^{15}]MG11, which is buried in the receptor cavity, from the N-terminal part that is pointing toward the extracellular domain of the receptor.[29] In the same previous study, we observed an increased interaction between the CCK2R and 1,4-Tz compound [^{175}Lu]Lu-**3** likely as the result of an additional cation−π interaction between the triazole ring and Arg^{356} of the receptor.[29] Similarly, the 1,5-Tz isomer [^{175}Lu]Lu-**6** showed an improved affinity toward the CCK2R in comparison to the reference compound [^{175}Lu]Lu-**1** (10 nM vs 15 nM). Even though the improvement of affinity of [^{175}Lu]Lu-**6** was not as pronounced as in the case of the 1,4-Tz analog [^{175}Lu]Lu-**3**, the presence of an aromatic ring between Tyr^{12} and Gly^{13} indeed seems beneficial for the receptor interaction. Despite showing good cell internalization, [^{175}Lu]Lu-**7** displayed a decreased affinity toward the CCK2R in comparison with its all-amide counterpart and its 1,4-Tz isomer (65 nM vs 15 nM and 21 nM, respectively). We conclude that the position between Ala^{11}-Tyr^{12} does not play a significant role in receptor binding due to its tolerance toward modifications.

Next, we studied the proteolytic degradation of compounds [^{177}Lu]Lu-**5**-**7** *in vitro* to determine if the use of 1,5-Tz benefits the stability of this regulatory peptide. The metabolic half-lives ($t_{1/2}$) of the conjugates were determined by incubation of the radiolabeled peptides in human blood plasma followed by quantification of their metabolites by γ-HPLC over 24 h

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(Table 1). The position of the amide-to-triazole switch utilizing 1,5-Tz had distinct effects on the metabolic stability of the resulting peptidomimetic conjugates in comparison to the previously studied 1,4-Tz-substituted minigastrins.[24,26,27] Peptidomimetics [^{177}Lu]Lu-**5** and [^{177}Lu]Lu-**7** showed an increased stability in comparison to reference compound [^{177}Lu]Lu-**1**, whereas the $t_{1/2}$ was decreased for compound [^{177}Lu]Lu-**6** (2.2 h vs 3.9 h). It appears that while the insertion of a Tz heterocycle can improve the affinity of the resulting peptidomimetic toward its receptor, it can also increase its susceptibility toward degradation by proteases. The degradation kinetics of 1,4- vs 1,5-Tz peptidomimetics of MG11 differed not only depending on the position of the heterocycle in the sequence but also on the substitution pattern of the triazole (Figure 5). Degradation of compound [^{177}Lu]Lu-**5** was

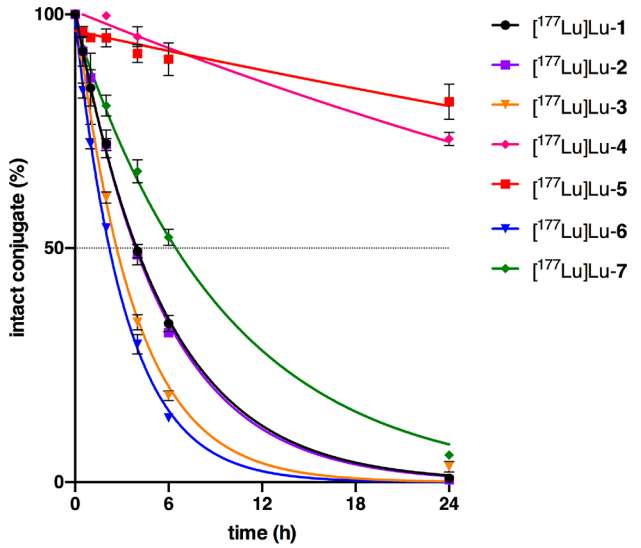

Figure 5. Degradation kinetics of ^{177}Lu-labeled peptide conjugates after incubation in human blood plasma for 24 h at 37 °C. Data points show the mean ± SD, n = 2−3, for values of $t_{1/2}$; see Table 1.

significantly slower than its 1,4-Tz counterpart [^{177}Lu]Lu-**2** ($t_{1/2}$ = 3.8 h vs 91.1 h). The behavior was reversed in the case of conjugate [^{177}Lu]Lu-**7**, which was metabolized faster than 1,4-Tz compound [^{177}Lu]Lu-**4** ($t_{1/2}$ = 51.4 h vs 6.7 h). Finally, degradation kinetics of compounds [^{177}Lu]Lu-**3** and [^{177}Lu]Lu-**6** were comparable (Figure 5). These results lead us to the conclusion that the contribution of differently substituted triazoles to the stability of a peptide may vary and does not follow a general pattern.

In vivo studies with mice bearing CCK2R-positive tumor xenografts were performed to investigate whether peptidomimetics [^{177}Lu]Lu-**6** and **7** retained the biodistribution profile of reference compound [^{177}Lu]Lu-**1** (see Supporting Information for details). Compound **5** was discarded for *in vivo* studies due to its reduced cell internalization and low affinity toward the CCK2R. Uptake of radioactivity in organs is expressed as % of injected dose per gram of organ or tissue (% ID/g). Female CD1 nu/nu mice were xenografted with A431-CCK2R cells and randomly assigned to groups of four mice. The results of the experiment are summarized in Figure 6 (see Supporting Information for complete data sets). At 4 h postinjection, all compounds showed typical biodistribution profiles of radiolabeled peptides with a fast clearance from the blood and unspecific uptake in the kidneys caused by renal excretion (Figure 6). Specific receptor-mediated uptake was demonstrated by co-injection with a 6000-fold excess of minigastrin (blocking), which led to a significant decrease of the uptake in the receptor-positive tumor and organs, e.g., the stomach (see Supporting Information). Compound [^{177}Lu]Lu-**6** showed the highest tumor uptake of the investigated 1,5-Tz peptidomimetics (3.9% ID/g). Similarly, a more than 2-fold increased uptake in the tumor was also previously observed for the analogous 1,4-Tz isomer [^{177}Lu]Lu-**3**. This is consistent with their improved CCK2R affinity and cell internalization in comparison with reference compound [^{177}Lu]Lu-**1** *in vitro* (Table 1). Tumor uptake of compound [^{177}Lu]Lu-**7** was inferior to its 1,4-Tz counterpart [^{177}Lu]Lu-**4** (1.7% ID/g vs 0.8% ID/g,). The difference in the tumor uptake of compounds **7** and **4** could be due to a decreased receptor affinity of compound **7** in comparison with compound **1** or **4** as well as to the superior proteolytic resistance of compound [^{177}Lu]Lu-**4**.

We herein report the use of 1,5-Tz heterocycles as metabolically stable mimics of *cis*-amide bonds in biologically active linear peptides. 1,5-Tz were placed in the backbone of the radiolabeled peptide [Nle^{15}]MG11 in the vicinity of a proposed β-turn. The obtained peptidomimetics were compared side-by-side with the all-amide bond reference compound [Nle^{15}]MG11 as well as with previously studied 1,4-Tz analogs, which adopt *trans*-conformations for the probed amide bonds.

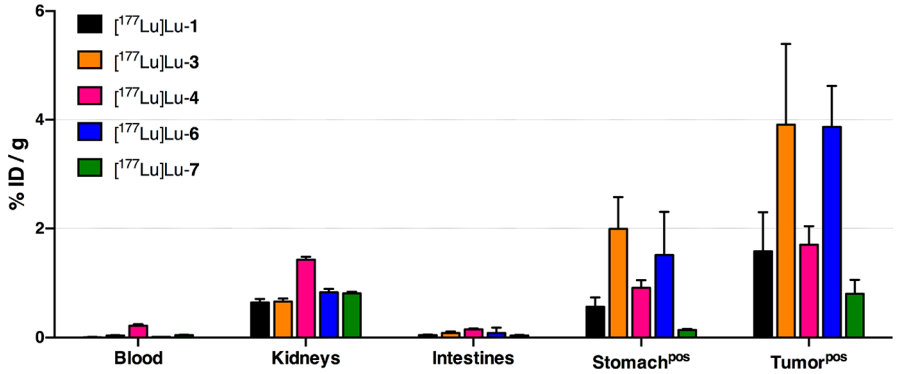

Figure 6. Comparison of uptakes of compounds [^{177}Lu]Lu-**1**, -**3**, -**4**, -**6**, and -**7** in selected organs of mice xenografted with A431-CCK2R positive cells at 4 h p.i. (n = 4). Data of compounds [^{177}Lu]Lu-**1**, -**3**, and -**4** are reproduced for comparison. Superscripted pos indicates receptor-positive tissue. Data points show the mean ± SD, n = 4, for values of all collected tissues and tumor-to-tissue ratios; see the Supporting Information.

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In comparison to the reference compound, some of the novel 1,5-Tz peptidomimetics showed improved biological properties such as enhanced plasma stability and affinity toward the CCKR2 *in vitro* or increased tumor uptake *in vivo*, thus providing another example for the successful application of 1,5-Tz as bioisosteres of amide bonds in bioactive peptides. Furthermore, the direct comparison of 1,4-Tz versus 1,5-Tz containing peptidomimetics revealed that a general prediction about the effect of the substitution pattern of the heterocycle on the biological properties of a peptide requires further investigations.

We encourage medicinal and peptide chemists who study 1,4-Tz-based peptidomimetics to consider also the use of 1,5-Tz as both heterocycles show high potential to enhance the pharmacological properties of bioactive peptides and thus expand the peptidomimetic toolbox for drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00636.

Synthesis and full characterization of peptides **5**−**7** and [^{177}Lu]**5**−**7** (UV- and γ-HPLC, mass spectrometric data) and detailed results of *in vitro* (internalization, competition binding, metabolic stability) and *in vivo* experiments (PDF)

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Author Contributions

I.E.V., T.L.M., and N.M.G. designed the compounds and planed the *in vitro* and *in vivo* experiments together with M.B., and I.E.V. performed the chemical synthesis of the compounds. N.M.G conducted radiolabeling, *in vitro* assays, and evaluated *in vitro* and *in vivo* data. M.B. and N.M.G. assisted with the *in vivo* experiments. R.S. contributed to interpretations of the results. I.E.V., N.M.G., and T.L.M. wrote and revised the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): R.S. and M.B. are inventors of patent WO201567473. T.L.M., M.B., R.S., I.E.V., and N.M.G. have submitted patent application WO 2019/057445 A1 as inventors.

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■ ABBREVIATIONS

ψ[Tz], a 1,2,3-triazole substituting an amide bond; BSA, bovine serum albumin; CCK2R, cholecystokinin-2 receptor; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; RP-HPLC, reverse-phase high-performance liquid chromatography; HR-MS, high-resolution mass spectrometry; ID, injected dose; MG, minigastrin; Nle, norleucine; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid

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