IN VIVO BEHAVIOR OF COPPER-64 LABELED Y3-OCTREOTATE USING A CROSS-BRIDGED CYCLAM LIGAND

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Keywords: Copper-64, cross-bridged macrocycle, somatostatin analog, microPET

Analogues of the macrocyclic chelator 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) are currently used as chelators of choice for labeling copper radionuclides to biological molecules. Recently, the "cross-bridged" cyclam complex, ^{64}Cu(II)-4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TE2A) was shown to have improved *in vivo* stability compared to ^{64}Cu(II)-TETA (1). We hypothesized that a bifunctional chelator of CB-TE2A would be a good candidate for radiolabeling copper to biological molecules for imaging and radiotherapy. However, it was postulated that both –COOH groups are required to stably bind Cu(II) to form a neutral, hexacoordinate complex; when CB-TE2A is conjugated to a peptide, one carboxylate is converted to an amide linkage. To evaluate our hypothesis, ^{64}Cu-CB-TE2A-Y3-octreotate (^{64}Cu-CB-TE2A-Y3-TATE) was synthesized and evaluated *in vivo* in a tumor-bearing rat model. **Methods:** CB-TE2A-Y3-TATE was synthesized by solid-phase methods using standard Fmoc chemistry and radiolabeled with ^{64}Cu in greater than 95% purity. Biodistribution and microPET (Concord Microsystems) experiments were performed in AR42J rat pancreatic tumor-bearing male Lewis rats. **Results:** Initial microPET imaging of ^{64}Cu-CB-TE2A-Y3-TATE in AR42J bearing rats showed the highest non-target uptake in the kidneys (1.76 $\pm$ 0.04 %ID/g at 1 h), with significant clearance by 24 h post-injection (0.32 $\pm$ 0.03 %ID/g). Liver uptake was 0.31 $\pm$ 0.02 %ID/g at 1 h and decreased to 0.051 $\pm$ 0.002 %ID/g by 24 h post-injection demonstrating that the primary mode of clearance is renal rather than hepatobiliary. Quantification of activity in rat tumor, liver, and kidney was similar to *in vivo* biodistribution results. In biodistribution experiments, somatostatin-receptor positive tissues displayed significantly higher uptake at all time points compared to non-target organs; this was blocked by co-injection of 150 g Y3-TATE (pancreas: 1 h = 6 $\pm$ 1 %ID/g, 1h block = 0.37 $\pm$ 0.03 %ID/g; AR42J tumor 1 h = 2.4 $\pm$ 0.4 %ID/g, 1 h block = 0.8 $\pm$ 0.1%ID/g). Blood clearance of ^{64}Cu-CB-TE2A-Y3-TATE compared to ^{64}Cu-TETA-Y3-TATE was found to be much more favorable. From 1 to 24 h post-injection, levels of ^{64}Cu-CB-TE2A-Y3-TATE in the blood decreased from 0.13 $\pm$ 0.01 to 0.014 $\pm$ 0.001% ID/g, while there was essentially no blood clearance of ^{64}Cu-TETA-Y3-TATE (0.09 $\pm$ 0.01 to 0.10 $\pm$ 0.01% ID/g) (2). This strongly suggests enhanced *in vivo* stability of the ^{64}Cu-CB-TE2A-Y3-TATE complex compared to ^{64}Cu-TETA-Y3-TATE. The enhanced blood clearance of ^{64}Cu-CB-TE2A-Y3-TATE gives rise to impressive tumor:blood ratios (156:1) at 4 h post-injection. **Conclusions:** These data indicate that CB-TE2A is a significantly improved bifunctional chelator compared to TETA for labeling ^{64}Cu to biological molecules such as the somatostatin analogue Y3-TATE and that only one –COOH group on CB-TE2A is required to bind Cu(II). **Acknowledgements:** The authors would like to thank Lynne Jones for technical assistance and acknowledge funding by NCI grant (CA93375). MicroPET imaging is supported by an NIH/NCI SAIRP grant (1 R24 CA83060) with additional support from the Small Animal Imaging Core of the Alvin J. Siteman Cancer Center at Washington University and Barnes-Jewish Hospital. The SAIC Core is supported by an NCI Cancer Center Support Grant # 1 P30 CA91842. The production of ^{64}Cu is supported by NCI R24 CA86307.

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IN VIVO STABILITY OF CU-64-AZAMACROCYCLIC COMPLEXES: COMPARISON OF TETA AND DOTA WITH CROSS-BRIDGED CHELATORS

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Keywords: copper-64, azamacrocycle, metabolism, bifunctional chelator

The increased use of copper radioisotopes in nuclear medicine has created a need for bifunctional chelators (BFCs) that form stable radiocopper complexes and allow covalent attachment to biomolecules. Azamacrocyclic chelators have been studied extensively for their ability to form stable Cu(II) complexes in biological systems due to their enhanced *in vivo* stability compared to acyclic chelators. The chelators most commonly utilized for labeling copper radionuclides to biological molecules are analogs of 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA); however, recent reports have communicated the *in vivo* instability of the radio-Cu(II)-TETA complexes. A new class of azamacrocycles, the "cross-bridged" cyclam (CB-cyclam) derivatives, form highly kinetically inert complexes with Cu(II), and therefore may be less susceptible to transchelation than their unbridged analogs (1).

Here we report results from experiments designed to characterize the *in vivo* metabolism and relative biological stabilities of four radiocopper complexes. A series of metabolism studies in normal rat liver have revealed that the ^{64}Cu complex of 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (^{64}Cu-CB-TE2A) results in significantly lower values of protein-associated ^{64}Cu than ^{64}Cu-TETA [26±9 % vs. 74±9 % at 4 h; 50±12 % vs. 92±5 % at 20 h]. A similar trend was observed for the corresponding cyclen analogs; the ^{64}Cu complex of 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (^{64}Cu-CB-DO2A) allowed less transchelation than the ^{64}Cu complex of 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (^{64}Cu-DOTA) [51±14 % vs. 87±1 % at 1 h; 61±14 % vs. 90.3±0.5 % at 4 h]. These data suggest that the unusually high *in vivo* stability of ^{64}Cu-CB-TE2A is likely due to a combination of two effects: the size of the macrocycle cavity, with cyclam being more hospitable for Cu(II) than cyclen, and the presence of a structurally-reinforcing crossbridge that enhances *in vivo* stability in both the cyclam and cyclen derivatives.

We have identified the major radiolabeled rat liver protein resulting from the dissociation of ^{64}Cu from azamacrocyclic complexes. Size-exclusion chromatography, SDS-PAGE, and immunoblot analyses of liver extracts have revealed that the radiolabeled protein is superoxide dismutase (SOD), a 32 kDa homodimeric enzyme containing one copper atom and one zinc atom in each subunit. This finding is consistent with a previous report demonstrating the dissociation of ^{64}Cu from a TETA-peptide conjugate in rat liver and subsequent binding to SOD (2).

The current study addresses critical questions regarding the *in vivo* stability of chelators used in biomedical applications of copper radionuclides. The metabolism data indicate that a BFC derivative of CB-TE2A is a highly desirable alternative for labeling copper radionuclides to biological molecules for diagnostic imaging and targeted radiotherapy. The data also indicate that a reinforced crossbridged azamacrocycle reduces metal loss to SOD, and that the azamacrocycle must also have an appropriate size to accommodate the Cu^{2+} ion.

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MICROPET IMAGING OF RADIOPHARMACEUTICALS LABELED WITH THREE COPPER RADIONUCLIDES

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Keywords: Cu-Radiopharmaceuticals, PET, positron range, iterative reconstruction, microPET

With the introduction of high-resolution small animal scanners, the positron range of several positron emitting radionuclides is larger than the intrinsic spatial resolution of the camera. In this study, data was acquired with the microPET-R4 (Concorde MicroSystems, Inc.). The first set of data was composed of phantom data consisting of a line-source surrounded by water. The line-source was filled with solution of either ^{60}Cu, ^{61}Cu, or ^{64}Cu and imaged in the microPET. Animal images were obtained utilizing two radiopharmaceuticals. Mice and rats were successively imaged with Cu-PTSM and Cu-DO3P labeled with the three nuclides listed above. Copper-PTSM is a flow agent[1] that will show uptake in all major organs while Cu-DO3P is a bone seeking agent[2] that will produce images of the skeleton. The amount of injected activity was such that the same numbers of positron decays occurred during each image acquisition. Images were reconstructed with standard reconstruction algorithms available from Concorde Microsystems, Inc. (FBP and OSEM) as well as with a Maximum A Posteriori (USC-MAP[3]) algorithm that includes a correction for the positron range. Measurements of the line-source width were used as a measure of spatial resolution recovery. Visual assessment of the image quality was performed on the animal images and spatial resolution improvement was evaluated by measuring thoracic vertebra widths on the Cu-DO3P images.

The line-source experiments showed that substantial improvement in spatial resolution could be achieved with MAP. The line-source imaged with ^{61}Cu reconstructed with FBP and OSEM produced a line spread function with a Full Width at Half Maximum (FWHM) of 2.8 mm and 2.2 mm, while MAP yielded a FWHM of 1.9 mm (without range correction) and 1.2 mm (with range correction). Inspection of the MAP reconstructed animal images showed a good recovery of contrast and general image quality. The widths of a vertebral bone using ^{61}Cu-DO3P decreased from 10.3 mm (with FBP or OSEM) to 4.2 mm with MAP (with range correction).

These initial results shows that MAP caused a distinct improvement in the image quality and that the implementation of the positron range correction yielded substantial improvement in the spatial resolution and image quality. Validation of the algorithm is presently underway and later releases will include a more accurate 3D model based-normalization and attenuation correction. Utilizing reconstruction algorithms that take into account the positron range, high-resolution images can be obtained even when using positron-emitting nuclides such as ^{60}Cu with high positron energy.

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A NEW BIOCLEARANCE AGENT WITH ANTIOXIDANT PROPERITES FOR POTENTIAL USE IN RADIOPHARMACEUTICAL APPLICATIONS

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Keywords: Non target tissues/organs, Bioclearance, Radiopharmaceuticals, Antioxidant

Efficient clearance of radioactivity (for rays and rays emitting radiopharmaceuticals) from blood and non target organs/tissue (e.g., Liver and Kidney) is of paramount importance in the safe and effective clinical applications of radiodiagnostic and therapeutic agents. Hydrophilic/Lipophilic properties dictate the pharmacokinetic properties and biodistribution profiles of specific radiopharmaceuticals. However, coinjection of exogenous agents can effectively reduce uptake of radioactivity from non target organs. We herein report the application of a novel trimeric aminoacid $P(CH_2NHCH(CH_3)COOH)_3$ (TRIALP) as a new generation bioclearance agent for use in nuclear medicine. TRIALP is a water-soluble and highly hydrophilic non toxic amino acid conjugate (**Figure 1**). In vivo studies in pigs, loaded with copper, have demonstrated that TRIALP effectively scavenge copper metal from kidney and liver. The pharmacokinetic and biodistribution data of ^{111}In-DOTA-Re(Arg11)CCMSH in B16/F1 C57 mice coinjected with TRIALP showed ~25% decrease in uptake of radioactivity in kidneys as compared to control set of mice which received no TRIALP.

TRIALP is also an effective antioxidant as evidenced by a high value of 18.81 μmol TE/g for scavenging peroxynitrite radicals. The dual properties of kidney clearance and antioxidant action of TRIALP make it a unique pharmacokinetic vector for use in Nuclear Medicine. Details on the chemistry, complexation properties, antioxidant studies and pharmacokinetic properties of TRIALP will be presented.

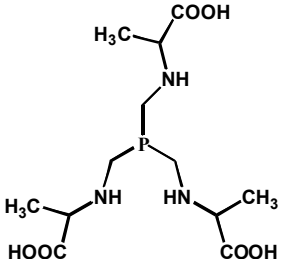

Figure 1. TRIALP

REGIOSELECTIVELY N-SUBSTITUTED CYCLENS: SYNTHESIS, BIODISTRIBUTION, LOG P AND MODELING

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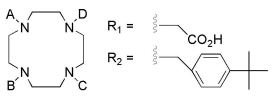
Keywords: cyclen, copper-64, biodistribution, log P, QSAR

Selective methods for the *N*-substitution of cyclen is a crucial step in most syntheses of new cyclen-based ligands and bifunctional chelating agents. We have developed synthetic methods for synthesizing variously substituted cyclens. (1) To obtain different substitution patterns, we utilize a strategy of regioselective protection/1st alkylation/deprotection/2nd alkylation.



1 A = B = C = D = R_2 **2** A = B = C = R_1, D = R_2
3 A = C = R_2, B = D = H **4** A = C = R_1, B = D = R_2
5 A = B = R_2, C = D = H **6** A = B = R_1, C = D = R_2
7 A = R_2, B = C = D = H **8** A = R_1, B = C = D = R_2

These macrocycles provide a common framework for investigating the *in vivo* behavior of Cu-64 complexes. A total of eight new regioselectively *N*-substituted cyclen derivatives with two different alkyl groups were prepared. 4-(*tert*-butyl)benzyl which is very hydrophobic, and the acetic acid group (CH_2CO_2H) were chosen as the varying substituents. These ligands were used for a systematic study of *in vivo* distribution examining the effects of charge and lipophilicity of copper complexes on blood levels and uptake in the liver and kidneys.

The eight complexes were successfully radiolabeled with ^{64}Cu by incubation of [Cu-64]$CuCl_2$ with the eight ligands in an ethanol/buffer mixture. Radiochemical purities of over 90% were achieved as determined by radio-TLC. Partition coefficients (log P) as measured by octanol/ammonium acetate buffer were determined to be between 1.27 and 3.19. Increasing the number of hydrophobic alkyl groups, 4-(*tert*-butyl)benzyls and decreasing the number of acetate groups, generally increased the lipophilicities. However, symmetry within the complexes seems to play more of a role in determining log P value. *trans*-Dialkylated cyclen (**3**) showed much higher log P value than its cis-dialkylated counterpart (**5**) (3.19 vs. 2.30).

The radioactive complexes were diluted with 0.1M ammonium acetate buffer (pH6.4) or saline for biodistribution studies (female Sprague-Dawley rats, n = 4 per 15min, 4h, and 24h postinjection). All eight Cu-64 complexes showed different retention and clearance patterns in blood, liver, and kidney. Positively charged complexes **1**, **3**, **5**, **7** showed high blood retention at 15 min. Isomers **3** and **5** showed very similar clearance patterns**,** as did their acetate counterparts **4** and **6**.

The complexes were modeled with a published force field in order to determine the most favorable conformations.(2) The lowest energy structures and the biological data were used to develop quantitative structure activity relationships(QSAR) using partial least squares analysis (PLS). A total of nine molecular descriptors, combining both 2D and 3D descriptors, were used in order to develop predictive models for blood, liver, kidney uptake and clearance. QSAR's for (%ID/g Blood) / (%ID/g Kidney) and (%ID/g Blood) / (%ID/g Liver) were developed at two time points 15 min (r^2's 0.56 and 0.65 respectively) and 24 hr (r^2's 0.98 and 0.89 respectively).

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SYNTHESIS AND EVALUATION OF C-TERMINALLY MODIFIED DERIVATIVES OF TYR^3-OCTREOTATE

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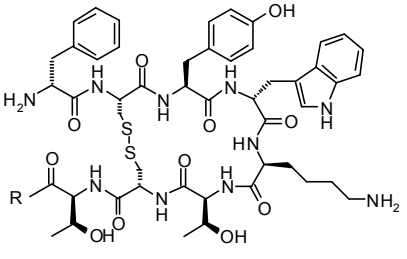
Keywords: Somatostatin, solid-phase synthesis, octreotate, C-terminal

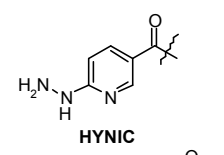
Somatostatin receptor (SSTR) selective peptides, in particular octreotide and octreotate have found widespread application as transporters for radioisotopes in nuclear medicine. The notable similarity in the traditional SPPS of octreotides is that they are all functionalised at the *N*-terminal. The main reason for this is that the *C*-terminal amino acid, threonine, is usually loaded onto the resin *via* its carboxylic acid moiety, thus, blocking this terminal for manipulation whilst still being attached to the resin. The aim of this work was to investigate the potential of conjugates of Tyr^3-octreotate linked at the C-terminal end of the peptide.

In order to access the C-terminal, *N*- -Fmoc-L-threonine allyl ester was synthesised and loaded onto Ellman's dihydropyran resin. The linear octapeptide Fmoc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(THP-resin)-OAllyl was synthesised stepwise using an Fmoc protocol. After cyclization and removal of the allyl ester protecting group, this peptide could be selectively functionalised at the *C*- or *N*-terminal.

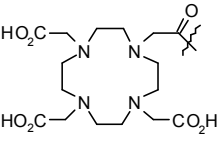


1 R = NHCH$_2$CH$_2$NH-DOTA
2 R = NHCH$_2$CH$_2$NH-HYNIC

Examples of compounds investigated



HYNIC



DOTA

To allow the determination of structure activity relationships for the C-terminal modification of Tyr^3-octreotate, receptor affinity profiles of the C-terminal modified Tyr^3-octreotate derivatives (IC_{50}) were obtained from competition binding experiments with ^{125}I-TOC on rat cortex membranes.

The loading of threonine to the DHP resin *via* its side chain alcohol group allows the Tyr^3-octreotate to be functionalised at both the *C*- and *N*-terminal, thus, introducing a new scope to the range of octreotate derivatives that can be synthesised using SPPS. The *C*-terminally functionalised octreotates revealed satisfactory receptor affinities.

substituent	IC_{50}	substituent	IC_{50}	substituent	IC_{50}
(Tyr^3-octreotate)	0.33 ± 0.04	-$(CH_2)_2NH_2$	12.60 ± 0.69	-$(CH_2)_2$NH-DOTA	8.95 ± 1.53
(octreotide)	2.78 ± 0.13	-$(CH_2)_6NH_2$	1.22 ± 0.29	-$(CH_2)_6$NH-(C=O)-CH_3	2.87± 0.07
-C_2H_5	6.24 ± 0.51	-$(CH_2)_2$NH-(C=O)-CH_3	7.53 ± 0.08	-$(CH_2)_6$NH-(C=O)-C_5H_{11}	3.17 ± 0.22
-C4H9	1.78 ± 0.13	-$(CH_2)_2$NH-(C=O)-C_5H_{11}	4.62 ± 0.04	-$(CH_2)_2$NH-Glu-DOTA	34.23 ± 5.63
-C6H13	1.09 ± 0.27	-$(CH_2)_2$NH-HYNIC	13.15 ± 0.06	-$(CH_2)_2$NH-Glu-Glu-Glu-DOTA	45.52 ± 3.01

Receptor affinities of the C-terminal modified Tyr^3-octreotate derivatives (IC_{50}, mean ± S.E.M).