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 Ab aggregation and its toxicity, as well as positron emission tomography (PET) imaging of Ab aggregates. Firstly, among the six compounds tested HYR-16 is shown to be capable to reroute the toxic Cu-mediated Ab 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/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.
Metal-Chelating Benzothiazole Multifunctional Compounds for the Modulation and $^{64}$Cu PET Imaging of Aβ Aggregation

Yiran Huang,† Hong-Jun Cho,† Nilantha Bandara,‡ Liang Sun,† Diana Tran,‡ Buck E. Rogers,*‡ and Liviu M. Mirica*†,v

† Department of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Avenue, Urbana, Illinois 61801, United States
‡ Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri 63108, United States
v Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110, United States

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ABSTRACT: 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 in vivo. Importantly, the $^{64}$Cu-labeled HYR-17 is shown to have a significant brain uptake of up to 0.99 ± 0.04 %ID/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 Aβ40 and Aβ42, containing 40 and 42 amino acids, respectively.8 Even though Aβ40 is present in the deposits in larger amounts, Aβ42 exhibits higher neurotoxicity and aggregates more easily.9-12 In the past two decades, soluble Aβ oligomers have been found to be the most toxic form among all Aβ species9-19 through their interactions with membrane and synaptic receptors8,10 that influence intracellular systems10 and affect neurotransmission,12-23 leading to neurodegeneration.

In addition, the amyloid deposits contain uncommonly high concentrations of metal ions such as Fe$^{3+}$, 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$\text{1/2}$ = 20.4 min and 109.8 min),39-45 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$\text{1/2}$ = 12.7 h, β$^-$ = 17%, β$^+$ = 39%, EC = 43%, E$_{\text{max}}$ = 0.656 MeV) are viewed as optimal PET imaging agents.46-47
Herein, we report a series of second-generation multifunctional compounds (MFCs) containing metal-binding and Aβ-interacting fragments that also exhibit additional key properties (Figure 1). In contrast to the first-generation MFCs, which were shown to promote the formation of neurotoxic oligomeric Aβ species, 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 \(^{64}\text{Cu}\) 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 \(^{64}\text{Cu}\)-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 \(^{64}\text{Cu}\) 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.** We have previously reported the first-generation multifunctional compounds (MFCs) that contain Aβ binding and metal chelating parts, and these MFCs were found to have the ability to modulate the aggregation of Aβ species, although some led to the formation of neurotoxic soluble Aβ oligomers. Moreover, some MFCs could be converted into PET imaging agents through pre-chelation with \(^{64}\text{Cu}\) and have with MFCs, PET imaging agents could be obtained for the diagnosis of Alzheimer’s disease. Following up on these 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, and thus we continued to use such molecular frameworks. Inspired by the structure of Pittsburgh compound B (PiB, Figure 1), a widely-used amyloid-binding compound with high Aβ binding affinity, we introduced a hydroxyl group in the 6-position of the benzothiazole aromatic ring (Figure 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.

Then, we have introduced a triazacyclononane (tacn) metal-chelating group attached to the benzothiazole aromatic ring in order to generate a MFC that can modulate the metal-Aβ interactions. 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. 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 bio-distribution studies, 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 (Figure 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-methoxy-benzothiazole 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-di-
methylation using paraformaldehyde and sodium cyano-
borohydride generated the 2-(4'-aniline-aryl)-benzothia-
zole derivatives. Deprotection of the methoxy group using
boron tribromide afforded the hydroxyl-benzothiazole de-
rivatives, while the last synthetic step for all compounds is
the Mannich reaction with paraformaldehyde and 2,4-di-
methyl-1,4,7-triazacyclononane under reflux to generate
the targeted MFCs (Scheme 1).

Scheme 1. Synthesis of the investigated MFCs HYR-1, -4, -14, -16, -17 and -18. The metal-binding and Aβ-interacting frag-
ments 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, O °C to rt, 1 h; (2d) BBr3,
DCM, rt, 24 h; (2e) Me2HTACN, (CH2O)n, MeCN, reflux, 24 h; (2f) MeH2TACN, (CH2O)n, MeCN, reflux, 24 h; (3a) NaOH
(1M), 90 °C, 3 h; (3b) (CH2O)n, NaBH4CN, 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; (3g)
Me2HTACN, (CH2O)n, MeCN, reflux, 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.
**ThT Fluorescence Competition Assays.** In order to measure the binding affinity of MFCs toward amyloid fibrils, ThT fluorescence competition assays were performed. The Aβ40 peptide was used in these experiments, since it is known that Aβ forms well-defined amyloid fibrils.\(^5\)\(^-\)\(^8\) Excitingly, HYR-1 and HYR-4 exhibit nanomolar affinities for the Aβ40 fibrils with \(K_i\) values of 11 ± 7 nM and 85 ± 9 nM, respectively, indicating that the MFCs can replace ThT efficiently and bind tightly to the Aβ40 fibrils (Figure 2). However, for the other MFCs it was difficult to obtain reproducible \(K_i\) values, likely due to the more significant structural differences between these compounds and ThT. In addition, we consider that probing the affinity of the MFCs toward native amyloid plaques by performing ex vivo binding studies with transgenic AD mice brain sections should provide more physiologically relevant results and also rule out any non-specific binding (see below).

![Figure 2](image-url) **Figure 2.** ThT fluorescence competition assays for MFCs HYR-1 and HYR-4 with Aβ fibrils ([Aβ] = 5 μM, [ThT] = 2 μM).

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

| MFC   | Conc. (μM) | MFC : Congo Red ratio |
|-------|-----------|-----------------------|
| HYR-1 | 25        | 5:1                   |
| HYR-4 | 25        | 5:1                   |
| HYR-14| 500       | 100:1                 |
| HYR-16| 250       | 50:1                  |
| HYR-17| 25        | 5:1                   |
| HYR-18| 25        | 5:1                   |

![Figure 3](image-url) **Figure 3.** Fluorescence microscopy images of 5xFAD mice brain sections incubated with MFCs HYR-1, -4, -14, -16, -17, and -18 (left panels), Congo Red (CR, middle panels), and merged images (right panels, \(R = \) Pearson's coefficient). Scale bar: 100 μm. The concentration of MFC and MFC:CR ratios used are listed in the top table.

Interestingly, incubation of the 5xFAD mouse brain sections with the 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 (Figure 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 (Figures 3 and S5, respectively). Also, HYR-1 can label other regions in wild type mice brain section (Figure S5), 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 Cu²⁺ complexes of HYR-4, -17 and -18 to label the amyloid plaques in 5xFAD mice brain sections (Figure 4). In this case, higher ligand to Congo Red ratios were used since the Cu²⁺ ions lead to some fluorescence quenching for our MFCs (Figure S5, S6). However, the Cu²⁺ complexes of HYR-4, -17 and -18 can still label the amyloid plaques efficiently (Figure 3), with the complexes of HYR-4 and -18 showing higher specificity compared with the Cu²⁺-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 Cu\(^{2+}\) complexes are able to specifically label the A\(\beta_{42}\) species (Figure 5).

| Cu complex | Conc. (μM) | Complex : CR ratio |
|------------|------------|--------------------|
| Cu-HYR-4   | 50         | 10:1               |
| Cu-HYR-17  | 50         | 10:1               |
| Cu-HYR-18  | 50         | 10:1               |

**Table 1.** Concentration of MFC and MFC:CR ratios used.

![Figure 4](image)

**Figure 4.** Fluorescence microscopy images of 5x:FAD mice brain sections incubated with the Cu\(^{2+}\) complexes of HYR-4, -17 and -18 (left panels), Congo Red (middle panels), and merged images (right panels, R = Pearson’s coefficient). The concentration of MFC and MFC:CR ratios used are listed in the top table. Scale bar: 100 μm.

![Figure 5](image)

**Figure 5.** Fluorescence microscopy images of 5x:FAD mice brain sections incubated with MFCs HYR-4, -17, and -18 or their Cu\(^{2+}\) complexes (left panels), CF594-6E10 antibody (middle panels), and merged images (right panels, R = Pearson’s coefficient). Scale bar: 100 μm.

**Modulation of Metal-free and Metal-induced A\(\beta\) Aggregation.** The ability of these MFCs to modulate the aggregation of A\(\beta_{42}\) was then explored, both in the absence or presence of Cu\(^{2+}\) ions. The A\(\beta_{42}\) peptide was used since it was shown to form neurotoxic soluble A\(\beta_{42}\) oligomers.\(^{12-13,16}\) Freshly prepared monomeric A\(\beta_{42}\) solutions were treated with MFCs, Cu\(^{2+}\), 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, Figure 6). The former analysis method reveals the presence of smaller, soluble A\(\beta\) aggregates and their MW distribution, while the latter method provides the characterization of the larger, insoluble A\(\beta\) aggregates that cannot penetrate the gel, and thus allowing us to visualize all A\(\beta_{42}\) aggregates of various types and sizes.

While the aggregation of A\(\beta_{42}\) in the absence of Cu\(^{2+}\) ions leads to well-defined A\(\beta_{42}\) fibrils – as confirmed by TEM (Figure 6c), the A\(\beta_{42}\) aggregation in the presence of Cu\(^{2+}\) ions generates a limited amount A\(\beta\) fibrils (Figure 6d), and native gel/Western blotting shows that the aggregation of A\(\beta_{42}\) with Cu\(^{2+}\) ions yields mostly soluble A\(\beta_{42}\) oligomers of various sizes (Figure 6b). These results are consistent with our previous studies which suggest that Cu\(^{2+}\) can stabilize the soluble A\(\beta_{42}\) oligomers and slow down the A\(\beta_{42}\) aggregation.\(^{13,16}\)

All MFCs HYR-1, -4, -14, -16 and -17 did not seem to significantly inhibit the A\(\beta_{42}\) aggregation in the absence of Cu\(^{2+}\), even though some morphological changes were observed for the A\(\beta_{42}\) fibrils (Figures 6b and 6c). Interestingly, the presence of HYR-16 had a dramatic effect on the Cu\(^{2+}\)-mediated oligomerization of A\(\beta_{42}\) and promoted the formation of larger A\(\beta_{42}\) aggregates, as observed by TEM (Figure 6d). Moreover, native gel/Western blotting analysis reveals the presence of large, insoluble A\(\beta_{42}\) aggregates at the top of the gel, which were not observed for the A\(\beta_{42}\) aggregation in presence of only Cu\(^{2+}\) ions. Thus, HYR-16 is expected to control the neurotoxicity of A\(\beta_{42}\) species by accelerating the aggregation of toxic A\(\beta_{42}\) oligomers into nontoxic A\(\beta_{42}\) aggregates. By comparison, the other MFCs do not show a dramatic effect on Cu\(^{2+}\)-mediated A\(\beta_{42}\) aggregation (Figure 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 (Figure 7). Among the different MFCs, HYR-4, -14, and -16 exhibit no appreciable cell toxicity (>80% cell viability) up to 20 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,33-34 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 (Figure 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 (Figure 8), suggesting that the 2-monomethylamino-pyridyl fragment found in HYR-16 is needed for the efficient alleviation of Aβ42 oligomer neurotoxicity.

Figure 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 absence or presence 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).

Figure 7. Toxicity 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.
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•). 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. 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, Figure 9a), with HYR-16 showing an antioxidant capacity similar to Trolox.

In addition, the ability of HYR-14 and HYR-16 to quench the Cu²⁺-induced hydroxyl radical (OH•) generation was measured by using coumarin-3-carboxylic acid (CCA) antioxidant assay. 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 CCA reacts with OH• to produce the fluorescent 7-hydroxy coumarin-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 µM, 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• (Figure 9b, c), while HYR-16 is able to limit the generation of OH• even at 40 µM (corresponding to a 1:3 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 Aβ₄₂ oligomers strongly suggest that HYR-16 may potentially exhibit therapeutic properties in animal studies.

**Radiolabeling and Log D<sub>oct</sub> 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<sub>oct</sub> were determined for the <sup>64</sup>Cu complexes of HYR-1, -4, -14, -16, -17 and -18. The obtained log D<sub>oct</sub> values for the <sup>64</sup>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. In contrast, the <sup>64</sup>Cu complexes of HYR-14 and -16 exhibit log D<sub>oct</sub> values of ~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.

| Ligand | MW (g·mol⁻¹) | log D<sub>oct</sub> |
|--------|--------------|--------------------|
| HYR-1  | 439.6        | 1.08 ± 0.11        |
| HYR-4  | 425.6        | 1.29 ± 0.19        |
| HYR-14 | 440.6        | 0.58 ± 0.05        |
| HYR-16 | 426.6        | 0.56 ± 0.17        |
| HYR-17 | 439.6        | 1.30 ± 0.15        |
| HYR-18 | 679.9        | 1.09 ± 0.11        |

Table 1. Molecular Weights for MFCs and log D<sub>oct</sub> values for the corresponding <sup>64</sup>Cu-labeled complexes.

When compared with WT brain sections that show a limited background intensity (Figure 10, first row), the <sup>64</sup>Cu-labeled complexes of HYR-4, -17 and -18 show an increased autoradiography intensity for the 5xFAD mouse brain sections (Figure 10, second row). The <sup>64</sup>Cu-labeled HYR-4 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 <sup>64</sup>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<sub>oct</sub> 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<sub>oct</sub> value and specific binding to amyloid plaques, we decided not to include HYR-18 in the in vivo biodistribution studies.

**Ex vivo Autoradiography Studies.** Ex vivo autoradiography studies using brain sections of transgenic 5xFAD mice were also performed to determine the specific binding to the amyloid plaques of the <sup>64</sup>Cu-labeled HYR-1, -4, -17 and -18. The brain sections were stained, washed, and radioimaged as described in the experimental section.

![Figure 10](image1.png)  
**Figure 10.** Autoradiography images of WT and 5xFAD mice brain sections using the <sup>64</sup>Cu-labeled HYR-1, -4, -17 and -18, and in the presence of a cold Aβ blocking agent.

![Figure 11](image2.png)  
**Figure 11.** Brain uptake (% injected dose/gram, %ID/g) results from the biodistribution studies in CD-1 mice, at 2, 60, and 240 min post injection.

![Figure 12](image3.png)  
**Figure 12.** Selected organs uptake (%ID/g) of <sup>64</sup>Cu-labeled HYR-4 and HYR-17 from the biodistribution studies in CD-1 mice, at 2, 60, and 240 min post injection.
Biodistribution Studies. Encouraged by the promising in vitro radiolabelling 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/gram (%ID/g) at 2 min post injection, which drops to 0.20 ± 0.01 %ID/g at 60 min (Figure 11 and Table 2). By comparison, $^{64}$Cu-HYR-4 shows a relatively low brain uptake of 0.16 ± 0.02 %ID/g at 2 min post injection, although $^{64}$Cu-HYR-4 shows a similar blood uptake to that of $^{64}$Cu-HYR-17 (Figure 12), and suggesting that the structure of HYR-17 containing the 4-hydroxyl 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 strongly 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 radiolabelled 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.

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 $^{64}$Cu 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 monomethylamino 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$\downarrow$ oligomer neurotoxicity and modulating Aβ aggregation. Thirdly, all investigated MFCs can be efficiently radiolabeled with the $^{64}$Cu radioisotope, and the $^{64}$Cu 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 tacn azamacrocycle on the benzothiazole ring has a dramatic effect on the BBB permeability of these $^{64}$Cu-labelled MFCs, with $^{64}$Cu-HYR-17 containing the 4-hydroxyl substituent and tacn azamacrocycle connected to the 5 position of the benzothiazole ring exhibiting high 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 the 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 $^{64}$Cu PET imaging agents for AD diagnosis.

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).

| Organ   | HYR-4          | HYR-17          |
|---------|----------------|-----------------|
|         | 2 min | 60 min | 240 min | 2 min | 60 min | 240 min |
| blood   | 2.85 ± 0.52 | 1.02 ± 0.12 | 0.86 ± 0.10 | 26.82 ± 1.59 | 3.70 ± 0.06 | 1.24 ± 0.08 |
| lung    | 3.77 ± 0.88 | 3.36 ± 0.25 | 4.47 ± 0.49 | 11.02 ± 0.52 | 3.65 ± 0.16 | 4.47 ± 0.49 |
| liver   | 32.48 ± 3.94 | 8.84 ± 0.98 | 9.57 ± 1.19 | 11.50 ± 0.69 | 14.79 ± 1.71 | 12.16 ± 1.28 |
| kidney  | 30.27 ± 0.44 | 9.39 ± 2.02 | 8.24 ± 1.95 | 15.96 ± 0.81 | 34.17 ± 2.87 | 21.40 ± 2.27 |
| muscle  | 0.37 ± 0.08 | 0.30 ± 0.02 | 0.30 ± 0.04 | 0.89 ± 0.04 | 0.62 ± 0.02 | 0.45 ± 0.01 |
| brain   | 0.16 ± 0.02 | 0.10 ± 0.01 | 0.15 ± 0.03 | 0.99 ± 0.04 | 0.20 ± 0.01 | 0.15 ± 0.02 |
| bone    | 0.54 ± 0.11 | 0.45 ± 0.01 | 0.59 ± 0.07 | 1.59 ± 0.07 | 0.59 ± 0.09 | 0.64 ± 0.07 |
| tail    | 15.91 ± 2.84 | 12.8 ± 5.38 | 3.69 ± 1.92 | 2.53 ± 0.16 | 3.20 ± 0.29 | 1.05 ± 0.05 |
ASSOCIATED CONTENT

Supporting Information. Detailed synthetic procedures, UV-vis and emission spectra of MFCs and Cu–MFC complexes, radio–HPLC traces, and NMR spectra of MFCs.

AUTHOR INFORMATION

Corresponding Author
* mirica@illinois.edu; b.rogers@wustl.edu

ORCID
Liviu Mirica: 0000-0003-0984-9508

Notes
The authors declare no competing financial interest.

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Metal-Chelating Benzothiazole Multifunctional Compounds for the Modulation and $^{64}$Cu PET Imaging of Aβ Aggregation

Yiran Huang,† Hong-Jun Cho,† Nilantha Bandara,‡ Liang Sun,† Diana Tran,‡ Buck E. Rogers,*‡ and Liviu M. Mirica*,†,Ⅴ

†Department of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Avenue, Urbana, Illinois 61801, United States
‡Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri 63108, United States
ⅤHope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110, United States

* mirica@illinois.edu; b.rogers@wustl.edu

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I. Experimental Procedures

General Methods. All reagents were purchased from commercial sources and used as received unless stated otherwise. 1,4-dimethyl-1,4,7-triazacyclononane (Me$_2$Htacn) and 1-methyl-1,4,7-triazacyclononane (MeH$_2$tacn) were synthesized according to reported procedures.$^{1-2}$ Solvents were purified prior to use by passing through a column of activated alumina using an MBRAUN SPS. All solutions and buffers were prepared using metal-free Millipore water that was treated with Chelex overnight and filtered through a 0.22 μm nylon filter. $^1$H (300.121 MHz) and $^{13}$C (151 MHz) NMR spectra were recorded on a Varian Mercury-300 spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane. UV−visible spectra were recorded on a Varian Cary 50 Bio spectrophotometer and are reported as $\lambda_{\text{max}}$, nm ($\varepsilon$, M$^{-1}$ cm$^{-1}$). TEM analysis was performed at the Nano Research Facility (NRF) at Washington University. HYR-1, -4, -14, -16, -17 and -18 were dissolved in DMSO to prepare 10.0 mM stock solutions.

Amyloid β Peptide Experiments. Aβ powder was prepared by dissolving commercial Aβ peptide (AnaSpec) in ammonia hydroxide solution (1%, v/v). The solution was then aliquoted out and lyophilized overnight. The resulting aliquoted powder was stored at −80 °C. Aβ monomers were generated by dissolving Aβ powder in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 1 mM) and incubating for 1 h at room temperature.$^3$ The solution was then evaporated overnight and dried by vacuum centrifuge to result monomeric films. Aβ fibrils were generated by dissolving monomeric Aβ films in DMSO, diluting into the appropriate buffer, and incubating for 24 h at 37 °C with continuous agitation (final DMSO concentration was < 2%).

Fluorescence Measurements. All fluorescence measurements were performed using a SpectraMax M2e plate reader (Molecular Devices). For ThT fluorescence studies, samples were diluted to a final concentration of 2.5 μM Aβ in PBS containing 10 μM ThT and the fluorescence measured at 485 nm ($\lambda_{\text{ex}} = 435$ nm). For ThT competition assays, a 5 μM Aβ fibril solution with 2 μM ThT was titrated with tiny amounts of compound and the ThT fluorescence measured ($\lambda_{\text{ex}}/\lambda_{\text{em}}$ = 435/485 nm). For calculating $K_i$ values, a $K_d$ value of 1.16 μM was used for the binding of ThT to Aβ fibrils.

Histological Staining of 5×FAD Mice Brain Sections. Eight-month-old 5×FAD transgenic mice brain sections were blocked with bovine serum albumin (2% BSA in PBS, pH 7.4, 10 min) and
covered with a PBS solution of compound and Congo Red (5 µM) for 60 min. The sections were treated with BSA again (4 min) to remove any compound non-specifically bound to the tissue. Finally, the sections were washed with PBS (3 × 2 min), DI water (2 min), and mounted with non-fluorescent mounting media. For antibody staining, the brain sections were incubated with CF594-conjugated anti-Aβ antibody (CF594-6E10 antibody) solution (1:1000 dilution in blocking solution) at room temperature for 1 h instead of Congo Red. The brain sections were then washed with PBS (3 × 2 min) and mounted with mounting media. The stained brain sections were imaged using a Zeiss LSM 7010 confocal fluorescent microscope.

Native Gel Electrophoresis and Western Blotting. All gels, buffers, membranes, and other reagents were purchased from Invitrogen and used as directed except where otherwise noted. Samples were separated on 10–20% gradient Tris-tricine mini gels. The gel was transferred to a nitrocellulose membrane in an ice bath and the protocol was followed as suggested except that the membrane was blocked overnight at 4 °C. After blocking, the membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-Aβ primary antibody (BioLegend) for 3 h followed by an alkalinephosphatase antimouse secondary antibody. The results were visualized using Invitrogen’s Western Breeze Chemiluminescent kit and the protein bands were imaged using a FUJIFILM Luminescent Image Analyzer LAS-1000CH.

Transmission Electron Microscopy (TEM). Glow-discharged grids (Formar/Carbon 300-mesh, Electron Microscopy Sciences) were treated with Aβ42 samples (25 µM, 7 µL) for 5 min at room temperature. The excess solution was removed using filter paper and the grids were rinsed with H2O (7 µL, 2 × 1 min). Then the grids were stained with uranyl acetate (1% w/v in H2O, 7 µL) for 2 min, rinsed with H2O (7 µL, 1 min), blotted with filter paper, and dried under vacuum overnight. Images were captured using a FEI G2 Spirit Twin microscope (60–80 kV, 6500–97000× magnification). TEM analysis was performed at the Nano Research Facility (NRF) at Washington University in St. Louis.

Trolox equivalent antioxidant capacity (TEAC) Assay. The antioxidant ability of multifunctional compounds was investigated by TEAC assay with the established protocol. ABTS (8.2 mg, 2,7 eq.) and K2S2O8 (1.6 mg, 1 eq.) were dissolved in 2 ml de-ionized water to prepare ABTS•• cation radicals. The reaction was proceeded overnight in the dark at room temperature. The ABTS•• solution was aliquoted (3-42 µL) into a transparent 96-well plate and diluted with
methanol to 300 μL that each volume was monitored at 470 nm via UV-vis spectroscopy to
determine the concentration of the radical solution displaying an absorbance around 0.7. The
absorbance of the ABTS** radicals increased linearly in this range and a volume of 36 μL was
selected for the following TEAC assay led an approximate absorbance around 0.7. Trolox,
glutathione, HYR-14 and HYR-16 were dissolved in methanol to prepare 1.5 mM stock solutions.
Each solution in different final concentrations (25, 50, 75, 100 μM) were added into 96-well plate
and diluted with methanol to a volume of 270 μL. After adding 36 μL ABTS solution into each
well, the absorbance at 470 nm was recorded at different time points (1, 3, 6, 15 min) immediately.

**Coumarin-3-carboxylic acid (CCA) antioxidant assay.** According to previous reports,⁵ CCA
was used to measure hydroxy radical production induced by Cu ions. The stock solutions (10 mM)
of CCA, CuSO₄, ascorbic acid, HYR-14 and HYR-16 were prepared in water. Each compound
was added into 96-well plate and diluted with PBS (1×, pH 7.4) to make final solution (final
concentration: CCA [100 μM], CuSO₄ [40 μM], ascorbic acid [400 μM], HYR-14 [0-160 μM]
and HYR-16 [0-160 μM], final volume: 200 μL). Then, the fluorescence of 7-hydroxycoumarin-
3-carboxylic acid was monitored under excitation at 395 nm and emission at 450 nm for 1 h using
a SpectraMax M2e plate reader (Molecular Devices, USA).

**Cytotoxicity Studies (Alamar Blue Assay).** Mouse neuroblastoma Neuro2A (N2A) cell lines
were purchased from the American Type Culture Collection (ATCC). Cells were grown in
DMEM/10% FBS, which is the regular growth media for N2A cells. N2A cells were plated to each
well of a 96 well plate (2.5 × 10⁴/well) with DMEM/10% FBS. The media was changed to
DMEM/N2 media 24 h later. After 1 h, the reagents (20 μM Aβ₄₂ species, compounds, and metals)
were added. Due to the poor solubility of compounds in water or media, the final amount of DMSO
used was 1% (v:v). After an additional incubation of 40 h, the Alamar blue solution was added in
each well and the cells were incubated for 90 min at 37 °C. Fluorescence intensity was measured
at 590 nm (excitation wavelength = 560 nm).

**Radiolabeling.** ⁶⁴Cu was produced by a (p,n) reaction on enriched ⁶⁴Ni on a TR-19 biomedical
cyclotron (Advanced Cyclotron Systems Inc, British Columbia, Canada) at Mallinckrodt Institute
of Radiology, Washington University School of Medicine, and purified with an automated system
using standard procedures.⁶ A stock solution of ⁶⁴CuCl₂ was diluted with a 10-fold excess of 0.1
M ammonium acetate (NH₄OAc), pH 7 for radiolabelling. Labelling of MFCs with ⁶⁴Cu was
achieved by adding 1 mM of compounds to 7.4 MBq (200 µCi) of $^{64}$CuCl$_2$ in 100 µL of 0.1 M NH$_4$OAc, pH 7. The reactions were incubated on a thermomixer with 800 rpm agitation at 45 °C for 20 - 60 min. Radiolabelled complexes were analysed by high-performance liquid chromatography (HPLC, Shimadzu 10Avp system) with a mobile phase of water (0.1% TFA) and acetonitrile (0.1% TFA), 0-100% acetonitrile over 10 min with a 1 mL/min flow rate. A radiochemical yield of greater than 95% was achieved for all labelled compounds and therefore they were used without further purification.

**Lipophilicity Studies.** The $^{64}$Cu-labeled complexes (5 µL, 0.37 MBq, 10 µCi) were added into an 1:1 (v:v) mixture of n-octanol and pH 7.4 PBS (500 µL/ea). The samples were vortexed at 1,000 rpm for 1 h, and then allowed for 30 min for the layers to separate. Aliquots (100 µL) from the aqueous and the n-octanol layers were removed and counted separately in an automated gamma counter. The distribution coefficients were calculated using the ratio of (activity detected in n-octanol)/(activity detected in aqueous layer) to get the $\log D_{oct}$ values. The experiment was conducted in six replicates, and the average of the different measurements was recorded as the final $\log D_{oct}$ value for each compound.

**Ex vivo Autoradiography Studies.** Brain sections of 10-month-old 5×FAD transgenic mice and aged-matched WT mice were immersed into a cryo-protectant solution. These sections were sorted and carefully removed using phosphate buffer in saline (PBS) with 1 % tween-20 solution and mounted onto an adhesive glass slide (CFSA 1X, Leica Bio Systems). Each section was washed with 100% PBS three times, and ~0.925 MBq (25 µCi) of $^{64}$Cu-labeled MFC in a 100 µL total volume was added to completely cover the brain section and incubate for 1 h at room temperature in a shielded bunker. After the incubation, brain sections were washed using PBS with five 1-minute cycles and briefly air-dried. The imaging slides were mounted on to a phosphor imaging screen plate (GE Healthcare Life Sciences) and were exposed for 1–5 minutes. The plates were then scanned using a biomolecule imager (Typhoon FLA 9500, GE) and the resulting images were processed using ImageQuant TL 8.1 (GE Healthcare Life Sciences).

**Biodistribution Studies.** All animal experiments were performed 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 School of Medicine. Initial biodistribution studies were conducted in wild type CD-1 female mice (Charles River
Laboratories) of age 5-7 weeks weighing 25.8 ± 2.1 g. The injection dose was prepared by diluting in to a 90 % saline solution. The uptake of $^{64}$Cu-labeled compounds was evaluated in mice that were injected via the tail vein with 0.22-0.37 MBq (6-10 µCi) of each compound per animal in 100 µL saline solution. After each time point (2, 60, and 240 min), mice were anesthetized with 1-2 % isoflurane and euthanized by cervical dislocation. Brain, blood, kidney, liver and other organs of interest were harvested and amount of radioactivity in each organ was counted on a gamma counter containing a NaI crystal. The data was corrected for radioactive decay and percent injected dose per gram (%ID/g) of tissue was calculated. All samples were calibrated against a known standard. Quantitative data were processed by Prism 7 (GraphPad Software, v 6.03, La Jolla, CA) and expressed as Mean ± SEM. Statistical analysis performed using one-way analysis of variance and Student’s t test. Differences at the 95% confidence level (p < 0.05) were considered statistically significant.
II. Synthesis and characterization of multifunctional compounds

Scheme S1. Synthesis of HYR-1.

S1b. 2-Amino-6-methoxy-benzothiazole S1a (10.0 g, 57.3 mmol) was suspended in 50% KOH (60 g KOH dissolved in 60 mL water) and ethylene glycol (13.3 mL). The suspension was heated to reflux for 48 h. Upon cooling to RT, toluene (100 mL) was added and the reaction mixture was neutralized with acetic acid (60 mL). The organic layer was separated and the aqueous layer was extracted with another 70 mL of toluene. The toluene layers were combined and washed with water and dried over MgSO$_4$. Evaporation of the solvent gave S1b as yellow solid (8.0 g, 90%). $^1$H NMR (acetone-$d_6$): $\delta$(ppm): 6.78 (d, 1H, $J$ = 2.7 Hz), 6.67 (d, 1H, $J$ = 8.7 Hz), 6.47 (dd, 1H, $J$ = 8.7, 3.0 Hz), 3.77 (s, 3H).

S1d. A mixture of S1b (0.75 g, 4.84 mmol) and S1c (0.72 g, 4.84 mmol) in DMSO (5 mL) was heated to 170 °C for 30 min. The reaction mixture was cooled to RT and poured into water. The organic compound was extracted with 50 mL of ethyl acetate 3 times. The combined layers were washed with water and dried over MgSO$_4$. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using hexane/ethyl acetate (6:1) to yield a yellow solid S1d (0.49 g, 35%). $^1$H NMR (acetone-$d_6$): $\delta$(ppm): 7.90 (d, 2H, $J$ = 9.0 Hz), 7.78 (d, 1H, $J$ = 9.0 Hz), 7.53 (d, 1H, $J$ = 2.5 Hz), 7.06 (dd, 1H, $J$ = 8.9, 2.6 Hz), 6.83 (d, 2H, $J$ = 9.0 Hz), 3.88 (s, 3H), 3.06 (s, 6H).

S1e. To a suspension of S1d (200 mg, 0.7 mmol) in CH$_2$Cl$_2$ (20 mL) was injected BBr$_3$ (1 M in CH$_2$Cl$_2$, 2.3 mL, 2.3 mmol). The reaction mixture was stirred at room temperature for 16 h. The reaction was quenched with water (20 mL) and the pH was adjusted to 4-7 with NaOH solution.
Precipitate was isolated by vacuum filtration, washed with water (3 × 5 mL), methanol (5 mL), CH$_2$Cl$_2$ (5 mL) and anhydrous ethanol (3 × 5 mL), and dried under vacuum to give a yellow solid S1e (116 mg, 61%). $^1$H NMR (DMSO-$d_6$): $\delta$ (ppm): 9.72 (s, 1H), 7.78 (d, 1H, $J = 8.7$ Hz), 7.71 (d, 2H, $J = 9.0$ Hz), 7.31 (d, 1H, $J = 2.4$ Hz), 6.90 (dd, 1H, $J = 8.7$, 2.5 Hz), 6.78 (d, 2H, $J = 9.0$ Hz), 3.01 (s, 6H).

**HYR-1.** Paraformaldehyde (8 mg, 0.27 mmol) was added to a solution of 1,4-dimethyl-1,4,7-triazacyclononane (28 mg, 0.18 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S1e (50 mg, 0.18 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using CH$_2$Cl$_2$/MeOH/NaOH (100:15:2) to yield a yellow solid (75 mg, yield 95%). $^1$H NMR (CDCl$_3$): $\delta$ (ppm): 7.89 (d, 2H, $J = 9.0$ Hz), 7.75 (d, 1H, $J = 8.7$ Hz), 7.02 (d, 1H, $J = 8.7$ Hz), 6.73 (d, 2H, $J = 9.0$ Hz), 4.02 (s, 2H), 3.04 (s, 6H), 2.95 (m, 4H), 2.68 (m, 4H), 2.59 (s, 4H), 2.41 (s, 6H). $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm): 167.34, 157.96, 154.41, 150.46, 137.78, 131.03, 124.50, 124.45, 119.35, 117.47, 114.41, 62.65, 60.21, 59.91, 55.67, 48.78, 42.86. ESI-MS: Calcd for [M+H]$^+$, 440.2479; Found, 440.2507.
Scheme S2. Synthesis of HYR-4 and HYR-18.

S2c. A mixture of S1a (2.97 g, 19 mmol) and S2b (2.89 g, 19 mmol) in DMSO (40 mL) was heated to 125 °C overnight. The reaction mixture was cooled to r.t. and poured into water (60 mL). The precipitate was filtered, washed with water, EtOH and dried to give a yellow solid S2c (4.9 g, 91%). $^1$H NMR (DMSO-$d_6$): $\delta$ (ppm): 8.37 (d, 2H, $J = 8.7$ Hz), 8.29 (d, 2H, $J = 9.0$ Hz), 8.02 (d, 1H, $J = 9.0$ Hz), 7.79 (d, 1H, $J = 2.6$ Hz), 7.19 (dd, 1H, $J = 9.0, 2.6$ Hz), 3.86 (s, 3H).

S2d. SnCl$_2$ (6.6 g, 35 mmol) was added to a solution of S2c (2 g, 7 mmol) in EtOH (60 mL) followed by the addition of Conc. HCl (3 mL). The solution was brought to reflux for 3 h and cooled to r.t. NaHCO$_3$ was added to adjust the pH to 8-9. The mixture was extracted with ethyl acetate ($3 \times 20$ mL). Then the combined layers were washed with water and dried over MgSO$_4$. The solvent was removed to give a yellowish solid S2d (1.38 g, 77%). $^1$H NMR (acetone-$d_6$): $\delta$ (ppm): 7.76 (d, 2H, $J = 8.7$ Hz), 7.75 (d, 1H, $J = 8.7$ Hz), 7.50 (d, 1H, $J = 2.7$ Hz), 7.02 (dd, 1H, $J = 9.0, 2.7$ Hz), 6.73 (d, 2H, $J = 8.7$ Hz), 5.26 (s, 2H), 3.86 (s, 3H).
S2e. NaOMe (0.65 g, 13 mmol) was added to the mixture of S2d (0.57 g, 2.2 mmol) and paraformaldehyde (0.4 g, 13 mmol) in methanol (60 mL). The solution was refluxed for 2 h. Ice bath was used to cool the mixture to 0 °C, then sodium borohydride (0.5 g, 13 mmol) was added. The mixture was refluxed again for 1 h to give an orange solution. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using hexane/ethyl acetate (4:1) to yield a yellow solid S2e (0.43 g, 72%).

S2f. To a solution of S2e (0.43 g, 1.6 mmol) in CH$_2$Cl$_2$ was added neat BBr$_3$ (1 M in CH$_2$Cl$_2$, 5 mL, 5 mmol) dropwise at r.t. The reaction was quenched with water and the pH was adjusted to 7 with saturated NaHCO$_3$ solution. Yellowish precipitate was isolated by vacuum filtration and then purified by silica gel column chromatography using hexane/ethyl acetate (2:1) to yield a yellow solid S2f (0.16 g, 38%). 1H NMR (DMSO-$d_6$): δ (ppm): 9.66 (s, 1H), 7.71 (d, 2H, $J = 8.7$ Hz), 7.67 (d, 1H, $J = 9.0$ Hz), 7.29 (d, 1H, $J = 2.4$ Hz), 6.88 (dd, 1H, $J = 8.7, 2.4$ Hz), 6.60 (d, 2H, $J = 8.7$ Hz), 6.35 (q, 1H), 2.72 (d, 3H, $J = 5.0$ Hz).

HYR-4. Paraformaldehyde (6 mg, 0.20 mmol) was added to a solution of 1,4-dimethyl-1,4,7-triazacyclononane (31 mg, 0.20 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S2f (50 mg, 0.20 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to give a yellowish residue that was purified by CombiFlash (reverse-phase) using MeCN/H$_2$O/TFA (30:70:1) to yield a yellow solution, which was then neutralized with saturated NaHCO$_3$ solution, extracted by CHCl$_3$ and dried to give a yellow solid (19 mg, yield 23%). 1H NMR (CD$_3$CN): δ (ppm): 7.81 (d, 2H, $J = 9.0$ Hz), 7.66 (d, 1H, $J = 8.7$ Hz), 7.00 (d, 1H, $J = 8.7$ Hz), 6.67 (d, 2H, $J = 9.0$ Hz), 4.98 (m, 1H), 4.04 (s, 2H), 2.84-2.89 (m, 4H), 2.82 (d, 3H, $J = 6.0$ Hz), 2.63-2.67 (m, 4H), 2.62 (s, 4H), 2.38 (s, 6H). 13C NMR (CDCl$_3$): δ (ppm): 167.33, 157.84, 153.75, 150.37, 138.00, 131.25, 125.57, 124.60, 119.38, 117.57, 114.70, 62.23, 59.48, 59.41, 55.16, 48.40, 33.02. HR-ESI-MS: Calcd for [M+H]$^+$, 426.2322; Found, 426.2352.

HYR-18. Paraformaldehyde (11 mg, 0.36 mmol) was added to a solution of 1-methyl-1,4,7-triazacyclononane (16 mg, 0.12 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S2f (60 mg, 0.23 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to give a yellowish residue that was purified by CombiFlash (reverse-phase) using MeCN/H$_2$O/TFA.
(30:70:1) to yield a yellow solution, which was then neutralized with saturated NaHCO$_3$ solution, extracted by CHCl$_3$ and dried to give a yellow solid (7 mg, yield 8%). $^1$H NMR (CD$_3$CN): $\delta$ (ppm): 7.82 (d, 4H, $J = 9.0$ Hz), 7.78 (d, 2H, $J = 9.0$ Hz), 7.05 (d, 2H, $J = 9.0$ Hz), 6.63 (d, 4H, $J = 9.0$ Hz), 3.99 (s, 4H), 3.05 (s, 4H), 2.90 (s, 6H), 2.83-2.85 (b, 4H), 2.66-2.68 (b, 4H), 2.50 (s, 3H).

HR-ESI-MS: Calcd for [M+H]$^+$, 680.2841; Found, 680.2850.
**Scheme S3.** Synthesis of HYR-14 and HYR-16.

**S3c.** A solution of S3a (1.0 g, 6.4 mmol) and S3b (1.6 g, 9.7 mmol) in NaOH (1 M in water, 18 mL) was stirred at 90 °C for 3 h. After the mixture was cooled to room temperature, the precipitate was collected by filtration, and S3c (1.6 g, 99%) was obtained as a green solid.

**S3d.** To a solution of S3c (515 mg, 2 mmol) and paraformaldehyde (300 mg, 10 mmol) in acetic acid (50 mL) was added NaCNBH$_3$ (189 mg, 6 mmol) in one portion at room temperature. The resulting mixture was stirred at room temperature overnight. After neutralization with NH$_4$OH, water was added, and the precipitate was collected by filtration to give S3d as a white solid (347 mg, 61%).

**S3e.** Intermediate S3e was synthesized according to the literature. To a solution of S3d (347 mg, 1.2 mmol) in CH$_2$Cl$_2$ (30 mL) was added neat BBr$_3$ (1 M in CH$_2$Cl$_2$, 4 mL, 4 mmol) dropwise at r.t. The reaction was quenched with water and the pH was adjusted to 7 with saturated NaHCO$_3$ solution. S3e was isolated by vacuum filtration as a yellow solid (325 mg, 99%). $^1$H NMR (DMSO-$d_6$): $\delta$ (ppm): 9.79 (b, 1H), 8.63 (d, 1H, $J = 2.5$ Hz), 8.07 (dd, 1H, $J = 9.1$, 2.5 Hz), 7.75 (d, 1H, $J = 8.8$ Hz), 7.35 (d, 1H, $J = 2.4$ Hz), 6.93 (dd, 1H, $J = 8.8$, 2.5 Hz), 6.81 (d, 1H, $J = 9.1$ Hz), 3.12 (s, 6H).
S3f. To a mixture of S3c (763 mg, 3 mmol) and paraformaldehyde (356 mg, 12 mmol) in MeOH (30 mL) was added CH$_3$ONa (1.6 g, 30 mmol). The mixture was stirred under reflux for 1 h. After the mixture was cooled, NaBH$_4$ (238 mg, 6 mmol) was added, and the mixture was brought to reflux again for 2 h. The reaction mixture was poured onto ice–water, and the precipitate was collected by filtration to obtain S3f as a white solid (484 mg, 60%). $^1$H NMR (DMSO-d$_6$): δ (ppm): 8.63 (d, 1H, $J = 2.4$ Hz), 7.96 (dd, 1H, $J = 8.7$, 2.5 Hz), 7.83 (d, 1H, $J = 9.0$ Hz), 7.65 (d, 1H, $J = 2.6$ Hz), 7.23 (d, 1H, $J = 5.1$ Hz), 7.07 (dd, 1H, $J = 8.9$, 2.6 Hz), 6.57 (d, 1H, $J = 8.8$ Hz), 3.83 (s, 3H), 2.85 (d, 3H, $J = 4.8$ Hz).

S3g. To a solution of S3f (484 mg, 1.8 mmol) in CH$_2$Cl$_2$ (32 mL) was added neat BBr$_3$ (1 M in CH$_2$Cl$_2$, 4.1 mL, 4.1 mmol) dropwise at r.t. The reaction was quenched with water and the pH was adjusted to 7 with saturated NaHCO$_3$ solution. S3g was isolated by vacuum filtration as a yellow solid (383 mg, 84%). $^1$H NMR (DMSO-d$_6$): δ (ppm): 9.75 (s, 1H), 8.53 (d, 1H, $J = 2.4$ Hz), 7.97 (dd, 1H, $J = 9.0$, 2.5 Hz), 7.71 (d, 1H, $J = 8.7$ Hz), 7.60 (b, 1H), 7.36 (d, 1H, $J = 2.4$ Hz), 6.93 (dd, 1H, $J = 8.8$, 2.5 Hz), 6.68 (d, 1H, $J = 8.9$ Hz), 2.83 (s, 3H).

HYR-14. Paraformaldehyde (8 mg, 0.27 mmol) was added to a solution of 1,4-dimethyl-1,4,7-triazacyclononane (28 mg, 0.18 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S3e (50 mg, 0.18 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to give a yellowish residue that was purified by CombiFlash (reverse-phase) using MeCN/H$_2$O/TFA (30:70:1) to yield a yellow solution, which was then neutralized by saturated NaHCO$_3$ solution, extracted by CHCl$_3$ and dried to give a yellow solid (34 mg, yield 43%). $^1$H NMR (CDCl$_3$): δ (ppm): 8.70 (d, 1H, $J = 2.4$ Hz), 8.08 (dd, 1H, $J = 9.0$, 2.4 Hz), 7.74 (d, 1H, $J = 8.7$ Hz), 7.09 (d, 1H, $J = 8.7$ Hz), 6.55 (d, 1H, $J = 9.0$ Hz), 4.00 (s, 2H), 3.15 (s, 6H), 2.88-2.91 (m, 4H), 2.67-2.69 (m, 4H), 2.66 (s, 4H), 2.43 (s, 6H). $^{13}$C NMR (CDCl$_3$): δ (ppm): 164.82, 162.42, 157.74, 150.18, 150.11, 138.11, 138.05, 124.74, 120.95, 119.52, 110.17, 108.13, 61.49, 58.49, 58.14, 54.36, 47.77, 40.78. HR-ESI-MS: Calcd for [M+H]$^+$, 441.2431; Found, 441.2472.

HYR-16. Paraformaldehyde (6 mg, 0.20 mmol) was added to a solution of 1,4-dimethyl-1,4,7-triazacyclononane (28 mg, 0.20 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S3g (50 mg, 0.20 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to
give a yellowish residue that was purified by CombiFlash (reverse-phase) using MeCN/H$_2$O/TFA (30:70:1) to yield a yellow solution, which was then neutralized by saturated NaHCO$_3$ solution, extracted by CHCl$_3$ and dried to give a yellow solid (9 mg, yield 11%). $^1$H NMR (CDCl$_3$): $\delta$ (ppm): 8.69 (d, 1H, $J = 2.1$ Hz), 8.11 (dd, 1H, $J = 8.7$, 2.4 Hz), 7.76 (d, 1H, $J = 8.7$ Hz), 7.03 (d, 1H, $J = 8.6$ Hz), 6.46 (d, 1H, $J = 8.7$ Hz), 4.89 (m, 1H), 4.03 (s, 2H), 3.00 (d, 3H, $J = 5.2$ Hz), 2.93-2.96 (m, 4H), 2.67-2.70 (m, 4H), 2.61 (s, 4H), 2.42 (s, 6H). $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm): 164.47, 162.96, 158.41, 150.45, 150.12, 138.52, 137.49, 124.61, 122.49, 119.67, 117.51, 108.68, 62.76, 60.41, 60.03, 55.78, 48.85, 31.69. HR-ESI-MS: Calcd for [M+H]$^+$, 427.2275; Found, 427.2309.
**Scheme S4. Synthesis of HYR-17.**

**S4b.** S4a (5 g, 27.7 mmol) was suspended in 50% KOH (29 g KOH dissolved in 29 mL water) and ethylene glycol (6.5 mL). The suspension was heated to reflux for 48 h. Upon cooling to room temperature, toluene (50 mL) was added and the reaction mixture was neutralized with acetic acid (30 mL). The organic layer was separated, and the aqueous layer was extracted with another 35 mL of toluene. The toluene layers were combined and washed with water and dried over MgSO₄. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using CH₂Cl₂/MeOH/NH₄OH (100:10:0.1) to yield S4b as an yellow oil (674 mg, 16%). ¹H NMR (DMSO-d₆): δ (ppm): 6.84 (d, 1H, J = 8.0 Hz), 6.68 (d, 1H, J = 7.9 Hz), 6.43 (t, 1H), 4.98 (s, 2H), 3.77 (s, 3H).

**S4d.** A mixture of S4b (200 mg, 1.3 mmol) and S4c (192 mg, 1.3 mmol) in DMSO was heated to 170 °C for 35 min. The reaction mixture was cooled to room temperature and poured into water. The organic compound was extracted with ethyl acetate. The combined organic layers were washed with water and dried over MgSO₄. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using hexane/ethyl acetate (4:1) to yield a yellow solid S4d (204 mg, 55%). ¹H NMR (DMSO-d₆): δ (ppm): 7.85 (d, 2H, J = 8.9 Hz), 7.57 (d, 1H, J = 8.0 Hz), 7.30 (t, 1H, J = 8.0 Hz), 7.01 (d, 1H, J = 8.1 Hz), 6.81 (d, 2H, J = 8.9 Hz), 3.96 (s, 3H), 3.01 (s, 6H).

**S4e.** To a solution of S4d (204 mg, 0.7 mmol) in CH₂Cl₂ (25 mL) was added neat BBr₃ (1 M in CH₂Cl₂, 2.5 mL, 2.5 mmol) dropwise at r.t. The reaction was quenched with water and the pH was adjusted to 7 with saturated NaHCO₃ solution. The organic compound was extracted with ethyl
acetate. The combined organic layers were washed with water and dried over MgSO$_4$. The solvent was removed to give a yellowish residue that was purified by silica gel column chromatography using hexane/ethyl acetate (3:1) to yield a yellow solid S4e (22.5 mg, 12%). $^1$H NMR (CDCl$_3$): $\delta$ (ppm): 7.92 (d, 2H, $J = 9.0$ Hz), 7.34 (d, 1H, $J = 8.0$ Hz), 7.19 (t, 1H, $J = 7.8$ Hz), 6.93 (d, 1H, $J = 7.9$ Hz), 6.74 (d, 2H, $J = 8.7$ Hz), 3.06 (s, 6H).

**HYR-17.** Paraformaldehyde (4 mg, 0.10 mmol) was added to a solution of 1,4-dimethyl-1,4,7-triazacyclononane (11 mg, 0.07 mmol) in MeCN (5 mL) and the resultant mixture was heated to reflux for 30 min. Then S4e (19 mg, 0.07 mmol) in MeCN (5 mL) was added, the solution was refluxed for an additional 24 h, and then cooled to room temperature. The solvent was removed to give a yellowish residue that was purified by CombiFlash (reverse-phase) using MeCN/H$_2$O/TFA (30:70:1) to yield a yellow solution, which was then neutralized by saturated NaHCO$_3$ solution, extracted by CHCl$_3$ and dried to give a yellow solid (8 mg, yield 27%). $^1$H NMR (CDCl$_3$): $\delta$ (ppm): 7.97 (d, 2H, $J = 9.0$ Hz), 7.24 (d, 1H, $J = 8.1$ Hz), 6.99 (d, 1H, $J = 8.1$ Hz), 6.73 (d, 2H, $J = 9.0$ Hz), 3.96 (s, 2H), 3.05 (s, 6H), 2.99-3.02 (m, 4H), 2.83-2.86 (m, 4H), 2.82 (s, 4H), 2.45 (s, 6H). $^{13}$C NMR (CDCl$_3$): $\delta$ (ppm): 170.54, 154.84, 151.78, 146.58, 137.74, 131.51, 129.70, 123.74, 121.59, 114.63, 114.31, 60.35, 57.11, 56.19, 54.22, 46.74, 42.81. HR-ESI-MS: Calcd for [M+H]$^+$, 440.2479; Found, 440.2503.
III. UV-vis and emission spectra of MFCs

Figure S1. UV-vis spectra of investigated MFCs (50 µM in ultrapure water with 0.5% DMSO).

Figure S2. Emission spectra of investigated MFCs in ultrapure water with <0.1% DMSO.
Table S1. Excitation and emission wavelengths of investigated MFCs.

| Compounds | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) |
|-----------|---------------------|---------------------|
| HYR-1     | 362                 | 450                 |
| HYR-4     | 352                 | 442                 |
| HYR-14    | 352                 | 420                 |
| HYR-16    | 344                 | 425                 |
| HYR-17    | 356                 | 450                 |
| HYR-18    | 344                 | 438                 |
IV. UV-vis and emission spectra of MFC-Cu(II) complexes

Figure S3. UV-vis spectra of MFC-Cu(II) complexes (50 µM in ultrapure water with 0.5% DMSO).

Figure S4. Emission spectra of MFC-Cu(II) complexes in ultrapure water with <0.1% DMSO.
Table S2. Excitation and emission wavelengths of MFC-Cu(II) complexes.

| Cu(II) complexes | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) |
|------------------|--------------------|--------------------|
| Cu-HYR-1         | 366                | 450                |
| Cu-HYR-4         | 358                | 436                |
| Cu-HYR-14        | 356                | 420                |
| Cu-HYR-16        | 352                | 418                |
| Cu-HYR-17        | 356                | 450                |
| Cu-HYR-18        | 350                | 434                |
V. Fluorescence staining of WT mouse brain sections

**Figure S5.** Representative fluorescence microscopy images of WT mouse brain sections stained with **HYR-1/HYR-4**. **HYR-1** shows non-specific binding to various biomolecules.
VI. Structure and Aβ fibril binding assays of blocking agent B1

![Structure of non-radiolabeled compounds used for blocking studies.](image)

**Figure S6.** Structure of non-radiolabeled compounds used for blocking studies.

![ThT competition assay of B1 with Aβ40 fibrils ([Aβ] = 2 µM, [ThT] = 1 µM).](image)

**Figure S7.** ThT competition assay of B1 with Aβ40 fibrils ([Aβ] = 2 µM, [ThT] = 1 µM).
VII. HPLC traces of $^{64}\text{Cu}$-radiolabeled MFCs

Figure S8. HPLC chromatographic profiles from $^{64}\text{Cu}$ radiolabeling. The retention times were 8.7, 8.2, 7.3, 5.3, 9.2, and 8.0 min for $^{64}\text{Cu-HYR-1}$, $^{64}\text{Cu-HYR-4}$, $^{64}\text{Cu-HYR-14}$, $^{64}\text{Cu-HYR-16}$, $^{64}\text{Cu-HYR-17}$ and $^{64}\text{Cu-HYR-18}$, respectively.
VIII. NMR spectra of MFCs

\(^1\text{H NMR of HYR-1}\)

\(^1\text{H NMR of HYR-4}\)
$^1$H NMR of HYR-14

$^1$H NMR of HYR-16
$^1$H NMR of HYR-17

$^1$H NMR of HYR-18
$^{13}$C NMR of HYR-1

$^{13}$C NMR of HYR-4
$^{13}$C NMR of HYR-14

$^{13}$C NMR of HYR-16
$^{13}$C NMR of HYR-17
IX. References

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