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Metal-chelating benzothiazole multifunctional compounds for the modulation and $^{64}$Cu PET imaging of Aβ aggregation

Reported herein are a series of multifunctional compounds (MFCs) with appreciable affinity for amyloid aggregates that can be potentially used for both the modulation of amyloid β (Aβ) peptide aggregation and its toxicity, as well as for positron emission tomography (PET) imaging of Aβ aggregates by pre-chelation of these MFCs with the longer-lived $^{64}$Cu radioisotope. By evaluating the various properties of these MFCs, valuable structure-activity relationships were obtained that should aid the design of improved therapeutic and diagnostic agents for AD.

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Metal-chelating benzothiazole multifunctional compounds for the modulation and $^{64}$Cu PET imaging of Aβ aggregation†

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While Alzheimer’s Disease (AD) is the most common neurodegenerative disease, there is still a dearth of efficient therapeutic and diagnostic agents for this disorder. Reported herein are a series of new multifunctional compounds (MFCs) with appreciable affinity for amyloid aggregates that can be potentially used for both the modulation of Aβ aggregation and its toxicity, as well as positron emission tomography (PET) imaging of Aβ aggregates. Firstly, among the six compounds tested HYR-16 is shown to be capable to reroute the toxic Cu-mediated Aβ oligomerization into the formation of less toxic amyloid fibrils. In addition, HYR-16 can also alleviate the formation of reactive oxygen species (ROS) caused by Cu$^{2+}$ ions through Fenton-like reactions. Secondly, these MFCs can be easily converted to PET imaging agents by pre-chelation with the $^{64}$Cu radioisotope, and the Cu complexes of HYR-4 and HYR-17 exhibit good fluorescent staining and radiolabeling of amyloid plaques both in vitro and ex vivo. Importantly, the $^{64}$Cu-labeled HYR-17 is shown to have a significant brain uptake of up to 0.99 ± 0.04 % ID per g. Overall, by evaluating the various properties of these MFCs valuable structure–activity relationships were obtained that should aid the design of improved therapeutic and diagnostic agents for AD.

Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disease, associated with loss of memory and cognitive decline.1 An estimated 5.8 million Americans of all ages are living with Alzheimer’s dementia currently.2 The presence of amyloid plaques and neurofibrillary tangles in the brain is the hallmark of AD.3 Amyloid β (Aβ) peptides, the main component of amyloid plaques,4,7 are formed from the cleavage of amyloid precursor protein (APP) by β- and γ-secretases. The main alloforms of Aβ are Aβ$_{40}$ and Aβ$_{42}$, containing 40 and 42 amino acids, respectively.5 Even though Aβ$_{40}$ is present in the deposits in larger amounts, Aβ$_{42}$ exhibits higher neurotoxicity and aggregates more easily.6–12 In the past two decades, soluble Aβ oligomers have been found to be the most toxic form among all Aβ species13–18 through their interactions with membrane and synaptic receptors18–20 that influence intracellular systems21 and affect neurotransmission,22,23 leading to neurodegeneration.

In addition, the amyloid deposits contain uncommonly high concentrations of metal ions such as Fe$^{2+}$, Cu$^{2+}$ and Zn$^{2+},24,25$ and it has been found that these metal ions promote the formation of neurotoxic Aβ aggregates.26–29 Cu and Fe ions can also cause the formation of reactive oxygen species (ROS), which exacerbates Aβ toxicity.25,30–32 Previously, we have reported that Cu$^{2+}$ ions can slow down Aβ fibrillization and stabilize Aβ oligomers,33,34 and thus small molecules that can inhibit the interaction between metal ions and Aβ peptides, can be used as potential therapeutic compounds for AD.35–38

Moreover, the development of novel diagnostic agents is essential for the prevention and treatment of AD. Recently, several positron emission tomography (PET) compounds have been approved by FDA and can be used to visualize amyloid plaques in AD patients. However, these radiolabeled agents are employing short-lived radionuclides, such as $^{11}$C and $^{18}$F ($t_{1/2} = 20.4$ min and $109.8$ min,39–42 respectively), thus limiting their widespread use. Thus, the development of longer-lived radiolabeled compounds is essential for further expanding the use of PET imaging in healthcare, and diagnostic agents employing longer-lived radionuclides such as $^{64}$Cu ($t_{1/2} = 12.7$ h, $\beta^- = 17\%$, $\beta = 39\%$, EC = 43%, $E_{max} = 0.656$ MeV) are viewed as optimal PET imaging agents.43–47

† Electronic supplementary information (ESI) available: Experimental section and Fig. S1–S8. See DOI: 10.1039/d0sc02641g
We have previously reported first-generation multifunctional compounds (MFCs) that contain Aβ binding and metal chelating fragments\(^{33,48}\) and these MFCs were found to have the ability to modulate the aggregation of Aβ\(_{12}\) species, although some led to the formation of neurotoxic soluble Aβ\(_{12}\) oligomers.\(^{33,48}\) Moreover, some MFCs could be converted into PET imaging agents through pre-chelation with \(^{64}\)Cu and have with MFCs, PET imaging agents could be obtained for the diagnosis of Alzheimer’s disease.\(^{45-50}\) Herein, we report a series of second-generation multifunctional compounds (MFCs) containing metal-binding and Aβ-interacting fragments that also exhibit additional key properties (Fig. 1). In contrast to the first-generation MFCs, which were shown to promote the formation of neurotoxic oligomeric Aβ species,\(^{33,48}\) these MFCs are capable of re-routing the neurotoxic metal-stabilized Aβ oligomers into less toxic aggregates, while also decreasing the formation of ROS. Moreover, these MFCs can be easily converted into PET imaging agents by chelation with the \(^{64}\)Cu radionuclide, and \textit{ex vivo} labeling studies using AD mouse brain sections reveal that the MFCs and their Cu complexes can clearly label the amyloid plaques. In addition, biodistribution studies show that the \(^{64}\)Cu-radiolabeled compounds cross the blood–brain barrier (BBB) efficiently and thus should be able to act as potential therapeutic or imaging agents \textit{in vivo}. Finally, structure–activity relationship (SAR) studies suggest that the presence of a monomethylamine group leads to increased specificity for binding to the Aβ aggregates, while the introduction of a pyridyl group is essential for modulating the neurotoxicity of metal–Aβ species. Most importantly, repositioning of the hydroxyl group and the metal-chelating azamacrocycle on the benzothiazole ring in HYR-17 has a dramatic effect on improving the brain uptake of the corresponding \(^{64}\)Cu complex, which could be a useful design approach for the development of improved PET imaging agents.

**Results and discussion**

**Design, synthesis and characterization of MFCs**

Following up on our previous results, we set out to develop second-generation MFCs aimed to better alleviate the metal-induced Aβ toxicity, while also obtaining valuable structure–activity relationships (SAR) that should give us insight into the design of improved therapeutic and imaging agents for AD.

Previously, 2-aryl-benzothiazole derivatives have been found to have appreciable Aβ binding affinity and fluorescence properties,\(^{17,55}\) and thus we continued to use such molecular frameworks. Inspired by the structure of Pittsburgh compound B (PIB, Fig. 1), a widely-used amyloid-binding compound with high Aβ binding affinity,\(^{29}\) we introduced a hydroxyl group in the 6-position of the benzothiazole aromatic ring (Fig. 1). In addition, dimethylamino and monomethylamino groups were introduced at the 4’ position of the phenyl ring, since such functional groups are present in many Aβ binding compounds such as Thioflavin T (ThT) and Florbetapir.\(^{43,51}\) Then, we have introduced a 1,4-dimethyl-1,4,7-triazacyclononane (tacn) metal-chelating group attached to the benzothiazole aromatic ring in order to generate a MFC that can modulate the metal–Aβ interactions. Importantly, previous studies have shown that the tacn-phenolate metal-chelating fragment can bind more tightly to Cu versus other metal ions such as Zn and Fe.\(^{52-54}\) Accordingly, HYR-1 and HYR-4 were developed, and we have also designed HYR-14 and HYR-16 as 3’-pyridyl analogues in order to probe the effect of a hydrogen-bond acceptor pyridyl group vs. a phenyl group.\(^{42,55}\) Moreover, it was previously found that a PIB derivative with a hydroxyl group in the 4-position of benzothiazole framework performed similarly as PIB in Aβ binding and biodistribution studies,\(^{33}\) and thus we have designed HYR-17, which contains a 4-hydroxyl substituent and has the tacn azamacrocycle connected to the 5 position of the benzothiazole ring (Fig. 1). Finally, the MFC HYR-18 that contains two 2-aryl-benzothiazole fragments attached to one tacn azamacrocycle was also synthesized, in order to probe whether additional Aβ binding fragments will improve the affinity for Aβ aggregates.

The synthesis of the MFCs HYR-1, -4, -14, -16, -17 and -18 follows a stepwise sequence of steps that typically includes an oxidative cyclization step between a 2-amino-methoxybenzenethiol derivative and a benzaldehyde or benzoic acid derivative to generate the 2-aryl-benzothiazole fragments (Scheme 1). In cases where the 2-amino-methoxybenzothiazole precursor was readily available, hydrolysis under basic conditions afforded the 2-amino-methoxybenzenethiol starting materials, while for the pyridyl derivatives 2-amino-5-(trifluoromethyl)pyridine was employed in the oxidative cyclization reaction. Reduction of the nitro group to the aniline derivative using tin(II) chloride (if needed) and subsequent N-monomethylation using paraformaldehyde and sodium borohydride or N-dimethylation using paraformaldehyde and sodium cyanoborohydride generated the 2-(4’-aniline-aryl)-benzothiazole derivatives. Deprotection of the methoxy group using boron tribromide afforded the hydroxyl-benzothiazole derivatives, while the last synthetic step for all
compounds is the Mannich reaction with paraformaldehyde and 2,4-dimethyl-1,4,7-triazacyclononane under reflux to generate the targeted MFCs (Scheme 1).

**ThT fluorescence competition assays**

In order to measure the binding affinity of MFCs toward amyloid fibrils, ThT fluorescence competition assays were performed. The Aβ_{40} peptide was used in these experiments, since it is known that Aβ_{40} forms well-defined amyloid fibrils.56,57 Excitingly, HYR-1 and HYR-4 exhibit nanomolar affinities for the Aβ_{40} fibrils with Kᵢ values of 11 ± 7 nM and 85 ± 9 nM, respectively, indicating that the MFCs can replace ThT efficiently and bind tightly to the Aβ_{40} fibrils (Fig. 2). However, for the other MFCs it was difficult to obtain reproducible Kᵢ values, likely due to the larger structural differences between these MFCs and ThT and thus the inability to compete with ThT for binding the Aβ_{40} fibrils.

![Scheme 1](image-url)  
**Scheme 1** Synthesis of the investigated MFCs HYR-1, -4, -14, -16, -17 and -18. The metal-binding and Aβ-interacting fragments are shown in blue and red, respectively. Reagents and conditions: (1a) KOH, H₂O, ethylene glycol, reflux, 48 h; (1b) DMSO, 170 °C, 30 min; (1c) BBr₃, DCM, rt, 24 h; (1d) Me₂HTACN, (CH₂O)ₙ, MeCN, reflux, 24 h; (2a) DMSO, 125 °C, overnight; (2b) SnCl₂, EtOH, conc. HCl, reflux, 3 h; (2c) (i) (CH₂O)ₙ, NaOMe, MeOH, reflux, 2 h; (ii) NaBH₄, 0 °C to rt, 1 h; (2d) BBr₃, DCM, rt, 24 h; (2e) Me₂HTACN, (CH₂O)ₙ, MeCN, reflux, 24 h; (2f) Me₂HTACN, (CH₂O)ₙ, MeCN, reflux, 24 h; (3a) NaOH (1 M), 90 °C, 3 h; (3b) (CH₂O)ₙ, NaBH₃CN, acetic acid, rt, overnight; (3c) BBr₃, DCM, rt, 24 h; (3d) Me₂HTACN, (CH₂O)ₙ, MeCN, reflux, 24 h; (3e) (i) (CH₂O)ₙ, NaOMe, MeOH, reflux, 2 h; (ii) NaBH₄, reflux, 1 h; (3f) BBr₃, DCM, rt, 24 h; (4a) KOH, H₂O, ethylene glycol, reflux, 48 h; (4b) DMSO, 125 °C, 30 min; (4c) BBr₃, DCM, rt, 24 h; (4d) Me₂HTACN, (CH₂O)ₙ, MeCN, reflux, 24 h.

![Fig. 2](image-url)  
**Fig. 2** ThT fluorescence competition assays for MFCs HYR-1 and HYR-4 with ThT-bound Aβ_{40} fibrils ([Aβ] = 5 μM, [ThT] = 2 μM).
the same binding site(s) on the amyloid fibrils and that not lead to an appreciable decrease in ThT fluorescence. In addition, we consider that probing the affinity of the MFCs toward native amyloid plaques by performing ex vivo binding studies with transgenic AD mouse brain sections should provide more physiologically relevant results and also rule out any non-specific binding (see below).

**Fluorescence imaging of amyloid plaques in 5×FAD mice brain sections**

Brain sections collected from 8 month-old 5×FAD transgenic mice were employed in these ex vivo Aβ binding studies. The transgenic 5×FAD mice overexpress mutant forms of the amyloid precursor protein (APP) and presenilin 1, develop amyloid plaque deposits at an younger age, and show progressive cognitive impairment similar to that found in AD in humans.\(^6\)

Interestingly, incubation of the 5×FAD mouse brain sections with different MFCs for 1 hour reveals significant fluorescent staining of the amyloid plaques, as confirmed by co-staining with Congo Red (CR), a well-known amyloid-binding fluorescent dye (Fig. 3). Among the MFCs, HYR-1, -4, -17, and -18 show significant amyloid staining at low concentration (25 μM), suggesting an appreciable Aβ binding affinity. The most specific amyloid-binding MFCs are HYR-4 and HYR-18 (and HYR-17 to a slightly lesser extent), which bind to the dense core of the amyloid plaques and thus show the best colocalization with CR. By contrast, HYR-1 seems to label other endogenous proteins, both in AD and WT brain sections (Fig. 3 and S5,\(^7\) respectively). Also, HYR-1 can label other regions in wild type mice brain section (Fig. S5f), which means its specificity is extremely low. By comparison, the pyridyl-containing MFCs HYR-14 and HYR-16 exhibit appreciable amyloid plaque staining only at high concentrations (250–500 μM), suggesting that their Aβ binding affinity is somewhat reduced and thus these two compounds were not employed in radiolabeling studies (see below).

We have also probed the ability of the Cu\(^{2+}\) complexes of HYR-4, -17 and -18 to label the amyloid plaques in 5×FAD mice brain sections (Fig. 4). In this case, higher ligand to Congo Red ratios were used since the Cu\(^{2+}\) ions lead to some fluorescence quenching for our MFCs (Fig. S5f). However, the Cu\(^{2+}\) complexes of HYR-4, -17 and -18 can still label the amyloid plaques efficiently (Fig. 3), with the complexes of HYR-4 and -18...
showing higher specificity compared with the Cu-HYR-17 complex. Finally, the fluorescently labeled CF594-6E10 antibody – which binds to a wide range of Aβ species, was employed to confirm that our MFCs and their Cu²⁺ complexes are able to specifically label the Aβ⁴₂ species (Fig. 5). It is important to note that we have previously determined the stability constants for the Cu²⁺ complex of another 1,4,7-triazacyclononane-phenolate ligand, which was shown to bind Cu²⁺ very tightly.⁴⁹,⁵⁰ Since the structural changes for the different MFCs described herein are remote from the Cu-binding site, we envision that the stability constants for the corresponding Cu²⁺ complexes will not vary significantly.

**Modulation of metal-free and metal-induced Aβ aggregation**

The ability of these MFCs to modulate the aggregation of Aβ⁴₂ was then explored, both in the absence or presence of Cu²⁺ ions. The Aβ⁴₂ peptide was used since it was shown to form neurotoxic soluble Aβ⁴₂ oligomers.¹²,¹³,¹⁸ Freshly prepared monomeric Aβ⁴₂ solutions were treated with MFCs, Cu²⁺, or both, and incubated for 24 hours at 37 °C, and the resulting samples were analysed by native gel electrophoresis/western blot analysis and transmission electron microscopy (TEM, Fig. 6). The former analysis method reveals the presence of smaller, soluble Aβ aggregates and their MW distribution, while the latter method

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**Fig. 5** Fluorescence microscopy images of 5xFAD mice brain sections incubated with MFCs HYR-4, -17 and -18 or their Cu(II) complexes (left panels), CF594-6E10 antibody (middle panels), and merged images (right panels). Scale bar: 100 μm.

**Fig. 6** Effects of the compounds on Cu²⁺-free and Cu²⁺-induced Aβ⁴₂ aggregation. (a) Scheme of the inhibition experiment: freshly prepared Aβ⁴₂ (25 μM) in the presence or absence of Cu²⁺ (25 μM) and with or without MFCs (25 μM) was incubated at 37 °C for 24 h with constant agitation. (b) Native gel/western blot analysis of the resulting Aβ⁴₂ species using the 6E10 anti-Aβ antibody. (c) Representative TEM images of the Aβ⁴₂ aggregates upon incubation with or without MFCs (scale bar, 200 nm). (d) Representative TEM images of the Aβ⁴₂ aggregates upon incubation with Cu²⁺ and with or without MFCs (scale bar, 200 nm).
provides the characterization of the larger, insoluble Aβ aggregates that cannot penetrate the gel, and thus allowing us to visualize all Aβ42 aggregates of various types and sizes.

While the aggregation of Aβ42 in the absence of Cu2+ ions leads to well-defined Aβ42 fibrils – as confirmed by TEM (Fig. 6c), the Aβ42 aggregation in the presence of Cu2+ ions generates a limited amount of Aβ fibrils (Fig. 6d), and native gel/western blotting shows that the aggregation of Aβ42 with Cu2+ ions yields mostly soluble Aβ42 oligomers of various sizes (Fig. 6b). These results are consistent with our previous studies which suggest that Cu2+ can stabilize the soluble Aβ42 oligomers and slow down the Aβ42 aggregation.13,29

All MFCs HYR-1, -4, -14, -16 and -17 did not seem to significantly inhibit the Aβ42 aggregation in the absence of Cu2+, even though some morphological changes were observed for the Aβ42 fibrils (Fig. 6b and c). Interestingly, the presence of HYR-16 had a dramatic effect on the Cu2+-mediated oligomerization of Aβ42 and promoted the formation of larger Aβ42 aggregates, as observed by TEM (Fig. 6d). Moreover, native gel/western blotting analysis reveals the presence of large, insoluble Aβ42 aggregates at the top of the gel, which were not observed for the Aβ42 aggregation in presence of only Cu2+ ions. Thus, HYR-16 is expected to control the neurotoxicity of Aβ42 species by accelerating the aggregation of toxic Aβ42 oligomers into nontoxic Aβ42 aggregates. By comparison, the other MFCs do not show a dramatic effect on Cu2+-mediated Aβ42 aggregation (Fig. 6b, left panels).

Cytotoxicity of MFCs and modulation of Aβ42 neurotoxicity

The neurotoxicity of the MFCs and their ability to alleviate the Cu-induced Aβ42 toxicity was performed using mouse neuroblastoma (N2a) cells. First, we examined the toxicity of all MFCs at various concentrations ranging from 2 to 20 μM (Fig. 7). Among the different MFCs, HYR-4, -14 and -16 exhibit no appreciable cell toxicity (>80% cell viability) up to 10 μM concentration, with HYR-16 showing no cell toxicity up to 20 μM concentration, and thus these three MFCs were employed in Aβ42-induced cytotoxicity studies. As we have shown before,23,24 the presence of both Cu2+ ions and Aβ42 leads to pronounced cell toxicity (~50% cell viability), likely due to the formation of neurotoxic Aβ42 oligomers (Fig. 8). Interestingly, HYR-16 can significantly alleviate the toxicity of Cu-stabilized Aβ42 oligomers and increase the cell viability up to 80% vs. a 1% DMSO control, which is consistent with in vitro results that HYR-16 can accelerate the aggregation of toxic Aβ42 oligomers into nontoxic Aβ42 aggregates. By comparison, HYR-4 and HYR-14 do not seem to significantly reduce the neurotoxicity of the Cu-Aβ42 species (Fig. 8), suggesting that the 2-monomethylamino-pyridyl fragment found in HYR-16 is needed for the efficient alleviation of Aβ42 oligomer neurotoxicity.

Antioxidant properties

Several previous reports have shown that the Cu2+ ions can interact with various Aβ species and lead to formation of reactive oxygen species (ROS) such as H2O2 and hydroxyl radicals (OH·).31,66 In this regard, the antioxidant capacity of the pyridine derivatives HYR-14 and HYR-16 – the least toxic MFCs investigated herein, was first evaluated via the Trolox equivalent antioxidant capacity (TEAC) assay.61 Both HYR-14 and HYR-16 showed better performance on scavenging the free radical ABTS+ (ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) than glutathione – a well-known antioxidant, at all the selected time points (1–15 min, Fig. 9a), with HYR-16 showing an antioxidant capacity similar to Trolox.

In addition, the ability of HYR-14 and HYR-16 to quench the Cu-induced hydroxyl radical (OH·) generation was measured by using coumarin-3-carboxylic acid (CCA) antioxidant assay.62 In this assay, the Cu2+ ions are reduced by ascorbic acid to Cu+ ions that then react with O2 to produce OH·. The non-fluorescent

![Fig. 7](image1)

**Fig. 7** Toxicty of MFCs at various concentrations in N2a cells, reported vs. a 1% DMSO control. The error bars indicate the standard deviations based on at least five samples per group.

![Fig. 8](image2)

**Fig. 8** The effect of MFCs on Cu2+-induced Aβ42 cytotoxicity, reported vs. a 1% DMSO control. N2a cells were treated with Aβ42 (20 μM), CuCl2 (20 μM), and MFCs (10 μM) and incubated for 40 h at 37 °C. The error bars indicate the standard deviations based on at least three samples per group, and the statistical analysis was evaluated according to one-way ANOVA (****p < 0.0001).
CCA reacts with OH\(^{-}\) to produce the fluorescent 7-hydroxycoumarin-3-carboxylic acid (CCA-OH), and CCA-OH formation can be monitored to evaluate the efficacy of ligand binding to Cu and inhibition of OH\(^{-}\) generation. At 80 \(\mu\)M, both of HYR-14 and HYR-16 show a strong Cu\(^{2+}\) binding affinity (corresponding to a 2 : 1 MFC : Cu ratio) and limit the generation of OH\(^{-}\) (Fig. 9b and c), while HYR-16 is able to limit the generation of OH\(^{-}\) even at 40 \(\mu\)M (corresponding to a 1 : 1 MFC : Cu ratio). Overall, these studies reveal that HYR-16 is the most potent anti-oxidant among the investigated MFCs, and along with its ability to efficiently destabilize the neurotoxic soluble \(\text{A}^\beta_{42}\) oligomers strongly suggest that HYR-16 may potentially exhibit therapeutic properties in animal studies.

### Radiolabeling and log \(D_{\text{oct}}\) Value Determination

One crucial factor for developing imaging agents for neurodegenerative diseases is that they should be able to effectively cross the blood–brain barrier (BBB). To determine the hydrophobicity of the radiolabeled compounds, the octanol/PBS partition coefficient values log \(D_{\text{oct}}\) were determined for the \(^{64}\text{Cu}\) complexes of HYR-1, -4, -14, -16, -17, and -18. The obtained log \(D_{\text{oct}}\) values for the \(^{64}\text{Cu}\)-radiolabeled complexes HYR-1, -4, -17 and -18 are in the range of 1.08–1.30 (Table 1), which supports their potential ability to cross the BBB, since log \(D\) values between 0.9 and 2.5 are considered optimal.\(^{43}\) In contrast, the \(^{64}\text{Cu}\) complexes of HYR-14 and -16 exhibit log \(D_{\text{oct}}\) values of \(\sim 0.6\), suggesting that 2-pyridyl-benzothiazole derivatives may be too hydrophilic to cross the BBB. Thus, only the MFCs HYR-1, -4, -17 and -18 were employed in the subsequent radiochemistry studies.

### Ex vivo Autoradiography Studies

Ex vivo autoradiography studies using brain sections of transgenic 5\(\times\)FAD mice were also performed to determine the
specific binding to the amyloid plaques of the $^{64}$Cu-labeled HYR-1, -4, -17 and -18. The brain sections were stained, washed, and radioimaged as described in the experimental section. When compared with WT brain sections that show a limited background intensity (Fig. 10, first row), the $^{64}$Cu-labeled complexes of HYR-4, -17 and -18 show an increased autoradiography intensity for the 5×FAD mouse brain sections (Fig. 10, second row). The $^{64}$Cu-labeled HYR-1 also exhibits non-specific binding as appreciable autoradiography intensity is observed for WT brain sections, which is consistent with the brain section fluorescent imaging studies described above. Finally, the specific binding to amyloid plaques of the $^{64}$Cu-labeled MFCs was confirmed by blocking with the non-radioactive blocking agent B₁ (Fig. S6†), which led to a markedly decreased autoradiography intensity (Fig. 10, third row). Overall, these autoradiography results strongly suggest that the $^{64}$Cu-labeled MFCs HYR-4, -17 and -18 have the necessary amyloid-binding specificity to be used as imaging agents in vivo. The MFC HYR-18, the bis-(2-phenylbenzothiazole) analogue of HYR-4, exhibits a slightly lower log $D_{\text{oct}}$ value than HYR-4, and it was also perceived to have a too large MW for an efficient brain uptake. Since HYR-4 already exhibits a promising log $D_{\text{oct}}$ value and specific binding to amyloid plaques, we decided to not include HYR-18 in the in vivo biodistribution studies.

**Biodistribution studies**

Encouraged by the promising in vitro radiolabeling and amyloid-binding studies, in vivo biodistribution experiments were performed to probe the uptake of $^{64}$Cu-labelled HYR-4 and -17 complexes using normal CD-1 mice. The retention and accumulation of the $^{64}$Cu-labeled complexes in selected organs was evaluated at 2, 60, and 240 minutes after tracer administration. Excitingly, $^{64}$Cu-HYR-17 shows an appreciable brain uptake of 0.99 ± 0.04 % injected dose per gram (%ID per g) at 2 min post injection, which drops to 0.20 ± 0.01 %ID per g at 60 min (Fig. 11 and Table 2). By comparison, $^{64}$Cu-HYR-4 shows a relatively low brain uptake of 0.16 ± 0.02 %ID per g at 2 min post injection, although $^{64}$Cu-HYR-4 shows a similar blood uptake to that of $^{64}$Cu-HYR-17 (Fig. 12), and suggesting that the structure of HYR-17 containing the 4-hydroxy substituent and tacn azamacrocycle connected to the 5 position of the benzothiazole ring should lead to improved brain uptake properties for the corresponding $^{64}$Cu-labeled complexes. Overall, these biodistribution studies strong suggest that the $^{64}$Cu-HYR-17 complex can efficiently cross the BBB and thus could serve as a PET imaging agent for the detection of Aβ aggregates in vivo. Importantly, the rapid clearance from the brain of WT mice suggest that these radiolabeled MFCs do not release $^{64}$Cu in the brain to an appreciable extent, and thus should not lead to a significant background PET signal in WT or healthy controls.

**Table 2** Overall biodistribution results of $^{64}$Cu-Labeled HYR-4 and HYR-17 for the three time points evaluated (2, 60, and 240 min; % injected dose/gram, mean ± SEM)

<table>
<thead>
<tr>
<th>Organ</th>
<th>HYR-4</th>
<th>HYR-17</th>
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<tr>
<td></td>
<td>2 min</td>
<td>60 min</td>
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<tr>
<td>Blood</td>
<td>2.85 ± 0.52</td>
<td>1.02 ± 0.12</td>
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<tr>
<td>Lung</td>
<td>3.77 ± 0.88</td>
<td>3.36 ± 0.25</td>
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<td>Liver</td>
<td>32.48 ± 3.94</td>
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<td>Kidney</td>
<td>30.27 ± 0.44</td>
<td>9.39 ± 2.02</td>
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<tr>
<td>Muscle</td>
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<td>0.30 ± 0.02</td>
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<tr>
<td>Brain</td>
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<td>Tail</td>
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Conclusions

Herein we report several metal-chelating benzothiazole multifunctional compounds (MFCs) and investigate their various biochemical, in vitro, and in vivo properties to bind to various Aβ species, modulate Aβ aggregation and its neurotoxicity, and potentially act as 64Cu PET imaging agents for in vivo detection of Aβ aggregates. During these studies, we have obtained important structure–activity relationships (SAR) that will guide us to develop improved MFCs as potential therapeutic or diagnostic agents for AD. Firstly, when comparing HYR-4 vs. HYR-1 and HYR-16 vs. HYR-14, we can conclude that the compounds containing a mono-methylamino vs. a dimethylamino group exhibit an increased specificity for the Aβ aggregates as well as reduced cytotoxicity. Secondly, the introduction of a pyridyl group in MFCs such as HYR-14 and HYR-16 dramatically reduces their cytotoxicity and improves their antioxidant properties. Taken together, the MFC HYR-16 containing the 2-monomethylamino-pyridyl fragment is most effective at alleviating the Aβ oligomer neurotoxicity and modulating Aβ aggregation. Thirdly, all investigated MFCs can be efficiently radiolabeled with the 64Cu radioisotope, and the 64Cu complexes of HYR-4, HYR-17, and HYR-18 exhibit acceptable lipophilicity and specific radiolabeling of amyloid plaques ex vivo.

Interestingly, the position of the hydroxyl group and the metal-chelating tac azamacrocycle on the benzothiazole ring has a dramatic effect on the BBB permeability of these 64Cu-labelled MFCs: the 64Cu complex of HYR-17 – which contains the 4-hydroxyl substituent and tac azamacrocycle connected to the 5 position of the benzothiazole ring, exhibits the highest brain uptake. Finally, our results suggest that employing HYR-18, the bis-(2-phenylbenzothiazole) analogue of HYR-4, does not improve the lipophilicity or specificity for amyloid plaques, while unnecessarily increasing the MW and thus possibly limiting its BBB permeability.

Overall, these detailed studies suggest that HYR-16 is the most effective MFC at alleviating the neurotoxicity of soluble Aβ oligomers, and thus it lends promise to the use of HYR-16 and its second-generation derivatives in future animal studies to evaluate their therapeutic properties. Moreover, HYR-17 exhibits the largest brain uptake and ex vivo specificity for native amyloid plaques, and derivatives of this MFC with similar positioning of the benzothiazole ring substituents will be used as lead compounds for microPET imaging studies in WT vs. AD transgenic mice, toward the development of improved 64Cu PET imaging agents for AD diagnosis.

Ethical statement

All animal studies were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) and were in compliance with the Guidelines for Care and Use of Research Animals established by the Division of Comparative Medicine and the Animal Studies Committee of Washington University in St. Louis. The animal protocol #20190073 including all the animal studies performed herein was reviewed and approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis.

Conflicts of interest

There are no conflicts to declare.

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Notes and references


