Synthesis of DOTA-pyridine chelates for $^{64}$Cu coordination and radiolabeling of αMSH peptide

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Abstract

**Background:** $^{64}$Cu is one of the few radioisotopes that can be used for both imaging and therapy, enabling theranostics with identical chemical composition. Development of stable chelators is essential to harness the potential of this isotope, challenged by the presence of endogenous copper chelators. Pyridyl type chelators show good coordination ability with copper, prompting the present study of a series of chelates DOTA-xPy (x=1-4) that sequentially substitute carboxyl moieties with pyridyl moieties on a DOTA backbone.

**Results:** We found that the presence of pyridyl groups significantly increases $^{64}$Cu labeling yield, with DOTA-2Py, -3Py and -4Py quantitatively complexing $^{64}$Cu at room temperature within 5 min ($10^{-4}$ M). [$^{64}$Cu]Cu-DOTA-xPy (x=2-4) exhibited good stability in human serum up to 24 hours. When challenged with 1000 eq. of NOTA, no transmetallation was observed for all three $^{64}$Cu complexes. DOTA-xPy (x=1-3) were conjugated to a cyclized $\alpha$-melanocyte-stimulating hormone ($\alpha$MSH) peptide by using one of the pendant carboxyl groups as a bifunctional handle. [$^{64}$Cu]Cu-DOTA-xPy-$\alpha$MSH retained good serum stability (>96% in 24 hours) and showed high binding affinity (Ki=2.1-3.7 nM) towards the melanocortin 1 receptor.

**Conclusion:** DOTA-xPy (x=1-3) are promising chelators for $^{64}$Cu. Further in vivo evaluation is necessary to assess the full potential of these chelators as a tool to enable further theranostic radiopharmaceutical development.

**Keywords:**
Copper-64, Chelating ligands, Radiolabeling, $\alpha$-Melanocyte-stimulating hormone, Pyridyl, DOTA
Background

Personalized medicine is predicated on a philosophy of tailoring disease diagnosis, monitoring and therapy for individual patients in order to ensure maximal medical benefit during disease management (Vogenberg et al. 2010). Theranostics is an approach in which a single compound can be used to both diagnose and treat disease, and is an important tool in the move toward personalized medicine (Yordanova et al. 2017). Quantitative target visualization of a therapeutic radiopharmaceutical can help predict if a patient will benefit from a therapy and avoid unnecessary or ineffective treatments. In many instances, modern radiotheranostics include isotopes used for therapy, such as yttrium ($^{90}$Y), lutetium-177 ($^{177}$Lu) and actinium-225 ($^{225}$Ac), and pairs them with compounds containing a different, imageable isotope for imaging, such as gallium-68 ($^{68}$Ga), fluorine-18 ($^{18}$F) and indium-111 ($^{111}$In) (Langbein et al. 2019; Yordanova et al. 2017). One key assumption is that the imaging tracer has identical biodistribution as the therapeutic radiopharmaceutical. This assumption is not always true considering differences in molar activity (activity per mole of compound), in vivo stability and inherent biological sensitivity to even the slightest perturbations in pharmaceutical structure (Di 2015). The above matched-pair philosophy is driven primarily by the fact that not many isotopes are capable of both imaging and therapy. Copper-64 ($^{64}$Cu, $t_{1/2} = 12.7$ h) decays via electron capture (branching ratio [BR] = 44%), $\beta^-$ emission (39%, 0.573 MeV), and $\beta^+$ emission (17%, $E_{\beta_{\text{avg}}} = 0.655$ MeV). This unique decay profile and half-life makes the radionuclide a versatile isotope for imaging, therapy or theranostics using small molecules, peptides, antibodies, and nanoparticle targeting and carrier platforms (Cai and Anderson 2014; Gutfilen et al. 2018). As one of the only copper isotopes that can be produced
via proton irradiation on lower energy medical cyclotrons, $^{64}$Cu is more readily available over other isotopes, making it more ideal for use in imaging (McCarthy et al. 1997).

The development of chelators for Cu is essential in order to harness its theranostic power. Cu is the third most abundant metal in the human body, and plays an important role in many biological functions, such as redox electron transfer, structure shaping, and catalysis (Gutfilen et al. 2018). As a result, there are many Cu chelating proteins in vivo and it is a challenge to develop thermodynamically and kinetically stable Cu chelators. DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (De León-Rodríguez and Kovacs 2008), TETA (1,4,8,11-tetraazacyclotradecane-1,4,8,11-tetraacetic acid) (Wadas and Anderson 2007) and their derivatives are commonly used, but they are unstable in vivo (Figure 1) (Smith 2004). Over time, NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) (Cooper et al. 2012), the cross-bridged cyclam (CB-TE2A) (Sprague et al. 2007), and sarcophagine-based chelators (Di Bartolo et al. 2006) have been developed and show improved stability (Anderson and Ferdani 2009; Cai and Anderson 2014).

The development of bifunctional chelators capable of complexing radiometals at ambient temperature, in short reaction times, and sub-micromolar concentrations is of increasing interest. Chelators containing pyridyl moieties, including pyridine, bipyridine and terpyridine, have been previously used to complex Cu (Czerwińska et al. 2017; Lee et al. 2011; Leussing and Hansen 1957), and as Cu(II) is a d9 metal of borderline hardness, it favors ligation with softer bases including amines and imines. We hypothesized for this study that the addition of pyridine moieties in place of the carboxylic acid prostheses on DOTA (referred to herein as DOTA-xPy, x=1-4; compounds 4-8 in Scheme 1) would maintain a higher affinity for the Cu(II) ion.
Furthermore, we present the application of novel DOTA-pyridine derivatives and the labeling of a peptide targeting the Melanocortin 1 receptor (MC1R) (referred to herein as DOTA-xPy-αMSH (x=1-3)). MC1R is overexpressed in primary and metastatic melanoma (López et al. 2007; Tafreshi et al. 2019). With low normal tissue expression, this receptor is an attractive target for molecular imaging and treatment of late-stage melanoma, which has a low long-term survival rate and no curative treatment options available (Andtbacka et al. 2015; Hertzman Johansson and Egyhazi Brage 2014; Hinrichs and Rosenberg 2014; Postow et al. 2015). We have extensively studied analogues of α-melanocyte-stimulating hormone (αMSH) for MC1R-targeted imaging and therapy. We have developed ⁶⁸Ga or ¹⁸F labeled cyclic αMSH (CCZ01048, CCZ01064, CCZ01096) for PET imaging in preclinical models of mouse or human melanoma (Zhang et al. 2020; Zhang et al. 2019; Zhang et al. 2018; Zhang et al. 2017). In all cases, tracers achieved good tumor visualization in PET images with excellent tumor-to-normal tissue ratios. Moreover, we recently evaluated ²²⁵Ac labeled αMSH derivatives, which also exhibited excellent tumor-to-normal tissue ratios (Ramogida et al. 2019; Yang et al. 2020). Knowing the potential of tumor accumulation and internalization, and low uptake in normal tissues, we were interested in labeling an αMSH peptide with ⁶⁴Cu to assess the unique theranostic power of ⁶⁴Cu. [⁶⁴Cu]Cu-αMSH peptide will allow for real-time monitoring of physiological processes during therapy studies, and when coupled with the longer half-life of ⁶⁴Cu over other PET isotopes (i.e. ⁶⁸Ga and ¹⁸F), it will enable later time points for imaging and biodistribution studies, and a more accurate assessment of the longer-term biological fate of both peptide and radionuclide.

Results and discussions
The chelator synthesis started with the cyclen-xPy (x=1-4, compounds 1-4, Scheme 1). All derivatives were synthesized using a single reaction followed by purification by HPLC. Each compound was then reacted with tert-butyl bromoacetate and deprotected to yield the final product DOTA-xPy (x=1-3, compounds 5-7)(Subat and König 2001; Veiga et al. 2013). Compounds cyclen-1Py (Aime et al. 1999; Aoki et al. 2004; Subat et al. 2007), cyclen-2Py (El Hajj et al. 2009), cyclen-4Py (Natrajan et al. 2010) and DOTA-1Py (Aime et al. 1999) are previously reported, while compounds cyclen-1Py, cyclen-3Py, DOTA-2Py and DOTA-3Py are new compounds to the best of our knowledge. DOTA-1Py and DOTA-3Py were obtained as a single compound, while DOTA-2Py maintained both cis- and trans- stereoisomer configurations. Although $^1$H NMR and $^{13}$C NMR did not clearly indicate the presence of a particular isomer, the labeled peptide $[^{64}$Cu]$^4$Cu-DOTA-2Py-$\alpha$MSH showed a double peak on HPLC gamma trace under isocratic conditions, leading us to presume the presence of both isomers.

Figure 1. Examples of commonly used $^{64}$Cu chelators.
Scheme 1. Synthesis of cyclen-xPy (x=1-4, compounds 1-4) and DOTA pyridine derivatives DOTA-xPy (x=1-3, compounds 5-7).

$^{64}$Cu was produced by proton irradiation of enriched $^{64}$Ni solid metal targets using TRIUMF’s TR13 cyclotron. After acid dissolution and purification by cation exchange resin (AG1-X8), $^{64}$Cu was obtained in dilute HCl. $^{64}$Cu labeling of DOTA, DOTA-1Py, DOTA-2Py, DOTA-3Py, and DOTA-4Py were examined at various ligand concentrations. The results (Table 1, Fig. 2 and Fig. 3) indicated that the subsequent addition of pyridine moiety served to enhance $^{64}$Cu labeling yields in general. While DOTA showed little complexation with $^{64}$Cu at ambient temperature even at ligand concentrations of $10^{-3}$ M, having one, two or three pyridyls significantly improved the labeling yields, with DOTA-3Py demonstrating efficient complexation of $^{64}$Cu (radiochemical yield, RCY close to 95%) at ligand concentrations of $10^{-5}$ M and at ambient temperature. The differences in $^{64}$Cu labeling performance between DOTA-2Py and DOTA-4Py were not significant.
Fig. 2. Radio-HPLC chromatogram showing the quantitative $^{64}\text{Cu}$ labeling of (a) DOTA-1Py (retention time, $t_R = 12.5$ min), (b) DOTA-2Py ($t_R = 12.7$ min), (c) DOTA-3Py ($t_R = 14.5$ min), and (d) DOTA-4Py ($t_R = 16.7$ min) at ligand concentration $10^{-3}$ M and ambient temperature.

Fig. 3. $^{64}\text{Cu}$ labeling yield at various ligand concentrations for DOTA, DOTA-1Py, DOTA-2Py, DOTA-3Py, and DOTA-4Py (n=3) at ambient temperature and 5 min reaction time.
We then studied the reaction kinetics for labeling DOTA-2Py, -3Py and -4Py at $10^{-4}$ M (DOTA-1Py not studied because the labeling was less efficient). Under the same conditions as described above, the reactions were monitored at 1, 5, 10, 20 and 30 min by radioTLC. All three chelators demonstrated near quantitative metal incorporation within 5 min at ambient temperature (Fig. 4).

Fig. 4. $^{64}$Cu labeling yield of DOTA-2Py, DOTA-3Py and DOTA-4Py at 1, 5, 10, 20 and 30 min performed with ligand concentration of $10^{-4}$ M and ambient temperature ($n = 3$).

The stability of [$^{64}$Cu]Cu-DOTA-2Py, -3Py and -4Py were examined in phosphate buffer and human serum at 1 hour and 24 hours at 37°C. All three ligands were largely stable (>94%) in both phosphate buffer and human serum in 24 hours (Table 1).
Table 1. Stability of $[^{64}\text{Cu}]\text{Cu-DOTA-}x\text{Py}$ (x=2-4) and $[^{64}\text{Cu}]\text{Cu-DOTA-}x\text{Py-MSH}$ (x=1-3) in phosphate buffer and human serum at 37°C.

| Radio-ligand                  | Phosphate buffer% | Human serum% |
|------------------------------|-------------------|--------------|
|                              | 1 h               | 24 h         | 1 h           | 24 h         |
| $[^{64}\text{Cu}]\text{Cu-DOTA-2Py}$ | 99.2 ± 0.1       | 99.1 ± 0.1    | 98.7 ± 0.3    | 94.6 ± 0.1  |
| $[^{64}\text{Cu}]\text{Cu-DOTA-3Py}$ | 99.4 ± 0.3       | 99.2 ± 0.2    | 99.0 ± 0.1    | 96.1 ± 0.2  |
| $[^{64}\text{Cu}]\text{Cu-DOTA-4Py}$ | 99.1 ± 0.1       | 99.0 ± 0.2    | 98.5 ± 0.1    | 97.2 ± 0.3  |
| $[^{64}\text{Cu}]\text{Cu-DOTA-1Py-\text{αMSH}}$ | 99.3 ± 0.1       | 98.2 ± 0.2    | 98.4 ± 0.2    | 96.5 ± 0.3  |
| $[^{64}\text{Cu}]\text{Cu-DOTA-2Py-\text{αMSH}}$ | 99.1 ± 0.04      | 98.6 ± 0.3    | 99.4 ± 0.2    | 98.3 ± 0.2  |
| $[^{64}\text{Cu}]\text{Cu-DOTA-3Py-\text{αMSH}}$ | 99.2 ± 0.2       | 99.3 ± 0.2    | 98.1 ± 0.1    | 97.7 ± 0.2  |

NOTA has been the widely used chelate for labeling with $^{64}\text{Cu}$ as it forms kinetically stable complexes (Cooper et al. 2012). For comparison, we examined the kinetic inertness of $[^{64}\text{Cu}]\text{Cu-DOTA-2Py}$, -3Py and -4Py using NOTA as the transchelation challenge reagent. After the formation of $[^{64}\text{Cu}]\text{Cu-DOTA-2Py}$, -3Py and -4Py at ligand concentration of $10^{-4}$ M, 1000 eq. of $p$-SCN-Bn-NOTA were added and the mixture was monitored by radio-HPLC over 4 hours. During this time, no transchelation was observed for all three $^{64}\text{Cu}$ complexes, indicating the kinetic inertness of $[^{64}\text{Cu}]\text{Cu-DOTA-2Py}$, -3Py and -4Py. As control experiments, $p$-SCN-Bn-NOTA was labeled with $^{64}\text{Cu}$, and 1000 eq. of DOTA-2Py, -3Py or -4Py were added and no transchelation was observed for up to 4 hours. Interestingly, when $^{64}\text{Cu}$ was added to an equimolar mixture ($10^{-4}$ M ligand) of DOTA-xPy (x = 2 – 4) and $p$-SCN-Bn-NOTA, $[^{64}\text{Cu}]\text{Cu-DOTA-2Py}$, -3Py and -4Py were formed with RCYs of 20%, 19% and 28%, while $[^{64}\text{Cu}]\text{Cu-}p$-SCN-Bn-NOTA formed at RCYs of 78%, 79% and 70%, respectively, indicating DOTA-xPy (2-4) chelators may have slower kinetics compared to NOTA.

With radiolabeling efficiency, stability and kinetic inertness of the Cu-complexes established, we attached DOTA-1Py, -2Py, -3Py to an αMSH peptide through a carboxylic moiety of each chelator (Fig. 5). Although DOTA-1Py-αMSH was expected to complex $^{64}\text{Cu}$ less efficiently, it
was included to study the trend with increasing pyridyls. The peptide was synthesized via the step-wise addition of amino acids followed by on-resin cyclization. The final Fmoc protecting group was removed by mixing with HOBT, HBTU and isopropyl ethylamine and agitated at ambient temperature overnight. The deprotection and purification by HPLC was done by standard procedures (Zhang et al. 2017) and characterized by mass spectrometry.

Fig. 5. Chemical structure of DOTA-3Py-αMSH

Fig. 6. Radio-HPLC chromatograms of (a) $[^{64}\text{Cu}]$Cu-DOTA-1Py-αMSH (RCY=66%), (b) $[^{64}\text{Cu}]$Cu-DOTA-2Py-αMSH, and (c) $[^{64}\text{Cu}]$Cu-DOTA-3Py-αMSH.
Radiolabeling was performed under similar conditions as discussed for the chelators themselves. $[^{64}\text{Cu}]\text{CuCl}_2$ was mixed with DOTA-xPy-\(\alpha\text{MSH}\) in MES buffer (0.4 M, pH 6.2) and allowed to sit at ambient temperature. At $10^{-5}$M peptide concentration, labeling with 3 MBq of $^{64}\text{Cu}$ was quantitative after 15 min for DOTA-2Py-\(\alpha\text{MSH}\) and DOTA-3Py-\(\alpha\text{MSH}\) and 66% for DOTA-1Py-\(\alpha\text{MSH}\) (Fig. 6). The resulting $[^{64}\text{Cu}]\text{Cu}$-DOTA-xPy-\(\alpha\text{MSH}\) (x=1-3) complexes were stable in both phosphate buffer and human serum at 37°C for 24 hours (radiochemical purity, RCP > 96%, Table 1).

Modifications of peptides may adversely impact target binding affinity (Di 2015). We studied the binding affinity of $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-xPy-\(\alpha\text{MSH}\) (x=1-3) and found that binding of all three peptides towards MC1R remained in the in low nanomolar level (Table 2), although lower than Ga-DOTA-\(\alpha\text{MSH}\) complex ($K_i = 0.31 \pm 0.06$ for CCZ01048) (Zhang et al. 2017).

Table 2. Inhibition constants ($K_i$) of $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-xPy-\(\alpha\text{MSH}\) (x=1-3) towards MC1R (n=3).

|              | $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-1Py-\(\alpha\text{MSH}\) | $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-2Py-\(\alpha\text{MSH}\) | $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-3Py-\(\alpha\text{MSH}\) |
|--------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| $K_i$ (nM)   | $2.34 \pm 0.91$                                              | $2.11 \pm 0.55$                                              | $3.72 \pm 1.38$                                              |

**Conclusion**

We have synthesized a new set of chelators DOTA-1Py, -2Py, -3Py and -4Py and demonstrated that substitution of the carboxylic groups with one or two pyridine groups on a DOTA backbone can significantly improve the binding affinity towards $^{64}\text{Cu}$. We covalently attached the chelators to an $\alpha\text{MSH}$ peptide and demonstrated that DOTA-xPy-\(\alpha\text{MSH}\) (x=1-3) can be quantitatively labeled by $^{64}\text{Cu}$ at ambient temperature. Moreover, all three $\text{n}\text{at}[^{64}\text{Cu}]$-DOTA-xPy-\(\alpha\text{MSH}\) (x=1-3)
retained good binding affinity towards MC1R. Metabolic stability and biodistribution evaluation of [64Cu]Cu-DOTA-xPy-αMSH (x = 1 – 3) is underway.

**Material and Methods**

**General**

All reactions were carried out with commercial solvents and reagents that were used as received. Concentration and removal of trace solvents was done via a Büchi rotary evaporator using dry ice/acetone condenser, and vacuum applied from an aspirator or Büchi V-500 pump. Nuclear magnetic resonance (NMR) spectra were recorded using D₂O or DMSO-d₆ or MeOD-d₄. Signal positions are given in parts per million from tetramethylsilane (δ = 0) and were measured relative to the signal of the solvent. ¹H NMR spectral data are tabulated in the order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet). NMR spectra were recorded on a Bruker 400 (400 MHz) or a Bruker 300 (300 MHz) spectrometer at 25°C unless otherwise noted. MS spectra was recorded on a Waters ZQ equipped with ESCI ion source (low resolution), a Kratos Concept IIHQ (high resolution) or a Bruker Autoflex MALDI-TOF. High performance liquid chromatography (HPLC) of radiolabeled samples was performed on an Agilent 1200 series instrument equipped with a diode array detector and Raytest GABI Star scintillation detector. Radioactivity was measured on a Capintec 55TR dose calibrator. RadioTLC was carried out using BioScan system 200 Image Scanner.

**Chelator synthesis**

Compounds cyclen-1Py (1) (Aime et al. 1999; Aoki et al. 2004; Subat et al. 2007), cyclen-2Py (2) (El Hajj et al. 2009), cyclen-4Py (4) (Natrajan et al. 2010) and DOTA-1Py (5) (Aime et al.
1999) are previously reported and we synthesized with a modified procedure. Compound 2' was co-produced with 2.

Synthesis of cyclen-1Py (1), cyclen-2Py (2, 2'), cyclen-3Py (3), and cyclen-4Py (4): To a suspension of cyclen (172.3 mg, 1 mmol) and K₂CO₃ (552 mg, 4 mmol) in anhydrous acetonitrile (25 mL), a solution of 2-(bromomethyl)pyridine hydrobromide (329 mg, 1.3 mmol) in 10 mL anhydrous acetonitrile was added dropwise over a period of 1 hr at 0 °C and the reaction mixture was allowed to stand at room temperature overnight. After the reaction was complete, the precipitate was filtered off and the solvent was removed by evaporation. The crude products were purified on reverse phase semi-preparative HPLC using Phenomenex Luna C18 (250 mm x 100 mm) column at 3 mL/min with the following method: A: H₂O with 0.1% TFA, B: CH₃CN with 0.1% TFA; 0-20 min, 100% A isocratic; 20-30min, 100%-85% A, 0%-15% B; 30-45min, 85% A, 15% B. The fraction at 6.3 min, 15.5 min and 33.0 min were collected and lyophilized to yield 1 as a colorless solid (40 mg, 15%), 2 (2') as a yellowish solid (63 mg, 18%) and 3 as a yellowish solid (76 mg, 17%), respectively.

1-(Pyridin-2-ylmethyl)-1,4,7,10-tetraazacyclododecane (cyclen-1Py, 1): ¹H NMR (300 MHz, D₂O) δ 8.75 (dd, J = 5.9, 1.6 Hz, 1H), 8.55 (td, J = 8.0, 1.6 Hz, 1H), 8.09 – 7.93 (m, 2H), 4.16 (s, 2H), 3.27 (t, J = 5.2 Hz, 4H), 3.20 – 3.10 (m, 4H), 3.01 – 2.87 (m, 8H). ¹³C NMR (126 MHz, MeOD) δ 158.56, 149.53, 140.87, 125.18, 125.09, 58.38, 51.06, 46.07, 44.19, 44.01. HR-MS: calcd. for C₂₄H₂₆N₅ ([M+H]⁺): 264.2188 ; found: 264.2112.

1,4-Di(pyridin-2-ylmethyl)-1,4,7,10-tetraazacyclododecane and 1,2-Di(pyridin-2-ylmethyl)-1,4,7,10-tetraazacyclododecane (cyclen-2Py, 2 and 2'): ¹H NMR (300 MHz, D₂O) δ 8.65 – 8.55 (m, 2H), 8.30 (td, J = 7.9, 1.7 Hz, 2H), 7.88 – 7.71 (m, 4H), 4.08 (s, 4H), 3.43 (s, 4H), 3.32 (dd, J = 6.7, 4.3 Hz, 4H), 3.08 – 2.92 (m, 8H). ¹³C NMR (126 MHz, MeOD) δ 161.92 (q, J = 36.5 Hz,
CF₃CO₂H) 154.44, 148.71, 141.43, 126.23, 125.61, 117.59 (q, J = 291.1 Hz, CF₃CO₂H) 57.24, 51.90, 51.51, 44.51, 43.80. ESI-MS calcd. for C₂₀H₃₁N₆ ([M+H]⁺): 355.3; found: 355.3.

1,4,7-Tri(pyridin-2-ylmethyl)-1,4,7,10-tetraazacyclododecane (cyclen-3Py, 3): ¹H NMR (500 MHz, MeOD) δ 8.63 (dd, J = 5.4, 1.6 Hz, 2H), 7.93 (td, J = 7.8, 1.7 Hz, 2H), 7.81 (td, J = 7.8, 1.8 Hz, 1H), 7.62 (d, J = 7.9 Hz, 2H), 7.57 (dd, J = 5.1, 1.6 Hz, 1H), 7.52 - 7.45 (m, 2H), 7.39 (d, J = 7.8 Hz, 1H), 7.23 (dd, J = 7.5, 4.9 Hz, 1H), 4.54 (s, 2H), 4.06 (s, 4H), 3.63 (t, J = 5.3 Hz, 4H), 3.42 (t, J = 5.1 Hz, 4H), 3.23 (t, J = 5.3 Hz, 4H), 3.09 – 2.96 (m, 4H). ¹³C NMR (126 MHz, MeOD) δ 162.02 (CF₃CO₂H), 161.73 (CF₃CO₂H), 161.44, 156.25, 150.49, 149.82, 148.33, 141.39, 139.42, 126.33, 125.64, 125.14, 118.65 (CF₃CO₂H), 116.35 (CF₃CO₂H), 58.18, 57.11, 52.65, 50.12, 43.73. HR-MS: calcd. for C₂₆H₃₆N₇ ([M+H]⁺): 446.3032; found: 446.2989.

Preparation of cyclen-4Py: A mixture of cyclen (35 mg, 0.2 mmol) and K₂CO₃ (414 mg, 3 mmol) in dry acetonitrile (15 mL) was placed in an ice bath and then a solution of 2-(bromomethyl)pyridine (190 mg, 0.75 mmol) in 10 mL dry CH₃CN was added dropwise over a period of 1 h. The resulting mixture was stirred for additional 10 h. The precipitate was filtered, and the filtrate concentrated in vacuo to give the crude product which was further purified by HPLC using the same method described above. ¹H NMR (300 MHz, D₂O) δ 8.57 (d, J = 5.3 Hz, 4H), 8.00 (td, J = 7.8 Hz, 1.1 Hz, 4H), 7.80 (d, J = 7.5 Hz, 4H), 7.66 (t, J = 6.3 Hz, 4H), 4.36 (s, 8H), 3.45 (s, 16H). ESI-MS: calcd. for C₃₂H₄₁N₈ ([M+H]⁺): 537.3; found: 537.3.

Preparation of DOTA-1Py: A solution of tert-butyl bromoacetate (59 mg, 0.3 mmol) in dry CH₃CN (5 mL) was added dropwise to a mixture of cyclen-1py (26 mg, 0.1 mmol), K₂CO₃ (83 mg, 0.6 mmol) and KI (10 mg, 0.06 mmol) in dry CH₃CN (10 mL). The resulting mixture was stirred at room temperature for 1h and then refluxed overnight. After cooling to room temperature, the mixture was filtered, and the residue washed with dry CH₃CN. The filtrate was collected and
evaporated to yield the title compound as a white solid. The solid was dissolved in 10 mL of mixed solvent containing TFA (9.5 mL), TIPS (0.25 mL) and H2O (0.25 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was removed by air flow and the residue was dissolved in CH3CN/H2O (1/1). The crude product was purified on reverse phase semi-preparative HPLC using Phenomenex Luna C18 (250 mm x 100 mm) column at 3 mL/min with the following method: A: H2O with 0.1% TFA, B: CH3CN with 0.1% TFA; 0-5min, 100% A isocratic; 5-25 min, 100%-60% A, 0%-40% B. The fraction at 6.7 min was collected and lyophilized to give the title compound as a colorless oil (32 mg, 74%). 1H NMR (600 MHz, D2O) δ 8.69 (ddd, J = 5.9, 1.6, 0.6 Hz, 1H), 8.46 (td, J = 7.9, 1.6 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.94 (ddd, J = 7.3, 5.9, 1.3 Hz, 1H), 4.09 (s, 2H), 3.89 (d, J = 15.8 Hz, 2H), 3.68 (d, J = 16.4 Hz, 4H), 3.47-3.44 (m, 4H), 3.35 (s, 2H), 3.31-3.28 (m, 2H), 3.15 (s, 2H), 3.06-3.02 (m, 2H), 2.92-2.86 (m, 4H). 13C NMR (151 MHz, D2O) δ 175.48, 169.03, 162.83 (q, J = 35.6 Hz, CF3CO2H), 150.13, 147.53, 143.16, 129.61, 128.39, 126.93, 116.26 (q, J = 291.7 Hz, CF3CO2H), 55.34, 53.60, 52.87, 52.05, 50.36, 48.15, 48.01. ESI-MS: calcd. for C20H32N5O6 ([M+H+]): 438.2; found: 438.2.

Preparation of DOTA-2Py: cyclen-2py (39 mg, 0.2 mmol) was dissolved in dry acetonitrile (10 mL), K2CO3 (55 mg, 0.4 mmol) and KI (7 mg, 0.04 mmol) was added to the solution. Then a solution of tert-butyl bromoacetate (35 mg, 0.1 mmol) in dry CH3CN (5 mL) was added dropwise. The mixture was stirred at room temperature for 1h and then refluxed overnight. After cooling to room temperature, the mixture was filtered, and the filtrate was evaporated to yield the title compound as a yellowish solid. The solid was dissolved in 10 mL of mixed solvent containing TFA (9.5 mL), TIPS (0.25 mL) and H2O (0.25 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was removed by air flow and the residue was dissolved in CH3CN/H2O (1/1) for further HPLC purification using the same method described above. The
fraction from 14-14.5 min was collected and lyophilized to give the title compound as colorless oil (36 mg, 77%). $^1$H NMR (500 MHz, MeOD) $\delta$ 8.44 (dd, $J = 5.3$, 1.7 Hz, 2H), 7.79 (td, $J = 7.7$, 1.7 Hz, 2H), 7.55 (s, 2H), 7.35 – 7.31 (m, 2H), 4.36- 4.08 (m, 4H), 3.73 (s, 4H), 3.53 – 2.95 (m, 18H, including MeOD). $^{13}$C NMR (75 MHz, DMSO) $\delta$ 170.77, 158.86 (CF$_3$COOH), 158.43 (CF$_3$COOH), 153.45, 149.07, 137.97, 124.36, 123.76, 119.20 (CF$_3$COOH), 115.25 (CF$_3$COOH), 57.12, 53.93, 50.00, 49.75, 49.44. HR-MS: calcd. for C$_{24}$H$_{35}$N$_6$O$_4$ ([M+H]$^+$): 471.2720; found: 471.2558.

Preparation of DOTA-3Py: To a suspension of cyclen-3py (45 mg, 0.1 mmol), K$_2$CO$_3$ (28 mg, 0.2 mmol) and KI (3 mg, 0.02 mmol) in dry acetonitrile (15 mL), a solution of tert-butyl bromoacetate (20 mg, 0.1 mmol) in dry CH$_3$CN (5 mL) was added. The resulting mixture was refluxed overnight. The precipitated solids were removed by filtration and the filtrate was concentrated to give the title compound as a yellowish solid. The solid was dissolved in 10 mL of mixed solvent containing TFA (9.5 mL), TIPS (0.25 mL) and H$_2$O (0.25 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was removed by air flow and the residue was dissolved in CH$_3$CN/H$_2$O (1/1) for further HPLC purification using the same method described above. The fraction at 15.2 min was collected and lyophilized to give the title compound as colorless oil (41 mg, 81%). $^1$H NMR (600 MHz, D$_2$O) $\delta$ 8.60 (d, $J = 5.7$ Hz, 2H), 8.50 (d, $J = 4.9$ Hz, 1H), 8.37-8.30 (m, 2H), 8.02 (s, 2H), 7.83 (td, $J = 7.8$, 1.6 Hz, 1H), 7.73 (t, $J = 7.1$ Hz, 2H), 7.45-7.39 (m, 1H), 7.32 (s, 1H), 4.32 (s, 2H), 4.16-3.91 (m, 4H), 3.83 (s, 2H), 3.73 – 3.42 (m, 8H), 3.30-2.94 (m, 8H). $^{13}$C NMR (151 MHz, D$_2$O) $\delta$ 178.19, 162.97 (q, $J = 34.7$ Hz, CF$_3$COOH), 156.69, 156.64, 147.95, 147.90, 147.82, 147.74, 147.64, 138.80, 138.78, 138.57, 124.37, 124.23, 124.20, 123.94, 123.65, 123.41, 116.3 (q, $J = 276.3$ Hz, CF$_3$COOH) 59.88, 59.68, 59.15, 58.40,
Production of $^{64}$Cu

Isotopically enriched $^{64}$Ni metal powder (50 mg) was dissolved in HCl. Aqueous ammonia was added to adjust the pH to $\sim$9. $^{64}$Ni is then electroplated onto a rhodium backing disk (35 mm diameter, 1 mm thickness) using a small custom plating apparatus overnight. The plated nickel layer was approximately 8 mm diameter x 110 µm thickness. The target was irradiated for 1 hour at 10 µA proton current 13 MeV and left in the cyclotron for 2 hours to allow for the decay of the short-lived isotopes. The major co-produced isotopes were $^{61}$Co (2.9%) and $^{103}$Pb (2.0%) after cool-down. The $^{64}$Ni layer of the target was dissolved in hot concentrated HCl. After evaporation to dryness, the residue was re-dissolved in 6 M HCl and loaded onto a 1 cm AG1-X8 solid extraction column pre-conditioned with 6 M HCl. The column was washed with 6 M HCl (10 mL) and $^{64}$Cu was eluted with milli-Q water. Radionuclide purity (>99%) was confirmed by gamma spectroscopy.

Labeling of DOTA, DOTA-1Py, DOTA-2Py, DOTA-3Py and DOTA-4py (cyclen-4Py) with $^{64}$Cu

To a solution of ligand (10 µL, $10^{-3}$M) containing MES buffer (83 µL, 0.4 M, pH = 6.2) in an Eppendorf tube was added $[^{64}$Cu$]$CuCl$_2$ (7 µL 3 MBq). Final concentration of the ligand was $10^{-4}$ M. The resulting mixture was kept at room temperature for 30 min. After the reaction, radiochemical purity of the radio-ligands was confirmed by both radio-HPLC and radio-iTLC. HPLC method: Phenomenex Luna C18 column (150 mm x 4.6 mm), 1 mL/min. A: H$_2$O with 0.1%
TFA, B: CH$_3$CN with 0.1% TFA, 0-5 min: 100% A isocratic; 5-25 min: 100%-60% A, 0-40% B.

$[^{64}\text{Cu}]\text{Cu-DOTA-1Py}, t_R = 12.5 \text{ min}; [^{64}\text{Cu}]\text{Cu-DOTA-2Py}, t_R = 12.7 \text{ min}; [^{64}\text{Cu}]\text{Cu-DOTA-3Py}, t_R = 14.5 \text{ min}; [^{64}\text{Cu}]\text{Cu-DOTA-4Py}, t_R = 16.7 \text{ min}$. Radio-iTLC: iTLC-SA plates as stationary phase, developed using 0.2 M EDTA (pH 5). Under these conditions uncomplexed $^{64}\text{Cu}^{II}$ migrates with the solvent front ($R_f = 1$), while the $^{64}\text{Cu}$-complex remains at the baseline ($R_f = 0$).

**Concentration dependence $^{64}\text{Cu}$ labeling**

To aqueous solution of ligands (10 µL, $10^{-2}$ M, $10^{-3}$ M, $10^{-4}$ M, $10^{-5}$ M, $10^{-6}$ M, $10^{-7}$ M) in MES buffer (83 µL, 0.4 M, pH = 6.2) was added $[^{64}\text{Cu}]\text{CuCl_2}$ (3 MBq, 7 µL of 0.01M HCl). Final concentrations of the ligand were $10^{-3}$ M, $10^{-4}$ M, $10^{-5}$ M, $10^{-6}$ M, $10^{-7}$ M, $10^{-8}$ M. The mixtures were kept at room temperature for 15 min and then analyzed by radio-HPLC and radio-iTLC using the conditions described above.

**Kinetics of radiolabeling studies**

To an aqueous solution of ligand (10 µL, $10^{-3}$M) in MES buffer (83 µL, 0.4 M, pH = 6.2) was added $[^{64}\text{Cu}]\text{CuCl_2}$ (3 MBq, 7 µL in 0.01M HCl). The reaction mixture was kept at room temperature. Aliquots of the mixtures were analyzed by radio-HPLC and radio-iTLC using the conditions described above to determine the radiolabeling yields.

**Ligand competition radiolabeling**

To equimolar aqueous mixtures of new ligand (10 µL, $10^{-3}$ M DOTA-xPy (x = 1 – 4)) and commercially available chelator $p$-SCN-Bn-NOTA (10 µL, $10^{-3}$ M NOTA-SCN) in MES buffer (73 µL, 0.4 M, pH = 6.2) was added $[^{64}\text{Cu}]\text{CuCl_2}$ (3 MBq, 7 µL in 0.01 M HCl). Final
concentrations of new ligand and NOTA-SCN were $10^{-4}$ M in the reaction mixtures. The mixtures were kept at room temperature for 15 min and then analyzed by radio-HPLC using the same method described above to determine the labeling ratio of radiolabeled new ligand and $[^{64}\text{Cu}]\text{Cu}$-NOTA-SCN ($t_R = 24.2$ min).

**Ligand challenge studies**

To a solution of ligand (10 µL, $10^{-3}$M) containing MES buffer (83 µL, 0.4 M, pH = 6.2) in an Eppendorf tube was added $[^{64}\text{Cu}]\text{CuCl}_2$ (7 µL 3 MBq). Final concentration of the ligand was $10^{-4}$ M. After 10 min, full complexation of the ligand was checked by radio-HPLC and radio-iTLC. NOTA-SCN (1000-fold excess) dissolved in water (50 µL) was subsequently added to the reaction solution, and stirred for 4 h at ambient temperature. The mixture was assayed by radio-HPLC to determine the decomposition. As an opposing experiment, an aqueous solution of NOTA-SCN (10 µL, $10^{-3}$M) and $[^{64}\text{Cu}]\text{CuCl}_2$ (3 MBq, 7 µL in 0.01 M HCl) were subsequently added into MES buffer (83 µL, 0.4 M, pH = 6.2). After full complexation, DOTA-xPy (x = 1 – 4, 1000-fold excess) dissolved in water (50 µL) was added to the complex solution and the mixture was stirred for 4 h. The mixture was analyzed by radio-HPLC to determine the decomposition.

**In vitro stability of radio-ligands**

Pre-formed $^{64}$Cu-labeled complexes (100 µL) were added to phosphate buffer (300 µL, pH 7.4) or human serum (300 µL). After 1 h or 24 h of incubation at 37 °C, the buffer samples were analyzed by semi-preparative HPLC. The serum samples were firstly treated with ethanol to precipitate and separate proteins (twice the volume of the mixture) and centrifuged for 1 min.
(13,200 rpm). The filtrate was decanted and further filtered (VWR sterile syringe filter, 0.2 µm PES), and subsequently analyzed by radio-iTLC using the conditions outlined above.

**Preparation of DOTA-1Py-αMSH, DOTA-2Py-αMSH and DOTA-3Py-αMSH**

Peptide synthesis for Fmoc-Pip-Nle-CycMSH<sub>hex</sub>-resin (Fmoc-αMSH-resin) was performed as described in previously published procedures (Zhang et al. 2017). Subsequently, Fmoc was removed. (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 5 eq.), hydroxybenzotriazole (HOBT, 5 eq.) and DOTA-xPy (x=1-3, 5 eq.) were dissolved in DMF (minimum), then added to the resin (1 eq.) and initiated by the addition of N,N-diisopropylethylamine (15 eq.). Coupling was carried out for ~16 h at room temperature with shaking. The resin was washed extensively with DMF and DCM and solvent removed in a flow of N<sub>2</sub>. The peptide was cleaved and deprotected by soaking the resin with a mixture of TFA/TIPS/water/phenol (90/2.5/2.5/5) and shaking at room temperature for 3 hours. After ether precipitation and filtration, the filtrate was collected and dried by N<sub>2</sub>. The residue was dissolved in CH<sub>3</sub>CN and water and purified by semi-preparative HPLC using Phenomenex Luna C18 (250 mm x 100 mm) column at 3 mL/min with the following method: A: H<sub>2</sub>O with 0.1% TFA, B: CH<sub>3</sub>CN with 0.1% TFA; 0-5 min, 90% A, 10% B; 5-25min, 90%-10% A, 10%-90% B. DOTA-1Py-αMSH, <i>t_R</i> = 14.9 min; DOTA-2Py-αMSH <i>t_R</i> = 15.1 min; DOTA-3Py-αMSH <i>t_R</i> = 15.4 min.

MALDI-TOF MS: DOTA-1Py-αMSH: calcd. 1540.8 for [C<sub>76</sub>H<sub>110</sub>N<sub>21</sub>O<sub>14</sub>]<sup>+</sup> ([M+H]<sup>+</sup>), found: 1541.9. DOTA-2Py-αMSH: calcd. 1573.9 for [C<sub>80</sub>H<sub>113</sub>N<sub>22</sub>O<sub>12</sub>]<sup>+</sup> ([M+H]<sup>+</sup>), found: 1574.9.

DOTA-1Py-αMSH: calcd. 1606.9 for [C<sub>84</sub>H<sub>116</sub>N<sub>23</sub>O<sub>10</sub>]<sup>+</sup> ([M+H]<sup>+</sup>), found: 1607.9.
Preparation of non-radioactive complexes (natCu-DOTA-Py-MSH, natCu-DOTA-2Py-MSH and natCu-DOTA-3Py-MSH)

A solution of peptide (200 µL, 10⁻³ M) and CuSO₄ (100 µL, 10⁻² M) in an Eppendorf tube was kept at room temperature for 2 h and subsequently purified by C-18 Sep-Pak (Waters). Non-radioactive title complexes were confirmed by MALDI-TOF-MS: natCu-DOTA-1Py-αMSH: calcd. 1603.8 for [C₇₆H₁₁₀₆₃CuN₂₁O₁₄]²⁺ ([M+H+6³Cu]⁺), found: 1604.1. natCu-DOTA-2Py-αMSH: calcd. 1636.8 for [C₈₀H₁₁₃₆₃CuN₂₂O₁₂]⁺ ([M+H+6³Cu]⁺), found: 1637.2. natCu-DOTA-1Py-αMSH: calcd. 1669.8 for [C₈₄H₁₁₆₆₃CuN₂₃O₁₀]⁺ ([M+H+6³Cu]⁺), found: 1670.2.

6⁴Cu labeling of bioconjugates DOTA-1Py-αMSH, DOTA-2Py-COOH-αMSH and DOTA-3Py-αMSH

To a solution of bioconjugate (10 µL, 10⁻⁴ M) in MES buffer (83 µL, 0.4 M, pH = 6.2) was added 6⁴CuCl₂ (3 MBq, 7 µL). Final concentration of the bioconjugate was 10⁻⁵ M. The resulting mixture was kept at room temperature for 15 min. After the reaction, radiochemical purity of the radiolabeled bioconjugate was confirmed by both radio-HPLC and radio-iTLC. HPLC method: Phenomenex Luna C18 column (150 mm x 4.6 mm), 1 mL/min. A: H₂O with 0.1% TFA, B: CH₃CN with 0.1% TFA, 0-5 min: 100% A isocratic; 5-25 min: 100%-0% A, 0-100% B. [⁶⁴Cu]Cu-DOTA-1Py-αMSH, tᵣ = 15.9 min; [⁶⁴Cu]Cu-DOTA-2Py-αMSH, tᵣ = 16.7 min; [⁶⁴Cu]Cu-DOTA-3Py-αMSH, tᵣ = 15.7 min. Radio-iTLC: SA plate, 0.2 M EDTA solution as mobile phase.

In vitro stability of radiolabeled peptides

Pre-formed ⁶⁴Cu-labeled bioconjugate (100 µL) was mixed in phosphate buffer (300 µL, pH 7.4) or human serum (300 µL). After 1 h or 4 h of incubation at 37 °C, the buffer samples were
analyzed by semi-preparative HPLC. The serum samples were firstly treated with ethanol to precipitate and separate proteins (twice the volume of the mixture) and centrifuged for 1 min (13,200 rpm). The filtrate was decanted and further filtered (VWR sterile syringe filter, 0.2 µm PES), and subsequently analyzed by radio-iTLC using the conditions outlined above.

**Binding affinity of natCu-DOTA-xPy towards MC1R**

$K_i$ was measured using the same method previously reported (Zhang et al. 2017). The measurements were repeated in three separate experiments and reported as mean ± standard deviation.

**List of abbreviations**

αMSH α-melanocyte-stimulating hormone

DOTA 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

EDTA ethylenediamine tetraacetic acid

HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt hydroxybenzotriazole

HPLC high performance liquid chromatography

iTLC instant thin layer chromatography

MC1R melanocortin 1 receptor

MES 2-(N-morpholino)ethanesulfonic acid

NOTA 1,4,7-triazacyclononane-triacetic acid

TFA trifluoroacetic acid

TIPS Triisopropyl silane
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Author contributions

HY, FG, FB, CR and PS designed the studies. HY, FG, BM, CZ, JZ carried out the experiments. HY, BM, CR, PS prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Not applicable.

Consent for publication
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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