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ARTICLE TYPE

Cu(I)-assisted Click Chemistry Strategy for Conjugation of Non-protected Cross-bridged Macrocyclic Chelators to Tumour-targeting Peptides

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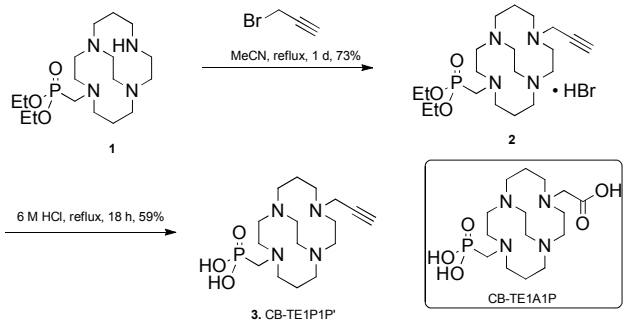
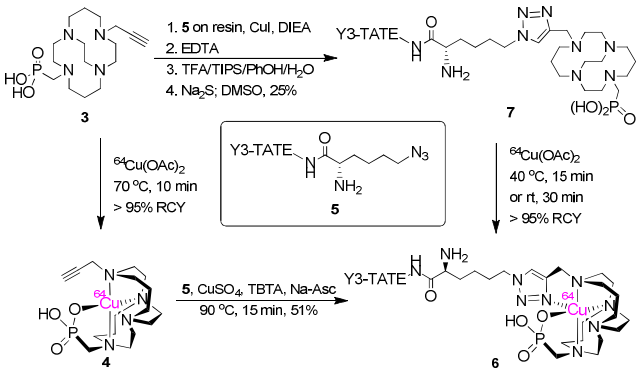
Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry has inherent challenges for copper-labeled radiopharmaceuticals. An azide-modified phosphonate-based cross-bridged macrocyclic chelator was synthesized for click chemistry conjugation with azide-modified Y3-TATE (a somatostatin analogue) on resin, without the need for protecting the chelator. The ^{64}Cu-labeled bioconjugate shows favourable in vitro and in vivo behaviour.

Copper-64 ($t_{1/2}$ = 12.7 h; β^+: 18%; β^-: 38%; electron capture: 44%) can be produced with a medical cyclotron in high-yield and high-specific activities.[1-3] It is well-suited for labeling a variety of molecules for positron emission tomography (PET) imaging and radiotherapy.[4] Because of the abundance of copper chelating proteins that can potentially displace the copper ion out of the chelator, the development of copper chelators that form stable intact copper chelates in vivo is both important and challenging in developing copper-based radiopharmaceuticals.[5, 6]

1,4,8,11-Tetraazabicyclo[6.6.2]hexadecane-4,11-diacetic acid (CB-TE2A)[7] has been explored extensively in copper-based nuclear medicine,[8] as it forms kinetically stable chelates with Cu(II).[9] However, somewhat harsh labeling conditions limit the application of this chelator to heat tolerant compounds. Changing one pendant arm from carboxylate to phosphonate resulted in CB-TE1A1P (Scheme 1), which can be labeled at room temperature in high-specific activity.[10] Because the direct amide coupling of CB-TE1A1P through its carboxylate to biomolecules is inefficient (< 7% overall yield to the N-terminus of Y3-TATE),[11] better conjugation strategies are needed. In addition, the in vivo stability and ease of labeling may be compromised after it is conjugated with biomolecules through its carboxylate pendant arm.[11] To circumvent this problem, the click-to-chelate strategy pioneered by Schibli,[12-14] and Todd and Watkinson[15, 16] may be advantageous, in part because the resulting triazole has been shown to coordinate with Cu(II).[15, 17] We have now synthesized a clickable phosphonate-based cross-bridged macrocyclic chelator, and explored different labeling and conjugation strategies, using a somatostatin receptor subtype 2 (sstr2) ligand, Y3-TATE, as a model peptide. Prior clickable



Scheme 1. Synthesis of azide-alkyne clickable phosphonate-based cross-bridged chelator, CB-TE1P1P' (**3**) and structure of CB-TE1A1P.



Scheme 2. Synthesis of ^{64}Cu-CB-TE1P1T-Y3-TATE (**6**).

chelators[14, 17] were all protected as t-butyl-esters. This phosphonate-based clickable chelator does not need to be protected. Furthermore, the clicked bioconjugate was labeled at rt within 30 min, which is among the mildest labeling conditions that can be achieved with cross-bridged chelators that form kinetically stable copper chelates. Imaging and biodistribution data of the CuAAC clicked bioconjugate are presented using a mouse xenograft model.

The azide-alkyne clickable phosphonate-based cross-bridged chelator **3** was synthesized efficiently in 43% overall yield from mono-phosphonate **1**[18] via N-alkylation with propargyl bromide,

Table 1. Biodistribution of ^{64}Cu-CB-TE1P1T-Y3-TATE (**6**) in blood, liver, kidney, muscle, bone, and sstr2-expressing organs (adrenal, pancreas, and tumour) at 1 h, 4 h, 4 h blocked with co-administration of Y3-TATE, and 24 h p.i. (n = 4).

	1h	4h	4h block	24 h
Blood	0.34 ± 0.13	0.20 ± 0.03	0.22 ± 0.04	0.15 ± 0.06
Liver	1.13 ± 0.16	1.30 ± 0.10	1.32 ± 0.30	1.05 ± 0.10
Kidney	15.87 ± 3.03	21.03 ± 2.84	16.13 ± 5.03	14.68 ± 2.53
Muscle	0.22 ± 0.15	0.07 ± 0.02	0.09 ± 0.03	0.06 ± 0.00
Bone	0.46 ± 0.16	0.50 ± 0.24	0.20 ± 0.04	0.35 ± 0.10
Adrenal	1.14 ± 0.46	1.24 ± 0.40	0.37 ± 0.05	1.82 ± 1.83
Pancreas	6.71 ± 0.22	4.41 ± 0.95	0.30 ± 0.07	1.84 ± 0.64
Tumour	4.73 ± 1.17	7.54 ± 1.43	0.69 ± 0.22	4.39 ± 1.71

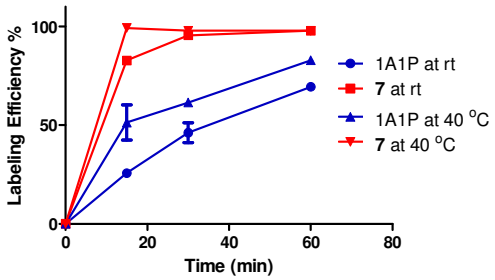


Figure 1. Labeling kinetics of the clicked CB-TE1P1T-Y3-TATE (**7**) and CB-TE1A1P-Y3-TATE (1A1P).

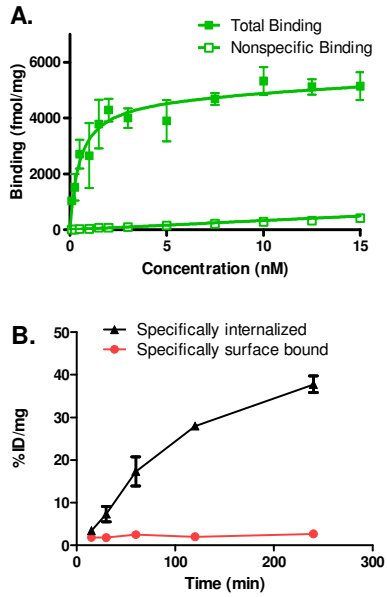


Figure 2. Saturation binding assay (A) and internalization assay (B) ^{64}Cu-CB-TE1P1T -Y3-TATE (**6**) with sstr2-transfected HCT116 cells.

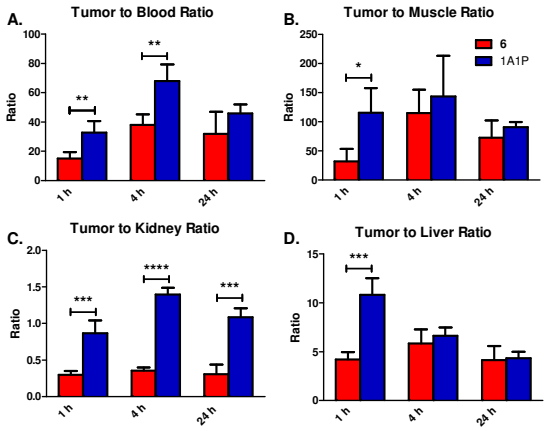


Figure 3. Tumour-to-blood ratio (A), tumour-to-muscle ratio (B), tumour-to-kidney ratio (C), and tumour-to-liver ratio (D) of ^{64}Cu-CB-TE1P1T-Y3-TATE (**6**) and ^{64}Cu-CB-TE1A1P-Y3-TATE (1A1P) at 1 h, 4 h, and 24 h p.i. * $P < 0.05$, n = 4.

followed by acid hydrolysis of the intermediate **2** (Scheme 1; further details are provided in the ESI). Two conjugation approaches were explored (Scheme 2). In the first approach, chelator **3** was labeled with ^{64}Cu(OAc)$_2$ at 70 °C in high-specific activity (1 mCi/mg) after 10 min. The ^{64}Cu-labeled chelate **4** was then added to a solution containing the azide-modified Y3-TATE **5**, cupric sulfate, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), and sodium ascorbate (Na-Asc). The click chemistry reaction between **4** and **5** was performed under argon at 90 °C for 15 min, resulting in 51% yield of bioconjugate **6** (Scheme 2). The precursors and ^{64}Cu-CB-TE1P1T-Y3-TATE (**6**) were easily separated by reversed-phase HPLC. This conjugation after radiolabeling approach may have broad applications in labeling a variety of biomolecules and nano-materials that do not tolerate prolonged heating.

For the second approach to prepare ^{64}Cu-labeled clicked bioconjugate **6**, labeling precursor **7** was synthesized via solid phase peptide synthesis (Scheme 2). Chelator **3** was clicked with **5** on resin using cuprous iodide as the catalyst. The excess catalyst was removed by repeated washing with EDTA. Compound **7** was cleaved from the solid support with a cocktail of trifluoroacetic acid, phenol, triisopropylsilane, and water. Sodium sulfide was added to precipitate any remaining copper salt. DMSO was used to quantitatively oxidize the cleaved thiol back to the cyclic peptidomimetic CB-TE1P1T-Y3-TATE (**7**). After HPLC purification, **7** was obtained in 25% overall yield and was labeled with ^{64}Cu at rt for 30 min or at 40 °C for 15 min in > 95% radiochemical yield (RCY), as confirmed by radio-HPLC. Consistent with results by Lebedev et al. with their clicked CB-TE2A analogue,[17] **7** was labeled more efficiently at rt and 40 °C than the corresponding CB-TE1A1P-Y3-TATE (1A1P) (Figure 1) (> 98% labeled at rt within 30 min and at 40 °C in under 15 min). Under the same conditions, the labeling efficiency of 1A1P was only 69 and 83% after 60 min at rt and 40 °C, respectively. These data further support that the triazole group facilitated labeling of **7** at lower temperature than the corresponding 1A1P, which bears an amide group.[11] The labelled peptidomimetic **6**

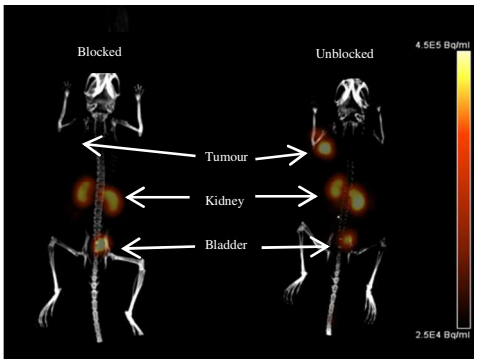


Figure 4. Representative 2 h p.i. PET/CT image of **6** with mice xenograft bearing subcutaneously implanted sstr2-transfected HCT116 cells. The blocked mouse was co-administered with Y3-TATE (10 mg/kg).

was used for biological studies without further purification (Scheme 2).

The in vitro saturation binding assays and internalization assays were performed following published protocols.[19] The binding affinity (K_d) of **6** was very high (0.52 ± 0.10 nM), with a maximum number of binding sites (B_{max}) of 4800 ± 190 fmol/mg (Figure 2A). Cellular internalization studies showed that **6** was quickly bound to the cell surface, and was steadily internalized, with 38%ID/mg protein internalized after 4 h. More than 95% ($P < 0.0001$, n = 3) of the surface bound, and thus internalized **6**, was blocked when the cells were pre-incubated with excess Y3-TATE, indicating that these processes were sstr2 receptor-mediated (Figure 2B). These data are consistent with our modelling study of sstr2, which indicated that there is a large pocket near the N-terminus of Y3-TATE when it is bound with sstr2.[19] The presence of this pocket allows modification of Y3-TATE at its N-terminus without decreasing the binding affinity and the maximum number of binding sites on the cell surface.

Next, we evaluated the in vivo performance of **6** in mice bearing subcutaneous sstr2-transfected HCT116 tumours. From the biodistribution data (Table 1), sstr2-expressing organs, such as adrenal, pancreas, and tumour showed high tracer uptake at 1, 4, and 24 h. The tumour uptake reached a maximum at 4 h post-injection (p.i.) (7.54 ± 1.43, n = 4) and cleared slowly to 4.39 ± 1.71 (n = 4) at 24 h p.i. Co-administration of the sstr2 agonist Y3-TATE blocked the uptake of **6** at 4 h p.i. in the sstr2-positive organs, with 70% ($P = 0.0051$, n = 4), 77% ($P = 0.0001$, n = 4), and 91% ($P < 0.0001$, n = 4) blocking for adrenal, pancreas, and tumour, respectively. Unlike the strain-promoted clicked Y3-TATE bioconjugates,[19] **6** was efficiently cleared from blood within 1 h. The contrast between tumour and other organs (except for kidney) was high (Figure 3), with a peak at 4 h p.i. (tumour-to-blood of 38 ± 7, n = 4; tumour-to-muscle of 115 ± 40, n = 4). The initial high uptake of **6** in the kidneys reflected its dominant route of excretion.[20] The consistently high uptake of **6** in kidneys may be due to the non-specific static interactions between the positively charged tracer **6** with the negatively charged podocytes and basement membranes,[21] which could be addressable by pre-injected amino acids.[22] The lower tumour uptake of **6** relative to the directly conjugated CB-TE1A1P-Y3-TATE (1A1P) might be from the 'sink effect' of the high uptake in kidneys, resulting in reduced availability of **6** to other organs. Thus, the tumour-to-blood ratios of **6** were significantly lower than those of 1A1P except at 24 h p.i. (Figure 3A). Even with lower uptake in tumour, the tumour-to-muscle ratio of **6** was not significantly lower than that of 1A1P (Figure 3B). As the liver uptake can reflect the in vivo stability of the ^{64}Cu-chelate,[23] the relatively low liver activity (less than 1.4%ID/g) throughout of the entire study indicated reasonable in vivo stability of the copper complex **6**. This liver activity of **6** was even lower than what we observed with the directly conjugated 1A1P at 4 h p.i. ($P = 0.0118$), though the tumour-to-liver ratios of **6** were not higher than 1A1P (Figure 3D).[19] PET/CT imaging at 2 h p.i. confirmed the excellent tumour uptake and contrast, with the exception of kidneys (Figure 4). The tumour standard uptake values (SUVs) (2.25 ± 0.49, n = 2) were significantly decreased after co-injection of Y3-TATE (0.20 ± 0.06, n = 2), confirming the receptor specific uptake of **6**.

Conclusions

In summary, an azide-alkyne clickable phosphonate-based cross-bridged macrocyclic chelator **3** was synthesized efficiently and radiolabeled with copper-64 under mild conditions in high-specific activity. Chelator **3** was conjugated efficiently to an azide-modified Y3-TATE through copper-catalyzed click chemistry before or after radiolabeling. The bioconjugate **6** was labeled at rt within 30 min, retained high affinity to sstr2, and was internalized rapidly and specifically by sstr2-transfected HCT116 cells. In vivo PET/CT imaging and biodistribution studies showed excellent tumour imaging, with relatively high contrast to non-targeted tissues. We anticipate broad applications of this clickable chelator **3** in labeling other biologically important molecules.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details, analytical and spectroscopic data of new compounds. See DOI: 10.1039/c000000x/.

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Table of content:

A novel phosphonate-based cross-bridged macrocyclic chelator was efficiently synthesized, clicked with an octreotate analogue, and evaluated as a ^{64}Cu-labelled PET radiopharmaceutical.