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The First Dual ChE/FAAH Inhibitors: New Perspectives for Alzheimer's Disease?

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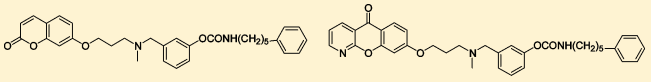
Supporting Information

ABSTRACT: The treatment of Alzheimer's disease (AD) still remains an area of significant unmet need, with drugs that only target the symptoms of the disease. Therefore, there is considerable need for disease-modifying therapies. The complex etiology of AD prompts scientists to develop multitarget strategies to combat causes and symptoms. To this aim, we designed, synthesized, and tested four new carbamates as dual cholinesterase-FAAH inhibitors. The dual activity of these compounds could lead to a potentially more effective treatment for the counteraction of AD progression, because they would allow regulation of both ACh and eCB signaling and improve neuronal transmission and/or counteract neuroinflammation.



FAAH = 50 nM
AChE = 74.9 nM
BuChE = 1.57 nM

FAAH = 40 nM
AChE = 89.5 nM
BuChE = 1.71 nM

KEYWORDS: *Alzheimer's disease, drug design, FAAH, AChE, BuChE, carbamate inhibitors*

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and its prevalence is increasing together with life expectancy. Although the etiology of AD is not completely known, histopathological hallmarks such as amyloid β (Aβ) deposits,[1] τ protein aggregation,[2] oxidative stress, inflammation, and dysfunction of acetylcholine (ACh) signaling in the basal forebrain seem to play significant roles. Indeed, the evidence that a selective loss of presynaptic cholinergic neurons occurred in AD patients led in the last decades to the development of cholinesterase inhibitors (ChEI),[3,4] which temporarily increase the amount of ACh in the neuronal synaptic cleft by inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), the enzymes responsible for ACh degradation. BuChE has received increasing attention in the past years since, as AD progresses, the activity of AChE decreases, while that of BuChE increases, in an attempt to modulate ACh levels in cholinergic neurons. Consequently, both enzymes are therapeutic targets at different stages of the pathology and, namely, for mild to moderate and for moderate to severe forms of AD, respectively.[5,6]

After more than three decades of research efforts in the field, the treatment of AD still remains an area of significant unmet need, with therapies based largely on the ChEI, rivastigmine, donepezil, and galantamine, with the only exception of memantine, an NMDA (*N*-methyl-D-aspartate) receptor antagonist. However, these drugs only target the symptoms of the disease without altering its progression.

To meet the need of disease-modifying drugs for AD, in recent years, new approaches have emerged in medicinal chemistry. In particularm the concept has recently been proposed that due to the multifactorial and complex etiology of AD, the modulation of a single factor might not be sufficient to produce the desired efficacy. Researchers are now turning to the design of structures that could be able to simultaneously interact with different targets involved in the pathogenic process.[7,8]

Recent advances in the field of the central nervous system (CNS) strongly suggest that glia (astroglia and microglia) play an important role in neurodegenerative diseases. Specifically, microglia, the resident macrophages in the brain, are activated in response to both Aβ and neuronal damage.[9,10]

Besides, there is an emerging interest in the relevance of the endocannabinoid system (eCB) to AD.[11,12] The eCB system consists of lipid signaling molecules that bind to two G-protein-coupled receptors, named CB_1 and CB_2: CB_1 receptors are mainly expressed in neurons, while CB_2 receptors can be found in a variety of immune cells, including activated microglia in the AD brain, most probably as a function of their inflammatory phenotype.[13] The selective expression of CB_2 receptors in regions of neuritic plaques suggests that this receptor plays a role in controlling the inflammation associated with AD. Specifically, CB_2 receptor expression may be an adaptive response to excessive inflammation induced in regions of Aβ deposition aimed at reducing microglia and astrocyte activation. Additionally, in regions of Aβ-enriched neuritic plaques, an increased activity of the enzyme fatty acid amide hydrolase (FAAH) was selectively demonstrated.[13] FAAH is an integral

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membrane enzyme that catalyzes the hydrolysis of several endogenous lipid messengers, including eCBs, and therefore, its inhibitors would allow regulation of eCB signaling and improve neuronal transmission and/or counteract neuroinflammation, via CB_1 and CB_2 receptors, respectively. Accordingly, early inhibition of eCB inactivation was found to reduce Aβ-induced gliosis, neuronal death, and memory retention loss.[14] Moreover, recent findings showed that anandamide levels are defective in cortical areas of postmortem AD brains and are directly correlated with the positive cognitive scores of the respective patients and negatively correlated with $A\beta_{42}$ accumulation.[15]

Our research groups have been involved for many years in the development of carbamate ChEIs and various FAAH inhibitors. Carbamate ChEIs, with the general formula shown in Figure 1, were designed as potential drug candidates for

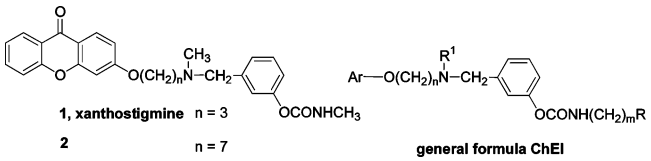


Figure 1. Lead molecules and general formula of carbamate ChEI.

AD.[16−20] The lead xanthostigmine **1**[17] and compound **2**[18] showed the highest activity toward human AChE (IC_{50} = 0.30 and 0.32 nM).

Taking into account the above-mentioned issues, in view of the fact that some carbamates are also known from the literature to be active as FAAH inhibitors[21,22] and of the recent finding that the eCB anandamide and some of its congeners potently inhibit human plasma BuChE,[23] we decided to test the library[16−20] of carbamates that was available in house on this new target.

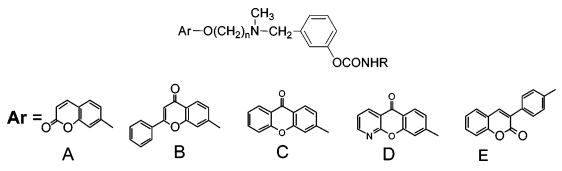
The inhibitory potencies of compounds showing significant activity, expressed as IC_{50} values, against anandamide hydrolysis by FAAH in rat brain membranes, are reported in Table 1 (inactive compounds not shown). These data highlight some important structure−activity relationships: (a) among the different aryl groups (Ar in the general formula of Figure 1 and Table 1), coumarin, azaxanthone, and xanthone are preferred; (b) the optimal chain length (n in the general formula) proves to be of three methylene units; and (c) the variation of the length of the carbamic N-substituent has a remarkable effect on the activity of the inhibitors, with longer chains yielding more potent inhibitors. Starting from these results, we have designed four new carbamates (Table 2): **13** and **14** carrying a coumarin core and **15** and **16** with an azaxanthone one, both substituted with an appropriate bulky group on the carbamate, in compliance with literature information on optimal FAAH ligands, but still maintaining the key features required for ChE inhibition, with the aim of obtaining multitarget compounds.

According to Scheme 1, compounds **13**−**16** were synthesized starting from 7-[*N*-methyl-*N*-(3-hydroxybenzyl)amino]-propoxy-2*H*-1-benzopyran-2-one[17] or 3-[*N*-methyl-*N*-(3-hydroxybenzyl)-amino]propoxy}-5-azaxanthen-9-one,[17] which were treated with the selected isocyanate in the presence of NaH.

As reported in Table 2, all new compounds appeared to inhibit anandamide hydrolysis by FAAH-containing rat brain membranes. Remarkably, compounds **13** and **15**, with the carbamic *N*-phenylpentyl substituent, showed activity in the submicromolar range (IC_{50} = 0.28 and 0.37 μM, respectively), which improved to nanomolar (IC_{50} = 50 and 40 nM) after preincubation, as expected from pseudoirreversible inhibitors. When tested on human recombinant FAAH, a remarkable drop in activity was seen for **13**, whereas both **15** and the reference compound URB597 showed a ∼13-fold decrease in activity.

Inhibitory activities of **13**−**16** against both cholinesterases were tested using the method of Ellman[24] (Table 2). All compounds showed a time-dependent pattern of ChEs inhibition, which is related to the formation of a carbamoylated covalent adduct with the Ser residue of the enzyme active site.[25,26] Inhibition increased until a steady state, generally reached within 60−100 and 5−10 min for human AChE and BuChE, respectively. Thus, IC_{50} values were determined using an incubation time suitable for the carbamoylation step to reach the steady state. Rivastigmine was used as a reference compound as it is the only marketed carbamate approved for

Table 1. Inhibitory Activities on FAAH[a]



	Ar	n	R	IC_{50} FAAH (μM) pit = 0	pit = 20 min
3	A	3	$(CH_2)_6CH_3$	0.62	0.17
4	B	3	$(CH_2)_3CH_3$	5.54	0.80
5	C	3	$(CH_2)_2CH_3$	9.28	0.10
6	D	3	$(CH_2)_6CH_3$	2.21	0.40
7	E	7	CH_3	9.20	NT
8	C	3	$(CH_2)_5CH_3$	4.29	0.29
9	C	3	$(CH_2)_6CH_3$	3.62	0.11
10	A	3	$(CH_2)_3CH_3$	8.68	NT
11	B	3	$(CH_2)_6CH_3$	6.81	NT
12	D	3	$(CH_2)_3CH_3$	4.23	1.4

[a]pit, preincubation time; NT, not tested; standard error of the mean within 5%.

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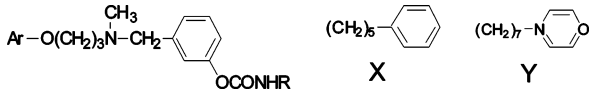
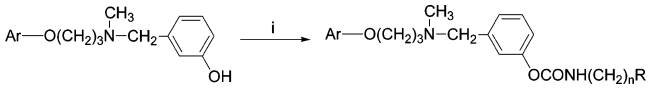
Table 2. Inhibitory Activities of New Designed Compounds against FAAH and Human Cholinesterases[a]



			FAAH				
			IC_{50} (nM)[b]		IC_{50} (nM)[c]	IC_{50} (nM)[d]	
	Ar	R	pit = 0	pit = 20 min	pit = 20 min	hAChE	hBuChE
13	A	X	280	50	2260	74.9	1.57
14	A	Y	5590	1820	14840	119	11.2
15	D	X	370	40	520	89.5	1.71
16	D	Y	4310	2710	39040	139	27.6
U				6.00	90		
R						1535	301

[a]pit, preincubation time; U = URB597; R = rivastigmine. [b]Rat brain FAAH. [c]Human recombinant FAAH. [d]Human recombinant AChE and BuChE; standard error of the mean within 5%.

Scheme 1. Synthesis of the Studied Compounds[a]



[a]Reagents and conditions: (i) 4-(7-Isocyanatoheptyl)-morpholine or 5-isocyanatopentylbenzene, NaH, room temperature, 24 h.

AD treatment. Results showed that all carbamates acted as potent butyryl-selective inhibitors with selectivity ranging from 5 (**16**) to 52.3 (**15**). Inhibitory potencies were in the low nanomolar range for hBuChE and in the high nanomolar range for hAChE inhibition. All compounds were also more potent cholinesterases inhibitors than the reference compound rivastigmine. The structural feature mostly affecting the inhibitory potency on both cholinesterases was the R substituent at the carbamic nitrogen as compounds **14** and **16**, differing at the aryl moiety, have similar inhibition profiles, while the replacement of the morpholino-heptyl chain of **14** and **16** with the phenyl-pentyl residue (to give **13** and **15**, respectively) increased the inhibitory potency on both AChE and BuChE, making **13** and **15** the most potent ChEI within this series.

As previously reported,[27] the inhibition of ChEs by carbamates involves the formation of a reversible complex, followed by carbamoylation of the enzyme and production of a covalent adduct (k_3, carbamoylation rate constant). The carbamoylated enzyme is then hydrolyzed by water to regenerate the free enzyme (k_5, decarbamoylation rate constant). After the reversible complex formation, the carbamoylation phase of the reaction is considerably more rapid than the decarbamoylation phase (i.e., $k_3 \gg k_5$), and the two phases can be characterized separately.[28,29] Therefore, we investigated the rate of inhibition by determining k_3, and the reactivation phase, by determining k_5, for two representative compounds of the new series bearing the phenyl-pentyl (**13**) and 7-morpholinoheptyl substituent (**14**) at the carbamic nitrogen, respectively, with the final aim to compare the mode of action of the new inhibitors.

The data obtained clearly indicate that the nature of the R substituent at the *N*-carbamoyl group plays a role in differentiating both the inhibitory potency and the kinetics of inhibition. In particular, the 7-morpholinoheptyl derivative **14** carbamoylated and decarbamoylated both cholinesterases more slowly (lower k_3 and k_5 values, see Table S1 in the Supporting Information) than the phenylpentyl analogue, in agreement with previous studies with other morpholino-alkyl carbamates.[18] Our data also show that independently from the R substituent, the inhibition kinetics is much faster on hBuChE than on hAChE (Figure S1 in the Supporting Information). Compound **13** showed the highest rate of carbamoyl-ChE formation (k_3 = 0.212 min^{-1} on AChE and 21.4 min^{-1} on BuChE), nearly 2 times higher than **14** on AChE and 24 times higher on BuChE (k_3 values for **14**, 0.109 min^{-1} on AChE and 0.893 min^{-1} on BuChE, Table S1 in the Supporting Information).

In parallel, the recovery of the enzyme activity after inhibition followed a similar trend; values of decarbamoylation constants k_5 indicate that velocity of the hydrolysis depends on the R residue and is higher for the phenylpentyl moiety on both cholinesterases. For a better understanding of the rate of the decarbamoylation process, it might be easier to compare the % of residual activity at a fixed time of dialysis. Indeed, after inhibition by **13**, 83% of initial hBuChE activity and only 30% of the initial hAChE activity were recovered after 48 h of dialysis. The k_5(**13**)/k_5(**14**) ratio is 1.87 on hAChE and 5.11 on hBuChE. These data are in agreement with results obtained by Perola[29] and with our docking studies.[18]

In summary, compound **13**, bearing the phenylpentyl substituent at the *N*-carbamoyl group, resulted the most potent and selective BuChE inhibitor in the series, and also one of the most potent BuChE inhibitors currently known. In the light of the recent hypothesis regarding a role for BuChE inhibitors in the treatment of AD, the inhibition profile of **13** might offer advantages in severe forms of AD. Some general considerations might be drawn about the physiological relevance of the kinetics profiles obtained in vitro by comparing data in Table S1 in the Supporting Information with those obtained for rivastigmine, a butyryl-selective anti-Alzheimer's drug that acts as ChEI and is structurally characterized by the presence of a carbamic function. The interaction of hChE with rivastigmine was extensively investigated by Bar-On[30] and partially re-evaluated in this study for a better comparison. Specifically, the k_3 value for rivastigmine on hAChE resulted 0.096 min^{-1}, which makes rivastigmine slightly slower than **13** and as fast as **14**.

Noteworthy, compounds **13** and **15** also exhibited high potency at inhibiting FAAH-catalyzed hydrolysis of anandamide by rat brain membranes. This dual activity renders these compounds a potentially more efficacious treatment for the counteraction of AD progression or, at least, of disorders

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characterized by defective cholinergic and endocannabinoid signaling.

In conclusion, from our in vitro studies on isolated cholinesterases, it might be stated that **13**–**16** show a similar kinetic behavior with respect to rivastigmine, being from 11 to 21 times more potent AChEI and from 11 to 192 times more potent BuChEIs. Furthermore, compounds **13** and **15** also exhibited high potency against rat brain FAAH, which, for **15**, was maintained in the submicromolar range on human recombinant FAAH. Further specific investigations will be needed to establish that the rational design of "dual" cholinesterase-FAAH inhibitors is feasible and applicable to the treatment of AD.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures of target compounds. 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.

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■ ABBREVIATIONS

AD, Alzheimer's disease; Aβ, β-amyloid peptide; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ChEI, cholinesterase inhibitors; FAAH, fatty acid amide hydrolase; eCBs, endocannabinoid system

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