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# Competition radioligand binding assays for the investigation of bispyridinium compound affinities to the human muscarinic acetylcholine receptor subtype 5 ($hM_5$)

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**Standard treatment of poisoning by organophosphorus (OP) nerve agents with atropine and oximes lacks efficacy with some nerve agents. Promising *in vitro* and *in vivo* results were obtained with the bispyridinium compound SAD-128 which was partly attributed to its interaction with nicotinic acetylcholine receptors. Previous studies indicate that bispyridinium compounds interact with muscarinic acetylcholine receptors as well. The muscarinic $M_5$ receptor is not well investigated compared to other subtypes, but could be important in the search for new drugs for treating nerve agent poisoning. A set of bispyridinium compounds structurally related to SAD-128 were tested in competition binding experiments with recombinant human $M_5$ muscarinic acetylcholine receptors. Five of the six investigated bispyridinium compounds interacted with the orthosteric binding site, with affinities in the low micromolar range. These data indicate that interaction of bispyridinium compounds with muscarinic receptors may contribute to their therapeutic efficacy. Copyright © 2012 John Wiley & Sons, Ltd.**

**Keywords:** muscarinic acetylcholine receptor; human $M_5$ receptor; bispyridinium compound; radioligand binding assay; [$^3$H] N-methylscopolamine

## Introduction

Muscarinic receptors responding to the natural ligand acetylcholine have a widespread tissue distribution and are involved in the control of numerous central and peripheral responses. They belong to the group of G-protein coupled receptors (GPCRs) because they are mediated by guanine nucleotide-dependent transducer proteins, the so-called G-proteins. $M_2$ and $M_4$ muscarinic receptor subtypes can couple to $G_{i/o}$ proteins, and $M_1$, $M_3$ and $M_5$ subtypes to $G_{q/11}$ proteins.[1] Coupling to the G-protein causes the receptors to convert to a high-affinity state for agonists.[2] The orthosteric binding pocket of the muscarinic receptor family is highly conserved, making the development of subtype selective agonists and antagonists very difficult.[3] The function of the $M_5$ mAChR is not yet clear, because the inability to clearly distinguish it from subtype $M_3$ has led to confusion about its physiological role. Determination of the precise distribution of $M_5$ receptors within different tissues is complicated by inadequate selectivity of radioligands as well as the low sensitivity/selectivity of polyclonal antisera in immunocytochemical studies. It appears that muscarinic $M_5$ receptors are selectively enriched in the *Substantia nigra* and ventral tegmental areas of the brain, suggesting that they may have a role in the modulation of dopaminergic transmission,[4] and stimulation of $M_5$ AChR results in an activation of dopaminergic pathways.[5] It has also been shown, however, that $M_5$ mAChRs are expressed ubiquitously throughout the brain and in non-neuronal tissues.[6] For example, the $M_5$ mAChR is required for cholinergic dilation of central blood arteries and arterioles.[7]

The role of $M_5$ mAChR in context of intoxication with organophosphorus (OP) nerve agents is not well investigated so far. Exposure to organophosphorus compounds has been associated with down-regulation of $M_5$ AChR related genes. Stimulation of $M_5$ AChR results in an activation of dopaminergic pathways which might explain the relationship between OP exposure and higher incidence of Parkinson's disease.[5] Although the physiological role of this receptor subtype has not been mapped comprehensively, investigations in the context of drug discovery for the treatment of nerve agent poisoning should not be neglected. Previous studies with the bispyridinium non-oxime SAD-128 demonstrated its therapeutic effect against soman *in vitro* and *in vivo* which was

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**Abbreviations:** *mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; GPCR, G-protein-coupled receptor; CHO, Chinese Hamster Ovary; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; AQ-RA 741, 11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one; 4-DAMP, 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide; NMS, N-methylscopolamine methyl chloride; VU 0238429, 1-(4-Methoxybenzyl)-5-trifluoromethoxyisatin*

partly attributed to its interaction with nicotinic acetylcholine receptors (nAChRs).[8–13] In addition, bispyridinium compounds have been shown to interact with muscarinic acetylcholine receptors, although primarily with the allosteric binding site.[14–18] In comparison to other mAChR subtypes, only a few binding studies have been performed with human $M_5$ mAChRs. Therefore, we initiated the present study to investigate interactions of non-oxime bispyridinium compounds with the $M_5$ mAChR subtype and focused on the orthosteric binding site. For the pharmacological characterization of this binding site, receptor binding experiments with $hM_5$ stably expressed in CHO cells were for example, phosphate buffer,[19–21] Tris–HCl buffer,[22] Krebs-Henseleit buffer,[23] and HEPES buffer,[24–27] and beyond the incubation time (1–5 h) and temperature (20-37 °C). In this present work, receptor binding experiments with [$^3$H]NMS as marker and 10 mM HEPES buffer as assay buffer were conducted. As an integral part of study, six novel SAD-128 analogues (Figure 1),[28] which showed promising effects in improving soman-impaired neuromuscular transmission,[29] were tested on membrane preparations of $hM_5$ mAChR/CHO-K1 cells applying a high-throughput binding assay with a custom-designed pipetting platform.

## Materials and methods

### Materials

CHO-K1 cell membrane preparations with human muscarinic receptors subtype $M_5$, and scopolamine methyl chloride, [N-methyl-$^3$H] with a specific activity of approximately 3 TBq/mmol, were purchased from Perkin Elmer, Jügesheim, Germany. AQ-RA 741, 4-DAMP, 5-methylfurmethiodide, oxotremorine M, pirenzepine, scopolamine hydrobromide and VU 0238429 were obtained from Tocris, Bristol, UK, atropine and carbamoylcholine chloride from Sigma-Aldrich, Taufkirchen, Germany. The bispyridinium compounds MB327, MB424, MB442, MB456, MB583 and MB770 (Figure 1) were synthesized at Dstl Porton Down, Salisbury, UK.[29] Stock solutions of bispyridinium compounds (1 mM), scopolamine hydrobromide (1 mM) and carbamoylcholine chloride (10 mM) were prepared in distilled water, atropine in ethanol (10 mM), and AQ-RA 741, 4-DAMP, 5-methylfurmethiodide, oxotremorine M and pirenzepine in 10% aqueous DMSO (1 mM), VU 0238429 in DMSO (1 mM) and stored at −20 °C until use.

### Radioligand binding

Radioligand binding experiments were performed according to described [$^3$H]NMS assay methods[30] with a few modifications. Pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-well plates, in assay binding buffer (10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 adjusted with NaOH at ambient temperature). An aliquot of the CHO/$hM_5$ mAChR membrane fraction was rapidly thawed and diluted in a 50-fold volume of cold binding buffer. Total protein amounted to 10 µg per well and total volume in each well was 250 µl. The membrane suspension was stirred (200 rpm) at +4 °C. After an incubation period of 120 min at 20 °C, bound and free [$^3$H]NMS were separated by rapid vacuum filtration using a cell harvester (Perkin Elmer, Jügesheim, Germany) onto GF/B filter plates (Perkin Elmer, Jügesheim, Germany), previously pre-soaked for 10 min in 0.1% polyethylenimine[31] to minimize non-specific binding[32] and rapidly washed eight times with ice-cold washing buffer (10 mM HEPES, pH 7,4 adjusted with NaOH at 1 °C). After 60 min drying at 50 °C, the membrane-containing filters were treated with a melt-on scintillator (MeltiLex B, Perkin Elmer-Wallac, Turku, Finland) for 4 min at 95 °C. Radioactivity was quantified using single photon counting on a MicroBeta scintillation counter (Perkin Elmer, Jügesheim, Germany) at ambient temperature. For considering the quench effects, concentrations of bound [$^3$H]NMS were calculated with a calibration curve (linear regression). To obtain the calibration curve, aliquots of the [$^3$H]NMS concentrations used in the assay were applied to the filter mate before melting of the solid scintillator.

In all experiments, total ligand binding never exceeded more than 10% of the added ligands in order to limit complications associated with depletion of the free radioligand concentration.[33,34]

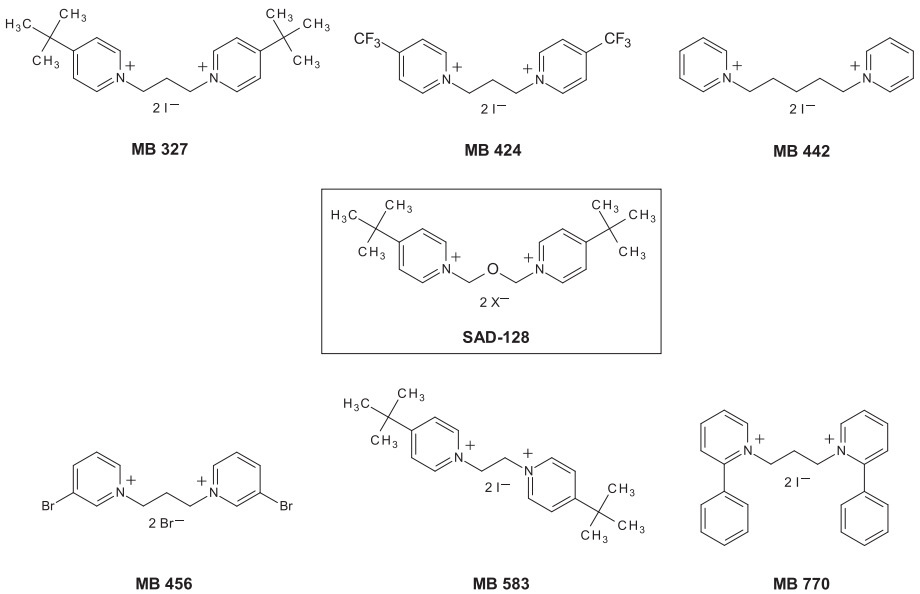


**Figure 1.** Structures of the tested bispyridinium compounds which are structurally related to SAD-128.

Total protein concentration was determined by the bicinchoninic acid method,[35] using bovine serum albumin as standard.

### [³H]NMS saturation binding

Binding was performed with assay concentrations of 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 4 nM, 7 nM and 10 nM [³H]NMS to obtain saturation binding curves. Non-specific binding was determined in the presence of 10 μM atropine. Aliquots of protein suspension (≈ 10 μg/well) were incubated at 20 °C with gentle agitation (≈ 300 rpm) for 120 min. Binding data for each concentration were obtained from triplicates.

### Competition assays

To obtain the binding affinity of unlabelled compounds to the orthosteric binding site, [³H]NMS competition experiments were performed at equilibrium. [³H]NMS was used at an assay concentration of 3 nM. Total binding was determined without competitor and non-specific binding in the presence of 10 μM unlabelled atropine in the assay. [³H]NMS was incubated in the presence of six indicated concentrations of the unlabelled compounds and protein suspension (≈ 10 μg/well) at 20 °C with gentle agitation for 120 min. The estimation of $K_i$ values included three independent experiments, each performed in six replicates.

### Data analysis

Specific binding was defined as the difference between total binding and non-specific binding. The concentration of a competing test compound that inhibited 50% of specific binding ($IC_{50}$) was calculated with nonlinear regression (Prism 5.0, GraphPad Software, San Diego, CA, USA) for sigmoidal dose–response curves obtained in competitive binding experiments (One site – Fit $K_i$). Top and bottom of the sigmoidal curve were constrained to values obtained for total binding (without competitor, top) or non-specific binding (in the presence of 10 μM atropine, bottom). $K_i$ values were determined according to the Cheng and Prusoff equation.[36] Values for the equilibrium dissociation constant ($K_D$) and the maximum density of binding sites ($B_{max}$) were calculated from binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.0). All data are expressed as means ± SEM (n = 3–6).

## Results and discussion

### Saturation assays

Depending on the incubation temperature, the membrane preparation revealed a slightly different maximum number of binding sites ($B_{max}$) that were occupied by mAChR antagonist [³H]NMS which addressed the orthosteric binding site. Figure 2 shows saturation isotherms with assay temperatures adjusted to 20 °C (Figure 2a) and 30 °C (Figure 2b). $B_{max}$ values of 6.93 ± 1.04 and 7.38 ± 1.36 pmol/mg protein were recorded at 20 °C and 30 °C, respectively. Considering the analytical uncertainty, there was no significant difference. However, the [³H]NMS dissociation constants ($K_D$) differed significantly depending on incubation conditions: $K_D$ values of 0.77 ± 0.11 nM and 5.06 ± 1.32 nM (n = 6) were calculated for 20 °C and 30 °C assay temperatures, respectively. During the complete study, the same batch of receptor membrane preparation aliquot for each assay was used to avoid inter-batch variations. A similar effect was observed when assays were performed in 10 mM HEPES buffer without $MgCl_2$.

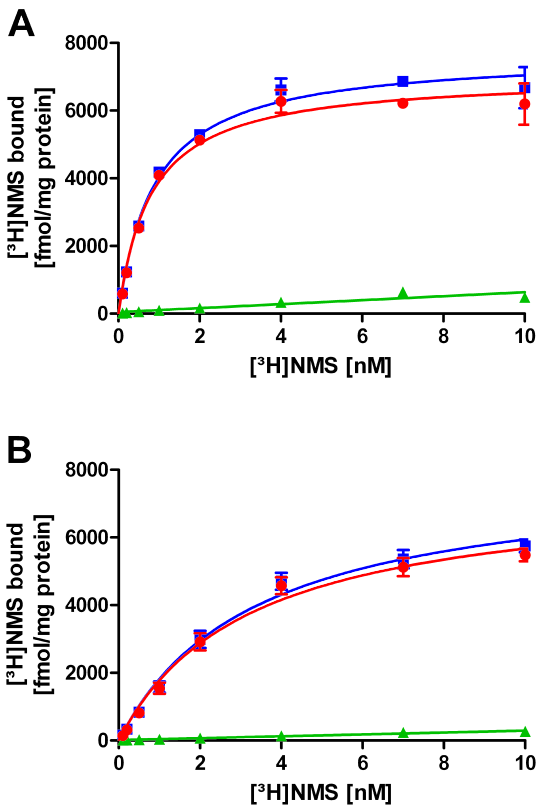


**Figure 2.** Saturation isotherms of [³H]NMS binding to $hM_5$ mAChR membrane fractions. (a) incubation temperature 20 °C. (b) incubation temperature 30 °C. Data are shown as means ± SEM (n = 3). Blue line and squares: total binding. Green line and triangles: non-specific binding. Red line and circles: specific binding.

Increasing of the incubation time to 4 h showed no significant altering of $K_D$ values.

Preliminary studies in our laboratory reflected an even more drastic influence of the assay temperature on the affinity of the weak agonist [³H]oxotremorine M. At an incubation temperature of 30 C the affinity of [³H]oxotremorine M decreased by several orders of magnitude relative to 20 C (data not shown). This effect is probably related to the fact that [³H]oxotremorine M selectively labels only a fraction of the high-affinity receptors – a phenomenon which was observed for several mAChR agonists.[37] It appeared that at temperatures higher than 20 °C, the muscarinic acetylcholine receptor converted from a high affinity G-protein coupled state to a low-affinity G-protein uncoupled state. It was found that the optimum temperature for monitoring muscarinic receptor-G protein interactions in ligand binding assays was 16–20 C.[2]

Equilibrium and rate constants are known to be temperature dependent, with equilibrium binding of antagonists tending to be entropy driven.[38] With G-protein-coupled histamine $H_3$ receptors, increased temperature resulted in higher $K_D$ values, but had no effect on $B_{max}$ values.[39]

The $K_D$ value of 0.77 ± 0.11 nM for [³H]NMS obtained in experiments with the $hM_5$ mAChR at the 20 °C incubation temperature was still slightly higher than $K_D$ values previously reported (0.11-0.48 nM).[19–27] As already mentioned in the introduction, buffers, incubation temperature and time were different. According to the literature data, there was no evidence that higher temperatures led to higher $K_D$ values.[23,24]

This is in contrast to the results achieved in this work. Rather, the different $K_D$ values may depend on the selected buffer. The pH of HEPES buffer is more temperature-dependent than the pH of phosphate buffers, but HEPES is compatible with $MgCl_2$ whereas phosphate or citrate containing buffers may chelate cations, such as $Mg^{2+}$, added in millimolar concentrations. $MgCl_2$ may promote agonist binding to G-protein-coupled receptors by favouring the formation of the high-affinity agonist-receptor-G-protein complex.[40] To avoid variations within assay conditions, HEPES buffer was adjusted to pH 7.4 at the temperature used during incubation and washing. Another reason could be the lower ionic strength compared to the buffers cited in literature. Higher ionic strength seems to increase affinities.[41] In conclusion, it is evident that experimental factors (particularly the buffer composition) may influence ligand binding affinity.

### Competition assays

Affinity constants measured for well-known probes of mAChRs and for the bispyridinium compounds, expressed as $K_i$ [nM] and their negative logarithm ($pK_i$), appear in Table 1. The $pK_i$ values obtained in this assay are in excellent agreement with previously recorded values in case of the antagonists 4-DAMP, pirenzepine, AQ-RA 741 and the agonist carbamoylcholine.[20,42–44] Figure 3 illustrates competition curves for selected agonists and antagonists. Affinity constants obtained under comparable assay conditions have not previously been published for scopolamine, oxotremorine M and 5-methylfurmethiodide. However, radioligand binding assays with recombinant $hM_1$-$hM_4$ mAChR yielded $pK_i$ 8.7-9.5 for scopolamine,[45] $pK_i$ 4.9-5.2 for oxotremorine M and $pK_i$ 4.6–4.9 for 5-methylfurmethiodide.[46] In view of the fact that these ligands are not subtype-selective, the binding affinities of these compounds are in the same range for the $M_1$, $M_2$, $M_3$ and $M_4$ mAChRs.

Surprisingly, atropine displayed an approximately 10-fold weaker affinity ($pK_i$ 8.5 ± 0.05) for the $hM_5$ mAChR than described in literature.[20,43] Similar results were obtained with other batches of atropine and [³H]NMS. Quality control by NMR spectroscopy and liquid chromatography-tandem mass spectrometry (LC-MS/MS) led to the conclusion that the original substance and the solutions were valid. Competition assays with $hM_1$ mAChR using identical solutions of atropine and [³H]NMS exhibited $pK_i$ 8.6 ± 0.07 which was in agreement with cited values of 8.5-9.3.[45,47,48] In functional affinity estimates, obtained for antagonists against carbamoylcholine-stimulated [³H]inositol phosphates accumulation in CHO-K1 cells expressing human recombinant muscarinic $M_5$ receptors, $pK_i$ values of 8.7 (atropine), 6.4 (pirenzepine), 6.1 (AQ-RA 741) and 8.6 (4-DAMP) were reported[49] which demonstrates an excellent agreement with the present data.

**Table 1.** Interaction of ligands with decreasing affinities at [³H]NMS binding sites of $hM_5$ mAChR. $K_i$ is the equilibrium dissociation constant, $pK_i$ is the negative logarithm of $K_i$. Data are means ± SEM (n = 3)

| Ligand | $K_i$ [nM] | $pK_i$ | $pK_i$ (cited) |
|---|---|---|---|
| Scopolamine | 0.96 ± 0.09 | 9.0 ± 0.05 | |
| 4-DAMP | 1.44 ± 0.16 | 8.8 ± 0.05 | 8.6-9.0 [20,42,43] |
| Atropine | 3.15 ± 0.36 | 8.5 ± 0.05 | 9.1-9.7 [20,43] |
| Pirenzepine | 102 ± 12 | 7.0 ± 0.05 | 6.2-7.1 [20,42,43] |
| AQ-RA 741 | 613 ± 120 | 6.2 ± 0.09 | 6.1 [42] |
| Oxotremorine M | 4350 ± 649 | 5.4 ± 0.07 | |
| 5-Methylfurmethiodide | 4470 ± 897 | 5.4 ± 0.09 | |
| Carbamoylcholine | 14900 ± 6220 | 4.8 ± 0.24 | 4.9 [44] |
| VU 0238429 | 19000 ± 7170 | 4.7 ± 0.21 | |
| MB770 | 1440 ± 261 | 5.8 ± 0.09 | |
| MB327 | 3300 ± 1120 | 5.5 ± 0.18 | |
| MB442 | 6620 ± 1390 | 5.2 ± 0.10 | |
| MB456 | 8550 ± 1800 | 5.1 ± 0.10 | |
| MB583 | 10800 ± 2700 | 5.0 ± 0.12 | |
| MB424 | > 100000 | < 4.0 | |

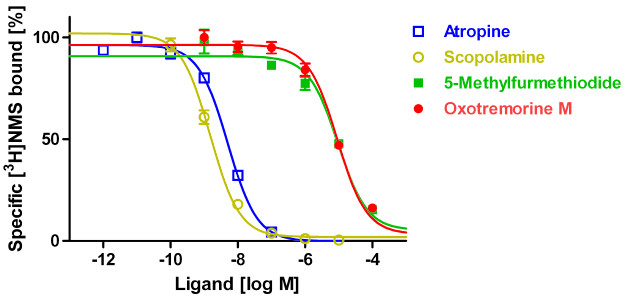


**Figure 3.** Competition binding curves with selected agonists and antagonists. Data are shown as means ± SEM (n = 6). Open symbols: antagonists. Filled symbols: agonists.

VU 0238429, described as a selective positive allosteric modulator of $M_5$ receptors,[50] revealed a weak activity (19 μM) at the $M_5$ orthosteric binding site.

The binding affinities of the tested bispyridinium compounds varied widely (Table 1). MB770, MB327, MB442, MB456 and MB583 exhibited $K_i$ values of 1.4 μM, 3.3 μM, 6.6 μM, 8.6 μM and 10.8 μM, respectively. Maximum affinity was observed for MB770 which contains a phenyl substituent like other high-affinity ligands such as scopolamine, 4-DAMP, atropine and pirenzepine. Shortening the alkane linker from C3 (MB327) to C2 (MB583) in the 4-*tert*-butyl series resulted in lower affinity. With MB424, the only compound among those shown in Figure 1 without pharmacological benefit in soman-poisoned guinea pig diaphragm preparations,[29] no interaction with the orthosteric binding site was detected. Fluorination apparently causes loss of activity considering that the only difference between this compound and the good ligand MB327 is the presence of $CF_3$ groups rather than *tert*-butyl groups at the 4-position of the pyridine rings.

Figure 4 shows exemplary the displacement curve for MB770, the investigated bispyridinium compound with the highest

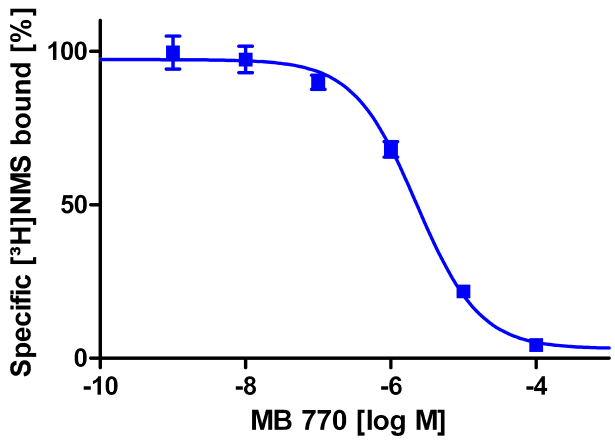


**Figure 4.** Competition binding curve of the bispyridinium compound MB770. Data are shown as means ± SEM (n = 6).

affinity. Compared to the [$^3$H]epibatidine binding sites of human α7 and *Torpedo* nicotinic acetylcholine receptors, the $K_i$ values of MB770, MB442 and MB450 are approximately 10–100-fold lower.[51] Interestingly, an additional phenomenon was observed with the bispyridinium compound MB442. Specific binding of [$^3$H]NMS was correlated positively to the MB442 concentration and reached a maximum of 150% of total binding at approximately 100 nM MB442, and decreased at higher ligand concentrations. This could indicate a mixed mode of competitive and allosteric interactions[52] since such effects are known for other mAChR subtypes.[53,54] To confirm this hypothesis, further investigations, preferably functional assays,[55] will have to be performed.

Muscarinic receptors require that agonists bear a positive charge under physiological conditions: an aspartic acid residue on the receptor macromolecule provides the negative charge for ligand binding. The high-affinity probes tested – scopolamine, 4-DAMP, atropine, pirenzepine, AQ-RA 741, oxotremorine M, 5-methylfurmethiodide and carbamoylcholine – all possess this feature. The bispyridinium compounds have two positive charges. Whether $M_5$ mAChR interacted with both positive charges remains unclear, since no analogous monomeric compounds were investigated yet. However, the $M_5$ mAChR differed between the compounds to extents that depend on their degree of separation (compare binding data for C3 and C2 4-*tert*-butyl analogues, MB327 and MB583, with that for the C5 bispyridinium analogue MB442 lacking the 4-*tert*-butyl groups). There may be scope for optimisation of bispyridinium ligands for the $hM_5$ mAChR by exploring more fully the effect of different ring substituents and linker lengths on binding affinity.

## Conclusion

The database on the binding of compounds to the human $M_5$ mAChR is small although this receptor is of relevance for the investigation of possible agents for the treatment of OP poisoning. Five of the six bispyridinium test compounds interacted with the orthosteric binding site, whereas one exhibited an atypical displacement curve that possibly indicated an allosteric or bitopic interaction with the $hM_5$ mAChR. Affinity of binding depended on two structural features in the bispyridinium compounds: the ring substituents and the length of the alkane linker separating the two rings. These preliminary results indicate that bispyridinium non-oximes interact with muscarinic as well as nicotinic receptors, and this interaction may contribute to their therapeutic efficacy in the treatment of OP poisoning *in vivo*.

#### Acknowledgements

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