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Toward a Rational Design of Multitarget-Directed Antioxidants: Merging Memoquin and Lipoic Acid Molecular Frameworks

Maria Laura Bolognesi,*[,†] Andrea Cavalli,[†,§] Christian Bergamini,[‡] Romana Fato,[‡] Giorgio Lenaz,[‡] Michela Rosini,[†] Manuela Bartolini,[†] Vincenza Andrisano,[†] and Carlo Melchiorre[†]

[†]*Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy,* [§]*Department of Drug Discovery and Development, Italian Institute of Technology, Via Morego 30, 16163 Genova, Italy, and* [‡]*Department of Biochemistry "G. Moruzzi", University of Bologna, Via Irnerio 48, 40126 Bologna, Italy*

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Novel multitargeted antioxidants **3**−**6** were designed by combining the antioxidant features, namely, a benzoquinone fragment and a lipoyl function, of two multifunctional lead candidates. They were then evaluated to determine their profile against Alzheimer's disease. They showed antioxidant activity, improved following enzymatic reduction, in mitochondria and T67 cell line. They also displayed a balanced inhibitory profile against amyloid-β aggregation and acetylcholinesterase, emerging as promising molecules for neuroprotectant lead discovery.

Experimental evidence has greatly expanded our understanding of the role of oxidative stress (OS[a]) in the pathogenesis of Alzheimer's disease (AD). This evidence comes from the established interconnections among OS and other key events of AD, such as apoptosis, amyloid-β (Aβ) processing and secretion, τ phosphorylation, and the disruption of Ca^{2+} homeostasis.[1] The brain exhibits particularly high sensitivity to OS, and the brains of AD patients reveal loss of synapses and OS damage by reactive oxygen species (ROS).[2] In transgenic mice, Aβ colocalizes with several OS markers confirming the in vivo link between Aβ deposition and oxidative damage.[2] OS promotes Aβ toxicity through the production of free radicals,[2] and the application of Aβ to neuronal cultures elevates intracellular H_2O_2.[2] Intriguingly, ROS generation is not just an outcome of the disease process but actually precedes extracellular amyloid deposition as one of the initial events in the AD cascade.[3] OS elevates β-secretase (BACE-1) expression and activity and Aβ levels in vivo[4] and triggers the amyloidogenic pathway in human cell lines.[5] These data imply that not only can Aβ induce OS but its generation is also increased as a consequence of OS, thus forming a vicious circle.

In this context, on the basis of what is defined as the OS theory of AD,[6] antioxidants may be key factors in fighting this disease. Coenzyme Q (CoQ) is an endogenous antioxidant and a powerful free radical scavenger in mitochondrial membranes. Synthesis of CoQ decreases with aging, and CoQ has potentially neuroprotective effects in aging and neurodegenerative diseases in which excessive OS is present.[7] Similarly, supplementation with α-lipoic acid (LA), which is a mitochondria-targeted antioxidant and a precursor of an essential cofactor for mitochondrial enzymes, may be useful for preventing or delaying mitochondrial decay, thus preventing or treating AD and related dementias.[8] However, the disappointing results of clinical trials of these and other antioxidants suggest that single antioxidants may not be adequate for the treatment of neurodegenerative diseases while optimal combinations of antioxidants may provide a more effective strategy.[9]

We have proposed that additive neuroprotective effects can be produced by a single molecule endowed with antioxidant properties and are able to act at different steps in the neurodegenerative process. This concept, termed multitarget-directed ligands (MTDLs) design strategy,[10,11] has been embodied in the development of two drug candidates memoquin (**1**) and lipocrine (**2**), which display a multitarget profile against AD. **1**, which bears the benzoquinone moiety of CoQ inserted into a polyamine chain, is able to modulate in vitro and in vivo different AD crucial molecular targets, such as acetylcholinesterase (AChE) and BACE-1 enzymes, Aβ, and oxidative processes.[12] A 9-amino-6-chloro-1,2,3,4-tetrahydroacridine moiety coupled with LA led to **2**. In addition to its AChE inhibitory activity, **2** also behaved as an antioxidant (Chart 1).[13]

Following the same rationale, we reasoned that merging the antioxidant features of **1** and **2** in a single chemical entity could, hopefully, lead to ligands (**3**−**6**) with multiple antioxidant mechanisms, hence a better potential for AD prevention and therapy. These hybrid molecules can be defined as multifunctional antioxidants, i.e., MTDLs with other pharmacological effects in addition to their antioxidant activity. Since LA[14] and CoQ[15] have shown antiaggregating properties, the new compounds **3**−**6** may also have intrinsic antiamyloidogenic capacities. Finally, since LA reduces CoQ to ubiquinol (the actual antioxidant form) by the transfer of a pair of electrons,[16] they should in principle possess a more effective antioxidant profile in vivo.

*To whom correspondence should be addressed. Phone: +39 0512099718. Fax: +39 0512099734. E-mail: marialaura.bolognesi@unibo.it.

[a] Abbreviations: AChE, acetylcholinesterase; LA, α-lipoic acid; AD, Alzheimer's disease; Aβ, amyloid-β; BChE, butyrylcholinesterase; BACE-1, β-secretase; CoQ, coenzyme Q; MTDLs, multitarget-directed ligands; NQO1, NAD(P)H/quinone oxidoreductase 1; OS, oxidative stress; ROS, reactive oxygen species; SMP, submitochondrial particles.

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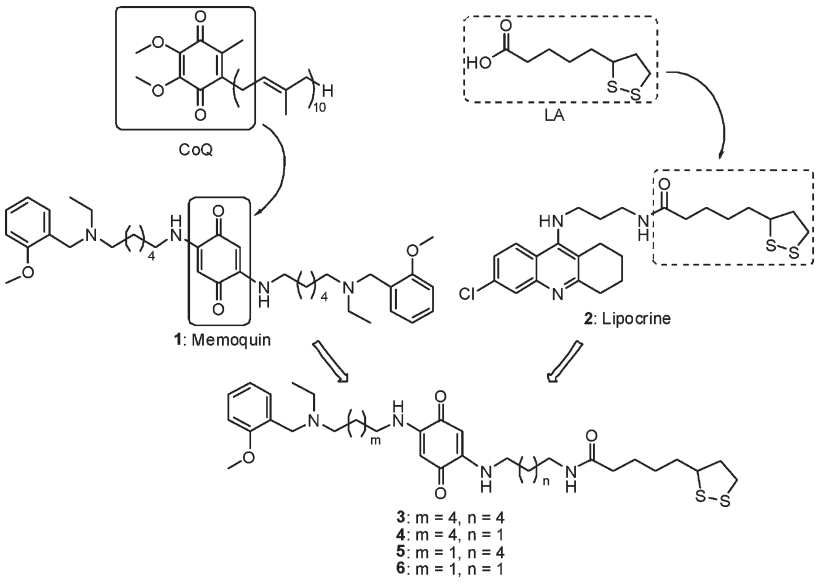
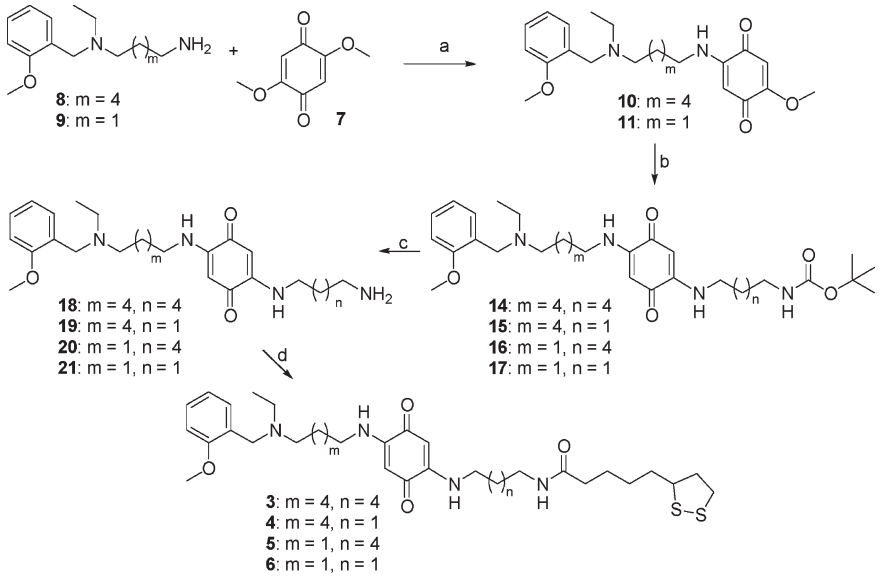
Chart 1. Design Strategy for Hybrids **3**−**6**



Scheme 1[a]



[a] Reaction conditions: (a) CH_2Cl_2, air, room temp, 6 h; (b) $NH_2(CH_2)_nNHBOC$ (**12**, $n = 3$; **13**, $n = 6$), CH_2Cl_2, room temp, overnight; (c) CF_3COOH, CH_2Cl_2, room temp, 4 h; (d) LA, EDCI, HOBt, NEt_3, CH_2Cl_2, room temp, overnight.

Results and Discussion

First, we replaced one terminal 2-methoxybenzyl function of **1** with an LA fragment to afford lipoamide-related compounds. LA bound in amide linkage is the essential cofactor in the mitochondrial oxoacid dehydrogenase complexes, and lipoamide was even more efficient than LA in protecting cells against oxidative insults.[8] Clearly, the same amido functionality is present in **2**. The possibility of having a cationic head, fundamental for recognizing some of the selected molecular targets (in cholinesterases molecular recognition is critically mediated by a Trp),[17] is guaranteed by the presence of a remaining benzylamino group in **3**−**6** (Chart 1). A three- or six-methylene spacer between the nitrogen atoms was used because this chain length was shown to confer a better MTDL profile in SAR studies on **1**.[17]

Because of the heterodimeric structure, **3**−**6** could not be synthesized following the efficient convergent synthetic protocol developed for **1** and analogues, which relies on the disubstitution reaction of diamines with 2,5-dimethoxy-1,4-benzoquinone (**7**).[17] Conversely, a stepwise linear synthesis approach was developed (Scheme 1). Monosubstitution of **7** was the key step of the new synthetic strategy. Thus, reaction of 8 equiv of **7** with diamines **8**[17] and **9**[17] gave monosubstituted derivatives **10** and **11** in acceptable yield. The second methoxy group could then participate in a subsequent substitution reaction with the monoprotected diamines **12** and **13**. The ready removal in mildly acidic conditions, compatible with the stability of the amino−quinone bond, made BOC a suitable protecting group to obtain **18**−**21**, by treating **14**−**17** with CF_3COOH. Since in linear synthesis the overall yield quickly drops with each step

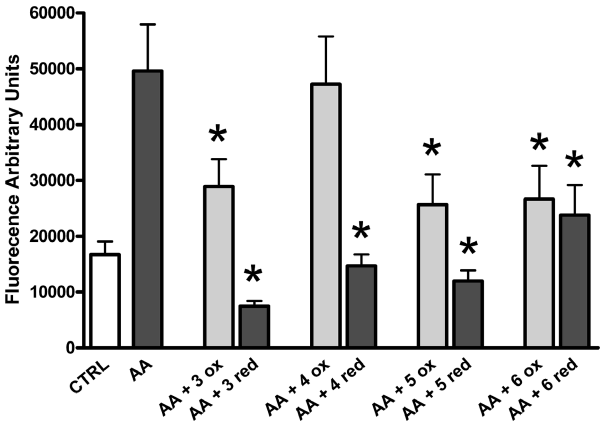


Figure 1. Effect of **3**−**6** on ROS production in SMP induced by 2 μM antimycin A (AA) using NADH as substrate. Experiments were performed with compounds in oxidized and reduced forms. Reduction was achieved by 30 min of preincubation with purified human NQO1 using NADH as substrate. After this time the reaction was stopped by adding 10 μM dicumarol: CTRL = control; (*) $p \leq 0.05$ with respect to AA treated samples.

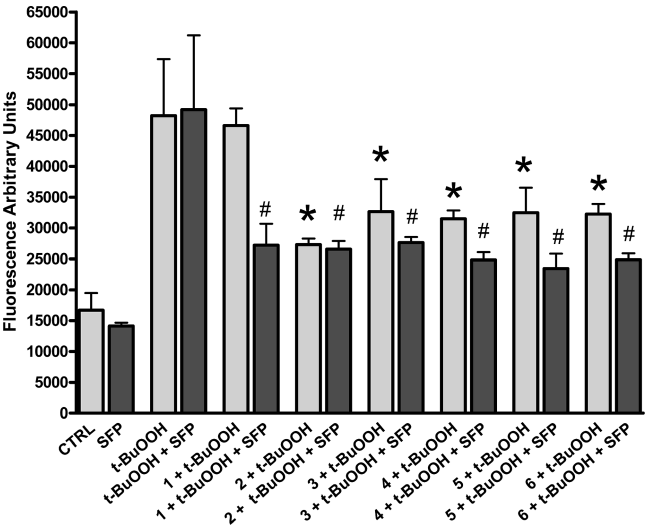


Figure 2. Effect of **1**−**6** on ROS formation in T67 cells. The antioxidant activity was evaluated against ROS formation induced by *t*-BuOOH. Experiments were performed with T67 cells treated or not with 2.5 μM sulforaphane (SFP): CTRL = control; (*) $p \leq 0.05$ with respect to *t*-BuOOH treated samples; (#) $p \leq 0.05$ with respect to *t*-BuOOH + SFP treated samples.

and since the method reported for **2**[13] was not very efficient, efforts were made to optimize the coupling conditions of **18**−**21** to LA. After variation of solvents and reaction times, it was found that addition of HOBT and NEt_3 gave the best yield to **3**−**6**.

The presence in **3**−**6** of the benzoquinone and lipoyl moieties of CoQ and LA, respectively, suggests that their antioxidant action may be targeting mitochondria, which are structurally and functionally damaged in AD.[18] Thus, their antioxidant activity was first tested on bovine heart submitochondrial particles (SMP). Addition of antimycin A to SMP treated with NADH induced a strong increase in ROS production. A 10 μM concentration of **3**−**6** in their quinone form significantly decreased ROS production. This was even more pronounced in their reduced form, after reduction by isolated NAD(P)H/quinone oxidoreductase 1 (NQO1) (Figure 1). This deserves comment. We have previously demonstrated that the antioxidant property of **1**, in analogy with CoQ, pertains mainly to its hydroquinone form.[17] NQO1, an inducible enzyme that catalyzes the two-electron reduction of quinones to hydroquinones, was shown to be responsible for the generation of the CoQ-reduced state, as well as that of **1**. More interestingly, NQO1 is increased in AD[19] in response to the OS typical of the pathology. In light of this, we advanced that, being specifically reduced by NQO1 into the corresponding hydroquinone, **1** may exert its antioxidant activity specifically in those brain regions affected by AD, where a colocalization of NQO1 activity with AD hallmarks coexists.[20] Therefore, since **1** and **3**−**6** share the same benzoquinone nucleus, their antioxidant action might be similarly mediated by NQO1, being able to convert them into the more-antioxidant hydroquinone form. This would explain the higher antioxidant potential of **3**−**6**, which was observed following NQO1 reduction (see Supporting Information (SI)). To further explore the antioxidant potential of **3**−**6**, cell viability and neuroprotective activity against OS were assayed in the human glioma cell line T67, following treatment with *t*-BuOOH and in the absence or presence of pretreatment with sulforaphane (Figure 2). As previously verified,[17] sulforaphane was able to increase NQO1 activity by 50% with respect to control cells (Figure 1 in SI). Figure 2 clearly shows that **3**−**6** (at 10 μM) in their oxidized form show a basal antioxidant activity, which is probably due to the LA fragment that does not require activation by NQO1. This activity was increased in cells pretreated with sulforaphane, confirming that NQO1 is involved in the activation of these compounds. As expected, the antioxidant activity of **2** is not influenced by the overexpression of NQO1.

Table 1. Inhibition of AChE and BChE Activities and Self-Induced Aβ Aggregation by **3**−**6** and Reference Compounds **1**, **2**, and Propidium

			IC_{50}[a] ± SEM (μM)		
compd	*m*	*n*	AChE	BChE	inhibition of Aβ aggregation[b] ± SEM (%)
1			$(1.5 \pm 0.11) \times 10^{-3}$	1.44 ± 0.1	66.8 ± 4.4[c]
2			$(0.253 \pm 0.016) \times 10^{-3}$	0.011 ± 0.003	< 10
3	4	4	0.10 ± 0.01	4.99 ± 0.13	45.4 ± 0.1
4	4	1	0.15 ± 0.01	21.0 ± 1.20	34.4 ± 1.8
5	1	4	0.16 ± 0.01	11.1 ± 0.80	31.8 ± 2.1
6	1	1	0.19 ± 0.01	212 ± 76	19.2 ± 2.3
propidium					61.1 ± 4.6[c]

[a] Human recombinant AChE and BChE from human serum were used. IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate. [b] Inhibition of self-induced Aβ(1−42) aggregation (50 μM) produced by the tested compound at 10 μM. SEM = standard error of the mean. [c] Data from ref 17.

In light of the antiaggregating properties of CoQ[15] and LA,[14] the ability of **2** and **3**−**6** to reduce $A\beta_{(1-42)}$ self-aggregation was also studied, using **1** and propidium as reference compounds. Data in Table 1 show that **3**−**6** at 10 μM inhibited Aβ aggregation in a range from 19% to 45%, whereas **2** at that concentration displayed a negligible activity, perhaps because, compared to LA, **2** lacks the free carboxylic group, an essential feature in some potent aggregation inhibitors.[21]

Since **1** and **2** are effective cholinesterases inhibitors, to achieve a wider picture of the multifunctional profile, **3**−**6** were evaluated for their activity against AChE, a validated molecular target in AD, and butyrylcholinesterase (BChE)

(Table 1). All compounds were fair inhibitors of AChE and BChE and markedly less potent than both prototypes, suggesting that the insertion of the lipoyl fragment at position 2 of the benzoquinone resulted in a highly negative effect on the interaction with the enzyme. This was not unexpected because the second protonated nitrogen of **1** is missed in **3**−**6**. However, we wanted to achieve a balanced multitarget profile rather than a highly potent AChE inhibitor. Where connections exist between two or more targets, multifunctional ligands with only modest activity at one or more targets may still produce superior in vivo effects compared to higher-affinity target-selective compounds.[22] Because most links in cellular networks are weak, a low-affinity MTDL may be enough to accomplish a significant modification.[23]

In conclusion, we discovered new multitargeted antioxidants that might represent a step forward in the search for new MTDLs against AD. **3**−**6** were superior to **1**, for the basal antioxidant activity (not dependent by NQO1 activation), and to **2** for their additional ability to inhibit Aβ aggregation. Moreover, they displayed balanced activity profiles, which are promising if properly corroborated by the definitive in vivo proof of principle.

All these considerations establish the new hybrids as promising molecular structures for the development of new AD agents that, beyond the modulation of OS, might have extra beneficial activities. In particular, the simultaneous modulation of brain levels of Aβ and ROS might break the vicious cycle that further promotes OS, elevates Aβ levels, and accelerates AD development. Clearly, proof of the concept will involve an investigation of their neuroprotectant profile in vivo.

The present study adds to the potential benefits of using rationally designed multitargeted antioxidants to study and ultimately address the complex mechanisms underlying neurodegeneration.

Experimental Section

Satisfactory elemental analysis results were obtained for all new compounds, confirming >95% purity.

Synthesis of 3. A solution of **18** (35 mg, 0.072 mmol) in CH_2Cl_2 (5 mL), NEt_3 (30 μL), hydroxybenzotriazole (HOBt) (15 mg, 0.12 mmol), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) (40 mg, 0.21 mmol), and additional NEt_3 (30 μL) were added successively to a solution of LA (16 mg, 0.077 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred overnight at 25 °C and diluted with water (30 mL). The product was extracted with CH_2Cl_2 (3 × 30 mL). Evaporation of the dried extracts afforded crude **3** that was purified by flash chromatography. Eluting with $CH_2Cl_2/CH_3OH/NH_3$ (9.5:0.5:0.05), **3** was obtained (red waxy solid, 83% yield).

Synthesis of 4. It was synthesized in 75% yield from LA (35 mg, 0.17 mmol) and **19** (60 mg, 0.14 mmol), following the procedure described for **3**.

Synthesis of 5. It was synthesized in 37% yield from LA (66 mg, 0.32 mmol) and **20** (130 mg, 0.3 mmol), following the procedure described for **3**.

Synthesis of 6. It was synthesized in 70% yield from LA (38 mg, 0.186 mmol) and **21** (70 mg, 0.17 mmol), following the procedure described for **3**.

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Supporting Information Available: Analytical characterization of **3**−**6**, experimental details of the synthesis of **10**−**21**, and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Smith, M. A.; Perry, G.; Pryor, W. A. Causes and consequences of oxidative stress in Alzheimer's disease. *Free Radical Biol. Med.* **2002**, *32*, 1049.
(2) Chong, Z. Z.; Li, F.; Maiese, K. Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog. Neurobiol.* **2005**, *75*, 207–246.
(3) Zhu, X.; Su, B.; Wang, X.; Smith, M. A.; Perry, G. Causes of oxidative stress in Alzheimer disease. *Cell. Mol. Life Sci.* **2007**, *64*, 2202–2210.
(4) Xiong, K.; Cai, H.; Luo, X. G.; Struble, R. G.; Clough, R. W.; Yan, X. X. Mitochondrial respiratory inhibition and oxidative stress elevate beta-secretase (BACE1) proteins and activity in vivo in the rat retina. *Exp. Brain Res.* **2007**, *181*, 435–446.
(5) Coma, M.; Guix, F. X.; Ill-Raga, G.; Uribesalgo, I.; Alameda, F.; Valverde, M. A.; Munoz, F. J. Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells. *Neurobiol. Aging* **2008**, *29*, 969–980.
(6) Markesbery, W. R. Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biol. Med.* **1997**, *23*, 134–147.
(7) Wadsworth, T. L.; Bishop, J. A.; Pappu, A. S.; Woltjer, R. L.; Quinn, J. F. Evaluation of coenzyme Q as an antioxidant strategy for Alzheimer's disease. *J. Alzheimer's Dis.* **2008**, *14*, 225–234.
(8) Holmquist, L.; Stuchbury, G.; Berbaum, K.; Muscat, S.; Young, S.; Hager, K.; Engel, J.; Munch, G. Lipoic acid as a novel treatment for Alzheimer's disease and related dementias. *Pharmacol. Ther.* **2007**, *113*, 154–164.
(9) Liu, J. The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview. *Neurochem. Res.* **2008**, *33*, 194–203.
(10) Cavalli, A.; Bolognesi, M. L.; Minarini, A.; Rosini, M.; Tumiatti, V.; Recanatini, M.; Melchiorre, C. Multi-target-directed ligands to combat neurodegenerative diseases. *J. Med. Chem.* **2008**, *51*, 347–372.
(11) Bolognesi, M. L.; Rosini, M.; Andrisano, V.; Bartolini, M.; Minarini, A.; Tumiatti, V.; Melchiorre, C. MTDL design strategy in the context of Alzheimer's disease: from lipocrine to memoquin and beyond. *Curr. Pharm. Des.* **2009**, *15*, 601–613.
(12) Cavalli, A.; Bolognesi, M. L.; Capsoni, S.; Andrisano, V.; Bartolini, M.; Margotti, E.; Cattaneo, A.; Recanatini, M.; Melchiorre, C. A small molecule targeting the multifactorial nature of Alzheimer's disease. *Angew. Chem., Int. Ed.* **2007**, *46*, 3689–3692.
(13) Rosini, M.; Andrisano, V.; Bartolini, M.; Bolognesi, M. L.; Hrelia, P.; Minarini, A.; Tarozzi, A.; Melchiorre, C. Rational approach to discover multipotent anti-Alzheimer drugs. *J. Med. Chem.* **2005**, *48*, 360–363.
(14) Ono, K.; Hirohata, M.; Yamada, M. Alpha-lipoic acid exhibits anti-amyloidogenicity for beta-amyloid fibrils in vitro. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 1046–1052.
(15) Ono, K.; Hasegawa, K.; Naiki, H.; Yamada, M. Preformed beta-amyloid fibrils are destabilized by coenzyme Q10 in vitro. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 111–116.
(16) Kozlov, A. V.; Gille, L.; Staniek, K.; Nohl, H. Dihydrolipoic acid maintains ubiquinone in the antioxidant active form by two-electron reduction of ubiquinone and one-electron reduction of ubisemiquinone. *Arch. Biochem. Biophys.* **1999**, *363*, 148–154.
(17) Bolognesi, M. L.; Banzi, R.; Bartolini, M.; Cavalli, A.; Tarozzi, A.; Andrisano, V.; Minarini, A.; Rosini, M.; Tumiatti, V.; Bergamini, C.; Fato, R.; Lenaz, G.; Hrelia, P.; Cattaneo, A.; Recanatini, M.; Melchiorre, C. Novel class of quinone-bearing polyamines as multi-target-directed ligands to combat Alzheimer's disease. *J. Med. Chem.* **2007**, *50*, 4882–4897.
(18) Reddy, P. H.; Beal, M. F. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends Mol. Med.* **2008**, *14*, 45–53.
(19) Raina, A. K.; Templeton, D. J.; Deak, J. C.; Perry, G.; Smith, M. A. Quinone reductase (NQO1), a sensitive redox indicator, is increased in Alzheimer's disease. *Redox Rep.* **1999**, *4*, 23–27.
(20) Bolognesi, M. L.; Cavalli, A.; Melchiorre, C. Memoquin: a multi-target-directed ligand as an innovative therapeutic opportunity for Alzheimer's disease. *Neurotherapeutics* **2009**, *6*, 152–162.
(21) Frid, P.; Anisimov, S. V.; Popovic, N. Congo red and protein aggregation in neurodegenerative diseases. *Brain Res. Rev.* **2007**, *53*, 135–160.
(22) Morphy, R.; Rankovic, Z. Fragments, network biology and designing multiple ligands. *Drug Discovery Today* **2007**, *12*, 156–160.
(23) Korcsmaros, T.; Szalay, M. S.; Böde, C.; Kovacs, I. A.; Csermely, P. How to design multi-target drugs: target search options in cellular networks. *Expert Opin. Drug Discovery* **2007**, *2*, 1–10.