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Multiple enzyme inhibitions by histamine H_3 receptor antagonists as potential procognitive agents

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Novel highly affine histamine H_3 receptor ligands with additional inhibitory effects on the main histamine metabolizing enzyme in the brain, *N*-methyltransferase, chemically show structural elements of the acetylcholinesterase inhibitor tacrine. H_3 receptor antagonism, inhibition of metabolisation of neuronal histamine as well as inhibition of hydrolysis of acetylcholine are each one believed to improve reduced cognitive functions, which is useful for symptomatic treatment of Alzheimer's disease. Some of the new compounds proved in a slightly modified colorimetric Ellmann's assay to be potent inhibitors of acetylcholinesterase and of butyrylcholinesterase which is another catalytic enzyme hydrolysing acetylcholine. Some compounds with (sub)nanomolar activities on the histamine-related targets are also active in the nanomolar concentration range on both cholinesterase targets being 5- to 40-times more potent than tacrine. Preliminary structure-activity relationships could already be drawn from the small number of compounds. The compounds acting as hybrid drugs simultaneously on four different targets to enhance cognitive functions via different pathways are promising lead structures for a new approach in the treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease is the most common neurodegenerative disease affecting some 20 million people worldwide. At least the initial phase of the disease can be perceived as 'cognitive disorder'. Due to aging of the population in industry nations this health problem will tremendously increase in the near future. Degeneration of cholinergic basal forebrain neurons innervating the cortex is believed to contribute substantially to the cognitive deficits. Therefore, acetylcholinesterase (AChE, EC 3.1.1.7) as the metabolizing enzyme of the neurotransmitter was detected as target. The first inhibitor of AChE marketed was tacrine (Crismon 1994). Despite the long-standing discussion on the benefit of AChE inhibitors recently reignited by the AD2000 study on donepezil (AD2000 Collaborative Group 2004; Giacobini 2003; Farlow 2002), primary symptomatic treatment of Alzheimer's disease is mainly based on AChE inhibitors.

Numerous other therapeutic approaches for the treatment of cognitive impairment are currently under development (Witkin and Nelson 2004). Acetylcholine is specifically hydrolyzed by AChE, but butyrylcholinesterase (BuChE, EC 3.1.1.8), also known as serum cholinesterase or pseudo-cholinesterase, also hydrolyzes the neurotransmitter in the brain. Since both enzymes are putatively involved in the (patho)physiological mechanisms related to neurodegenerative disorders, such as Alzheimer's disease and dementia with Lewy bodies, both of them are suitable targets to increase cholinergic neurotransmission. Tacrine and rivastigmine share these dual properties inhibiting both enzymes in contrast to donepezil and galantamine which have different pharmacological properties (Ballard 2002).

A different approach to fight cognitive disorders is taken with the stimulation of the central histaminergic system. Histamine H_3 receptor antagonists stimulate the histaminergic system by presynaptic inhibition of a negative feedback mechanism for the inhibition on the synthesis and the release of histamine. H_3 receptor antagonists have been proven to have preventive effects in scopolamine- and MK-801 (dizocilpine)-induced cognitive impairment as well as to have numerous other procognitive and arousal effects (Stark

Abbrevations

AChE acetylcholine esterase,
BuChE butyrylcholine esterase,
CI confidence interval,
DTNB 5,5′-dithiobis-(2-nitrobenzoic acid),
HMT histamine *N*-methyltransferase,
TNB^- 5-thio-2-nitrobenzoate

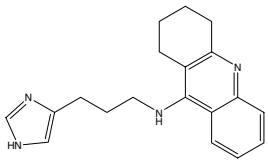
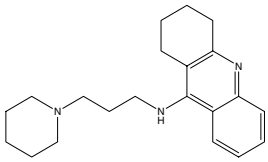
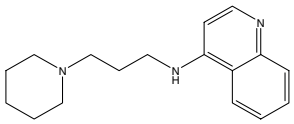
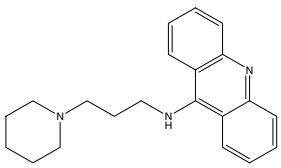
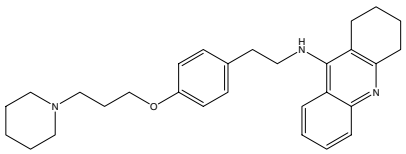
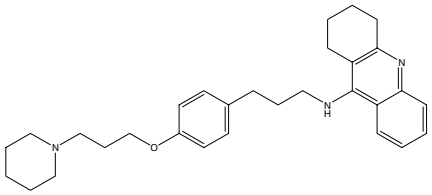
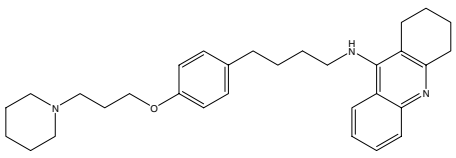
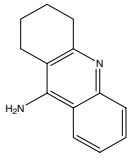
2003). Interestingly, tacrine was shown to inhibit also histamine *N*-methyltransferase (HMT, EC 2.1.1.8) (Cumming and Vincent 1992; Morisset et al. 1996), the main histamine metabolizing enzyme in the human brain. This led to the development of a new class of potentially procognitive hybrid compounds combining histamine H_3 receptor antagonist and HMT inhibitory potencies (Apelt et al. 2002; Grassmann et al. 2003). Since these compounds have similar structural features to tacrine it was of crucial interest to test selected compounds for their inhibitory activities on AChE and BuChE. These integrated pharmacological features of hybrid compounds should act additively or synergistically on the improvement of cognitive functions.

Selected compounds of different lead developments were tested on their inhibitory activity on AChE and on BuChE using a slightly modified colorimetric Ellmann's method. The data were compared with the data previously obtained on histamine H_3 receptor affinity and HMT inhibitory potency.

2. Investigations, results and discussion

The novel compounds showed poor to good inhibitory potencies on AChE as well as on BuChE (Table). At maximum concentration full inhibition of esterases was achieved with all compounds tested. Since most of the classic histamine H_3 receptor antagonists are imidazole-

Table: Chemical structures and pharmacological screening results of novel tacrine derivatives

Cmpd.	Structure	hH_3 K_i [nM][a]	HMT IC_{50} [nM][b]	AChE IC_{50} [nM] (95% CI)[c]	BuChE IC_{50} [nM] (95% CI)[d]
1 FUB770		93	35	111 (96–125)	127 (78–176)
2 FUB734		34	45	269 (252–285)	67 (52–82)
3 FUB735		85	64	40,000 (37,000–43,000)	25,400 (23,000–28,000)
4 FUB816		188	110	1,900 (1,700–2,000)	3,500 (3,000–4,000)
5 FUB833		0.33	48	2.6 (2–3.2)	8.8 (5.5–12.0)
6 FUB834		1.4	95	8.6 (7.8–9.5)	10 (6.8–13.2)
7 FUB835		1.8	48	3.1 (2.8–3.4)	9.4 (6.8–12.0)
Tacrine		n.d.[e]	110	105 (50–160)	64 (34–93)

[a] [^{125}I]iodoproxyfan binding assay to human histamine H_3 receptors stably expressed in CHO cells (Apelt et al. 2002; Grassmann et al. 2003); [b] HMT assay on isolated enzyme from rat kidneys (Apelt et al. 2002; Grassmann et al. 2003); [c] AChE assay on whole blood samples from humans (Worek et al. 1999), CI confidence interval; [d] BuChE assay on heparinized human blood (Worek et al. 1999), CI confidence interval; [e] n.d. not determined

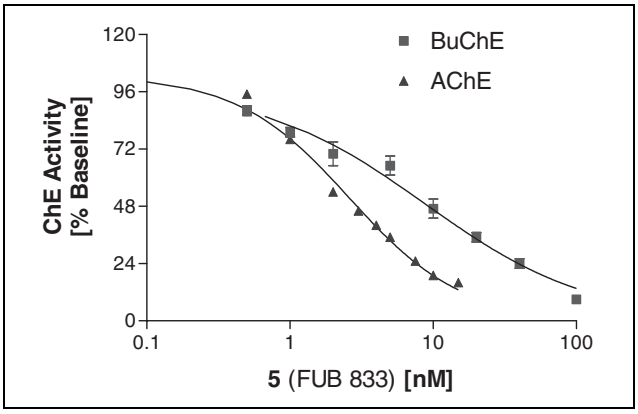


Fig.: Inhibitory activities of **5** on AChE and BuChE

containing compounds we started with compound **1** for initial screening, which showed inhibitory potencies on cholinesterases comparable to those of the reference compound tacrine. In **1** the tacrine moiety is connected by a three methylene linker to an imidazole ring. Replacement of the imidazole aromate by a piperidino group to **2** as performed in state-of-the-art antagonists (Meier et al. 2001; Stark 2003) maintained inhibitory activity with a slight decrease for AChE and slight increase for BuChE and hH_3 receptors as compared to that of **1**. HMT activity stayed almost in the same range. Variation of the tacrine moiety in the piperidinopropyl series by deletion of the four ring methylene groups (**3**) or introduction of additional double bounds to another aromatic ring (**4**) led to a drastic decrease (**4**) or even a loss of inhibitory cholinesterase activities (**3**). Therefore, further variations were performed on the linking group between a conserved tacrine element and piperidino group. Chain elongation by an oxyphenylalkyl group led to compounds with high inhibitory potencies on the cholinesterases as well as on hH_3 receptors. The inhibitory potencies on HMT were nearly the same for compounds **1**, **2**, **5**, and **7**, and were for these substances about twice as good as that of tacrine or **6**. Here, compound **5** is the most promising lead given that it displays subnanomolar affinity for hH_3 receptors, low nanomolar IC_{50} values for both cholinesterases (Fig.) and good affinity for HMT. Since compound **5** displayed different slopes in esterase inhibitions (cf. Fig.; AChE: −0.57; BuChE: −0.34: expressed as difference in activities %/log[C_2] – log[C_1])) additional properties cannot be excluded. The high affinity hH_3 receptor ligands tested for their affinity have also been shown to act as inverse agonist (antagonist) on a functional screening in rats (Apelt et al. 2002). Since these enzymes and the G-protein coupled receptor display different densities and different signalling behaviour within different areas of the brain it is not clear what would be the best balanced activity profile for a procognitive agent (Stark 2004). Further *in vivo* studies on these compounds or on further developments are eagerly awaited and clearly needed to clarify these topics. Nevertheless, it is clear that these potential drugs can simultaneously act at multiple targets. The application of a hybrid drug has clear pharmacokinetic advantages since in contrast to a cocktail of drugs one has less potential metabolic pathways and only one half-life and t_{max} to follow.

Cholinesterase inhibitors have produced the best evidence of clinical efficacy for treating Alzheimer's patients. Since both AChE and BuChE hydrolyse acetylcholine, inhibition of both enzymes should result in higher neurotransmitter levels than by one inhibition only. Despite the inactivation of acetylcholine the enzymes may display multiple, unrelated biological functions (e.g. neuritogenesis, cell adhesion, synaptogenesis, modulation of dopaminergic neurons, amyloid fibre assembly, haematopoesis (Giacobini 2001; Soreq and Seidmann 2001; Darvesh et al. 2003), which may be beneficial in the treatment of cognitive deficiencies. It may be of further interest if the compounds presented here display any effect on β-amyloid aggregation or not (Dorronsoro et al. 2003).

Previously we have shown that these compounds act also as histamine hH_3 receptor antagonists and inhibitors of HMT. The additional inhibitory activities at both cholinesterases add pharmacological properties, which clearly benefit the supposed potential therapeutic target of cognitive impairment. Two structurally related compounds having combined high hH_3 receptor antagonist and inhibitory HMT potencies have previously been screened *in vivo* on their potency to modulate brain histamine levels after oral application to mice (Apelt et al. 2002). Unfortunately, these compounds showed only moderate or missing effect on brain histamine levels in this assay. Presently, it is unclear if pharmacokinetic problems related to absorption or distribution or problems of pharmacodynamic receptor cross-talk may be the reasons for the missing proof of the starting working hypothesis. These compounds were excluded from the investigations presented here because they have failed under *in vivo* conditions. Maybe other neurotransmitter receptors or metabolic pathways must be taken into consideration for further *in vitro* evaluation of these lead structures.

Taken these results together one may conclude that the requirement for activity at multiple different pharmacological targets for the treatment of cognitive impairment is satisfied with the design, synthesis and *in vitro* testing of compounds **5**–**7** and with lower potencies for compounds **1** and **2**. All these compounds act simultaneously at hH_3, HMT, AChE and BuChE. Despite these positive results additional (behavioural) experiments on their *in vivo* effects on cognitive disorders have to be performed to prove the concept of these hybrid compounds as procognitive agents.

3. Experimental

3.1. Pharmacology

3.1.1. Acetylcholinesterase assay

The AChE activity was measured in diluted whole blood samples from human volunteers in the presence of the selective BuChE inhibitor ethopropazine as previously described (Worek et al. 1999). The assay, which is based on Ellman's method (Ellmann et al. 1961), measures spectrophotometrically the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellmann's reagent) to 5-thio-2-nitrobenzoate (TNB^-) by thiocholine, the product of acetylthiocholine hydrolysis. Freshly drawn venous blood samples were diluted in 0.1 M phosphate buffer (pH 7.4) and incubated with DTNB (10 mM) and ethopropazine (6 mM) for 20 min at 37 °C prior to addition of acetylthiocholine. The change in the absorbance of DTNB was measured at 436 nm. The AChE activity was calculated using an absorption coefficient of TNB^- at 436 nm ($e = 10.6\ mM^{-1}$ cm). The values were normalized to the haemoglobin (Hb) content (determined as cyanmethaemoglobin) and expressed as mU/μmol Hb (Van Kampen and Zijlstra 1961). Blood samples from human volunteers were used (n = 5, 2 males; 3 females). None of the volunteers was on any drugs. Enzyme activities were determined in the absence of (baseline value) and then after addition of increasing concentrations of the test substance (a minimum of eight different concentrations were used). All enzyme activities measured in the presence of an inhibitor were expressed as percentage of baseline value (100%). For the IC_{50} calculation the SlideWrite (Advanced Graphics Software Inc, Encinitas, CA/USA) software was applied (user defined equation $y = a_0/[1 + (x/a_1) \exp a_2]$) where the coefficient a_1 corresponds to the IC_{50} value. The IC_{50} values given are mean value of five separate measurements with the 95% confidence intervals (CI).

3.1.2. Butyrylcholinesterase assay

BuChE activity was measured in human plasma obtained from heparinized blood after centrifugation (10 min, 500 × g). Plasma was analyzed immediately or kept frozen in 1 ml aliquots until analysis. Plasma from human volunteers was used (n = 5, 2 males; 3 females). None of the volunteers was on any drugs. The assay which is based on Ellman's method (Ellmann et al. 1961), measures spectrophotometrically the reduction of DTNB to TNB^- by thiocholine, the product of butyrylthiocholine hydrolysis at 37 °C. The change in the absorbance of DTNB was measured at 436 nm. The BuChE activity was calculated using an absorption coefficient of TNB^- at 436 nm ($e = 10.6\ mM^{-1}$ cm). The values were expressed as μM/min (Worek et al. 1999). Further details are according to the conditions described in the AChE assay.

3.2. Chemicals and reagents

All chemicals and reagents used for the pharmacological assays were purchased from Sigma-Aldrich Chemie, Munich, Germany. The novel test compounds were prepared by H.S., Walter Schunack and Joachim Apelt.

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