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ARTICLE TYPE

Effects of structural modifications on the metal binding, anti-amyloid activity, and cholinesterase inhibitory activity of chalcones

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As the number of individuals affected with Alzheimer's disease (AD) increases and the availability of drugs for AD treatment remains limited, the need to develop effective therapeutics for AD becomes more and more pressing. Strategies currently pursued include inhibiting acetylcholinesterase (AChE) and targeting amyloid-β (Aβ) peptides and metal-Aβ complexes. This work presents the design, synthesis, and biochemical evaluation of a series of chalcones, and assesses the relationship between their structures and their ability to bind metal ions and/or Aβ species, and inhibit AChE/BChE activity. Several chalcones were found to exhibit potent disaggregation of pre-formed *N*-biotinyl $A\beta_{1-42}$ ($bioA\beta_{42}$) aggregates *in vitro* in the absence and presence of Cu^{2+}/Zn^{2+}, while others were effective at inhibiting the action of AChE.

Introduction

Alzheimer's disease (AD) is a deadly progressive neurodegenerative disorder that manifests itself by the decline in cognitive function and the loss of memory. It is the most common form of dementia and the sixth leading cause of death in the United States, affecting one in nine older Americans.[1] The socioeconomic burden associated with AD is estimated to a staggering $172 billion per year in the USA.[2, 3] There are currently no medications that can cure AD or stop its progression; only a few have been approved to provide temporary symptomatic relief.[4-8] Further research efforts are thus needed to develop AD therapeutics. Pathologically, AD is characterized by the deposition of amyloid β-peptide (Aβ)-rich plaques and the accumulation of hyperphosphorylated tau protein as neurofibrillary tangles in the brain of afflicted patients,[9-13] as well as the degeneration of neurons and synapses due to the increased activity of cholinesterases.[13] These hallmarks thus represent potential pharmacological targets for the development of AD therapeutics.

Acetylcholinesterase (AChE) inhibitors are among the commonly investigated treatments for AD.[14-18] Four of the six drugs currently approved for treating AD (tacrine, donepezil, rivastigmine, and galantamine) increase the amount of acetylcholine neurotransmitter in the brain by inhibiting the action of AChE. However, they are only modestly effective at alleviating AD symptoms. Aβ accumulation in the cerebral cortex has been suggested to be an early event in AD pathogenesis,[19] and attempts to prevent Aβ oligomerization or disrupt existing Aβ aggregates have attracted considerable efforts aiming at slowing down the progression of AD.[10, 20, 21] Metal ions such as Cu^{2+} and Zn^{2+}, which concentrate in senile plaques,[22, 23] have been shown to interact with Aβ peptides and promote their assembly into toxic Aβ oligomers[24, 25] as well as the formation of reactive oxygen species (ROS).[26-28] This suggests that small molecule metal chelators capable of targeting Aβ species and inhibiting cholinesterases such as acetylcholinesterase (AChE) and/or butyrylcholinesterase (BChE) could be valuable AD therapeutics.

Chalcones are 1,3-diaryl-2-propen-1-ones with a wide range of interesting biological activities.[29] A large number of chalcones have been reported as Aβ-imaging tracers with high brain uptake.[30-32] Chalcones with tertiary amines in their structures, especially the *N*,*N*-dimethyl amino group, have also been shown to exhibit substantial affinities for Aβ plaques.[33] Combining these observations into a single molecule, chalcone **3a** (Fig. 1A), containing a metal chelation site and a dimethylamino group, was synthesized. Chalcone **3a** is capable of regulating both metal-free and metal-mediated Aβ aggregation.[34] However, its ability to inhibit the action of cholinesterases has never been investigated.

Herein, we present the design, synthesis, metal binding capabilities, and *in vitro* metal-free and metal-induced reactivity towards Aβ and cholinesterase enzymes of a series of chalcones, allowing us to evaluate the structure-activity relationship of chalcones capable of interacting with metal ions and/or Aβ peptides, and inhibiting cholinesterases.

Results and discussion

Integrated design and synthesis of chalcones

Chalcones **3a-i** (Fig. 1A) were designed to contain a metal chelation site and an Aβ-binding moiety. This was achieved *via* an integrated approach, whereby part of the known Aβ-interacting chalcone scaffold was replaced by the appropriate feature, with minimal structural changes. The 2-pyridyl ketone moiety (Fig. 1C) was chosen as the metal chelation site because it

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had previously been installed in other Aβ self-assembly inhibitors with favourable results.[34, 35] Moreover, installation of the *N*,*N*-dimethylamino group at the *para*-position in ring II (Fig. 1A) has been shown to improve binding affinity to Aβ plaques,[31, 33] and thus any additional substitution was localized on ring I. A methyl group or a bromine atom was thus attached at various positions of ring I of the chalcones **3b-i** (Fig. 1A) to evaluate their effects on metal binding, Aβ modulating properties, and/or cholinesterase inhibitory capabilities. To further investigate the necessity of a metal chelation site in the modulation of metal-Aβ complexes, chalcones **6a-h** and **7a**, **b**, and **h** (Fig. 1B), with a 2-acyl phenol and a 2-acyl aniline moiety that can form intramolecular hydrogen bonds (Fig. 1C) and prevent metal coordination, respectively, were prepared.

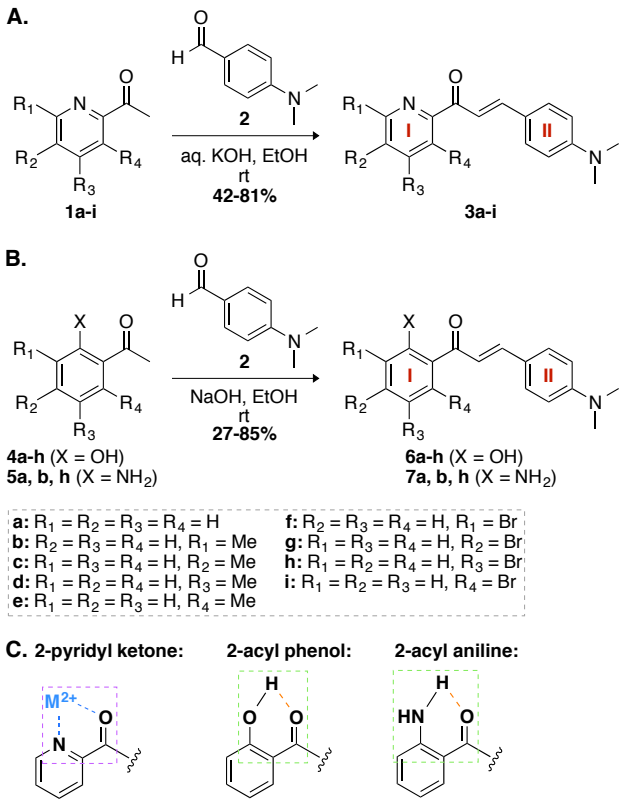

Fig. 1. Synthetic scheme for the formation of **A.** chalcones **3a-i**, and **B.** chalcones **6a-h**, **7a**, **b**, and **h**. **C.** Representation of metal (M^{2+}) (note either Cu^{2+} or Zn^{2+}) chelation by the 2-pyridyl ketone moiety of **3a-i**, as well as intramolecular hydrogen bonding preventing metal chelation to the 2-acyl phenol and 2-acyl aniline moieties of **6a-h** and **7a**, **b**, and **h**.

The target chalcones were synthesized according to the known Claisen-Schmidt condensation (Fig. 1). 4-(Dimethylamino)benzaldehyde, **2**, was reacted with various 2-acetylpyridines/acetophenones (**1**, **4**, or **5**) in the presence of a base (KOH or NaOH) in ethanol at room temperature. This afforded the chalcones **3a-i**, in yields ranging from 42 to 81%, and the chalcones **6a-h**, **7a-b**, and **h** in 27 to 85%.

Study of the metal (Cu^{2+} and Zn^{2+}) binding properties of synthesized chalcones

Cu^{2+} binding: The ability of **3a-i**, **6a-h**, **7a**, **b**, and **h** to bind Cu^{2+} ions was investigated by UV-Visible spectroscopy (Fig. 2). Upon addition of 1-5 equivalents of $CuCl_2$ to an ethanolic solution of chalcone **3a**, a distinctive shift of the optical band from ~440 nm to ~560 nm was observed, suggesting Cu^{2+} binding of **3a** at the metal chelation site formed by the nitrogen and the oxygen atoms from the 2-pyridyl ketone moiety (Fig. 1C), as previously reported.[34] When a methyl group was added at the R_1 position in **3b**, the optical band at 440 nm did not completely shift to 560 nm, even in the presence of 5 equivalents of $CuCl_2$, implying that the ability to chelate Cu^{2+} in this case was less efficient. This can be attributed to the steric hindrance caused by the methyl group, which perturbs the metal chelation site. This was confirmed when the methyl group was moved further away from the metal chelation site, as in **3c** and **3d**, and the Cu^{2+} binding properties of these chalcones were regained. Also, replacing R_4 with the methyl group in **3e** gave similar results as **3b**, although with a less profound decrease in Cu^{2+} interaction.

Substitution of the methyl group by a bromine atom lessened the Cu^{2+} interaction of the chalcone in all cases. No optical shift was observed for **3f** and **3i**, although incomplete in the corresponding methyl counterparts **3b** and **3e**, respectively. Moreover, while it took 2 equivalents of $CuCl_2$ to **3c** and **3d** to completely shift the optical band at 440 nm and thus completely bind Cu^{2+} ions, **3g** and **3h** required up to 5 equivalents of $CuCl_2$. These results stem from the electron-withdrawing effect of the bromine atom. As a matter of fact, Br is a good electronegative atom and pulls electrons away from the nitrogen-donor atom on ring I, thus disrupting the metal chelation site.

As expected, replacement of the 2-pyridyl ketone moiety in chalcones **3a-i** by a 2-acyl phenol (**6a-h**) or a 2-acyl aniline (**7a**, **b**, and **h**) moiety resulted in a complete loss of Cu^{2+} binding property. Intramolecular hydrogen bonding in **6a-h**, **7a**, **b**, and **h** (Fig. 1C) prevents the formation of the characteristic metal chelation site observed in **3a-i**, and hence their ability to interact with Cu^{2+}. Overall, these results demonstrate Cu^{2+} interaction of chalcones **3a-e**, **3g** and **3h**, suggesting that the 2-pyridyl ketone moiety, or another scaffold capable of forming a metal chelation site, is essential for binding metal ions. Furthermore, disruption of the metal chelation site either by steric hindrance of bulky substituents (**3b** and **3e**) or by decreasing the electron density with electron-withdrawing substituents (**3f** and **3i**), reduces or nullifies the metal binding capability of chalcones.

Zn^{2+} binding: We also investigated the Zn^{2+} binding properties of **3a**, **3d**, **3h**, **6a**, and **7a**. Altough Cu^{2+} binding was studied by UV-Visible spectroscopy, which enabled us to perform a quantitative analysis, no optical changes could be observed in this assay when $ZnCl_2$ was used. Therefore, the standard qualitative ^1H NMR spectroscopy assay was performed to study the Zn^{2+} binding properties of our chalcones (Fig. 3). Upon addition of 3 equivalents of $ZnCl_2$ to a CD_3CN solution of **3a**, **3d**, and **3h**, distinctive downfield shifts of the peaks corresponding to the pyridyl protons were observed. This implies that the nitrogen atom on ring I is involved in Zn^{2+} binding. It is also noticeable that the peaks associated with protons e and f were displaced, suggesting that the oxygen atom might participate in metal chelation. The effect of the involvement of the oxygen atom in the coordination of metal ions was further extended to ring II as protons g and h also experienced some chemical shifts (Fig. 3; panels A, B, and C). These results confirm that the 2-pyridyl

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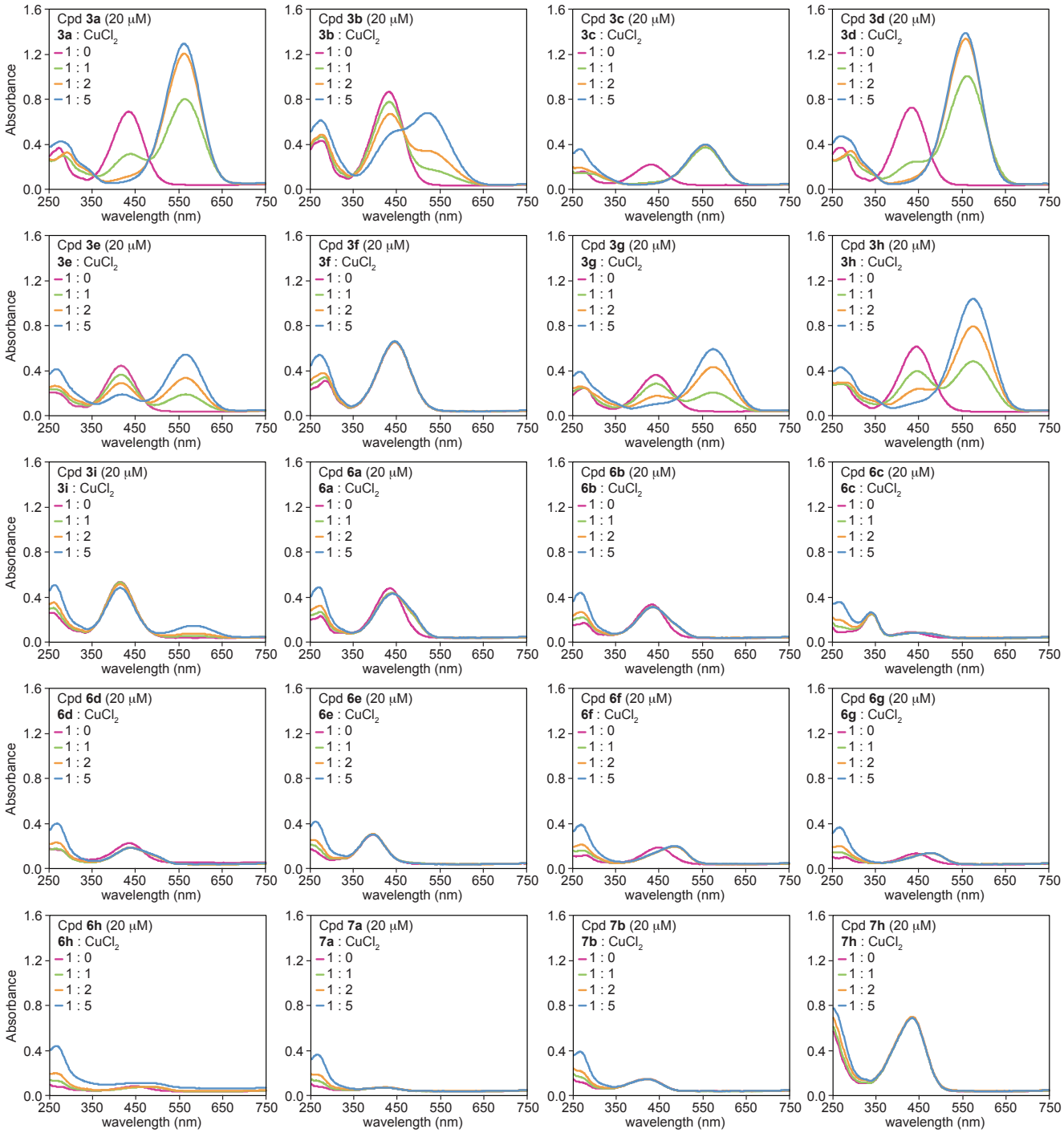

Fig. 2. Cu^{2+} binding studies by UV-Vis using 0, 1, 2, and 5 equivalents of $CuCl_2$ with 20 μM of chalcones **3a-i**, **6a-h**, and **7a**, **b**, and **h** dissolved in EtOH at room temperature and incubated for 3 min.

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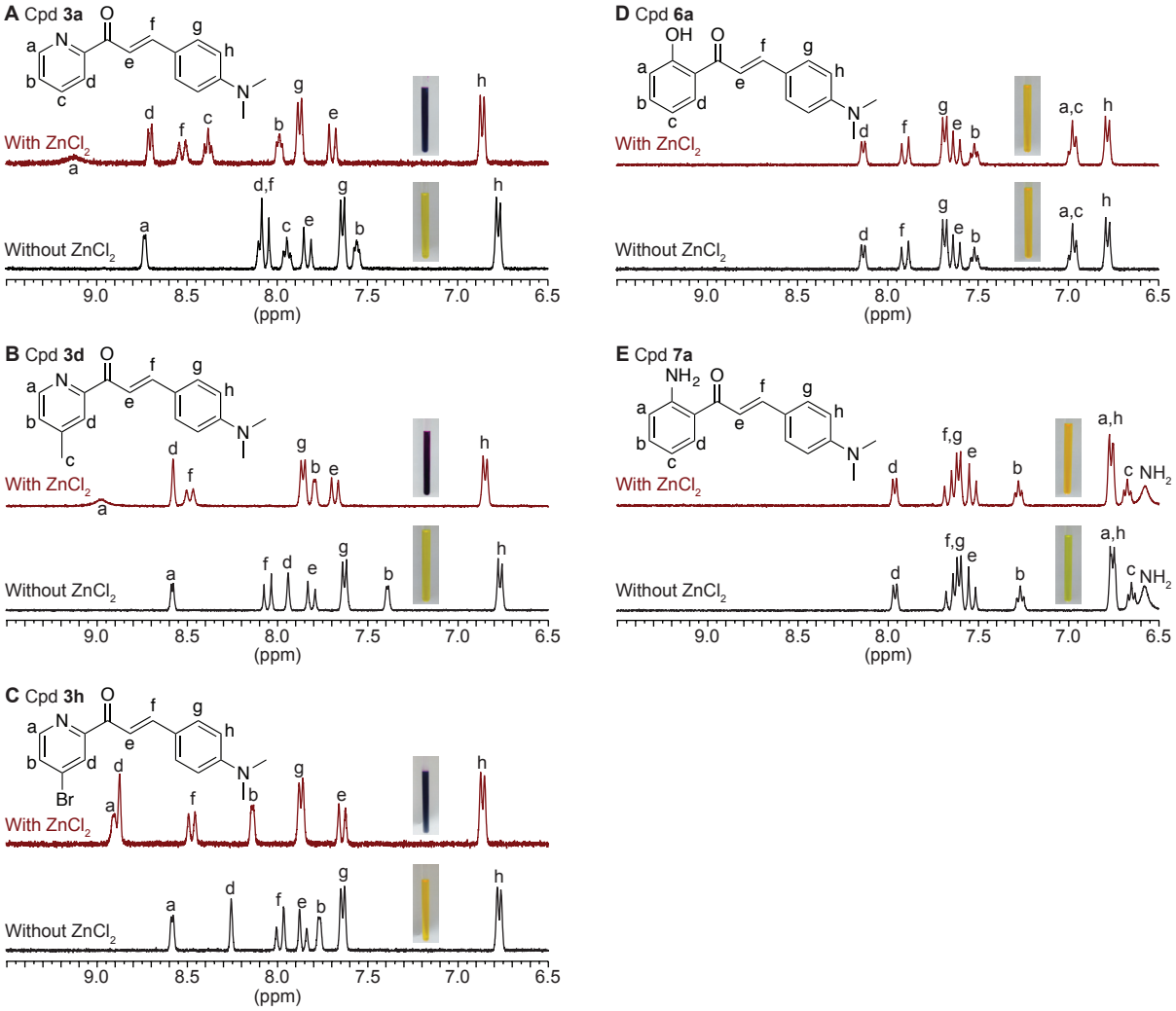

Fig. 3. Zn^{2+} binding studies of selected chalcones by ^1H NMR spectroscopy in CD_3CN at room temperature. NMR spectra of chalcone **A. 3a** without (black, 4 mM) and with $ZnCl_2$ (red, 12 mM), **B. 3d** without (black, 4 mM) and with $ZnCl_2$ (red, 12 mM), **C. 3h** without (black, 4 mM) and with $ZnCl_2$ (red, 12 mM), **D. 6a** without (black, 4 mM) and with $ZnCl_2$ (red, 12 mM), and **E. 7a** without (black, 4 mM) and with $ZnCl_2$ (red, 12 mM). *Note*: Pictures of NMR tubes for each chalcone without and with $ZnCl_2$ are presented above their respective spectra. The change in color from yellow to purple is observed for chalcones that bind Zn^{2+}.

ketone moiety bears a metal chelation site formed by the nitrogen and the oxygen atoms (Fig. 1C). Interaction of **3a**, **3d**, and **3h** with Zn^{2+} could also be reaffirmed by the color change of the NMR sample from yellow to deep purple upon addition of $ZnCl_2$.

On the other hand, treatment of a CD_3CN solution of **6a** and **7a** with 3 equivalents of $ZnCl_2$ did not exhibit any distinct chemical shift of protons (Fig. 3, panels D and E), suggesting that **6a** and **7a**, with a 2-acyl phenol and a 2-acyl aniline moiety, respectively, do not interact with Zn^{2+} ions. This was further confirmed as no discrete color change was noticeable upon addition of $ZnCl_2$ to the CD_3CN solution of **6a** and **7a**. This is in agreement with the observations made in the presence of Cu^{2+} and confirms that the intramolecular hydrogen bonding in **6a** and **7a** (Fig. 1C) prevents the formation of the characteristic metal chelation site. In light of these results, chalcone **3a**, with a 2-pyridyl ketone moiety, and any of its derivatives bearing a substituent at the preferred R_3 positin, such as **3d** and **3h**, are capable of binding Cu^{2+} and

Solution speciation studies

To further evaluate the metal ion interaction with our chalcones, we performed UV-visible variable-pH titration of chalcones **3a**, **3d**, **3h**, **6a**, and **7a** in the absence and presence of Cu^{2+}. The acidity constants (pK_as) of **3a**, **3d**, **3h**, **6a**, and **7a** were first determined from the solution speciation diagrams obtained from the corresponding UV-visible variable-pH titration spectra in the absence of Cu^{2+} (Fig. 4). For chalcone **3a**, the pK_{a1} (protonation of the pyridyl nitrogen) was found to be 2.87 ± 0.03 and the pK_{a2} (protonation of the *N*,*N*-dimethylamino nitrogen) was 4.33 ± 0.03, which match previously reported values (pK_{a1} = 3.23 and pK_{a2} = 4.00).[34] The solution speciation diagram we obtained for **3a** was also in agreement with the literature,[34] and indicated the presence of three species in solution: neutral (S), monoprotonated (SH), and diprotonated (SH_2). For chalcone **3d**, pKa_1 = 3.11 ± 0.07 and pKa_2 = 3.33 ± 0.05 were obtained. Indeed, as the methyl group donates electrons, the basicity of the pyridyl ring increases from **3a** to **3d**, and thus the pK_{a1} value should also increase from **3a** to **3d**. Similarly, an electron-withdrawing group such as Br would decrease the pK_{a1} value, as it was observed in chalcone **3h**

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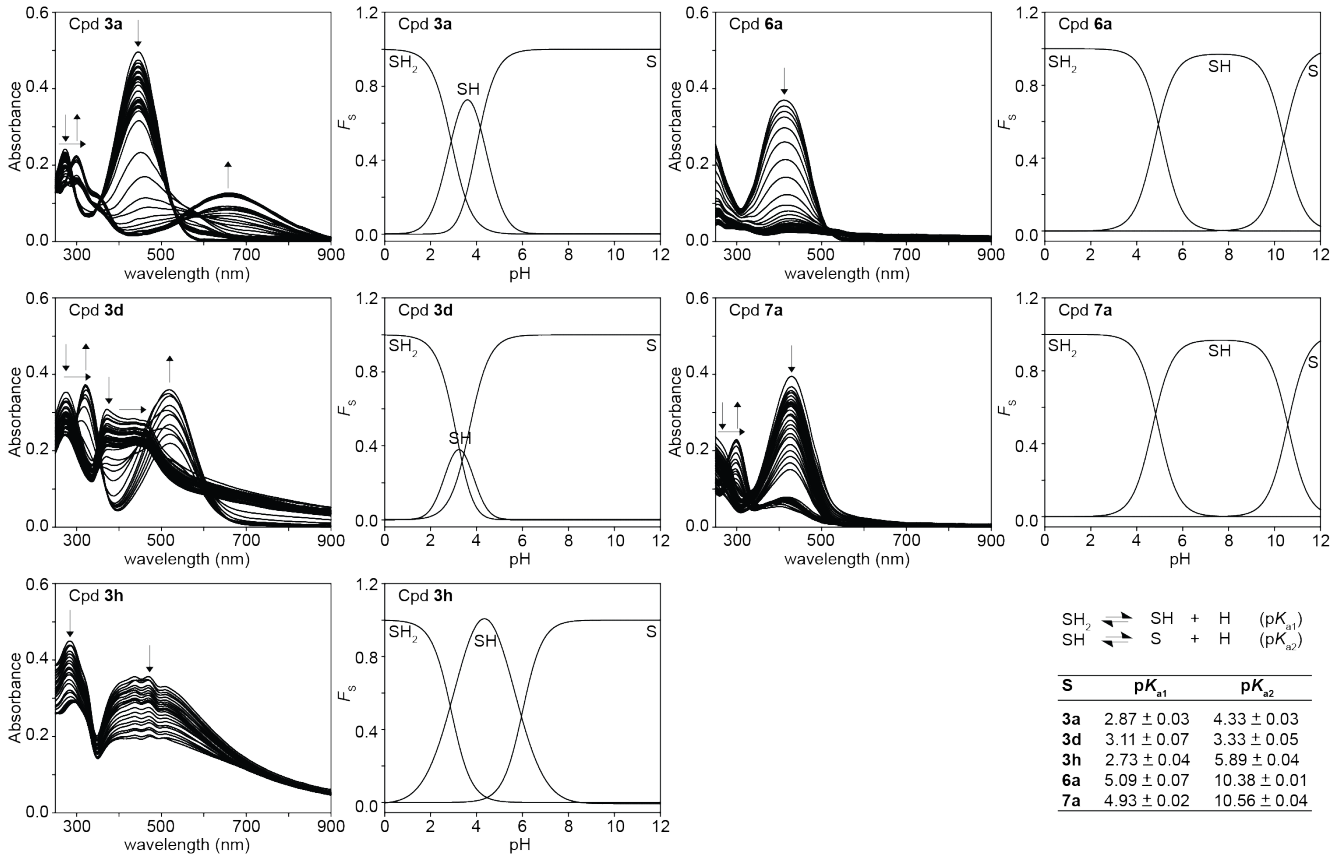

$SH_2 \rightleftharpoons SH + H$ (pK_{a1})
$SH \rightleftharpoons S + H$ (pK_{a2})

S	pK_{a1}	pK_{a2}
3a	2.87 ± 0.03	4.33 ± 0.03
3d	3.11 ± 0.07	3.33 ± 0.05
3h	2.73 ± 0.04	5.89 ± 0.04
6a	5.09 ± 0.07	10.38 ± 0.01
7a	4.93 ± 0.02	10.56 ± 0.04

Fig. 4. Solution speciation studies of chalcones (S) **3a**, **3d**, **3h**, **6a**, and **7a**. UV-visible variable-pH titration spectra (left) and solution speciation diagrams (right) of chalcones (S) **3a** (20 μM), **3d** (50 μM), **3h** (50 μM), **6a** (20 μM), and **7a** (20 μM) at room temperature. Titrations were done from pH 12-2. F_S represents the fraction of species present in solution at the given pH. Acidity constants (pK_as) of chalcones **3a**, **3d**, **3h**, **6a**, and **7a** are summarized in the table at the bottom right.

(pKa_1 = 2.73 ± 0.04 and pKa_2 = 5.89 ± 0.04). These results thus suggest that **3a**, **3d**, and **3h** will be present as neutral species at physiological pH (7.4). On the other hand, the p*K*a values of **6a** and **7a** were determined as follows: **6a**; pKa_1 = 5.09 ± 0.07 and pKa_2 = 10.38 ± 0.01; and **7a**, pKa_1 = 4.93 ± 0.02 and pKa_2 = 10.56 ± 0.04. In this case, the pKa_1 value was relevant to the protonation of the *N*,*N*-dimethylamino nitrogen, while the pKa_2 value was a measure of the ability to protonate the hydroxyl oxygen and aniline nitrogen.

Once the acidity constants of chalcones **3a, 3d, 3h, 6a,** and **7a** were determined, we then performed their spectrophotometric titrations in the presence of $CuCl_2$ (Fig. 5 and Fig. S53). While various differences were noticeable in the UV-visible variable-pH titration spectra of **3a**, **3d**, and **3h** obtained in the absence (Fig. 4) and presence (Fig. 5) of $CuCl_2$, the spectra for **6a** and **7a** were similar in both cases (Fig. 4 and Fig. S53). For **3a**, we noticed a decrease in value of the maximum absorbance (at 440 nm) from 0.49 to 0.40 in the presence of $CuCl_2$. A new peak also appeared at 570 nm in the pH-titration spectrum of **3a** in the presence of $CuCl_2$. Moreover, there was a subtle shift of the spectrum when the chalcone **3d** was titrated in the presence of $CuCl_2$. Finally, the shape of the pH-titration curve of **3h** changed, resulting from a more pronounced decline in absorbance of the chalcone in the presence of $CuCl_2$ as the pH decreased. Indeed, the absorbance of **3h** decreased from 0.45 to 0.28 at 280 nm, and from 0.37 to 0.20 at 470 nm in the absence of $CuCl_2$. Meanwhile, the absorbance of **3h** decreased from 0.46 to 0.13 at 280 nm, and from 0.36 to 0.06 at 470 nm in the presence of $CuCl_2$. All these results support our previous observations that chalcones with a 2-pyridyl ketone moiety, such as **3a**, **3d**, and **3h**, would interact with Cu^{2+} ions, while chalcones with a 2-acylphenol moiety (**6a**) or a 2-acylaniline moiety (**7a**) do not bind Cu^{2+}.

Based on the determined pK_a values of chalcones **3a**, **3d**, and **3h** above, we were able to find the stability constants (logβ) of the complexes formed between Cu^{2+} and our chalcones. For **3a**, $log\beta_1$ = 4.63 and $log\beta_2$ = 4.66; for **3d**, $log\beta_1$ = 4.06 and $log\beta_2$ = 4.17; and for **3h**, $log\beta_1$ = 5.10 and $log\beta_2$ = 5.13. The similarity in $log\beta_1$ and $log\beta_2$ values explains the fact that protonation of the neutral chalcone species first occurs at the *N*,*N*-dimethylamino nitrogen and should not prevent Cu^{2+} from binding at the proposed

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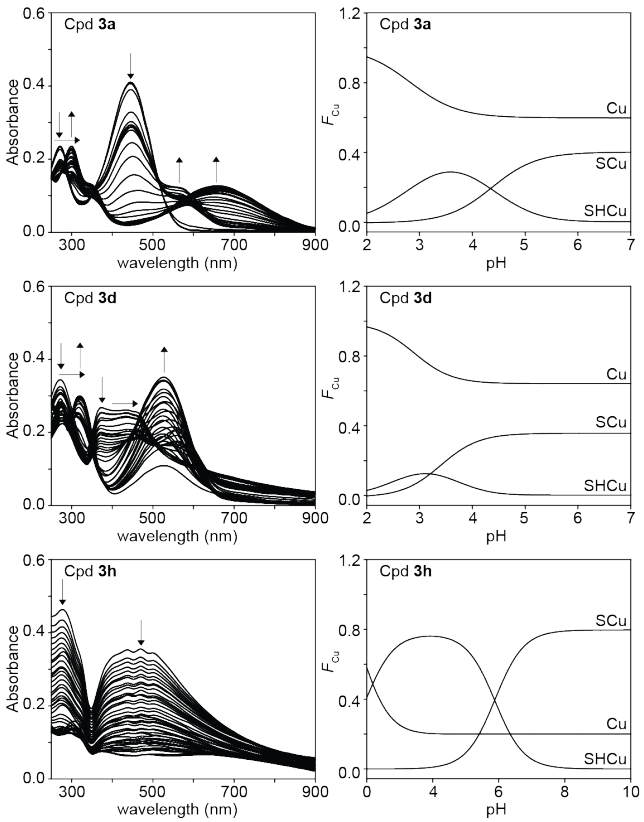
chelation site of the 2-pyridyl ketone moiety. Based on these logβ values, the stability of the Cu^{2+}-chalcone complex was increasing in the order **3d**, **3a**, and **3h**, although the variation was not substantial. We were also able to plot the solution speciation diagrams based on these logβ values (Fig. 5, right columns) and found out that free Cu^{2+}, SCu, and SHCu were present in all the chalcone solution. At pH 7, the SCu complexes were present in solution at about 40%, 40%, and 75% for **3a**, **3d**, and **3h**, respectively.



				logβ		
				S = 3a	3d	3h
SHCu	⇌	SH	+ Cu	4.63	4.06	5.10
SCu	⇌	S	+ Cu	4.66	4.17	5.13

Fig. 5. Solution speciation studies of chalcones (S) **3a**, **3d**, and **3h** in the presence of $CuCl_2$. UV-visible variable-pH titration spectra (left) and solution speciation diagrams (right) of Cu^{2+}-**3a**, Cu^{2+}-**3d**, and Cu^{2+}-**3h**. 20 μM (**3a**) or 50 μM (**3d** and **3h**) of the chalcone (S) was incubated for 30 minutes with $CuCl_2$ ([Cu^{2+}]/[S]). Titrations were then performed at room temperature from pH 7-2 for **3a** and **3d**, and from pH 12-2 for **3h**. F_{Cu} represents the fraction of free Cu and SCu complexes present in solution at the given pH. The stability constants (logβ) of Cu^{2+}-**3a**, Cu^{2+}-**3d**, and Cu^{2+}-**3h** complexes are summarized in the table at the bottom.

In vitro study of the effect of chalcones on Aβ assembly and dissociation in the absence and presence of metal ions

The ability of **3a** to regulate metal-free and metal-induced Aβ aggregation and dissociation has previously been demonstrated.[34] In these studies, Aβ fibrils, which have been proposed to be an important causal agent of AD, were employed. However, soluble Aβ oligomeric species have recently been shown to cause more potent neurotoxicity than fibrils.[36] This inspired us to evaluate the effects of our chalcones on Aβ oligomers assembly and disaggregation (Fig. 6). Although $Aβ_{1-40}$ and $Aβ_{1-42}$ are the major forms of Aβ peptides, $Aβ_{1-42}$ is the most abundant[37, 38] and neurotoxic.[39-41] $Aβ_{1-42}$ in its *N*-biotinylated form ($bioAβ_{42}$) was thus used in our anti-oligomer assays because it readily assembles into oligomers at near-physiologic nanomolar concentrations, while $Aβ_{1-40}$ forms oligomers only very weakly.[42]

As previously noted, metal ions such as Cu^{2+} and Zn^{2+} have been observed to interact with Aβ and promote peptide aggregation.[22-25] The ability of chalcones **3a-i**, **6a-h**, **7a**, **b**, and **h** to thus modulate not only Aβ species, but also Aβ-metal complexes was examined.

$BioAβ_{42}$ oligomer aggregation: The influence of the synthesized chalcones on the self-assembly of $bioAβ_{42}$ monomers into oligomers in the absence and presence of metal ions was first examined (Fig. 6A). The inhibition experiment was performed to determine whether chalcones **3a-i**, **6a-h**, **7a**, **b**, and **h** were able to control the formation of metal-free and metal-associated $bioAβ_{42}$ oligomers using a quantitative $bioAβ_{42}$ single-site streptavidin-based assay.[43, 44] Among these chalcones, **3d**, **6f**, and **6g** were the only ones to demonstrate oligomer assembly inhibition (EC_{50} <50 μM; Table 1). However, in the absence of metals, they displayed EC_{50} values that were 6-, >36-, and 14-fold greater than that of the known assembly inhibitor clioquinol (EC_{50} = 1.4 μM).[44] Interestingly, **6g**, which does not bind Cu^{2+} ions (Fig. 2), exhibited the greatest reactivity with Aβ monomers, both in the absence or presence of metal ions. The presence of $ZnCl_2$ or $CuCl_2$ does not seem to affect the inhibitory capability of **3d** and **6g**. $CuCl_2$, on the contrary, increases the reactivity of **6f** while metal-free or Zn^{2+}-induced Aβ oligomer assembly were unaffected by the compound. Moreover, chalcone **3a**, which was previously found to exhibit antifibrillogenic activity,[34] appears not to be efficacious at the oligomeric level. This thus suggests that the ability of chalcones to interact with metal ions might not be an indispensable feature in the prevention the of $bioAβ_{42}$ oligomers formation.

Inhibition experiments

A. ASSEMBLY:

Chalcone (50-0.78 μM) +/- $CuCl_2$ or $ZnCl_2$ (25 μM) —5 min, rt→ + monomeric $bioAβ_{42}$ (10 nM) —1. 30 min, rt; 2. Tween 20→ measure oligomer content

B. DISSOCIATION:

Chalcone (50-0.78 μM) +/- $CuCl_2$ or $ZnCl_2$ (25 μM) —5 min, rt→ + pre-formed oligomeric $bioAβ_{42}$ (2.8 nM) —18 h, rt→ measure oligomer content

Fig. 6. Schematic representation of the inhibition experiments of assembly and dissociation of $bioAβ_{42}$ in the presence or absence of metal salts.

$Aβ_{42}$ oligomer dissociation: The influence of the synthesized chalcones on the dissociation of pre-formed Aβ oligomers in the absence and presence of metal ions was also investigated (Fig. 6B). The experiment was performed, in this case, to assess the ability of chalcones **3a-i**, **6a-h**, **7a**, **b**, and **h** to disassemble pre-formed $bioAβ_{42}$ oligomers in the absence or presence of metal ions. $BioAβ_{42}$ oligomers, of size similar to that of $Aβ_{42}$ oligomers from AD brain,[45] were used in this study. In general, all the chalcones were better at disassembling $bioAβ_{42}$ oligomers than preventing their formation from monomers (Table 1). Chalcones

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3a-i, which bear the 2-pyridyl ketone moiety, showed greater reactivity in the absence of metal ions. Incubation of these chalcones with metal ions prior to the addition of pre-formed bioAβ_{42} oligomers may allow them to first interact with the metal ions, which could then distort the structural backbone known to bind Aβ species, and thus disassemble less effectively pre-formed bioAβ_{42} oligomers. Furthermore, while the EC_{50} values of the chalcones obtained in the presence of $ZnCl_2$ were comparable (although slightly higher) to those in metal-free conditions, there was a significant increase in EC_{50} values of the chalcones in Cu^{2+}-treated bioAβ_{42} oligomer dissociation. Cu^{2+} ions thus seem to provide a greater barrier to chalcones than Zn^{2+} ions in the dissociation of bioAβ_{42} oligomers, suggesting that these chalcones may interact better with Cu^{2+} than Zn^{2+}. This may result from the fact that chelation of Zn^{2+} is more pronounced in nitrogen-rich environment.[46, 47] The metal ions could also be binding to the Aβ oligomers stabilizing their structure.

Table 1. EC_{50} values (in μM) for chalcones in the absence or presence of metal ($ZnCl_2$ or $CuCl_2$) for prevention of bioAβ_{42} oligomers self-assembly and for dissociation of pre-formed bioAβ_{42} oligomers.

Prevention of assembly of bioAβ_{42} oligomers[a]			
Cpd	**No metal**	**$ZnCl_2$**	**$CuCl_2$**
3d	19.25 ± 0.96	20.00 ± 0.01	19.88 ± 0.63
6f	>50	>50	16.63 ± 5.68
6g	8.93 ± 0.38	9.25 ± 0.96	9.83 ± 0.62
Dissociation of pre-formed bioAβ_{42} oligomers			
Cpd	**No metal**	**$ZnCl_2$**	**$CuCl_2$**
3a	1.04 ± 0.09	1.10 ± 0.07	2.63 ± 0.93
3b	3.13 ± 0.61	4.30 ± 0.84	>50
3c	1.25 ± 0.10	1.58 ± 0.29	2.45 ± 0.44
3d	1.70 ± 0.41	4.14 ± 1.47	>50
3e	1.53 ± 0.39	1.45 ± 0.30	3.70 ± 0.49
3f	6.50 ± 1.05	5.38 ± 0.81	8.25 ± 0.50
3g	9.33 ± 1.67	11.13 ± 0.63	18.50 ± 2.38
3h	10.50 ± 2.52	11.50 ± 2.52	33.25 ± 0.96
3i	10.25 ± 0.29	11.00 ± 0.71	13.75 ± 1.50
6a	1.15 ± 0.06	1.88 ± 0.15	1.93 ± 0.30
6b	2.05 ± 0.06	2.25 ± 0.19	2.08 ± 0.10
6c	2.68 ± 0.49	3.68 ± 0.62	3.20 ± 0.58
6d	2.33 ± 0.15	2.43 ± 0.26	2.53 ± 0.46
6e	2.85 ± 0.31	2.55 ± 0.17	2.93 ± 0.41
6f	2.35 ± 0.44	2.40 ± 0.08	2.38 ± 0.62
6g	1.28 ± 0.17	1.45 ± 0.10	3.78 ± 1.30
6h	2.58 ± 0.39	2.50 ± 0.36	2.53 ± 0.42
7a	>50	>50	>50
7b	>50	>50	>50
7h	15.50 ± 2.38	>50	>50

[a] All compounds not presented displayed EC_{50} values >50 μM.

In both the absence and presence of metal ions, chalcones **3f-i**, with a Br substituent on the pyridyl moiety, were less efficient at dissociating bioAβ_{42} oligomers than their methyl counterparts **3b-e**. Since the former are poorer metal chelating agents than their methyl counterparts, they would be expected to bind bioAβ_{42} oligomers better in accordance with previous observations. This reinforces the fact that assembly of Aβ species is a complex process. Finally, no direct correlation was observed between the substitution pattern on ring I of these chalcones and their ability to dissociate bioAβ_{42} oligomers.

Chalcones **6a-h** exhibited EC_{50} values that were quite constant both in metal-free and metal-treated studies. Also, substitution on ring I only seems to slightly reduce the efficacy of the chalcones (**6a** *versus* **6b-h**). When compared to **3a-i,** chalcones **6b**, **6f**, **6g**, and **6h** demonstrated better reactivity at disassembling bioAβ_{42} oligomers than their 2-pyridyl ketone counterparts in the absence and presence of metal ions.

Chalcones **7a**, **b**, and **h** were the least potent of the synthesized molecules as they showed poor reactivity (EC_{50} >50 μM). **7h** was only able to dissociate bioAβ_{42} oligomers in the absence of metal ions.

Overall, chalcones **3a**, **3c**, **3d**, **3e**, **6a**, and **6g** were the best dissociators in the absence of metal ions and they displayed EC_{50} values that were within 2-fold of the EC_{50} value of the dissociator 2,5-dihydroxybenzoic acid (0.7 μM).[48] **6b**, **6f**, **6g**, and **6h** also had great effect on the *in vitro* modulation of metal-free and metal-induced bioAβ_{42} dissociation.

Taken together, the results from bioAβ_{42} oligomer aggregation and bioAβ_{42} oligomer dissociation studies reveal that **3d** and **6g** are better than the assembly inhibitor clioquinol (that does not work on dissociation)[44] and the dissociator 2,5-dihydroxybenzoic acid (that has no effect on assembly).[48]

In vitro cholinesterase inhibition

AChE and BChE inhibition: Cholinesterases represent another valuable target in the discovery of AD therapeutics. To that effect, we evaluated the potential cholinesterase inhibitory activity of our chalcones **3a-i**, **6a-h**, **7a**, **b** and **h** by determining their individual IC_{50} values against AChE from *Electrophorus electricus* (*Ee*AChE) and BChE from equine serum (*es*BChE) (Table 2, Fig. 54) according to Ellman's method.[49] All our tested chalcones displayed micromolar IC_{50} values that were in the range of the IC_{50} value previously observed for the FDA-approved AChE inhibitor rivastigmine.[50] The IC_{50} values ranged from 9.55 ± 1.95 μM to >200 μM for derivatives **3a-i** with the 2-pyridyl ketone moiety, and 1.61 ± 0.62 μM to >200 μM for derivatives **6a-h** with the 2-acylphenol moiety, while **7a**, **7b**, and **7h**, with the 2-acyl aniline moiety, had IC_{50} values of 4.00 ± 1.20 μM, 1.44 ± 0.48 μM, and 0.503 ± 0.154 μM, respectively. The known chalcone **3a** revealed potent AChE inhibitory activity with IC_{50} = 14.8 ± 5.6 μM. Methyl substitution at any position of the pyridyl ring does not appear to improve the inhibitory activity of **3a** against AChE. Meanwhile, the attachment of a bromide atom at the R_1 (**3f**: IC_{50} = 9.67 ± 3.00 μM) or R_2 (**3g**: IC_{50} = 9.55 ± 1.95 μM) position seems to enhance its inhibitory activity. For chalcones **6a-h**, substitution tends to enhance the inhibitory activity of **6a** against AChE (IC_{50} = 13.5 ± 4.4 μM), except in the case of a methyl substitution at the R_2 position (**6c**: IC_{50} >200 μM) and a bromo substitution at the R_3 position (**6h**: IC_{50} >200 μM). Finally, methyl substitution at the R_1 position of chalcone **7a** lowers its IC_{50} value by 2-fold (**7a**: IC_{50} = 4.00 ± 1.20 μM *versus* **7b**: IC_{50} = 1.44 ± 0.48 μM) and bromo substitution at R_3 position lowers its IC_{50} value by 8-fold (**7a**: IC_{50} = 4.00 ± 1.20 μM *versus* **7h**: IC_{50} = 0.503 ± 0.154 μM). Overall, chalcones with the 2-acyl aniline moiety (**7a**, **7b**, and **7h**) were better *in vitro* AChE inhibitors than the chalcones with the 2-acyl phenol moiety (**6a-h**), which in turn were more potent than **3a-i**. Furthermore, when compared to other chalcones reported in the literature as potent AChE inhibitors,[50] **6e**, **6g, 7a**, **7b**, and **7h** displayed improved inhibitory activities with $IC_{50} \leq 4$ μM.

Since BChE is another cholinesterase enzyme associated with Aβ plaques,[18] we tested the inhibitory activity of our synthesized

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chalcones towards BChE. Unfortunately, our tested compounds were in general less effective against BChE than AChE. Nevertheless, **3a**, **3c**, **3e**, **3h**, **6d**, **6f**, **6g**, and **7b** exhibited some potency against BChE, with **3c**, **3e**, and **6f** even showing some improvement in activity when compared to AChE.

Our synthesized chalcones thus appear to be more active against AChE than BChE, and as a result their inhibitory effect on AChE was also examined in the presence of metal ions.

Table 2. Inhibition (IC_{50} values (in μM)) of the activity of AChE alone and in the presence of $ZnCl_2$ and $CuCl_2$, and BChE alone by chalcones **3a-i**, **6a-h**, and **7a**, **b**, and **h**.

Cpd	AChE	AChE + $ZnCl_2$	AChE + $CuCl_2$	BChE
3a	14.8 ± 5.6	>200	>200	14.3 ± 2.8
3b	16.3 ± 6.3	>200	>200	>200
3c	12.3 ± 1.3	>200	>200	8.24 ± 1.20
3d	>200	--[a]	--[a]	>200
3e	35.9 ± 7.6	>200	>200	16.4 ± 3.1
3f	9.67 ± 3.00	>200	>200	>200
3g	9.55 ± 1.95	>200	>200	>200
3h	14.9 ± 0.9	>200	>200	39.0 ± 9.8
3i	55.8 ± 16.8	>200	>200	>200
6a	13.5 ± 4.4	9.27 ± 2.28	15.3 ± 7.7	>200
6b	10.1 ± 3.8	3.50 ± 0.81	5.78 ± 1.87	>200
6c	>200	--	--	>200
6d	7.14 ± 1.60	>200	>200	34.8 ± 5.0
6e	1.61 ± 0.62	>200	>200	>200
6f	7.76 ± 2.77	>200	>200	2.82 ± 0.29
6g	2.85 ± 0.89	>200	>200	4.32 ± 1.05
6h	>200	--	--	>200
7a	4.00 ± 1.20	0.688 ± 0.159	20.2 ± 8.9	>200
7b	1.44 ± 0.48	2.07 ± 0.61	22.5 ± 9.0	3.92 ± 0.90
7h	0.503 ± 0.154	>200	>200	>200

[a] These were not detemined as the IC_{50} value against AChE alone was already >200 μM.

Effect of metals on AChE inhibition: The effect of Cu^{2+} and Zn^{2+} on AChE inhibition was tested and, while most of our chalcones suffered a decrease in their activity, displaying IC_{50} values >200 μM, **6a**, **6b**, **7a**, and **7b** were still potent, with IC_{50} values ranging from 5.78 ± 1.87 μM to 22.5 ± 9.0 μM in the presence of $CuCl_2$, and from 0.688 ± 0.159 μM to 9.27 ± 2.28 μM in the presence of $ZnCl_2$. Cu^{2+} increased the IC_{50} value of all the chalcones but **6b**. On the other hand, Zn^{2+} actually improved the inhibitory activity of **6a**, **6b**, and **7a**. It thus appears that chalcones bearing a 2-acyl aniline moiety could be important AChE inhibitors.

Conclusions

Three main classes of chalcones have been synthesized and biochemically evaluated *in vitro*. These compounds showed ability to chelate metal ions and/or potent dissociation of preformed bioAβ_{42} aggregates. Chalcones **3a-i** showed the ability to bind metal ions such as Cu^{2+} and Zn^{2+}. Additionally, **3d** and **6g** effectively modulated the assembly and disassembly of bioAβ_{42} oligomers in the presence and absence of metal ions, showing a broader reactivity on Aβ modulation than clioquinol and 2,5-dihydroxybenzoic acid. Chalcones **7a**, **7b**, and **7h**, with a 2-acyl aniline moiety, also displayed potent AChE inhibitory activities and thus appear to be a class of compounds worthy of additional studies. Finally, **6g** seems to be a multifunctional compound as it exhibited metal-Aβ modulator capability and inhibited AChE activity. Further optimization studies are currently underway in our laboratory.

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures for the synthesis and characterization of all novel compounds generated along with their NMR spectra and elemental analysis data, for Cu^{2+} and Zn^{2+} binding studies at various pH, for assays for bioAβ_{42} oligomer assembly and dissociation, and for cholinesterase inhibition in the presence and absence of $CuCl_2$ and $ZnCl_2$ studies are provided. See DOI: 10.1039/b000000x/

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