Letter

Neuroprotective Tri- and Tetracyclic BChE Inhibitors Releasing Reversible Inhibitors upon Carbamate Transfer

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Ⓢ Supporting Information

ABSTRACT: Tri- and tetracyclic nitrogen-bridgehead compounds were designed and synthesized to yield micromolar cholinesterase (ChE) inhibitors. Structure–activity relationships identified potent compounds with butyrylcholinesterase selectivity. These compounds were selected as starting points for the design and synthesis of carbamate-based (pseudo)irreversible inhibitors. Compounds with superior inhibitory activity and selectivity were obtained and kinetically characterized also with regard to the velocity of enzyme carbamoylation. Structural elements were identified and introduced that additionally showed neuroprotective properties on a hippocampal neuronal cell line (HT-22) after glutamate-induced intracellular reactive oxygen species generation. We have identified potent and selective pseudoirreversible butyrylcholinesterase inhibitors that release reversible inhibitors with neuroprotective properties after carbamate transfer to the active site of cholinesterases.

R =

IC$_{50}$ (BChE) = 60 nM / K$_C$ = 13 nM, no inhibition of AChE at 100 μM.
R = H (formed after carbamoylation of BChE's active site): strongly reduced ROS-mediated cytotoxicity on hippocampal neuronal cells (HT-22)

KEYWORDS: Alzheimer's disease, butyrylcholinesterase, carbamates, neuroprotection
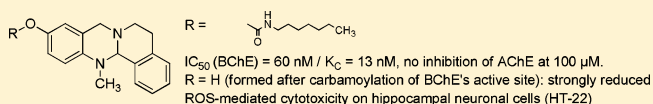
Alzheimer's disease (AD) is one of the most often occurring and most devastating neurodegenerative diseases. Huge efforts have been made to investigate the pathophysiology of AD, but to make a long story short, the disease is still incurable.[1] The number of approved drugs is extremely limited to one NMDA antagonist (memantine) and three acetylcholinesterase (AChE) inhibitors (rivastigmine, donepezil, and galantamine). Despite their purely symptomatic mode of action to improve cognition and memory, their clinical effectiveness was proven in a number of studies.[2] While galantamine and donepezil are reversible cholinesterase (ChE) inhibitors, rivastigmine (Exelon) represents an irreversible inhibitor transferring a carbamate moiety to the serine unit in the active center of AChE and butyrylcholinesterase (BChE), respectively, the second cholinesterase in the human body.[3,4] The carbamates' mode of action is termed pseudoirreversible since the carbamate moiety slowly hydrolyses from the enzyme, yielding the active enzyme, again in contrast to irreversibly inhibiting organophosphates. Rivastigmine, a compound derived from the alkaloid physostigmine, is of special interest because in clinical and pharmacological investigations, it was shown to be of high effectiveness, which some authors attributed to its ability to inhibit also BChE with high efficiency (Chart 1).[4,5] There has been postulated a putative beneficial role in the therapy of AD through BChE inhibition; for example, it was found that the amount of AChE decreases in later stages of AD (when AChE inhibitors become clinically ineffective), whereas the amount of BChE stays the same or is even increased.[6,7] Also, it was shown that with a decreasing amount of AChE, BChE seems to be able to compensate for AChE loss, resulting in improved cognition.[7] BChE's role in the human body is not yet fully understood; it is responsible for detoxification of xenobiotics, and it influences lipoprotein

Chart 1. Structures of the ChE Inhibiting Alkaloid Physostigmine, the Tetracyclic Lead Structure (5a, cf. Scheme 1), and the AD Drug Rivastigmine

Physostigmine

Rivastigmine
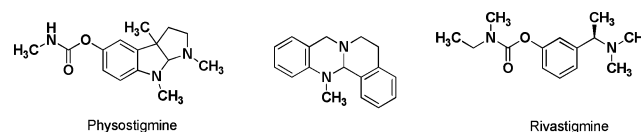
metabolism and may also play a role in neuronal differentiation.[3–7]

For the benefit of developing more effective AD drugs and to investigate BChE's physiological functions, there have been efforts in developing selective BChE inhibitors. Gilmer's group has described a very interesting class of pseudoirreversible carbamate inhibitors based on an isosorbide template with remarkable potency and selectivity.[8] Our group has used two alkaloids (deoxyvasicine and dehydroevodiamine) that had been described as moderately active and unselective ChE inhibitors as lead structures to identify a series of N-bridgehead tri- and tetracyclic compounds as reversible and competitive inhibitors with improved inhibitory activities and BChE selectivity (Chart 1).[9,10] The greatest BChE selectivities were achieved with quinazolinimine structures.[10] By applying the bivalent compound approach and the design of hybrid molecules, their pharmacological activities were further improved and enlarged.[11–14]
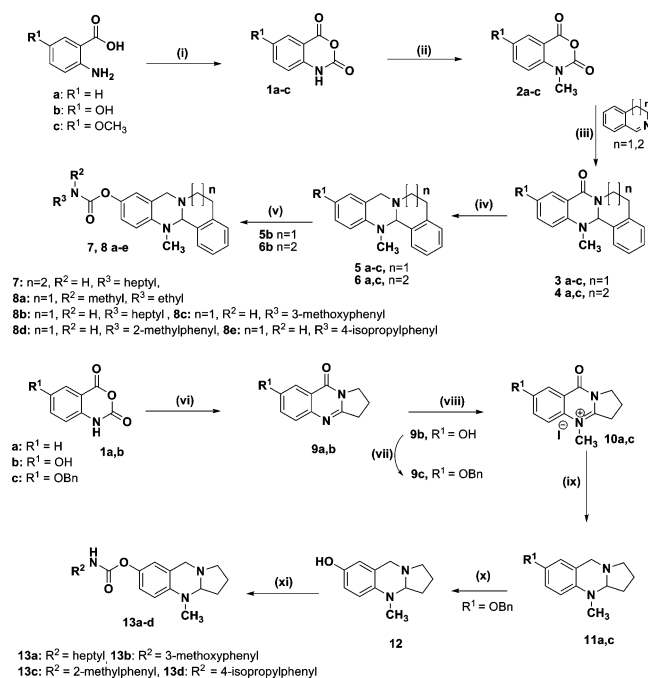
Because even the best ChE inhibitors stay symptomatically acting compounds, there has been considerable interest in applying antioxidant compounds as disease-modifying drugs in AD drug development. Oxidative stress and the formation of reactive oxygen species (ROS) are key processes in AD and result in neuronal damage and subsequent cell death.[15,16] It was therefore tried to combine AChE inhibitors with compounds possessing antioxidant properties, for example, by forming covalently connected hybrids or physiologically more labile codrugs.[11,12,17,18] Albeit highly promising compounds, hybrids also suffer from some difficulties, such as their inherent high molecular mass and therefore reduced bioavailability.[11]

The vast majority of recent work on cholinesterase inhibitors, mainly hybrids, has been done on the basis of a surprisingly small number of chemical templates, especially tacrine.[12] There is a constant search for novel structural templates for ChE inhibitors, especially from natural products.[19−21] Our rationale in this present work was to use a previously described tetracyclic lead structure as a template for the development of novel pseudoirreversible inhibitors in which the heterocyclic structure serves as a kind of carrier into the active center of ChEs. Such compounds would release reversible inhibitors after transfer of the carbamate moiety to the enzymes, ideally enabling a more pronounced efficiency of inhibition. Structure−activity relationships (SARs) were investigated for the heterocyclic template, in particular to find out whether the introduction of a phenolic hydroxyl group to enable connection with a carbamate unit is tolerated. Additionally, SARs of the resulting carbamates with the aim to achieve higher selectivity and inhibitory activity toward BChE were investigated. A second rationale of this work was to incorporate antioxidant properties into our target compounds, but not by connecting them covalently to antioxidant moieties as it is the case for hybrid molecules, but to use the heterocyclic phenolic structure of the reversible inhibitor itself as an antioxidant. Weinstock and Groner have used such an approach by combining carbamate structures with MAO-B inhibitors.[22] Phenolic structures are key factors for antioxidant and neuroprotective properties in a number of natural products, such as quercetin, ferulic acid, and silibinin.[17,18]

We assumed that the compounds released after carbamate transfer represent antioxidants (since, for example, cyclic quinonimines could be formed after oxidation) and could therefore enlarge the biological spectrum of the compounds without increasing their molecular mass. For assaying antioxidant properties, we used two different assays: the first one, the oxygen radical absorbance capacity (ORAC) assay, determines the ability of compounds to protect fluorescein from destruction by peroxyl radicals and, therefore, directly assesses their antioxidant physicochemical properties.[14,18] The second assay is cell-based and uses murine HT-22 hippocampal cells that lack ionotropic glutamate receptors to study intracellular oxidative stress-induced neurotoxicity.[17,23,24] In this neuronal oxidative stress-induced toxicity (oxytosis), cell death is induced by extracellular glutamate challenge in a nonreceptor-mediated "oxidative" pathway. Glutamate toxicity is mediated by the cystine/glutamate antiporter system, which—after exposure to high extracellular glutamate concentrations—results in inhibition of cystine uptake, leading to intracellular cysteine and therefore glutathione depletion, which induce ROS accumulation and cell injuries.[23−25] Administration of antioxidants such flavonoids can effectively prevent oxidative neuronal death in this cell line.[26,27]

For the investigation of SARs concerning the tri- and tetracyclic N-bridgehead compounds, the six-membered ring system containing compound 3,4-dihydroisoquinoline and the seven-membered one 4,5-dihydro-3H-benzo[c]azepine were reacted with isotoic acid anhydride, a reactive form of anthranilic acid formed after reaction with triphosgene (Scheme 1). Preparation of isotoic acid anhydrides also provides an easy

Scheme 1. Synthesis of Tetra- and Tricyclic N-Bridgehead Compounds[a]

7: n=2, R² = H, R³ = heptyl,
8a: n=1, R² = methyl, R³ = ethyl
8b: n=1, R² = H, R³ = heptyl , 8c: n=1, R² = H, R³ = 3-methoxyphenyl
8d: n=1, R² = H, R³ = 2-methylphenyl, 8e: n=1, R² = H, R³ = 4-isopropylphenyl

13a: R² = heptyl, 13b: R² = 3-methoxyphenyl
13c: R² = 2-methylphenyl, 13d: R² = 4-isopropylphenyl

[a]Reagents: (i) (Cl₃CO)₂CO, dry THF, 40−50 °C, 3 h. (ii) CH₃I, N,N-diisopropylethylamine, N,N-dimethylacetamide, 40 °C, 24 h. (iii) Toluene/reflux, 24 h. (iv) LiAlH₄, THF, 70 °C, 3 h. (v) Ethyl(methyl)carbamic chloride, NaH, THF, rt or isocyanate, Et₃N, CH₂Cl₂, rt. (vi) Pyrrolidin-2-one, MW 100−200 W, 130 °C, 1 h. (vii) Benzylbromide, K₂CO₃, acetone, 70 °C, 24 h. (viii) CH₃I, dioxane, 90 °C, 24 h. (ix) LiAlH₄, THF, 70 °C, 3 h. (x) H₂, Pd/C, ethanol, rt, 24 h. (xi) Isocyanate, CH₂Cl₂, Et₃N, rt, 1−3 h.

method for selective monomethylation of anthranilic acid's amino group. Seven- and six-membered rings were synthesized to investigate their influence on BChE selectivity (previous work on related quinazolinimines showed higher selectivity with increasing ring size).[10,13,14]

Reduction of the amide group of compounds 3a−c and 4a,c by lithium aluminum hydride afforded the desired tetracyclic quinazoline compounds 5a−c and 6a,c, respectively. Compound 6b was synthesized by demethylation of 6c using boron tribromide. Compound 5b was treated with ethyl(methyl)-carbamic chloride in the presence of sodium hydride to afford 8a in 60% yield. The reaction of n-heptylisocyanate with 6b in the presence of sodium hydride gave 7 in 75% yield. Carbamoylation of 5b with the appropriate isocyanate in dichloromethane in the presence of triethylamine gave the carbamates 8b−e in good yields (72−88%) (Scheme 1). Unsubstituted tetracyclic compounds 5a and 6a were prepared, and compounds bearing a methoxy (5c and 6c) and a hydroxyl group (5b and 6b) to test whether substitution in this position is tolerated. Determination of IC₅₀ values at AChE and BChE

confirmed that the introduction of −OCH$_3$ or −OH groups has no influence on the inhibitory activities and, therefore, proved that this position of the heterocycle is ideal for introduction of the carbamoyl unit (Table 1). Interestingly, for the seven-membered ring-containing compounds, the inhibition data were considerably modified: As expected from the results with tricyclic quinazolinimines, activity at BChE was slightly higher, whereas inhibitory activities at AChE were slightly lower, resulting in increased selectivity toward BChE (>8).[10] Again, introduction of a methoxy or hydroxyl group did not result in a significant alteration of binding behavior. It has to be stated though that all compounds are only 1−2 digit micromolar inhibitors of BChE, and selectivities were never higher than 10.

We also synthesized analogous tricyclic quinazoline compounds to investigate their binding profiles. Their synthesis was accomplished as illustrated in Scheme 1. The fusion of isatoic anhydrides **1a,b** with pyrrolidin-2-one using a microwave reactor at 100−200 W power and 100 °C temperature afforded **9a,b** in good yields (79−80%). Benzylation of **9b** by benzylbromide in the presence of K$_2$CO$_3$ afforded **9c**. Quaternization reaction of **9a,c** with methyl iodide gave **10a,c**. Reduction of **10a,c** by lithium aluminum hydride afforded the desired tricyclic quinazoline compounds **11a,c** in good yields (74−78%). Debenzylation reaction of **11c** gave **12**. Finally, carbamoylation of **12** to yield the different carbamates **13a−d** was accomplished as reported for the preparation of **8b−e** in good yields (68−83%) (Scheme 1).

For investigation into the SARs of this class of compounds, several structurally diverse carbamate groups were introduced at the phenolic hydroxyl group. Several aliphatic and aromatic moieties were used in previous work on carbamates: the methyl ethyl carbamate occurs in rivastigmine (Chart 1) and, therefore, served as our first structure. Previous medicinal research has been done using a physostigmine or rivastigmine-based heterocyclic core:[28,29] The compounds synthesized inhibited both ChEs in the nanomolar range. A heptyl group was identified that led to high acitivity, in the case of conformationally restricted rivastigmine analogues with 16-fold selectivity [heptylphysostigmine (eptastigmine) shows 4-fold selectivity].[29] The synthesis of phenylcarbamates has been described, again connected to a physostigmine-related heterocyclic core.[29] In this case, modulation of selectivity could be achieved by fairly minor structural modifications, for example, a methyl group in the *o*-position led to AChE selectivity, whereas an isobutyl substituent in the *p*-position led to BChE selectivity.[28] We therefore also applied these carbamate structures to our heterocyclic templates to investigate SARs.

Tri- as well as tetracyclic target compounds bear a chiral C atom attached to two N atoms, but at this stage, no enantioseparation was performed. The stability of carbamoylated compounds under assay conditions was investigated by incubation of **8a** and **8b** in buffer and control by LC-ESI-MS, and no decomposition was observed.

The tetracyclic carbamate compound **8a** bearing methyl and ethyl substituents at the carbamate group (like rivstigmine) shows significant improvement in binding activities over noncarbamate compounds, moderately pronounced at AChE (6-fold more potent as compared to the unsubstituted compound **5a**) and very pronounced at BChE (100-fold more potent as compared to the unsubstituted compound **5a**), leading to submicromolar activities at BChE and 54-fold selectivity over AChE (Table 1). The introduction of a heptyl substituent as in heptylphysostigmine for compounds **7** and **8b**

Table 1. AChE and BChE Inhibition Results, Resulting ChE Selectivity, and Antioxidant Capacities Expressed as Trolox Equivalents (Concentration Range, 0.5−7.5 μM)

| compound | IC$_{50}$ (pIC$_{50}$ ± SEM), μM | | Selectivity[b] | Trolox equiv. |
	AChE[a]	BChE[a]		
Physostigmine	0.090	0.042	2.1	
5a	145.2 (3.84 ± 0.13)	44.0 (4.36 ± 0.06)	3.3	0.92 ± 0.26
5b	110.3 (3.96 ± 0.09)	44.3 (4.35 ± 0.04)	2.5	2.87 ± 0.61
5c	232.5 (3.63 ± 0.13)	55.6 (4.26 ± 0.13)	4.2	0.51 ± 0.06
6a	>200[c]	23.7 (4.63 ± 0.09)	>8.4	
6b	37.8 (4.42 ± 0.07)	7.30 (5.14 ± 0.08)	5.2	2.48 ± 0.30
6c	>200[d]	29.7 (4.53 ± 0.07)	>6.7	1.20 ± 0.16
7	>200[e]	0.046 (7.33 ± 0.03)	>4,000	0.86[f] ± 0.03
8a	24.4 (3.63 ± 0.13)	0.450 (4.61 ± 0.057)	54.2	0.65 ± 0.06
8b	No inhibition	0.060 (7.23 ± 0.02)	>15,000	0.30[f] ± 0.12
8c	>200[g]	0.218 (6.66 ± 0.104)	>900	
8d	>200[h]	0.144 (6.84 ± 0.10)	>1,350	
8e	>200[i]	0.131 (6.88 ± 0.06)	>1,500	
11a	130.8 (3.88 ± 0.18)	38.9 (4.41 ± 0.08)	3.4	
12	30.6 (4.52 ± 0.035)	41.0 (4.39 ± 0.04)	0.75	3.43 ± 0.35
13a	32.3 (4.49 ± 0.12)	1.95 (5.71 ± 0.02)	16.6	1.03 ± 0.23
13b	40.2 (4.40 ± 0.05)	7.52 (5.12 ± 0.06)	5.3	
13c	66.3 (4.18 ± 0.07)	4.11 (5.39 ± 0.03)	16.1	
13d	>200[j]	3.00 (5.53 ± 0.04)	>66.0	

[a]AChE from electric eel and BChE from equine serum; data are the means of at least three independent determinations. [b]Selectivity ratio [IC$_{50}$(AChE)/IC$_{50}$(BChE)]. [c]Inhibition at 100 μM, 26% inhibition. [d]21% inhibition. [e]33% inhibition. [f]Concentration range, 1.0−5.0 μM. [g]8% inhibition. [h]18% inhibition. [i]1% inhibition. [j]24% inhibition.

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dx.doi.org/10.1021/ml3001825 | ACS Med. Chem. Lett. 2012, 3, 914−919

lead to a further 10-fold increase in activity at BChE and a very remarkable decrease in activity at AChE. For compound **8b**, no activity at all could be observed even at 100 μM. So, two-digit nanomolar inhibitors of BChE were obtained lacking any activity at AChE. The tricyclic heptyl carbamate **13a** only showed moderate activity and selectivity with IC$_{50}$ (AChE) = 32.3 μM and IC$_{50}$ (BChE) = 2.0 μM.

We also studied the effect of phenylcarbamates on ChE inhibition activity and selectivity. Tetracyclic 3-methoxyphenyl-, 2-methylphenyl-, and 4-isopropylphenylcarbamates **8c**, **8d**, and **8e**, respectively, showed submicromolar activities at BChE with 900−1500-fold selectivity over AChE (Table 1). Tricyclic 3-methoxyphenyl-, 2-methylphenyl-, and 4-isopropylphenyl carbamates **13b**, **13c**, and **13d**, respectively, only showed moderate activity and selectivity with IC$_{50}$ (AChE) ranging from 40 to lower than 200 μM and IC$_{50}$ (BChE) ranging from 3 to 8 μM (Table 1).

Kinetic studies were performed to get more detailed information about the time course of inhibition and therefore more precise data about the equilibrium constant of inhibition (for experimental details, cf. Supporting Information). The equilibrium constant of the inhibitor−ChE complexes (K_C) and the carbamoylation rate constants (k_3) of two representative target compounds **8a** and **8b** and physostigmine as a positive control were determined (Figure 1). The K_C value calculated
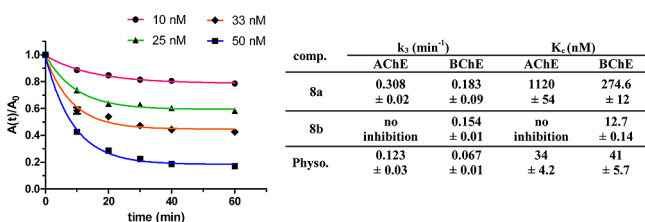

Figure 1. (A) Time-dependent pattern of inhibition of BChE by compound **8b** (10−50 nM). (B) Stability constants of the inhibitor−ChE complex (K_C) and rate constants of carbamoyl−ChE formation (k_3) of **8a,b** and physostigmine, respectively.

for physostigmine as positive control is in agreement with the value reported in the literature.[29] For compound **8a** at AChE, the actual K_C value is even smaller than determined as the IC$_{50}$ values with K_C = 1.1 μM and at BChE with K_C = 274 nM, resulting in 4-fold selectivity. The rate constants of 0.31 min^{-1} (AChE) and 0.18 min^{-1} (BChE) show rapid carbamoylation, albeit slower than for physostigmine (Figure 1). For compound **8b**, K_C (BChE) is even lower as for determination of the IC$_{50}$ value with K_C = 12.7 nM, and no carbamoylation could be observed at AChE for the concentrations tested. The velocity of carbamoylation was fairly rapid with k_3 = 0.15 min^{-1}. Compound **8b** shows a time-dependent pattern of inhibition characterized by an increase until a steady state after 60 min (Figure 1).

The tetracyclic heptyl carbamate **8b** represents a highly potent pseudoirreversible BChE inhibitor with K_C = 12.7 nM without any activity at AChE and fast onset of action.

For evaluation of antioxidant potencies, in a first assay, the target compounds' radical scavenging capacities were determined by means of their ability to reduce the amount of peroxyl radicals (ORAC assay). Results are expressed in "trolox" (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble vitamin E derivative) equivalents.[14] As can be seen from Table 1, the phenolic compounds (**5b**, **6b**, and **12**) are by far

the most potent compounds with antioxidant capacities ranging from 2.5 to 3.4 trolox equivalents, meaning that they are far more potent than the very active positive control trolox. The methoxy compounds **5c** and **6c** show lower potencies with 0.5 and 1.2 trolox equivalents, respectively. The unsubstituted compound **5a** is among the least potent compounds. Finally, the carbamates tested show only very moderate antioxidant properties (0.65 trolox equiv for the ethyl methyl carbamate **8a**, 0.30 trolox equiv for the tetracyclic heptyl carbamate **8b**, and 1.03 equiv for the tricyclic heptyl carbamate **13a**). None of these compounds lacks radical scavenging properties completely, though, and for structurally related compounds, there can be significant differences in their capacities.[14] As a general rule, it can be seen that the hydroxyl compounds released after carbamate transfer to the enzymes' serine unit represent highly potent antioxidants with slightly higher capacities for the tricyclic compound (Table 1).

In a second assay assessing the target compounds' antioxidant properties, their ability to prevent oxycytotic cell death of murine hippocampal HT-22 cells after glutamate exposure and subsequent intracellular ROS formation was evaluated. The six- and seven-membered ring tetracyclic hydroxyl compound **5b** and **6b**, as well as the tricyclic hydroxyl-compound **12** formed after carbamate transfer to the enzyme, showed very pronounced neuroprotective properties (tested at 10 μM) to the same extent as the potent positive control quercetin (tested at 25 μM) and much stronger than the flavolignan silibinin.[17] The six-membered ring heterocyclic methoxy compound **5c** showed activity in the same range, although performing these experiments at various concentrations revealed that the hydroxy compound exhibits neuroprotection already at 5 μM, when the methoxy compound is not active (cf. Supporting Information for data at 5 μM). The unsubstituted compound **5a** showed no activity. Surprisingly, all tested carbamates **7**, **8b**, and **13a** showed potent antioxidant capacities to the same extent as quercetin does, and only the tricyclic compound **13a** showed slightly lower activities at a concentration of 5 μM. The methyl ethyl-substituted carbamate **8a** did not show neuroprotective activity at 10 μM (Figure 2).
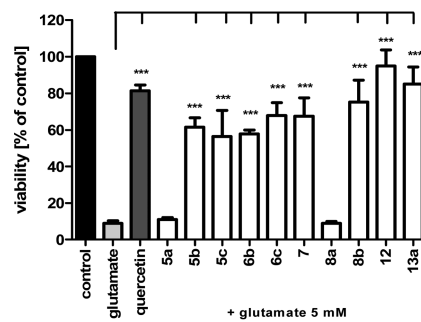

Figure 2. Evaluation of neuroprotection of unsubstituted, phenolic, methoxy-substituted, and selected carbamylated compounds at 10 μM against glutamate-induced oxidative stress on HT-22 cells (cf. the Supporting Information for details).

The phenolic hydroxyl group, which is also formed after carbamate transfer to the enzyme, led to potent ROS scavenging molecules exhibiting up to 3.5 trolox equiv in a physicochemical ORAC assay. Interestingly, evaluation of the antioxidant properties of the target compounds on glutamate-challenged neuronal HT-22 cells (and therefore increased

intracellular ROS production) showed very potent neuroprotective properties not only for the hydroxyl compounds but also for the carbamates in the same or higher range than for the positive control quercetin. These results are surprising since we expected high neuroprotection only for the hydroxyl compounds, but obviously, in the cell-based assay also, the carbamate compounds are very potent neuroprotectants in their own regard.

Into a novel heterocyclic template consisting of basic tri- and tetracyclic N-bridgehead compounds that inhibit ChEs, a phenolic hydroxyl group was introduced, and SARs showed that the −OH group as well as respective phenol ethers are tolerated without loss of activity. Introduction of carbamate units not only increased inhibitory activities into the submicromolar range, but SARs on the carbamate moiety led to nanomolar inhibitors with ≫15000-fold selectivity toward BChE over AChE. Kinetic studies on selected carbamates proved pseudoirreversible inhibition and—depending on the concentration of inhibitors—a comparatively fast onset of action. Kinetic studies revealed an even higher stability constant (K_C) for compounds **8a** and **8b** as reflected by the IC$_{50}$ value alone.

We are currently performing computational and docking studies to explain the very pronounced changes in enzyme inhibitory activity and selectivity by only minor changes in the chemical structure, and results will be presented at a later stage.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Elemental analysis and spectral data, detailed synthetic and pharmacological procedures, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

AD, Alzheimer's disease; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ROS, reactive oxygen species; ORAC, oxygen radical absorbance capacity

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