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Novel carbamate cholinesterase inhibitors that release biologically active amines following enzyme inhibition

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ABSTRACT

Conjugation of the phenol derived from rivastigmine with amphetamines gave access to novel carbamate cholinesterase inhibitors. All compounds possessed increased affinity and selectivity for AChE compared to rivastigmine and were orally bioavailable. Compound **4a**, incorporating *d*-amphetamine, caused significant inhibition of cholinesterase in vivo at doses that were well tolerated. Release of amphetamine from **4a** was demonstrated following in vitro and in vivo inhibition of cholinesterase. Compound **4a** was also effective in alleviating scopolamine induced amnesia in a rat passive avoidance model.

Cholinesterase inhibitors (ChEIs) of the carbamate type ('stigmines') have been known for decades. Towards the end of the 19th century, physostigmine found medicinal use in the treatment of glaucoma. More recently, physostigmine has been explored for treatment of Alzheimer's disease.[1] However, the severity of the side effects associated with high doses of physostigmine has spurred the search for other carbamate cholinesterase inhibitors that are safer and better tolerated. Examples of such stigmines are the approved AD drug rivastigmine[2] (marketed as Exelon®) and the experimental AD drug phenserine[3] (Fig. 1). Although these second generation cholinesterase inhibitors are better tolerated than physostigmine, their application is still hampered by limited efficacy and a narrow therapeutic window.[4]

Stigmines inhibit AChE by transferring their carbamoyl group to a serine residue in the active site[5] (semi-irreversible inhibition, see Fig. 2). The covalently bound carbamate is slowly hydrolyzed to reconstitute the active enzyme. During this process, a carbamic acid is released that in turn dissociates into carbon dioxide and an amine. In known stigmines, this amine is a small molecule that is considered pharmacologically inactive. We hypothesized that this mechanism could be leveraged to release a biologically active amine during the process of carbamic acid dissociation. Thus, these stigmines could provide both cholinesterase inhibition and actions at additional relevant targets in a single molecule, potentially leading to increased efficacy and tolerability compared to known cholinesterase inhibitors.

Attempts to provide ChEIs with additional pharmacology have been published.[6–10] In contrast to our model, the published approaches required significant structure–activity modifications to retain both ChE inhibition and the secondary pharmacology within a single molecule. We have developed a general approach towards ChE inhibitors with dual activity. By relying on the release of a known amine with beneficial pharmacological properties, we expect to gain access to molecules incorporating a variety of activities with minimal structural optimization.

In this Letter, we present the synthesis and in vitro and in vivo inhibitory activity of a number of novel carbamate ChEIs. Rates of decarbamylation of AChE following inhibition by the novel conjugates are determined. Amine release following inhibition of cholinesterase is demonstrated in vitro as well as in vivo. Finally, behavioral and cognitive effects are described.

Initial efforts were directed towards synthesis of rivastigmine-based conjugates of amphetamine isomers. Rivastigmine was chosen as starting point for the novel compounds because it has proven clinical utility in the management of Alzheimer's disease and because the corresponding phenol is chemically well characterized, accessible and stable. Amphetamines were selected due to their attractive and well characterized pharmacological properties.

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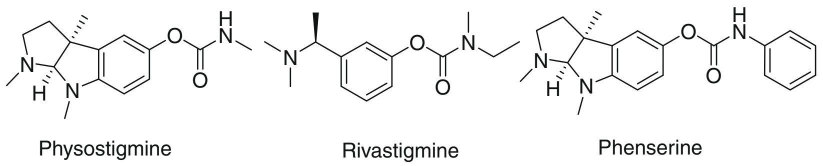


Figure 1. Representative examples of carbamate cholinesterases ('stigmines').

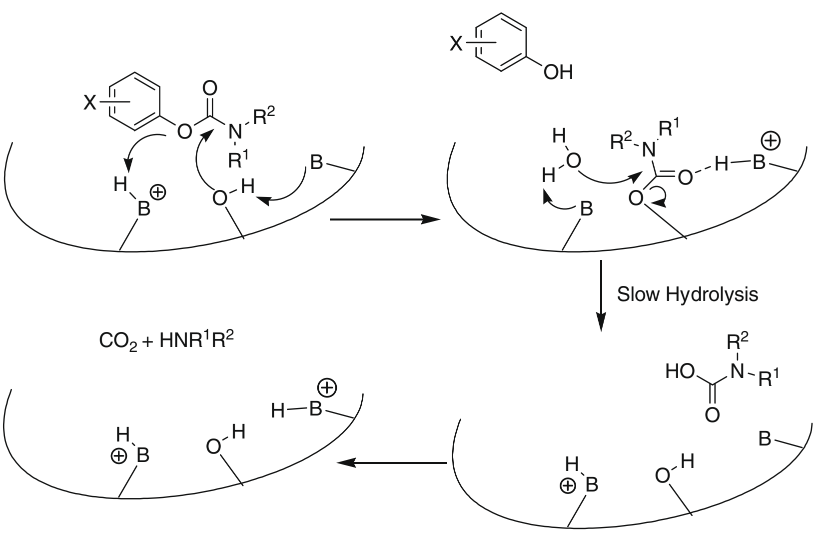


Figure 2. Schematic mechanism of action for cholinesterase inhibition by carbamates.

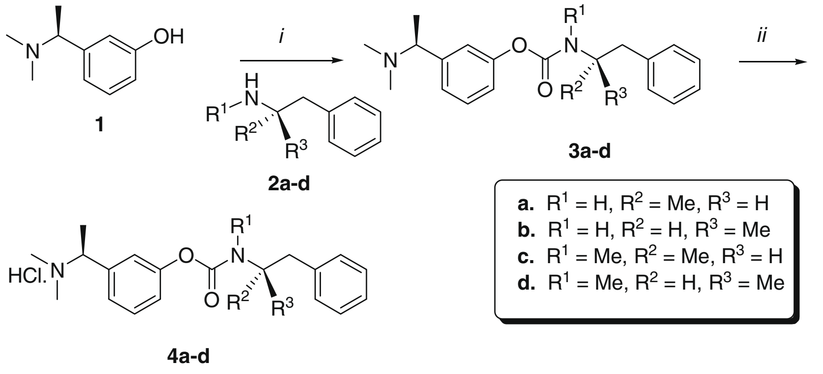
Amphetamines are neurotransmitter uptake inhibitors[11] with stimulant properties[12,13] which could be beneficial in the treatment of (geriatric) depression and fatigue, two symptoms that often accompany Alzheimer's disease. In addition, *d*-amphetamine is a well known cognitive enhancer[14–19] and it has recently been shown that *l*-isomers of the amphetamines are potent cognitive enhancers possessing only limited stimulant properties.[20]

Synthesis of the target compounds was accomplished as depicted in Scheme 1. Thus, (−)-3-hydroxyphenylethyldimethylamine[21] **1** was reacted with carbonyl diimidazole in dry ethyl acetate to form the activated imidazolide. Addition of the amphetamine isomers (**2a–d**) gave access to the target carbamates **3a–d** in good yields (40–60% after purification by column chromatography). Free base carbamates **3a–d** were converted into the corresponding hydrochloride salts **4a–d** by addition of hydrogen chloride in diethyl ether followed by removal of the solvents. The identity and purity of the resulting white solids was firmly established by ^{1}H NMR and HPLC analysis.

Having carbamates **4a–d** in hand, attention was focused on their inhibitory potential against AChE (human recombinant) and BuChE (human, purified from serum). Cholinesterase activity was determined spectrophotometrically by a modified Ellman procedure.[22] The results of these experiments are provided in Table 1 and demonstrate that the novel carbamates were all more potent inhibitors of acetylcholinesterase than the parent structure (rivastigmine).

Interestingly, all novel carbamates had less affinity for butyrylcholinesterase than rivastigmine. Consequently, all four novel inhibitors were more selective AChE inhibitors than rivastigmine. The increase in AChE selectivity was especially pronounced for compounds **4a** (incorporating *d*-amphetamine, which was 225-fold more selective for AChE than rivastigmine) and **4d** (incorporating *l*-methamphetamine, which was 1200-fold more selective than rivastigmine).

No clear structure–activity rules can be established from the data in Table 1. For example, the compound incorporating the *d*-isomer of amphetamine (**4a**) is 13-fold more selective than that incorporating the *l*-isomer (**4b**), whereas incorporation of the *d*-isomer of methamphetamine (**4c**) results in a compound with 20-fold less selectivity than in case of incorporation of the *l*-isomer



Scheme 1. Reagents and conditions: (i) carbonyldiimidazole, then **2a–d**, 40–60%; (ii) HCl, Et_2O, quant.

Table 1

Inhibitory concentrations (IC_{50}'s) of cholinesterases **4a–d** and the parent structure rivastigmine against recombinant human acetylcholinesterase and purified human butyrylcholinesterase (plasma)

Compd	Amine	rhAChE IC_{50}[a] (nM)	hBuChE IC_{50}[a] (nM)	Selectivity[b]
Rivastigmine	N/A	2615	179	1:15
4a	*d*-Amphetamine	508	7322	14:1
4b	*l*-Amphetamine	404	416	1:1
4c	*d*-Methamphetamine	131	334	3:1
4d	*l*-Methamphetamine	302	24400	81:1

[a] Values represent the mean of two or three independent experiments, each performed in triplicate. Standard deviations were typically within 10% of the IC_{50} value.

[b] Selectivity (IC_{50} hBuChE/IC_{50} rhAChE).

(**4d**). The addition of a methyl substituent on the *d*-amphetamine backbone results in a fourfold loss of selectivity (compare **4a** and **4c**), whereas the same substitution results in a 70-fold increase in selectivity in case of *l*-amphetamines **4b** and **4d**. The observed IC_{50} reflects a complicated interplay between affinity for the enzyme, rate of enzyme carbamylation and rate of reconstitution of enzymatic activity. Although more detailed studies are required to fully explain the observed SAR, it is clear that replacement of the methylethylamine moiety in rivastigmine with pharmacologically active amines can be tolerated.

Having established the in vitro inhibitory potential of the novel stigmines, we next evaluated whether these conjugates were effective in vivo inhibitors of ChE. Initially, the Maximum Tolerated Dose (MTD) was determined for each compound. The MTD was the dose at which clear, yet reversible and non-life threatening cholinergic effects were observed. The data in Table 2 clearly demonstrate that the novel ChEIs were better tolerated than their parent structure rivastigmine. Especially in case of *d*-amphetamine derivative **4a**, side-effects were very mild. At the highest dose tested (100 mg/kg), only moderate cholinergic effects were observed (mild fasciculations, some salivation). For comparison, rivastigmine (5 mg/kg, po) resulted in increased diarrhea, and caused seizures and severe fasciculation, salivation and lacrimation. It should also be noted that even after the highest dose of **4a**, corresponding to 41 mg/kg *d*-amphetamine, no stimulant effects (e.g., locomotor stimulation) were observed, suggesting that initial release of *d*-amphetamine following oral dosing of **4a** is low.

To establish the in vivo cholinesterase inhibition properties of the novel conjugates, rats were dosed orally with saline solutions of the test compounds at the MTD (or at the highest dose tested in case of **4a** and **4c**). Following administration, rats were sacrificed and blood and brain samples were collected, processed and ChE activity was determined spectrophotometrically as described above. Initially, cholinesterase inhibition was quantified at 30, 60 and 180 min following oral dosing of rivastigmine to assess time-dependence. As there was no consistent difference in the level of inhibition achieved between these times (Bonferroni *t*-test), which is in accord with reports of its long duration of pseudo-irreversible inhibition,[23] plasma and brain levels of cholinesterase inhibition of all other compounds were determined at a single time point (60 min). All compounds resulted in significant inhibition of cholinesterase following oral dosing in both plasma and brain, demonstrating the oral bioavailability and blood–brain barrier penetration of compounds **4a–d**.

Table 2

Inhibition of total brain cholinesterase and total plasma cholinesterase 60 min following oral dosing of compounds **4a–d** and rivastigmine at the maximum tolerated dose or highest dose tested

Compd	Amine	MTD (mg/kg)	Plasma ChEI[a] (%)	Brain ChEI[a] (%)
Rivastigmine[b]	N/A	5	39	56
4a	*d*-Amphetamine	>100	59	72
4b	*l*-Amphetamine	32	26	25
4c	*d*-Methamphetamine	>64	50	50
4d	*l*-Methamphetamine	10	45	64

[a] Values represent the mean of $n = 4$ animals per group. Standard deviations were typically within 10% of the determined cholinesterase activity.

[b] Rivastigmine data is the mean of three measurements between 30 and 180 min following dosing.

The data in Table 2 reveal that there is no clear correlation between plasma or brain ChE inhibition and maximum tolerated dose. Compound **4a**, incorporating *d*-amphetamine, resulted in significant inhibition of both brain (72%) and plasma (59%) ChE without causing severe side effects (as assessed by salivation, lacrimation and overt behavior). On the other hand, significant side effects were observed following a dose of 32 mg/kg of compound **4b**, associated with limited inhibition of brain (25%) and plasma (26%) ChE. Although the increase in MTD for **4a–d** could be explained by poor oral bioavailability, the high level of brain ChE inhibition without severe side effects following dosing with **4a** suggests that there are other factors that influence the MTD as well.

Next, the release of amphetamine from inhibited cholinesterase was investigated indirectly by monitoring reconstitution of enzyme activity as described above. Recombinant human cholinesterase was incubated with an excess of carbamates **4a–d**, resulting in >80% inhibition of enzyme activity. The enzyme was separated from small organic molecules (carbamate and possible degradation products) by size exclusion chromatography over a Sephadex column. Purified, inhibited enzyme was incubated in phosphate buffer at 37 °C, aliquots were drawn at various time points and the enzymatic activity was determined as described above. The data were plotted as the natural logarithm of the percent inhibition versus time according to a first-order kinetics model. The decarbamylation rate constant k was determined as the slope of this line. The half-life $t_{1/2}$ is directly related to k ($k = -\ln(0.500)/t_{1/2}$).

The results of the decarbamylation experiments are presented in Table 3. Reconstitution of activity was significantly faster for carbamates of primary amines (**4a** and **4b**) than for carbamates of secondary amines (rivastigmine, **4c** and **4d**). Hydrolysis of *d*-amphetamine from the enzyme was especially rapid. A rapid decarbamylation rate raises concerns about insufficient duration of cholinesterase inhibition. However, following oral doses of 16–64 mg/kg of **4a**, the in vivo cholinesterase inhibition at 3 h post-dosing was only 10–20% less than at 1 h post-dosing.

Based on the data presented above, compound **4a** was selected for further characterization. Compound **4a** shows potent and selective inhibition of AChE in vitro and is a potent inhibitor of brain ChE following oral dosing. More interestingly, doses of **4a** associated with high levels of ChE inhibition are very well tolerated. The relatively rapid rate of reconstitution of ChE activity following inhibition by **4a** suggests that relatively high levels of amine may be released following doses of this compound.

Table 3

Decarbamylation rates (k) and decarbamylation half-lives ($t_{1/2}$) following inhibition of rhAChE by compounds **4a–d** and rivastigmine

Compd	Amine	k (h^{-1})	$t_{1/2}$ (h)
Rivastigmine	N/A	0.007	>24
4a	*d*-Amphetamine	0.558	1.2
4b	*l*-Amphetamine	0.057	12
4c	*d*-Methamphetamine	0.003	>24
4d	*l*-Methamphetamine	0.011	>24

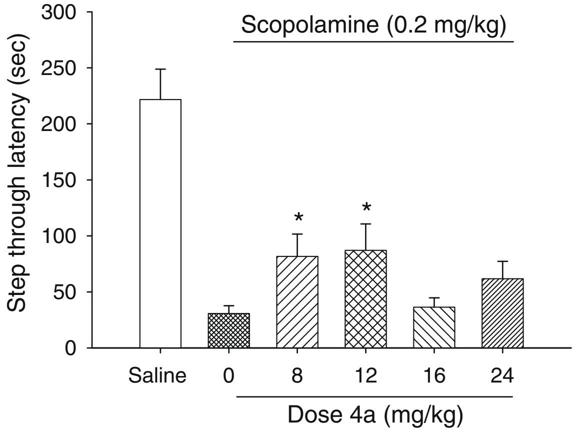


Figure 3. Step-through latencies in a rat passive avoidance model. Rats received a dose of 0.2 mg/kg scopolamine sc 40 min before training and doses of 0, 8, 12, 16 or 24 mg of **4a** ip 30 min before training.

In order to unequivocally demonstrate that enzyme reconstitution results in release of amphetamine, aliquots from the decarbamylation experiments with **4a** were analyzed for levels of amphetamine using an LC/MS/MS method.[24] It was demonstrated that aliquots drawn at $t = 0$ did not contain amphetamine, whereas aliquots drawn 4 h later (corresponding to 80% reconstitution) contained 53 ng/mL of *d*-amphetamine. Similarly, plasma collected from rats 1 h following oral doses (64 mg/kg) of **4a** contained significant amounts of amphetamine (17 ng/mL).

Finally, in order to determine whether compound **4a** had any memory enhancing properties, we investigated its effects in a scopolamine model of passive avoidance.[25] Prior to training, rats were injected with scopolamine hydrobromide (0.2 mg/kg, sc, 40 min before training) and with either saline or compound **4a** (8, 12, 16 or 24 mg/kg, ip, 30 min prior to training). The animals received a retention test 24 h following training. The retention test was identical to training except that no foot-shock or drug was delivered. Latency to enter the dark chamber was recorded. As shown in Figure 3, pre-training administration of scopolamine produced a robust and statistically significant amnesia ($p < 0.0001$). This amnesia could be alleviated by doses of 8 and 12 mg/kg of compound **4a** ($p < 0.01$). These results clearly demonstrate that compound **4a** is able to improve mnemonic performance in rats. Moreover, the effectiveness of **4a** in this model was comparable to the effect of rivastigmine. Interestingly, the approved AD drug donepezil was less effective in this model than **4a**, and only showed a trend toward reversal of amnesia ($p = 0.09$).

In conclusion, we have shown that bifunctional cholinesterase inhibitors can be prepared through combination of a pharmacologically active amine and the phenol moiety of a known cholinesterase inhibitor in a single molecule. The resulting hybrid molecules retain their ability to inhibit cholinesterase, both in vitro and in vivo, and as demonstrated for **4a** release pharmacologically active amines following decarbamylation of the inhibited enzyme.

The high level of brain and plasma cholinesterase inhibition in absence of severe side effects following oral doses of **4a** is unprecedented and suggests that novel bifunctional cholinesterase inhibitors may have a greater therapeutic window than currently known cholinesterase inhibitors. Since it can be expected that peripheral cholinergic effects are antagonized by the release of adrenergic agents, the increased tolerability may be a reflection of in vivo pharmacological effects of released *d*-amphetamine. However, further research is warranted to confirm this hypothesis.

Acknowledgments

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