Supporting Information

Novel Bipharmacophoric Inhibitors of the Cholinesterases with Affinity to the Muscarinic Receptors M_1 and M_2

Regina Messerer[a], Clelia Dallanoce[b], Carlo Matera[b], Sarah Wehle[a], Lisa Flammini[c], Brian Chirinda[d], Andreas Bock[e], Matthias Irmen[d], Christian Tränkle[d], Elisabetta Barocelli[c], Michael Decker[a], Christoph Sotriffer[a], Marco De Amici[b], Ulrike Holzgrabe[a, *]

[a] *Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany*

[b] *Dipartimento di Scienze Farmaceutiche, Sezione di Chimica Farmaceutica "Pietro Pratesi", Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milano, Italy*

[c] *Dipartimento di Farmacia, Università degli Studi di Parma, Parco Area delle Scienze, 27/A, 43124 Parma, Italy*

[d] *Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Straße 3, 53121 Bonn, Germany*

[e] *Institute of Pharmacology and Toxicology, University of Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany*

Table of Contents

Table S1: *Numerical estimates of parameters from binding experiments characterizing the interaction of the test compounds with the inverse orthosteric agonist NMS at muscarinic M_2 receptors.*

	[^{3}H]NMS-dissociation kinetics		[^{3}H]NMS-equilibrium binding	
Compound	$LogK_{X,diss}$ X⭘NMS-M_2	n slope	$logIC_{50}$ X⭘M_2	n slope
10-C7	-6.36* ±0.04	-1.17 ±0.14	-8.38 ±0.08	-0.63 ±0.05**
10-C10	-6.94 ±0.04	-0.67 ±0.05**	-8.24* ±0.09	-0.98 ±0.16
7b-C10	-7.18* ±0.05	-0.77 ±0.08	-8.53 ±0.04	-0.76 ±0.05**
7a-C10	-5.57* ±0.07	-0.88 ±0.13	-8.42 ±0.05	-0.63 ±0.04**
7a-C6	-4.93*, § ± 0.03	-0.89 ±0.05	-6.03* ±0.02	-0.98 ±0.05

$logK_{X,diss}$, log binding constant of the allosteric agent X for NMS-bound receptors measured as the concentration of the test compound ligand that reduces the dissociation rate constant of [^{3}H]NMS dissociation by 50%; n: slope factor of the curve; $logIC_{50}$: log concentration of the test compound X reducing the specific binding of the orthosteric radioligand [^{3}H]NMS in the absence of X by 50%. The data shown are mean values ± S.E.M. of three to four experiments carried out in duplicate (dissociation) or triplicate (equilibrium binding). *, value was determined with curve slope fixed to unity; **, value deviates significantly from unity (F-test, P<0.05). **§,** value was taken from Bock et al. 2014. For further details see references Fang et al. 2010 and detailed pharmacological procedures.

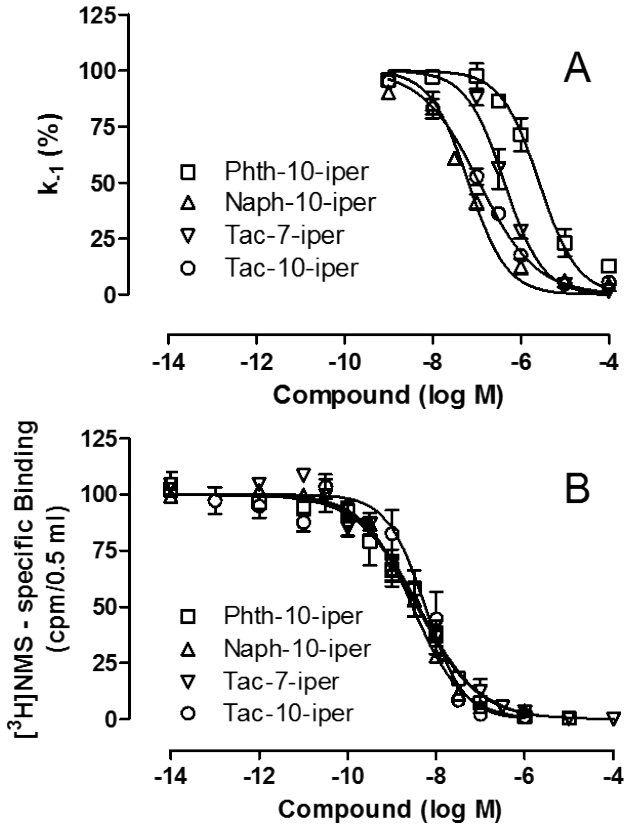
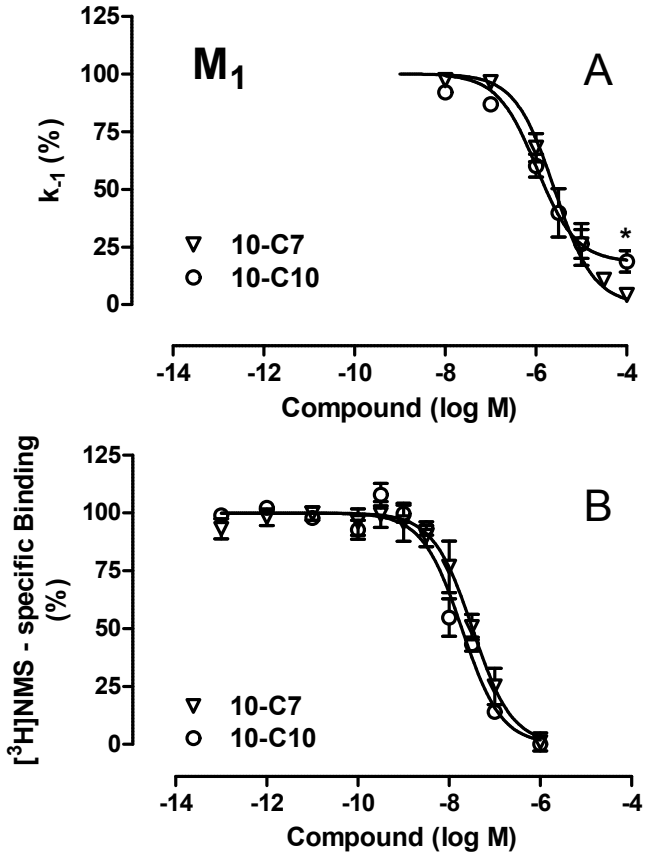


Figure S1: (A) Retardation of NMS dissociation expressed as concentration-effect curves of selected test-compounds on the dissociation rate constant k_{-1} of the radioligand [^{3}H]NMS to determine $logK_{X,diss}$ as an affinity measure of the test compounds at M_2 receptors that are orthosterically blocked by NMS. (B) Test-compound induced inhibition of specific [^{3}H]NMS (0.2 nM) equilibrium binding to estimate corresponding affinity measures at orthosterically unliganded M_2 receptors. Curves were obtained by logistic curve fitting. The data illustrated are mean values ± S.E.M. of three to four experiments carried out as (A) duplicate or (B) triplicate determinations.

Table S2: Numerical estimates of parameters from binding experiments characterizing the interaction of selected test compounds with the inverse orthosteric agonist NMS at muscarinic M_1 receptors.

	[^{3}H]NMS-dissociation kinetics		[^{3}H]NMS-equilibrium binding	
Compound	$LogK_{X,diss}$ X⭘NMS-M_1	n slope	$logIC_{50}$ X⭘M_1	n slope
10-C7	-5.59* ±0.08	-0.81 ±0.12	-7.49* ±0.06	-0.98 ±0.14*
10-C10	-5.98*,§ ±0.11	-0.89§ ±0.20	-7.72* ±0.05	-1.05 ±0.11

$logK_{X,diss}$, log binding constant of the allosteric agent X for NMS-bound receptors measured as the concentration of the test compound ligand that reduces the dissociation rate constant of [^{3}H]NMS dissociation by 50%; n: slope factor of the curve; $logIC_{50}$: log concentration of the test compound X reducing the specific binding of the orthosteric radioligand [^{3}H]NMS in the absence of X by 50%. The data shown are mean values ± S.E.M. of three to four experiments carried out in duplicate (dissociation) or quadruplicate (equilibrium binding). *, value was determined with curve slope fixed to unity; §, values derived with bottom plateau significantly different from zero (F-test, P<0.05). For further details see references Fang et al. 2010 and detailed pharmacological procedures.



104

105 *Figure S2: (A) Retardation of NMS dissociation expressed as concentration-effect curves of selected*
106 *test-compounds on the dissociation rate constant k_{-1} of the radioligand [^{3}H]NMS to determine*
107 *log$K_{X,diss}$ as an affinity measure of the test compounds at M_1 receptors that are orthosterically*
108 *blocked by NMS. (B) Test-compound induced inhibition of specific [^{3}H]NMS (0.2 nM) equilibrium*
109 *binding to estimate corresponding affinity measures at orthosterically unliganded M_1 receptors.*
110 *Curves were obtained by logistic curve fitting. The data illustrated are mean values ± S.E.M. of three*
111 *to four experiments carried out as (A) duplicate or (B) quadruplicate determinations.*, bottom*
112 *plateau deviates significantly from zero (F-Test, P< 0.05).*

113

114

115

116

117

118

119

120

121

Table S3: Experimentally determined logk` and calculated logP values of selected reference substances.

Reference substance	logk`	logP[1]
Biphenyl	1.08	4.01
Ethylbenzene	0.87	3.15
Chlorobenzene	0.62	2.89
Toluene	0.66	2.73
Benzene	0.41	2.13
2-Phenylethanol	-0.01	1.36
Acetanilide	-0.22	1.00
2-Butanone	-0.35	0.30

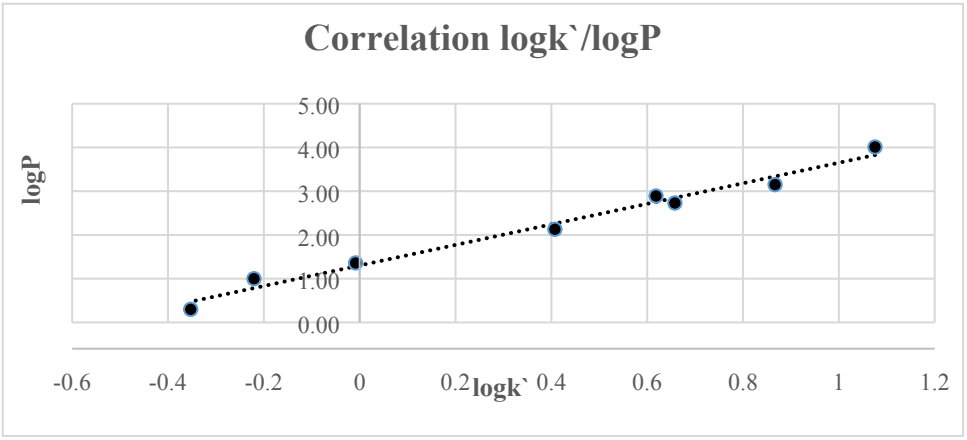


Table S4: Experimentally determined k` and logk` values and calculated logP values of the hybrid compounds.

Compound	k`	logk`	logP
7a-C7	0.60	-0.22	0.79
7a-C8	0.60	-0.22	0.78
7a-C9	0.60	-0.22	0.79
7a-C10	0.61	-0.21	0.80
8a-C4	1.27	0.11	1.55
8a-C6	0.74	-0.13	0.99
8a-C8	0.63	-0.20	0.83
6a-C6	0.63	-0.20	0.84
6b-C6	1.26	0.10	1.54
7b-C7	1.47	0.17	1.70
7b-C8	1.35	0.13	1.61
7b-C9	1.46	0.16	1.69
7b-C10	1.58	0.20	1.77
10-C10	6.96	0.84	3.28
10-C7	2.08	0.32	2.05

Sequence alignment

The identity and homology between *ee*AChE and *h*AChE amounts to 88% and 93%, respectively. Therefore, *ee*AChE can be used to replace *h*AChE in enzyme kinetic measurements. *Tc*AChE shows an identity and homology of about 57% and 73%, respectively, to both *ee*AChE and *h*AChE. The binding site of these three isoforms is highly conserved, with the replacement of Phe330 in *ee*AChE and *Tc*AChE by Tyr337 in *h*AChE as the main difference. Thus, *Tc*AChE can savely be used as model system for docking studies even though enzyme kinetic data may be obtained with other AChE isoforms.

Table S5: Sequence alignment of electric eel, torpedo californica and human AChE with identity (left) and homology (right).

	Identity [%]			**Homology [%]**		
	*Tc*AChE	*ee*AChE	*h*AChE	*Tc*AChE	*ee*AChE	*h*AChE
*Tc*AChE	100	57.7	56.5	100	73.4	73.8
*ee*AChE	-	100	88.0	-	100	93.0
*h*AChE	-	-	100	-	-	100

Method:

Pairwise sequence alignment was carried out with the Needle program, using the EBLOSUM62 matrix of EMBOSS v.6.3.1.[2] The PDB-IDs 4EY7 (human),[3] 1C2O (electric eel)[4] and 2CKM (torpedo californica)[5] were taken for comparison.

References

1. C. Hansch, A. Leo and D. Hoekman, *Exploring QSAR: Volume 2: Hydrophobic, Electronic and Steric Constants, American Chemical Society*, Waschington DC, 1995.
2. P. Rice, I. Longden and A. Bleasby, *Trends Genet.*, 2000, **16**, 276-277.
3. J. Cheung, M. J. Rudolph, F. Burshteyn, M. S. Cassidy, E. N. Gary, J. Love, M. C. Franklin and J. J. Height, *J. Med. Chem.*, 2012, **55**, 10282-10286.
4. Y. Bourne, J. Grassi, P. E. Bougis and P. Marchot, *J. Biol. Chem.*, 1999, **274**, 30370-30376.
5. E. H. Rydberg, B. Brumshtein, H. M. Greenblatt, D. M. Wong, D. Shaya, L. D. Williams, P. R. Carlier, Y. P. Pang, I. Silman and J. L. Sussman, *J. Med. Chem.*, 2006, **49**, 5491-5500.