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Multitarget therapeutic leads for Alzheimer's disease. Quinolizidinyl derivatives of bi- and tri-cyclic systems as dual inhibitors of cholinesterases and Aβ aggregation

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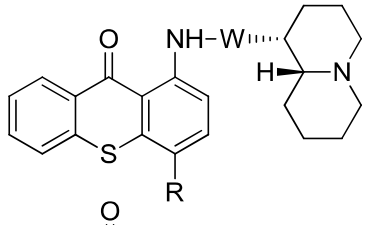
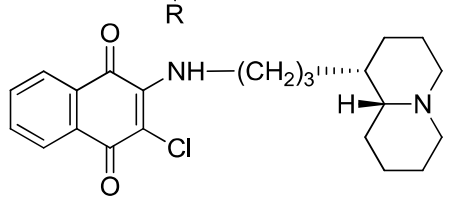
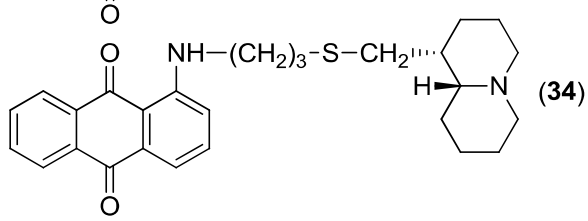


		IC_{50} (μM) AChE	BChE	Aβ
R= CH_3, W= $(CH_2)_2$	(**2**)	5.2	0.46	**0.84**
R= CH_3, W= $(CH_2)_3$-S-CH_2	(**4**)	0.18	**0.088**	4.3
R= NH_2, W= $(CH_2)_3$	(**17**)	2.0	0.48	1.3



(**28**) **0.011** 12.0 (43% at 100μM)



(**34**) 0.84 1.1 8.3

donepezil	0.020	1.9	/
quercetin	/	/	0.82

Multitarget Therapeutic Leads for Alzheimer's Disease: Quinolizidinyl Derivatives of Bi- and Tri-cyclic Systems as Dual Inhibitors of Cholinesterases and Aβ Aggregation

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Abstract

Based on the models of compounds capable of maintaining or restoring cell protein homeostasis and of inhibiting Aβ oligomerization, 37 thioxanthen-9-one, xanthen-9-one, naphto- and anthraquinone derivatives were tested for the direct inhibition of Aβ(1–40) aggregation and for the inhibition of eeAChE and hsBChE. These compounds are characterized by basic side chains, mainly quinolizidinylalkyl moieties, linked to various bi- and tri-cyclic (hetero)aromatic systems. With very few exceptions, these compounds displayed inhibitory activity on both AChE and BChE and on the spontaneous aggregation of β-amyloid. In most cases IC_{50} values were in the low micromolar and sub-micromolar range, but single compounds even reached nanomolar potency. The time course of amyloid aggregation in the presence of the thioxanthenone **2** (the most active compound with IC_{50}= 0.84 μM) revealed that these compounds may act as destabilizers of mature fibrils rather than mere inhibitors of fibrillization. Many compounds inhibited one or both ChEs and Aβ

aggregation with similar potency, a fundamental requisite for the possible development of therapeutics exhibiting a multitarget mechanism of action.

Keywords: acetyl- and butyrylcholinesterases; amyloid-beta peptides; quinolizidine derivatives; single-entity multitarget agents; thioxanthenone and quinonic derivatives.

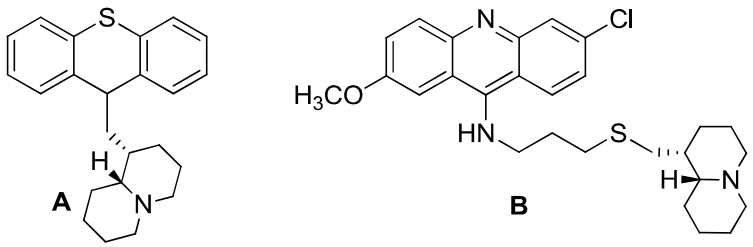
Abbreviations: Aβ, β-amyloid; Aβ(1-40), β-amyloid with 40 amino acids; Aβ(1-42), β-amyloid with 42 amino acids; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; AP: apical; Ape-1, apurinic endonuclease-1; BChE, butyrylcholinesterase; BL: basolateral; CD, circular dichroism; ChEs, cholinesterases; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); FBS: fetal bovine serum; FD4: fluorescein isothiocyanate-dextran; HFIP, hexafluoroisopropanol; MDCKII-MDR1: Madin-darby Canine Kidney cells, retrovirally transfected with human multidrug resistance gene 1; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PAS, peripheral anionic site; PBS, phosphate buffer solution; SI, selectivity index; TEER: trans-epithelial electrical resistance; TEM, transmission electron microscopy; ThT, thioflavin T.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of aggregates of β-amyloid (Aβ) and hyperphosphorylated tau proteins leading to the formation of extracellular amyloid plaques and intracellular fibrillary tangles, respectively. Aβ exists as a mixture of two prevalent peptides, Aβ(1-40) and Aβ(1-42), the latter being the most prompt to aggregate and the most cytotoxic in humans. Nowadays, it is accepted that soluble, low-molecular-weight oligomers formed as intermediate species in the aggregation process of Aβ are responsible for neuronal damage and ultimately cell death. These events have been challenged by the "cholinergic hypothesis" that associates the main cognitive and behavioral symptoms of AD to the loss of cholinergic neurons and the consequent low synaptic levels of the neurotransmitter acetylcholine (ACh).[1] Actually, current symptomatic therapies of AD rely on the restoration of ACh levels through the inhibition of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of ACh at its catalytic anionic site (CAS). Indeed, it has been shown[2] that AChE, at its peripheral anionic binding site (PAS), may promote the aggregation of Aβ; therefore, dual binding site inhibitors of AChE may be beneficial in relieving cognitive and behavioral symptoms of AD by reducing both ACh hydrolysis and Aβ aggregation. In progressive AD, AChE levels in the brain decline while butyrylcholinesterase (BChE) levels progressively increase; BChE is also able to

hydrolyze ACh, even if at lower rate. Selective BChE inhibitors have been reported to increase the ACh level in the brain and also reduce the formation of Aβ.[3]

To obtain novel dual cholinesterase (ChE) inhibitors, or rather BChE selective inhibitors, a few years ago[4] we prepared a number of compounds derived from phenothiazine and other related tricyclic systems linked by different kinds of spacers to the bulky, lipophilic and strongly basic quinolizidine ring. It is worth noting that even simple derivatives of the quinolizidine ring (that differentiates the novel compounds from the classic BChE inhibitors such as ethopropazine and Astra1397[5]) are endowed with some anti-ChE activity.[6] All the examined compounds[4] exhibited activity against both ChEs, but inhibition of BChE was generally stronger, with sub-micromolar IC_{50} values for most of them. 9-[(Quinolizidin-1-yl)methyl]thioxanthene (9-lupinylthioxanthene; **A**) was the most potent and selective inhibitor for BChE (IC_{50}= 0.15 μM; SI= 47; SI is the selectivity index measured as the ratio IC_{50}AChE/IC_{50}BChE). The elongation of the spacer improved the inhibition of AChE; however, this prevailed over BChE inhibition only for 6-chloro-2-methoxy-9-{N-[3-(quinolizidin-1-yl)methylthio]propylamino}acridine **B** (IC_{50}= 0.22 μM; SI= 0.32).



Aside from the valuable inhibitory activity of ChEs, several of the studied compounds have shown additional pharmacological properties that could reinforce central cholinergic activity or improve certain cognitive performances in AD. Pursuing the aim to obtain ChE inhibitors with additional pharmacological properties of potential utility in the control of symptoms and/or progression of AD, we turned our attention to quinolizidinyl derivatives of: a) other tricyclic systems as thioxanthen-9-one and xanthenone, and b) bi- and tricyclic quinoid compounds (naphtho- and anthraquinones). Our main goal was the identification of new and potent ChE inhibitors exhibiting also a direct inhibition of Aβ self-aggregation.

The first subgroup of examined compounds is structurally related to lucanthone (**C**), a drug largely used in the past for the treatment of schistosomiasis,[7] which since 1980 has been under study as an antitumor agent.[8] Its activity has been related to DNA intercalation, the inhibition of nucleic acid biosynthesis, topoisomerase II and apurinic endonuclease-1 (Ape-1) without affecting its redox activity.[9] More recently, lucanthone has also been shown to disrupt lysosomal function and to

inhibit cell autophagy.[10] Autophagy is a degradative process that eliminates anomalous proteins and protein aggregates, thereby maintaining protein homeostasis. Under metabolic and/or hypoxic stress conditions, cells may undergo self-digestion to generate ATP and other essential molecules, thus promoting their own survival. On the other hand, it has been shown that Aβ can be generated in autophagic vacuoles,[11] thus counterbalancing the neuroprotective potential of autophagy. While inhibition of autophagy has been demonstrated to play a role in cancer therapy (by accelerating death of cancer cells typically in a state of nutrient and oxygen deprivation), adequate autophagy modulation might reduce the production of Aβ in AD. Besides the possible reduction of Aβ production by autophagy modulation, of particular relevance for the present work is lucanthone's potent inhibition of horse serum BChE (IC_{50}= 0.15 μM)[12] associated with only a modest inhibition of electric eel AChE (25-40% at 10 μM) rendering this drug an interesting multitarget lead for AD therapy. The second subgroup of compounds is related to several naphtho- and anthraquinones that have been shown to affect important biological targets. Specifically, compound E3330 and related naphthoquinones[9b,13] inhibit (in contrast with lucanthone) the redox function of Ape-1 while, more importantly, other naphthoquinones, as juglone (**D**), inhibit Aβ oligomerization[14] without inhibiting fibrillization. Oligomerization and fibrillization might be seen as independent pathways and soluble, low-molecular-weight amyloid oligomers are the primary toxic species in neurodegenerative diseases.[15] The linking of a naphthoquinone moiety to the amino group of tryptophan produced potent inhibitors (**E**) of both oligomerization and fibrillization.[16] Antitumor anthraquinones such as rubicins (**F**)[17] and xanthrones (e.g., mitoxantrone (**G**) and pixantrone (**H**)[18]) proved to be effective Aβ oligomerization inhibitors, with the latter also reducing the cellular toxicity of Aβ peptide.

Interestingly, poly-hydroxyanthraquinones (as emodin (**I**), 1,2,5,8-tetrahydroxyantraquinone, and rubicins) are also able to inhibit tau protein aggregation and even to dissolve preformed aggregates, precluding neurofibrillary tangle formation in AD.[19] Other anthraquinones substituted with (ω-*tert*-amino)acylamino chains, such as 2,7-bis[(3-*tert*-aminopropionyl)amino]anthraquinones (**J**), exhibit high cytotoxicity against a panel of human cancer cell lines, by specifically targeting G-quadruplex and inhibiting telomerase.[20] The association of telomerase shortening and cellular senescence is well demonstrated *in vitro* and evidence is growing for such an association also *in vivo*.[21] Telomerase knockout mice exhibit reduced neurogenesis, loss of neurons, and short-term memory deficit. On the contrary, in aging APP23 transgenic mice, telomerase shortening has been shown to reduce the progression of amyloid plaque pathology and improve spatial learning ability.[22]

Very recently hybrid compounds, bearing naphtoquinone (**K**)[23] or anthraquinone(rhein) moieties (**L**, **M**)[24,25] linked to the well known cholinesterase inhibitors tacrine or huprine, have been reported as promising disease-modifying anti-Alzheimer drug candidates.

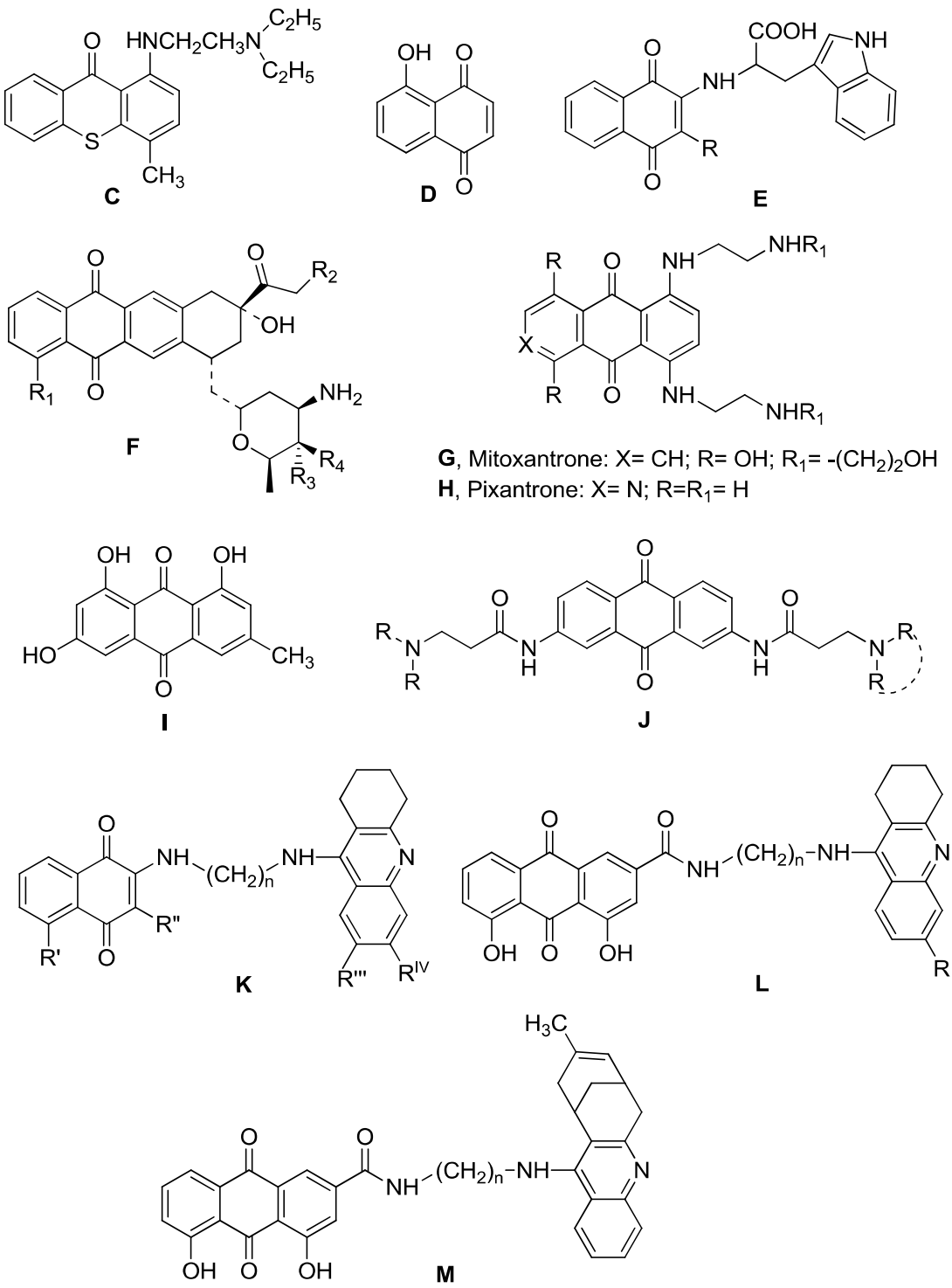


Chart 1

Results and discussion

Chemistry

Based on these premises, we have now combined structural features of the previously studied ChE inhibitors[4] with those of thioxanthenone and quinoid derivatives in an attempt to obtain "single entity multitarget" agents of potential interest in the treatment of AD. The entire set of studied compounds is represented in Charts 2 and 3.

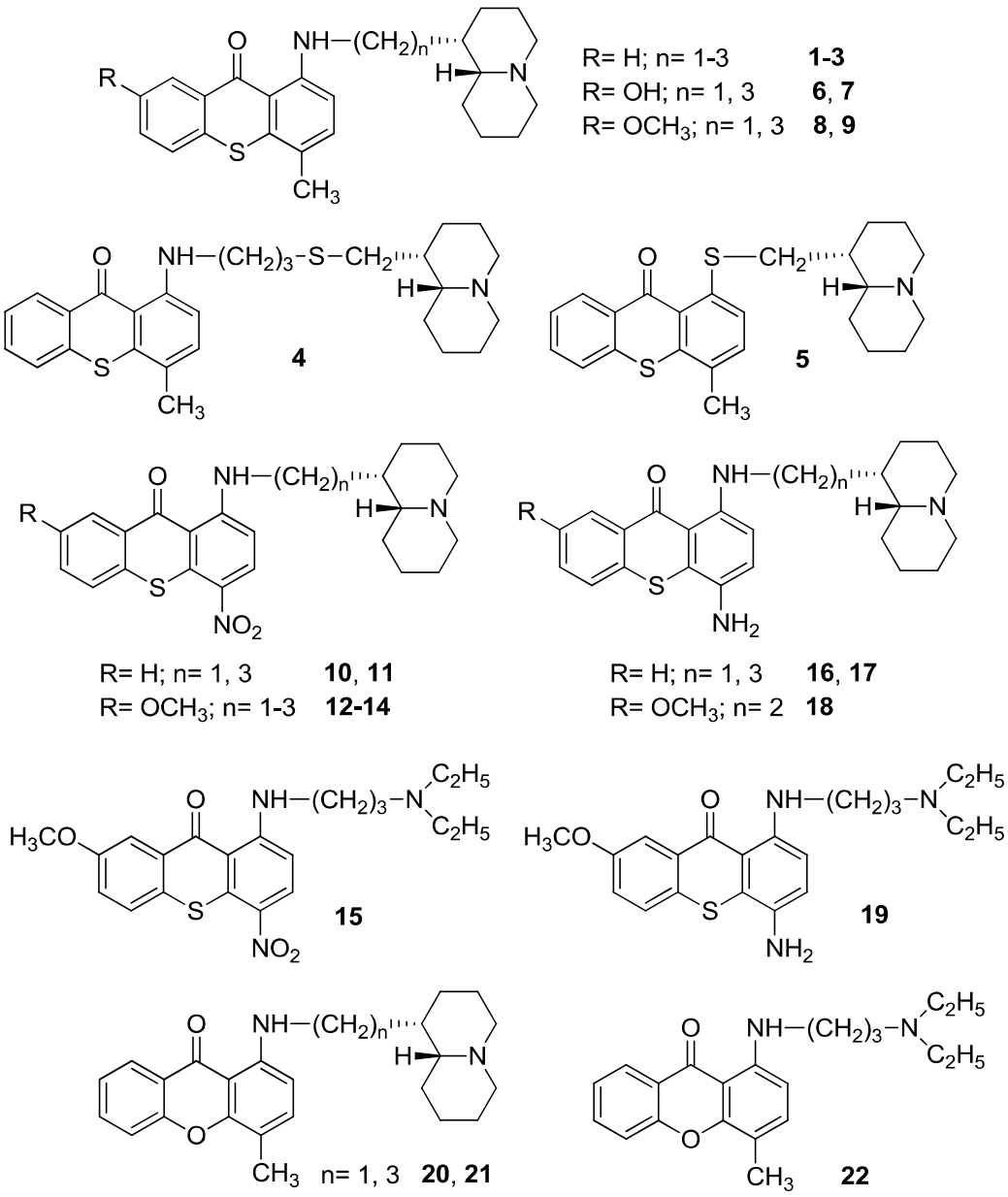


Chart 2

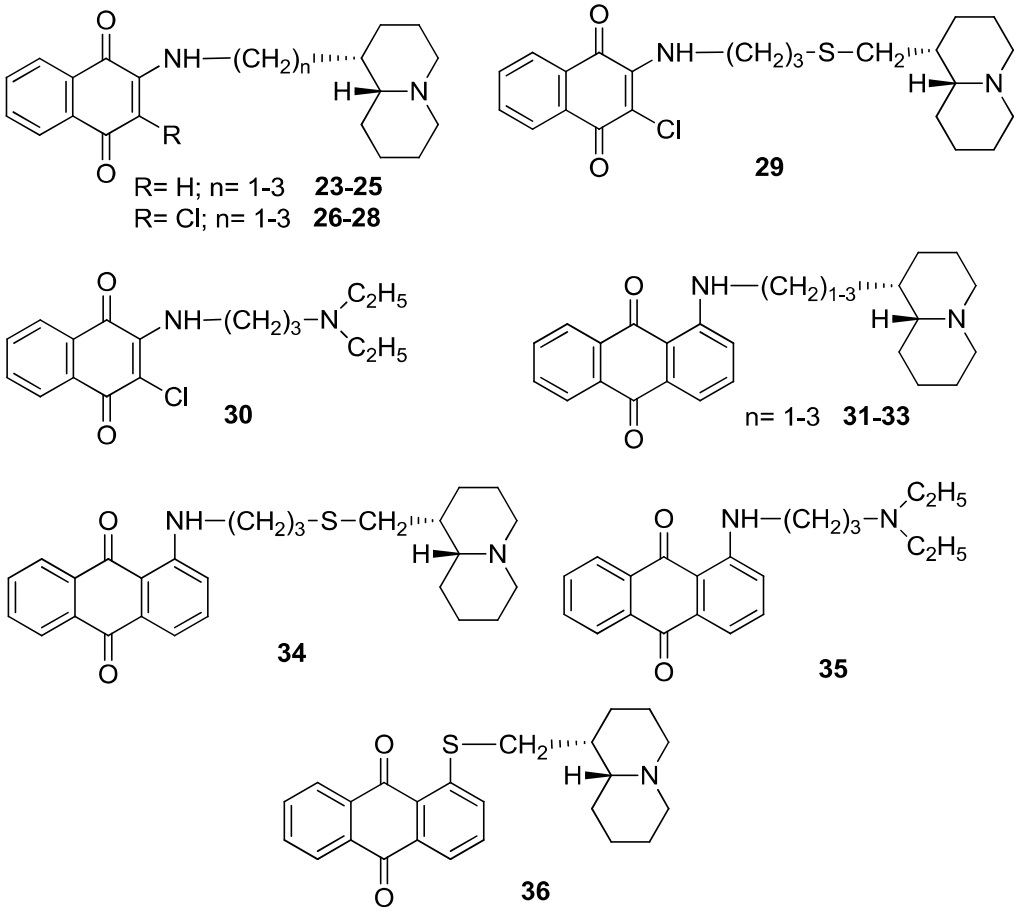


Chart 3

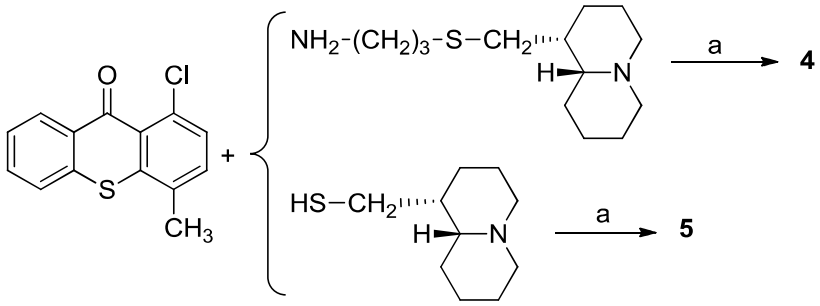
In most of these compounds a quinolizidine ring is linked to an aromatic amino group through a polymethylene chain of variable length. The effect of the length of the linker on biological activities has been explored on the basis of previous findings of ours and others,[26-29] coming from the study from homo- and hetero-dimeric dual binding site ChE inhibitors; the optimal distance between the two moieties, interacting at the catalytic and peripheral anionic sites, was associated to the presence of 6-8 methylenes tethering the two moieties.

The elongation of the spacer between the quinolizidine ring and the aromatic system requires the preparation of long chain ω-(quinolizidin-1-yl)alkylamines. However, for compounds with more than 3 methylenes, the synthesis may results time consuming and we resorted to the use of ω-{[(quinolizidin-1-yl)methyl]thio}alkylamines, taking into account the well known bioisosterism of a CH_2 unit to an S atom.

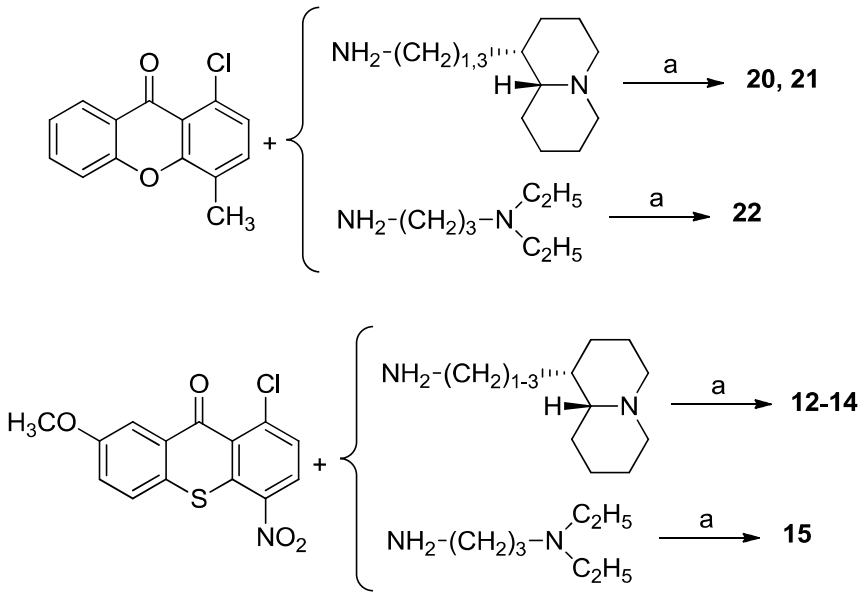
Twenty of the evaluated compounds were already described by some of us (**1-3**, **6-11**, **16**, **17**;[30] **23-28**, **31-33**[31]) and assayed as antileukemic[30,31] and antimicrobial agents.[32] Compound **30** was previously described by Elslager et al.[33]

The fifteen novel compounds (**4**, **5**, **12-15**, **18-22**, **29**, **34-36**) were prepared with the same methods used for the preparation of the above known compounds, with minor modifications. Thus, for the synthesis of compounds **4** and **5**, the 1-chloro-4-methylthioxanthen-9-one[34] was reacted with 3-{[(1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methyl]thio}propan-1-amine[35] or (1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methanthiole (thiolupinine)[36] in Dowtherm A in the presence of Cs_2CO_3 (Scheme 1). 1-Chloro-4-methylxanthen-9-one, prepared according to[7a], was reacted with the suitable quinolizidinylalkylamine[37] or *N,N*-diethyl-1,3-propylenediamine to give **20-22** (Scheme 2).

For the synthesis of compounds **12-15**, it was necessary to first prepare 1-chloro-7-methoxy-4-nitrothioxanthen-9-one[38] by reacting 4-methoxythiophenol with 2,6-dichloro-3-nitrobenzoic acid and then forming the thioxanthenone ring by refluxing the formed diphenyl thioether with trifluoroacetic acid and trifluoroacetic anhydride. Finally the side chains were introduced, as usual, by reacting the obtained chlorothioxanthenone with the quinolizidinylalkylamines[37] or N,N-diethyl-1,3-propylenediamine (Scheme 2).



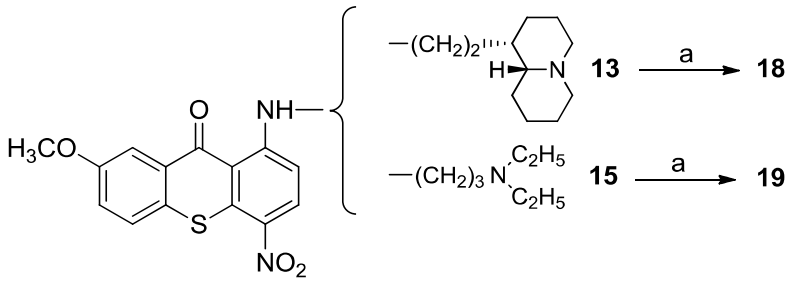
Scheme 1. Reagents and conditions: a) Dowtherm A, Cs_2CO_3, 170°C, 5h.



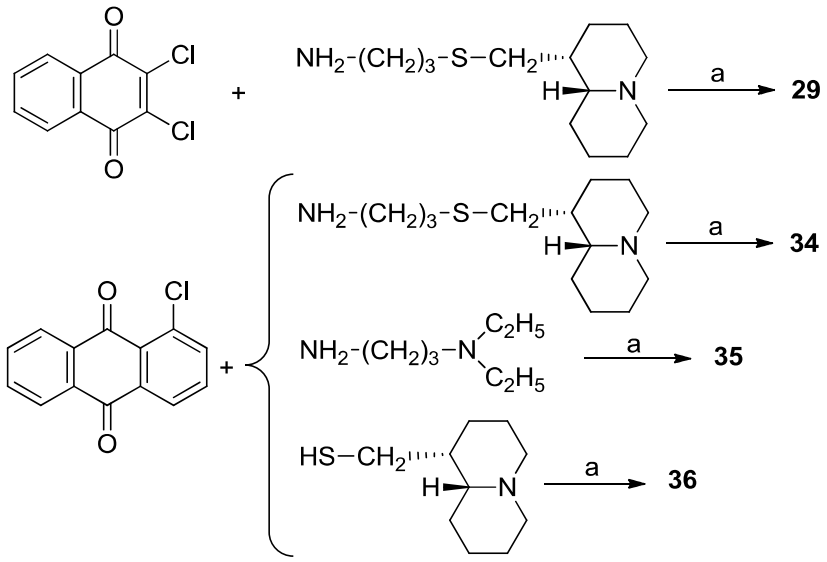
Scheme 2. Reagents and conditions: a) chlorocompound/amine ratio 1:2, 170°C, 6h.

The nitro compounds **13** and **15** were reduced, in ethanol solution, with $SnCl_2$ and conc. HCl to the corresponding amino compounds **18** and **19** (Scheme 3).

2,3-Dichloro-1,4-naphthoquinone and 1-chloro-9,10-anthraquinone were heated with 3-{[(1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methyl]thio}propan-1-amine[35], or *N,N*-diethyl-1,3-propylenediamine, or (1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methanthiole (thiolupinine)[36] to obtain the naphthoquinone **29** and the anthraquinones **34-36** (Scheme 4).



Scheme 3. Reagents and conditions: a) $SnCl_2$, conc. HCl, EtOH, reflux 6h.



Scheme 4. Reagents and conditions: a) chlorocompound/amine ratio 1:1, 170°C, 6h.

Biological assays

Inhibitory activities on AChE (either from electric eel and recombinant human) and BChE (from equine serum) were determined by the spectrophotometric method of Ellman[39] and are reported in Tables 1 and 2 as IC_{50} (μM) for the most active compounds, or as percentage of inhibition at 10 μM for low active (i.e., <50%) compounds.

In vitro inhibition of Aβ(1–40) aggregation was assessed following a previously reported ThT fluorescence-based method involving the use of hexafluoroisopropanol (HFIP) as aggregation enhancer.[40] For the most active compounds (≥ 80% Aβ aggregation inhibition) IC_{50} values were determined under the same assay conditions as already described;[40] data are reported in Table 1. In order to confirm the activity of these new classes of compounds, compound **2** was tested for its inhibitory activity also on Aβ(1–42) aggregation.

Transmission electron microscopy (TEM) and circular dichroism (CD) spectroscopy

Aggregation kinetics were followed simultaneously for a free incubation sample of Aβ(1–40), 50 μM, and a co-incubated sample with the most active inhibitor **2** at 25 μM concentration. Incubation conditions were slightly modified from those used in the screening assay, in order to achieve a slow and complete Aβ aggregation within seven days, avoiding the use of HFIP, in PBS at the temperature of 37 °C. DMSO was replaced with ethanol as the co-solvent (5% v/v).

TEM analysis was performed at 48,000-fold magnification for samples of coincubated Aβ(1–40) (50 μM) with compound **2** (25 μM), compared with a reference free incubation sample of Aβ. CD

spectra were recorded in the spectral range 195–250 nm, following the conformational random coil to β-sheet transition as revealed by the increase of the negative peak of CD signal at 217 nm.

Structure-activity relationships of ChEs and Aβ aggregation inhibition

The inhibitory activities of lucanthone and compounds **1-36** toward AChE, BChE and Aβ(1–40) aggregation are listed in Table 1, together with those of donepezil[41] and quercetin[42] used as reference compounds.

Table 1. Inhibitory activities of lucanthone and compounds 1-36[a]

Compound	eeAChE		hsBChE		Aβ aggregation	
	% at 10μM	IC_{50} (μM)	% at 10μM	IC_{50} (μM)	% at 100μM	IC_{50} (μM)
Lucanthone		5.7		0.72		24
1	34			6.1		28
2		5.2		0.46		0.84
3		8.3		0.93		4.3
4		0.18		0.088		4.3
5		0.82		0.57	46	
6		5.8		1.9		5.9
7		4.9		0.72		2.9
8		7.6		0.94		4.3
9		4.5		0.43		2.5
10		3.3		0.30		20
11		1.1		0.98		13
12		0.77		0.60		11
13		2.0		0.15		9.1
14		0.12		2.0		7.9
15		0.5		0.88		17
16		4.8		0.41		14
17		2.0		0.48		1.3
18		1.9		0.15		13
19		4.8		0.45		21
20		2.7		2.4		48
21		0.14		0.37		13
22		9.4		0.75	55	
23		5.8	36		27	
24		0.46	25		35	
25		0.16		7.5	9	
26		1.5	47		0	
27		0.93		9.8	44	
28		0.011		12	43	
29		0.41		4.1		61
30		2.5	18		47	
31		1.3		2.9		6.4
32		1.9		1.6		31
33		0.84		1.3		9.7
34		0.84		1.1		8.3
35		1.9		1.6	66	
36		3.8		3.4		61
Donepezil		0.020		1.9	0	
Quercetin	9		0			0.82

[a] values are means of two/three independent experiments; SEM always < 10%.

Typical dose-response curves are reported for compound **2** in Figure 1.

As a common option in the screening of small chemical libraries of Aβ aggregation inhibitors, we decided to use Aβ(1–40) instead of the more toxic and aggregation-prone Aβ(1–42). To confirm the results reported in Table 1, we also decided to test the derivative **2** in the inhibition of Aβ(1–42) aggregation, obtaining the same inhibitory response (Fig. 3B).

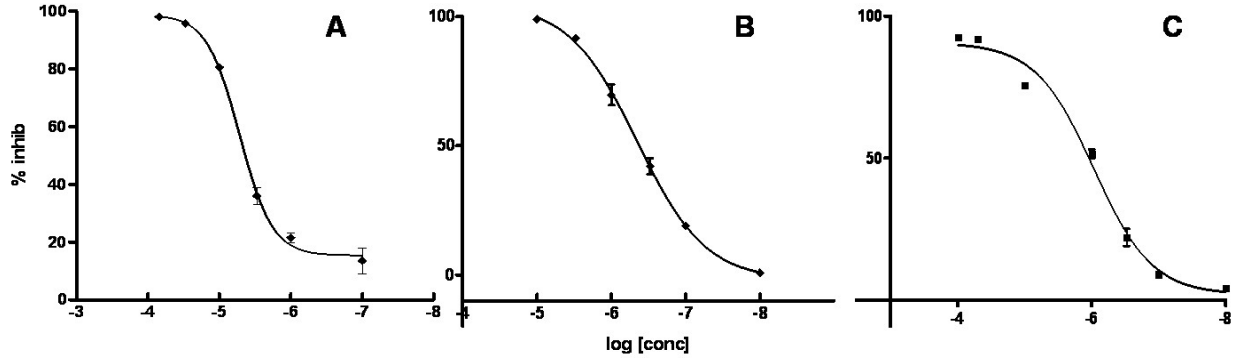


Figure 1. Inhibition dose-response curves of compound **2** for AChE (A), BChE (B), and Aβ(1-40) (C).

All tested compounds exhibited interesting inhibitory potencies on both AChE and BChE. The IC_{50} values were in the low micromolar or sub-micromolar range for at least one enzyme, but often for both of them. It is worth noting that two compounds even reached nanomolar potency: the long side chain thioxanthenone derivative **4,** which exhibited an IC_{50} for BChE = 88 nM, and the naphthoquinone derivative **28**, showing an IC_{50} for AChE = 11 nM, which resulted comparable to or even more potent than donepezil (IC_{50}= 20 nM; for rat brain AChE: IC_{50}= 6.7 nM[41]). Along with the most potent Aβ inhibitor **2**, compound **28** was also tested on human AChE (hAChE), and inhibitory potencies (reported in Table 2) were in the activity range determined for electric eel AChE (eeAChE).

Table 2. Inhibitory activities on hAChE[a]

Compound	IC_{50} (μM)
2	3.5 ± 0.1
28	0.040 ± 0.006
Donepezil	0.019 ± 0.005

[a] values are means of two independent experiments ± SEM.

Evaluation of inhibition kinetics of **28** on hAChE indicated a reversible, mixed-mode type, as expected for dual binding site inhibitors of AChE,[43] with a K_i equal to 0.116 μM (Figure 2).

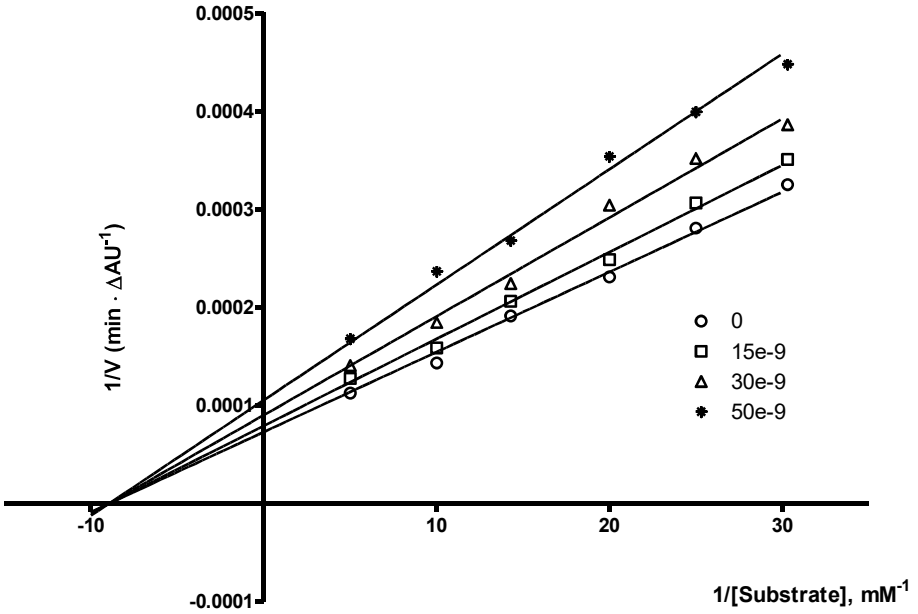


Figure 2. Lineweaver–Burk plot of inhibition kinetics of **28**: reciprocals of enzyme activity (human AChE) vs. reciprocals of substrate (S-acetylthiocholine) concentration in the presence of different concentrations (0-50 nM) of inhibitor.

Indeed, all the tricyclic thioxanthenone derivatives are dual, though generally BChE-preferring inhibitors (with the exception of compounds **14** and **15**), while the naphthoquinone derivatives are dual AChE-preferring inhibitors, reaching a 1000-fold selectivity in cited compound **28**. The tricyclic anthraquinone derivatives exhibited a balanced dual inhibitory activity, with an alternant slight preference for AChE or BChE.

At a concentration of 100 μM all compounds, except two (**25** and **26**), significantly inhibited the aggregation of Aβ; more importantly, 24 of 37 compounds displayed IC_{50} values in the range of 0.84-31 μM, with a mean of 11.4 μM. Of these compounds, 19, 1 and 4 were thioxanthenone, xanthenone, and anthraquinone derivatives, respectively.

The 10 compounds for which it was not possible to calculate the IC_{50} (seven are naphthoquinone derivatives), still inhibited Aβ aggregation by 27-66% at a 100 μM concentration. Once more, the size of the aromatic substructures (tri- versus bi-cyclic ring system) seems to have a higher influence on the biological activity than their chemical nature. It is worth noting that compound **2** exhibited a potency (IC_{50}= 0.84 μM) close to that of quercetin (IC_{50}= 0.82 μM), a reference anti-amyloidogenic compound.

The inhibition of Aβ aggregation is commonly associated (lucanthone and compounds **2**-**4**, **6**-**21**, **29**, **31**-**34** and **36**) with the simultaneous elevated inhibition of AChE and BChE, the latter of which is generally higher than the former (20 of 26 compounds). Moderate inhibition of Aβ aggregation was still observed for compound **1**, even if only the BChE was moderately inhibited (IC_{50}= 6.1 μM) compared to AChE (34% inhibition at 10 μM).

In contrast, when AChE inhibition (IC_{50} range 0.011-5.8 μM) largely prevailed over BChE inhibition (affinity ratio from 10 to 1000), as observed for naphthoquinones (**23**-**30**), Aβ aggregation was poorly inhibited or not affected at all. Two compounds (**5** and **22**) endowed with dual ChE inhibition, and specifically with sub-micromolar IC_{50} for BChE inhibition, were poorly active against Aβ aggregation (46% and 55% inhibition, respectively, at 100 μM), indicating that other factors play a role in determining this biological activity. However, it is important to emphasize that some of the presently studied compounds inhibited the ChEs and Aβ aggregation with relatively close potencies, which is a basic requisite for the potential development of therapeutic agents exhibiting multitarget activity. Thus, the most relevant inhibitors compared favorably with the reference compounds donepezil and quercetin, which are among the most potent compounds for the inhibition of ChEs and Aβ aggregation, respectively. Donepezil, a more potent AChE inhibitor than BChE, is practically devoid of inhibitory activity on direct Aβ aggregation, whereas a weak inhibition resulted from its binding at the PAS of AChE (22% inhibition at 100 μM).[44] On the other hand, quercetin (to which some of the thioxanthenone derivatives are comparable in potency as Aβ aggregation inhibitors) was inactive as a ChE inhibitor (Table 1).

Concerning further aspects of structure-activity relationships, it has already been observed that the tricyclic compounds (thioxanthenone, xanthenone, and anthraquinone derivatives) exhibited rather close patterns of biological activity (dual, but BChE-preferring, and Aβ aggregation inhibitory activities). In particular, anthraquinone derivatives resembled the thioxanthenone and xanthenone derivatives more than the chemically similar, but smaller, bicyclic naphthoquinone derivatives, which exhibited dual, but AChE-preferring inhibitory activity and quite modest activity on Aβ aggregation. The tricyclic (hetero)aromatics are most likely capable of establishing stronger hydrophobic, π–π stacking and electrostatic interactions with the sequence of amino acids $H_{14}QKLVFF_{20}$ of Aβ, with the highest propensity to aggregate,[45] than the bi-cyclic system. The same tricyclic systems seemed to guarantee better interactions at the external site of BChE than on the PAS of AChE.

Within the subset of tricyclic compounds, it was important to define the influence of the sulfur bridge that differentiates the thioxanthenone derivatives from the isosteric xanthenones. It is worth

noting that a number of natural[46,47] and synthetic[48-50] xanthenone derivatives have been shown to possess potent ChE inhibitory activity. However, all of them are characterized by the presence of several hydroxyl/methoxy groups or even carbamic ester function, and therefore are not directly comparable with lucanthone and the thioxanthenone derivatives considered herein. The comparison of compounds **1**, **3** and **C** (lucanthone) with the corresponding compounds **20-22** indicated that the sulfur bridge consistently enhances the inhibition of Aβ aggregation, while the oxygen bridge seems more suited to the inhibition of ChEs, particularly AChE.

The compounds bearing quinolizidine moieties generally display higher activities than the open dialkylaminoalkyl analogues (compare the following groups of compounds: **1-4** vs. **C**; **12-14** vs. **15**; **18** vs. **19**; **20**; **21** vs. **22**; **26-29** vs. **30** and **31-34** vs. **35**). The few exceptions to such a trend mainly regard AChE and, more occasionally, BChE inhibition, suggesting that other dialkylaminoalkyl chains might give rise to more valuable activities in ChE inhibition. On the other hand, the bulky and highly lipophilic quinolizidine moiety appears particularly suited to promote the inhibition of Aβ aggregation, besides that of the ChEs.

Among the thioxanthenone and xanthenone derivatives it has been observed that the increase of the length of the linker between the 1-amino group and the quinolizidine nucleus produced an increase of activity. Examples of the few exceptions to this trend are represented by the greater BChE inhibition on the part of 4-nitro-1-(quinolizidinylmethylamino)thioxanthenone **10** (IC_{50}= 0.3 μM) versus 1-(quinolizidinylpropylamino) homolog **11** (IC_{50}= 0.98 μM) and, more so, by the higher inhibition of Aβ aggregation by 4-methyl-1-(quinolizidinylethylamino)thioxanthenone (**2**, IC_{50}= 0.84 μM) compared to homologs **3** and **4** (IC_{50}= 4.3 μM). In addition, among the quinone compounds the polymethylene linker elongation tended to enhance the potencies of ChE inhibition; however, a remarkable 37-fold reduction of AChE inhibition was observed when the tri-methylene linker of **28 (**IC_{50} = 0.011 μM) was further elongated (**29**, IC_{50}= 0.41 μM).

Concerning the inhibition of Aβ aggregation, the increasing length of the linker in the anthraquinone derivatives **31**, **33**, and **34** (1→3→5 atoms, respectively), produced only a moderate effect (IC_{50}= 6.4, 9.7, and 8.3 μM, respectively), whereas an unexpected net reduction of potency was observed when the linker was constituted of two methylene groups (**32**, IC_{50} = 31 μM).

The nature of the substituent in position 4 of the thioxanthenone ring is important for biological activity. 4-Nitro and 4-amino are less favorable substituents than 4-methyl for ChE inhibitory activity, while the influence is somewhat more variable when Aβ aggregation is accounted for. The additional presence of a 7-methoxy group often improves the activity, though several exceptions have been observed. The introduction of a chlorine atom to the naphthoquinone derivatives (**26-29**)

clearly enhanced the potency of ChE inhibition, while its effect on Aβ aggregation was rather modest.

Finally, the exchange of the imino group on position 1 of thioxanthenone for a sulfur bridge (compare **1** with **5**) improved the inhibitory activity on the ChEs; however, this effect did not hold for Aβ aggregation. The same structural modification in anthraquinone derivatives consistently produced negative effects (compare **31** with **36**).

Kinetic studies of Aβ aggregation

The inhibitory behavior of compound **2** in the Aβ(1-40) aggregation cascade was investigated by means of circular dichroism (CD), thioflavin-T (ThT) fluorescence spectroscopy and transmission electron microscopy (TEM) analysis of aggregates. Aliquots from a coincubated sample of Aβ (50 μM) and **2** (25 μM) in PBS containing 5% ethanol were collected and analyzed through seven days of incubation at 37 °C; a free incubation sample of Aβ was used as reference. Besides its inhibitory potency (0.84 μM, i.e. very similar to that of quercetin), compound **2** was also selected for its fair solubility, since it emerged among the most potent inhibitors (Table 1) as the most soluble in the assay medium in a preliminary UV-based solubility screening (data not shown).

The ThT fluorescence data (Fig. 3A) revealed a net effect of **2** in Aβ(1-40) fibrillization, with a massive inhibition of the final fibril content. The time course of aggregation exhibited the typical sigmoidal shape, with an exponential growth of ThT fluorescence between days 1 and 3, whereas the coincubated sample revealed a similar, though considerably flattened, sigmoidal shape. The apparent difference in inhibitory potency of **2** (80% of inhibition of ThT fluorescence at 25 μM concentration) might be compatible with its submicromolar value of IC_{50}, since incubation experimental conditions for time course experiment were significantly different from those used in the preliminary screening.

To confirm the antifibrillogenic effect of **2**, we performed the same ThT fluorescence experiment with Aβ(1-42) under the same assay conditions (Fig. 3B). Because the aggregating propensity of Aβ(1-42) is substantially higher than Aβ(1-40), we detected a sudden increase of fluorescence in the first 24 h for the free incubation sample, with the loss of the initial lag phase. Satisfactorily, coincubation with **2** resulted also in this case in a strong decrease of fluorescence that reached only 20% of the control value.

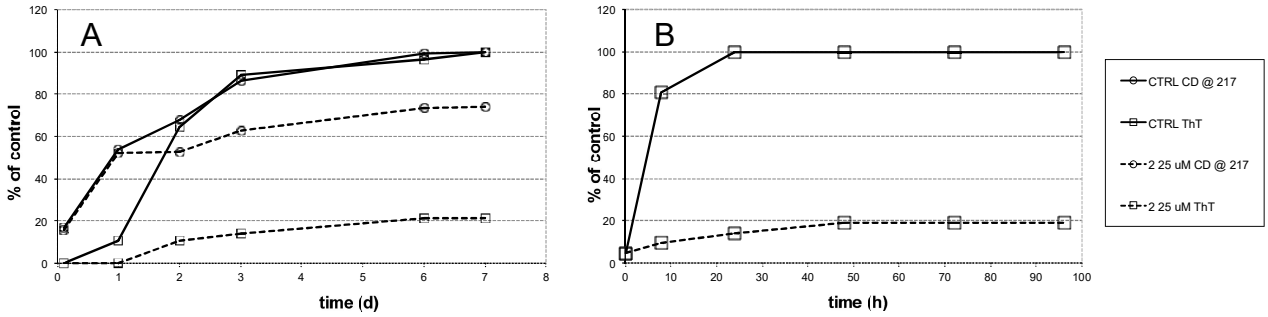


Figure 3. Typical time course experiment of aggregation kinetics of Aβ(1-40) (A) and Aβ(1-42) (B): controls (full lines) and co-incubated with **2** (dotted lines) in PBS/5% ethanol at 37 °C. Squares and circles represent ThT fluorescence and CD ellipticity at 217 nm, respectively. Values are displayed as percent of maximal values obtained from the controls.

CD spectra outlined a net β-sheet arrangement for both Aβ(1-40) free and Aβ/**2** coincubated samples, reaching almost 60% of the final β-sheet content in the first 2 days of incubation, as shown by the negative ellipticity band centered at 217 nm. CD spectra (not reported) of both samples accounted for a stepwise rearrangement of the secondary structure from initial random coil to final β-sheet, with a small difference for coincubated Aβ/**2**, displaying a residual content of random coil structure and a lower (80%) intensity of the β-sheet band. These data demonstrate that the antifibrillogenic activity displayed by this class of compounds is not related to the inhibition of the β-sheet arrangement, but rather to a different mechanism that we investigated by means of TEM to obtain visual proof of the shape and dimension of eventual aggregates.

The evolution of fibril formation for the same samples described above was detected at three time points: i.e., at the start (day 0), the third, and finally seventh day of incubation (Figure 4). Samples at day 0 were homogeneously devoid of any prefibrillar aggregate, both in the control (Fig. 4A) and in the coincubated sample (Fig. 4D). At day 3, a network of fibrillar species was already visible in both control and coincubated specimens (Figs. 4B, 4E), leading at day 7 to the massive formation of tangles (Figs. 4C, 4F). The amount of fibrillar species formed also in the presence of **2** suggested that the quenching of ThT fluorescence cannot be explained by the mere inhibition of fibril formation, but rather with a different structural arrangement that hampers ThT staining.

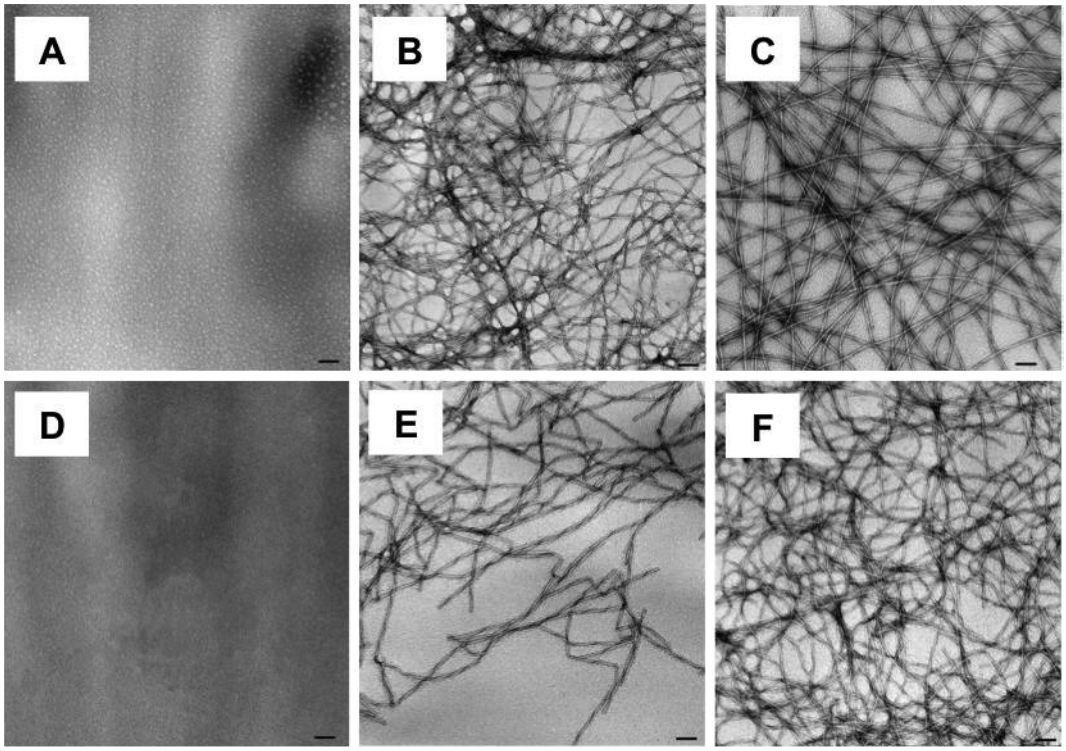


Figure 4. TEM micrographs of samples from aggregation kinetics of 50 μM Aβ(1-40) alone (A-C) or coincubated with 25 μM **2** (D-F) in PBS/5% ethanol at 37 °C. Samples were collected at time point zero (A, D), 3 (B, E) and 7 days of incubation (C, F). Black scale bars indicate 100 nm.

For a sound explanation of these results, a statistical analysis was performed to determine the average diameter of fibrils formed at 3 and 7 days of incubation for both the control (*d*) and coincubated (*d'*) samples. At day 3, average *d* and *d'* were 13.3 ± 2.4 nm and 10.5 ± 2.3 nm, respectively. At day 7, the thickness of control fibrils slightly increased to 13.8 ± 3.1 nm, while for the coincubated sample it was 9.4 ± 1.7 nm. Such average diameters are fully compatible with those reported in the literature;[51] worthy of note is the difference of approximately 4 nm between *d* and *d'* of fibrils finally formed after 7 days. Visual inspection revealed that the fibrillar structures formed in the presence of the inhibitor appeared at any time to be more rigid, inhomogeneous and to curl compared to those of the control specimen. The loss of ThT staining could therefore be ascribed to a destabilizing effect of the inhibitor in the correct evolution and assembly of prefibrillar species into mature fibrils.

In vitro Blood-brain Barrier permeation assays

Even if all tested compounds have molecular weight lower than 500Da and logD from 2 to 4 (ACDLabs, software V11.02), which should not challenge their ability to cross cell membranes, two compounds (**2** and **28**) representative of thioxanthenone and naphthoquinone subsets of compounds, were investigated for their ability to permeate, by passive diffusion, the Blood-Brain Barrier (BBB)

and to interact with P-glycoprotein (P-gp), which plays an important role in the efflux transport of drugs. To this purpose, transport studies involving these compounds were performed on MDCKII-MDR1 cells, which are characterized by a high expression of P-gp, and represent a well established *in vitro* method mimicking BBB.[52,53] Transport studies were conducted both in apical (AP) to basolateral (BL) and basolateral to apical direction and results are reported in Table 3.

Compounds **2** and **28** showed efflux ratio (ER) equal to 0.95 and 0.78, respectively, suggesting that they are able to permeate the monolayer with permeability at least comparable to diazepam (ER= 0.79).

Table 3. Bi-directional Transport Across MDCKII-MDR1 Cells of Compounds **2** and **28**

Compd	P_{app} AP(cm/sec)	P_{app} BL(cm/sec)	ER[a] P_{app}BL/P_{app}AP
2	$6.40*10^{-5}$	$6.08*10^{-5}$	0.95
28	$3.02*10^{-5}$	$2.35*10^{-5}$	0.78
Diazepam	$1.56*10^{-5}$	$1.23*10^{-5}$	0.79
FD-4[b]	$1.13*10^{-6}$	$2.68*10^{-7}$	0.23

[a] Efflux ratio (ER) was calculated using the following equation: ER = P_{app}, BL-AP / P_{app}, AP-BL, where P_{app}, BL-AP is the apparent permeability of basal-to-apical transport, and P_{app}, AP-BL is the apparent permeability of apical-to-basal transport. An efflux ratio greater than 2 indicates that a test compound is likely to be a substrate for P-gp transport. [b]FD-4= Fluorescein isothiocyanate-dextran.

Cytotoxicity assays

The assessment of safety profile is fundamental for drugs supposed to be used for long time as it is required for AD treatment. Thus in order to achieve a preliminary assessment of the neurotoxicity of the studied compounds, cytotoxicity assays were carried out against the human neuroblastoma cell line SH-SY5Y, commonly used in neurobiological studies. Compounds **2** and **28** were used in these experiment as representative of the thioxanthenone and naphthoquinone subsets of compounds respectively. The thioxanthenone **2** was the most potent inhibitor of Aβ aggregation, while the naphthoquinone **28** was the most potent inhibitor of AChE.

As shown in Table 4 they had a comparable cytotoxicity effect after 24h of incubation, with IC_{50} values of 7.2 ± 0.1 μM and 3.6 ± 0.2 μM, respectively. For compound **28**, the IC_{50} value for cytotoxicity was even 327 fold higher than the IC_{50} for AChE inhibition; however, on the whole, the results of Table 4 indicate a rather narrow safety margin.

Table 4. Cytotoxicity and inhibitory activities of compounds **2** and **28**.

Compd	Cytotox.[a] IC_{50} (μM)	AChE[b] IC_{50} (μM)	Ratio IC_{50}Tox/ IC_{50}AChE	BChE[b] IC_{50} (μM)	Ratio IC_{50}Tox/ IC_{50}BChE	Aβ aggreg.[b] IC_{50} (μM)	Ratio IC_{50}Tox/ IC_{50}Aβ
2	7.2 ± 0.1	5.2	1.38	0.46	15.65	0.84	8.52
28	3.6 ± 0.2	0.011	327	12	0.3	/	

[a]Concentration inducing 50% cell survival inhibition for compounds tested against human neuroblastoma cell line SH-SY5Y, *in vitro* after 24 h of incubation. [b]See Table 1.

On the other hand, based on the results of the testing for antileukemic activity,[30,31] only a low-moderate level of *in vivo* toxicity is expected for the present compounds. Indeed, no mortality was observed when several of these compounds were injected i.p. at the dose of 50-200 mg/Kg, once a day for five consecutive days, in a group of six mice, previously inoculated with leukemia P388 cells. Compounds **1** (the lower homolog of **2**) at the dose of 200 mg/Kg for nine days even prolonged the mice survival time of 24%; thus its i.p. LD_{50} should be quite superior to the indicated dosage. Donepezil and galantamine, two current drugs for AD, have been shown to exhibit higher toxicity in mice, with oral LD_{50} equal to 45 and 18.7 mg/Kg, respectively.[54,55]
For a more meaningful assessment of the value of the present compounds, the issue of their *in vitro* and *in vivo* toxicity, in comparison to established drugs for AD, should be further investigated.

Conclusions

Based on the models of compounds previously described as capable of maintaining or restoring cell protein homeostasis and of inhibiting Aβ oligomerization, 37 thioxanthen-9-one, xanthen-9-one, naphtho- and anthraquinone derivatives were tested for the direct inhibition of Aβ aggregation and the inhibition of AChE and BChE. These compounds are characterized by the presence of basic side chains, mainly quinolizidinylalkyl moieties, that have been recently shown to produce potent ChE inhibitors when linked to various bi- and tri-cyclic (hetero)aromatic systems.[4]
With very few exceptions, all these compounds were able to display inhibitory activity on both AChE and BChE and on the spontaneous aggregation of Aβ. In this regard, the time course of amyloid aggregation in the presence of **2** revealed that these compounds might act as destabilizers of mature fibrils rather than mere inhibitors of fibrillization. Their potential utility in the control of symptoms and/or progression of AD would therefore be confirmed only after an extensive

biophysical evaluation of prefibrillar, oligomeric species (i.e., the true neurotoxic species of Aβ) interfered during the aggregation process.

Most compounds exhibited IC_{50} values in the low micromolar or sub-micromolar range in the different biological activities; yet, single compounds even reached nanomolar potency (e.g., thioxanthenone derivative **4**, with IC_{50}= 88 nM for the inhibition of BChE, and chloronaphthoquinone derivative **28** with IC_{50}= 11 nM for AChE inhibition).

More importantly, many compounds inhibited one or both ChEs and Aβ aggregation with similar potencies, thus fulfilling a fundamental requirement for a multitarget mechanism of action. Among these compounds, the thioxanthenone derivatives **2**, **4**, **7**, and **9**, and most of all **17**, as well as anthraquinones **33** and **34** warrant further investigations.

Exploratory investigations on compounds **2** and **28**, as representative of the thioxanthenone and naphthoquinone subsets of compounds, have indicated that they may penetrate the BBB with a permeability comparable or even superior to that of diazepam.

In vitro assays for cytotoxicity against the neuroblastoma cell line SH-SY5Y, indicated for the two compounds a rather narrow safety margin, which seems, however, somewhat conflicting with the low-moderate *in vivo* toxicity previously observed.[27,28] Thus, this issue deserves further investigations in order to establish the value of the present compounds as potential agents for the treatment of AD.

Experimental Section

Chemistry

Chemicals, solvents and reagents used for the syntheses were purchased from Sigma-Aldrich, Fluka or Alfa Aesar, and were used without any further purification. Column chromatography (CC): neutral alumina (Al_2O_3), activity 1 (Merck). Mps: Büchi apparatus, uncorrected. ^{1}H NMR spectra: Bruker Avance DPX-300 or Varian Gemini-200 spectrometers; $CDCl_3$; δ in ppm rel. to Me_4Si as internal standard. *J* in Hz. Elemental analyses were performed on a Carlo Erba EA-1110 CHNS-O instrument in the Microanalysis Laboratory of the Department of Pharmacy of Genoa University.

General procedure for the synthesis of compounds 4, 5, 12-15, 20-22, 29 and 34-36

A mixture of the suitable reactive chloroderivative (1-chloro-4-methyl-9H-xanthen-9-one; 1-chloro-7-methoxy-4-nitro-9H-thioxanthen-9-one; 2,3-dichloro-1,4-naphthoquinone; 1-chloro-9,10-anthraquinone) with the suitable aminocompound [(1*S*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)alkylamines;[37] 3-{[(1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methyl]thio}propan-1-amine;[35] (1*R*,9a*R*)-(octahydro-2*H*-quinolizin-1-yl)methanthiole (thiolupinine);[36] *N,N*-diethyl-1,3-

propylenediamine] was heated at 170°C in a closed tube for 6h. The ratio between chlorocompound/amine was 1:2 for compounds **12-15** and **20-22**, while was 1:1 for compounds **29** and **34-36**.

To synthesize compounds **4** and **5**, 1-chloro-4-methyl-9H-thioxanthen-9-one (2.3 mmol) was reacted with 3-{[(1*R*,9a*R*)-(octahydro-2H-quinolizin-1-yl)methyl]thio}propan-1-amine or thiolupinine in a ratio 1:1 and in solution of Dowtherm A (2 mL) and in the presence of Cs_2CO_3 (2.3 mmol), heating at 170°C for 5h.

1-Chloro-4-methyl-9H-xanthen-9-one and 1-chloro-4-methyl-9H-thioxanthen-9-one were used as mixtures with the unreactive isomers as indicated in[7a] and[34].

In all cases at r.t. the mixture was triturated with 2N HCl and extracted with Et_2O; then the acid solution was basified with 6N NaOH and exhaustively extracted with Et_2O or CH_2Cl_2. The organic layer was washed with water and, after drying (Na_2SO_4), evaporated affording a residue that was crystallized from the indicated solvent or purified by CC (Al_2O_3/Et_2O or CH_2Cl_2). In the case of compounds **4**, **22**, **34** and **35** the oily residue was converted into the corresponding monohydrochloride with 1N ethanolic solution of HCl.

4-Methyl-1-{3-[(1R,9aR)-(octahydro-2H-quinolizin-1-yl)methylthio]prop-1-yl-amino}-9H-thioxanthen-9-one (4)

Oil. CC(Al_2O_3/CH_2Cl_2); yield: 43%; ^{1}H NMR (200MHz, $CDCl_3$):δ=0.94-2.22 (m, 16H, 14H of Q and 2H of CH_2*CH_2*CH_2), 2.36 (s, CH_3-Ar), 2.62-2.99 (m, 6H, 2Hα near N of Q, 2H of CH_2CH_2*CH_2*-S and 2H of SCH_2), 3.34-3.55 (m, 2H, NH*CH_2*), 6.61 (d, *J*= 7.6, 1 arom. H), 7.21-7.68 (m, 4 arom. H), 8.52 (dd, *J*= 7.6, 0.9, 1 arom. H), 10.12 (s, NH, collapses with D_2O); Anal. calcd for $C_{27}H_{34}N_2OS_2$: C 69.49, H 7.34, N 6.00, S 13.74, found: C 69.71, H 7.07, N 6.28, S 13.45; monohydrochloride: mp: 152-154°C (EtOH/Et_2O); Anal. Calcd for $C_{27}H_{34}N_2OS_2$+HCl: C 64.45, H 7.01, N 5.57, S 12.75, found: C 64.33, H 7.09, N 5.37, S 12.00.

4-Methyl-1-{[(1R,9aR)-(octahydro-2H-quinolizin-1-yl)methyl]thio}-9H-thioxanthen-9-one (5)

Yellow crystals (CH_2Cl_2); yield: 66%; mp: 113-115°C; ^{1}H NMR (200MHz, $CDCl_3$): δ=1.04-2.33 (m, 14H of Q), 2.50 (s, CH_3-Ar), 2.79-3.38 (m, 4H, 2Hα near N of Q and 2H of SCH_2), 7.24-7.82 (m, 5 arom. H), 8.60 (d, *J*= 7.6, 1 arom. H); Anal. calcd for $C_{24}H_{27}NOS_2$: C 70.38, H 6.64, N 3.42, S 15.65, found: C 70.39, H 6.67, N 3.47, S 15.26

7-Methoxy-4-nitro-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)methyl]amino}-9H-thioxanthen-9-one (12)

Dark yellow crystals (Et_2O/pentane); yield: 57%; mp: 194-196°C; ^{1}H NMR (200MHz, $CDCl_3$):δ=1.11-2.42 (m, 14H of Q), 2.91-3.12 (m, 2Hα near N of Q), 3.62-3.80 (m, 2H, NH*CH_2*),

3.97 (s, 3H, OCH_3), 6.78 (d, *J*= 9.2, 1 arom. H), 7.26-7.38 (m, 1 arom. H), 7.63 (d, *J*= 8.6, 1 arom. H), 7.97 (d, *J*= 2.8, 1 arom. H), 8.56 (d, *J*= 9.2, 1 arom. H), 12.03 (s, NH-Ar, collapses with D_2O); ^{13}C NMR (200MHz, $CDCl_3$):δ=19.8, 23.8, 24.2, 26.8, 28.7, 37.3, 41.2, 54.6, 56.2, 63.6, 106.4, 108.0, 110.0, 121.6, 126.4, 127.8, 129.3, 129.4, 131.3, 141.3, 156.8, 158.2, 181.5; Anal. calcd for $C_{24}H_{27}N_3O_4S$: C 63.56, H, 6.00, N 9.26, S 7.07, found: C 63.42, H 5.96, N 9.64, S 6.85.

7-Methoxy-4-nitro-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)ethyl]amino}-9H-thioxanthen-9-one (13)

Red crystals (Et_2O/pentane); yield: 67%; mp: 170-173°C; ^1H NMR (300MHz, $CDCl_3$): δ=1.20-2.04 (m, 16H, 14H of Q and 2H of $NHCH_2CH_2$), 2.77-2.93 (m, 2Hα near N of Q), 3.22-3.48 (m, 2H, $NHCH_2CH_2$), 3.96 (s, 3H, OCH_3), 6.50 (d, *J*= 6.0, 1 arom. H), 7.00 (d, *J*= 6.0, 1 arom. H), 7.10-7.21 (m, 1 arom. H), 7.46 (d, *J*= 6.0, 1 arom. H), 7.98 (d, *J*= 2.0, 1 arom. H), 9.77 (s, NH-Ar, collapses with D_2O); Anal. calcd for $C_{25}H_{29}N_3O_4S$: C 64.22, H 6.25, N 8.99, S 6.86, found: C, 64.19, H 6.42, N 8.99, S 6.49.

7-Methoxy-4-nitro-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)prop-1-yl]amino}-9H-thioxanthen-9-one (14)

Dark yellow crystals (Et_2O/pentane); yield: 46%; mp: 112-114°C; ^1H NMR (300MHz, $CDCl_3$): δ= 1.12-2.18 (m, 18H, 14H of Q and 4H of $CH_2CH_2CH_2$-Q), 2.78-2.94 (m, 2Hα near N of Q), 3.28-3.45 (m, 2H, $NHCH_2$), 3.87 (s, 3H, OCH_3), 6.57 (d, *J*= 6.0, 1 arom. H), 7.18-7.27 (m, 1 arom. H), 7.52 (d, *J*= 6.0, 1 arom. H), 7.85 (d, *J*= 2.0, 1 arom. H), 8.44 (d, *J*= 6.0, 1 arom. H), 11.86 (s, NH, collapses with D_2O); Anal. calcd for $C_{26}H_{31}N_3O_4S$: C 64.84, H 6.49, N 8.72, S 6.66, found: C 64.97, H 6.39, N 8.37, S 6.87.

1-{[3-(N,N-Dimethylamino)prop-1-yl]amino}-7-methoxy-4-nitro-9H-thioxanthen-9-one (15)

Dark yellow crystals (Et_2O/pentane); yield: 66%; mp: 101-103°C; ^1H NMR (200MHz, $CDCl_3$): δ= 1.19 (t, *J* = 7.0, 6H, $N(CH_2CH_3)_2$), 2.00-2.24 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.60-2.94 (m, 6H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 3.42-3.60 (m, 2H, $NHCH_2CH_2CH_2N(C_2H_5)_2$)), 3.96 (s, 3H, OCH_3), 6.68 (d, *J*= 9.8, 1 arom. H), 7.20-7.32 (m, 1 arom. H), 7.57 (d, *J*= 8.8, 1 arom. H), 7.89 (d, *J*= 2.8, 1 arom. H), 8.49 (d, *J*= 9.8, 1 arom. H), 11.96 (s, NH, collapses with D_2O); Anal. calcd for $C_{21}H_{25}N_3O_4S$: C 60.70, H 6.06, N 10.11, S 7.72, found: C 60.64, H 6.00, N 10.18, S 7.41.

4-Methyl-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)methyl]amino}-9H-xanthen-9-one (20)

Yellow crystals (Et_2O); yield: 67%; mp: 109-111°C; ^1H NMR (200 MHz, $CDCl_3$): δ=1.08-2.24 (m, 14H of Q), 2.36 (s, CH_3-Ar), 2.81-2.96 (m, 2Hα near N of Q), 3.30-3.60 (m, 2H of $NHCH_2$), 6.42 (d, *J*= 9.8, 1 arom. H), 7.24-7.72 (m, 4 arom. H), 8.24 (d, *J*= 9.2, 1 arom. H), 9.38 (s, NH, collapses with D_2O); Anal. calcd for $C_{24}H_{28}N_2O_2$: C 76.56, H 7.50, N 7.44, found: C 76.21, H 7.38, N 7.68.

4-Methyl-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)prop-1-yl]amino}-9H-xanthen-9-one (21)

Yellow crystals (Et_2O); yield: 40%; mp: 82-84°C; ^1H NMR (200 MHz, $CDCl_3$): δ=1.05-2.00 (m, 18H, 14H of Q and 4H of $CH_2CH_2CH_2$-Q), 2.37 (s, CH_3-Ar), 2.86-3.14 (m, 2Hα near N of Q), 3.18-3.36 (m, 2H, $NHCH_2$), 6.36 (d, *J*= 8.4, 1 arom. H), 7.23-7.74 (m, 4 arom. H), 8.24 (d, *J*= 7.8, 1 arom. H), 9.38 (s, NH, collapses with D_2O); ^{13}C NMR (200MHz, $CDCl_3$):δ=13.9, 20.1, 23.9, 26.7, 27.0, 37.2, 42.3, 56.3, 64.8, 102.0, 105.8, 108.9, 116.2, 121.0, 122.3, 124.9, 132.8, 136.5, 149.3, 154.3, 154.4, 179.0; Anal. calcd for $C_{26}H_{32}N_2O_2$: C 77.19, H 7.97, N 6.92, found: C 76.95, H 8.20, N 7.05.

1-{[3-(N,N-Diethylamino)prop-1-yl]amino}-4-methyl-9H-xanthen-9-one (22)

Oil, CC(Al_2O_3/Et_2O); yield: 58%; ^1H NMR (200MHz, $CDCl_3$): δ=1.07 (t, *J* = 7.4, 6H, $N(CH_2CH_3)_2$), 1.80-2.20 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.35 (s, CH_3-Ar), 2.40-2.78 (m, 6H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 3.17-3.39 (m, 2H, $NHCH_2CH_2CH_2N(C_2H_5)_2$)), 6.36 (d, *J*= 9.8, 1 arom. H), 7.20-7.71 (m, 4 arom. H), 8.23 (d, *J*= 9.4, 1 arom. H), 9.37 (s, NH, collapses with D_2O); monohydrochloride: mp 176-179°C (EtOH/Et_2O); Anal. Calcd for $C_{21}H_{26}N_2O_2$+HCl: C 67.28, H 7.26, N 7.47, found: C 67.29, H 7.51, N 7.63.

3-Chloro-2-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)methylthio]prop-1-ylamino}-1,4-naphthoquinone (29)

Yellow crystals, CC(Al_2O_3/CH_2Cl_2); yield: 63%; mp: 96-97°C; ^1H NMR (200MHz, $CDCl_3$): δ=0.94-2.24 (m, 16H, 14H of Q and 2H of $CH_2CH_2CH_2S$), 2.64 (t, *J* = 6.4, 2H, $CH_2CH_2CH_2S$), 2.72-3.04 (m, 4H, 2Hα near N of Q and 2H of SCH_2), 3.88-4.12 (m, 2H, $NHCH_2$), 6.23 (s, NH, collapses with D_2O), 7.59-7.82 (m, 2 arom. H), 7.98-8.24 (m, 2 arom. H), 9.82 (s, NH, collapses with D_2O); Anal. calcd for $C_{23}H_{29}ClN_2O_2S$: C 63.80, H, 6.75, N 6.47, S 7.41, found: C 64.10, H 6.49, N 6.42, S 7.08.

1-{3-[(1R,9aR)-(octahydro-2H-quinolizin-1-yl)methylthio]prop-1-ylamino}-9,10-anthra-quinone (34)

Oil, CC(Al_2O_3/CH_2Cl_2); yield: 57%, ^1H NMR (200MHz, $CDCl_3$): δ=0.94-2.26 (m, 16H, 14H of Q and 2H of $CH_2CH_2CH_2$), 2.57-3.05 (m, 6H, 2Hα near N of Q, 2H of $CH_2CH_2CH_2$-S and 2H of SCH_2), 3.38-3.62 (m, 2H, NH-$CH_2CH_2CH_2$), 7.12 (dd, *J*= 9.2, 1.6, 1 arom. H), 7.43-7.88 (m, 4 arom. H), 8.16-8.40 (m, 2 arom. H), 9.82 (s, NH, collapses with D_2O); Anal. calcd for $C_{27}H_{32}N_2O_2S$: C 72.29, H 7.19, N 6.24, S 7.15, found: C 72.03, H 6.98, N 6.47, S 7.42; monohydrochloride (higroscopic): Anal. calcd for $C_{27}H_{32}N_2O_2S$+HCl+$2H_2O$: C 62.23, H 7.16, N 5.38, S 6.15, found: C 62.48, H 7.48, N 5.60, S 6.15.

1-{[3-(N,N-Dimethylamino)prop-1-yl]amino}-9,10-anthraquinone (35)

Oil, CC(Al_2O_3/Et_2O); yield: 70%, ^{1}H NMR (300MHz, $CDCl_3$): δ=1.46 (t, *J* = 7.4, 6H, $N(CH_2\mathit{CH_3})_2$), 2.26-2.44 (m, 2H, $CH_2\mathit{CH_2}CH_2N(C_2H_5)_2$), 3.10-3.36 (m, 6H, $CH_2CH_2\mathit{CH_2}N(\mathit{CH_2}CH_3)_2$), 3.54 (t, *J*= 7.4, 2H, $NH\mathit{CH_2}CH_2CH_2N(C_2H_5)_2$)), 7.06 (d, *J*= 8.8, 1 arom. H), 7.52-7.81 (m, 4 arom. H), 8.22 (d, *J*= 8.6, 2 arom. H), 12.24 (s, NH, collapses with D_2O); ^{13}C NMR (200MHz, $CDCl_3$):δ=7.5, 22.5, 24.2, 39.3, 45.7, 48.5, 112.3, 115.3, 116.7, 125.6, 131.8, 132.1, 132.9, 133.5, 133.6, 134.5, 149.9, 182.3, 184.1; monohydrochloride: mp: 224-226°C (EtOH/Et_2O); Anal. calcd for $C_{21}H_{24}N_2O_2$+HCl: C 67.64, H 6.76, N 7.51, found: C 67.44, H 7.11, N 7.44.

1-{[(1R,9aR)-(octahydro-2H-quinolizin-1-yl)methyl]thio}-9,10-anthraquinone (36)

Yellow crystals (EtOH); yield: 64%, mp: 182-184°C; ^{1}H NMR (200MHz, $CDCl_3$): δ=0.96-2.28 (m, 14H of Q), 2.71-2.98 (m, 2Hα near N of Q), 3.06-3.21 (m, 2H of SCH_2), 7.62-7.97 (m, 4 arom. H), 8.09-8.46 (m, 3 arom. H); Anal. calcd for $C_{24}H_{25}NO_2S$: C 73.62, H 6.44, N 3.58, S 8.19, found: C 73.80, H 6.06, N 3.22, S 8.01.

General procedure for the synthesis of compounds 18 and 19.

To a stirred soln. of the 4-nitroderivative **13** or **15** (0.96 mmol) in EtOH (20 ml), a solution of $SnCl_2{\cdot}2H_2O$ (0.76 g, 3.36 mmol) in conc. HCl (5 ml) was slowly added. The mixture was refluxed for 6 h and then concentrated *in vacuo*. The residue was taken up in H_2O, alkalized with 6N NaOH and repeatedly extracted with Et_2O. After drying (Na_2SO_4), the solvent was removed and the oily residue converted into the dihydrochloride.

4-Amino-7-methoxy-1-{[(1S,9aR)-(octahydro-2H-quinolizin-1-yl)ethyl]amino}-9H-thio-xanthen-9-one (18)

Red oil; yield: 61%; ^{1}H NMR (300MHz, $CDCl_3$): δ=1.10-2.06 (m, 16H, 14H of Q and 2H of $NHCH_2\mathit{CH_2}$), 2.75-2.90 (m, 2Hα near N of Q), 3.08-3.42 (m, 4H, 2H of $NH\mathit{CH_2}CH_2$ and 2H of NH_2-Ar, collapse with D_2O), 3.86 (s, 3H, OCH_3), 6.47 (d, *J*= 6.0, 1 arom. H), 6.95 (d, *J*= 6.0, 1 arom. H), 7.08-7.20 (m, 1 arom. H), 7.37 (d, *J*= 6.0, 1 arom. H), 7.93 (d, *J*= 2.0, 1 arom. H), 9.78 (s, NH-Ar, collapses with D_2O); dihydrochloride: mp: 235-238°C (EtOH); Anal. calcd for $C_{25}H_{31}N_3O_2S$+2HCl: C 58.82, H 6.52, N 8.23, S 6.28, found: C 59.10, H 6.73, N 8.08, S 6.36.

4-Amino-1-{[3-(N,N-dimethylamino)prop-1-yl]amino}-7-methoxy-9H-thioxanthen-9-one (19)

Red oil; yield: 65%; ^{1}H NMR (200MHz, $CDCl_3$): δ=1.19 (t, *J* = 7.2, 6H, $N(CH_2\mathit{CH_3})_2$), 1.92-2.14 (m, 2H, $CH_2\mathit{CH_2}CH_2N(C_2H_5)_2$), 2.57-2.98 (m, 6H, $CH_2CH_2\mathit{CH_2}N(\mathit{CH_2}CH_3)_2$), 3.22-3.56 (m, 4H, 2H of $NH\mathit{CH_2}CH_2CH_2N(C_2H_5)_2$ and 2H of NH_2-Ar, collapse with D_2O), 3.94 (s, 3H, OCH_3), 6.55 (d, *J*= 8.8, 1 arom. H), 7.04 (d, *J*= 8.8, 1 arom. H), 7.17 (dd, *J*= 8.8, 3.0, 1 arom. H), 7.46 (d, *J*= 8.8,

1 arom. H), 8.00 (d, *J*= 2.8, 1 arom. H), 9.86 (s, NH, collapses with D_2O); dihydrochloride: mp: 230-233°C (EtOH); Anal. calcd for $C_{21}H_{27}N_3O_2S$+2HCl: C 55.02, H 6.38, N 9.17, S 6.99, found: C 55.20, H 6.43, N 9.08, S 7.37.

Biological tests

All reagents and enzymes were purchased from Sigma-Aldrich Italy, unless otherwise specified. For title compounds, the concentration which determined 50% inhibition of the tested activity (IC_{50}) was determined by testing in duplicate 5–7 concentrations, ranging from 100 to 0.01 μM, and calculated by nonlinear regression of the response/log(concentration) curve, using GraphPad Prism® v. 5.01 software (La Jolla, CA, USA). Values were obtained as the mean from two/three independent experiments.

Inhibition of cholinesterases

The in vitro inhibition assays of AChE, either from electric eel (463 U/mg) and human recombinant (2770 U/mg), and BChE from equine serum (13 U/mg) were run in phosphate buffer 0.1 M, pH 8.0. Acetyl- and butyrylthiocholine iodides were used, respectively, as substrates, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used as the chromophoric reagent.[39] Inhibition assays were carried out on an Agilent 8453E UV-visible spectrophotometer equipped with a cell changer. Solutions of tested compounds were prepared starting from 10 mM stock solutions in DMSO, which were diluted with water to a final content of organic solvent always lower than 1%. AChE inhibitory activity was determined in a reaction mixture containing 100 μL of a solution of AChE (0.9 U/mL in 0.1 M phosphate buffer, pH 8.0), 100 μL of a 3.3 mM solution of DTNB in 0.1 M phosphate buffer (pH 7.0) containing 6 mM $NaHCO_3$, 100 μL of a solution of the inhibitor (six to seven concentrations ranging from 1×10^{-8} to 1×10^{-4} M), and 600 μL of work buffer. After incubation for 20 min at 25 °C, acetylthiocholine iodide (100 μL of 5 mM aqueous solution) was added as the substrate, and AChE-catalyzed hydrolysis was followed by measuring the increase of absorbance at 412 nm for 5.0 min at 25 °C. BChE inhibitory activity was assessed similarly using butyrylthiocholine iodide as the substrate.

Kinetic studies of hAChE inhibition

Kinetic studies were performed under the same incubation conditions above described, using six concentrations of substrate (from 0.033 to 0.2 mM) and four concentrations of inhibitor **28** (0–50 nM). Apparent inhibition constant and kinetic parameters were calculated within the 'Enzyme kinetics' module of Prism.

Thioflavin T fluorescence spectroscopy analysis

To obtain batches of Aβ(1–40) and Aβ(1–42) free from preaggregates, commercial peptides (purity >95%; EzBiolab, Carmel, USA) were dissolved in HFIP, lyophilized and stored at -20 °C. The solution of ThT (25 μM) used for fluorimetric measures was prepared in phosphate buffer 0.025 M, pH 6.0, filtered through 0.45 μm nylon filters and stored at 4 °C. For Aβ(1–40) inhibition, compounds were first tested at 100 μM; test samples were prepared in phosphate buffered saline (PBS; 0.01 M, NaCl 0.1 M, pH 7.4), with 30 μM Aβ peptide concentration, and contained 2% HFIP and 10% DMSO. Blank samples were prepared for each concentration, devoid of peptide, and their fluorescence value subtracted from the corresponding fluorescence values of coincubation samples. As the control, a sample of peptide was incubated in the same PBS/2% HFIP/10% DMSO buffer, without inhibitor. Incubations were run in triplicate at 25 °C for 2 h. Fluorimetric measures were performed in a 700 μL cuvette with a Perkin–Elmer LS55 spectrofluorimeter, using FLWinlab program. 470 μL of ThT solution were mixed with 30 μL of sample, and the resulting fluorescence measured with parameters set as follows: excitation at 440 nm (slit 5 nm); emission at 485 nm (slit 10 nm); integration time 2 s. Biological activity was determined as percent of inhibitory activity V_i for each concentration, according to the formula:

$$V_i = 100 - [(F_i - F_b)/F_0] \times 100$$

where F_i is the fluorescence value of the sample, F_b its blank value, and F_0 the fluorescence value of Aβ control (already subtracted of its blank).

Aβ aggregation kinetics

Samples for time course experiments of Aβ(1–40) and Aβ(1–42) aggregation inhibition were prepared in PBS with 5% ethanol as co-solvent and incubated up to seven days at 37 °C. Final concentrations of Aβ and **2** were 50 μM and 25 μM, respectively. A control sample of self-aggregating Aβ was prepared in the same buffer/cosolvent conditions.

TEM studies

The same samples prepared as in Section 4.2.3 were used for TEM analysis. For each sample, a little drop (20 μL) of incubated sample solution was applied to carbon coated copper/rhodium grid (400 mesh; TAAB Laboratories Equipment Ltd, Aldermaston, Berks, GB). The coated grid was floated for 2 min on the sample drop and rinsed with 200 μL of double distilled water. Negative staining was performed with 200 μL of 2% w/v uranyl acetate solution (TAAB Laboratories

Equipment Ltd). After draining off the excess of staining solution by means of a filter paper, the specimen was transferred for examination in a Philips Morgagni 282D transmission electron microscope, operating at 60 kV. Electron micrographs of negatively stained samples were photographed on Kodak electron microscope film 4489 (Kodak Company, New York, USA) and analyzed with Image 1.38 software. Statistics of fibril diameters were obtained with Kaleidagraph (Sinergy Software) calibration software.

CD spectroscopy analysis

CD spectra were recorded in the spectral range 195-250 nm, by using 0.1 cm path length quartz cells (280 μL internal volume, from Hellma GmbH & Co KG, Milan, I) with a CD Jasco J-810 single beam spectropolarimeter. Control Aβ and Aβ/inhibitor coincubation samples were the same as in TEM studies. CD spectra were recorded at room temperature at 0.1 nm intervals with 4 nm bandwidth and 100 nm/min scan speed. The baseline was recorded with the buffer/ethanol blank or, for co-incubation experiments, with buffer/ethanol solution of inhibitor blank, and automatically subtracted from corresponding spectra.

Bi-directional Transport Studies on MDCKII-MDR1 monolayers

Apical to basolateral (P_{app}, AP) and basolateral to apical (P_{app}, BL) permeability of compounds **2** and **28** were measured using Madin-Darby Canine Kidney (i.e., MDCK) cells, retrovirally transfected with the human MDR1 cDNA (MDCKII-MDR1). These cells were cultured in DMEM medium and seeded at a density of 100,000 cell/cm^2 onto polyester 12 well Transwell inserts (pore size 0.4 μm, 12 mm diameter, apical volume 0.5 mL, basolateral volume 1.5 mL). MDCKII-MDR1 cell barrier function was verified prior to the described transport experiments by means of trans-epithelial electrical resistance (TEER) using an EVOM apparatus, and the measurement of the flux of Fluorescein isothiocyanate-dextran (FD4, Sigma Aldrich, Italy) (200 μg/mL) and Diazepam (75 μM) as paracellular and transcellular markers, respectively, of cell monolayers integrity and as an internal control to verify tight junction integrity during the assay. The TEER was measured in growth media (DMEM) at room temperature and calculated as the measured resistance minus the resistance of an empty Transwell (blank without cells). Cell monolayers with TEER values 800 Ohm cm^2 were used. Following the TEER measurements, the cells were equilibrated in transport medium in both the apical and basolateral chambers for 30 minutes at 37 °C. The composition of transport medium was as follows: 0.4 mM K_2HPO_4, 25 mM $NaHCO_3$, 3 mM KCl, 122 mM NaCl, 10 mM glucose and the pH was 7.4, and the osmolarity was 300 mOsm as determined by a freeze point based osmometer. At time 0, culture medium was aspirated from both the apical (AP) and

basolateral (BL) chambers of each insert, and cell monolayers were washed three times (10 min per wash) with Dulbecco`s Phosphate Buffered Saline (DPBS) pH = 7.4. Finally, a solution of compounds diluted in transport medium was added to the apical or basolateral chamber. For AP-to-BL or BL-to-AP flux studies, the drug solution was added in the AP chamber or in the BL chamber, respectively. Except for FD4, which was solubilized directly in the assay medium at a concentration of 200 μg/mL, the other compounds were first dissolved in DMSO and then diluted with the assay medium to a final concentration of 75 μM. Next, the tested solutions were added to the donor side (0.5 mL for the AP chamber and 1.5 mL for the BL chamber) and fresh assay medium was placed in the receiver compartment. The percentage of DMSO never exceeded 1% (v/v) in the samples. The transport experiments was carried out under cell culture conditions (37 °C, 5% CO_2, 95% humidity). After incubation time of 120 min, samples were removed from the apical and basolateral side of the monolayer and then stored until further analysis.

Quantitative analysis of compounds **2**, **28** and diazepam, were performed through UV–visible (Vis) spectroscopy using a PerkinElmer double-beam UV–visible spectrophotometer Lambda Bio 20 (Milan, Italy), equipped with 10 mm path-length-matched quartz cells. Standard calibration curves were prepared at maximum absorption wavelength of each compound using PBS as solvent and were linear (r^2 =0.999) over the range of tested concentration (from 5 to 100 μM). The FD4 samples were analyzed with a Victor3 fluorometer (Wallac Victor3, 1420 Multilabel Counter, Perkin-Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. Each compound was tested in triplicate, and the experiments were repeated three times.

The apparent permeability, in units of cm/sec, was calculated using the following equation:

$$P_{app} = \left(\frac{V_A}{area\ x\ time}\right) x \left(\frac{[drug]_{acceptor}}{[drug]_{initial}}\right)$$

Where “VA” is the volume in the acceptor well, “area” is the surface area of the membrane, “time” is the total transport time, “$[drug]_{acceptor}$” is the concentration of the drug measured by UV-spectroscopy and “$[drug]_{initial}$” is the initial drug concentration in the AP or BL chamber. Efflux ratio (ER) was calculated using the following equation: ER = P_{app}, BL-AP / P_{app}, AP-BL, where P_{app}, BL-AP is the apparent permeability of basal-to-apical transport, and P_{app}, AP-BL is the apparent permeability of apical-to-basal transport. An efflux ratio greater than 2 indicates that a test compound is likely to be a substrate for P-gp transport.

Cytotoxicity Assays

Cytotoxicity assays were carried out against human neuroblastoma cell line SH-SY5Y. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 in DMEM (Lonza) nutrient supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cytotoxicity of compounds is expressed as IC_{50} values, the concentrations that cause 50% growth inhibition. The results were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were dispensed into 96-well microtiter plates at a density of 10,000 cells/well. Following overnight incubation, cells were treated with a range of compounds concentrations (0.1-100 µM). Then the plates were incubated at 37 °C for 24 h. An amount of 10 µL of 0.5% w/v MTT was further added to each well and the plates were incubated for an additional 3 h at 37 °C. Finally the cells were lysed by addition of 100 µL of DMSO/EtOH 1:1 (v/v) solution. The absorbance at 570 nm was determined using a Perkin Elmer 2030 multilabel reader Victor TM X3.

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