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**Ionophoric polyphenols selectively bind Cu²⁺, display potent
antioxidant and anti-amyloidogenic properties, and are non-toxic
toward *Tetrahymena thermophila***

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In memory of Roberto A. Sánchez-Delgado

ABSTRACT

21 Alzheimer's disease (AD) is the most common form of dementia affecting more than 28 million
22 people in the world. Only symptomatic treatments are currently available. Anticipated tri-fold
23 increase of AD incidence in the next 50 years has established the need to explore new possible
24 treatments. Accumulation of extracellular amyloid- β ($A\beta$) plaques, intracellular tangles in the
25 brain, and formation of reactive oxygen species (ROS) are the major hallmarks of the disease.
26 The active role of some metal ions, especially Cu^{2+} , in promoting both $A\beta$ aggregation and
27 reactive oxygen species formation has rendered ionophoric drugs as a promising treatment
28 strategy. In this work, a series of 5 disease-modifying and multi-target ionophoric polyphenols
29 (**1-5**), inspired on the structure of natural resveratrol, have been synthesized and characterized.
30 All compounds bind Cu^{2+} selectively over other biologically relevant metal ions. They form 2:1
31 (compound: Cu^{2+}) complexes with association constants $\log K_a$ 12-14 depending on the molecular
32 design. Our results indicate that compounds **1-5** possess excellent antioxidant properties: they
33 inhibit the Cu^{2+} -catalyzed reactive oxygen species production between 47% and 100%, and they
34 scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) and AAPH (2,2'-azobis(2-amindino-propane)
35 dihydrochloride) free radicals in general better than clioquinol, resveratrol and ascorbic acid. In
36 addition, compounds **1-5** interact with $A\beta$ peptides and inhibit both the Cu^{2+} -catalyzed
37 aggregation and the self-assembly of $A\beta$ (1-40) up to a ~92% extent. Interestingly, **1-5** are also
38 able to disaggregate up to ~91% of pre-formed $A\beta$ (1-40) aggregates. Furthermore, cytotoxic
39 studies show remarkably low toxicity of **1-5** toward *Tetrahymena thermophila* with LD_{50} values
40 higher than 150 μM , comparable to non-toxic natural resveratrol.

KEYWORDS: Alzheimer's disease, polyphenol, ionophoric compound, amyloid-beta aggregation, multi-target compound, reactive oxygen species, antioxidant, Tetrahymena

1. Introduction

Alzheimer's disease (AD) is the most common and incurable form of dementia, affecting more than 28 million people worldwide.^{1,2} It is most often diagnosed in people over 65 years old, although early-onset AD can also occur. These numbers are expected to increase rapidly due to the aging population, the rise in life expectancy in both the developed and developing countries, and the lack of any effective treatment.^{3,4} While the etiology and pathological mechanisms of AD are still under debate, the accumulation of extracellular amyloid- β (A β) plaques, intracellular tangles in the brain, and formation of reactive oxygen species (ROS) are the major hallmarks of the disease that lead to an uncontrolled neuronal death.⁵⁻¹⁰ Fragments A β (1-40) and A β (1-42) are the typical components of A β plaques.^{9,10} The production of A β is a normal physiological process occurring in the brain and cerebrospinal fluid throughout life.^{11,12} However, according to the amyloid cascade hypothesis,^{7,8} an increased A β production and/or accumulation leads first to the formation of A β oligomers, then to protofibrils and ultimately to fibrils that are the principal constituent of the plaques.

Therapeutic targets for the treatment of AD have been classified into two groups: 1) symptomatic therapies, and 2) disease-modifying targets. Current approved treatments (Figure 1) are old, ineffective and based on symptomatic therapies, i.e. drugs that only attenuate the symptoms but do not interdict the underlying causal pathobiology. They involve four acetylcholinesterase inhibitors (tacrine, rivastigmine, galantamine and donepezil) and an NMDA receptor antagonist (memantine). The rationale for the use of these drugs is based on the cholinergic hypothesis, one

of the earliest and most extensively researched theories regarding AD.¹³ However, the benefits from their use are very limited and none of them clearly shows to delay or halt the progression of AD. In addition, the use of anticholinergic drugs has been recently linked to higher risk of dementia¹⁴ and, thus, the search for novel chemotherapies for Alzheimer's disease clearly requires urgent attention.

Figure 1. Five approved AD treatments (tacrine, rivastigmine, galantamine, donepezil and memantine), examples of chelating agents that reached clinical trials in AD (desferrioxamine, clioquinol and PBT2), and polyphenol resveratrol.

Metal ions are thought to be involved in AD in different ways, and consensus arises in the case of Cu^{2+} . A wealth of evidence for this comes from two facts: 1) Cu^{2+} has been detected in elevated concentrations in A β plaques,^{12,15} and 2) Cu^{2+} binding to A β is aberrant and does not

87 take place under healthy conditions. Essential copper (Cu, referring to its different oxidation
88 states) is normally present in proteins related to electron and oxygen transport, although it can
89 also be found in synaptic vesicles as “free” ions (loosely bound to low affinity ions, for instance
90 glutamate).¹⁶ Importantly, there is evidence indicating that Cu homeostasis is altered in AD
91 brains, resulting in abnormal concentrations of its metal ions in the synaptic cleft.¹⁷⁻²⁰ The
92 binding of aberrant concentrations of “free” Cu to A β is then able to promote aggregation into
93 toxic and neurodegenerative forms of the protein. In addition, redox active metal ions like Cu²⁺
94 are crucial for the production of reactive oxygen species (ROS) and oxidative stress that
95 ultimately lead to neuronal death.²¹ Proof has been provided that the complex with A β (Cu²⁺-A β)
96 might be directly involved in the production of ROS.^{22,24} This mounting evidence supporting the
97 role of metal ions, specifically Cu²⁺, in the pathophysiology of AD has rendered metal
98 ionophoric therapy as a promising treatment strategy. In vitro studies have shown that production
99 of ROS via interaction of Cu²⁺ with A β is significantly attenuated by the presence of
100 diethylenetriaminepentaacetic acid (DTPA), triethylenetetraamine (TETA) and desferrioxamine
101 (DFO),²⁵ known as potent metal chelators. Furthermore, the latter, DFO, clioquinol (CQ) and
102 second generation PBT2 (Figure 1) have shown indication of slowing neurological decline in
103 early stage clinical trials.²⁵⁻²⁹ Therefore, the potential shown by this family of compounds has
104 opened a promising line of research and, although some groups have lately reported their work
105 on small molecules able to chelate Cu²⁺ and Zn²⁺,³⁰⁻³⁴ research on this issue clearly requires
106 further attention.

107 Promising strategies in the treatment of AD also involve the use of natural products capable of
108 crossing the blood brain barrier (BBB) to display neuroprotective properties by either
109 detoxification of ROS, anti-amyloidogenic potential or interaction with relevant proteins closely

related to AD.³⁵ Prominent examples are resveratrol, curcumin or flavonoids.³⁶⁻³⁹ Among them, resveratrol (3,5,4'-trans-trihydroxystilbene, Figure 1), mostly found in berries and grapes, has currently completed phase II clinical trials in 120 patients with possible AD diagnosis due to its ability to modify important AD biomarkers.⁴⁰ Several health-promoting properties have been reported for resveratrol due to its intrinsic antioxidant capacity. Interestingly, this natural compound is able to penetrate the blood-brain barrier (BBB) and exert strong neuroprotective effects,⁴¹ even at low concentrations. It has proven to fight the neuronal dysfunction caused in AD by several proposed mechanisms.^{42,43} Very importantly, resveratrol also exerts its neuroprotective activity by acting as an anti-amyloidogenic and fibril-destabilizing polyphenol,^{44,45} and by detoxifying metal-A β induced ROS, although it is not sufficient to fully block metal-A β toxicity, likely because of its inability to effectively compete with toxic forms of A β peptides for the binding of AD-related metal ions.⁴⁶

Due to the complexity of AD and the identification of many potential targets, the design of disease-modifying multi-target compounds has recently attracted the attention of researchers in the pursuit of improved drugs.^{47,48} In line with this, herein we present the synthesis of a family of multi-functional polyphenols (compounds **1-5** in Schemes 1 and 2), inspired on the structure of natural resveratrol, that additionally incorporate a Cu²⁺-selective metal ionophoric motif in their structure for enhanced activity. We also report: 1) their Cu²⁺ binding properties and ion selectivity, 2) their ability to both inhibit the Cu²⁺-A β (1-40) induced ROS formation and scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) and AAPH (2,2'-azobis(2-amindino-propane) dihydrochloride) free radicals, 3) their potential to inhibit the aggregation of A β (1-40) into pathological forms of the peptide, and 4) their cytotoxicity toward healthy eukaryotic cells of *Tetrahymena thermophila*.

2. Results and discussion

2.1. Drug design and drug likeliness

Ionophoric polyphenols **1-5** (Schemes 1 and 2) were designed inspired on clinically tested resveratrol, because of its proven anti-AD properties and lack of toxicity. Importantly, the resveratrol-like structures were provided with a Cu²⁺ selective ionophoric motif for increased anti-AD potential resulting from the combination. Thus, compounds **1-5** were expected to maintain and/or enhance all intrinsic antioxidant and anti-amyloidogenic properties of natural polyphenols, and display the added benefit of selective Cu²⁺ chelation (Scheme 1). In general, two major issues must be tackled when designing anti-AD ionophoric agents: 1) finding the right balance between activity, selectivity and metal ion affinity, and 2) achieving blood brain barrier (BBB) permeability. On one hand, ligands displaying a high affinity and/or low selectivity for metal ions might present undesired side effects. In this context, the molecular design incorporates a low-medium affinity and selective chelating motif (bidentate NH-OH or N-OH) into the structure of a polyphenol-like compound (see section 2.3 below for expanded discussion and results on metal ion selectivity). On the other hand, failure to cross the BBB is a major impediment for the design of effective drugs in anti-AD therapy.⁴⁹ BBB permeability of molecules can be predicted from Lipinski's rules, a set of criteria that evaluate molecular properties important for drug pharmacokinetics.^{50,51} These rules are based on the observation that most of the orally active drugs satisfy certain standards regarding molecular weight, lipophilicity (cLogP), number of hydrogen bond donors (HBD) and acceptors (HBA), and polar surface area (PSA). As observed in Table 1, the compounds herein presented satisfy the drug-like criteria comparably to both clinically tested resveratrol and CQ. Importantly for us, the calculation of

logBB for predicted BBB penetration ability (see equation on footnote of Table 1) using Lipinski's rules confirms probable brain distribution for all compounds.

Scheme 1. Design of ionophoric polyphenols combining the properties of natural polyphenols and ionophoric agents. Chelating motif in grey shade.

Table 1. Physicochemical properties of the ionophoric polyphenol compounds **1-5**, CQ and resveratrol.

	1	2	3	4	5	Resveratrol	CQ	Lipinski's Rules
MW	215.25	215.25	263.30	231.25	279.30	228.25	305.5	≤450
cLogP	1.755	1.755	3.59	1.158	2.924	2.833	3.729	≤5.0
HBA	3	3	3	4	4	3	2	≤10
HBD	3	3	2	4	3	3	1	≤5
PSA	52.49	52.49	52.82	72.72	73.05	60.69	32.59	≤90
LogBB	-0.30	-0.30	-0.11	-0.77	-0.51	-0.27	0.21	*

* Calculated according to $\log BB = -0.0148 \times PSA + 0.152 \times clogP + 0.130$. Drugs with logBB below -1 are expected to be poorly distributed in the brain.^{50,51}

MW, molecular weight; cLogP, calculated logarithm of the octanol-water partition coefficient; HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; PSA, polar surface area.

One last consideration in the molecular design is concerned with the nature of the nitrogen functional group. In line with this, imino compounds **3** and **5** were expected to display different electron delocalization properties than amino **1**, **2** and **4** when scavenging free radicals. In addition, we anticipated that reduced compounds **1**, **2** and **4** would present better solubility and, to some extent, different metal ion affinity or selectivity. Thus, the synthesis of both types was of interest in order to establish structure-activity relationships.

2.2. Chemistry

Target compounds **1**, **2** and **4** were synthesized by means of a typical two-step reductive amination, similarly to procedures previously described in the literature.^{52,53} In the first step, the amino group of 2-aminophenol was condensed with the aldehyde group of 4-hydroxybenzaldehyde, 3-hydroxybenzaldehyde or 3,4-dihydroxybenzaldehyde, respectively, in refluxing dry methanol through a Schiff base reaction. In the second step, the resulting imine was reduced at 0-2°C by addition of NaBH₄ to produce the desired product in good yields. Compounds **3** and **5** were synthesized by a Schiff base condensation of 2-hydroxy-1-naphthaldehyde with 2-aminophenol or 4-aminoresorcinol, respectively, in refluxing dry methanol, and isolated as stable imino compounds (Scheme 2).

Regarding stereochemical considerations, compounds **3** and **5** were obtained in their E conformation for the C-N double bond, as confirmed by ¹H-¹H NOESY experiments showing a diagnostic signal between the proton in the CH of the imino group and the hydrogen in ortho of the phenol group (See compound **3** as example in Supplementary Information, Figure S1). Additionally, energy optimization calculations revealed a difference of 10.8 and 14.6 kcal/mol between Z and E configurations (Z displaying higher energy than E) for **3** and **5**, respectively.

192 Imino intermediates of **1**, **2** and **4** were also isolated, but NMR showed decomposition in
 193 presence of water or D₂O and, therefore, only the reduced amino derivatives were used for
 194 further testing.

206 **Scheme 2.** Synthesis of compounds **1-5**. **a**, **b** and **d**) i. Reflux, 2h, MeOH; ii. NaBH₄, MeOH, 0-
 207 2°C; **c** and **e**) Reflux, 5h, MeOH.

208

209 2.3. Metal ion binding properties and selectivity

210 Although metal chelation represents a promising strategy for the treatment of AD, long term use
 211 of ionophoric agents might affect the homeostasis of numerous biometals and the normal
 212 physiological functions of biomolecules that require of those metal ions.^{29,31} For this reason, it is
 213 of critical importance that ligands targeting toxic concentrations of specific ions present both an
 214 ion-selective binding rather than systemic, and a moderate-low ion affinity. This would allow to

215 compete with A β for metal binding (A β forms 1:1 complexes with Cu²⁺ with LogK_a values in the
216 range 8-10, depending on experimental conditions^{17,54}), but not with metalloenzymes, therefore,
217 resulting in a combination that could promote restoration of ionic balance rather than total
218 removal of metal ions.⁵⁵ Given the active role of Cu²⁺ in promoting A β aggregation and ROS
219 production, compounds **1-5** were studied spectrophotometrically and compared to CQ and
220 resveratrol to determine 1) their ability to selectively chelate Cu²⁺ versus other biologically
221 relevant metal ions, such as Zn²⁺, Fe²⁺, Ca²⁺, Mn²⁺ and Mg²⁺, and 2) their Cu²⁺ binding
222 properties, including stoichiometry and association constants of the compound-Cu²⁺ adducts. CQ
223 (used here as a control) is known to form 2:1 complexes with Cu²⁺ with low association
224 constants in the range of LogK_a 10-16,^{56,57} depending on buffer conditions;^{57,58} these values are
225 lower than those observed for other 8-hydroxyquinolines.⁵⁹ Because of its low metal binding
226 affinity, literature argues that CQ has the potential not to interfere with intracellular metal-
227 binding enzymes⁵⁷ and, therefore, CQ is claimed to be able to remove metal ions from A β and
228 redistribute them within the brain regions where they can promote synaptic function.⁵⁵ However,
229 severe myelo-optic acute neuropathy on patients treated with CQ has been associated to lack of
230 tissue and/or ion selectivity and, most especially, to the ability to chelate Zn²⁺.²⁹ Our results for
231 CQ metal binding agree, in general, with these latter findings; CQ displayed an average LogK_a of
232 15.58, but exhibited a poor selectivity for Cu²⁺ with respect to Zn²⁺, Fe²⁺, Ca²⁺, Mn²⁺ and Mg²⁺
233 (Figure S2 in Supplementary Information). On the other hand, natural resveratrol showed a
234 selective binding for Cu²⁺ and Fe²⁺ over Zn²⁺, Ca²⁺, Mn²⁺ or Mg²⁺ (Figure S2 in Supplementary
235 Information), but displayed a much lower association constant for Cu²⁺ (logK_a= 6.70, on
236 average) likely insufficient to compete with A β for metal binding and, therefore, to prevent AD
237 Cu toxicity.

The interaction of **1-5** with metal ions was spectrophotometrically followed by changes in the absorption spectra upon interaction with metal ions. For metal ion selectivity, buffered solutions of compounds **1-5** were prepared and treated with the metal ion under study (Zn^{2+} , Fe^{2+} , Ca^{2+} , Mn^{2+} and Mg^{2+}). After 5 minutes incubation, the optical response was observed, followed by addition of Cu^{2+} and consequent analysis of further spectral changes (see Experimental Section for more details). Results for compounds **1-5** (only **1** and **3** are shown in Figure 2 as representative examples of amino and imino compounds; see the behavior of compounds **2**, **4**, **5**, CQ and resveratrol in Supplementary Information, Figure S2) show that these ionophoric polyphenols bind Cu^{2+} with a remarkably better selectivity than clinically tested CQ; this feature could have important implications in preventing side effects. Binding stoichiometry and apparent association constants for Cu^{2+} binding were calculated from spectrophotometric titrations of **1-5** upon stepwise additions of a Cu^{2+} solution (see Figure S3 in Supplementary Information). These experiments revealed that all the ionophoric polyphenols form 2:1 complexes with Cu^{2+} (compound: Cu^{2+} ; binding through the chelating motif NH-OH for amino compounds, or N-OH for imino compounds is proposed. See Scheme 1), similarly to what has been observed for CQ. Interestingly, compounds **1-5** displayed low values for LogK_a (see Table 2), as proposed in the molecular design (Section 2.1), adequate to prevent competition with cuproenzymes. When compared within the series of ionophoric polyphenols, amino compounds **1**, **2** and **4** showed lower association constants and slightly worse metal selectivity than imino derivatives **3** and **5**. The experiments described in the following sections were aimed at correlating Cu^{2+} binding properties with the compounds' ability to inhibit ROS production and $\text{A}\beta(1-40)$ aggregation.

Figure 2. Relative changes in optical response (absorbance intensity) when compounds **1** (A) and **3** (B) interact with metal ions (see Figure S2 in Supplementary Information for results on compounds **2**, **4**, **5**, CQ and resveratrol). Black bars represent normalized affinity for metal ions M ($M = Zn^{2+}$, Fe^{2+} , Ca^{2+} , Mn^{2+} or Mg^{2+}), while blue bars indicate affinity for Cu^{2+} in presence of M.

Table 2. Apparent association constants for Cu^{2+} , and binding affinity to $\text{A}\beta(1-40)$ peptide (calculated from tyrosine fluorescence quenching in titration curves; Figure S7 in Supplementary Information) of compounds **1-5**, CQ and resveratrol.

Compound	Apparent Association Constant for Cu^{2+} (LogK_a)	Binding Affinity for $\text{A}\beta(1-40)$ ($\times 10^5 \text{ M}^{-1}$)
CQ	15.58 ± 0.34	3.69 ± 0.59
Resv	6.70 ± 0.25	1.65 ± 0.17
1	12.82 ± 0.40	3.27 ± 0.65
2	13.07 ± 0.40	1.55 ± 0.15
3	14.01 ± 0.19	1.91 ± 0.19
4	12.62 ± 0.12	3.55 ± 0.52
5	14.29 ± 0.35	2.46 ± 0.13

2.4. Antioxidant ability: Inhibition of Cu^{2+} - $\text{A}\beta(1-40)$ hydroxyl radical production and scavenging of DPPH and AAPH radicals

Evidence indicates that one of the main causes of neuronal cell loss in AD is due to excessive free radical generation. ROS, such as $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$, which are produced under oxidative stress, seem to play a role in the neurotoxicity of $\text{A}\beta$, and research has shown that at least trace amounts of metals are necessary for the formation of such toxic species.⁶⁰⁻⁶² A causative link between the Cu^{2+} - $\text{A}\beta$ complex and oxidative stress is supported by a body of evidence: 1) sequestering Cu^{2+} from $\text{A}\beta$ induces a significant decrease in $\text{A}\beta$ toxicity, 2) only Cu-containing $\text{A}\beta$ aggregates are toxic to primary neurons, and 3) Cu-containing fibrils are more toxic to PC12 cells than other

forms of the peptide.¹⁷ The complex Cu^{2+} -A β is known to catalyze the generation of $\text{O}_2^{\cdot-}$, H_2O_2 and $\text{OH}^{\cdot}$ through standard Fenton reactions in presence of molecular oxygen and a reducing agent.⁶³ This becomes relevant given that the brain environment is well supplied in oxygen and several reducing agents (ascorbate, L-dopa, dopamine, cholesterol, etc) are easily available at relatively high concentrations (for instance, brain concentration of ascorbate ranges between 200 μM and 10 mM).

In this work we have measured the ability of compounds **1-5** to inhibit the Cu^{2+} -A β (1-40) catalyzed formation of hydroxyl radical ($\text{OH}^{\cdot}$), one of the most toxic radicals in biological systems. The effect of resveratrol and CQ was also analyzed for comparison purposes. Hydroxyl radical production was promoted through Fenton reaction of Cu^{2+} -A β (1-40) with dissolved O_2 in presence of ascorbate and 2 eq. of the compounds under study (see Experimental Section for more details). Furthermore, time course plots (Figure S4 in Supplementary Information) were used to calculate the apparent rate of ROS formation by determining the slope of the linear segment of the graphs. All results are displayed in Figure 3 and Table 3, and show that, in general, compounds **1-5** both inhibit and slow down the Cu^{2+} -A β (1-40) catalyzed generation of hydroxyl radical better than parent resveratrol. Importantly, compounds **3** and **5** completely suppress the Cu^{2+} -A β (1-40) catalyzed ROS formation, better than clinically tested resveratrol and CQ, most likely due to their higher Cu^{2+} binding affinity. Results for CQ agree with previously reported experiments.⁶³

Figure 3. Percent $\text{OH}^\bullet$ generation by the adduct $\text{Cu}^{2+}\text{-A}\beta(1\text{-}40)$ in presence of 2 eq. of compounds **1-5**, CQ and resveratrol. ROS generation by the $\text{Cu}^{2+}\text{-A}\beta(1\text{-}40)$ adduct was taken as 100% positive control. See time course production of hydroxyl radical by the adduct $\text{Cu}^{2+}\text{-A}\beta(1\text{-}40)$ in presence of compounds **1-5** in Supplementary Information, Figure S4.

In addition, the total antioxidant capacity of compounds **1-5** was also determined in absence of Cu^{2+} ions by the DPPH assay⁶⁴ and a modification of the ORAC (oxygen radical absorbance capacity) assay.^{65,66} The DPPH assay is based on the reduction of the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Because of its unpaired electron, DPPH gives a strong absorption maxima at 517 nm (purple color), as the odd electron of the radical becomes paired off in presence of a radical scavenger, the absorption intensity decreases and the discoloration is stoichiometric with the number of electrons captured.⁶⁷ The DPPH assay is commonly used to establish the antioxidant activity of phenolic compounds as well as natural plant extracts.⁶⁵ On the other hand, assays based on the scavenging of 2,2'-azobis(2-amindino-propane) dihydrochloride (AAPH), like the ORAC assay, are said to be more relevant because they use a

biologically significant radical source. This method determines the ability of antioxidants to protect fluorescein (FS) from damage by free radicals. It consists of measuring the decrease in fluorescence of FS when it suffers oxidative damage caused by a source of peroxy radical (ROO[•]) such as AAPH. This is important given that the presence of oxygen radicals in AD brains is a common feature.

Table 3. Antioxidant properties of compounds **1-5**. Ability to inhibit Cu²⁺-A β (1-40) catalyzed hydroxyl radical production and to scavenge DPPH and AAPH free radicals.

Compound	% inhibition of Cu ²⁺ -A β (1-40) catalyzed ROS production	Rate of radical formation (a.u./min)*	IC ₅₀ for DPPH radical** (r.a.)	IC ₅₀ for AAPH radical** (r.a.)
Cu ²⁺ -A β (1-40)	0	13.0 $\pm$ 0.2	--	--
CQ	87.7 $\pm$ 0.9	1.2 $\pm$ 0.4	40.6 $\pm$ 6.3 (0.4)	9.72 $\pm$ 0.42 (0.2)
Resv	9.0 $\pm$ 3.3	11.2 $\pm$ 0.4	17.8 $\pm$ 0.8 (1.0)	1.70 $\pm$ 0.04 (1.0)
Ascorbic Acid	--	--	12.8 $\pm$ 0.8 (1.4)	62.2 $\pm$ 0.2 (0.02)
1	47.3 $\pm$ 1.0	6.8 $\pm$ 0.6	6.27 $\pm$ 0.59 (2.8)	1.38 $\pm$ 0.13 (1.2)
2	51.1 $\pm$ 1.3	5.4 $\pm$ 0.8	5.87 $\pm$ 0.48 (3.0)	1.24 $\pm$ 0.15 (1.4)
3	100.0	0.00	38.5 $\pm$ 5.8 (0.5)	11.5 $\pm$ 0.5 (0.1)
4	59.9 $\pm$ 2.4	4.0 $\pm$ 0.2	3.05 $\pm$ 0.10 (5.8)	0.61 $\pm$ 0.08 (2.8)
5	98.5 $\pm$ 0.2	0.03	13.5 $\pm$ 0.3 (1.3)	3.15 $\pm$ 0.08 (0.5)

*Calculated as the slope of the lineal part of the time course plot fluorescence intensity vs. time. Figure S4 in Supplementary Information.

**All values in μ M concentration.

r.a. (relative activity): IC₅₀ resveratrol/IC₅₀ compound.

352 The DPPH assay was performed as previously published by others.⁶⁴ IC₅₀ values for the
353 scavenging of DPPH radicals were calculated and compared to those of resveratrol, CQ and
354 ascorbic acid used as controls. All results are shown in Table 3 and Figure S5 in Supplementary
355 Information (dose vs. response curves), and reveal that amino compounds **1**, **2** and **4** are between
356 2.8 and 5.8 better at scavenging DPPH radicals than resveratrol, and between 2 and 4.2 times
357 more active than ascorbic acid. On the other hand, compound **5** proved to have a better activity
358 than resveratrol and comparable to that of ascorbic acid, while compound **3** and CQ showed poor
359 DPPH scavenging properties. Our results for ascorbic acid agree with previously reported values
360 measured under the same conditions,⁶⁴ whereas values for resveratrol have been reported at
361 different concentrations or buffer solutions and cannot be directly compared.^{65,66} Our
362 experimental conditions were chosen to ensure spectrophotometric accuracy at 517 nm
363 (absorbance between 0.1-1.0).

364 To measure the ability of compounds **1-5** to scavenge peroxyl radicals, we used a modification of
365 the ORAC assay.^{65,66} The protective effect on FS of the ionophoric polyphenols was then
366 compared to that of resveratrol, CQ and ascorbic acid used as controls. Results, shown in Table 3
367 and Figure S6 in Supplementary Information (dose vs. response curves), generally follow a
368 similar trend to that observed in the DPPH assay. Amino compounds **1**, **2** and **4** display a better
369 ability to reduce the peroxyl radical AAPH in absence of metal ions. Importantly, compound **4** is
370 up to 2.8 times better scavenger than resveratrol under the studied conditions. In general,
371 compounds **3**, **5** and CQ are less efficient than resveratrol, while ascorbic acid proved to have a
372 low peroxyl radical protecting ability when compared to the polyphenols, as previously reported
373 by others.^{66,69}

Results obtained for compounds **3** and **5** in all antioxidant tests herein performed seem to indicate that the activity observed in the hydroxyl radical inhibition experiment (Figure 3) was mainly due to their better ability to sequester Cu^{2+} ions as a consequence of a higher Cu^{2+} binding affinity, whereas amino compounds **1**, **2** and **4**, despite having a lower Cu^{2+} affinity and therefore being worse inhibitors of fenton-produced radicals, they are better antioxidant agents in absence of metal ions. We hypothesize then that this behavior could be partly due to radical scavenging by hydrogen transfer from the amino group. In particular, all antioxidant tests taken together show that compound **4** displays the best overall antioxidant activity of our ionophoric polyphenols, inhibiting to a 60% extent the Cu^{2+} -A β (1-40) catalyzed production of hydroxyl radicals, while being able to reduce and scavenge DPPH and AAPH free radicals better than both resveratrol and ascorbic acid. These observations might be of importance in the rational design of future generations of anti-AD agents with antioxidant properties.

2.5. Inhibition of Cu^{2+} -induced A β (1-40) aggregation

The amyloid cascade hypothesis suggests that increased production in A β peptides leads first to the formation of A β amorphous oligomers that evolve then to protofibrils and ultimately to fibrils, the main constituents of the amyloid plaques.^{9,10} There is evidence indicating that Cu^{2+} promotes A β aggregation into neurodegenerative forms of the protein in a pH dependent fashion. It has been reported that Cu^{2+} -catalyzed aggregation is more significant at slightly acidic pH (6.6-6.8) than at 7.4,⁷⁰ and this becomes relevant given that altered proton homeostasis, known as cerebral acidosis, is hypothesized in AD.^{71,72} Thus, the ability of **1-5** to inhibit the Cu^{2+} -induced A β (1-40) aggregation was tested and compared with that of CQ and resveratrol at pH=6.60. Solutions of A β (1-40) were incubated up to 18 days with 1 eq. of Cu^{2+} and 2 eq. of compounds

1-5. The amount of aggregates was then measured after 3, 7 and 18 days by means of the thioflavin-T (ThT) and turbidity assays. ThT exhibits a moderately strong fluorescence emission at 485 nm upon interaction with mature A β fibrils and, therefore, the ThT assay is used as a key diagnostic for the formation of this kind of aggregates. On the other hand, the turbidity assay measures light scattering due to the presence of protein aggregates in solution and, therefore, it can determine the total amount of aggregates, including both the initial amorphous oligomeric form and mature fibril aggregates that form A β plaques. However, in agreement with what other researchers have suggested,⁷³ we observed that amorphous aggregates exhibited remarkably higher turbidity than mature fibrils and, therefore, in this work turbidity results were taken mostly as evidence of misfolded structures of A β (1-40) in the oligomeric form. Whether the oligomeric or the fibril is the most toxic form of A β aggregates still remains controversial.⁷⁴ Figure 4 shows results when ThT fluorescence and turbidity were monitored up to 18 days.

Figure 4. Inhibition of Cu^{2+} -induced $\text{A}\beta(1-40)$ aggregation by compounds **1-5**, CQ and resveratrol studied by ThT (**A**) and turbidity (**B**) assays. No significant changes were observed in ThT fluorescence after 18 days of incubation, and, therefore, that data is not shown for clarity. All values of fluorescence and percent inhibition of fibril aggregation can be found in Table S1 in Supplementary Information.

441 Several interesting observations can be made when turbidity and ThT assays results are taken
442 together: 1) Previously reported results by ThT, AFM and electron microscopy⁷⁵⁻⁷⁸ indicate that
443 the presence of Cu^{2+} exacerbates the formation of amorphous aggregates (when compared to A β
444 self-assembly) and inhibits the fibrilization of A β . The increase in turbidity and decrease of ThT
445 fluorescence observed when A β (1-40) was treated with Cu^{2+} in our experiments (see Figure 4)
446 agree with previous findings and validate our observation of higher turbidity for amorphous
447 forms of A β (1-40) aggregates. The Cu^{2+} ion effect has been attributed to its preferential binding
448 to high affinity protein sites of the N-terminal side of the A β peptide that are crucial for β -sheet
449 formation and consequent stabilization of non-amyloidogenic conformations. A slight decrease
450 of turbidity combined with an increase in fluorescence is observed for control Cu^{2+} -A β (1-
451 40) from 3 to 18 days incubation, which suggests that amorphous aggregates might be evolving
452 to mature A β (1-40) fibrils with time. 2) ThT and turbidity assays for resveratrol and CQ show
453 significantly distinct behavior for the two controls. The natural polyphenol attenuates the
454 appearance of mature A β (1-40) fibrils to an~ 85% extent (see Table S1 in Supplementary
455 Information for all values of fluorescence and percent inhibition) by promoting the aggregation
456 into amorphous forms, a phenomenon that has been previously observed for resveratrol⁷⁹ and
457 several other natural polyphenols like morin, quercetin, ferulic acid or gossypol.⁷³ It is
458 hypothesized that fibrillar structures possess structural features in which aromatic residues of the
459 A β peptide engage in stacking interactions that are absent in the oligomeric forms. It is then
460 suggested that resveratrol and other polyphenols can interact with aromatic chains of A β , which
461 thus explains the fact that such polyphenols seem to selectively destabilize the A β fibril
462 population.⁷⁹ and refs. therein In particular, it has been found that resveratrol remodels the A β
463 aggregation to form non-toxic high-molecular weight oligomeric aggregates.⁷⁹ The formation of

such aggregates would agree with the increase of turbidity observed in our experiments. On the other hand, CQ directs and enhances the Cu^{2+} -induced aggregation process into assembly to mature $\text{A}\beta(1-40)$ fibrils (~195-250% fibrils with respect to the Cu^{2+} - $\text{A}\beta(1-40)$ control are detected in our experiments; see Table S1 in Supplementary Information), a phenomenon previously reported and associated to two main factors: Firstly, metal removal from the $\text{A}\beta(1-40)$ protein by CQ seems to restore the aggregational properties of the native peptide. Secondly, the interaction of CQ with hydrophobic domains of $\text{A}\beta(1-40)$ could stimulate protein conversion into mature fibrils⁸⁰ (in section 2.6 the ability of CQ to interact with $\text{A}\beta(1-40)$ has been tested). 3) Compound **3** prevents the formation of Cu^{2+} -induced total aggregates by inhibiting the misfolding of $\text{A}\beta(1-40)$ into its amorphous form up to a ~50% extent, better than resveratrol and slightly better than CQ, and the fibrillary assembly up to a ~67% extent. 4) ThT assay reveals that all compounds inhibit the formation of mature $\text{A}\beta(1-40)$ fibrils between a ~67% and a ~92% extent, comparably to resveratrol and much better than CQ; and 5) compounds **1**, **2**, **4** and **5** destabilize the $\text{A}\beta(1-40)$ fibril formation by initially promoting amorphous aggregates. As previously stated, literature on polyphenols, as well as on small molecules and peptides, suggests that this kind of compounds might redirect the aggregation cascade instead of inhibiting it completely.⁸¹⁻⁸³ Importantly, the high similarity with the results observed for resveratrol allows us to hypothesize that our ionophoric polyphenols **1-5** might be exerting a comparable effect to that of the natural polyphenol⁷⁹ and remodeling the aggregation towards non-toxic high molecular weight oligomeric forms of $\text{A}\beta$, although this statement cannot be fully confirmed with the data at hand. In addition, our results show that prolonged exposure to compounds **1-5** (up to 18 days were studied in this work) induces a decrease in the amount of amorphous aggregates without increasing the fibril population. The differential turbidity pattern observed for

compound **3** with respect to the rest of ionophoric polyphenols could be partly explained by its higher Cu^{2+} affinity and thus better ability to counteract Cu^{2+} effect, although lack of the same pattern for compound **5** cannot be explained with the data at hand. Comparison of **1-5** with both controls CQ and resveratrol leads to postulate that our ionophoric polyphenols prevent $\text{A}\beta(1-40)$ aggregation by mechanisms different from both, and that the observed anti-amyloidogenic effect is probably consequence of Cu^{2+} removal from $\text{A}\beta(1-40)$ as well as fibril destabilizing interactions of **1-5** (or their respective Cu^{2+} complexes) with the peptide. It is worth highlighting that these results confirm the initial postulate of molecular design; compounds **1-5** were conceived hypothesizing that the combination of both ionophoric and polyphenol moieties in one single compound would result in a cooperative effect able to enhance the activity of the parent drugs. Our results also show that no significant difference is observed within the three amino compounds (**1**, **2** and **4**), suggesting that the position and number of hydroxy groups in these compounds do not play an important role in the observed activity. A final remark concerns with increase of $\text{A}\beta(1-40)$ fibril concentration with time. It is theorized that Cu^{2+} -induced $\text{A}\beta$ oligomeric aggregates are not a final product and that those oligomeric forms can eventually evolve to mature fibrils. 47% increase in Cu^{2+} -induced $\text{A}\beta(1-40)$ fibrils formation between 3 and 7 days combined with a decrease of turbidity in the same time period seem to support this fact. Importantly, this conversion is also inhibited by compounds **1-5**.

2.6. Binding affinity to $\text{A}\beta(1-40)$ and inhibition of self-induced $\text{A}\beta(1-40)$ aggregation

Molecular self-assembly is an ubiquitous process in biological systems and crucial to the function of cells. Lipids assemble to form membranes, individual strands of nucleic acids do it to form the double helix of DNA and the assembly of proteins results in quaternary structures with

specific functions. However, molecular self-assembly processes of inappropriately folded peptides into amyloid fibers are responsible for the formation of insoluble protein aggregates that have been associated with several serious human ailments, most especially, neurodegenerative diseases.^{9,10} Because of the low Cu²⁺ binding affinity and distinct Cu²⁺-induced aggregation profile to CQ we have postulated mechanism(s) additional to ion chelation to explain the Cu²⁺-induced aggregation inhibition of compounds **1-5**. These observations, therefore, provided the rationale to further investigate the ability of our ionophoric polyphenols to interact with Aβ(1-40) and inhibit its self-induced aggregation in absence of metal ions.

The interaction of **1-5**, CQ and resveratrol with Aβ(1-40) was studied by fluorimetric titration, monitoring the quenching of Aβ(1-40) tyrosine fluorescence upon consecutive additions of compound solutions. Results of binding affinity for **1-5**, collected in Table 2, are comparable to those described for other small ionophoric compounds⁸⁴ and similar to both controls CQ and resveratrol despite their different Aβ(1-40) aggregation inhibition profile. These observations taken together suggest that the compound-protein interactions although of same magnitude must be of different nature.

Figure 5. Inhibition of self-induced A β (1-40) aggregation by compounds **1-5**, CQ and resveratrol studied by ThT (**A**) and turbidity (**B**) assays. No significant changes were observed in ThT fluorescence after 18 days of incubation, and, therefore, that data is not shown for clarity. All values of fluorescence and percent inhibition of fibril aggregation can be found in Table S2 in Supplementary Information.

In a different set of experiments, the ability of **1-5**, resveratrol and CQ to inhibit self-induced A β (1-40) aggregation was studied by the ThT and turbidity assays. A solution of A β (1-40) was incubated for 3-18 days in absence or presence of 2 eq. of compounds **1-5**, resveratrol or CQ at pH=6.60 for comparison purposes with the Cu²⁺-induced experiment. Results in Figure 5 show that compounds **1-5** inhibit between a ~77% and a ~96% extent the A β (1-40) self-assembly into fibril aggregates in the time frame studied (see Table S2 in Supplementary Information for all values of fluorescence and percent inhibition). In general, results for compounds **1-5** in absence of Cu²⁺ display consistent trends to those in presence of Cu²⁺, confirming that the activity of the ionophoric polyphenols must be explained beyond metal chelation. In our interpretation of turbidity and ThT results, and similarly to what we previously discussed, resveratrol and the ionophoric polyphenols seem to destabilize fibril formation by initially re-directing aggregation to non-toxic high molecular weight amorphous populations⁷⁹ that end up re-dissolving upon long-term exposure to **1-5**, which translates into an overall decrease of the aggregate population. Importantly, the fact that compound **3** does not show a distinctive behavior in absence of Cu²⁺ ions could confirm that the pattern observed in Cu²⁺-containing experiments might be partly due to its stronger metal ion affinity. Interestingly, CQ again stimulates the conversion to misfolded structures of A β (1-40) peptide in both amorphous and fibril forms of the protein, as reported by others,^{80,85} and consistently proves to have poor anti-amyloidogenic properties. On the other hand, resveratrol follows a parallel pattern to that observed in presence of Cu²⁺, suggesting that its effect on A β (1-40) aggregation is metal ion independent, in good agreement with its low metal binding affinity. The effect of resveratrol, curcumin and some other polyphenols on A β aggregation has been previously reported^{37,73,79} and generally agree with our findings.

2.7. Disaggregation of pre-formed A β (1-40) aggregates

It has been described that resveratrol,⁷⁹ curcumin and some flavonoids seem to exert their anti-amyloidogenic activity by preferably binding amyloid fibrils rather than primary monomeric A β structures and, therefore, the ability of curcumin -and other drugs like CQ- to promote A β disaggregation of pre-formed aggregates at different levels has been studied.^{73,86,87} In line with this, we have observed that compounds **1-5** suppress the formation of A β (1-40) fibrils but they require long times of incubation to produce an inhibitory effect on amorphous aggregates, suggesting that these potential drugs might also interact preferably with some type of pre-formed A β aggregates or with certain structural patterns present only in mature fibrils. In this context, thus, it was relevant to test the ability of **1-5** to disaggregate mature A β (1-40) forms of the peptide after aggregation had been initiated both in presence and absence of Cu²⁺ ions.

First, A β (1-40) peptide was incubated for 7 days with 1 eq. of Cu²⁺ to allow aggregate formation. After that time, amorphous and fibril A β (1-40) aggregates were measured by turbidity and ThT assays, followed by addition of 2 eq. of compounds **1-5** and incubation for 12 more days. Results are depicted in Figure 6. Cu²⁺-A β (1-40) control behaved as previously observed in section 2.5. Fluorescence ThT assay confirms that addition of CQ also promotes fibril aggregation under these experimental conditions. On the other hand, results on turbidity for CQ do not significantly differ from the control, which in turn translates in an overall increase of the aggregate population. These results for CQ are consistent with all our observations although they contrast with some reported studies.⁸⁵ Interestingly, resveratrol affects fibrilization of pre-formed Cu²⁺-A β (1-40) aggregates only after long periods of incubation, while not changing the turbidity profile with respect to the control. The ionophoric polyphenols display different trends.

Compounds **1** and **2** show a decrease in turbidity similar to that of the Cu²⁺-Aβ(1-40) control, while stopping the aggregation into mature fibrils, resulting in a lower overall amount of aggregates. Best results are obtained for compound **3**, which actually shows ability to disaggregate up to a ~57% extent Cu²⁺-Aβ(1-40) fibrils and lowers up to a ~22% the total turbidity of Aβ(1-40) aggregates after 12 days incubation, while compounds **4** and **5** seem to be redirecting aggregation from mature fibrils to amorphous aggregates (see Table S3 in Supplementary Information for all values of fluorescence and percent inhibition).

Disaggregation experiments of self-assembled Aβ(1-40) aggregates were carried out following the same experimental conditions as for the Cu²⁺-induced experiment, but lacking Cu²⁺ ions. Results are shown in Figure 7 and reveal that fibrilization is remarkably reduced between ~73% and ~91% extent after 4 or 12 days of treatment with compounds **1-5** (see Table S4 in Supplementary Information for all values of fluorescence and percent inhibition). Turbidity experiments are consistent with our previous observations. Resveratrol and compounds **1-5** seem to remodel mature Aβ fibrils into high molecular weight oligomeric amorphous aggregates, as previously reported,⁷⁹ which also disaggregate when exposed to the ionophoric polyphenols **1-5** for long periods of time (i.e. ~46% turbidity reduction when treated with compound **2**, ~25% when treated with compound **3** for 12 days) without raising the amount of mature fibrils. Therefore, ThT and turbidity assays taken together indicate a remarkable overall decrease of pre-formed Aβ(1-40) aggregates upon treatment with ionophoric polyphenols **1-5**. Comparison with clinically tested controls CQ and resveratrol show a significant better anti-amyloidogenic profile for our family of polyphenols. Lastly, we speculate that differences observed in presence and absence of Cu²⁺ ions might be a consequence of competition between, at least, three types of

interactions: Cu^{2+} -compound, Cu^{2+} - $\text{A}\beta(1-40)$ and compound- $\text{A}\beta(1-40)$. This fact highlights once more the importance of Cu^{2+} in $\text{A}\beta$ aggregation.

Figure 6. Disaggregation of Cu^{2+} -induced $\text{A}\beta(1-40)$ aggregates by compounds **1-5**, CQ and resveratrol studied by ThT (**A**) and turbidity (**B**) assays. All values of fluorescence and percent inhibition can be found in Table S3 in Supplementary Information.

Figure 7. Disaggregation of self-induced A β (1-40) aggregates by compounds **1-5**, CQ and resveratrol studied by ThT (**A**) and turbidity (**B**) assays. All values of fluorescence and percent inhibition can be found in Table S4 in Supplementary Information.

3. Cytotoxicity on *Tetrahymena thermophila*

For any compound designed to display biological activity it is crucial to establish whether they also produce undesirable toxic effects that may limit their therapeutic potential. In order to prove this important aspect, we studied the cytotoxic effect of compounds **1-5** on healthy *Tetrahymena thermophila* (*T. thermophila*) in direct comparison with resveratrol and CQ. *Tetrahymena* is a fresh-water protozoan that has been extensively used as cellular models for a wide variety of studies. Because these cells have many features comparable to humans and other animals, they are valuable for studies such as membrane transport, cell physiology, cellular structure and function, and cell biochemistry. *T. thermophila* and other *Tetrahymena* species have also been commonly used for toxicity studies such as that of nanoparticles,⁸⁸ heavy metals⁸⁹ and other environmental factors and drugs.^{90,91}

Table 4. LD₅₀ of compounds **1-5**, CQ and resveratrol on *T. thermophila* by the MTT assay.

Compound	LD ₅₀ (μM)
CQ	239.68 ± 19.45
Resv	274.26 ± 8.07
1	210.57 ± 6.94
2	180.50 ± 9.96
3	157.57 ± 15.76
4	354.41 ± 43.66
5	264.79 ± 14.28

Cytotoxicity of compounds **1-5** was determined using the MTT (3-(4,5-dimethyl-tiazol-2-yl)-2,5 diphenyl-tetrazolium bromide) assay on *T. thermophila*. Results are collected in Table 4 (and Figure S8 in Supplementary Information) and show that all compounds are generally non-toxic toward healthy eukaryotic cells, comparably to resveratrol or CQ, with lethal doses higher than 150 μ M in all cases. Comparison with resveratrol is again of particular interest given that this natural polyphenol is present in a number of dietary sources and its chemopreventive properties and lack of toxicity have been unambiguously ascertained.

4. Conclusions

In this work we have synthesized a family of 5 multi-target compounds (**1-5**) that incorporate a selective Cu^{2+} ion binding motif into the structure of a natural-like polyphenol. Our results show that the resulting ionophoric polyphenols **1-5** are able to combine and enhance the antioxidant and anti-amyloidogenic properties of natural polyphenols with the anti-AD benefits of selective metal ionophoric agents. Two molecular designs have been explored; reduced amino compounds **1**, **2** and **4**, and imino derivatives **3** and **5** for structure-activity comparison purposes. Resveratrol and CQ (two drugs that have completed phase II clinical trials in patients with AD) have been used as controls. Our results show that compounds **1-5** bind Cu^{2+} selectively over other biologically relevant metal ions (Zn^{2+} , Fe^{2+} , Ca^{2+} , Mn^{2+} and Mg^{2+}), forming 2:1 (compound: Cu^{2+}) complexes with low-moderate association constants. Remarkably, compounds **1-5** decrease from a 47% up to a 100% extent the Cu^{2+} -A β (1-40) catalyzed formation of ROS while being able to scavenge DPPH and AAPH free radicals better than ascorbic acid, resveratrol and CQ in most cases. Our results also show excellent anti-amyloidogenic ability: compounds **1-5** inhibit both the Cu^{2+} -induced and self-induced A β (1-40) fibril aggregation to an extent that ranges from 67% to 96%, while disaggregating pre-formed A β (1-40) mature fibrils up to a 57%

and a 91% extent in presence and absence of Cu^{2+} , respectively. Inhibition of amorphous $\text{A}\beta(1-40)$ aggregation is, in general, moderate and only after longer exposure to the ionophoric polyphenols, suggesting that **1-5** preferably interact and destabilize mature fibril forms of $\text{A}\beta(1-40)$. All results taken together show that the ionophoric polyphenols herein presented display a remarkably better overall profile than both controls -validating our multi-target approach- and demonstrate the promising potential of this family of compounds. In an attempt to establish some general structure-activity correlations, we observed that: 1) imino compounds **3** and **5** displayed higher Cu^{2+} affinity and slightly better Cu^{2+} selectivity than amino compounds **1**, **2** and **4**; 2) imino compounds **3** and **5** inhibited the Cu^{2+} -catalyzed hydroxyl radical production better than amino compounds **1**, **2** and **4** (likely due to the higher Cu^{2+} affinity), but the latter were better antioxidant agents in absence of Cu^{2+} ions; and 3) compounds **1-5** all displayed comparable anti-amyloidogenic profile in presence or absence of Cu^{2+} ions. In addition, cytotoxicity of compounds **1-5** was investigated *in vitro* on *T. thermophila* unicellular organisms (used as models of healthy eukaryotic cells) and compared to that of resveratrol and CQ. Our results show that the ionophoric polyphenols herein presented display a comparable lack of toxicity to that of resveratrol. Because of the observed properties, all compounds are currently being subject of further *in vivo* testing in *Drosophila melanogaster* (fruit fly) models of Alzheimer's disease for toxicity and behavioral changes.

5. Experimental section

All manipulations were carried out under N_2 using common Schlenck techniques. Solvents (analytical grade, Aldrich) were dried and degassed immediately prior to use by means of an Innovative Technology solvent purification unit; resveratrol, CQ, DPPH, AAPH, fluorescein and other reagents (Aldrich and Fisher Scientific) were used as received. Human $\text{A}\beta(1-40)$ was

purchased from CreoSalus (Louisville, Kentucky) and used as received. Elemental Analyses were performed at Atlantic Microlab, INC. Norcross, GA. NMR spectra were obtained using an AVANCE III HD Bruker 300 MHz instrument; chemical shifts are expressed in ppm relative to residual proton or carbon signals in the deuterated solvents. Mass spectrometry was performed at Brooklyn College, using an Agilent 6220 Accurate-Mass TOF instrument and electrospray ionization. Spectrophotometric studies experiments were performed on a Varian Cary Scan 100 double beam spectrophotometer equipped with temperature control accessory. Steady-state fluorescence measurements were carried out using a Shimadzu RF-5301PC.

5.1. Synthesis of compounds 1-5

5.1.1. General procedure for the synthesis of compounds 1, 2 and 4

The synthesis of compounds **1**, **2** and **4** was performed by a slight modification of standard procedures found in the literature.^{52,53} A solution of the corresponding hydroxybenzaldehyde (4-hydroxybenzaldehyde, 3-hydroxybenzaldehyde or 3,4-dihydroxybenzaldehyde, 5.39 mmol) in dry methanol (10 ml) was added in a dropwise fashion over a period of 10 minutes at room temperature to another solution of 2-aminophenol (587.9 mg, 5.39 mmol) also prepared in dry methanol (15 ml). The mixture was then refluxed under N₂ for 2 hours. After that time, the methanolic solution was evaporated to dryness. The resulting oil/solid was washed with chloroform (3 x 5 ml), filtered off and dried under vacuum to yield the imino-intermediate. This product was then suspended/dissolved in dry methanol (10 ml) and cooled down to 0-2 °C in an ice bath. Another suspension of NaBH₄ (400.0 mg, 10.57 mmol) was prepared in dry methanol (10 ml) and added dropwise to the imino-intermediate solution. Addition was stopped when decoloration of the solution (for compounds **1** and **2**) or complete dissolution of the suspension

(for compound **4**) was observed. The mixture was quenched with water (50 ml) and the product extracted with portions of diethyl ether (3 x 25 ml). The organic fractions were combined, dried over MgSO₄ and the solvent evaporated to dryness. The resulting solid/oil was washed with chloroform (3 x 10 ml), filtered and dried under vacuum to yield the final product.

5.1.1.1. 2-((4-hydroxybenzyl)amino)phenol, 1

Yellow-orange solid (74%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.23 (s, 2H), 7.15 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 8.4 Hz, 2H), 6.65-6.56 (m, 2H), 6.40 (m, *J* = 15.0, 7.7, 1.4 Hz, 2H), 4.98 (t, *J* = 6.0 Hz, 1H), 4.15 (d, *J* = 5.4 Hz, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 156.54, 144.46, 137.71, 130.85, 128.84, 120.00, 116.16, 115.48, 113.78, 110.56, 46.69. ESI-MS (5% ACN, 0.1% Formic Acid in H₂O) *m/z* calculated for C₁₃H₁₃NO₂: 215.0946; *m/z* found: 216.1025[M+H]⁺. Elemental Anal. Calculated %: C, 72.54; H, 6.09; N, 6.51. Found %: C, 72.45; H, 6.11; N, 6.55

5.1.1.2. 2-((3-hydroxybenzyl)amino)phenol, 2

Orange solid (65%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.30 (s, 1H), 9.28 (s, 1H), 7.15 – 7.03 (m, 1H), 6.81 – 6.70 (m, 2H), 6.70 – 6.48 (m, 3H), 6.44 – 6.29 (m, 2H), 5.21 (t, *J* = 6.1 Hz, 1H), 4.20 (d, *J* = 6.0 Hz, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 157.86, 144.47, 142.58, 137.66, 129.66, 119.99, 118.03, 116.15, 114.14, 113.95, 113.79, 110.49, 46.99. ESI-MS (5% ACN, 0.1% Formic Acid in H₂O) *m/z* Calculated for C₁₃H₁₃NO₂: 215.0946; *m/z* found: 216.1087[M+H]⁺. Elemental Anal. Calculated %: C, 72.54; H, 6.09; N, 6.51. Found %: C, 72.59; H, 6.04; N, 6.45

5.1.1.3. 4-(((2-hydroxyphenyl)amino)methyl)benzene-1,2-diol, **4**

Yellow solid (55%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.25 (s, 1H), 8.81 (s, 1H), 8.71 (s, 1H), 6.75 (d, *J* = 2.0 Hz, 1H), 6.71 – 6.52 (m, 4H), 6.44 – 6.33 (m, 2H), 5.00 (s, 1H), 4.11 (s, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 145.58, 144.45, 144.41, 137.71, 131.57, 119.99, 118.43, 116.12, 115.83, 115.04, 113.74, 110.56, 46.89. ESI-MS (5% ACN, 0.1% Formic Acid in H₂O) *m/z* calculated for C₁₃H₁₃NO₃: 231.0895; *m/z* found: 232.0967[M+H]⁺. Elemental Anal. Calculated %: C, 67.52; H, 5.67; N, 6.06. Found %: C, 67.61; H, 5.72; N, 6.10

5.1.2. General procedure for the synthesis of Schiff bases **3** and **5**

The synthesis of compounds **3** and **5** was performed following standard procedures found in the literature.^{52,53} A solution of 2-hydroxy-1-naphtaldehyde (531.4 mg, 3.08 mmol) in dry methanol (10 ml) was added in a dropwise fashion to another solution of 2-aminophenol or 4-aminoresorcinol hydrochloride (3.09 mmol) also in dry methanol (15 ml) over a period of 10 minutes at room temperature. This mixture was refluxed for 5 hours. The solution was then cooled down to room temperature and concentrated to 5-10 ml to favor precipitation of the final product, which was then washed with chloroform (3 x 5 ml) and dried under vacuum.

5.1.2.1. (E)-1-(((2-hydroxyphenyl)imino)methyl)naphthalen-2-ol, **3**

Orange solid (63%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 15.74 (s, *J* = 9.6 Hz, 1H), 10.33 (s, 1H), 9.50 (d, *J* = 9.5 Hz, 1H), 8.39 (d, *J* = 8.4 Hz, 1H), 7.94 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.81 (d, *J* = 9.4 Hz, 1H), 7.68 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.49 (ddd, *J* = 8.4, 7.0, 1.5 Hz, 1H), 7.27 (ddd, *J* = 7.9, 7.0, 1.0 Hz, 1H), 7.12 (ddd, *J* = 8.5, 7.2, 1.5 Hz, 1H), 7.04 – 6.90 (m, 2H), 6.79 (d, *J* = 9.3 Hz, 1H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 178.02, 149.90, 148.88, 138.37, 134.38, 129.44, 129.06,

128.54, 127.19, 126.29, 125.54, 123.48, 120.26, 120.18, 118.04, 116.41, 108.15. ESI-MS (5%
ACN, 0.1% Formic Acid in H₂O) m/z calculated for C₁₇H₁₃NO₂: 263.0946; m/z found:
264.0998[M+H]⁺. Elemental Anal. Calculated %: C, 77.55; H, 4.98; N, 5.32. Found %: C, 77.57;
H, 5.01; N, 5.28

5.1.2.2. (E)-4-(((2-hydroxynaphthalen-1-yl)methylene)amino)benzene-1,3-diol, **5**

Orange solid (61%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 15.65 (s, 1H), 10.46 (s, 1H), 9.69 (s, 1H),
9.52 (s, 1H), 8.43 (d, *J* = 8.5 Hz, 1H), 7.87 (d, *J* = 9.4 Hz, 1H), 7.82 (d, *J* = 8.8 Hz, 1H), 7.75 (d,
J = 7.9 Hz, 1H), 7.52 (t, *J* = 7.7 Hz, 1H), 7.31 (t, *J* = 7.5 Hz, 1H), 6.92 (d, *J* = 9.4 Hz, 1H), 6.51
(d, *J* = 2.3 Hz, 1H), 6.39 (dd, *J* = 8.8, 2.5 Hz, 1H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 173.76,
157.93, 150.64, 149.60, 137.47, 134.02, 129.41, 128.47, 126.52, 123.99, 123.41, 121.09, 120.36,
119.41, 107.98, 107.65, 103.30. ESI-MS (5% ACN, 0.1% Formic Acid in H₂O) m/z calculated
for C₁₇H₁₃NO₃: 279.0895; m/z found: 280.0976[M+H]⁺. Elemental Anal. Calculated %: C,
73.11; H, 4.69; N, 5.02. Found %: C, 73.02; H, 4.61; N, 4.96

5.2. DFT-based energy calculations

Structures of E and Z isomers of compounds **3** and **5** were optimized using B3LYP^{93, 94} density
functional method as implemented in the *Gaussian 03* suite of quantum chemical programs.⁹⁵ All
atoms were treated with a 6-21G(d,p) basis set.⁹⁶ The relative energies of spatial isomers were
estimated based on full optimizations employing a gas-phase model.

5.3. Metal ion binding selectivity and Cu²⁺ binding properties

Experiments for the determination of metal ion binding selectivity were carried adapting a procedure previously described³⁰ as follows. 4.0 mM stock solutions of CuCl₂, ZnCl₂, FeCl₂, CaCl₂, MnCl₂ and MgSO₄ in MQ water, and 5.0 mM of compounds **1-5**, CQ and resveratrol in ethanol were freshly prepared prior to use. For all compounds, a 3 ml working solution in buffer (20 mM HEPES, 150 mM NaCl, pH=7.40) was prepared containing the right amount of every stock solution to achieve a 20 µM concentration in ionophoric agent, and treated with 0.5 equivalents of ZnCl₂, FeCl₂, CaCl₂, MnCl₂ and MgSO₄. Spectra were recorded after 5 minutes incubation at 25°C. The metal binding selectivity was assessed by then adding 0.5 equivalents of CuCl₂ solution and incubating for 5 additional minutes at 25°C. Selectivity was quantified by comparing and normalizing the absorbance values of the maximum in each case with the absorbance of the solution at the same wavelength after addition of CuCl₂.

Titration of compounds **1-5**, resveratrol and CQ with Cu²⁺ were performed as follows. Aliquots of a 4 mM solution of CuCl₂ were added (0.1 equivalents/addition) over 2.5 ml of a 20 µM solution of the corresponding compound in 20 mM HEPES buffer pH=7.40 (10 µM for compounds **3** and **5**). The decrease in the intensity of the absorption band between 400-500 nm for **3** and **5**, and between 300-400 nm for resveratrol and CQ was monitored until saturation was observed. For compounds **1**, **2** and **4**, the increase of a band between 300-400 nm was monitored under the same conditions. Binding stoichiometry was determined from the intercept of tangents on both arms of the graphs plotted between absorbance and CuCl₂ concentration.

Association constants Cu²⁺-Ligand (Ligand=compounds **1-5**, CQ and resveratrol) were calculated using the equilibrium constant expression $K_a = C[1-X]/[X(nX)^n]$, where C is the

concentration of complex, X is the degree of dissociation and n represents the stoichiometry $\text{Cu}^{2+}\text{-Ligand}_n$. The degree of dissociation was calculated using $X=(E_m-E_s)/(E_m)$, where E_m is the absorbance value obtained from the intercept of tangents on both arms of the curve absorbance vs. Cu^{2+} concentration, and E_s is the absorbance at equilibrium concentrations of Cu^{2+} and ligand obtained by intercept of absorbance curve by the line parallel to y axis through E_m .

5.4. A β (1-40) sample preparation

A β (1-40) sample was prepared immediately prior to use as follows. 2.500 mg of the peptide were fully dissolved in 780 μl of NaOH solution 10 mM. 2.107 ml of 20 mM HEPES buffer, 150 mM NaCl pH=6.60 were then added to obtain 2.887 ml of a 200 μM A β (1-40) concentration solution (866 $\mu\text{g}/\text{ml}$). Aliquots of this solution were taken as needed for the following experiments.

Both NaOH solution and HEPES buffer were filtered through a 0.2 μm cellulose acetate filter prior to preparing the A β (1-40) stock solution.

5.5. Inhibition of Cu^{2+} -A β (1-40) ROS production

The production of ROS (hydroxyl radical) was measured following a procedure previously described.⁶³ Briefly, the hydroxyl radical concentration was monitored by the formation of fluorescent 7-hydroxy-coumarin-3-carboxylic acid (7-hydroxy-CCA) (excitation wavelength=395 nm, emission wavelength=450 nm) from the reaction of coumarin-3-carboxylic acid (CCA) with hydroxyl radicals in buffer (20 mM HEPES, 150 mM NaCl, pH=7.40). A 3 ml working solution was prepared 10 μM in Cu^{2+} -A β (1-40), 1 μM in deferoxamine (DFO, necessary to chelate trace amounts of metals present in the buffer), 10 mM in CCA and 20 μM in

the compound under study when necessary. In short, buffer, DFO, Cu^{2+} , A β (1-40) peptide (from 200 μM stock solution prepared as described above) and the compound under study were mixed and incubated for 1 hour prior addition of CCA. The formation of ROS was then started by addition of ascorbate to a final concentration of 300 μM , and the fluorescence of 7-hydroxy-CCA followed over a period of 60 minutes. The concentration of coumarin-3-carboxylic acid was maintained 3 orders of magnitude higher than that of the corresponding ionophoric polyphenol in order to prevent possible interferences between the absorption of compounds and the emission of 7-hydroxy-coumarin-carboxylic acid. All experiments were done in triplicate.

5.6. Scavenging of DPPH radical

DPPH assay was performed as previously described.⁶⁴ In short, a stock solution of DPPH was prepared in methanol buffered with acetic acid (0.10 M, pH=5.50). Buffered methanol was obtained by mixing 40% in volume of 0.10 M acetic acid buffer (pH=5.50) and 60% of methanol. Stock solutions of the compounds (5 mM) in buffered methanol or ethanol were freshly prepared prior to use. Different aliquots of these stock solutions were taken and diluted with the proper amount of buffered methanol to produce 2 mL working solutions with compound concentrations ranging from 5 μM to 25 μM . The reaction was started by addition of 42 μL of a 2.391 mM DPPH solution in buffered methanol (the final DPPH concentration in the working solution was 50 μM). The samples were then incubated at 30 °C for 30 minutes and the DPPH absorbance was measured at 517 nm. All experiments were done by triplicate and results were averaged. IC_{50} values were calculated from the dose-response curves. The method was validated by using ascorbic acid as standard.

5.7. Scavenging of AAPH radical

Scavenging of AAPH radical was measured by a modification of the ORAC assay.^{65,66} Briefly, 3 mL working solutions were prepared with a 3 nM concentration of fluorescein and a concentration of compound under study ranging from 1 to 12 μ M (1-16 μ M for compound **3** and 1-96 μ M for ascorbic acid) in phosphate buffer 75 mM (pH=7.40). The samples were pre-incubated for 30 min at 37 °C, then AAPH was rapidly added from a stock solution 150 mM to reach a final concentration 19 mM in each sample. The mixtures were then incubated for 60 more minutes at 37 °C. Emission of fluorescein was then measured at 514 nm (excitation = 495 nm). All experiments were prepared in triplicate and results were averaged. IC₅₀ values were calculated from the dose-response curves.

5.8. ThT assay for A β (1-40) fibril quantification

Thioflavin T (ThT) is a benzothiazol salt used to visualize mature A β fibrils. Upon binding to A β fibrils, its excitation and emission wavelengths are 440 and 485 nm, respectively, and fluorescent signal is directly proportional to amount of aggregates formed.^{73,87}

For Cu²⁺-induced A β (1-40) aggregation, 1 ml samples at an A β (1-40) peptide concentration of 50 μ M in HEPES buffer pH=6.60 were prepared in presence of 1 equivalent of Cu²⁺ (50 μ M) and 2 equivalents of the compound under study (100 μ M) as follows: buffer, Cu²⁺ (from 4.0 mM stock solution in MQ water) and compound (from 5.0 mM stock solution in EtOH) were mixed for 5 minutes prior addition of the A β (1-40) peptide (from 200 μ M stock solution prepared as described above), and then incubated at 37°C for 18 days without agitation.

878 For self-induced A β (1-40) aggregation, 1 ml samples at an A β (1-40) peptide concentration of 50
879 μ M in HEPES buffer pH=6.60 were prepared in presence of the compound under study (100
880 μ M) as follows: buffer, compound (from 5.0 mM stock solution in EtOH) and A β (1-40) peptide
881 (from 200 μ M stock solution prepared as described above) were mixed and incubated at 37°C for
882 18 days without agitation.

883 For A β (1-40) fibril quantification, a 10 μ M ThT solution was prepared in 50 mM glycine/NaOH
884 buffer pH=8.50. 50 μ l aliquots of the samples incubated as described above were dissolved in 1.5
885 ml of the 10 μ M ThT solution in glycine/NaOH buffer and fluorescence at 485 nm (excitation at
886 440 nm) was measured 2 minutes after mixing. Absorbance of **1-5** at 440 nm and emission at 485
887 nm were tested by dissolving 50 μ l aliquots of the same A β (1-40) solutions containing
888 compounds **1-5** in 1.5 ml of ThT solution in 50 mM glycine/NaOH buffer to rule out possible
889 interferences. Absorption of compounds **1-5**, CQ and resveratrol at the studied concentration (3.3
890 μ M) was negligible compared to the absorption of ThT at the excitation wavelength, (see Figure
891 S9 in Supplementary Information). All experiments were performed in triplicate and results were
892 averaged.

893 To further rule out any possible bias in the ThT assay by either fluorescence interference,
894 interaction of the compounds **1-5**, resveratrol or CQ with ThT, or competition for binding with
895 A β , the following experiment was performed based on work previously published.⁹² A 1 ml
896 sample of A β (1-40) was incubated in HEPES buffer pH=6.60 as described before for 7 days at
897 37°C to allow mature fibril formation. After incubation, 50 μ l aliquots of this sample were
898 immediately added to a freshly prepared 1.5 ml solution 3.3 μ M of each compound in 10 μ M
899 ThT solution in glycine/NaOH buffer and fluorescence at 485 nm (excitation at 440 nm) was

measured 2 minutes after mixing, as previously described in our experiments. None of the compounds interfered with ThT fluorescence under these conditions (see Figure S10 in Supplementary Information), suggesting that no interaction between the compounds and ThT or competition for A β binding was present at the studied concentration. This experiment was done in duplicate.

Re-dissolution experiments of pre-formed A β (1-40) aggregates were performed as follows: 1 ml samples of A β (1-40) peptide 50 μ M were incubated in HEPES buffer pH=6.60 for 7 days at 37°C in absence or presence of 1 equivalent of Cu²⁺ (50 μ M). After that time, the amount of A β (1-40) fibrils was quantified using the ThT assay as previously described. Then, compounds **1-5**, CQ and resveratrol (from 5.0 mM stock solution in EtOH) were added to achieve a final concentration of 100 μ M (1:2 ratio A β peptide:compound) and the mixtures were incubated for 12 more days at 37°C. The amount of A β (1-40) fibrils was monitored by the ThT assay after 4 and 12 days. All experiments were performed in triplicate.

5.9. Turbidity assay for total A β (1-40) aggregates quantification

A β (1-40) 50 μ M peptide samples prepared as described above for ThT assay (for all, Cu²⁺-induced A β (1-40) aggregation, self-induced A β (1-40) aggregation, as well as disaggregation experiments) were also analyzed for turbidity at 550 nm using 500 μ l volume cuvettes, in an adaptation of a previously described procedure.⁷³ The readings were corrected with blanks prepared by centrifugation of every sample and analysis of the supernatant. All measurements were taken in triplicate.

5.10. Binding affinities to A β (1-40) peptide

Binding affinities of **1-5** to A β (1-40) were measured by a similar procedure to that describe by others.⁸⁴ In short, dilutions of A β (1-40) peptide (3 μ M) were prepared in 20 mM HEPES buffer, 150 mM NaCl, pH=7.40 and titrated with consecutive additions of **1-5**, CQ and resveratrol stock solutions 0.5 mM until saturation was reached. Emission was monitored for the quenching of tyrosine (excitation 278 nm, emission 306 nm) and corrected with blanks of the compounds. Finally, data was fitted to the equation $E=(E_0 + E_{\infty}K[C])/1 + K[C]$ for a 1 binding site using a non-linear least square fitting, where E_0 is the initial emission, E_{∞} represents the emission at saturation, E the emission at each point of the titration and K is the conditional association constant. All experiments were performed in triplicate.

5.11. Cytotoxicity on *Tetrahymena thermophila*

Cytotoxicity was determined using MTT (3-(4,5-dimethyl-tiazol-2-yl)-2,5 diphenyl-tetrazolium bromide) assay. The assay is based on the cleavage of MTT, a yellow tetrazolium salt, by viable cells. Cleaved MTT results in formation of water-insoluble dark blue formazan crystals. Cells were incubated in 24 well plates for 24 h after which they were exposed to different concentrations of each compound, 50, 100, 200, 300, 400, and 500 μ M, for 48 h at 30 °C with continuous shaking. At the end of the incubation period 100 μ l of cells were extracted from each well and added in triplicates to a 96 well plate. 10 μ l of freshly prepared MTT (5mg/ml in PBS) were added to each well. After 4 h incubation, 110 μ l of MTT fixative solution in DMSO were added to each well. Plates were incubated for about 15 min after which absorbance was measured at 570 nm.

Cell survival graphs were generated for each compound (see Figure S8 in Supplementary Information) and analyzed using best fit curve to calculate LD₅₀ values. Statistical analysis was completed using GraphPad InStat. Graphs and curves were prepared using Microsoft Office Excel.

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