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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 4252–4261
Bioorganic & Medicinal Chemistry

Design, synthesis and pharmacological evaluation of hybrid molecules out of quinazolinimines and lipoic acid lead to highly potent and selective butyrylcholinesterase inhibitors with antioxidant properties

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Received 7 December 2007; revised 19 February 2008; accepted 26 February 2008
Available online 29 February 2008

Abstract—A set of hybrid molecules were synthesized out of lipoic acid, α,ω-diamines of different lengths serving as spacers, and cholinesterase (ChE) inhibiting [2,1-*b*]quinazolinimines. Depending on the length of the alkylene spacer the amide hybrids are inhibitors of acetylcholinesterase (AChE) with inhibitory activities of 0.5–4.6 μM and inhibitors of butyrylcholinesterase (BChE) with activities down to 5.7 nM, therefore greatly exceeding the inhibitory activities of the parent quinazolinimines by factors of up to 1000. Due to increasing activity at BChE with increasing length of the alkylene spacer ~100-fold selectivity toward BChE is reached with a hepta- and an octamethylene spacer. Kinetic measurements reveal competitive and reversible inhibition of both ChEs by the hybrids. Furthermore, cell viability and antioxidant activity (using the ORAC-fluorescein assay) of several hybrids were evaluated, showing cytotoxicity at concentrations from 3.7 to 10.2 μM and antioxidant properties are in the range of 0.4–0.8 Trolox equivalents (lipoic acid = 0.6).

1. Introduction

Different forms of dementia, which involve impairment of cognitive functions, like Alzheimer's disease (AD), are characterized by reduced levels of acetylcholine (ACh) in the cortex and hippocampus.[1] The approved therapeutic options to AD are extremely limited to only four acetylcholinesterase (AChE) inhibitors and the NMDA receptor antagonist memantine. Since AChE activity decreases progressively in certain brain regions during the course of AD to reach 10–15% of the initial value, while the activity of the less specific butyrylcholinesterase (BChE) stays the same or is increased,[2–4] we have focused on the development of BChE selective inhibitors and identified quinazolinimines as a novel class of ChE inhibitors, which can either inhibit both cholinesterases or are highly selective for BChE with low micromolar or higher inhibitory activities (**1**, Fig. 1).[5,6] Design and synthesis of (homo-) bivalent quinazolinimines led to nanomolar inhibitors, the ones with larger sizes of the respective alicycle retained BChE selectivity, whereas heterocycles containing a five-membered alicycle were as potent on AChE as on BChE (**2**, Fig. 1).[7]

Since the antioxidant system of elderly people loses to a certain degree its full activity,[8] and oxidative damage was observed before the formation of AD-specific pathological β-amyloid plaques,[9] antioxidants may be applied as neuroprotective agents in AD. Although in vitro-data for antioxidants like vitamin E are very promising,[10,11] the outcome of clinical trials has been ambiguous, for example, applying vitamin E as supplement.[12,13] Nevertheless, especially for mild cognitive impairment (MCI) as an early stage of AD, the positive role of antioxidants is unambiguous.[14,15]

Keywords: Cholinesterase inhibition; Tricyclic[2,1-*b*]quinazolinimines; Butyrylcholinesterase (BChE) selectivity; Lipoic acid; Hybrids; Antioxidants.
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doi:10.1016/j.bmc.2008.02.083

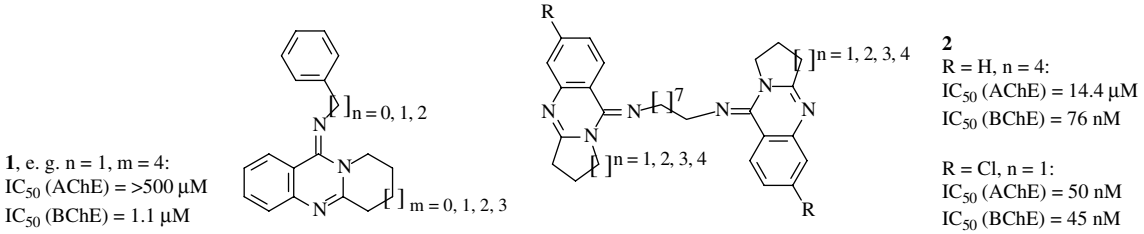


Figure 1. BChE selective imine-N-substituted quinazolinimines (**1**) containing an additional benzene ring at the imine-*N* and highly potent homobivalent quinazolinimines (**2**).

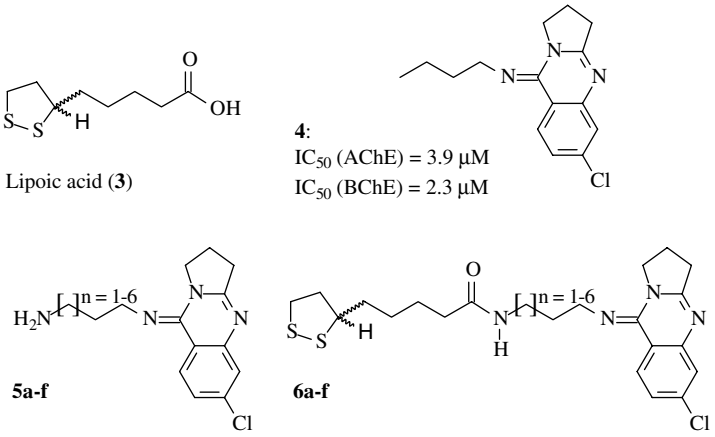


Figure 2. The antioxidant and cytoprotective lipoic acid (**3**), an unselective monovalent quinazolinimine (**4**), which can be regarded as a 'monomer' or 'univalent' form of bivalent quinazolinimines (**2**), and target compounds **5a**–**f** and **6a**–**f**.

Lipoic acid (**3**, Fig. 2) represents such a versatile antioxidant,[16,17] especially because it was shown that it is able to protect neurons against amyloid-induced cell death through the Akt signaling pathway.[18] There are also some (although limited) data for its positive effects on disease progression in AD patients.[19]

Recently, the potent AChE inhibitor tacrine was connected with lipoic acid[20] and melatonin,[21] respectively, to form hybrid molecules combining AChE inhibiting with antioxidant and radical scavenging properties. From the medicinal chemical point of view such hybrids are of special interest, because these compounds do not only preserve the pharmacological properties of their components, but also show improved properties: on the one hand some melatonin–tacrine hybrids exhibited nanomolar AChE-inhibiting properties with higher AChE selectivity maintaining their antioxidant properties.[21] On the other hand also some tacrine–connected to lipoic acid hybrids increased their activity at AChE and—of special importance—also showed β-amyloid aggregation-inhibiting properties,[20] indicating interaction with the peripheral anionic site (PAS), which accelerates amyloid fibril formation.[22,23] Both kinds of hybrids improved their activity toward BChE only to a very low or no extent.[20,21]

In order to combine BChE-inhibiting activities with antioxidant ones, we wanted to synthesize hybrids out of quinazolinimines and lipoic acid (**6a**–**f**, Fig. 2). Because of the fact that homobivalent quinazolinimines showed strong activity increases,[7] also the primary amines (**5a**–**f**) yielded as synthesis intermediates were examined, in which a simple amino-group replaces one basic quinazolinimine moiety (Fig. 2).

N-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]-1-butanamine has previously been identified as an inhibitor of both AChE and BchE with inhibitory activities of 3.9 μM and 2.3 μM, respectively.[7] It represents a potent univalent compound in the series of quinazolinimines synthesized (compounds bearing an additional phenyl-group in the alkyl moiety (**1**) are more potent, but are less suitable for coupling and functionalization).[6] This quinazolinimine moiety was selected for coupling in order to evaluate the influence of functionalization and coupling with lipoic acid on ChE inhibition and BChE selectivity in particular. To find a reasonable concentration range for further cellular in vitro testings some representatives of the hybrids were tested for cytotoxicity in a murine microglial cell line. The antioxidant activity of the hybrids was evaluated in the ORAC-fluorescein assay.[24]

2. Results and discussion

2.1. Synthesis

N-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]alkane-α,ω-diamines could be synthesized in a straightforward four-step synthesis (Scheme 1): 2-pyrro-

lidinone (**7**) was activated using *Meerwein* salt (triethyloxonium tetrafluoroborate). The resulting 5-ethoxy-3,4-dihydro-2*H*-pyrrole (**8**, the respective iminium ether) reacted with 4-chloro-anthranilic acid to 6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-one (**9**).[7] This quinazolinone was transformed into the thione (**10**) using Lawesson's reagent.[5,6] Thione (**10**) reacted in the presence of mercury(II) bromide in moderate yields with excess α,ω-diamines to the primary amines (**5a–f**, *N*-[6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]alkane-α,ω-diamines) suitable for further coupling with lipoic acid.

In order to obtain hybrid molecules, the latter compounds (**5a–f**) were connected to (±)-lipoic acid (**3**) after its activation with *N*,*N*′-carbonyldiimidazole (CDI) to yield the target amides (**6a–f**).

To evaluate the effect of increasing alicyclic ring size on BChE inhibition,[6,7] *N*-[6,7,8,9,10,11-hexahydro-13*H*-azocino[2,1-*b*]quinazolin-13-ylidene]-1,8-octanediamine (**12**) -obtained from the eight-membered-ring thione[6] (**11**)- and its respective hybrid with lipoic acid (**13**) were also synthesized (Scheme 2).

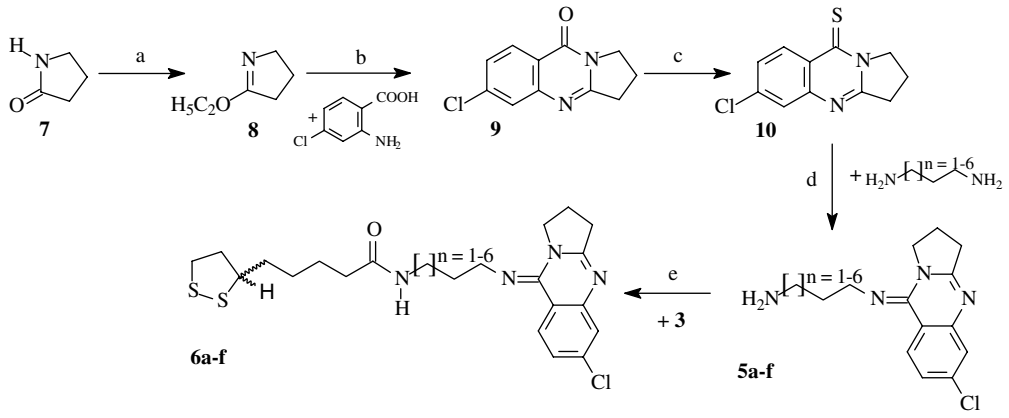
2.2. Pharmacology

Inhibitory activities of quinazolinimines bearing a primary amine moiety (**5a–f**, **12**) and of the lipoic acid hybrids (**6a–f**, **13**) were measured at AChE (E.C. 3.1.1.7, type VI-S, from Electric Eel) and BChE (E.C. 3.1.1.8, from equine serum) using the Ellman assay.[5,6,25] Although there are of course a number of species-dependent differences between these enzymes and the human ones, the enzymes used show sufficient homology in the amino acid sequence to the human enzymes (e.g., 88% for equine serum BChE).[26]

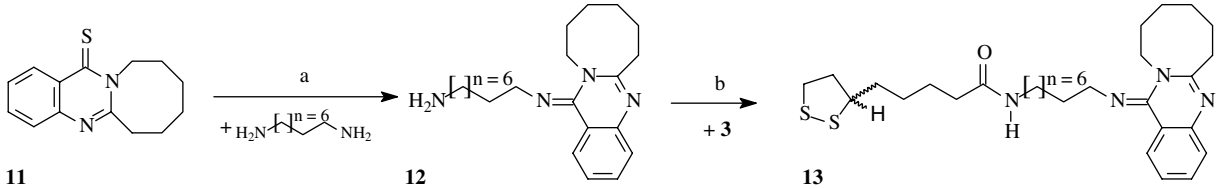
The inhibition profiles obtained reveal some very interesting results (Table 1): concerning the amines, only the primary propylene amine (**5e**) shows lower activities at AChE than the *n*-butyl parent quinazolinimine (**4**)—the heptamethylene amine (**5e**) shows the same activity as compound **4**. The butylene (**5b**), the pentylene (**5c**) and the octylene (**5f**) compounds show activities of 0.48 μM, 0.17 μM, and 0.13 μM, respectively. The latter two compounds exhibit ~20-fold activity increases compared to compound **4** at AChE.

At BChE all activities measured are higher than 1 μM apart from the ones of the propylene compound (**5a**). The octamethylene compound (**5f**) shows the highest activity of 12 nM. No relevant selectivity toward one cholinesterase can be observed, the highest one (11-fold) with the octamethylene compound (**5f**). No prominent trend can be observed for the activity development apart from the activity increase for **5f**, which is the most active compound in this series surpassing the parent compound by a factor of ~200 (Table 1).

Concerning the amide hybrids, activities from 0.47 to 4.6 μM were measured at AChE with activities comparable to the amines (Table 1). The activity development at BChE is very remarkable, because there is a

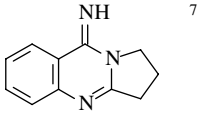
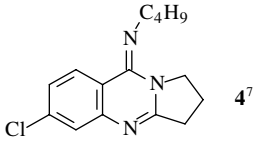


Scheme 1. Synthesis of *N*-[6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]alkane-α,ω-diamines **5a–f** and lipoic acid-quinazolinimine hybrids **6a–f**. Reagents and conditions: (a) 1—$[(C_2H_5)_3O]BF_4$, chloroform, rt, 15 h; 2—cold NaOH, CH_2Cl_2 extraction; (b) acetone, 10 °C, 2 h → 60 °C, 4 h; (c) Lawesson's reagent, toluene, reflux, 12 h; (d) 3 equiv NEt_3, 2.5 equiv $HgBr_2$, toluene, reflux, 14 h; (e) 1 equiv CDI-activated (±)-lipoic acid, CH_2Cl_2, rt, 4 h.



Scheme 2. Synthesis of *N*-[6,7,8,9,10,11-hexahydro-13*H*-azocino[2,1-*b*]quinazolin-13-ylidene]-1,8-octanediamine (**12**) and its (±)-lipoic acid hybrid **13**. Reagents and conditions: (a) 3 equiv NEt_3, 2.5 equiv $HgBr_2$, toluene, reflux, 14 h; (b) 1 equiv CDI-activated (±)-lipoic acid, CH_2Cl_2, rt, 4 h.

Table 1. AChE and BChE inhibition results[a]

Compound	AChE[b] IC_{50}, μM (pIC_{50} ± SEM)	BChE[c] IC_{50}, μM (pIC_{50} ± SEM)	Selectivity IC_{50}(AChE)/IC_{50}(BChE)
Galanthamine	0.64 (6.197 ± 0.052)	8.40 (5.076 ± 0.033)	0.08
 **7**	13.16 (4.881 ± 0.061)	7.16 (6.145 ± 0.031)	1.8
 **4**[7]	3.93 (5.406 ± 0.085)	2.29 (5.639 ± 0.083)	1.7
5a (n = 1)	19.00 (4.721 ± 0.139)	10.23 (4.990 ± 0.229)	1.9
5b (n = 2)	0.48 (6.315 ± 0.089)	0.72 (6.142 ± 0.198)	0.7
5c (n = 3)	0.17 (6.780 ± 0.063)	0.19 (6.731 ± 0.113)	0.9
5d (n = 4)	1.49 (5.828 ± 0.104)	0.38 (6.416 ± 0.184)	3.9
5e (n = 5)	4.06 (5.391 ± 0.216)	0.71 (6.148 ± 0.091)	5.7
5f (n = 6)	0.13 (6.893 ± 0.103)	0.012 (7.926 ± 0.052)	10.7
12	3.4 (5.474 ± 0.065)	0.046 (7.336 ± 0.079)	73.9
6a (n = 1)	2.84 (5.547 ± 0.159)	2.58 (5.589 ± 0.149)	1.1
6b (n = 2)	4.58 (5.339 ± 0.125)	0.17 (6.773 ± 0.097)	27.1
6c (n = 3)	4.38 (5.358 ± 0.065)	0.053 (7.275 ± 0.087)	83.0
6d (n = 4)	0.74 (6.129 ± 0.054)	0.026 (7.589 ± 0.168)	28.8
6e (n = 5)	1.94 (5.712 ± 0.222)	0.014 (7.840 ± 0.052)	133.8
6f (n = 6)	0.468 (6.330 ± 0.114)	0.0057 (8.247 ± 0.040)	82
13	<10% enzyme inhibition at 10^{-5} M	0.023 (7.630 ± 0.059)	>5000

See Figure 2 and Schemes 1 and 2 for structures.
[a] Values are means of at least three independent determinations.
[b] E.C. 3.1.1.7, type VI-S, from Electric Eel.
[c] E.C. 3.1.1.8, from equine serum.

continuous increase in activity from the propylene compound with an activity of IC_{50}(BChE) = 2.6 μM (**6a**) and a very high activity of IC_{50}(BChE) = 5.7 nM for the octamethylene compound (**6f**) (Table 1). Therefore a concomitant increase in selectivity toward BChE can be observed reaching 80- and 130-fold selectivities for the octa- and heptamethylene compounds, respectively (Table 1). Compared to the 'parent compound' (**4**) the octamethylene compound (**6f**) shows a ~10-fold activity increase toward AChE and ~1000-fold increase toward BChE.

Compared to the tacrine–lipoic acid hybrids prepared in Melchiorre's group,[20] a number of relevant differences can be observed: largely independent of spacer length these compounds were one to two digit nanomolar inhibitors with no or only moderate selectivity toward BChE. The most potent compound based on 6-chlorotacrine was connected to lipoic acid by a propylene spacer and it shows 40-fold AChE selectivity (lipocrine). It is able to inhibit β-amyloid fibril formation, which was proved by kinetic measurements showing mixed type of inhibition by interacting with both the active site and the PAS and also directly by a thioflavin T-based fluorimetric assay.[20]

In order to investigate the binding mode of the hybrid molecules synthesized and explain the remarkable differences compared to tacrine hybrids, the most potent octamethylene compound (**6f**) was selected for kinetic measurements. The inhibition mechanisms toward both cholinesterases were analyzed by recording substrate–velocity curves in the presence of varying concentrations of the inhibitor. The inhibitor concentrations selected were slightly higher and lower than the respective IC_{50} value (Fig. 3).

Figure 3 shows the resulting substrate–velocity curves for BChE and AChE, respectively, and the respective Lineweaver–Burk plots, that is, reciprocal rates versus reciprocal substrate concentrations for the different inhibitor concentrations. K_m values (i.e., the negative reciprocal of the X intercept) differ, but in contrast the V_{max} values (i.e., the reciprocal of the Y intercept) do not significantly change with different inhibitor concentrations at both cholinesterases.

These kinetic results indicate reversible and competitive inhibition at the active site of both AChE and BChE. In contrast to lipocrine no interaction with the PAS of AChE can be assumed. Also at BChE there seems to be a reversible and competitive inhibition with the substrate molecule, which means that the high affinity of the hybrid toward BChE cannot be explained by its interaction with a second binding site, which would lead to an altered kinetic profile. To validate this assumption, molecular modeling (docking studies at BChE) applying this molecule would be advantageous.

Since very high activities at BChE could be achieved with the hybrid molecules and a C8-spacer between the imine-nitrogen and the amide-nitrogen, this spacer

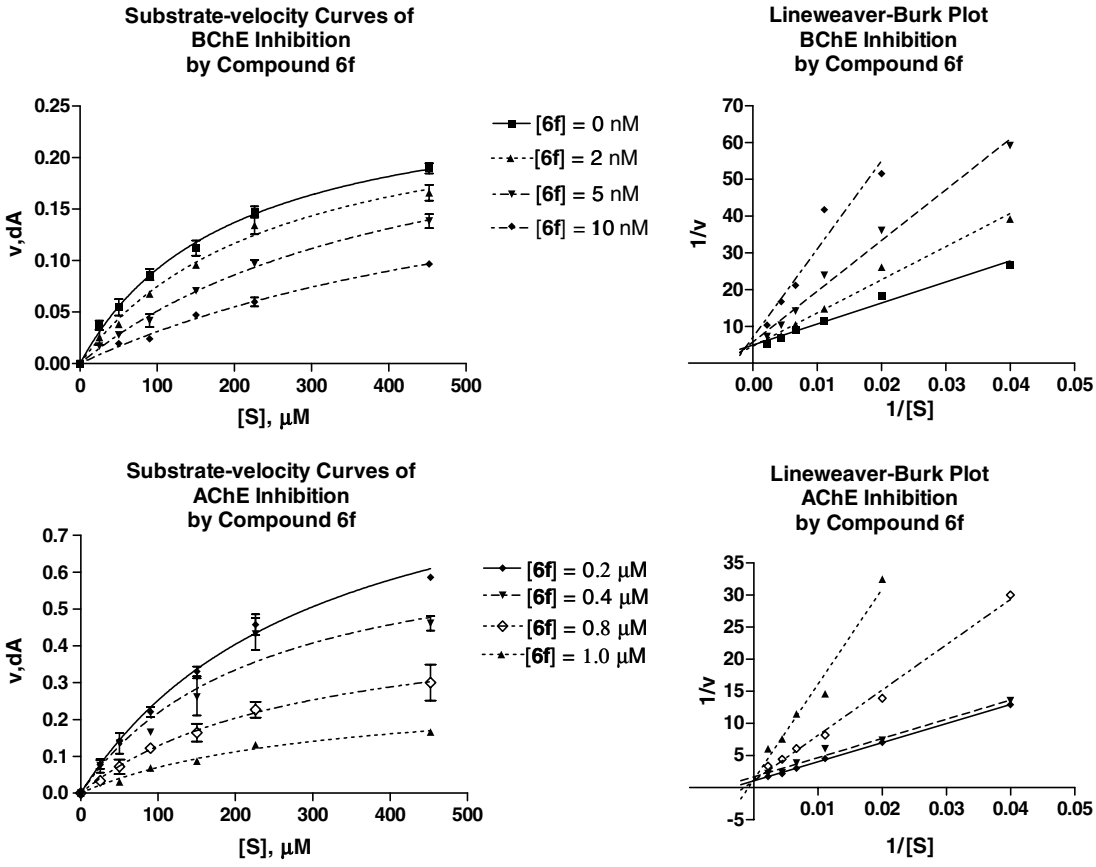


Figure 3. Substrate–velocity curves and corresponding Lineweaver–Burk plots of BChE (upper graphs) and AChE activity (lower graphs) with different substrate concentrations (25–450 µM) in the presence of varying concentrations of hybrid **6f**.

length was also applied to a quinazolinimine with an eight-membered alicyclic ring-system, which previously proved successful in achieving BChE-selectivity using homobivalent quinazolinimines and quinazolinimines bearing additional benzene rings at the imine-*N*. Indeed, these compounds proved to be highly BChE-selective: the amine compound **12** is an inhibitor of BChE with an IC_{50}-value of 46 nM, therefore showing 74-fold selectivity. The respective hybrid is also very potent (IC_{50} = 23 nM), which shows >5000-fold selectivity. Even at a concentration of 10 µM, no significant inhibition of AChE activity could be observed.

Selected compounds were measured on their influence to their cytotoxic effects on murine microglial cell, in which all compounds tested showed an IC_{50} value between 3.7 and 10.2 µM, proving their low cytotoxic potential (Table 2). Microglia cells are of high importance in the central nervous system, especially with regard to neuron homeostasis, therefore this cell line was used in the cytotoxicity assay. Interestingly, the compound with the lowest dose for toxic effects was the amine compound **12**, which is not a hybrid. The other amines tested (**5d**, **5f**) do not show a higher toxicity, but in each case the IC_{50} values are lower than for the hybrids. This might be regarded as a hint for reduced toxicity due to introduction of the lipoic acid molecule. There is definitely no evidence for a higher toxicity (Table 2).

In order to prove the antioxidant capacities of the hybrid molecules, the ability of selected compounds to reduce the amount of peroxyl radicals (ORAC assay) was determined, in which lipoic acid itself served as a standard (Table 3). The compounds' ability to scavenge

Table 2. Cell viability measured in a photometric MTT assay

Compound	IC_{50} (µM)	SD
5d (amine, *n* = 4)	6.01	±1.35
5f (amine, *n* = 6)	6.27	±0.29
6a (hybrid, *n* = 1)	7.51	±0.10
6f (hybrid, *n* = 6)	10.17	±0.15
12	3.74	±1.05
13	6.54	±0.15

See Figure 2 and Schemes 1 and 2 for structures.

Table 3. Oxygen radical absorbance capacity (ORAC, Trolox equiv, 10 µM) by selected quinazolinime-hybrids and their primary amines

Compound	Trolox equivalents	SD
5d (amine, *n* = 4)	0.2	±0.03
5f (amine, *n* = 6)	0.1	±0.01
6a (hybrid, *n* = 1)	0.8	±0.10
6f (hybrid, *n* = 6)	0.5	±0.10
12	0.1	±0.01
13	0.4	±0.10
Lipoic acid	0.6	±0.01

See Figure 2 and Schemes 1 and 2 for structures.

radicals is expressed as Trolox equivalent, that is, their relative ability (at a concentration of 10 μM) compared to the highly potent compound Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). This concentration seems to be relatively high when compared to the cell viability data, but it has to be taken into account that it is much lower (up to 10-fold) in comparison to previously published work concerning the in vitro antioxidant capacities of lipoic acid derivatives,[20,27] and they do not necessarily reflect the concentrations necessary in vivo to act beneficially[16,19] (for a detailed review about antioxidant, chelating and other pharmacological properties of lipoic acid and the correlation of in vitro and in vivo data see Ref. 17). Nevertheless, some relative correlations between the properties of the different compounds tested can be given (Table 3): all compounds tested (including the amines **5f** and **12**) are weak or moderate antioxidants in comparison to the strong activity of natural polyphenols.[24] In the ORAC test the antioxidant activity of lipoic acid is in low concentrations (up to 10 μM) significant, but moderate (0.6 Trolox equivalents). The antioxidant activity of the hybrid molecules is in the same range in comparison to lipoic acid and thus antioxidant activity is clearly related to this substructure, whereas the amines are not active. According to literature,[27] the activity of lipoic acid further increases in higher concentrations (>10 μM, data not shown), but due to the high inhibitory activity of the hybrid compounds at AChE and BChE a more potent antioxidant seems to be more suitable to be connected to the active enzyme-inhibiting structures synthesized/identified.

3. Conclusion

In conclusion, it was possible to combine two distinct pharmacologically active moieties, that is, antioxidant lipoic acid and a micromolar unselective ChE-inhibiting quinazolinimine, to hybrid molecules that retain their respective pharmacological behavior. Furthermore, the activity toward AChE increased and using optimized alkylene spacer lengths the activity toward BChE could be dramatically increased by a factor of ~1000 yielding ~100-fold BChE selectivity. Utilizing the information about obtaining selectivity by modifying the heterocycle, it was possible to obtain an inhibitor with high nanomolar activity at BChE without any AChE-inhibiting properties at all. Kinetic measurements revealed competitive and reversible binding at the active sites of both ChEs. The concentration range to observe antioxidant properties in vitro (also for the parent compound lipoic acid as well) is much higher than for the ones necessary for enzyme inhibition, a problem that often encounters with the design of hybrid molecules.[28] Here the complex antioxidant behavior of lipoic acid in vitro and in vivo has to be taken into account. Nevertheless, the compounds synthesized prove that just by the appropriate design of hybrid molecules it is possible to greatly improve pharmacological properties, which means that a hybrid molecule can be designed to be much superior to its components. It seems more reasonable though to apply other, more potent antioxidants to yield hybrids that are able to exhibit their pharmacological activity in the same concentration range.

4. Experimental protocols

4.1. General

Melting points are uncorrected and were measured in open capillary tubes, using a Gallenkamp melting point apparatus. ^{1}H NMR spectral data were obtained from a Bruker Advance 250 spectrometer (250 MHz). Elemental analyses were performed on a Hereaus Vario EL apparatus. TLC was performed on silica gel F254 plates (Merck). For detection, iodine vapour or UV light (254 nm), respectively, was used. EI-MS-Spectra were recorded using LCQ Advantage by ThermoElectron. Silica gel column chromatography utilized silica gel 60 63–200 μm (Baker). UV-measurements were performed on a Jasco V-570 UV/VIS/NIR spectrophotometer.

4.2. Chemistry

Preparation and spectroscopic data of 6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazoline-9(1*H*)-thione (**10**) have been described recently.[7]

4.2.1. General procedure for the preparation of *N*-[6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]-alkane-α,ω-diamines (5). A mixture of 472 mg (2.0 mmol) of 6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazoline-9(1*H*)-thione (**10**), 1.21 g (12 mmol) of triethylamine, and 12 mmol of the respective α,ω-diamine in 150 mL of toluene was heated to ~100 °C. To the warmed solution, 1.8 g (5 mmol) of well triturated mercury(II) bromide was slowly added in small portions. The suspension was heated at reflux for 14 h, in which time a black solid was formed. After cooling, the toluene solution was decanted and the solvent removed in vacuo. The crude product was purified by column chromatography using CH_2Cl_2/MeOH/aqueous NH_3 (90:9:1) as eluent.

4.2.1.1. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]propane-1,3-diamine (5a). Beige powder. 161 mg (29% yield). Mp 108 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.77 (2H, br s, NH_2), 1.83 (2H, qui, J = 7.5 Hz, CH_2CH$_2$N), 1.94 (2H, qui, J = 5.0 Hz, CH_2CH$_2$NH$_2$), 2.62 (2H, t, J = 7.5 Hz, C(3a)CH_2), 2.75 (2H, t, J = 7.5 Hz, CH_2N), 3.54 (2H, t, J = 5.0 Hz, CH_2NH$_2$), 3.83 (2H, t, J = 5.0 Hz, CH_2N=), 7.12 (1H, dd, J = 9, 2.5 Hz, C(7)*H*), 7.29 (1H, d, J = 2.5 Hz, C(8)*H*), 7.94 (1H, d, J = 9 Hz, C(5)*H*) ppm. IR (KBr): 2933, 2851, 1629, 1587, 1464, 1310, 1169, 884 cm^{-1}. EI-MS *m*/*z* 278, 246, 233, 218, 206, 178, 163, 136, 30. Anal. Calcd for $C_{14}H_{17}ClN_4$: C, 60.8; H, 6.2; N, 20.2. Found: C, 60.4; H, 6.1; N, 20.4.

4.2.1.2. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]butane-1,4-diamine (5b). Beige powder. 244 mg (42% yield). Mp 240 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.49 (2H, br s, NH_2), 1.58–1.70 (4H, m, CH$_2$CH_2CH_2CH$_2$), 2.12 (2H, qui, J = 6.75 Hz,

CH_2CH$_2$N), 2.75 (2H, t, J = 6.75 Hz, C(3a)CH_2), 2.94 (2H, t, J = 6.75 Hz, CH_2N), 3.76 (2H, t, J = 6.75 Hz, CH_2NH$_2$), 3.94 (2H, t, J = 6.75 Hz, CH_2N=), 7.11 (1H, dd, J = 8, 2.5 Hz, C(7)*H*), 7.42 (1H, d, J = 2.5 Hz, C(8)*H*), 7.99 (1H, d, J = 8 Hz, C(5)*H*) ppm. IR (KBr): 2917, 2851, 1635, 1594, 1467, 1290, 913 cm^{-1}. EI-MS *m*/*z* 292, 273, 260, 246, 232, 218, 204, 197, 83, 30. Anal. Calcd for $C_{15}H_{19}ClN_4$: C, 62.0; H, 6.6; N, 19.3. Found: C, 61.6; H, 6.4; N, 19.6.

4.2.1.3. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]pentane-1,5-diamine (5c). Beige powder. 103 mg (17% yield). Mp 86 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.31 (2H, br s, NH_2), 1.42–1.60 (4H, m, CH_2CH_2CH$_2$NH$_2$), 1.62–1.75 (2H, m, CH_2CH$_2$N=), 2.07 (2H, qui, J = 6.5 Hz, CH_2CH$_2$N), 2.66 (2H, t, J = 6.5 Hz, C(3a)CH_2), 2.92 (2H, t, J = 6.5 Hz, CH_2N), 3.69 (2H, t, J = 7 Hz, CH_2NH$_2$), 3.88 (2H, t, J = 7 Hz, CH_2N=), 7.07 (1H, dd, J = 9, 2.5 Hz, C(7)*H*), 7.37 (1H, d, J = 2.5 Hz, C(8)*H*), 7.93 (1H, d, J = 9 Hz, C(5)*H*) ppm. IR (KBr): 2933, 2848, 1635, 1591, 1401, 1280, 907 cm^{-1}. EI-MS *m*/*z* 306, 288, 260, 246, 232, 220, 203, 197, 30. Anal. Calcd for $C_{16}H_{21}ClN_4 \times 1/2H_2O$: C, 61.2; H, 7.1; N, 17.9. Found: C, 61.6; H, 7.5; N, 17.6.

4.2.1.4. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]hexane-1,6-diamine (5d). Beige powder. 160 mg (25% yield). Mp 167 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.35–1.55 (8H, m, (CH_2)$_3$CH$_2$NH_2), 2.70 (2H, m, CH_2CH$_2$N=), 2.12 (2H, qui, J = 8 Hz, CH_2CH$_2$N), 2.69 (2H, t, J = 8 Hz, C(3a)CH_2), 2.98 (2H, t, J = 8 Hz, CH_2N), 3.69 (2H, t, J = 6.5 Hz, CH_2NH$_2$), 3.94 (2H, t, J = 6.5 Hz, CH_2N=), 7.13 (1H, dd, J = 9, 2.5 Hz, C(7)*H*), 7.43 (1H, d, J = 2.5 Hz, C(8)*H*), 8.14 (1H, d, J = 9 Hz, C(5)*H*) ppm. IR (KBr): 2927, 2849, 1638, 1591, 1470, 1423, 910, 660 cm^{-1}. EI-MS *m*/*z* 320, 302, 288, 232, 220, 197, 30. Anal. Calcd for $C_{17}H_{23}ClN_4$: C, 64.0; H, 7.3; N, 17.6. Found: C, 64.2; H, 7.5; N, 17.6.

4.2.1.5. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]heptane-1,7-diamine (5e). Beige powder. 313 mg (30% yield). Mp 86 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.25–1.50 (8H, m, (CH_2)$_4$CH$_2$NH_2), 1.58–1.66 (2H, m, CH_2CH$_2$N=), 2.07 (2H, qui, J = 7.5 Hz, CH_2CH$_2$N), 2.62 (2H, t, J = 8 Hz, C(3a)CH_2), 2.93 (2H, t, J = 9 Hz, CH_2N), 3.68 (2H, t, J = 7.5 Hz, CH_2NH$_2$), 3.89 (2H, t, J = 7.5 Hz, CH_2N=), 7.08 (1H, dd, J = 10, 2.5 Hz, C(7)*H*), 7.38 (1H, d, J = 2.5 Hz, C(8)*H*), 7.93 (1H, d, J = 10 Hz, C(5)*H*) ppm. IR (KBr): 2930, 2854, 1638, 1587, 1467, 1423, 1277, 913, 663 cm^{-1}. EI-MS *m*/*z* 334, 302, 288, 258, 232, 219, 197, 30. Anal. Calcd for $C_{18}H_{25}ClN_4 \times 1/2H_2O$: C, 63.2; H, 7.7; N, 16.4. Found: C, 62.9; H, 7.5; N, 16.6.

4.2.1.6. *N*-[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1H)-ylidene]octane-1,8-diamine (5f). Beige powder. 298 mg (43% yield). Mp 176 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.30–1.48 (10H, m, (CH_2)$_5$CH$_2$NH_2), 1.63–1.72 (2H, m, CH_2CH$_2$N=), 2.12 (2H, qui, J = 7 Hz, CH_2CH$_2$N), 2.67 (2H, t, J = 7 Hz, C(3a)CH_2), 2.98 (2H, t, J = 7 Hz, CH_2N), 3.73 (2H, t, J = 7 Hz, CH_2NH$_2$), 3.95 (2H, t, J = 7 Hz, CH_2N=), 7.14 (1H, dd, J = 10, 2.5 Hz, C(7)*H*), 7.43 (1H, d, J = 2.5 Hz, C(8)*H*), 8.15 (1H, d, J = 10 Hz, C(5)*H*) ppm. IR (KBr): 2923, 2851, 1635, 1591, 1404, 1280, 913, 660 cm^{-1}. EI-MS *m*/*z* 348, 316, 311, 288, 246, 232, 220, 197, 30. Anal. Calcd for $C_{19}H_{27}ClN_4$: C, 65.8; H, 7.9; N, 16.2. Found: C, 65.9; H, 7.5; N, 16.6.

4.2.1.7. *N*-(6,7,8,9,10,11-Hexahydro-13*H*-azocino[2,1-*b*]quinazolin-13-ylidene)octane-1,8-diamine (12). Pale white powder. 207 mg (30% yield). Mp 113 °C. ^{1}H NMR (250 Hz, $CDCl_3$) δ 1.30–2.01 (22H, m, alicycle- and alkylene-CH_2, NH_2), 2.67 (2H, t, J = 6 Hz, C(3a)CH_2), 2.91 (2H, t, J = 6 Hz, CH_2N), 3.80 (2H, t, J = 6 Hz, CH_2NH$_2$), 4.30 (2H, br s, CH_2N=), 7.12–7.19 (1H, m, C(2)*H*), 7.43 (2H, m, C(1, 3)*H*), 8.15 (1H, d, J = 8.5 Hz, C(4)*H*) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 24.40, 26.64, 26.85, 27.39, 28.45, 29.48, 29.55, 30.64, 33.49, 33.88, 36.14, 42.27, 43.87 (*C*H$_2$N), 51.13 (imine-N-*C*H$_2$), 119.73 (C13a), 123.41 (C4), 126.40 (C2), 127.92 (C1), 131.28 (C3), 145.21 (C4a), 147.46 (*C*=N), 160.36 (C5a) ppm. IR (KBr): 2920, 2848, 1625, 1575, 1176, 1141, 764, 669 cm^{-1}. EI-MS *m*/*z* 355, 274, 243, 229, 198, 172, 145, 115. Anal. Calcd for $C_{22}H_{34}N_4$: C, 74.5; H, 9.7; N, 15.8. Found: C, 74.7; H, 9.8; N, 15.5.

4.2.2. General procedure for the preparation of *N*-{[6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]-amino}alkyl-5-(1,2-dithiolan-3-yl)pentanamides (6). To a solution of 103 mg of (±)-lipoic acid (0.5 mmol) in 20 mL of dichloromethane was added 304 mg (0.6 mmol) of *N*,*N*′-carbonyldiimidazole (CDI). The solution was moderately stirred for 3 h, in which time CO_2 evolution could be observed. To this solution of activated lipoic acid a solution of 0.5 mmol of the respective *N*-[6-chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]alkane-α,ω-diamine in 5 mL of dichloromethane was added drop wise. The mixture was allowed to stand for 4 h. Afterward the solution was washed four times with water, the organic phase was dried over sodium sulfate and the solvent removed under reduced pressure.The crude product was purified by column chromatography using EtOAc/MeOH 1/1 as eluent.

4.2.2.1. *N*-(8-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}propyl)-5-(1,2-dithiolan-3-yl)pentanamide (6a). Pale yellow crystals. 88 mg (38% yield). Mp 158 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.30–1.65 (6H, m), 1.82 (1H, sex, J = 6 Hz), 1.88–2.02 (4H, m), 2.08 (2H, t, J = 7 Hz, CH_2C), 2.36 (1H, sex, J = 6.5 Hz), 2.62 (2H, t, J = 7 Hz, CH_2N), 3.02–3.10 (2H, m), 3.32 (2H, q, J = 6.3 Hz), 3.46 (1H, qui, J = 6.5 Hz, SC*H*), 3.54 (2H, t, J = 5.5 Hz, CH_2NH), 3.8 (2H, t, J = 5.5 Hz, CH_2N=), 6.3 (1H, br s, amide-N*H*), 7.14 (1H, dd, J = 10, 2.5 Hz, C(7)*H*), 7.27 (1H, d, J = 2.5 Hz, C(8)*H*), 7.96 (1H, d, J = 10 Hz, C(5)*H*) ppm. IR (KBr): 3307, 2917, 2854, 1641, 1581, 1546, 1309, 1074 cm^{-1}. EI-MS *m*/*z* 464, 431, 405, 246, 232. Anal. Calcd for $C_{22}H_{29}ClN_4OS_2$: C, 56.8; H, 6.3; N, 12.1. Found: C, 56.6; H, 6.2; N, 12.2.

4.2.2.2. *N*-(7-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}butyl)-5-(1,2-dithiolan-3-yl)-pentanamide (6b). Pale yellow oil. 77 mg (32% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.42–1.73 (11H, m), 1.90 (1H, sex, J = 6 Hz), 2.15–2.23 (4H, m), 2.43 (1H, sex, J = 6.5 Hz), 3.00 (2H, t, J = 8 Hz, CH_2N), 3.11–3.16 (1H, m), 3.33 (2H, q, J = 6 Hz), 3.66 (1H, qui, J = 5 Hz, SCH), 3.78 (2H, t, J = 6 Hz, CH_2NH), 3.95 (2H, t, J = 7 Hz, CH_2N=), 5.71 (1H, br s, amide-NH), 7.17 (1H, dd, J = 9, 2.5 Hz, C(7)H), 7.45 (1H, d, J = 2.5 Hz, C(8)H), 7.98 (1H, d, J = 9 Hz, C(5)H) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 18.63 (alicycle-$CH_2CH_2CH_2$), 25.45, 27.48, 28.90, 30.36, 32.84, 34.60, 36.60, 38.44, 39.41, 40.24, 48.38 (CH_2N), 50.09 (imine-N-CH_2), 56.44 (CHS), 118.16 (C8a), 124.10 (C5), 126.41 (C7), 129.12 (C8), 137.49 (C–Cl), 144.78 (C=N), 150.29 (C4a), 161.31 (C3a), 172.59 (C=O) ppm. IR (KBr): 3291, 2933, 2854, 1635, 1581, 1584, 1423, 1283, 910, 663 cm^{-1}. EI-MS *m*/*z* 479, 445, 362, 280, 234. Anal. Calcd for $C_{23}H_{31}ClN_4OS_2$: C, 57.7; H, 6.5; N, 11.7. Found: C, 57.9; H, 6.2; N, 11.8.

4.2.2.3. *N*-(6-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}pentyl)-5-(1,2-dithiolan-3-yl)-pentanamide (6c). Pale yellow oil. 62 mg (25% yield). ^{1}H NMR (250 MHz, MeOD) δ 1.40–1.81 (12H, m), 1.85 (1H, sex, J = 6 Hz), 2.14–2.25 (4H, m), 2.41 (1H, sex, J = 6.5 Hz), 2.95–3.07 (4H, m), 3.19 (2H, t, J = 6 Hz), 3.52 (1H, qui, J = 5 Hz, SCH), 3.77 (2H, t, J = 6 Hz, CH_2NH), 3.95 (2H, t, J = 7 Hz, CH_2N=), 7.22 (1H, dd, J = 9, 2 Hz, C(7)H), 7.33 (1H, d, J = 2 Hz, C(8)H), 8.08 (1H, d, J = 9 Hz, C(5)H) ppm. IR (KBr): 3291, 2927, 2858, 1638, 1591, 1423, 1287, 910, 660 cm^{-1}. EI-MS *m*/*z* 492, 459, 433, 288, 273, 246, 232, 218, 197, 41. Anal. Calcd for $C_{24}H_{33}ClN_4OS_2$: C, 58.5; H, 6.8; N, 11.4. Found: C, 58.9; H, 6.9; N, 11.0.

4.2.2.4. *N*-(5-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}hexyl)-5-(1,2-dithiolan-3-yl)-pentanamide (6d). Pale yellow crystals. 95 mg (38% yield). Mp 129 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.42–1.80 (16H, m), 1.91 (1H, sex, J = 6 Hz), 2.13–2.18 (3H, m), 2.42 (1H, sex, J = 6.5 Hz), 2.99 (2H, t, J = 7.5 Hz, CH_2N), 3.11–3.26 (3H, m), 3.52 (1H, qui, J = 6.5 Hz, SCH), 3.54 (2H, t, J = 6.5 Hz, CH_2NH), 3.95 (2H, t, J = 7 Hz, CH_2N=), 5.52 (1H, br s, amide-NH), 7.14 (1H, dd, J = 9, 2.5 Hz, C(7)H), 7.43 (1H, d, J = 2.5 Hz, C(8)H), 7.98 (1H, d, J = 9 Hz, C(5)H) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 18.61 (alicycle-$CH_2CH_2CH_2$), 25.42, 26.76, 26.91, 28.86, 29.71, 32.86, 32.99, 34.58, 36.52, 38.43, 39.45, 40.21, 48.33 (CH_2N), 50.44 (imine-N-CH_2), 58.19 (CHS), 120.00 (C8a), 123.97 (C5), 126.33 (C7), 129.14 (C8), 139.11 (C–Cl), 146.26 (C=N), 152.08 (C4a), 163.13 (C3a), 174.34 (C=O) ppm. IR (KBr): 3307, 2927, 2851, 1635, 1594, 1467, 1283, 913, 660 cm^{-1}. EI-MS *m*/*z* 508, 473, 447, 288, 232, 220, 155, 123, 95. Anal. Calcd for $C_{25}H_{35}ClN_4OS_2 \times 1/2$ EtOAc: C, 58.8; H, 7.1; N, 10.2. Found: C, 58.9; H, 6.8; N, 10.6.

4.2.2.5. *N*-(4-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}heptyl)-5-(1,2-dithiolan-3-yl)-pentanamide (6e). Pale yellow crystals. 78 mg (30% yield). Mp 89 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.30–1.78 (17H, m), 2.16 (3H, q, J = 7.5 Hz), 2.32 (1H, t, J = 6.5 Hz), 2.46 (1H, sex, J = 6.5 Hz), 2.99 (2H, t, J = 8 Hz, CH_2N), 3.10–3.29 (3H, m), 3.59 (1H, qui, J = 6.5 Hz, SCH), 3.74 (2H, t, J = 8 Hz, CH_2NH), 3.95 (2H, t, J = 8 Hz, CH_2N=), 5.54 (1H, br s, amide-NH), 7.17 (1H, dd, J = 7, 2 Hz, C(7)H), 7.43 (1H, d, J = 2 Hz, C(8)H), 7.99 (1H, d, J = 7 Hz, C(5)H) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 18.62 (alicycle-$CH_2CH_2CH_2$), 25.43, 26.96, 27.22, 28.86, 29.15, 29.64, 32.86, 33.06, 34.59, 36.53, 38.43, 39.54, 40.22, 48.33 (CH_2N), 51.50 (imine-N-CH_2), 56.42 (CHS), 118.26 (C8a), 123.97 (C5), 126.29 (C7), 129.18 (C8), 137.32 (C–Cl), 144.43 (C=N), 150.27 (C4a), 161.40 (C3a), 172.63 (C=O) ppm. IR (KBr): 3319, 2924, 2848, 2357, 1635, 1591, 1283, 913, 660 cm^{-1}. EI-MS *m*/*z* 522, 487, 461, 302, 232, 220, 123, 95. Anal. Calcd for $C_{26}H_{37}ClN_4OS_2$: C, 59.9; H, 7.2; N, 10.8. Found: C, 59.7; H, 6.9; N, 11.2.

4.2.2.6. *N*-(3-{[6-Chloro-2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene]amino}octyl)-5-(1,2-dithiolan-3-yl)-pentanamide (6f). Pale yellow crystals. 91 mg (34% yield). Mp 132 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.28–1.70 (20H, m), 1.87 (1H, sex, J = 6 Hz), 2.17–2.18 (3H, m), 2.40 (1H, sex, J = 6.5 Hz), 2.93 (2H, t, J = 8 Hz, CH_2N), 3.05–3.18 (3H, m), 3.49 (1H, qui, J = 6.5 Hz, SCH), 3.68 (2H, t, J = 7 Hz, CH_2NH), 3.92 (2H, t, J = 7 Hz, CH_2N=), 5.40 (1H, br s, amide-NH), 7.09 (1H, dd, J = 9, 2.5 Hz, C(7)H), 7.37 (1H, d, J = 2.5 Hz, C(8)H), 7.94 (1H, d, J = 9 Hz, C(5)H) ppm. IR (KBr): 3322, 2920, 2848, 2363, 1635, 1594, 1540, 1404, 1287, 910, 663 cm^{-1}. EI-MS *m*/*z* 535, 501, 473, 419, 234, 206, 137. Anal. Calcd for $C_{27}H_{39}ClN_4OS_2$: C, 60.6; H, 7.3; N, 10.5. Found: C, 60.6; H, 7.3; N, 10.7.

4.2.2.7. 5-(1,2-Dithiolan-3-yl)-*N*-[8-(6,7,8,9,10,11-hexahydro-13*H*-azocino[2,1-*b*]quinazolin-13-ylideneamino)-octyl]pentanamide (13). Pale yellow crystals. 166 mg (61% yield). Mp 101 °C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.22–1.90 (27H, m), 2.22 (1H, t, J = 4 Hz), 2.45 (1H, sex, J = 6 Hz), 2.88 (2H, t, J = 4 Hz, CH_2NH), 3.12–3.30 (6H, m), 3.74 (1H, qui, J = 6.5 Hz, SCH), 3.82 (2H, t, J = 5 Hz, CH_2N=), 5.45 (1H, br s, amide-NH), 7.18 (1H, m, C(2)H), 7.46–7.48 (2H, m, C(1, 3)H), 8.04 (1H, d, J = 5 Hz, C(4)H) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 24.29, 24.45, 25.44, 26.66, 26.90, 27.34, 28.50, 28.87, 29.35, 29.37, 29.69, 30.65, 33.44, 34.62, 36.09, 36.55, 38.45, 39.56, 40.23, 43.92 (CH_2N), 51.09 (imine-N-CH_2), 56.43 (CHS), 119.72 (C13a), 123.40 (C4), 126.38 (C2), 127.93 (C1), 131.31 (C3), 145.26 (C4a), 147.43 (C=N), 160.41 (C5a), 172.55 (C=O) ppm. IR (KBr): 3313, 2920, 2848, 1638, 1581, 1559, 1236, 761, 669 cm^{-1}. EI-MS *m*/*z* 543, 509, 485, 325, 254, 240, 226, 198. Anal. Calcd for $C_{30}H_{46}N_4OS_2$: C, 66.4; H, 8.5; N, 10.3. Found: C, 66.6; H, 8.4; N, 10.7.

4.3. Pharmacology

AChE (E.C.3.1.1.7, Type VI-S, from Electric Eel) and BChE (E.C.3.1.1.8, from equine serum) were purchased from Sigma–Aldrich (Steinheim, Germany) with a pur-

ity of $\geqslant 60\%$ protein by biuret. 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB), acetylthiocholine (ATC) and butyrylthiocholine iodides (BTC) were obtained from Fluka (Buchs, Switzerland).

As references for ChE inhibition the established drug galanthamine was used. Galanthamine hydrobromide was purchased from JANSSEN-CILAG GmbH, Neuss, Germany.

The assay was performed as described in the following procedure[5,25]: a stock solution of the test compound was prepared in 50% aqueous ethanol under addition of equimolar amounts of 1 N hydrochloric acid, and the highest concentration of the test compounds applied in the assay was 10^{-4} M (final concentration). In order to obtain an inhibition curve, at least five different concentrations (normally in the range of 10^{-4}–10^{-9} M) of the test compound were measured at 25 °C at 412 nm, each concentration in triplicate.

For buffer preparation, 1.36 g of potassium dihydrogen phosphate (10 mmol) was dissolved in 100 mL of water and adjusted with NaOH to pH 8.0 ± 0.1. Enzyme solutions were prepared to give 2.5 U/mL in 1.4 mL aliquots (with a unit hydrolyzing 1.0 mmol of ACh to choline and acetate per min at pH 8.0 at 37 °C according to the manufacturer's specification). Furthermore, 0.01 M DTNB solution, 0.075 M ATC and BTC solutions, respectively, were used. A cuvette containing 3.0 mL of phosphate buffer, 100 μL of the respective enzyme, and 100 μL of the test compound solution was allowed to stand for 5 min, then 100 μL of DTNB was added, and the reaction was started by addition of 20 μL of the substrate solution (ATC/BTC). The solution was mixed immediately, and exactly 2.5 min after substrate addition the absorption was measured. For the reference value, 100 μL of water replaced the test compound solution. For determining the blank value, additionally 100 μL of water replaced the enzyme solution.

The inhibition curve was obtained by plotting percentage enzyme activity (100% for the reference) versus logarithm of test compound concentration.

For the kinetic measurements the following substrate concentrations were used: 0, 25, 50, 90, 150, 266, 452 μM. As inhibitor concentrations 0.2, 0.4, 0.8, and 1.0 μM of compound **6f** were applied for determining AChE, and 0, 2, 5, and 10 nM were applied for BChE substrate–velocity curves. In contrast to the above-described affinity measurements the reaction time was extended to 4 min before measurement of the absorption. V_{max} and K_m values, respectively, of the Michealis–Menten kinetics were calculated by nonlinear regression out of the substrate velocity curves. Linear regression was used for calculating the Lineweaver–Burk plots.

All cell culture studies were done using the microglial cell line N11 (kindly provided Dr. G. Münch, University of Leipzig, Germany), which was derived from microglia isolated from embryonic mouse brain and infected with a retrovirus.[29] Cells were kept under standard cell culture conditions using Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum (FCS) and 2 mM glutamine (Life Technologies, Karlsruhe, Germany).

Cell viability was evaluated by an MTT assay.[30] In brief, cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured for 24 h. Subsequently cells were incubated for another 24 h either with medium only (without phenolred, 1% FCS), medium supplemented with the hybrid molecules or supplemented with solvent only. After treatment, 10 μL MTT (Sigma, Schnelldorf, Germany) solution (5 mg/mL in PBS) was added to each well and cells were incubated for another 3 h at 37 °C. Subsequently 100 μL lysis buffer (10% SDS, pH 4.1) was added and formazan allowed to dissolve overnight. Absorbance at 550 nm was determined with a multiwell plate photometer (TiterTek Plus, ICN, Meckenheim, Germany).

The antioxidant activity was determined by the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay,[31] recently modified by Dávalos et al.[24] The ORAC-assay measures antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C.

The reaction was carried out in 75 mM phosphate buffer (pH 7.4) and the final reaction mixture was 200 μL. Antioxidant (20 μL) and fluorescein (120 μL, 300 nM final concentration) were placed in the wells of a black 96-well plate and the mixture was incubated for 15 min at 37 °C. Then AAPH (Sigma, Steinheim Germany) solution (60 μL; 12 mM final concentration) was added rapidly. The plate was immediately placed into a Spectrafluor Plus plate reader (Tecan, Crailsheim, Germany) and the fluorescence was measured every 60 s for 90 min with exitation at 485 nm and emission at 535 nm. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, Steinheim Germany) was used as standard (1–8 μM, final concentration). A blank (FL + AAPH) using phosphate buffer instead of antioxidant and Trolox calibration were carried out in each assay. The samples were measured at different concentrations (0.5–10 μM). All reaction mixtures were prepared fourfold and at least four independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (without antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=90} f_i/f_0 \qquad (1)$$

where f_0 is the initial fluorescence at 0 min and f_i is the fluorescence at time i.

The net AUC for a sample was calculated as follows:

$$\text{Net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}} \qquad (2)$$

The ORAC-FL values were calculated:

$$[(\text{AUC sample} - \text{AUC blank})/(\text{AUC Trolox} - \text{AUC blank})] \times [(\text{concentration of Trolox/concentration of sample})] \quad (3)$$

and expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were in μM of Trolox equivalent/μM of pure compound.

Acknowledgments

Financial support for M. Decker by the 'Fonds der Chemischen Industrie' (FCI) is gratefully acknowledged. Appreciation is expressed to Petra Wiecha and Monika Listing (both Lehrstuhl für Pharmazeutische/Medizinische Chemie, Friedrich-Schiller-Universität Jena) and Gabi Brunner (Lehrstuhl für Pharmazeutische Biologie, Universität Regensburg) for excellent technical assistance.

References and notes

1. Francis, P. T.; Palmer, A. M.; Snape, M.; Wilcock, G. K. *J. Neurol. Neurosur. Ps.* **1999**, *66*, 137.
2. Perry, E. K.; Tomlinson, B. E.; Blessed, G.; Bergmann, K.; Gibson, P. H.; Perry, E. H. *Brit. J. Med.* **1978**, *2*, 1457.
3. Perry, E. K.; Perry, R. H.; Blessed, G.; Tomlinson, B. E. *Neuropath. Appl. Neuro.* **1978**, *4*, 273.
4. Giacobini, E.; Spiegel, R.; Enz, A.; Veroff, A. E.; Cutler, R. E. *J. Neural Transm.* **2002**, *109*, 1053.
5. Decker, M. *Eur. J. Med. Chem.* **2005**, *40*, 305.
6. Decker, M.; Krauth, F.; Lehmann, J. *Bioorg. Med. Chem.* **2006**, *14*, 1966.
7. Decker, M. *J. Med. Chem.* **2006**, *49*, 5411.
8. Floyd, R. A.; Hensley, K. *Neurobiol. Aging* **2002**, *23*, 795.
9. Moreira, P. I.; Siedlak, S. L.; Aliev, G.; Zhu, X.; Cash, A. D.; Smith, M. A.; Perry, G. *J. Neural Transm.* **2005**, *112*, 921.
10. Sung, S.; Yao, Y.; Uryu, K.; Yang, H.; Lee, V. M.-Y.; Trojanowski, J. Q.; Pratico, D. *FASEB J.* **2004**, *18*, 323.
11. Kontush, A.; Schekatolina, S. *Ann. N.Y. Acad. Sci.* **2004**, *1031*, 249.
12. Petersen, R. C.; Thomas, R. G.; Grundmann, M.; Bennett, D.; Doody, R.; Ferris, S.; Galasko, D.; Jin, S.; Kaye, J.; Levey, A.; Pfeiffer, E.; Sano, M.; van Dyck, C. H.; Thal, L. J. *N. Eng. J. Med.* **2005**, *352*, 2379.
13. Zandi, P. P.; Anthony, J. C.; Khachaturian, A. S.; Stone, S. V.; Gustafson, D.; Tschanz, J. T.; Norton, M. C.; Welsh-Bohmer, K. A.; Breitner, J. C. S. *Arch. Neurol.* **2004**, *61*, 82.
14. Mecocci, P.; Mariani, E.; Cornacchiola, V.; Polidori, M. *Neurol. Res.* **2004**, *26*, 598.
15. Hajieva, P.; Behl, C. *Curr. Pharm. Des.* **2006**, *12*, 699.
16. Tirosh, O.; Sen, C. K.; Roy, S.; Kobayashi, M. S.; Packer, L. *Free Radic. Biol. Med.* **1999**, *26*, 1418.
17. Biewenga, G. P.; Haenen, G. R.; Bast, A. *Gen. Pharmacol.* **1997**, *29*, 315.
18. Zhang, L.; Xing, G. Q.; Barker, J. L.; Cahng, Y.; Maric, D.; Ma, W.; Li, B. S.; Rubinow, D. R. *Neurosci. Lett.* **2001**, *312*, 125.
19. Hager, K.; Marahrens, A.; Kenklies, M.; Riederer, P.; Munch, G. *Arch. Gerontol. Geriatr.* **2001**, *32*, 275.
20. Rosini, M.; Andrisano, V.; Bartolini, M.; Bolognesi, M. L.; Hrelia, P.; Minarini, A.; Tarozzi, A.; Melchiorre, C. *J. Med. Chem.* **2005**, *48*, 360.
21. Rodriguez-Franco, M. I.; Fernandez-Bachiller, M. I.; Perez, C.; Hernandez-Ledesma, B.; Bartolome, B. *J. Med. Chem.* **2006**, *49*, 459.
22. Bartolini, M.; Bertucci, C.; Cavrini, V.; Andrisano, V. *Biochem. Pharmacol.* **2003**, *65*, 407.
23. Inestrosa, N. C.; Alvarez, A.; Perez, C. A.; Moreno, R. D.; Vicente, M.; Linker, C.; Casanueva, O. I.; Soto, C.; Garrido, J. *Neuron* **1996**, *16*, 881.
24. Dávalos, A.; Gómez-Cordovés, C.; Bartolomé, B. *J. Agric. Food Chem.* **2004**, *52*, 48.
25. Ellman, G. L.; Courtney, K. D.; Aandres, V., Jr.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
26. Savini, L.; Gaeta, G.; Fattorusso, C.; Catalanotti, B.; Campiani, G.; Chiasserini, L.; Pellerano, C.; Novellino, E.; McKissic, D.; Saxena, A. S. *J. Med. Chem.* **2003**, *46*, 1.
27. Detsi, A.; Bouloumbasi, D.; Prousis, K. C.; Koufaki, M.; Athanasellis, G.; Melagraki, G.; Afantitis, A.; Igglesi-Markopoulou, O.; Kontogiorgis, C.; Hadjipavlou-Litina, D. J. *J. Med. Chem.* **2007**, *50*, 2450.
28. Decker, M. *Mini-Rev. Med. Chem.* **2007**, *7*, 221.
29. Righi, M.; Letari, O.; Sacerdote, P.; Marangoni, F.; Miozzo, A.; Nicosia, S. *J. Neurochem.* **1995**, *64*, 121.
30. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
31. Ou, B.; Hampsch-Woodill, M.; Prior, R. *J. Agric. Food Chem.* **2001**, *49*, 4619.