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# Hybrid Molecules from Xanomeline and Tacrine: Enhanced Tacrine Actions on Cholinesterases and Muscarinic $M_1$ Receptors

Lei Fang,[‡,†,⊥] Sabine Jumpertz,[∥] Yihua Zhang,[†] Dorothea Appenroth,[§] Christian Fleck,[§] Klaus Mohr,[∥] Christian Tränkle,*[,∥] and Michael Decker*[,‡]

[†]*Center of Drug Discovery, China Pharmaceutical University, 210009 Nanjing, P. R. China,* [‡]*Lehrstuhl für Pharmazeutische/Medizinische Chemie, Institut für Pharmazie, Friedrich-Schiller-Universität Jena, D-07743 Jena, Germany,* [§]*Institut für Pharmakologie und Toxikologie, Friedrich-Schiller-Universität Jena, D-07740 Jena, Germany, and* [∥]*Pharmazeutisches Institut, Rheinische Friedrich-Wilhelms-Universität Bonn, D-53121, Germany.* [⊥] *Present address: School of Chemistry and Chemical Engineering, Southeast University, 211189 Nanjing, P. R. China.*

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A set of amide- and amine-linked hybrid molecules comprising moieties of the orthosteric $M_1$ muscarinic receptor agonist xanomeline and the cholinesterase inhibitor and allosteric receptor modulator tacrine were prepared with varying spacer length of 10−17 atoms. The hybrids inhibited acetylcholinesterase with similar or higher potency compared to tacrine. $M_1$ receptor binding affinity was similar or higher relative to xanomeline and far higher relative to tacrine. Affinities hardly changed when the receptors' orthosteric site was occupied by an inverse agonist ligand. When occupied by the orthosteric activator acetylcholine, affinity for the hybrids declined to unmeasureably low levels. Hybrids did not activate $M_1$ receptors. In vivo studies assaying cognition impairment in rats induced by scopolamine revealed pronounced enhancement of scopolamine action. Taken together, instead of dualsteric (simultaneous allosteric/orthosteric) binding, the hybrids seem to prefer purely allosteric binding at the inactive $M_1$ receptor.

## Introduction

The search for novel agents for the treatment of cognitive deficits in Alzheimer's disease (AD[a]) is guided by the "cholinergic hypothesis" which correlates cognitive deficits in AD patients with the decline of the number of cholinergic neurons. Consequently, an established therapeutic approach is to increase the amount of the neurotransmitter acetylcholine (ACh) in the brain by inhibiting the degrading enzyme acetylcholinesterase (AChE).[1]The AChE inhibitor tacrine (tetrahydroaminoacridine, **2** in Chart 1) represents an interesting pharmacological tool because it also binds to the allosteric site of muscarinic (M) receptors where it acts as an atypical modulator yielding steep concentration-effect curves.[2,3] Being devoid of intrinsic efficacy, tacrine modulates the binding and action of ligands that attach to the orthosteric acetylcholine site.[4]

In addition to AChE inhibitors, M receptor ligands, in particular of the $M_1$ subtype, have gained great relevance for the development of anti-Alzheimer drugs. Neurotransmission via muscarinic $M_1$ receptors can be stimulated either directly by muscarinic (orthosteric or allosteric) agonists or indirectly by allosteric agents that act as ACh enhancers.[5] $M_1$ stimulation seems to open a more than symptomatic treatment option because it can directly influence pathophysiological hallmarks of AD by decreasing A$\beta$42 and $\tau$ pathologies via activation of protein kinase C and formation of soluble forms of amyloid precursor protein among other pathways.[6,7] Unfortunately, the high homology of the orthosteric binding sites among the M receptor subtypes may result in pronounced side effects of the orthosteric $M_1$ agonists developed so far.[8] The less conserved allosteric binding site of the $M_1$ receptor seems to be a suitable alternative target,[9,10] although allosteric and $M_1$-selective enhancers of ACh binding described to date show comparatively weak potencies.[11,12]

Xanomeline (compound **1** in Chart 1) is a functionally selective $M_1$ agonist with promising antidementive properties in vivo,[13] but it suffers from unfavorable side effects that might be due to poor selectivity of pharmacologically active metabolites.[14] The molecular mode of xanomeline binding to the $M_1$ receptor is complex and not yet fully understood; besides an orthosteric interaction there is a wash-resistant component that seems to result from binding at a second site instead of a simple hydrophobic interaction of xanomeline's *O*-hexyl chain with the membrane.[15,16] There is an obvious need to exploit additional ways to achieve $M_1$-selective receptor activators.[12]

Notably, the hybrid molecule approach in which two distinct pharmacological (and chemical) classes of compounds are connected covalently in one molecule has been successfully applied to the design of antidementive agents.[17,18] Designed appropriately, such compounds not only maintain their pharmacological properties but may surpass the potencies of the parent compounds and sometimes even exhibit novel pharmacological activities.[17−19] Simultaneous orthosteric/allosteric binding of hybrid ligands to G-protein-coupled receptors can be achieved as has been reported for muscarinic acetylcholine receptors.[20−22] Notably, the rational design of

*To whom correspondence should be addressed. For C.T.: phone, +49-228-73-9104; fax, +49-228-73-9215; e-mail, traenkle@uni-bonn.de. For M.D.: phone, +49-3641-949826; fax, +49-3641-949802; e-mail: m.decker@uni-jena.de.

[a] Abbreviations: AD, Alzheimer's disease; (h)ACh(E), (human) acetylcholine(esterase); BChE, butyrylcholinesterase; CHO, Chinese hamster ovary; DMF, dimethylformamide; EATCM, extended allosteric ternary complex model; GTP$\gamma$S, guanosine 5′-*O*-[$\gamma$-thio]triphosphate; NMS, *N*-methylscopolamine; TBDPSCl, *tert*-butylchlorodiphenylsilane; THF, tetrahydrofuran.

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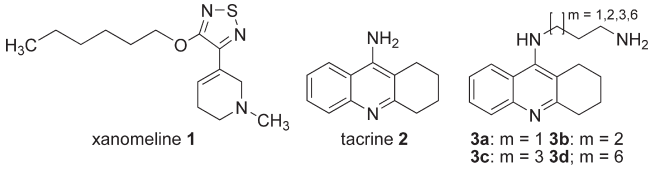
such dualsteric ligands yielded $M_2$ receptor activators with receptor subtype selectivity and signaling pathway specificity.[20,22−24] Combining tacrine and xanomeline moieties within one molecule can lead to compounds activating the $M_1$ receptor directly (xanomeline part) and indirectly by increasing the amount of neurotransmitter (tacrine part). Since this effect might be most pronounced at the site of the $M_1$ receptors, a higher selectivity may result in vivo. Furthermore, a dualsteric compound concomitantly binding to the ortho- and the less conserved allosteric binding sites might create more potent and selective ligands on the receptor level.[21,22] Hybrid molecule design has been successfully used to optimize the binding profile of at least one of the hybrid components, in most cases the ChE inhibiting part.[17−19]

Within this work we designed and synthesized a set of hybrid molecules comprising moieties of tacrine and xanomeline connected by distinct chemical spacer structures (amines and amides) and a broad range of spacer lengths. Furthermore, the connecting amide group was located at different positions within the spacer. We investigated two issues: First, a novel type of antidementive compound would emerge if the AChE inhibiting properties of tacrine are combined with the $M_1$ agonist properties of xanomeline. Second, topologically, the allosteric binding properties of tacrine that are known to affect both ACh binding proteins (AChE[25,26] and M receptors[2,3]) might be linked to the orthosteric binding properties of xanomeline if a suitable spacer length for a bivalent interaction could be identified. These compounds might show increased affinity and even isoenzyme or/and receptor-subtype selectivity. We studied the interaction of the newly synthesized compounds with the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as with $M_1$ receptors, aiming in particular at elucidating the binding topography of the hybrid compounds.

**Chart 1.** $M_1$ Agonist Xanomeline **1**, AChE Inhibitor/Atypical Allosteric Modulator Tacrine **2**, and Aminoalkyltacrines **3a−d**

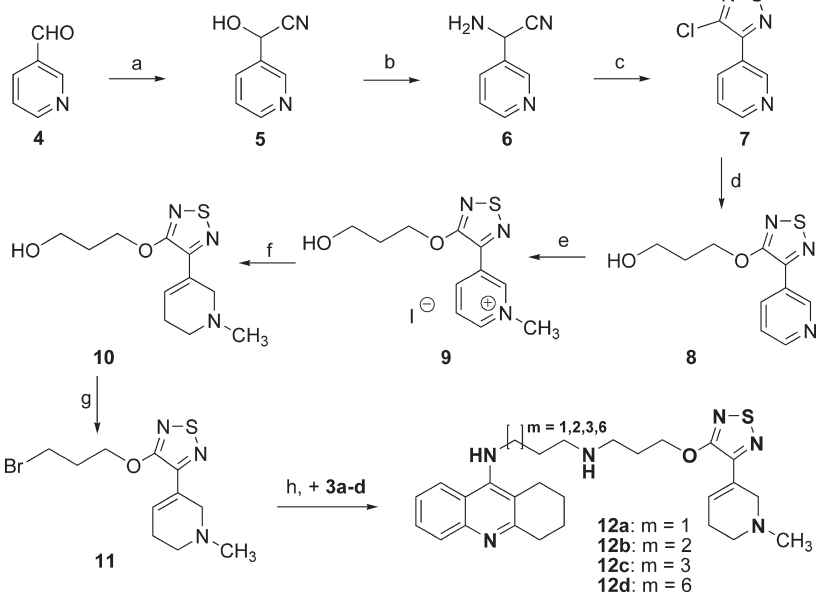



## Results and Discussion

**Chemistry.** For the synthesis of the xanomeline−tacrine hybrids (both linked by an amine bond and amide bond) the tacrine-part key intermediates aminoalkylamino-1,2,3,4-tetrahydroacridines **3a−d** (i.e., tacrine connected to a aminoalkyl spacer) were synthesized as previously described:[27] Anthranilic acid and cyclohexanone were condensed to yield 9-chloro-1,2,3,4-terahydroacridine,[26,27] which in turn reacted with α,ω-diamines of variable length (three, four, five, and eight carbon atoms, respectively) to yield compounds **3a−d** (Chart 1).

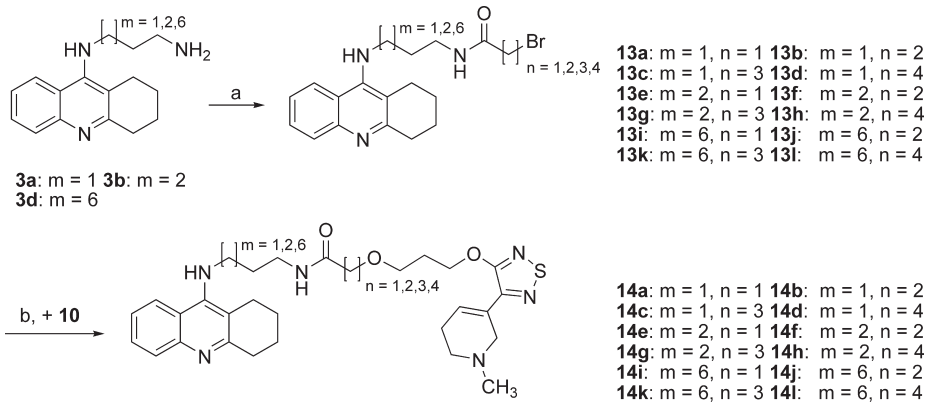
As the second key intermediate the xanomeline derivative **10** with a hydroxy function in the alkyl moiety was applied (Scheme 1). Compound **10** was synthesized out of 3-pyridinecarbaldehyde **4** which yields in a Strecker synthesis 2-amino-2-(pyridine-3-yl)acetonitrile **6** after reaction of the alcohol **5** with ammonia.[16,28] Compound **6** can be cyclized with sulfur monochloride in DMF to 3-(3-chloro-1,2,5-thiadiazol-4-yl)pyridine **7**.[16,28] Propane-1,3-diol reacted in the presence of sodium hydride with the chloro compound **7** to 3-[(4-pyridin-3-yl-1,2,5-thiadiazol-3-yl)oxy]propan-1-ol **8** (Scheme 1). In a recently described synthesis for preparation of xanomeline derivatives in which the *O*-hexyl chain was functionalized, one of the hydroxyl groups was protected with *tert*-butylchlorodiphenylsilane (TBDPSCl) which was not necessary in our synthesis.[16] Methylation of the pyridine-N followed by reduction using sodium borohydride led to key intermediate 3-(4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yloxy)propan-1-ol **10** (Scheme 1). The primary hydroxy group of **10** was replaced by a bromine

**Scheme 1.** Synthesis of Amine-Bridged Xanomeline−Tacrine Hybrids **12a−d**[a]




[a] Reagents: (a) KCN, $H_2O$, AcOH, 10 °C, 18 h; (b) $NH_3$(aq), $NH_4Cl$, room temp, 18 h; (c) $S_2Cl_2$, DMF, 10 °C, 1.5 h; (d) (i) NaH, propane-1,3-diol, THF, reflux, 1 h; (ii) +**7**, reflux, 24 h; (e) $CH_3I$, acetone, room temp, 18 h; (f) $NaBH_4$, abs MeOH, room temp, 24 h; (g) 3 eq.uiv of $PPh_3$, 6.5 equiv of $CBr_4$, $CH_2Cl_2$, room temp, 24 h; (h) $K_2CO_3$, cat. KI, $CH_2Cl_2$, room temp, 18 h.

**Scheme 2.** Synthesis of Amide-Bridged Xanomeline−Tacrine Hybrids **14a**−**l**[a]




[a] Reagents: (a) $Br(CH_2)_nCOCl$, $CH_2Cl_2$, −5 °C, 0.5 h; (b) $K_2CO_3$, cat. KI, $CH_2Cl_2$, room temp, 18 h.

atom using tetrabromomethane and triphenylphosphine in methylene chloride to yield **11**, which could be coupled directly by alkylation with aminoalkylamino-1,2,3,4-tetrahydroacridines **3a**−**d** to amine-bridged hybrids **12a**−**d** with 7, 8, 9, and 12 spacer atoms (not counting the tacrine N and xanomeline O atoms) (Scheme 1).

Amide bridged xanomeline−tacrine hybrids with variable spacer length of 10−18 spacer atoms (not counting the tacrine N and xanomeline O atoms) were also synthesized (Scheme 2). Since both the alkylene spacer length of the aminoalkylamino-1,2,3,4-tetrahydroacridines **3a**−**d** and the length of the connecting carboxylic acid were varied, identical spacer lengths could be realized with different positions of the connecting amide bond within the spacer: compounds **14b** and **14e** with 11, **14c** and **14f** with 12, **14d** and **14g** with 13 spacer atoms, respectively (Scheme 2). In the latter compounds the amide bond was one atom further down the spacer from the direction of the tacrine-like moiety. The amide-bridged hybrids were synthesized out of bromoacyl chlorides with aminoalkylamino-1,2,3,4-tetrahydroacridines **3a**,**b**,**d** yielding the halogenated intermediates **13a**−**l**.[27] Compounds **13a**−**l** could in turn be coupled with the xanomeline part key intermediate 1,2,5,6-tetrahydropyridine **10** in the presence of potassium carbonate and potassium iodide to yield amide-bridged hybrids **14a**−**l** (Scheme 2).

**Pharmacological Results.** All hybrid compounds were measured in vitro in a colorimetric assay for their ability to inhibit AChE and BChE (Table 1).[19,29] Remarkably, all compounds were very potent inhibitors of both cholinesterases irrespective of the considerable difference in spacer length (10−18 atoms). In addition, the type of the spacer (amine or amide with concomitant differences in physicochemical properties) was of minor importance. In a comparison of the two compounds with a 10-atom spacer (amine **12d** and amide **14a**), the amide showed only a slightly higher inhibitory activity (Table 1). Apart from the seven-atom spacer hybrid **12a**, all hybrids displayed the same or significantly higher inhibitory activity toward AChE than tacrine (the most potent compound **14l** with $pIC_{50}$ = 8.21 and the least potent compound **12b** with $pIC_{50}$ = 7.36). The highest activities were obtained with higher spacer lengths (with the only exception of compound **14j** with 16 spacer atoms); there was a coherent development of anti-AChE activity with increasing spacer length from $pIC_{50}$ = 7.20 (**12a**) to $pIC_{50}$ = 8.21 (**14l**),

**Table 1.** Inhibition of AChE and BChE ($pIC_{50}$ Values) and Selectivity Expressed as the Ratio of the Resulting $IC_{50}$ Values

| | $pIC_{50} \pm SEM$[a] | | |
|---|---|---|---|
| compd | AChE[b] | BChE[b] | selectivity ratio[c] |
| tacrine | 7.34 ± 0.06 | 8.29 ± 0.08 | 8.8 |
| **12a** | 7.20 ± 0.03 | 7.54 ± 0.03 | 2.2 |
| **12b** | 7.36 ± 0.02 | 7.79 ± 0.03 | 2.6 |
| **12c** | 7.66 ± 0.04 | 7.91 ± 0.03 | 1.8 |
| **12d** | 7.58 ± 0.03 | 7.87 ± 0.04 | 1.9 |
| **14a** | 7.83 ± 0.03 | 8.01 ± 0.05 | 1.5 |
| **14b** | 7.87 ± 0.03 | 7.97 ± 0.03 | 1.3 |
| **14c** | 7.97 ± 0.03 | 8.00 ± 0.02 | 1.1 |
| **14d** | 7.99 ± 0.03 | 8.05 ± 0.01 | 1.1 |
| **14e** | 7.92 ± 0.04 | 7.97 ± 0.05 | 1.1 |
| **14f** | 7.97 ± 0.03 | 8.04 ± 0.05 | 1.2 |
| **14g** | 8.07 ± 0.04 | 8.17 ± 0.04 | 1.2 |
| **14h** | 8.06 ± 0.04 | 8.23 ± 0.05 | 1.5 |
| **14i** | 8.18 ± 0.06 | 8.29 ± 0.04 | 1.3 |
| **14j** | 7.64 ± 0.06 | 7.88 ± 0.04 | 1.7 |
| **14k** | 8.19 ± 0.03 | 8.30 ± 0.04 | 1.3 |
| **14l** | 8.21 ± 0.03 | 8.23 ± 0.05 | 1.0 |

[a] Data are the mean values of at least three determinations. $pIC_{50}$ = $-\log IC_{50}$. [b] AChE from electric eel and BChE from equine serum were used. [c] Selectivity ratio = ($IC_{50}$ of AChE)/($IC_{50}$ of BChE).

resulting in a 10-fold increase of potency. The distance of the amide bond from the tacrine moiety did not have any effect on inhibitory activity or AChE selectivity, strongly indicating that the amide bond does not specifically interact with amino acids in the mid-gorge of the enzyme.

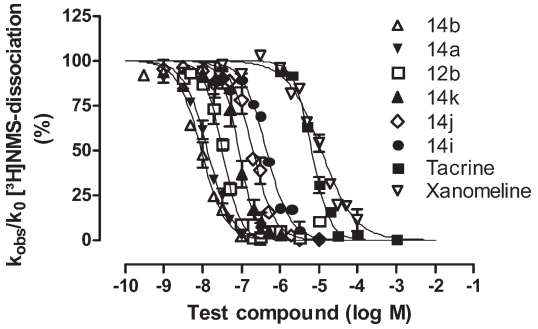
Similar relationships could be observed for the inhibitory activities at BChE, although the differences were less pronounced because of generally high activities (Table 1). Since the activity increase was less pronounced for BChE than for AChE, tacrine's moderate BChE selectivity was lost with the hybrids; they acted at both enzymes with almost the same inhibitory activity. Taken together, the increase in affinity might favor the view that in AChE the hybrids are able to interact simultaneously with both the substrate (ACh) binding site and the peripheral (allosteric) site. Such binding topography has been described for a series of tacrine dimers (bivalent tacrines).[26,30] It appears unlikely that the xanomeline part of the hybrids interacts with the ACh binding site (catalytic binding site) of the enzymes, as there is no evidence in the literature for an interaction of xanomeline with the catalytic site of AChE or BChE.[31] With respect to tacrine Pang et al. showed in docking studies that tacrine dimers

(with a suitable spacer length) are likely to interact by their tacrine moieties with both the catalytic and the peripheral site in hAChE.[30] Therefore, it is tempting to speculate that our hybrids bind with their tacrine moiety at the catalytic site and with their xanomeline moiety at the peripheral site.

In human $M_1$ receptors stably expressed in CHO cells, we aimed at determining the affinity of the test compounds for unliganded receptors using the orthosteric radioligand [$^3$H]*N*-methylscopolamine ([$^3$H]NMS) as a probe. Muscarinic allosteric ligands typically retard the dissociation of [$^3$H]NMS by allosteric binding to [$^3$H]NMS-occupied receptors, thereby prolonging the incubation time needed for reaching [$^3$H]NMS equilibrium binding. Therefore, we first checked whether and to which extent the hybrid compounds inhibit [$^3$H]NMS dissociation.

Dissociation of [$^3$H]NMS proceeded monophasically both under control conditions ($t_{0.5off}$ = 15.6 ± 0.2 min, mean ± SEM, $n$ = 10) and in the presence of the test compounds. All compounds induced an allosteric inhibition of [$^3$H]NMS dissociation. Applying two point kinetic experiments,[32] we obtained concentration-effect curves, the inflection points of which indicate the affinity of the test compounds for NMS-bound $M_1$ receptors (Chart 2). These affinities (log $K_{X,diss}$) and the slope factor $n$ of the respective concentration-effect curves are compiled in Table 2. The measured affinities log $K_{X,diss}$ of the hybrids for NMS-bound receptors surpassed those of their respective building blocks tacrine and xanomeline (Table 2). For instance, compared to xanomeline the log $K_{X,diss}$ of the most potent hybrid **14b** was more than 3 log units higher. The steep concentration-effect curves of the hybrids implies that allosteric binding is governed by the tacrine moiety, as the slope of the concentration-effect curve (slope factor $n$) was normal for xanomeline but high for tacrine and the hybrids ($F$-test, $P < 0.05$). As mentioned above, steep curves are a characteristic of the allosteric action of tacrine. Thus, the residue being attached to tacrine in the hybrid including xanomeline and the linker chain appears to increase binding affinity by enlarging the area of contact with the allosteric receptor site. Worth mentioning, it appears unlikely that the receptor area mediating wash-resistant xanomeline binding is involved in the allosteric binding topography; in particular, the minimum length (in C atoms) of the aliphatic side chain of xanomeline ($C_7$) to achieve wash-resistant binding amounts to $C_4$ whereas the hybrids contain $C_3$ only.[15]

**Chart 2.** Concentration-Effect Curves of Selected Test Compounds on the Rate Constant $k_{-1}$ of [$^3$H]NMS Dissociation To Determine the Affinity of the Test Compounds to $M_1$ Receptors That Are Orthosterically Blocked by NMS[a]




[a] Curves were fitted individually using Prism 5.02 for Windows (GraphPad Software, Inc., San Diego, CA) by applying a built-in standard four-parameter logistic equation.

Worth mentioning, a considerable increase of affinity for the allosteric site of $M_2$ receptors has recently been reported for tacrine−gallamine hybrids[33] as well as for tacrine−tacrine dimers (bis-tacrines).[34] The chemical variations applied (Schemes 1 and 2) induced incremental increases of affinity (Chart 2, Table 2). The affinity did not generally correlate with the length of the spacer in the hybrid molecule because the most potent hybrid **14b** (11-atom spacer) was not the shortest of the series tested at the $M_1$ receptors (Chart 2); **14a** (10 atoms) and **12b** (8 atoms) were even shorter. The data obtained with **12b** indicate that a loss of an ether bridge and a switch from an amide to an amine bond can retain a high affinity log $K_{X,diss}$ at the allosteric site of NMS-bound receptors.

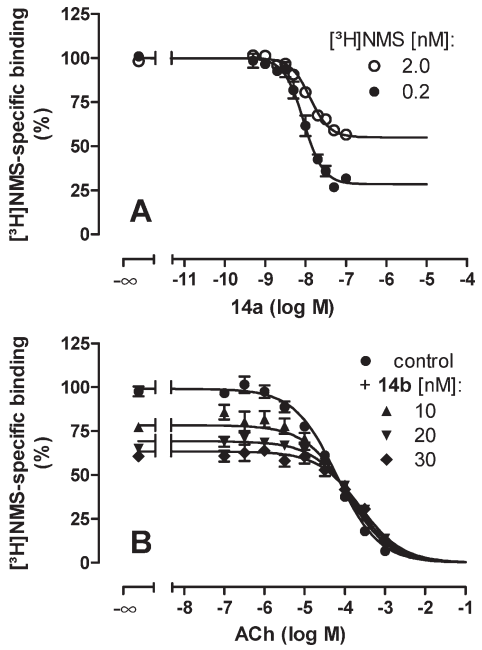
The log $K_{X,diss}$ values were used to calculate the incubation time necessary for reaching equilibrium binding of [$^3$H]NMS in the presence of test compound.[36] The concentration-dependent effect of the hybrids on [$^3$H]NMS binding was measured at two concentrations of tracer rather than at one

**Table 2.** Parameters from Binding Experiments Characterizing the Interaction of the Test Compounds with the Inverse Orthosteric Agonist NMS and the Endogenous Orthosteric Agonist ACh at Muscarinic $M_1$ Receptors[a]

| | [$^3$H]NMS dissociation | | [$^3$H]NMS equilibrium binding | | | | |
|---|---|---|---|---|---|---|---|
| compd | log $K_{X,diss}$, X → NMS-$M_1$ | $n$ slope | log $K_X$, X → $M_1$ | log α, X/NMS/$M_1$ | $n$ slope | log($\alpha K_X$), X → NMS-$M_1$ | log $\beta$, X/ACh/$M_1$ |
| xanomeline | 4.94 ± 0.04 | −1.10 ± 0.09 | 7.40 ± 0.18 | −4.57 ± 1.42 | −0.93 ± 0.04 | 2.83 ± 1.29 | < −3 |
| tacrine | 5.18 ± 0.02 | −1.91 ± 0.15* | 5.86 ± 0.04 | −1.30 ± 0.04 | −1.73 ± 0.18* | 4.56 ± 0.02 | < −3 |
| **12b** | 7.49 ± 0.02 | −1.89 ± 0.19* | 8.13 ± 0.02 | −0.65 ± 0.07 | −5.80 ± 2.79 | 7.48 ± 0.07 | < −3 |
| **14a** | 7.87 ± 0.03 | −1.43 ± 0.12* | 8.10 ± 0.11 | −0.63 ± 0.02 | −2.00 ± 0.18* | 7.47 ± 0.11 | < −3 |
| **14b** | 8.03 ± 0.03 | −1.41 ± 0.13* | 8.09 ± 0.08 | −0.63 ± 0.08 | −1.35 ± 0.35 | 7.46 ± 0.16 | < −3 |
| **14i** | 6.35 ± 0.03 | −1.43 ± 0.16* | 6.88 ± 0.08 | −1.44 ± 0.12 | −1.11 ± 0.11 | 5.44 ± 0.15 | < −3 |
| **14j** | 6.70 ± 0.03 | −1.55 ± 0.17* | 7.57 ± 0.23 | −1.48 ± 0.15 | −1.11 ± 0.11 | 6.09 ± 0.14 | < −3 |
| **14k** | 7.06 ± 0.03 | −1.94 ± 0.25* | 7.38 ± 0.04 | −0.80 ± 0.14 | −1.64 ± 0.15* | 6.58 ± 0.06 | < −3 |

[a] log $K_{X,diss}$, log affinity constant of the allosteric agent X for NMS-bound receptors measured as the concentration of the allosteric ligand that reduces the dissociation rate constant of [$^3$H]NMS dissociation by 50%; $n$, slope factor of the curve; log $K_X$, log affinity constant of the allosteric agent X for $M_1$ receptors derived from its cooperative interaction with the orthosteric ligand NMS; α, cooperativity factor of the allosteric interaction between X and NMS (with log α < 0 indicating negative cooperativity) log($\alpha K_X$), log affinity constant for the binding of the allosteric agent to the NMS-occupied $M_1$ receptors, derived from the allosteric ternary complex model. The model predicts that this is the log affinity of the allosteric ligand for inhibiting the [$^3$H]NMS dissociation rate constant. $\beta$, cooperativity factor of the allosteric interaction between X and ACh (with log $\beta$ < 0 indicating negative cooperativity);. The data shown are mean values ± SEM of three to four experiments carried out in triplicate. log $K_L$, which was needed for the curve fitting, was derived from homologous inhibition experiments according to ref 36 and amounted to 9.42 ± 0.05. The maximal receptor number $R_t$ was 3.43 ± 0.29 pmol/mg protein. The asterisk (∗) indicates that the value is larger than unity (F-test, P < 0.05). For further details, see refs 35 and 36 and detailed pharmacological procedures.

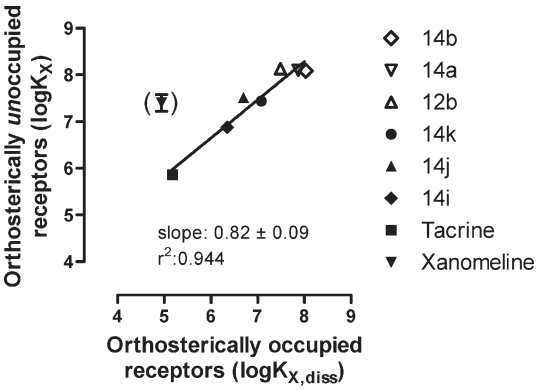
**Chart 3.** (A) Inhibition of [$^3$H]NMS (0.2 and 2.0 nM) Equilibrium Binding by Hybrid **14a** to Determine the Affinity of the Test Compounds at Orthosterically Unliganded $M_1$ Receptors and Their Cooperativity with NMS and (B) Effect of Increasing Concentrations of Hybrid **14b** on the Inhibition of [$^3$H]NMS (0.2 nM) Equilibrium Binding by ACh To Measure the Cooperativity of the Hybrids with ACh[a]




[a] Note that the negative cooperativity of the hybrid with NMS and ACh is reflected by the decrease of the upper plateau and the rightward shift of the ACh inhibition the curve, respectively. Data sets in (A) and (B), respectively, were fitted globally using Prism 5.02 by applying in (A) eq 2 from ref 43 and in (B) eq 34 from ref 36. Data are the mean values of at least three experiments performed as triplicate determinations. For details, refer to detailed pharmacological procedures.

in order to allow for a global nonlinear regression data analysis and a reduced analytical error of the numerical parameter values. All test compounds inhibited [$^3$H]NMS equilibrium binding (Chart 3), thus displaying negative cooperativity with the orthosteric radioligand. Data were analyzed applying an extended allosteric ternary complex model (EATCM) as described by Lazareno and Birdsall, which allows characterization of interactions of an allosteric ligand with both an unlabeled orthosteric ligand (here acetylcholine) and an orthosteric probe (here [$^3$H]NMS).[36] The model describes that the allosteric agent X can bind to the orthosterically free $M_1$ receptor, forming a binary complex, and to the orthosterically occupied $M_1$ receptor (in the current study either NMS or ACh), forming a ternary complex. Binary complex formation ($X \rightarrow M_1$) is characterized by the affinity $K_X$. Ternary complex formation with the NMS-bound receptor ($X \rightarrow$ NMS-$M_1$) is characterized by $\alpha K_X$ (corresponding to $K_{X,diss}$). The extent and direction of affinity change of the allosteric agent for ternary complex formation ($X \rightarrow NMS/M_1$ or $X \rightarrow ACh/M_1$) compared to binary complex formation ($X \rightarrow M_1$) are an allosteric measure and quantified by the cooperativity factor $\alpha$. It is noted that $\alpha$ is reciprocal in nature; i.e., the orthosteric ligand is subject to the identical affinity shift. Worth mentioning, the affinity of the allosteric agent for orthosterically liganded receptors (measured as $K_{X,diss}$ or $\alpha K_X$ depending on the assay) depends on the structure of the orthosteric ligand

**Chart 4.** Correlation of Affinity Measures of the Hybrids and of Tacrine Obtained in Free ($\log K_X$, Chart 3A) and NMS Occupied ($\log K_{X,diss}$, Chart 2) $M_1$ Receptors[a]




[a] The line was obtained by linear regression analysis excluding the data point of xanomeline illustrated in parentheses.

bound and may thus be different with different ligands (e.g., NMS or ACh).

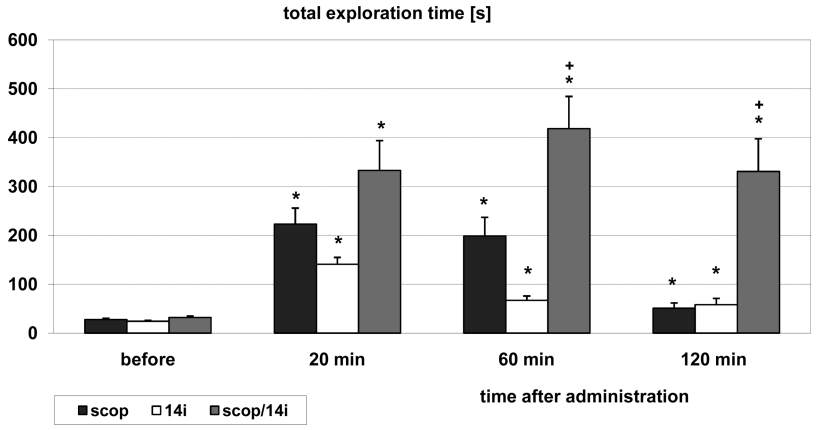
The affinities ($\log K_X$) of the test compounds for orthosterically free receptors and their binding cooperativities with NMS ($\log \alpha$) are compiled in Table 2.

With respect to molecule length, the $M_1$ receptor affinities $\log K_x$ at orthosterically unliganded receptors of the hybrid molecules **14b** (11 spacer atoms, $\log K_x = 8.09$), **14i** (15 spacer atoms, $\log K_x = 6.88$), and **14k** (17 spacer atoms, $\log K_x = 7.38$) and the affinity of xanomeline ($\log K_x = 7.40$) lie within 1.2 log concentration units (Table 2). Explicitly, **12b** ($\log K_x = 8.13$), **14a** ($\log K_x = 8.10$), and **14b** ($\log K_x = 8.09$) with a smaller spacer length of 8, 10, and 11 atoms, respectively, exceed the binding affinity of xanomeline slightly by about a factor of 3, whereas a long spacer of 17 atoms as in **14k** has a lower binding affinity relative to **12b**, **14a**, and **14b** and a similar affinity relative to xanomeline. In unoccupied $M_1$ receptors, slope factors $n$ were nonuniform resulting in steep curves for tacrine, **14a**, and **14k** only. Taken together, there is no coherent influence of the middle chain length on the affinity of the hybrids for the free receptor.

The log of the affinity constant of test compound binding to NMS-occupied $M_1$ receptors, $\log K_{X,diss}$ determined in dissociation experiments (Chart 2), can be predicted by means of the EATCM from the parameters determined in the equilibrium binding experiments as $\log(\alpha K_X)$ (Table 2). The match was perfect with **12b** ($\Delta$0.01), rather acceptable with the other hybrids, but poor with xanomeline ($\Delta$2.11), the deviation in the last case probably being caused by the very low affinity for NMS-bound receptors and the resulting wide scatter of $\log \alpha$.

In order to elucidate the interaction of the hybrids with the endogenous transmitter acetylcholine, the effect of increasing concentrations of ACh on [$^3$H]NMS equilibrium binding was measured in the absence and presence of several concentrations of the test compounds (Chart 3B). In these experiments, the concentration-dependent decline of the upper curve plateau reflects the inhibiting action of the hybrids on [$^3$H]NMS binding as already measured separately in detail in Chart 2. Further ACh-induced inhibition of radioligand binding indicates competition between these orthosteric ligands. The test compounds (such as **14b** in Chart 2) induced a rightward shift of the ACh inhibition curve, indicating inhibition of ACh binding. In terms of the

**Chart 5.** Effect of Scopolamine (0.05 mg/100 g Body Weight), Hybrid Molecule **14i** (2 $\mu$mol/100 g Body Weight), and Scopolamine plus **14i** (Coadministration) on Total Exploration Time of Rats (10−15 Test Animals Each) To Find All Food Pellets in an Eight-Arm Radial Maze Cognition Experiment before and after Test Compound Administration at the Indicated Intervals[a]




[a] (∗) Significantly different from control period in untreated animals. (+) Significantly different from scopolamine (scop) and **14i**-treated groups (single administration), respectively.

ETCAM, all hybrids showed a strong negative cooperativity with ACh ($\log\beta < -3$; Table 2). Thus, negative cooperativity of the hybrids with ACh is much more pronounced than with NMS ($\log\alpha$ values in Table 2); in other words, the affinity of the hybrids for ACh-occupied active receptors is much lower than at inverse agonist (NMS) occupied inactive $M_1$ receptors. Similar findings have been made with alkane-bis-ammonium-type allosteric modulators at $M_2$ receptors.[37,38]

We compared the affinity of the hybrids for free ($\log K_x$) and for NMS-liganded ($\log K_{x,diss}$) $M_1$ receptors and found an excellent correlation ($r^2 = 0.94$, Chart 4). This suggests that the hybrids stick to the allosteric binding topography even in the orthosterically unoccupied $M_1$ receptor. Thus, the tacrine moiety seems to prevent the xanomeline moiety from interacting with the orthosteric binding cavity of the receptor. This action of tacrine may involve, first, steric hindrance and, second, a "functional misfit" of receptor conformations. First, tacrine probably binds to the allosteric core region which is located next to the entrance to the orthosteric binding cavity,[39] thereby hindering xanomeline from gaining access to the orthosteric site. Second, tacrine, being devoid of intrinsic activity for receptor activation, stabilizes the receptor in an inactive conformation, thereby imprinting a conformation on the orthosteric site that is unfavorable for binding of the agonist xanomeline. This "misfit" of receptor conformations is also a likely explanation for the strong mutual inhibition of receptor binding between tacrine and the agonist acetylcholine. In any case, the hybrids do not undergo dualsteric, i.e., simultaneous allosteric/orthosteric, receptor binding. Worth mentioning, screening-type [$^{35}$S]GTP$\gamma$S binding experiments did not provide evidence for $M_1$-receptor activation by the hybrids.

In order to check how the properties of the hybrids, to interact with muscarinic receptors and cholinesterases as determined in vitro, would translate into behavioral effects, we tested the compounds in vivo in a cognition assay using rats in an eight-arm radial maze.[27b,40−42] In this model, reversible amnesia (deteriorated information acquisition) is induced by scopolamine administration, and therefore, this assay has been used to test procognitive effects of both AChE inhibitors and $M_1$ agonists.[27b,40] Tacrine and xanomeline are able to reduce cognition impairment induced by scopolamine (data not shown).[27b] Hybrid **14i**, the weakest allosteric modulator in the hybrid series but one of the most potent AChE inhibitors, was tested in this assay. Without coadministration of scopolamine, **14i** induced an increase in total exploration time, possibly reflecting an antagonistic action at M receptors (Chart 5). Upon coadministration with scopolamine, cognition was impaired in a more-than-additive fashion. These findings reflect the results obtained from in vitro characterization: as the compounds are characterized by a more pronounced negative binding cooperativity with the agonist acetylcholine compared with the scopolamine-like antagonist, the interplay at the muscarinic receptor between the endogenous transmitter and scopolamine is likely to be shifted in favor of the antagonist. As a consequence, the cognition impairing action of scopolamine is augmented.

Without prior administration of scopolamine, increase in total exploration time indicating antagonist action at M receptors could be observed (Chart 5). After scopolamine application, the effects of scopolamine were significantly enhanced. These findings are in line with the results obtained from in vitro characterization, i.e., the loss of $M_1$-receptor activation and allosteric retardation of [$^3$H]NMS dissociation. A similar effect has previously been observed using this assay for quinazolinimines that act as BChE inhibitors and allosteric modulators of M receptors.[42]

Taken together, hybrid molecules composed of a xanomeline-like building block and a tacrine one were generated by using appropriate spacers. Compared with their parent compounds, these hybrids show in vitro improved AChE inhibition and enhanced $M_1$ allosteric affinity. Structure−activity relationships suggest that the hybrids prefer a purely allosteric binding topography even in orthosterically free receptors. In $M_1$ receptors, the hybrid modulators inhibit binding of the endogenous transmitter ACh. These findings are supported by in vivo studies investigating scopolamine-induced memory impairments in rats in which hybrid **14i** was able to significantly enhance scopolamine action, thereby outbalancing its high ChE-inhibiting activity. Thus, our initial hypothesis to obtain dualsteric compounds or compounds acting directly and indirectly at the $M_1$ receptor proved incorrect for these hybrids, and therefore, in disorders associated with cholinergic hypofunction such as in conditions of Alzheimer's disease, the pronounced tacrine-like

allosteric character of the $M_1$ receptor interaction of the hybrids described would counterbalance their improved activity as AChE inhibitors. For the design of hybrid molecules incorporating allosteric modulators, special attention has to be given to the interplay of molecule components at the receptor level with special regard to receptor activation. For potentially procognitive agents the use of positive allosteric modulators (even without ChE inhibiting properties) seems to be more promising.

## Experimental Section

**General Synthetic Methods.** Melting points are uncorrected and were measured in open capillary tubes using a Gallenkamp melting-point apparatus. $^1$H NMR and $^{13}$C NMR spectral data were obtained from Bruker Avance 250 (250 and 75 MHz). Elemental analyses were performed on a Vario EL III apparatus (Firma Elementar Analysensysteme GmbH, Germany). ESI mass spectra were recorded using LCQ Advantage by Thermo-Electron. Silica gel column chromatography utilized silica gel 60, 63−200 $\mu$m (Baker). As determined by elemental analysis, the purity of all target compounds is >95%.

**3-[(4-Pyridin-3-yl-1,2,5-thiadiazol-3-yl)oxy]propan-1-ol (8).** Sodium hydride (0.72 g, 30 mmol) was suspended in 30 mL of freshly dried THF and cooled to 0 °C by ice bath. Propane-1,3-diol (1.1 mL, 15 mmol) was dropped into the mixture slowly. The mixture was then refluxed for 1 h. Compound **7**[16,28] (1.4 g, 7 mmol) dissolved in 10 mL of dry THF was added dropwise in 5 min. The reaction solution continued to reflux for 24 h. The reaction was terminated by adding 10 mL of water to the cooled solution. The solvent was removed under reduced pressure, and the residue was extracted by $CH_2Cl_2$ (3 × 10 mL). The combined organic phases were dried over $Na_2SO_4$ and concentrated under reduced pressure to give the crude product. Purification was performed by column chromatography (eluent $CH_2Cl_2$/MeOH = 10/1, v/v) to give **8** as a yellow oil (1.04 g, 63%). $^1$H NMR ($CDCl_3$): $\delta$ 9.35 (s, 1H, arom), 8.65−8.62 (m, 1H, arom), 8.39−8.35 (m, 1H, arom), 7.40−7.35 (m, 1H, arom), 4.58 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$CH$_2$), 3.75 (t, $J$ = 7.5 Hz, 2H, CH$_2$-C$\underline{H_2}$OH), 2.53−2.48 (m, 2H, CH$_2$C$\underline{H_2}$CH$_2$) ppm.

**3-[4-(3-Hydroxypropoxy)-1,2,5-thiadiazol-3-yl]-1-methylpyridinium Iodide (9).** $CH_3I$ (0.31 mL, 5 mmol) was added to a solution of compound **8** (0.47 g, 2 mmol) in 5 mL of acetone. The reaction solution was stirred at room temperature for 18 h, and a yellow deposit formed. The deposit was filtered off, washed with diethyl ether, and dried under reduced pressure to give **9** as a yellow powder (0.67 g, 89%). Mp 164−166 °C. $^1$H NMR ($CD_3OD$): $\delta$ 9.15 (s, 1H, arom), 8.76 (d, $J$ = 7.5 Hz, 1H, arom), 8.49 (d, $J$ = 7.5 Hz, 1H, arom), 7.79−7.72 (m, 1H, arom), 4.33 (s, 3H, NC$\underline{H_3}$), 4.28 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$CH$_2$), 3.32 (t, $J$ = 7.5 Hz, 2H, CH$_2$C$\underline{H_2}$OH), 2.43−2.39 (m, 2H, CH$_2$C$\underline{H_2}$CH$_2$) ppm.

**3-{[4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl]oxy}propan-1-ol (10).** Compound **9** (0.85 g, 2.25 mmol) was dissolved in 15 mL of absolute methanol and cooled to −5 °C by ice−salt bath. $NaBH_4$ (0.25 g, 6.5 mmol) was carefully added to the solution under strong gas formation. The reaction solution was stirred for 24 h at room temperature. Then the reaction was terminated by adding 10 mL of water. The sovent was removed under reduced pressure, and the residuce was extracted with $CH_2Cl_2$ (3 × 10 mL). The combined organic phases were dried over $Na_2SO_4$ and concentrated under reduced pressure to give the crude product. The purification was performed by column chromatography (eluent $CH_2Cl_2$/MeOH = 10/1, v/v) to give **10** as a yellow oil (0.46 g, 81%). $^1$H NMR ($CDCl_3$): $\delta$ 7.02−6.99 (m, 1H, C=C$\underline{H}$), 4.58 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$CH$_2$), 3.75 (t, $J$ = 7.5 Hz, 2H, CH$_2$C$\underline{H_2}$OH), 3.42 (s, 2H, $\underline{H_2}$CC=CH), 2.59−2.54 (m, 4H, =CHC$\underline{H_2}$CH$_2$, =CHCH$_2$C$\underline{H_2}$), 2.43 (s, 3H, NC$\underline{H_3}$), 2.10−2.02 (m, 2H, C$\underline{H_2}$CH$_2$CH$_2$) ppm.

**5-[4-(3-Bromopropoxy)-1,2,5-thiadiazol-3-yl]-1-methyl-1,2,3,6-tetrahydropyridine (11).** Compound **10** (0.25 g, 1 mmol) and $PPh_3$ (0.8 g, 3 mmol) were dissolved in 15 mL of dry $CH_2Cl_2$ and cooled to −5 °C. $CBr_4$ (2.2 g, 6.5 mmol) dissolved in 5 mL of dry $CH_2Cl_2$ was dropped into the reaction solution slowly, controlling the inside temperature to prevent it from increasing above 5 °C. The reaction was continued for another 24 h at room temperature. The reaction solution was then extracted by 10% HCl aqueous solution (3 × 15 mL), and the organic phases were discarded. The aqueous phase was basified to pH 10 with 10% NaOH aqueous solution. The solution was extracted with $CH_2Cl_2$ (3 × 15 mL). The combined organic phases were dried over $Na_2SO_4$ and solvent was removed under reduced pressure to give the crude product. The purification was performed by column chromatography (eluent $CH_2Cl_2$/MeOH = 10/1, v/v) to give **11** as a yellow oil (0.13 g, 42%). $^1$H NMR ($CDCl_3$): $\delta$ 6.92−6.88 (m, 1H, C=C$\underline{H}$), 4.51 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$CH$_2$), 3.51 (t, $J$ = 7.5 Hz, 2H, CH$_2$C$\underline{H_2}$Br), 3.42 (s, 2H, $\underline{H_2}$CC=CH), 2.61−2.55 (m, 4H, =CHC$\underline{H_2}$CH$_2$, =CHCH$_2$C$\underline{H_2}$), 2.37 (s, 3H, NC$\underline{H_3}$), 2.10−2.02 (m, 2H, CH$_2$-C$\underline{H_2}$CH$_2$) ppm.

**General Procedure for the Synthesis of 12a−d.** Compounds **3a−d**[25] (2 mmol), compound **11** (0.64 g, 2 mmol), potassium carbonate (0.28 g, 2 mmol), and catalytic amounts of potassium iodide (0.1 g) were added to 5 mL of $CH_2Cl_2$ and stirred for 18 h at room temperature. The mixture was filtered and the filtrate was concentrated under reduced pressure to give the crude product. The purification was performed by column chromatography (eluent $CH_2Cl_2$/MeOH = 7/3, v/v, plus 10 mL 25% ammonia aqueous solution per 1000 mL) to give **12a−d**. Full spectral data for compounds **12a−d** are presented in the Supporting Information.

***N*-(3-{[4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl]oxy}propyl)-*N*′-(1,2,3,4-tetrahydroacridin-9-yl)-butane-1,4-diamine (12b).** Brown oil, yield 45%. $^1$H NMR ($CDCl_3$): $\delta$ 8.11−8.08 (m, 1H, arom), 7.75−7.73 (m, 1H, arom), 7.55−7.52 (m, 1H, arom), 7.37−7.33 (m, 1H, arom), 6.93 (br, 1H, C=C$\underline{H}$), 4.66 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$), 3.57−3.54 (m, 2H, ArNHC$\underline{H_2}$), 3.34 (s, 2H, $\underline{H_2}$CC=CH), 3.04 (br, 2H, C4-$\underline{H_2}$), 2.77 (br, 2H, C1-$\underline{H_2}$), 2.60 (t, $J$ = 7.5 Hz, 2H, NHC$\underline{H_2}$CH$_2$CH$_2$O), 2.53−2.30 (m, 6H, C$\underline{H_2}$NH, =CHC$\underline{H_2}$CH$_2$, =CHCH$_2$C$\underline{H_2}$), 2.16 (s, 3H, NC$\underline{H_3}$), 2.02−1.98 (m, 2H, C$\underline{H_2}$CH$_2$O), 1.84−1.54 (m, 8H, C2-$\underline{H_2}$, C3-$\underline{H_2}$, ArNHCH$_2$CH$_2$C$\underline{H_2}$, ArNHCH$_2$C$\underline{H_2}$) ppm.

**General Procedure for the Synthesis of 14a−l.** Compounds **13a−l**[27] (2 mmol), compound **10** (0.52 g, 2 mmol), potassium carbonate (0.28 g, 2 mmol), and catalytic amounts of potassium iodide (0.1 g) were added to 5 mL of $CH_2Cl_2$ and stirred for 18 h at room temperature. The mixture was filtered and the filtrate was concentrated under reduced pressure to give the crude product. The purification was performed by column chromatography (eluent $CH_2Cl_2$/MeOH = 7/3, v/v, plus 10 mL 25% ammonia aqueous solution per 1000 mL) to give **14a−l**. Full spectral data for compounds **14a−l** are presented in the Supporting Information.

**2-(3-{[4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl]oxy}propoxy)-*N*-[8-(1,2,3,4-tetrahydroacridin-9-ylamino)octyl]acetamide (14i).** Yellow oil, yield 34%. $^1$H NMR ($CDCl_3$): $\delta$ 8.36−8.32 (m, 1H, arom), 7.81−7.77 (m, 2H, arom), 7.57−7.53 (m, 1H, arom), 6.98 (br, 1H, C=C$\underline{H}$), 4.71 (t, $J$ = 7.5 Hz, 2H, C$\underline{H_2}$OC=N), 3.85 (s, 2H, OC$\underline{H_2}$CO), 3.72 (t, $J$ = 7.5 Hz, 2H, OC$\underline{H_2}$CH$_2$CH$_2$), 3.42 (s, 2H, =CC$\underline{H_2}$N), 3.40−3.29 (m, 4H, ArNHC$\underline{H_2}$, C$\underline{H_2}$NHCO), 3.01 (br, 2H, C4-$\underline{H_2}$), 2.74−2.68 (m, 4H, C1-$\underline{H_2}$, =CHC$\underline{H_2}$CH$_2$), 2.51−2.48 (m, 5H, =CHC$\underline{H_2}$-CH$_2$, NC$\underline{H_3}$), 2.04−2.01 (m, 2H, C3-$\underline{H_2}$), 1.93−1.40 (m, 16H, ArNHCH$_2$(C$\underline{H_2}$)$_6$, C2-$\underline{H_2}$, C$\underline{H_2}$CH$_2$O) ppm.

**Experimental Procedures for the Pharmacological Investigations. Acetyl- and Butyrylcholinesterase Inhibition Assay.** 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). DTNB (Ellman's reagent) and ATC and BTC iodides were obtained from Fluka (Buchs, Switzerland).

The assay was performed as described in the following procedure:[19,27] stock solutions of the test compounds were

prepared in ethanol, 100 μL of which gave a final concentration of $10^{-3}$ M when diluted to the final volume of 3.32 mL. The highest concentration of the test compounds applied in the assay was $10^{-4}$ M (10% EtOH in the stock solution did not influence enzyme activity). In order to obtain an inhibition curve, at least five different concentrations (normally $10^{-4}-10^{-9}$ M) of the test compound were measured at 25 °C and 412 nm, each concentration in triplicate.

For buffer preparation, an amount of 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 units $mL^{-1}$ in 1.4 mL aliquots. Furthermore, 0.01 M DTNB solution and 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 a total of 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 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 the percentage enzyme activity (100% for the reference) versus logarithm of test compound concentration.

**$M_1$ Receptor Binding. Cell Culture and Preparation of Membranes.** Chinese hamster ovary cells (CHO-cells) stably transfected with the human $M_1$-receptor gene (generously provided by Dr. N. J. Buckley, University of Leeds, U.K.) were grown, harvested, homogenized, and processed as described elsewhere.[43] The resulting membrane pellets were washed twice in 20 mM HEPES, 0.1 mM $Na_4EDTA$, pH 7.4, 4 °C ("storage buffer"), and the final pellets were stored as a membrane suspension in storage buffer at −80 °C. Protein content was determined by the Lowry method and amounted to 1.7−2.5 mg/mL.

**Binding Assays.** The [$^3$H]NMS filtration binding assay was carried out as described before.[35] The buffer was 4 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, pH 7.4 at 23 °C ("Na/K/$P_i$ buffer"). [$^3$H]NMS equilibrium binding assays used 0.2 nM and 2.0 nM [$^3$H]NMS and 50 μg protein/mL membranes. Nonspecific [$^3$H]NMS binding was determined in the presence of 1 μM atropine and did not exceed 5% of total binding. Specific binding of [$^3$H]NMS under control conditions was characterized by a negative log equilibrium dissociation constant, p$K_L$, and a maximal receptor number $R_t$. All inhibition experiments with [$^3$H]NMS and the test compounds were carried out in a 0.3 mL volume in 1.2 mL deep well plates (Abgene House, Epsom, U.K.). The incubation time in homologous and heterologous competition experiments with [$^3$H]NMS was 2 and 6−17 h, respectively. To measure the interaction of [$^3$H]NMS and acetylcholine with the test compounds, the incubation time amounted to 2−8 h. The incubation time necessary to equilibrate [$^3$H]NMS binding in the presence of an allosteric modulator was calculated according to the following equation:

$$t_{0.5obs} = t_{0.5off}\left(1+\frac{1}{K_{X,diss}}[X]\right) \quad (1)$$

$t_{0.5obs}$ is an estimate of [$^3$H]NMS association half-life in the presence of allosteric modulator X, $t_{0.5off}$ is the half-life of [$^3$H]NMS dissociation in the absence of allosteric modulator, and $K_{X,diss}$ indicates the modulator concentration at which the half-life of [$^3$H]NMS dissociation is doubled. Equilibrium was assumed to be reached after $5t_{0.5obs}$.

For dissociation experiments, membranes were incubated with the respective radioligand for 60 min at 23 °C. Thereafter, aliquots of the mixture were added to excess unlabeled ligand in buffer over a total period of 120 min followed by simultaneous filtration of all samples. To determine the effect of the test compounds on the dissociation of [$^3$H]NMS, dissociation was measured by addition of 1 μM atropine in combination with the respective test compounds. Three-point kinetic experiments were performed in analogy to two-point kinetic experiments[32] with measurements of [$^3$H]NMS binding at $t = 0$, $t = 24$ min, and $t = 27$ min, respectively.

Receptor bound radioactivity was separated by filtration on a Tomtech 96-well Mach III harvester (Wallac) using glass fiber filtermats (Filtermat A, Wallac, Turku, Finland) which had been pretreated with 0.2% polyethyleneimine. Filtration was followed by two rapid washing steps (0.4 mL Na/K/$P_i$ incubation buffer at 4 °C). Filtermats were dried for 3 min at 400 W in a microwave oven. Thereafter, scintillation wax (Meltilex A, Wallac, Turku, Finland) was melted for 1 min at 90 °C onto the filtermat using a Dri-Block DB-2A (Techne, Duxford Cambridge, U.K.). The filters were placed in sample bags (Wallac, Turku, Finland), and filter bound radioactivity was measured using a Microbeta Trilux-1450 scintillation counter (Wallac, Turku, Finland).

**Data Analysis.** The binding data from individual experiments were analyzed by computer-aided nonlinear regression analysis using Prism 5.02 (GraphPad Software, San Diego, CA).

[$^3$H]NMS dissociation data were analyzed assuming a monoexponential decay as described previously.[43] The slowing actions of the allosteric agents on [$^3$H]NMS dissociation were expressed as the percent reduction of the apparent rate constant for [$^3$H]NMS dissociation. The concentration-effect curves for reduction of the [$^3$H]NMS dissociation rate constant by allosteric agents were fitted to a four-parameter logistic function. The parameter "top" was the value of $k_{-1}$ measured in the absence of test compound and was fixed at 100%, whereas the "inflecion point" and slope factors $n$ were set as variables. The parameter "bottom" was checked whether it, as a variable, yielded a significantly better fit compared with it being fixed at 0%. This was not the case in all experiments ($P > 0.05$, $F$-test). Finally we tested whether the slope factors of the curves were different from unity ($F$-test, $P < 0.05$ being taken as the level of statistical significance).

Homologous competition data obtained with [$^3$H]NMS were analyzed using a four-parameter logistic function to yield estimates of the bottom and top plateaux, the inflection point ($IC_{50}$), and the slope factor, $n$, of the curve. If the observed slope factors did not differ significantly from unity ($F$-test, $P > 0.05$), the $IC_{50}$ values were estimated with $n$ constrained to 1. The $K_L$ and $R_t$ values of the radioligand were calculated according to DeBlasi et al.[35]

Analyses of the data for the effect of the test compounds on the specific binding of the orthosteric radioligand [$^3$H]NMS were based on the allosteric ternary complex model.[36] We used eq 2 from Lazareno and Birdsall (ref 36) in which $R_t$ was replaced by eq 8 of the same study and a slope factor, $n$, was included:

$$B_{LX} = B_0\frac{(1+K_L[L])(1+\alpha(K_X[X])^n)}{1+(K_X[X])^n+K_L[L](1+\alpha(K_X[X])^n)} \quad (2)$$

$B_{LX}$ and $B_0$ denote the specific binding of the radioligand L ([$^3$H]NMS) in the presence and absence of the cooperatively interacting agent X, respectively. Note that in eq 2 $K_L$ and $K_X$ are the equilibrium affinity constants for the binding of L and X, respectively, at the unliganded receptors. α is the cooperativity factor for the allosteric interaction between X and L (with $\log\alpha > 0$, $\log\alpha < 0$, $\log\alpha = 0$ indicating positive, negative, and neutral cooperativity, respectively). The factor $n$ represents the slope factor of the curve. When [X] is plotted as the independent variable and $B_{LX}$ as the dependent variable, a global fit of the data with [L] and $K_L$ as known constants yielded estimates for $K_X$ and α.

Analyses of the data for the effect of the test compounds on the inhibition of specific [$^3$H]NMS binding by the endogenous

agonist acetylcholine A were based on eq 34 from Lazareno and Birdsall:[36]

$$\text{bound radioligand} = R_t \frac{[L]K_L(1+\alpha[X]K_X)}{1+[X]K_X+([A]K_A)(1+\beta[X]K_X)+[L]K_L(1+\alpha[X]K_X)} \quad (3)$$

In addition to the parameters contained already in eq 2, see above, $R_t$ denotes the maximal receptor number and $K_A$ the equilibrium affinity constants for the binding of A at the unliganded receptors. $\beta$ denotes the cooperativity factor $\beta$ of the allosteric interaction between A and X (with $\log\beta > 0$, $\log\beta < 0$, $\log\beta = 0$ indicating positive, negative, and neutral cooperativity, respectively). Plotting [A] as the independent variable and "bound radioligand" and [X] as the dependent variables, a global fit of the data with [L] and $K_L$ as known constants yielded estimates for $K_X$, $\alpha$, $K_A$, and $\beta$.

**Drugs.** [$^3$H]NMS (specific activity 70−84 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Homburg, Germany). Atropine sulfate and tacrine hydrochloride were purchased from Sigma Chemicals, Munich, Germany.

**In Vivo Studies. Animals.** Investigations were performed on 2-month-old female Wistar rats (Han:Wist) of our institute's own out-bred stock. Rats were housed in a room that was automatically maintained on a (12 h light)/(12 h dark) cycle at 25 °C and proper humidity. Rats were given food (Altromin 1326, Altromin, Lage, Germany) and tap water ad libitum. The permission of the animal protection commission of the State of Thuringia was given.

**Substances and Dose.** Scopolamine hydrobromide was obtained from Sigma Chemicals, Munich, Germany, and 0.05 mg was dissolved in 1 mL of saline. The substance **14i** was dissolved at a concentration of 2 $\mu$mol/mL.

**Behavioral Studies.** We examined whether **14i** could improve spatial memory impairment induced by scopolamine using performance in an eight-arm radial maze (RAM).[41,42] Each arm (44 cm length, 30 cm height, 14 cm width) radiated from an octagonal platform that served as a starting point. A food cup (3 cm diameter) was located at the end of each arm. All the arms and food cups were painted grey and placed in a dark and calm room. Animal behavior was monitored by a video camera. Image analysis and pattern recognition from the monitor were performed by a VideoMot 2 program (video tracking, motion analysis, and behavior recognition system) provided by TSE Systems (Bad Homburg, Germany). The computerized recording systems were located in the same room.

After a short handling period in which the rats were in close contact with the laboratory staff, three rats experienced free movement and feeding in the RAM once a day for 5 days to adapt to the maze. The baits (dustless precision rodent pellets, Bilaney Consultants Ltd., Sevenoaks, Kent, U.K.) were scattered in all arms. After handling and adaptation of the rats to the maze, food was restricted to reduce the rat's body weight by 10%.

**Trainig Trials.** Each rat was placed once daily in the center of the RAM to visit all eight arms and eat all reward food baits in each food cup. Each trial was performed until the rat entered and ate all the pellets in the eight arms or made 16 errors (re-entry into an arm that has been previously visited) or 10 min elapsed.

**Memory Impairment by Scopolamine.** This was induced in rats trained to the criteria of trials at 3 consecutive days. Before the administration of substances, a control run was performed. Thus, each animal was its own control. The following parameters were registered 20, 60, and 120 min after scopolamine: number of errors and time needed to visit all arms (total exploration time). The trial was finished after the rat had eaten all pellets or 10 min had elapsed or 16 errors were made.

**Experimental Groups.** Experimental groups were characterized as follows: (1) 0.05 mg of scopolamine/100 g body weight (scop); (2) 2 $\mu$mol of **14i**/100 g body weight (**14i**); (3) 5 min after scop administration and coadministration of compound **14i** (scop/**14i**). Both solutions were injected intraperitoneally (ip).

**Statistics.** Each group of rats consisted of 10−15 animals. Arithmetic mean ± SEM values were calculated. Student's *t* test was used to assess significant differences ($p \leq 0.05$).

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**Supporting Information Available:** Results from elemental analysis and synthesis/spectroscopic data of intermediates and target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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