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Multitargeted drugs discovery: Balancing anti-amyloid and anticholinesterase capacity in a single chemical entity

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ABSTRACT

Memoquin (**1**) is a lead compound multitargeted against Alzheimer's disease (AD). It is an AChE inhibitor, free-radical scavenger, and inhibitor of amyloid-β (Aβ) aggregation. A new series of **1** derivatives was designed and synthesized by linking its 2,5-diamino-benzoquinone core with motifs that are present in the structure of known amyloid binding agents like curcumin, the benzofuran derivative SKF64346, or the benzothiazole bearing compounds KHG21834 and BTA-1. The weaker AChE inhibitory potencies and the concomitant nearly equipotent anti-amyloid activities of the new compounds with respect to **1** resulted in a more balanced biological profile against both targets. Selected compounds turned out to be effective Aβ aggregation inhibitors in a cell-based assay. By properly combining two or more distinct pharmacological properties in a molecule, we can achieve greater effectiveness compared to single-targeted drugs for investigating AD.

In the fight against Alzheimer's disease (AD), great gains in basic science knowledge have not yet resulted in clinically effective drugs. A contributing factor to this lack of translational progress is the difficulty in defining an appropriate drug discovery strategy that parallels the complexity of the AD pathology. The only available drugs are acetylcholinesterase (AChE) inhibitors and memantine, both of which target neurotransmission with modest palliative clinical effects. Currently, AD drug development targets the production or aggregation of amyloid-β peptide (Aβ) into oligomers, which are responsible for disrupting neuronal synaptic plasticity and for the resulting early cognitive impairment associated with AD.[1] However, no potential drug compound has successfully completed clinical trials. A careful re-examination of current strategies is therefore warranted.

Since AD is a complex neurodegenerative disorder resulting from multiple molecular abnormalities, and not from a single target/gene defect, we and others have proposed a move from the 'one protein, one target, one drug' strategy to a strategy of developing drugs that simultaneously affect multiple targets.[2–5] This approach has led us to a new paradigm in medicinal chemistry, the 'multitarget-directed ligand' (MTDL) design strategy.[6] In the past decade, by following this strategy, we have developed several ligands able to simultaneously hit multiple AD biological targets.[7] Memoquin (**1**; Fig. 1) is a quinone-bearing polyamine currently in preclinical investigation: it inhibits self- and AChE-induced Aβ aggregation or its formation, by inhibiting the beta secretase enzyme (BACE-1), and acts an antioxidant.[8,9] Its development offered the proof of concept of the effectiveness of the MTDL drug discovery approach, with in vivo confirmation of its multitarget mechanism of action.[10]

In the search for novel memoquin analogues, we focused on MTDLs with a better and a more balanced anti-amyloid/anticholinesterase profile. The view that Aβ is one of the (main) factors, and not the only factor underlying AD pathogenesis, is more consistent with current knowledge,[11] and might account for the failure of purely anti-amyloid strategies. Moreover, it further supports the rationale that MTDLs could be more therapeutically effective if they have other biological properties in addition to the anti-amyloid one. To this end, we have rationally manipulated the structure of **1**. Our starting point was the assumption that its planar 2,5-diamino-benzoquinone (BQ) scaffold could be a privileged motif for modulating protein–protein interactions (PPIs) and an optimal central core for the design of bivalent ligands.[9,12–15] The terminal 2-methoxybenzyl groups of **1** were replaced with several amyloid binding fragments from known amyloidophilic agents, as depicted in Figure 1. Curcumin (Curc) directly binds Aβ, blocking aggregation and fibril formation in vitro and in vivo.[16] The vanillic (Van) ring seems to be critically involved in the interaction.[17] Several compounds containing a benzofuran (BFur) moiety (e.g., SKF64 346) have been identified as inhibitors of Aβ fibril formation.[18,19] Benzothiazole (BTh) derivatives are extremely interesting molecules for neurodegenerative drug development. As an example, the benzothiazole derivative

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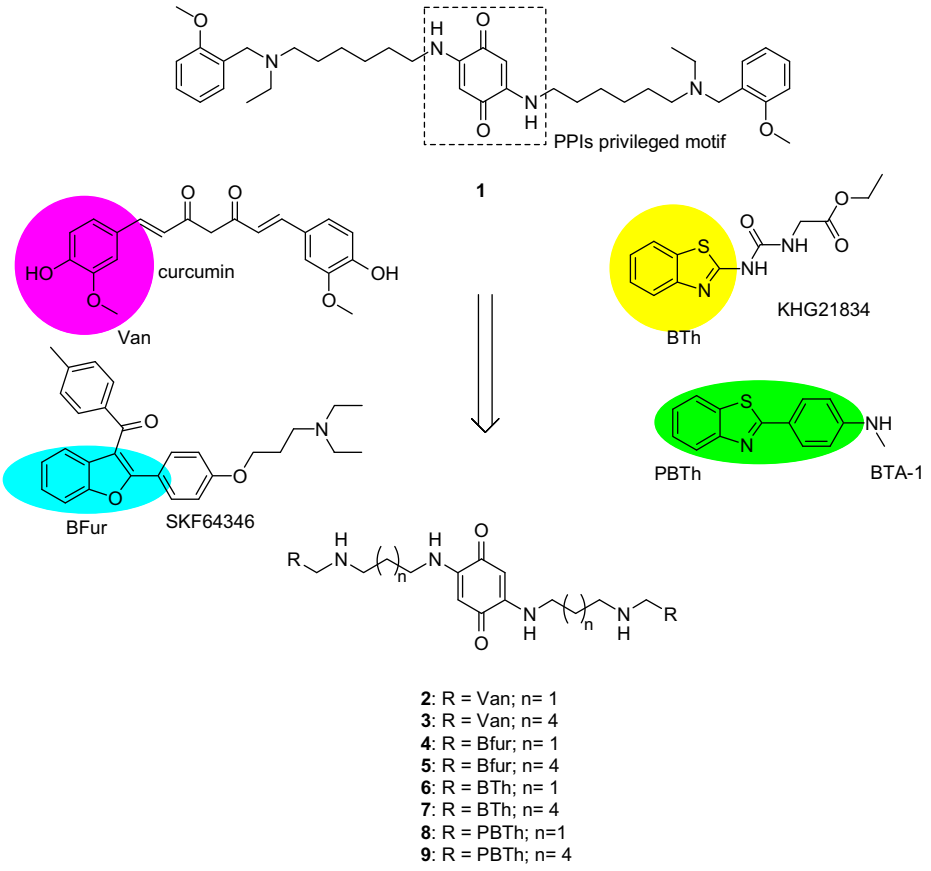


2: R = Van; n= 1
3: R = Van; n= 4
4: R = Bfur; n= 1
5: R = Bfur; n= 4
6: R = BTh; n= 1
7: R = BTh; n= 4
8: R = PBTh; n=1
9: R = PBTh; n= 4

Figure 1. Design strategy for compounds **2**–**9**.

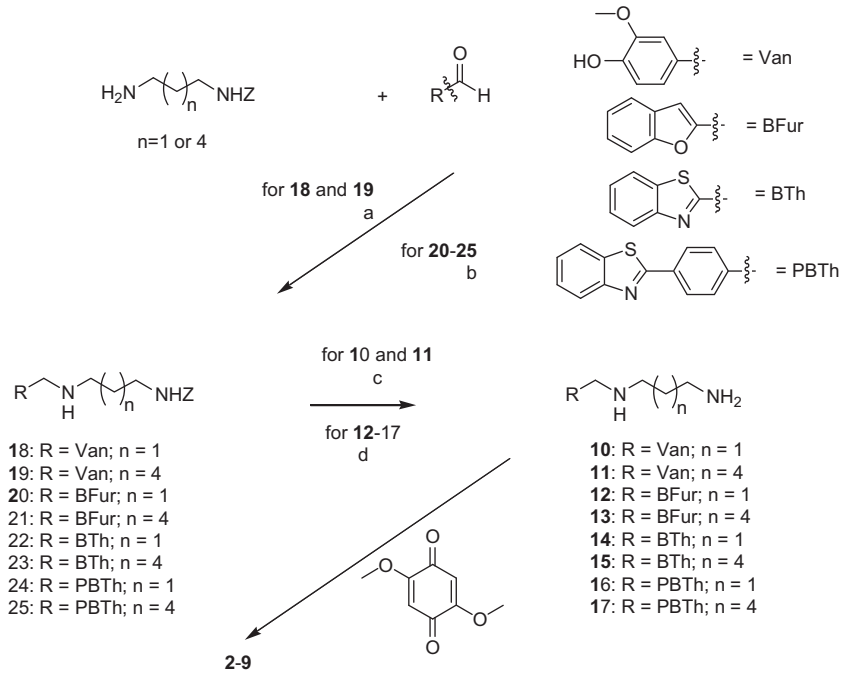
KHG21834 is neuroprotective against the Aβ-induced degeneration of neuronal cells in vitro and in vivo.[20,21] Furthermore, starting from the 2-phenylbenzothiazole (PBTh) dye thioflavin T (ThT), several amyloid imaging agents were developed. It was reported that the uncharged ThT analogue BTA-1[22] shows very good brain entry and a specific binding to Aβ deposits in AD brain while the ^{11}C-labelled *N*-methyl 6-hydroxy derivative of BTA-1 ([^{11}C]PIB), also known as Pittsburgh compound B, is the most commonly used agent for the positron emission tomography (PET) imaging of Aβ plaques.[23] The selected amyloid recognition motifs were linked to the BQ nucleus through proper spacers, that is, a diaminopropane or a diaminohexane chain, which conferred a better MTDL profile in previous SAR studies on **1** derivatives.[9]

From the synthetic standpoint, a retrosynthetic analysis revealed that, as with **1**, the target compounds can be obtained from substitution reaction of 2,5-dimethoxy-1,4-benzoquinone with the appropriate diamines **10–17** (Scheme 1).[9,24] Compounds **12**–**17**, in turn, were synthesized by reductive amination of an aldehyde with *N*-*Z*-diaminohexane or *N*-*Z*-diaminopropane (**20**–**25**), followed by cleavage of the N-protecting group with HBr. A milder reductive amination protocol was used for vanillin-derived diamines **10** and **11**. The reaction using trimethylorthoformate as solvent required only 1 equiv of the respective protected diamine, and corresponding equivalents of sodium cyanoborohydride together with 5% glacial acetic acid. This gave high yields of the expected product without protection of the phenolic group. In this case, removal of the *Z* group was achieved by hydrogenolysis.

First, we tested the ability of **2**–**9** to inhibit AChE and butyrylcholinesterase (BuChE) catalytic activity in comparison with **1**. As reported in Table 1, the nature of the R residue strongly influenced the ability to bind and inhibit cholinesterase activity. In contrast to **1**, which inhibits AChE in the nanomolar range, **2**–**9** were weaker inhibitors with IC_{50} values in the micromolar range.

Previous results demonstrated that quinone-bearing polyamines can be potent AChE inhibitors only when the terminal protonable nitrogen atom bears an ethyl substituent, with the des-ethyl-memoquin nearly 100 times less active than **1**.[9] This could also account for the decreased activity observed for **2**–**9**. However, **2** and **3**, carrying the vanillic moiety, showed IC_{50} values in the submicromolar range on hAChE (0.198 and 0.102 μM, respectively). A submicromolar inhibitory potency was also observed for compound **9**. These levels of inhibition are relatively modest in comparison to **1**, yet it is important to note that inhibition of acetylcholine cleavage by current drugs, such as rivastigmine[25] and donepezil,[26] is not dissimilar.

The activity of **2**–**9** towards the inhibition of $A\beta_{1-42}$ self-induced aggregation was then investigated using a ThT-based fluorometric assay to quantify Aβ fibril formation in the presence and absence of inhibitor (Table 1).[27] The similar inhibition percentages provided by **2**–**9** with respect to **1** point to the conclusion that the presence of different amyloid binding motifs is not so relevant against Aβ self-induced aggregation. However, the fact that all the derivatives at 10 μM concentration inhibited amyloid aggregation at a similar extent to the known anti-aggregator curcumin (curc) reinforce the rationale that the BQ is a privileged motif for bivalent ligands targeting PPI. Furthermore, the length of the spacer appears to play a role, since the potency of all tested compounds (except **2** and **3**) was always higher for the three methylene-spaced compounds than for the corresponding six methylene ones. Compound **3**, bearing the vanillic fragment and a 6-methylene chain, maintained an inhibitory potency in the same range as the reference compound **1**, and was the most potent inhibitor of the series.



Scheme 1. Reagents and conditions: (a) 1 equiv RCHO, trymethylorthoformate, rt, 1 h, then $NaBH_3CN$, 5% CH_3COOH, rt, overnight; (b) 1.1 equiv RCHO, toluene, Dean–Stark trap, reflux, 3 h, then EtOH, $NaBH_4$, rt, overnight; (c) H_2, 10% Pd/C (10% w/w), MeOH, rt,2 h; (d) 30% HBr, AcOH, rt, overnight; (e) 0.5 equiv 2,5-dimethoxybenzoquinone, EtOH, 50 °C for 3 h, then rt, overnight.

Table 1
Inhibitory activity of human AChE and BuChE and amyloid self-aggregation by **2**–**9** and reference compounds **1** and curcumine (Curc)

Compd	IC_{50} hAChE (μM)[a] ± SEM	IC_{50} hBuChE (μM)[a] ± SEM	Aβ self-aggregation (%)[b] ± SEM
1[c]	0.00155 ± 0.00011[c]	1.44 ± 0.10[c]	66.8 ± 4.4
2	0.198 ± 0.008	8.24 ± 0.43	22.0 ± 1.6
3	0.102 ± 0.014	1.60 ± 0.01	50.4 ± 0.6
4	21.8 ± 0.1	2.60 ± 0.19	27.2 ± 2.0
5	>>10	84.2 ± 0.2	18.4 ± 1.8
6	>>10	>>10	16.2 ± 1.4
7	22.0 ± 2.5	>>10	18.5 ± 1.0
8	31.4 ± 0.2	35.3 ± 2.5	41.4 ± 6.8
9	0.305 ± 0.009	27.3 ± 0.4	25.7 ± 1.1
Curc	nd[d]	nd[d]	34.4 ± 1.1

[a] Human recombinant AChE and BuChE 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 duplicate; IC_{50} values were determined by following Ellman's method; SEM = standard error of the mean.

[b] % inhibition of 50 μM Aβ(1–42) self-induced aggregation by 10 μM compound. The Aβ(1–42)/inhibitor ratio was equal to 5/1. Values are the mean of two independent experiments, each performed in duplicate.

[c] From Ref. 9.

[d] nd = not determined.

It should be also mentioned that PBTh derivatives may displace ThT from amyloid fibrils.[28] This may translate in an overestimation of the inhibitory activity in the ThT-based assay. Therefore, to ensure that the fluorescence decrease observed for compounds **8** and **9** was exclusively due to the inhibition of fibril formation, control experiments were performed adding compounds **8** and **9** (10 μM) to aggregated Aβ samples after dilution with ThT. Samples in which compounds **8** and **9** were added to aggregated Aβ samples diluted with the ThT solution showed the same fluorescence output as those containing only aggregated Aβ and ThT, providing convincing evidence that no displacement of ThT occurred in the used experimental conditions. Therefore, any observed reduction in fluorescence intensity can be exclusively attributed to the inhibition of Aβ fibril formation by the tested compounds.

The neuroprotective effects of selected compounds were also determined against the $A\beta_{1-42}$ induced toxicity in human neuronal-like SH-SY5Y cells using a colorimetric MTT assay.[19] The 3-methylene-spaced compounds were tested because of their greater compliance with Lipinski's rules (MW and lipophilicity) and also because preliminary results showed that they were generally less cytotoxic than the higher homologues (data not shown). Thus, intrinsic cell toxicity of newly synthesized compounds was first evaluated. Treating SH-SY5Y cells with **2** and **6** (1–50 μM) did not lead to modified neuronal viability, whereas, 24 h treatment of SH-SY5Y cells with **1** and **4** decreased neuronal viability with IC_{50} (concentration of compound resulting in 50% inhibition of neuronal viability) values of 19.4 μM and 14.8 μM, respectively. Notably, curcumin exhibited a similar toxicity profile (IC_{50} = 12.8 μM).[30] We then assessed the protective effects of **1**, **2**, **4**, and **6** against neurotoxicity induced by $A\beta_{1-42}$ oligomers in SH-SY5Y cells. We used a range of concentrations of tested compounds (0.1–1 μM for **1**, **4**, and **6**; 0.1–10 μM for **2**) that did not affect neuronal viability. As shown in Figure 2, compounds **2**, **4**, and **6** at 1 μM partially inhibited the $A\beta_{1-42}$ oligomer-induced neurotoxicity (25–36%), while **1** exerted a stronger inhibition (70%) at the same concentration. However, **2**, at higher concentration (10 μM), produced a neuroprotective effect similar to that of **1** (∼ 80%).

In conclusion, an appropriate decoration of the BQ nucleus of **1** allowed us to identify novel multitargeted hit compounds. Although the cholinesterase activities decreased significantly, the new compounds were endowed with more balanced biological profiles. In principle, novel MTDLs active in the micromolar range, such as **2** and **4**, represent interesting starting points for further development. In fact, where connections exist between two or more targets, as seems to be the case for AChE and Aβ, MTDLs with only moderate activities are expected to produce superior in vivo effects compared to higher-affinity single-targeted compounds. In this context, because most links in cellular networks are weak, low-affinity MTDLs such as those developed herein, might well accomplish a significant modification of the AD neurotoxic cascade.

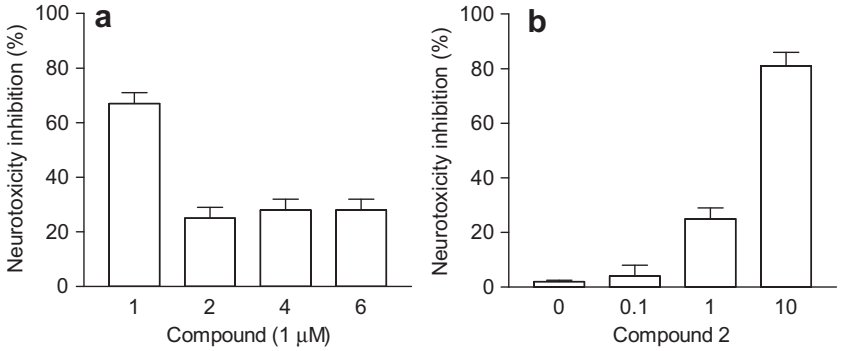


Figure 2. (a) Effects of compounds (1 μM) on neurotoxicity induced by $A\beta_{1-42}$ oligomers in SH-SY5Y cells. (b) Effects of various concentrations of **2** (0.1–10 μM) on neurotoxicity induced by $A\beta_{1-42}$ oligomers in SH-SY5Y cells. The neuronal viability in SH-SY5Y cells was determined by the MTT assay, after 3 h of incubation with $A\beta_{1-42}$ aggregated to oligomers in presence of compounds.[29] The results are expressed as a percentage of inhibition of neurotoxicity induced by $A\beta_{1-42}$ oligomers and shown as mean ± SD of at least two independent experiments.

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24. *Spectra data of selected compounds.* Compound **22**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.70–1.87 (m, 2H + 1H exchangeable with D_2O), 2.79–2.87 (m, 2H), 3.33–3.42 (m, 2H), 4.23 (s, 2H), 5.12(s, 2H), 5.42 (br s, 1H exchangeable with D_2O), 7.17–7.41 (m, 7H), 7.84–7.88 (m, 1H), 7.96–8.00 (m, 1H) ppm. Compound **14**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.55–1.65 (br s, 3H exchangeable with D_2O). 1.69–1.79 (m, 2H), 2.82–2.88 (m, 4H), 4.25 (s, 2H), 7.28–7.45 (m, 2H), 7.86–8.00 (m, 2H) ppm. Compound **6**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.74–1.80 (br s, 2H exchangeable with D_2O), 1.87–1.93 (m, 4H), 2.89 (t, J = 6.4, 4H), 3.30–3.40 (m, 4H), 4.27 (s, 4H), 5.40(s, 2H), 7.08 (br s, 2H exchangeable with D_2O), 7.43–7.52 (m, 4H), 7.89–8.00 (m, 4H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.38, 41.00, 47.12, 51.63, 92.80, 121.82, 122.71, 124.85, 125.93, 151.23, 153.34, 172.93, 178.23 ppm; EI/MS 546.
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