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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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KEYWORDS

1,2,3-Triazoles, Peptidomimetics, Structure-Activity Relationships, Radiopharmaceuticals, Tumor Targeting, Cancer

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 [Nle¹⁵]MG11 (DOTA-DGlu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH₂) as a model compound since it is known that the cholecystinin-2 receptor (CCK2R) is able to accommodate turn conformations. Analogs of [Nle¹⁵]MG11 incorporating 1,5-Tz in the backbone were synthesized, radiolabeled with lutetium-177, and their pharmacological properties (cell internalization, receptor binding affinity and specificity, plasma stability, and biodistribution)

were evaluated and compared with [Nle¹⁵]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 [Nle¹⁵]MG11 with nanomolar CCK2R-binding affinity and twofold 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.

LETTER

Five-membered N-heterocycles have attracted attention as amide bond mimics for the last twenty 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 the only 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

1,2,3-triazoles have been seldomly used as peptidomimetics until 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 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*-methyl amide 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 [Nle¹⁵]MG11 (compound **1**, Figure 3) labeled with lutetium-177 (¹⁷⁷Lu).^{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 D¹⁰Glu¹⁰ to Trp¹⁴ of compound **1** while, 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 Based on 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 obtained following published procedures (see Supporting Information (SI)).^{24, 27} 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 SI). Cp*RuCl(PPh $_3$) $_2$ and reaction temperatures of 60 °C^{40, 41} afforded the cycloaddition products in solution within 2-8 h (see SI). 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 separation of the diastereomers 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 SI).

Compounds **5** to **7** were radiolabeled with [¹⁷⁷Lu]LuCl₃ to obtain radioconjugates [¹⁷⁷Lu]Lu-**5-7** (Table 1) in high radiochemical yields and purities of ≥95%, with specific molar activities between 25-50 MBq/nmol (not optimized).^{29, 30} Representative chromatograms of quality controls by γ-HPLC can be found in the SI.

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 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 SI). With the exception of compound [¹⁷⁷Lu]Lu-**5**, the other 1,5-Tz analogs of compound **1** were able to bind and internalize into receptor-positive A431-CCK2R cells. Conjugates [¹⁷⁷Lu]Lu-**6** and [¹⁷⁷Lu]Lu-**7** reached similar or higher cell internalizations than reference compound **1** (51 to 30% versus 32%, respectively), and the internalization rates and kinetics resembled closely the ones of their 1,4-substituted counterparts [¹⁷⁷Lu]Lu-**3** and -**4**, respectively (54 and 30% respectively, Table 1, Figure 4).

The affinity (IC₅₀, Table 1) of the novel triazolopeptides toward the CCK2R was determined by a competition-binding assay. Briefly, the established CCK2R-ligand [¹⁷⁷Lu]Lu-PP-F11N (DOTA-(DGLu)₆-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH₂)⁴³ was added to A431-CCK2R cells and displaced by increasing concentrations of non-radioactive ¹⁷⁵Lu-labeled peptidomimetics **5-7** for 1 h. The cells were washed and lysed to determine the bound fraction of [¹⁷⁷Lu]Lu-PP-F11N as a function of the concentration of added competitors (see Table 1 and the SI). As anticipated from the internalization assays, compound [¹⁷⁵Lu]Lu-**5** with the lowest cell internalization had the lowest affinity for its target (IC₅₀ ~450 nM). Compounds [¹⁷⁵Lu]Lu-**6** and [¹⁷⁵Lu]Lu-**7** showed IC₅₀ values of 11 nM and 65 nM, respectively, with only conjugate [¹⁷⁵Lu]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 Gly¹³-Trp¹⁴ 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 Gly¹³. This observation confirms our previous hypothesis that Gly¹³ separates the C-terminal part of [Nle¹⁵]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 Arg³⁵⁶ 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 Tyr¹² and Gly¹³ 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 Ala¹¹-Tyr¹² does not play a significant role in receptor binding due to its tolerance towards 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 (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 [¹⁷⁷Lu]Lu-5 and [¹⁷⁷Lu]Lu-7 showed an increased stability in comparison to reference compound [¹⁷⁷Lu]Lu-1, whereas the $t_{1/2}$ was decreased for compound [¹⁷⁷Lu]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 towards its receptor, it can also increase its susceptibility towards degradation by proteases. The degradation kinetics of 1,4- versus 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 [¹⁷⁷Lu]Lu-5 was significantly slower than its 1,4-Tz counterpart [¹⁷⁷Lu]Lu-2 ($t_{1/2}$ = 3.8 h vs. 91.1 h). The behavior was reversed in the case of conjugate [¹⁷⁷Lu]Lu-7, which was metabolized faster than 1,4-Tz compound [¹⁷⁷Lu]Lu-4 ($t_{1/2}$ = 51.4 h vs. 6.7 h). Finally, degradation kinetics of compounds [¹⁷⁷Lu]Lu-3 and [¹⁷⁷Lu]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 [¹⁷⁷Lu]Lu-6 and 7 retained the biodistribution profile of reference compound [¹⁷⁷Lu]Lu-1 (see SI 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 4 mice. The results of the experiment are summarized in Figure 6 (see SI for complete data sets). At 4 h post-injection, 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 SI). Compound [¹⁷⁷Lu]Lu-6 showed the highest tumor uptake of the investigated 1,5-Tz peptidomimetics (3.9 % ID/g). Similarly, a more than two-fold increased uptake in the tumor was also previously observed for the analogous 1,4-Tz isomer [¹⁷⁷Lu]Lu-3. This is consistent with their improved CCK2R affinity and cell internalization in comparison with reference compound [¹⁷⁷Lu]Lu-1 *in vitro* (Table 1). Tumor uptake of compound [¹⁷⁷Lu]Lu-7 was inferior to its 1,4-Tz counterpart [¹⁷⁷Lu]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 compounds **1** or **4** as well as to the superior proteolytic resistance of compound [¹⁷⁷Lu]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 MG11 in the vicinity of a proposed β-turn. The obtained peptidomimetics were compared side-by-side with the all-amide bond reference compound [Nle¹⁵]MG11 as well as with previously studied 1,4-Tz analogs, which adopt *trans*-conformations for the probed amide bonds.

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 towards 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.

NOTES

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.

ASSOCIATED CONTENT

Supporting Information. Synthesis and full characterization of peptides **5-7** and [¹⁷⁷Lu]**5-7** (UV- and γ -HPLC, mass spectrometric data) and detailed results of *in vitro* (internalization, competition binding, metabolic stability) and *in vivo* experiments can be found in the Supporting Information.

AUTHOR INFORMATION

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CONTRIBUTIONS

I.E.V., T.L.M., and N.M.G. designed the compounds and planned the *in vitro* and *in vivo* experiments together with M.B.; 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 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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ABBREVIATIONS

ψ [Tz], stands for a 1,2,3-triazole substituting an amide bond; BSA, bovine serum albumin; CCK2R, cholecystinin-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, minigastirin; Nle, norleucine; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

ACKNOWLEDGEMENTS

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FIGURES

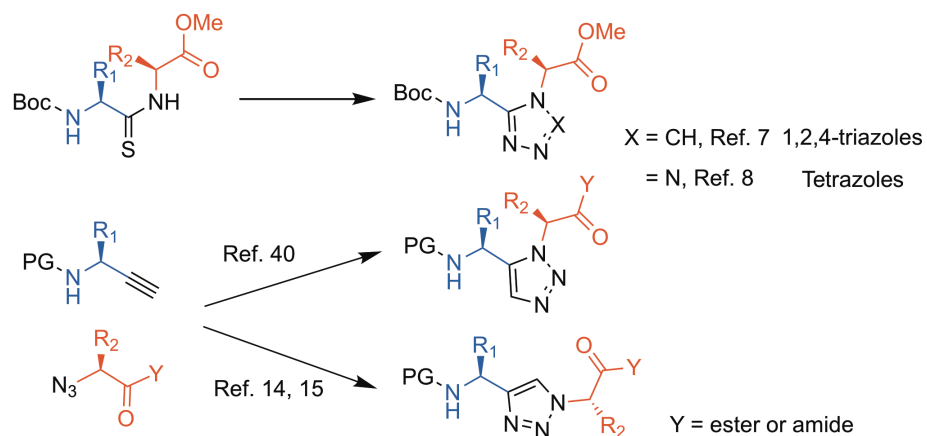


Figure 1. Mimetics of amide bonds based on *N*-heterocycles.

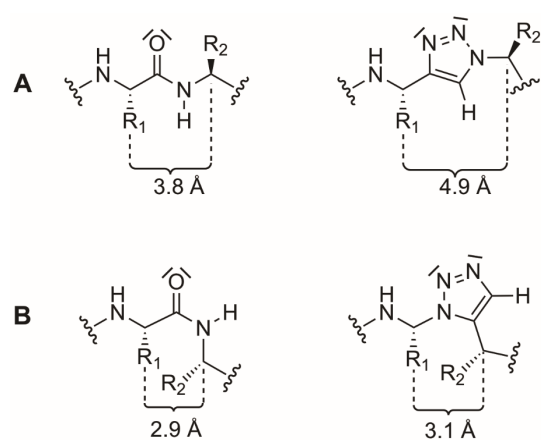
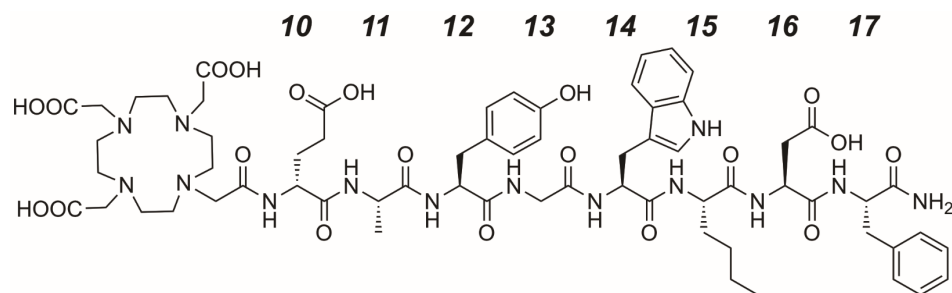
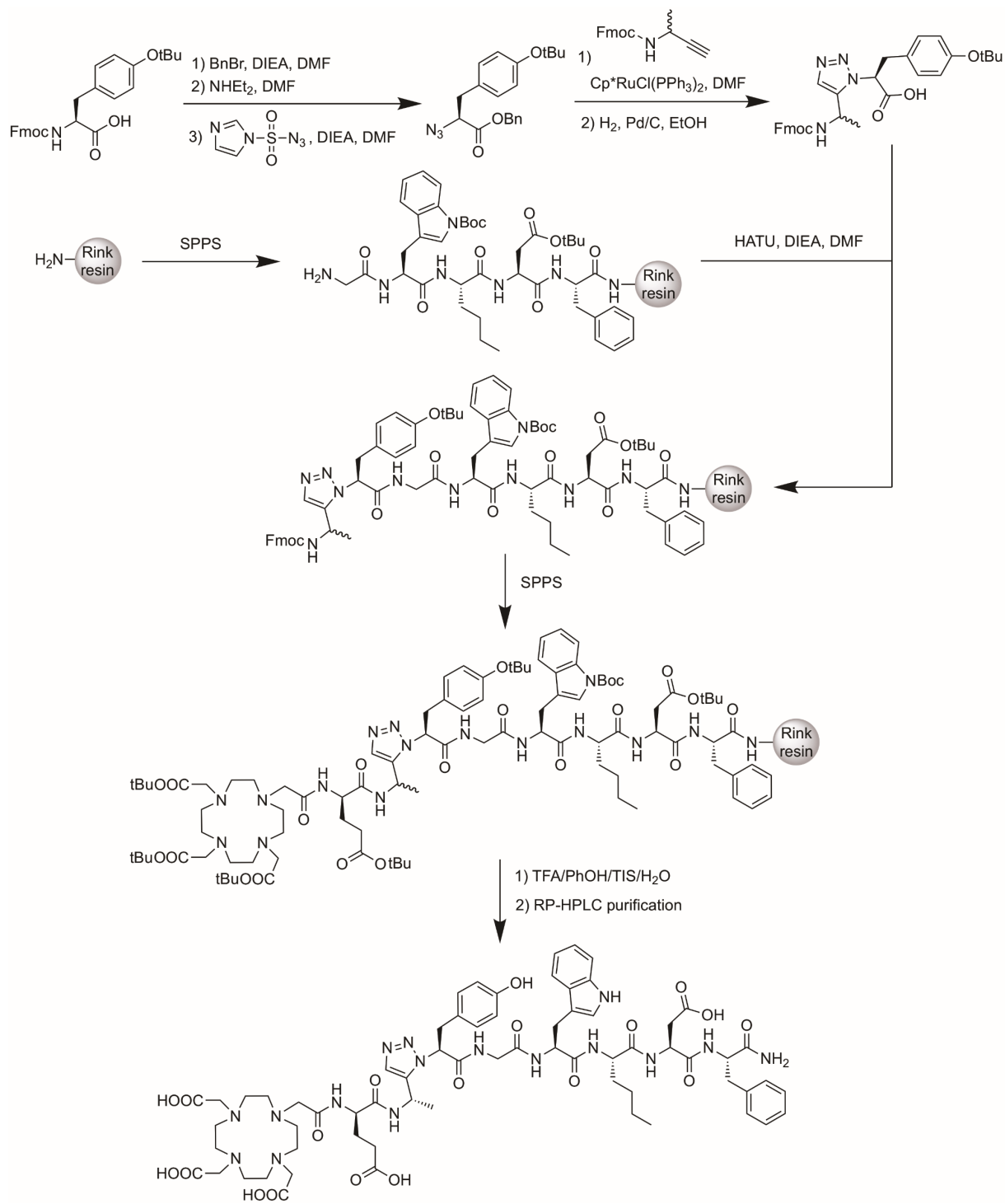


Figure 2. Comparison between *trans*-amide and *cis*-amide bonds and 1,4- (A) and 1,5-disubstituted 1,2,3-triazoles (B).



Compound 1 : [Nle¹⁵]MG11

Figure 3. Structure of [Nle¹⁵]MG11 with numbering of the amino acid residues.



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

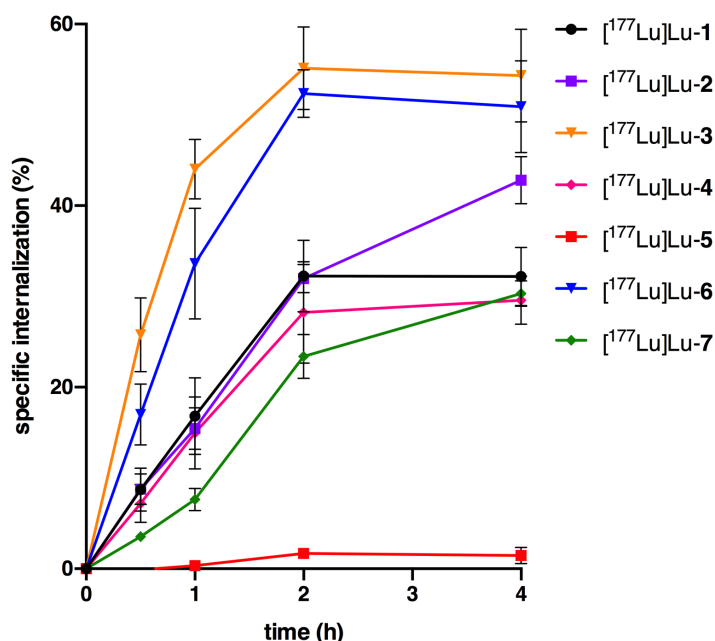


Figure 4. Specific internalization kinetics of conjugates [¹⁷⁷Lu]Lu-1 to 7 in A431-CCK2R cells at 37 °C. Data of previously reported compounds [¹⁷⁷Lu]Lu-1-4²⁹ are included for comparison (n = 3 in triplicates).

Table 1. Summary of in vitro results of compounds [¹⁷⁷Lu]Lu-1-7.

Compound	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-Gly Ψ [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-Tyr Ψ [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-Ala Ψ [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-Gly Ψ [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-Tyr Ψ [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-Ala Ψ [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 [¹⁷⁷Lu]Lu-1 and 1,4-Tz analogs [¹⁷⁷Lu]Lu-2-4 are reproduced for comparison.²⁹ ^b Competition experiments were performed with non-radioactive analogs of the compounds 1 to 7 labeled with ¹⁷⁵Lu.

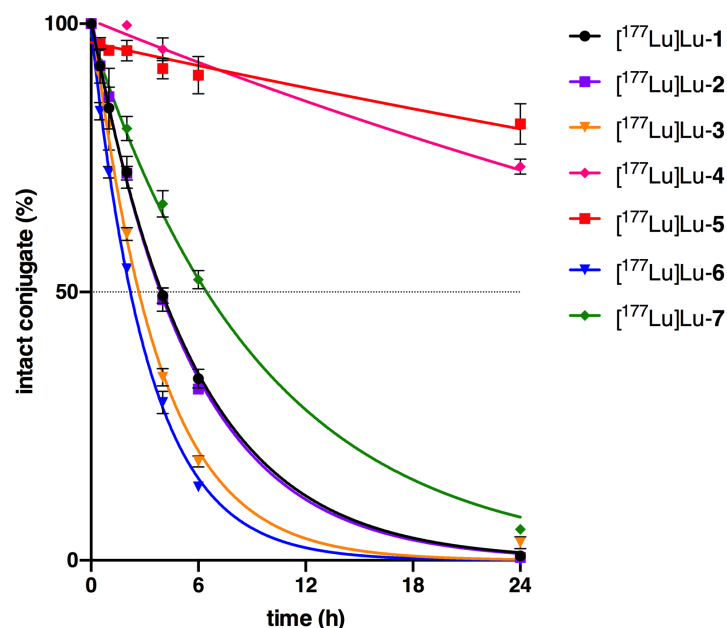


Figure 5. Degradation kinetics of ^{177}Lu -labeled peptide conjugates after incubation in human blood plasma for 24 h at 37 °C. Data points show mean \pm SD. $n = 2-3$, for values of $t_{1/2}$, see Table 1.

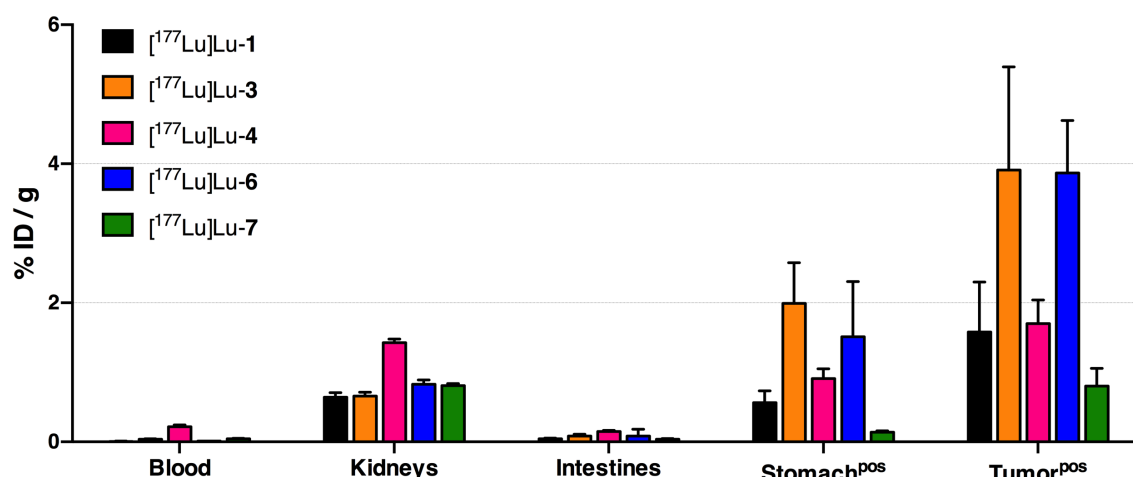


Figure 6. Comparison of uptakes of compounds ^{177}Lu Lu-1, -3, -4, -6, and -7 in selected organs of mice xenografted with A431-CCK2R positive cells at 4 h p.i. ($n = 4$). Data of compounds ^{177}Lu Lu-1, -3, and -4 are reproduced for comparison. Superscripted pos indicates receptor-positive tissue.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

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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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1,5-disubstituted 1,2,3-triazole-based analogs of CCK2 receptor radioligand

