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Atypical muscarinic allosteric modulation: cooperativity between modulators and their atypical binding topology in muscarinic M_2 and M_2/M_5 chimeric receptors

Christian Tränkle, Andreas Dittmann, Uwe Schulz, Oliver Weyand, Stefan Buller, Kirstin Jöhren, Eberhard Heller, Nigel J. M. Birdsall, Ulrike Holzgrabe, John Ellis, Hans Dieter Höltje and Klaus Mohr

Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Str. 3, D-53121 Bonn, Germany;

Institute of Pharmaceutical & Medicinal Chemistry, Heinrich Heine-University of Düsseldorf, Universitätsstr. 1, Building 26.23, 40225 Düsseldorf;

Pharmaceutical Chemistry, Institute of Pharmacy, University of Würzburg, Am Hubland, 97074 Würzburg;

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK;

Departments of Psychiatry and Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania, USA.

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Running title page

Running title: Atypical muscarinic allosteric modulation

Corresponding author: Christian Tränkle

Tel.: +49-228-739104; Fax.: +49-228-739215; e-mail: traenkle@uni-bonn.de

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Abbreviations: M_2 receptor, M_2 subtype of the muscarinic acetylcholine receptor; NMS, (-)-N-methylscopolamine; dimethyl-W84, N,N´-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2H-isoindol-2-yl)propyl]-N,N,N´,N´-tetramethyl-1,6-hexanediaminium diiodide; Duo3, 4,4´-bis-[(2,6-dichloro-benzyloxy-imino)-methyl]-1,1´-propane-1,3-diyl-bis-pyridinium dibromide; WDuo3, 1,3-bis[4-(phthalimidomethoxyimino-methyl)-pyridinium-1-yl] propane dibromide; tacrine dimer, 1,6-bis[amino-1,2,3,4-tetrahydroacridinyl]-hexane.

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Abstract

The binding and function of muscarinic acetylcholine receptors can be modulated allosterically. Some allosteric muscarinic ligands are 'atypical', having steep concentration-effect curves and not interacting competitively with 'typical' allosteric modulators. For atypical agents a second allosteric site has been proposed. Different approaches have been used to gain further insight into the interaction with M_2 receptors of two atypical agents, tacrine and the bispyridinium compound, Duo3. Interaction studies, using radioligand binding assays and the allosteric ligands obidoxime, Mg^{2+}, and the new tool hexamethonium to antagonise the allosteric actions of the atypical ligands, showed different modes of interaction for tacrine and Duo3 at M_2 receptors. A negatively cooperative interaction was observed between hexamethonium and tacrine (but not Duo3). A tacrine dimer, which exhibited increased allosteric potency relative to tacrine but behaved like a typical allosteric modulator, was competitively inhibited by hexamethonium. M_2/M_5-receptor mutants revealed a dependence of tacrine and Duo3 affinity on different receptor epitopes. This was confirmed by docking simulations using a 3D model of the M_2 receptor. These showed that the allosteric site could accommodate two molecules of tacrine simultaneously but only one molecule of Duo3 that binds in different mode from 'typical' allosteric agents. Therefore the atypical actions of tacrine and Duo3 involve different modes of receptor interaction, but their sites of attachment seem to be the 'common' allosteric binding domain at the entrance to the orthosteric ligand binding pocket of the M_2-receptor. Additional complex behaviour may be rationalised by allosteric interactions transmitted within a receptor dimer.

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A rapidly increasing number of G protein coupled receptors (GPCRs) have been discovered to be sensitive to allosteric modulation (Christopoulos and Kenakin, 2002). Potentially favourable features for a clinical application of such modulation include the enhancement of the binding of endogenous ligands (but also exogenous agonists and antagonists), absolute subtype selectivity of action and self-limiting effects on receptor function (see Christopoulos and Kenakin, 2002 for review).

Over the past decades allosteric interactions at muscarinic acetylcholine receptors have been intensively studied (e.g. Ellis, 1997; Mohr et al., 2003, Birdsall and Lazareno, 2005). All five muscarinic receptor subtypes are sensitive to allosteric modulation (Ellis et al., 1991) but many allosteric modulators have their highest affinity for the M_2 subtype (e.g. Ellis et al., 1991; Buller et al., 2002; Jakubík et al, 2005), a fact that has been used to gain insight into the binding topology of allosteric ligands. The effects of many of these ('typical') allosteric agents have been shown to be mediated via a "common allosteric site" of the M_2 receptor (Ellis and Seidenberg, 1992; Tränkle et al., 1997; Lanzafame et al., 1997; Ellis and Seidenberg, 2000; Tränkle et al., 2003). Site directed mutagenesis has revealed for some common site allosteric agents that the amino acids M_2 ^{177}Tyr and M_2 ^{423}Thr (Buller et al., 2002; Voigtländer et al., 2003) fully account for the M_2/M_5 selectivity of the ligands. According to a 3-dimensional model of the M_2 receptor, the allosteric site is located at the entrance of the ligand binding pocket in a cleft-like vestibule that is linked by a narrow corridor with the orthosteric, acetylcholine binding site (Voigtländer et al., 2003).

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There is another group of muscarinic allosteric agents termed 'atypical' allosteric modulators. Tacrine (Flynn and Mash, 1989; Potter et al., 1989) and Duo3 (Fig.1; Tränkle and Mohr, 1997) represent archetypal members of this group (Fig.1). These agents have concentration-effect curves with slope factors greater than 1 (Potter et al., 1989, Tränkle and Mohr 1997). Furthermore, the competitive ligand at the common allosteric site, obidoxime (Ellis and Seidenberg, 1992), inhibits the actions of Duo3 only weakly and in a non-competitive fashion (Tränkle and Mohr, 1997). Additionally, the affinity of Duo3 is much less sensitive to the buffer composition compared with ´typical´ agents that are also bis-cationic but competitive with obidoxime (Tränkle et al., 1996; Schröter et al., 2000). From these data it was suggested that another allosteric site might be involved in the action of Duo3 (Tränkle and Mohr, 1997) as has also been suggested for derivatives of staurosporine and WIN 62,577 (Lazareno et al., 2000, 2002).

Tacrine hydrochloride is a comparatively small mono-cationic compound (Fig.1) whose binding is known to be sensitive to obidoxime (Ellis and Seidenberg, 1992) but the mechanism of its antagonism with muscarinic receptors has not yet been elucidated. Studies on M_2/M_5-chimeric receptors (Ellis and Seidenberg, 2000) are compatible with ^{177}Tyr and ^{423}Thr of the M_2 receptor also being involved in the binding of tacrine but this has not been directly investigated. The epitope dependency of Duo3 binding has not been studied.

Our study is aimed at gaining further insight into the molecular events and interactions underlying the allosteric actions of the ´atypical´ agents, Duo3 and

tacrine, in comparison to the ´typical´ allosteric agent WDuo3 (Fig.1). Three structurally different allosteric ligands, obidoxime, hexamethonium (Eglen et al., 1989) and Mg^{2+} (Burgmer et al., 1998) have been used to probe for differences in their interactions with WDuo3, Duo3 and tacrine. Obidoxime and hexamethonium resemble the central chains of Duo3 and WDuo3 (Fig 1) and of alkane-bisammonio-type allosteric agents (Mohr et al., 2003), respectively. Interactions with a newly synthesized tacrine-dimer (Fig.1) were also investigated. Point-mutated M_2 receptors and chimeric M_2/M_5-receptors have been used to explore the different binding modes of the chemically related typical and atypical allosteric ligands. Docking simulations using a model of the M_2 receptor (Jöhren and Höltje, 2002; Voigtländer et al., 2003), suggested that the allosteric site can easily accommodate two molecules of tacrine simultaneously. Duo3 also fits into the cleft-like allosteric site, but does not come as close as typical allosteric agents to the entrance of the orthosteric site.

Our findings suggest that some ´atypical´ allosteric agents may interact with the ‘common’ allosteric domain of the M_2 receptor protein but in an ´atypical´ fashion.

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Materials and Methods

Materials. [^{3}H]N-methylscopolamine ([^{3}H]NMS, specific activity 70-82 Ci/mmol) was purchased from NEN-Dupont, Bad Homburg, Germany. The synthesis of the radiolabeled compound [^{3}H]dimethyl-W84 (specific activity 154-168 Ci/mmol) was carried out by Amersham Life Science (Braunschweig, Germany) using the method described by Tränkle et al., 1998. Atropine sulfate, (-)scopolamine methylbromide, were obtained from Sigma Chemicals, München, Germany. Obidoxime dichloride was generously provided by Merck KG, Darmstadt, Germany. The allosteric modulators dimethyl-W84, Duo3, and WDuo3 were synthesized as described elsewhere (Mohr et al., 2003 and references therein). Hexamethylene-linked bis-tacrine: tacrine hydrochloride (5.0 mMol, 1.17 g) was suspended in 100 ml dry THF and cooled to -70°C (ethanol dry ice). *n*-butyllithium (30 mMol) was added and stirred for 15 min at -70°C. Dibromohexane (2.5 mMol, 0.61 g) was then added and the solution was stirred for 2 h at -70°C. The temperature was then allowed to rise up to -30°C. Water (10 ml) were added and the solution was stirred for a further 30 min at 20°C. The solvent was evaporated and the residue was diluted with 50 ml of water. The solid was filtered off and dried. The crude product was refluxed for 30 min in 20 ml EtOH and filtered hot. The filtrate was crystallized at +4°C to yield 0.72 g (60%) of a colourless solid. Mp 225°C (dec). ^{1}H-NMR- and MS-data are in accord to Hu et al. 2002, respectively.

W84 is commercially available from Tocris Cookson Inc. (Ellisville, MO). Hams F12 medium, DMEM, fetal calf serum, penicillin G, streptomycin, glutamine, geneticin disulfate, trypsin-EDTA-solution and HEPES were purchased from

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Sigma-Aldrich Chemie, Steinheim, Germany. Sodium butyrate was from Acros Organics, Geel, Belgium.

Receptor mutagenesis and expression.

The human M_2/M_5 chimeric receptors used in this study have been described previously (Wess et al., 1992, Ellis et al., 2000); schematic sketches of the chimeric receptors are shown in Fig. 6. The exact sequences are as follows: CR1: hM_2 1-69, hM_5 77-532; CR2 hM_5 1-76, hM_2 70-155, hM_5 163-532; CR3 hM_5 1-162, hM_2 156-300, hM_5 336-532; CR_6: hM2 1-69, hM_5 77-445, hM_2 391-466. In addition two M_2 receptors were used in which single amino acids were mutated: M_2 ^{423}Thr→His and M_2 ^{177}Tyr→Gln plus ^{423}Thr→His (Voigtländer et al., 2003). Plasmids containing the human M_2 or M_5 wild-type or mutated receptor genes were purified from bacterial cultures and transiently transfected into COS-7 cells by calcium phosphate precipitation. Cells were harvested 72h after transfection by scraping into 5 mM Na,K,P_i buffer, pH 7.4.

Membrane preparation.

Porcine cardiac membranes. Membranes were prepared as described previously (Tränkle and Mohr, 1997). All steps were carried out at 4°C. Briefly, ventricular tissue (40 g) of porcine hearts, obtained from the local slaughterhouse, was homogenized in a 0.32 M sucrose solution and centrifuged for 11 min at 300·g (2,000 rpm in a Beckman rotor 35). The supernatant was centrifuged for 40 min at 80,000·g (32,000 rpm in a Beckman rotor 35) and the resulting pellet resuspended in 4 mM Na_2HPO_4, 1mM KH_2PO_4, pH 7.4 (4 ml/g original tissue wet weight). Aliquots (0.5 ml) were shock frozen in liquid nitrogen

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and stored at -80°C, the protein content (3.9 ± 0.7 mg/ml) was measured according to Lowry et al. (1951) with human serum albumin as a standard.

COS-7 membrane preparations. COS-7 cells were grown in DMEM medium containing 100 units/ml penicillin G, 0.1 mg/ml streptomycin, 1 mM L-glutamine**,** 10 % fetal calf serum until confluent and harvested after addition of trypsin-EDTA by scraping in 4 ml ice cold Na, K, P_i-buffer (5 mM). Cells were homogenized by means of a Polytron-homogenizer (three times at "level 6" for 10 seconds on ice). Membranes were centrifuged (Avanti J25 centrifuge, JA 25.50 rotor, Beckman, Palo Alto, CA) at 40.000 x g (18.000 rpm, 30 min, 4 °C) and the pellets were resuspended in Na, K, P_i-buffer (5 mM). Membranes were stored in 1 ml aliquots at –80°C until use.

[^{3}H]NMS binding assays.

Interaction studies on cardiac membranes. Porcine cardiac membranes (200-450 µg protein/ml) were incubated with 0.2 nM [^{3}H]NMS in 5 mM Na,K,Pi-buffer, pH 7.4 at 23°C. Nonspecific binding was determined in the presence of 1 µM of atropine. Specific binding of [^{3}H]N-methylscopolamine under control conditions was characterized by a pK_D = 10.04 ± 0.16 and B_{max} = 104 ± 12 fmol/mg protein (means ± S.E.M., n = 12). In order to measure the kinetics of radioligand dissociation, the assays were prepared in a larger volume. Membranes were preincubated with radioligand for 30 min, before the time course of [^{3}H]NMS dissociation was initiated by adding 1µM atropine. Test compounds were added together with atropine, either alone or combined with an additional allosteric ligand. At specified time intervals 1 ml aliquots were

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removed and filtered rapidly (glass fibre filters No.6, Schleicher and Schüll, Dassel, FRG). The filters were washed twice with 5 ml ice cold incubation buffer, placed into scintillation vials and the radioactivity was determined in the presence of 5 ml Ready Protein® (Beckman) by liquid scintillation counting in a Beckman LS 6500.

Binding studies with membranes from cultured cells.

COS-7 cell membranes. Equilibrium binding and kinetic experiments with [^{3}H]NMS and using membranes from COS-7 cells were performed in a 1 ml volume in reaction tubes and filtered using a 48 place Brandel cell harvester and Whatman GF/B filters (Brandel Part # FBP-148L, preincubated with 0.1 % PEI for 30 min). After separation of the membranes, filters were washed twice with 5 ml ice cold 40 mM Na, K, P_i-buffer. For two point kinetic dissociation experiments (Kostenis and Mohr, 1996), membranes were pre-incubated with [^{3}H]NMS for 30 minutes. Thereafter, aliquots of the mixture were added to excess unlabelled atropine (final concentration, 3μM) in buffer, alone, or in the presence of an allosteric agent over a total period of up to 120 min followed by simultaneous filtration of all samples.

CHO cell membranes. CHO cells expressing hM_2 receptors were prepared for binding studies using [^{3}H]dimethyl-W84 as described previously (Tränkle et al., 2003). Briefly, Chinese hamster ovary cells (CHO-cells) stably transfected with the human M_2-receptor gene (generously provided by Dr. N. J. Buckley, University of Leeds, U.K.) were grown in a medium consisting of nutrient-mixture - Hams F12, 10 % fetal calf serum, 100 units/ml penicillin G, 0.1 mg/ml

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streptomycin, 1 mM L-glutamine and 0.2 mg/ml of G418 in a humidified atmosphere at 37 °C and 5 % CO_2. Sixteen hours prior to cell harvesting (cell-confluence approximately 80 %) cells were treated with 5 mM sodium butyrate. The cells were lysed and harvested by scraping in a cold homogenisation buffer (20 mM HEPES [N-[2-hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid], 10 mM Na_4EDTA, pH 7.4, 4 °C). Cells were homogenized with a Polytron-Homogenizer (PT 10-35, Kinematica AG, twice at "level 6" for 6 seconds). The membranes were pelleted by centrifugation at 40.000 x g (18.000 rpm, 10 min, 4°C) in an Avanti J25 centrifuge with a JA 25.50 rotor (Beckman, Palo Alto, CA) and washed twice in 20 mM HEPES, 0.1 mM Na_4EDTA, pH 7.4, 4°C ('storage buffer'). The final pellets were resuspended and stored as a membrane suspension in storage buffer (approx. 1.4 mg protein /ml) at –80°C.

[^{3}H]dimethyl-W84 binding assay.

Binding experiments using the allosteric radioligand [^{3}H]dimethyl-W84 were carried out as described previously (Tränkle et al., 2003). Briefly, all handling of [^{3}H]dimethyl-W84 (specific activity 154-168 Ci/mmol) was carried out in 20 mM NaCl / 0.01% bovine serum albumin (bovine serum albumin). [^{3}H]Dimethyl-W84 (1.5 - 2.0 nM) and membranes containing CHO hM_2 receptors (100 µg protein/ml) were incubated in a buffer consisting of 10 mM HEPES, 20 mM NaCl, and 0.01% bovine serum albumin, pH 7.4 at 23°C (´HEPES buffer´). [^{3}H]Dimethyl-W84 binding experiments were carried out in a 0.3 ml volume in 1.2 ml deep well plates (Abgene House, Epsom, U.K.) at 23°C temperature for 2 h. Nonspecific [^{3}H]dimethyl-W84 binding was determined in the presence of 10 µM gallamine. Specific binding of [^{3}H]dimethyl-W84 under control conditions

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was characterized by a pK_D = 8.47 ± 0.04 (K_D = 3 nM) and B_{max} 511 ± 45 fmol/mg protein (mean ± S.E.M., n = 22). The receptor bound radioligand was filtered on a Tomtech 96-well Mach III Harvester (Wallac®) and the filter (Filtermat A®, Wallac, Turku, Finland) washed once (0.8 ml 100 mM NaCl, 4 °C, 1.7 s) and dried 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).

Homology Modelling.

The model is based on the X-ray structure of bovine rhodopsin (PDB code no. 1F88/1HZX; Palczewski et al., 2000) and the sequence of the human M_2 receptor (SwissProt code P08172; Bonner et al., 1987). Transmembrane regions of the M_2 receptor were detected using so-called pinpoints, identified by Baldwin et al. (1997). The extracellular and intracellular loops were created employing a combination of different methods: secondary structure prediction and/or application of a loop search routine based on homology aspects as implemented in the Homology module of Insight II (Insight II 2000, Accelrys Inc., San Diego, CA, USA). 3D-coordinates for the N and C termini were built in analogy to the X-ray structure of bovine rhodopsin. A detailed description of the modelling procedure has been given elsewhere (Jöhren and Höltje, 2002).

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Docking. In order to dock the ligands into their binding sites within the loop region of the receptor model, FlexX (Kramer et al., 1999) was used. As a prerequisite a 3-D structure of the target protein is needed. In the case reported here a validated protein model was used. In addition, the active site has to be defined accurately. A particularly important feature of FlexX is that the ligands are treated as flexible molecules. This is accomplished by a 3-step procedure. Firstly a basic part of the molecule, 'the base fragment', is selected and placed inside the binding pocket. Subsequently, the complete molecule is constructed in a stepwise manner. For the search of various placements of the base fragment inside the binding pocket two algorithms are used. One superposes triples of interaction centers of a base fragment with triples of compatible interaction points in the active site. If a base fragment has fewer than three interaction centers or if the number of placements is too low, another algorithm, called line matching, is implemented. This procedure matches pairs of interaction centers with pairs of interaction points. Because of geometry ambiguity, multiple placements are generated by rotation around the axis defined by the interaction points and centers. Both placement algorithms generate a large number of solutions which are reduced by clash tests and clustering. Ranking of the docking results is performed employing a modified scoring function (Böhm, 1994). Algorithms and scoring functions are described in more detail elsewhere (Böhm, 1994; Rarey, 1997; Kramer et al., 1999).

Data analysis.

The data of individual experiments were analysed by non-linear regression analysis using the Prism software (Ver. 4.0, Graph Pad®, San Diego, USA). The

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dissociation data were fitted to a monoexponential decay; bi-exponential curve fitting did not yield better results (F-test, $P \geq 0.05$, data not shown). Concentration effect curves for the reduction of the observed rate constant of dissociation, k_{obs}, were analysed using a four parameter logistic function (except for hexamethonium; see below). The parameters "inflection point" and "slope factor" n were variables, the upper plateau of the curve was the control value of k_{obs} and was set $k_0 = 100\%$. Whether the lower plateau of the curve yielded a better fit with k_{obs} as a variable > 0 compared with $k_{obs} = 0\%$ was tested (F-test). If this was not the case the lower plateau of the curve was fixed at $k_{obs} = 0\%$. In addition we tested whether the slope factors of the curves were different from unity (i.e., n = 1) by statistically comparing the fits obtained by nonlinear regression analysis, using a partial F-test. A p-value < 0.05 was taken as the criterion for significance. In the case of hexamethonium, a two-site fit was used, in which it was assumed that each site of the fit exhibited a slope factor of unity (n = 1).

Analysis of interactions between allosteric ligands. The interaction of one allosteric ligand with a second allosteric agent at [^{3}H]NMS-occupied receptors was analysed using a method described by Lazareno and Birdsall (1993). The procedure can be regarded as a condensed form of the Schild method (Arunlakshana and Schild, 1959) to analyse the action of an antagonist. It requires the simultaneous analysis of two sets of data. The first data-set is the concentration-effect curve of the test allosteric agent, A, alone. The second data-set is an antagonist concentration-effect curve for the attenuation of the action of a single, fixed concentration of A by varying concentrations of a

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second allosteric antagonist, B. In a complementary fashion, we measured a concentration-effect curve for A in the presence of a fixed concentration of B to determine whether B causes a parallel shift of the concentration-effect curve for A. In the current study the observed effect monitored was the retardation of [^{3}H]NMS dissociation by the test allosteric agent in the absence and presence of (B), e.g. hexamethonium.

In order to analyse the antagonist action of one allosteric ligand on the binding of a second allosteric ligand, the effect of the first ligand on [^{3}H]NMS dissociation was eliminated from the analysis by normalization. [For details see Results]. Using hexamethonium as an example, the dose-response curves for (a) the test allosteric modulator alone (b) the test allosteric modulator in the presence of a high concentration of hexamethonium and (c) the effect of hexamethonium on [^{3}H]NMS dissociation in the presence of a fixed concentration of test modulator were simultaneously fitted using two independent variables, the concentrations of the test modulator A and of the additional allosteric antagonist B (e.g. hexamethonium), respectively. This analysis was based on the following equation (Lazareno and Birdsall, 1993) using Sigma Plot® for Windows (version 8.0, Jandel Scientific Software):

$$\text{Effect} = \frac{(E_{max} - \text{basal})}{\left(1 + \left\{\frac{EC_{0.5,control}}{[A]} \cdot \left(1 + [B]^s \cdot K_B\right)\right\}^n\right)} + \text{basal} \qquad (1)$$

[A] is the concentration of the allosteric agent, E_{max} and basal denote the maximum and the minimum effects of A, respectively, n is the slope factor of the curve (corresponding to the Hill slope factor) for A alone, $EC_{0.5,control}$ indicates

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the concentration at which A produces a half-maximal effect, [B] is the concentration of the antagonist, K_B denotes the equilibrium affinity constant of B, and s corresponds to the Schild slope factor.

Curvilinear Schild data from antagonist studies were fitted according to Lazareno and Birdsall (1995), applying the following equation:

$$\text{Effect} = \frac{(E_{max} - \text{basal})}{\left(1 + \left\{\frac{EC_{0.5,control}}{[A]} \cdot \frac{1 + [B] \cdot K_B}{1 + \alpha \cdot [B] \cdot K_B}\right\}^n\right)} + \text{basal} \qquad (2)$$

K_B is the equilibrium affinity constant for the binding of allosteric agent B at the NMS-liganded receptor. α is the cooperativity factor for the interaction between A and B at NMS-occupied M_2 receptors ($\alpha > 1$, $\alpha < 1$, $\alpha = 1$ indicating positive, negative and neutral cooperativity, respectively). Fits applying equation 1 with a Schild slope of unity, were tested versus equation 1 with a variable Schild slope (F-Test); if the latter fitted the data better, this fit was compared with a fit to equation 2. Since equation 1 and 2 possess the same number of variables, the equation which produced the lower sum of squares determined by nonlinear regression analysis was designated the better fit to the data.

Results

Sensitivity of M_2 receptors to allosteric antagonism. The hypothesis of a common allosteric site in M_2 muscarinic acetylcholine receptors was derived from experiments in which the orthosteric site of the receptors was occupied by the conventional orthosteric antagonist [^{3}H]N-methylscopolamine ([^{3}H]NMS). Under these conditions, the binding of allosteric agents is reflected by an allosteric modulation (usually reduction) of the observed dissociation rate constant of [^{3}H]NMS. Structurally different allosteric agents have been shown to share an equal sensitivity to the competitive antagonist action of obidoxime on [^{3}H]NMS dissociation, a finding that is compatible with obidoxime and the allosteric ligands binding competitively (Ellis and Seidenberg, 1992; Tränkle and Mohr, 1997). Here we introduce hexamethonium as an additional allosteric antagonist tool to obidoxime with the potential to provide useful additional information.

[^{3}H]NMS dissociation from porcine heart M_2 receptors under control conditions occurred monoexponentially (data not shown) as reported previously (Tränkle et al., 1996) with $t_{1/2,control}$ = 4.30 ± 0.15 min (means ± S.E.M, n=105). Under all conditions examined, [^{3}H]NMS dissociation remained monoexponential in the presence of hexamethonium and WDuo3, Duo3, tacrine and the tacrine dimer (Fig. 1). This finding validates the use of two-point estimations of rate constants, as performed in the mutagenesis experiments (see below). As shown in Fig. 2, hexamethonium interacts with the [^{3}H]NMS-occupied M_2 receptor in an allosteric fashion but with submaximum efficacy, i.e. at high concentrations it does not totally abolish the dissociation of [^{3}H]NMS from the receptor. The

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concentration-effect curve for the allosteric effect of hexamethonium on the rate of dissociation of [^{3}H]NMS, illustrated in Fig. 2, levels off at 15 ± 3 %, i.e. the maximal inhibition of the [^{3}H]NMS dissociation rate constant is ca. 7 fold. The $pEC_{0.5}$, slope factor and lower plateau for a global fit of the combined data were 4.11 ± 0.08, -0.57 ± 0.06 and 0.12 ± 0.04, respectively. Nonlinear regression analysis revealed that a two site model yielded a significantly better fit to the data compared with a one site model (F-Test, $p<0.05$). The parameters characterizing the high (H) and low affinity (L) components were $pEC_{0.5,high}$ = 5.03 ± 0.23, capacity %H = 47 ± 0.1%; $pEC_{0.5,low}$ = 3.55 ± 0.21, capacity %L = 53 ± 0.1% (mean ± S.E.M, n=42).

The antagonist behaviour of hexamethonium on the allosteric actions of tacrine and Duo3 in porcine cardiac M_2 receptors is illustrated in Fig. 3 and the parameters obtained from the data analysis are compiled in Table 1. The concentration effect curves for the retarding actions of tacrine and Duo3 on [^{3}H]NMS dissociation in the absence of hexamethonium are shown by open circles in Fig. 3A and 3B, respectively. Their allosteric potencies (Duo3 $pEC_{0.5,control}$ = 6.08 ± 0.04, tacrine $pEC_{0.5,control}$ = 5.28 ± 0.03, n = 16, respectively) and their steep curve slopes (Duo3 n = 1.92 ± 0.26, tacrine n = 1.82 ± 0.18) were in accord with published data (Tränkle et al., 1996). Antagonism by increasing concentrations of hexamethonium was measured at submaximally effective concentrations of tacrine (20 μM) and Duo3 (2 μM), which slowed [^{3}H]NMS dissociation by about 90%. The rate constant k_0 for these combination experiments was always normalized to the allosteric action of hexamethonium alone to compensate for the hexamethonium induced

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slowing of [^{3}H]NMS dissociation; in other words, k_{obs} observed in the presence of hexamethonium alone was set at k_0 = 1.0. A 6 fold slowing of [^{3}H]NMS dissociation was produced by the highest concentration of hexamethonium used (10 mM).

As can be seen from Fig. 3, hexamethonium inhibited the allosteric actions of Duo3 and tacrine concentration-dependently (filled circles in Fig. 3). The antagonism by hexamethonium resulted in a *speeding up* of [^{3}H]NMS dissociation in the presence of the fixed concentrations of Duo3 and tacrine. The increase in observed rate constant varied from 2.6 fold (0.1 mM hexamethonium) to 7.4 fold (3 mM hexamethonium) for Duo3 and 1.9 fold (0.1 mM hexamethonium) to 6.7 fold (10 mM hexamethonium) in case of tacrine.

To check whether the antagonism by hexamethonium results in parallel shifts of the concentration-response curves, a second complete curve for each allosteric agent was measured in the presence of a high concentration of hexamethonium (3 mM; Fig. 3, filled triangles); the top plateaux of these curves were not different from a level of 100% inhibition of [^{3}H]NMS-dissociation (F-Test, $p>0.05$). All sets of three curves were included in the respective global nonlinear regression analyses to characterize the type of antagonism. The results of the global nonlinear regression analysis of the data shown in Fig. 3 were used to display an array of curves shifted in parallel to the right (not shown) which allowed dose-ratios DR = $EC_{0.5,antagonist}/EC_{0.5,control}$ to be calculated where $EC_{0.5,control}$ and $EC_{0.5,antagonist}$ are the concentrations of allosteric agent to induce a half maximum inhibition of [^{3}H]NMS-dissociation either in the absence or

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presence, respectively, of the antagonist (in this example, hexamethonium). Schild plots of the data are shown in Figures 4A,B (diamonds) to illustrate the different modes of interaction between these ligands and hexamethonium. For comparison the interaction of hexamethonium with the conventional allosteric agent WDuo3 was also measured (Fig. 4C, diamonds, Table 1).

WDuo3 exhibited the simplest interaction pattern with hexamethonium. The potency of WDuo3 in the absence of hexamethonium ($pEC_{0.5,control}$ = 8.30 ± 0.30) and a slope not different from unity (n = 1.04 ± 0.06; mean ± S.E.M., n= 9) were in line with published data (Tränkle et al., 1996; Tränkle and Mohr, 1997). The global fit yielded a Schild slope for the interaction of hexamethonium with WDuo3 which was not significantly different from unity (F-Test, $p>0.05$) indicating a formally competitive interaction with a calculated $logK_B$ of 4.32 ± 0.09.

The interaction of tacrine with hexamethonium (Fig. 3A, 4A) was best described by equation 2 suggesting a cooperative interaction between these ligands, log α = -0.92 ± 0.03, and a $logK_B$ for hexamethonium of 3.93 ± 0.06 (mean ± S.E.M., n=16). The interaction of Duo3 with hexamethonium (Fig. 3B) was best described by equation 1 with a Schild factor s = 0.68 ± 0.06, which was different from unity (F-Test, $p<0.05$), and a pA_2 = 3.59 ± 0.19 (means ± S.E.M., n=16). Thus, the hexamethonium/Duo3 interaction was non-competitive.

The antagonist actions of obidoxime and Mg^{2+} on the allosteric interactions of tacrine, Duo3, and WDuo3 with [^{3}H]NMS were also studied. The findings are

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illustrated in Fig. 4. Obidoxime (open triangles in Fig. 4) has been shown previously to be a rather weak and non-competitive antagonist against Duo3 (pA_2 = 3.00 ± 0.08; s = 0.51 ± 0.04), identifying Duo3 as an atypical allosteric agent (Tränkle and Mohr, 1997). Tacrine was also sensitive to obidoxime (Ellis and Seidenberg, 1992), but the present data additionally reveal that the interaction does not appear to be competitive (s = 0.63 ± 0.02, cf. Table 1). In other words, tacrine also behaves like an atypical allosteric agent. As shown previously (Tränkle and Mohr, 1997), the conventional allosteric agent WDuo3 is antagonized by obidoxime in a competitive fashion ($\log K_B$ = 4.16 ± 0.07; s not different from unity) and has therefore been classified as a common site allosteric agent.

Mg-ions (filled circles in Fig. 4) only inhibited the allosteric actions of Duo3 on [^{3}H]NMS dissociation at very high concentrations, whereas the actions of tacrine were considerably more sensitive to Mg^{2+}. Surprisingly, the type of antagonism between tacrine and Mg^{2+} (analogous to the tacrine/hexamethonium interaction) was clearly cooperative ($\log\alpha$ = -0.77 ± 0.09, $\log K_B$ = 2.64 ± 0.04). WDuo3 was antagonized by Mg^{2+} in an apparently competitive fashion, with the potency of Mg^{2+} ($\log K_B$ = 3.20 ± 0.06) comparable with the potency of Mg^{2+} against the common site allosteric agent W84 ($\log K_B$ = 3.40 ± 0.09; Burgmer et al.,1998).

Taken together, according to their sensitivities to the three allosteric antagonists, WDuo3 can be classified as a common site allosteric agent, whereas Duo3 and tacrine behave atypically. Notably, hexamethonium and

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Mg^{2+} are negatively cooperative with tacrine; i.e., tacrine appears to have the propensity to bind simultaneously with these antagonists to the M_2 receptor.

Effects of a dimerized tacrine. In order to investigate whether the allosteric site has room for more than one molecule of tacrine, two molecules of tacrine were linked by a hexamethylene chain, yielding the "tacrine dimer" (Fig. 1). If the allosteric site can accommodate one molecule of the dimer instead of two molecules of tacrine, then the dimer should have an increased affinity compared with tacrine and a normal instead of a steep concentration-effect-relationship. [^{3}H]NMS-dissociation experiments using porcine cardiac M_2 receptors revealed that this was the case: dimerisation increased the allosteric potency 20 fold ($pEC_{0.5}$ = 6.56 ± 0.08, means ± S.E.M., n=18) compared with tacrine ($pEC_{0.5}$ = 5.27 ± 0.01, means ± S.E.M., n=3, Fig. 5A), with the slope factor ($n_{tacrine}$ = -1.86 ± 0.08) decreasing, as predicted by the hypothesis, to a value ($n_{tacrine\ dimer}$ = -1.14 ± 0.15) that was not significantly different from unity (F-Test, p>0.05). Hexamethonium was used to antagonize the allosteric action of the dimer (Fig. 5A, inset) because it is cooperative with tacrine and has a higher potency than Mg^{2+}. Global nonlinear regression analysis of the data for the antagonist action of hexamethonium against the dimer (3 μM) and the data for the concentration-effect curve of the dimer in the presence of 3 mM hexamethonium indicated that equation 1 was sufficient to describe the data with s = 0.78 ± 0.14 being not different from unity (F-Test, P>0.05) and pK_B = 3.48 ± 0.06 (means ± S.E.M., n=18). Thus, in contrast to tacrine, the tacrine dimer acted in a formally competitive manner with hexamethonium in porcine cardiac M_2 receptors.

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In order to monitor the binding of the tacrine-dimer and tacrine to M_2 receptors whose orthosteric site is not occupied by NMS, receptors were labelled with the allosteric radioligand [^{3}H]dimethyl-W84 (Fig. 5B). In this assay we used membranes of CHO cells stably expressing human M_2 receptors, because measurement of [^{3}H]dimethyl-W84 binding (Fig. 5B) is facilitated by the higher receptor density than that found in porcine cardiac membranes (Fig. 5A; Tränkle et al., 2003). Control experiments did not reveal a difference in allosteric potency between porcine cardiac M_2 and CHO hM_2 receptors (data not shown). The tacrine-dimer (pK_i = 6.94 ± 0.04, means ± S.E.M., n=5) inhibited [^{3}H]dimethyl-W84 binding with about 50-fold higher affinity than tacrine (pIC_{50} = 5.21 ± 0.03; Tränkle et al., 2003). Furthermore, the inhibition curve of the dimer had a slope factor (n = -1.05 ± 0.09) not different from unity whereas the slope of the tacrine curve (n = -1.41 ± 0.15) is significantly greater than unity, as reported previously (Tränkle et al., 2003). Thus dimerization of tacrine considerably increases the binding affinity compared with tacrine, both at the [^{3}H]NMS-occupied and the unoccupied M_2 receptor, suggesting that the allosteric site may have room for more than one tacrine molecule. In principle, the finding does not allow one to differentiate whether the full length of the dimer is bound within the site or whether binding of only the additional hexamethylene chain accounts for the observed increase in binding affinity. Structure-activity-relationships in W84 derivatives suggest that the hexamethylene middle chain would not fully explain the observed increase in affinity (Mohr et al., 2004). Furthermore, dimerization eliminates the atypical features of tacrine action, in that the concentration-effect curves of the dimer have slopes not different from unity and the interaction with the antagonist

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hexamethonium changes from cooperative to competitive. It should be noted that the experiments shown in Fig. 5B were conducted under somewhat different buffer conditions from those in Fig. 5A and elsewhere in this manuscript in order to allow [^{3}H]dimethyl-W84 binding to be accurately measured (Tränkle et al., 2003). As allosteric actions are sensitive to the buffer conditions, the resulting affinities obtained from the data in the two panels are not strictly comparable.

Sensitivity of binding of the allosteric ligands to M_2/M_5-receptor mutations. In order to shed light on possible differences in the receptor epitopes involved in the binding selectivity between M_2 and M_5 receptors of the atypical ligands Duo3 and tacrine, compared to the conventional ligands WDuo3 and the tacrine dimer, we investigated the allosteric actions of these ligands on a number of human M_2/M_5 chimeras (Ellis et al., 2000) and two mutants of the human M_2 receptor, M_2 ^{423}Thr → His (Buller et al., 2002) and the double point mutant M_2 ^{177}Tyr → Gln + ^{423}Thr → His (Voigtländer et al., 2003). All receptors were expressed in transiently transfected COS-7 cells.

Previously, we have shown that, for some typical, “common site” allosteric agents, two amino acids, M_2 ^{177}Tyr and M_2 ^{423}Thr may account entirely for the M_2/M_5-selectivity in receptors whose orthosteric site was occupied by NMS (Voigtländer et al., 2003). The starting point for the identification of these amino acids were studies on chimeric M_2/M_5 muscarinic receptors.

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The chimeric receptors represent the M_5 receptor with parts of the amino acid sequence being replaced by the corresponding amino acids of the M_2 receptor. Thus, stretches of amino acids in M_2 that increase the binding affinity of allosteric agents above the level of M_5 can be identified. All of these mutants and chimeras are depicted in Fig. 6.

The allosteric inhibition of [^{3}H]NMS dissociation was used as the measure of receptor binding of the test compounds. For all four modulators, the observed affinity was higher at M_2 than at M_5 receptors. The M_2/M_5 subtype selectivity was large in the case of WDuo3 (140 fold), and considerably lower for the tacrine dimer (16 fold), Duo3 (15 fold), and for tacrine (13 fold) (Table 2). Another notable feature of the inhibition curves of [^{3}H]NMS dissociation from M_5 receptors is that these ligands were only capable of generating a maximum 2-4 fold slowing of the rate constant. This contrasts with the ability of the same ligands to essentially abolish [^{3}H]NMS dissociation from M_2 receptors. Of the chimeras used in this study, only CR6 consistently had this aspect of the M_2 behaviour for the ligands examined. This suggests the importance of a region of the o3 loop, the N-terminus and possibly TM1/TM6/TM7 in regulating [^{3}H]NMS dissociation from [^{3}H]NMS-receptor-allosteric ligand complexes. Another surprising result is that the potency of Duo3 to inhibit [^{3}H]NMS dissociation is higher at the human M_2 receptor expressed in COS-7 cells than at the porcine heart M_2 receptor. The reason for this is not known.

The high affinity of WDuo3 for M_2 receptors was reduced by a factor of 3 in M_2 ^{423}Thr (Fig. 6A), while the double point mutation reduced the affinity of WDuo3

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to the level of its M_5 affinity, suggesting a critical role of M_2 ^{177}Tyr. Thus, WDuo3 behaves in the same manner as the common site allosteric agents W84, dimethyl-W84, and diallylcaracurine (Voigtländer et al., 2003). In the case of Duo3, however, these point mutations hardly affected its affinity (Fig. 6B). The affinity of WDuo3 was clearly raised compared with M_5 only in the CR3 chimera (Fig. 6C,E). This is the only chimera to contain the M_2 ^{177}Tyr.

In contrast, Duo3 gained affinity in CR1 and also in CR2, CR3, and CR6 (Fig. 6D,F). The gain in affinity seen in CR1 and CR2 indicates that the M_2/M_5-selectivity of Duo3, but not WDuo3, involves epitopes in the N-terminal region of the M_2 receptor.

The affinity of tacrine for M_2 was essentially unaltered in the M_2 ^{423}Thr → His single point mutant, but was clearly reduced in the double point mutant (Fig.7A), although the affinity for the double point mutant did not fully reach that of M_5. The affinity of tacrine was increased in CR3 and CR6 relative to M_5 (Fig. 7C) and unchanged in CR1 and CR2 (Fig. 7E). These results match the findings obtained previously in the same chimeric receptors (Ellis et al., 2000). Thus, the epitope dependency of tacrine resembles that of the common site agent WDuo3.

The binding of the tacrine-dimer was unchanged in the M_2 ^{423}Thr → His mutant but decreased at the M_2 double point mutant (Fig. 7B), thus resembling tacrine. Furthermore, similar to tacrine, the dimer gained affinity relative to M_5 in CR3

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and, to a lesser extent, in CR6 (Fig. 7D), but not in CR1 (Fig. 7F). In contrast to tacrine however, the dimer gained affinity in CR2 (Fig. 7F).

Taken together, the epitope dependencies of the atypical allosteric agents Duo3 and tacrine are different. The epitope dependence of tacrine resembles that of common site allosteric modulators, whereas the affinity of Duo3 is sensitive to epitopes in both CR1 and CR2 that contain the N-terminal elements of the M_2 receptor protein.

Docking simulations in the model of the M_2 receptor. As reported previously, this model is based on the 3D structure of bovine rhodopsin in the inactive state (Voigtländer et al., 2003). Docking simulations were carried out for the human M_2 receptor whose orthosteric site is occupied with NMS, i.e. the receptor is fixed in an inactive state similar to the crystallized bovine rhodopsin (Palczewski et al., 2000) used as a template for the model. WDuo3 is chemically closely related to the common site allosteric ligand W84. As might be predicted, this leads to a W84-like binding mode of WDuo3. For both ligands one of the lateral phthalimide residues is close to the epitopes ^{423}Thr and ^{177}Tyr (Fig. 8A). These amino acids line the end of the allosteric binding cleft at the place where, in the unliganded M_2 receptor, a corridor leads to the orthosteric site (Jöhren and Höltje, 2002).

For tacrine, FlexX predicts two different placements for this rather small molecule: one next to ^{177}Tyr and ^{423}Thr between o2 and o3, and another close

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to o1 and the N terminus (Fig. 8B). The distance between the two locations is about 12 Å and therefore large enough to accommodate the hexamethylene spacer in the structure of the tacrine-dimer. The docking geometry of tacrine-dimer found by FlexX is illustrated in Fig. 8C.

Although hexamethonium is a relatively small and very flexible ligand, there appears to be one preferred position inside the common allosteric binding site. In this binding mode hexamethonium forms cation-π interactions with ^{177}Tyr (not shown). Another but less favourable docking geometry locates hexamethonium closer to the N terminus. In this binding mode an additional tacrine molecule can be placed simultaneously inside the allosteric binding cavity (Fig. 8D).

For Duo3, the docking simulations yield a position in which the molecule does not come close to the end of the allosteric binding cleft. This contrasts with the docked positions of WDuo3 and W84 (Fig. 8E, A).

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Discussion

Current evidence points to there being at least two allosteric ligand binding sites on muscarinic receptors, the 'common allosteric site' or 'gallamine' site, to which 'typical' allosteric ligands such as gallamine, alcuronium, W84 and strychnine bind, and the 'WIN' site that binds staurosporine and WIN62,577 (Birdsall and Lazareno, 2005). Such ligands, binding to their respective sites, interact in a 'simple' fashion with the binding of ligands to the orthosteric site, obeying the predictions of the allosteric ternary complex model in equilibrium, kinetic and functional studies. Interaction studies using gallamine or strychnine and an analogue of WIN 62,577 indicate that there is often neutral cooperativity at the two allosteric sites (Lazareno et al., 2002). It is therefore possible to generate muscarinic receptor complexes with *both* allosteric sites simultaneously occupied by two *different* ligands.

Another class of allosteric ligand, termed 'atypical', of which tacrine (Potter et al., 1989), Duo3 (Tränkle and Mohr, 1997) and pentacyclic carbazolones (Gharagozloo et al., 2002) are examples, bind with positive cooperativity (slope factors > 1, and generally ≤ 2), even with the orthosteric site occupied. This implies the possibility of *both* allosteric sites being occupied simultaneously by the *same* ligand, with positive cooperativity being generated between the binding of the ligand to the two sites.

A third class of ligand, exemplified by obidoxime and also shown in this study by hexamethonium, exhibit slope factors less than 1 for inhibiting [^{3}H]NMS] dissociation from M_2 receptors. This indicates heterogeneous binding or

homotropic negative cooperativity (in contrast to the homotropic positive cooperativity shown by atypical ligands). Furthermore, and crucial to the present study, obidoxime and hexamethonium, in contrast to 'typical' allosteric ligands, do not fully inhibit [^{3}H]NMS] dissociation from M_2 receptors. This means that [^{3}H]NMS] can dissociate from the M_2 orthosteric site when the allosteric site(s) are occupied by these ligands. A similar behaviour to obidoxime and hexamethonium is also shown by Mg^{2+} (Burgmer et al., 1998).

This study explores the complex pattern of behaviour of these ligands at the allosteric sites using interaction studies, mutant receptors and docking studies.

Using cardiac M_2 receptors our studies investigated two 'atypical' ligands, tacrine and Duo3 and two related ligands, WDuo3 and tacrine dimer, which are 'typical' in their ability to inhibit [^{3}H]NMS] dissociation. Their interactions with the third class of allosteric ligands (probe ligands) were studied. Three different interactions were observed, a competitive effect (linear Schild plot, slope 1), cooperative (non-linear, plateauing, Schild plot) and 'non-competitive' (complex, linear Schild plot, slope < 1). It should be noted that what might be interpreted as a competitive interaction may be an allosteric action with high negative cooperativity. In addition, the intercept at log(DR-1)=0 gives the pA_2 estimate (or pK_B if the slope factor equals 1) of the probe ligands. Any differences in pA_2 estimates of the probe ligands points towards the presence of multiple binding sites.

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All three probes (hexamethonium, Mg^{2+}, and obidoxime) appeared to be competitive with WDuo3. In contrast, Duo3 was affected in a non-competitive fashion by hexamethonium and obidoxime. Furthermore, Duo3 was less sensitive than WDuo3 to these probes and was almost insensitive to Mg^{2+}. Duo3 is clearly binding differently from WDuo3.

Compared to Duo3, tacrine was affected by lower concentrations of obidoxime, but also in a non-competitive way. In contrast to Duo3, however, tacrine was antagonised by hexamethonium and Mg^{2+} in an allosteric (negatively cooperative) fashion, with obidoxime and possibly hexamethonium being more potent against tacrine than against Duo3. Duo3 appears to be binding differently from tacrine. It appears as if hexamethonium and Mg^{2+} are capable of binding to a receptor-NMS-tacrine complex.

Studies employing mutated human M_2 receptors expressed in COS-7 cells and docking simulations in a M_2 receptor model have shown that $M_2{}^{177}$Tyr and $M_2{}^{423}$Thr account for the M_2/M_5-selectivity of typical allosteric agents (Voigtländer et al., 2003). The present study shows that the M_2/M_5-selectivity of the typical allosteric agent WDuo3 can also be fully explained by these epitopes.

The binding of tacrine is also somewhat sensitive to these epitopes, despite tacrine behaving as an atypical allosteric agent in the interaction studies. This apparent paradox was resolved by the docking simulations which show that the allosteric binding cleft has sufficient room for a simultaneous binding of

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hexamethonium and tacrine (Fig.8D). Docking also allows two molecules of tacrine to bind simultaneously in the allosteric binding cleft, as predicted by Potter et al., (1989), with one of these molecules being located close to the epitopes M_2^{177}Tyr and M_2^{423}Thr deep in the allosteric binding cleft (Fig.8B) with the other molecule being bound near the entrance of the allosteric domain. Thus, the allosteric actions of tacrine involve ´common site' epitopes, but the interaction of tacrine with hexamethonium is atypical because both agents may bind simultaneously to the allosteric site. Docking experiments further suggest that two molecules of hexamethonium may bind simultaneously but with different affinities within the allosteric binding cleft (not shown). This could explain the biphasic effect of hexamethonium on [^{3}H]NMS-dissociation (Fig. 2) via an interaction solely within the allosteric binding domain.

It may be speculated that the WIN compounds and the relatively bulky staurosporine interact with the second binding location of tacrine but do not reach the location that accommodates the "first" tacrine molecule or one end of W84 and WDuo3.

The "tacrine dimer" explores whether the allosteric site can accommodate two molecules of tacrine linked by a hexamethylene spacer present in several typical bisquaternary modulators (Nassif-Makki et al., 1999). Similar tacrine dimers are potent inhibitors of acetylcholinesterase (e.g. Pang et al., 1996). Analogously, the affinities of the "tacrine dimer" for unoccupied and [^{3}H]NMS-occupied cardiac M_2 receptors are considerably higher (20-60 fold) than those of tacrine (Fig 5), suggesting additional binding interactions. However the

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positive homotropic cooperativity is absent in the dimer. The competitive interaction of the tacrine dimer with hexamethonium suggests that the allosteric site cannot accommodate both modulators simultaneously.

For Duo3, however, there is no evidence from interaction nor from docking studies for the simultaneous binding of two molecules. According to the 3D model, the long Duo3 molecule almost fills the allosteric binding cavity (Fig.8E) but it does not reach as deep into the cavity as W84 or WDuo3 (Fig.8A). We found a small (15-fold) COS7-hM_2/M_5 subtype selectivity of Duo3 (Table 2) relative to that found in the case of WDuo3 (140-fold). As the affinity for the M_5 subtype is nearly identical for the two compounds (Table 2), the small M_2/M_5-selectivity of Duo3 is the consequence of its relatively low affinity for M_2 receptors. According to the 3D model, Duo3 does not come as close as WDuo3 and W84 to the amino acids M_2^{177}Tyr and M_2^{423}Thr which are important for the M_2/M_5 subtype selectivity of these typical allosteric agents. This finding is in accord with the affinity of Duo3 for M_2 only being slightly reduced in the double point mutant. Furthermore, Duo3 stands out in that its affinity is increased over the level of M_5 in all the chimeric receptors, with the greatest gain found in CR1. None of the other agents have increased affinity in CR1, which contains the N-terminal domain of the M_2 receptor.

Taken together, the mutagenesis studies and the 3D model both suggest that Duo3 binds to the allosteric binding cavity but in an atypical mode. Yet, this does not directly explain the atypical features of its allosteric action such as the apparently non-competitive interplay with obidoxime and hexamethonium and

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its steep slope factors. These results do not support the former hypothesis that a distinct second allosteric site is involved in the atypical action of Duo3.

Although atypical, the postulated binding of Duo3 and tacrine within the 'common' allosteric binding cavity does explain our previous observation that both agents did not affect the dissociation of the 'typical' allosteric ligand [^{3}H]dimethyl-W84 from M_2 receptors (Tränkle et al., 2003): when the allosteric binding cavity is occupied by the radioligand, the binding site of the atypical agents is occluded.

It has been argued that atypical allosteric agents, including Duo3, might modulate receptor dimerisation or generate a positive homotropic interaction transmitted via the receptor-receptor dimer interface (Gharagozloo et al., 2002, Tränkle et al., 2003). We cannot exclude this explanation at present. In fact, the 'flat' curve of hexamethonium (and obidoxime) for inhibiting [^{3}H]NMS dissociation (Fig 2) could represent homotropic negative cooperativity across the receptor dimer and the steep curve for Duo3 inhibiting [^{3}H]NMS dissociation represent the equivalent positive cooperativity. The apparent 'non-competitive' nature of the interaction of obidoxime and hexamethonium with tacrine and Duo3 (Table 1) could also be explained by complex interplay between a positive and negative cooperative system that is not accounted for in the model used to analyse the data. Such an interpretation does not exclude the possibility of 2 tacrine molecules bound/monomer (4 per dimer) as suggested by the docking studies. The divergent binding modes of the atypical ligands (and one would predict obidoxime and hexamethonium) may be responsible for generating

conformational changes at the receptor dimer interface that cause the positive and negative homotropic cooperativities.

In conclusion, this study shows for the first time that atypical muscarinic allosteric agents can have different binding modes, but that their atypical actions may, nevertheless, be mediated via the 'common' allosteric binding domain of muscarinic receptors.

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Footnotes

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reprint address:

Dr. Christian Tränkle, Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Str. 3, D-53121 Bonn, Germany.

Legends for Figures

Figure 1: Structures of the allosteric ligands. The molecules are shown in an energetically favourable conformation.

Figure 2: Concentration-dependent effect of hexamethonium on the apparent rate constant of [^{3}H]NMS dissociation from porcine cardiac M_2 receptors. Ordinate: ratio of the observed rate constant of [^{3}H]NMS dissociation in the presence of allosteric agent + atropine (k_{obs}) to the rate constant of [^{3}H]NMS dissociation in the presence of atropine alone (k_0). Data points are derived from complete dissociation curves. Illustrated are the mean values ± S.E.M. of 42 experiments. Error bars are only shown when they exceed the size of the symbols. Curve fitting was based on a two site model.

Figure 3: Retardation of [^{3}H]NMS dissociation from porcine cardiac M_2 receptors by increasing concentrations of tacrine (panel A) and Duo3 (panel B) (i) alone (open circles), (ii) in the presence of a fixed concentration of hexamethonium (filled triangles). The antagonist action of hexamethonium on the effect of a fixed concentration of the respective test compound (filled circles) is also shown. Ordinate: Inhibition of [^{3}H]NMS dissociation ($1 – k_{obs}/k_0$, c.f. legend to Fig. 2); k_0 for the experiments in the presence hexamethonium was always normalized to the allosteric action of hexamethonium alone. Abscissa: log concentration of the respective allosteric agent (retardation curves) or of hexamethonium (inhibition curve). Experiments were carried out in ´Na, K, P_i-buffer´ using porcine cardiac membranes. Simultaneous nonlinear fitting of the three curves of panel A and panel B, respectively, was carried out according to Lazareno and Birdsall (1993).

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For details see Methods. Mean values of 8 to 18 independent experiments yielding complete dissociation curves are shown.

Figure 4: Antagonism by obidoxime, hexamethonium, and Mg^{2+} of the effects of tacrine (panel A), Duo3 (panel B), and WDuo3 (panel C) displayed in the form of Schild-plots. The results of the global nonlinear regression analysis, as shown in Fig. 3, were used to display an array of curves shifted in parallel to the right (not shown) which allowed dose ratios DR = $EC_{0.5,antagonist}/EC_{0.5,control}$ to be calculated, where $EC_{0.5}$ is the concentration of allosteric agent that induces a half maximum inhibition of the [^{3}H]NMS dissociation rate constant in the presence ($EC_{0.5,antagonist}$) and in the absence of antagonist ($EC_{0.5,control}$), respectively. Similarly, the results of the global analysis allowed lines with Schild slopes s of unity for WDuo3 and s = 0.68 for Duo3 to be constructed (see Table 1). In the case of tacrine, the curve was based on the ternary complex model of allosteric interactions and drawn according to Lanzafame et al. (1996). For details see text.

Figure 5: (A) Inhibition of [^{3}H]NMS dissociation by tacrine (open circles) and the tacrine dimer (filled circles) at muscarinic M_2 receptors in porcine cardiac membranes. Ordinate as in Fig.2. Abscissa: log concentrations of the allosteric agents. Inset: Schild plot of the antagonist action of hexamethonium on the allosteric action of the tacrine dimer on [^{3}H]NMS dissociation from porcine cardiac M_2 receptors. Data were analysed as in Fig. 3 and displayed as described in the legend to Fig. 4. (B) Inhibition of specific [^{3}H]dimethyl-W84 binding by tacrine and the tacrine dimer in membranes from CHO cells expressing human M_2 receptors. Non-specific [^{3}H]dimethyl-W84 binding was defined in the presence of 10 μM gallamine. Specific binding of [^{3}H]dimethyl-W84 (1.5 nM) in the absence of

inhibitor was constrained to 100%. For comparison, data obtained with tacrine from Tränkle et al. (2003) was included in (B) as a dashed line. Error bars are shown when they exceed the size of the symbols. Curve fitting was based on a one site model. Mean values ± S.E.M. of three to eight experiments are shown; dissociation experiments were carried out in duplicate whereas binding experiments were performed as triplicate determinations.

Figure 6: Concentration-effect curves for the allosteric retardation of [^{3}H]NMS dissociation (A-F) induced by WDuo3 (left panel) and Duo3 (right panel) at the indicated wild-type and mutant receptors, using membranes from transiently transfected COS-7 cells. After prelabeling the receptors with 0.2 nM [^{3}H]NMS, dissociation was measured in the absence or presence of the allosteric modulator (see Methods). Ordinate as in Fig.2. Abscissa: log concentrations of the allosteric agents. Indicated are mean values ± S.E.M of three to seven separate experiments performed as duplicate determinations. G: schematic sketches of the wildtype and mutant receptors used in the current study.

Figure 7: Allosteric effects of tacrine (left panel) and the tacrine dimer (right panel) at the indicated wild-type and mutated receptors. Experiments were carried out and analysed as described in the legend to Fig. 6. Mean values ± S.E.M. of three to six separate experiments carried out in duplicate are shown.

Figure 8: Docking simulations using a human M_2 receptor model in which the orthosteric site is occupied by NMS. View from the membrane. Protein: helices, red; o1 and N terminus, grey; o2, cyan; EDGE (residues 172–175 in M_2) green; o3, yellow; disulfide bridge, magenta; ^{177}Tyr / ^{423}Thr: carbon, green; nitrogen, dark

blue; oxygen, red; hydrogen, white. Volumes of the binding sites: grey grid. Allosteric modulator/orthosteric NMS: carbon, white; nitrogen, dark blue; oxygen, red; hydrogen, cyan. (A) WDuo3 and W84 inside the allosteric binding site (top) of the M_2 receptor whose orthosteric site (bottom of panel A) is liganded with NMS. W84: carbon, orange; nitrogen, dark blue; oxygen, red; hydrogen, cyan. (B) – (E): Docking at the allosteric site of (B) two tacrine molecules simultaneously, (C) the tacrine dimer, (D) hexamethonium (left) and tacrine (right) simultaneously, (E) Duo3.

Tables

Table 1: Parameters characterizing the actions of the indicated allosteric agents and allosteric antagonists at porcine heart M_2 receptors with the orthosteric site blocked by NMS.

		descriptors of allosteric agent action			descriptors of antagonist action			
antagonist	**allosteric agent**	**$pEC_{0.5\ diss}$**	**n**	**type of antagonism**	**s**	**logα**	**logK_B**	**N**
hexamethonium	Duo3	6.08 ± 0.04	1.92 ± 0.26	non-competitive[b]	0.68 ± 0.06	-	3.59 ± 0.19[e]	16
	tacrine	5.28 ± 0.01	1.74 ± 0.06	cooperative[c]	-	-0.92 ± 0.03	3.96 ± 0.06	17
	tacrine dimer	6.56 ± 0.08	1.14 ± 0.15[n.s]	competitive[a]	0.78 ± 0.14[n.s]	-	3.48 ± 0.06	18
	WDuo3	8.30 ± 0.30	1.04 ± 0.59 [n.s]	competitive[a]	0.73 + 0.18[n.s.]	-	4.32 ± 0.09	9
Mg^{2+}	Duo3	6.12 ± 0.02	1.32 ± 0.11	competitive[a]	0.89 ± 0.20[n.s.]	-	1.21 ± 0.07	9
	tacrine	5.17 ± 0.02	1.93 ± 0.12	cooperative[c]	-	-0.77 ± 0.09	2.64 ± 0.04	10
	WDuo3	8.25 ± 0.02	0.97 ± 0.04[n.s]	competitive[a]	1.02 + 0.13[n.s.]	-	3.20 ± 0.06	9
obidoxime	Duo3	5.88 ± 0.02[d]	2.63 ± 0.41[d]	non-competitive[d]	0.51 ± 0.04[d]	-	3.00 ± 0.08[e]	22
	tacrine	5.17 ± 0.02	1.87 ± 0.12	non-competitive[b]	0.63 ± 0.02	-	3.89 ± 0.10[e]	11
	WDuo3	7.77 ± 0.04[d]	1.10 ± 0.09[n.s.,d]	competitive[d]	0.94 ± 0.06[n.s.,d]	-	4.16 ± 0.07[d]	16

$pEC_{0.5\ diss}$: - log equilibrium dissociation constant of the allosteric agent: concentration causing a half maximal reduction of the observed rate constant k_{obs} of [^{3}H]NMS dissociation in the absence of antagonist. *n*: slope factor of the corresponding curve. *Type of antagonism*: best fit model (see Methods): [a]: equation 1 (condensed Schild analysis, Lazareno & Birdsall, 1993) with a

Schild slope s not different from unity, [b]: equation 1 with a linear Schild plot but a slope different from unity, [c]: equation 2 (allosteric ternary complex model, Lazareno & Birdsall, 1995). Parameters n and s were constrained to 1, respectively, when they did not differ from unity (F-test, $p \geq 0.05$) *s*: Schild slope. $\log\alpha$: log factor of cooperativity as a measure of the reciprocal effect on affinity between the respective antagonist and the respective allosteric agent (with $\log\alpha < 0$ indicating negative cooperativity). $LogK_B$: log equilibrium affinity constant of the allosteric antagonist; N: number of independent experiments; [d] data taken from Tränkle et al., 1997; [e] value of pA_2; n.s.: not significantly different from unity.

Table 2: Potency of the allosteric agents to slow the rate of [^{3}H]NMS dissociation from the indicated wild-type and mutant human muscarinic receptors as a measure of the agents' binding affinity.

		M_2	$M_2^{423}T\rightarrow H$	$M_2^{177}Y\rightarrow Q + {}^{423}T\rightarrow H$	M_5	CR1	CR2	CR3	CR6
WDuo3	$pEC_{0,5\ diss}$	7.83 ± 0.06	7.31 ± 0.03	5.41 ± 0.04	5.69 ± 0.15	5.49 ± 0.12	5.78 ± 0.17	6.61 ± 0.13	5.76 ± 0.06
	N	4	3	3	7	4	3	5	4
Duo3	$pEC_{0,5\ diss}$	7.05 ± 0.09	6.93 ± 0.04	6.70 ± 0.04	5.89 ± 0.12	6.68 ± 0.19	6.40 ± 0.04	6.50 ± 0.05	6.40 ± 0.10
	N	3	3	3	6	4	3	4	3
tacrine-dimer	$pEC_{0,5\ diss}$	7.13 ± 0.08	7.09 ± 0.03	6.63 ± 0.06	5.92 ± 0.11	5.91 ± 0.07	6.31 ± 0.03	7.15 ± 0.09	6.27 ± 0.09
	N	3	3	3	6	4	3	4	3
tacrine	$pEC_{0,5\ diss}$	5.48 ± 0.04	5.18 ± 0.03	4.82 ± 0.02	4.38 ± 0.03	4.47 ± 0.04	4.39 ± 0.05	5.06 ± 0.04	5.02 ± 0.04
	N	3	3	3	6	4	3	4	3

$pEC_{0,5\ diss}$: -log concentration of allosteric agent which reduces the observed rate constant k_{obs} of [^{3}H]NMS dissociation half maximally. N: number of independent experiments

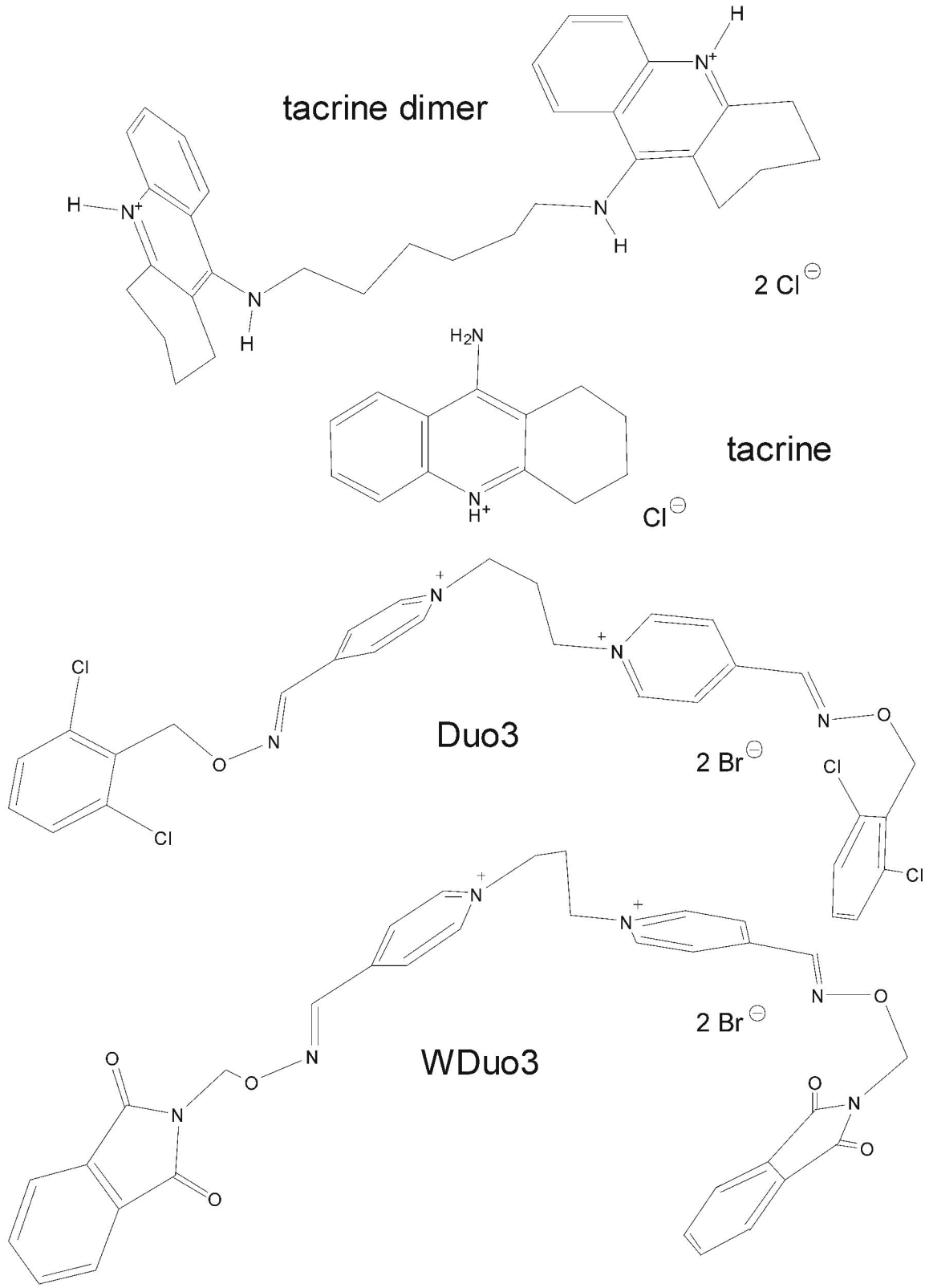


Figure 1 MOLPHARM/2005/017707

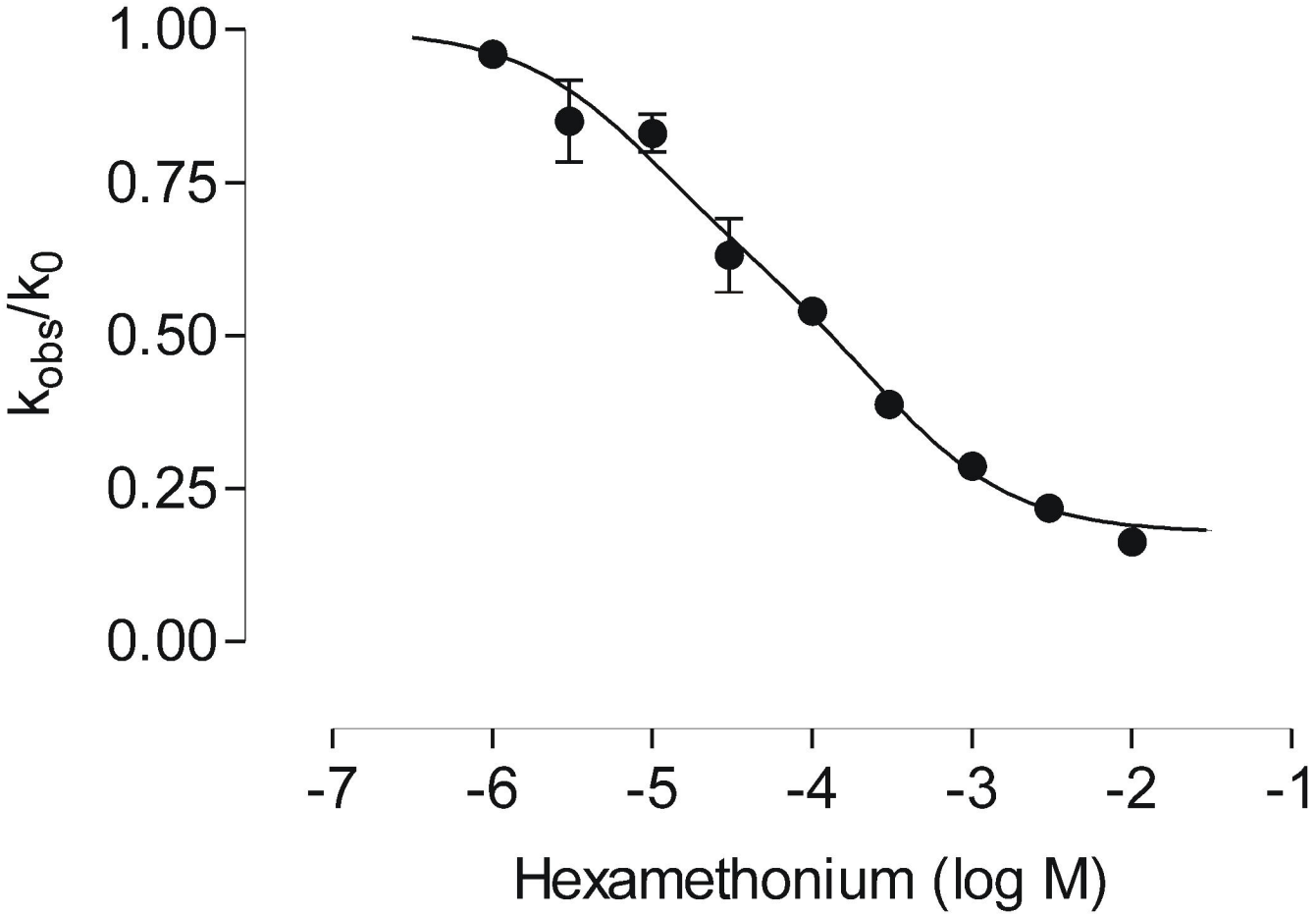
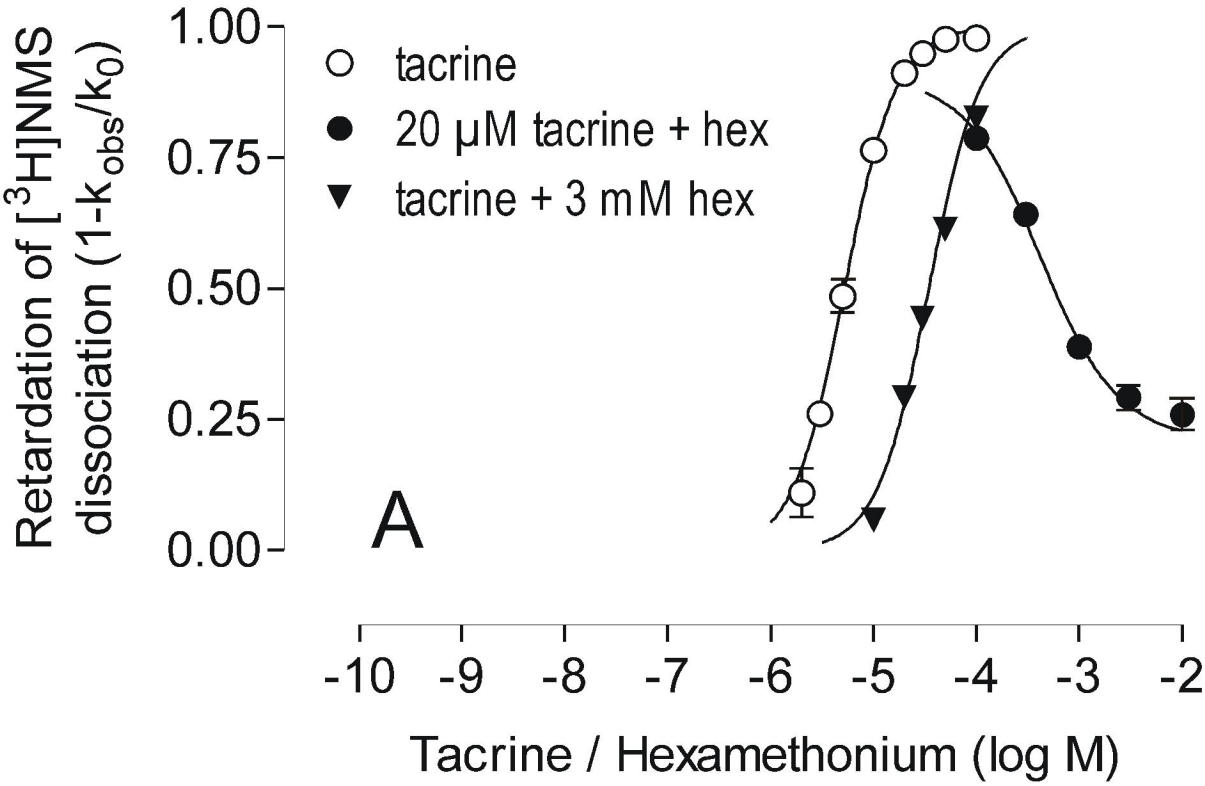
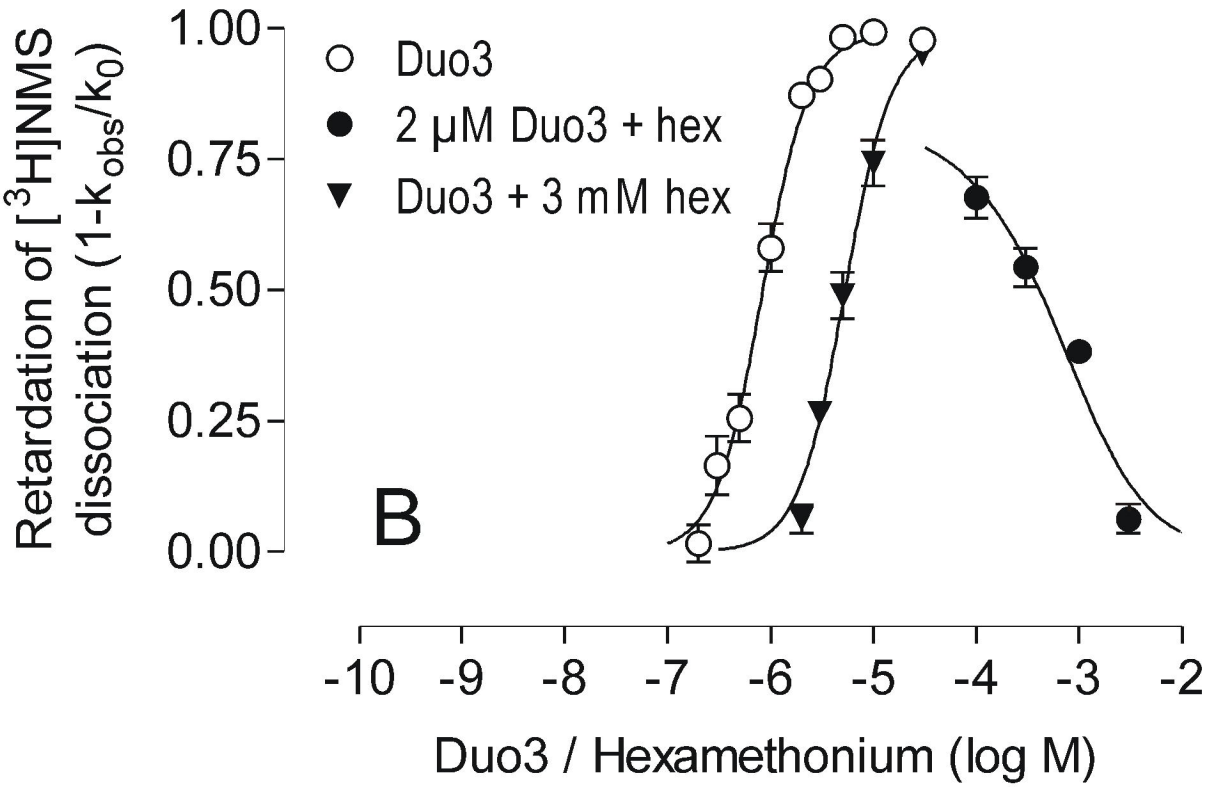


Figure 2 MOLPHARM/2005/017707


Retardation of [3H]NMS dissociation (1-kobs/k0)
1.00
0.75
0.50
0.25
0.00
tacrine
20 µM tacrine + hex
tacrine + 3 mM hex
A
-10 -9 -8 -7 -6 -5 -4 -3 -2
Tacrine / Hexamethonium (log M)


Retardation of [3H]NMS dissociation (1-kobs/k0)
1.00
0.75
0.50
0.25
0.00
Duo3
2 µM Duo3 + hex
Duo3 + 3 mM hex
B
-10 -9 -8 -7 -6 -5 -4 -3 -2
Duo3 / Hexamethonium (log M)

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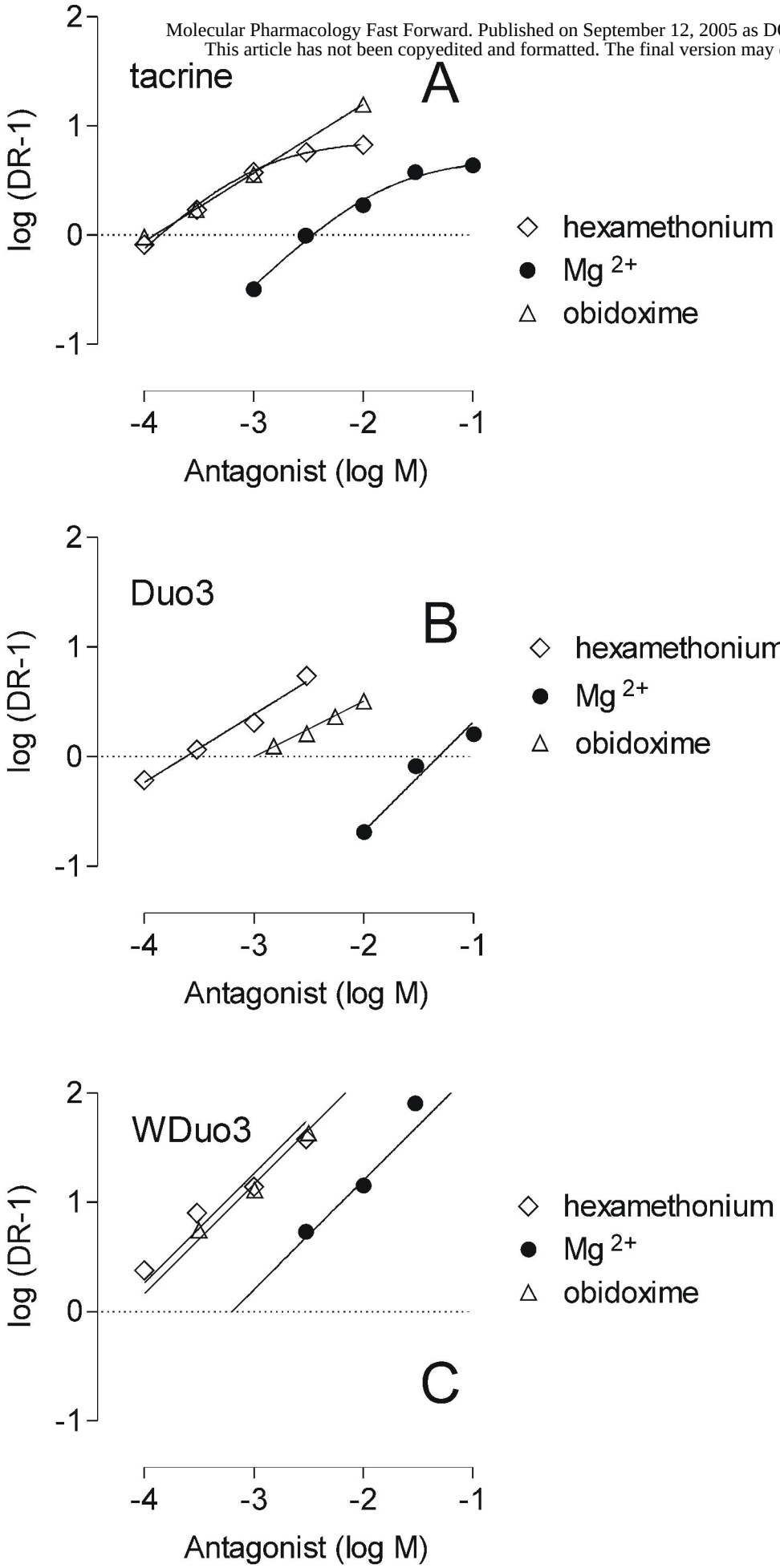

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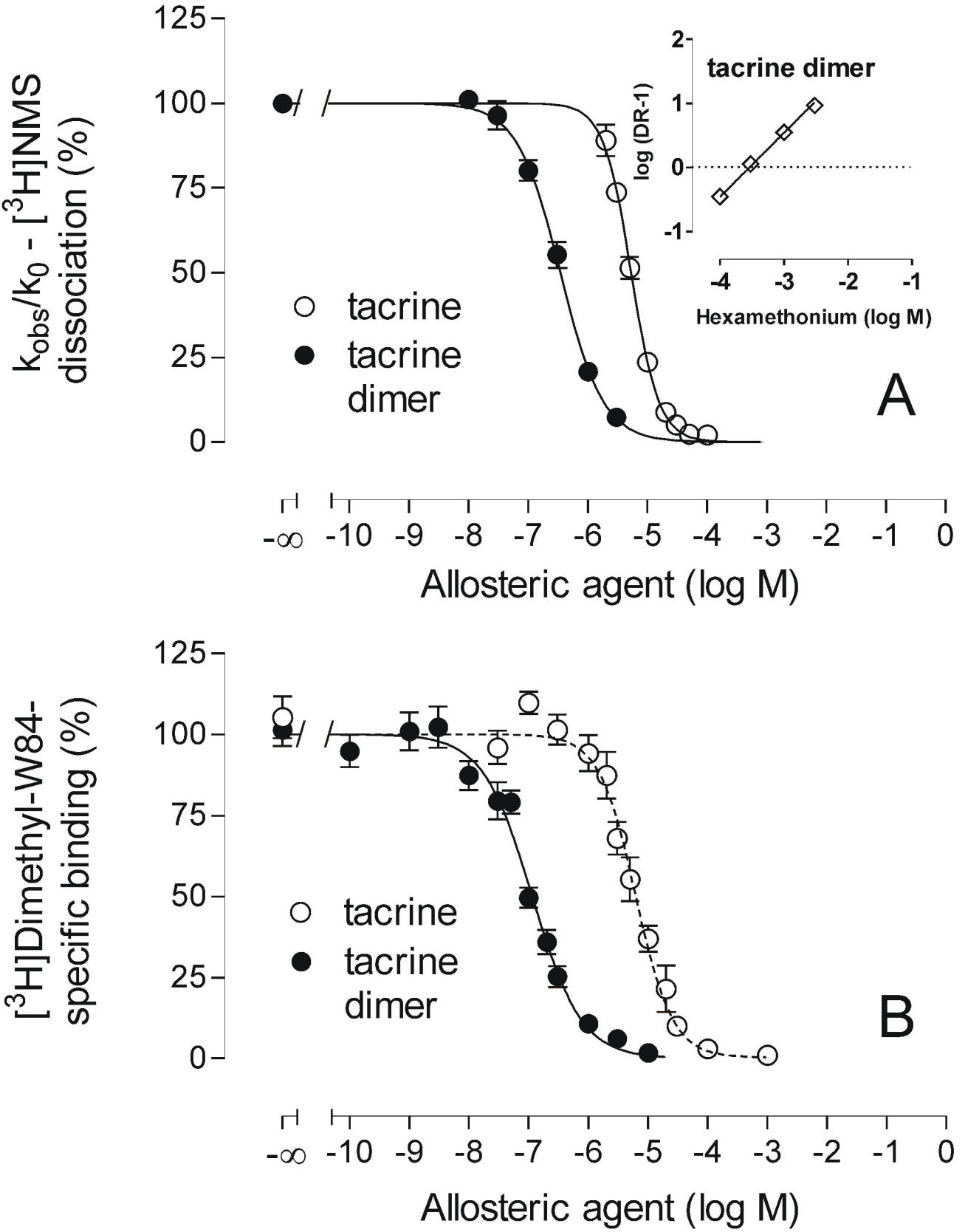

Figure 5 MOLPHARM/2005/017707

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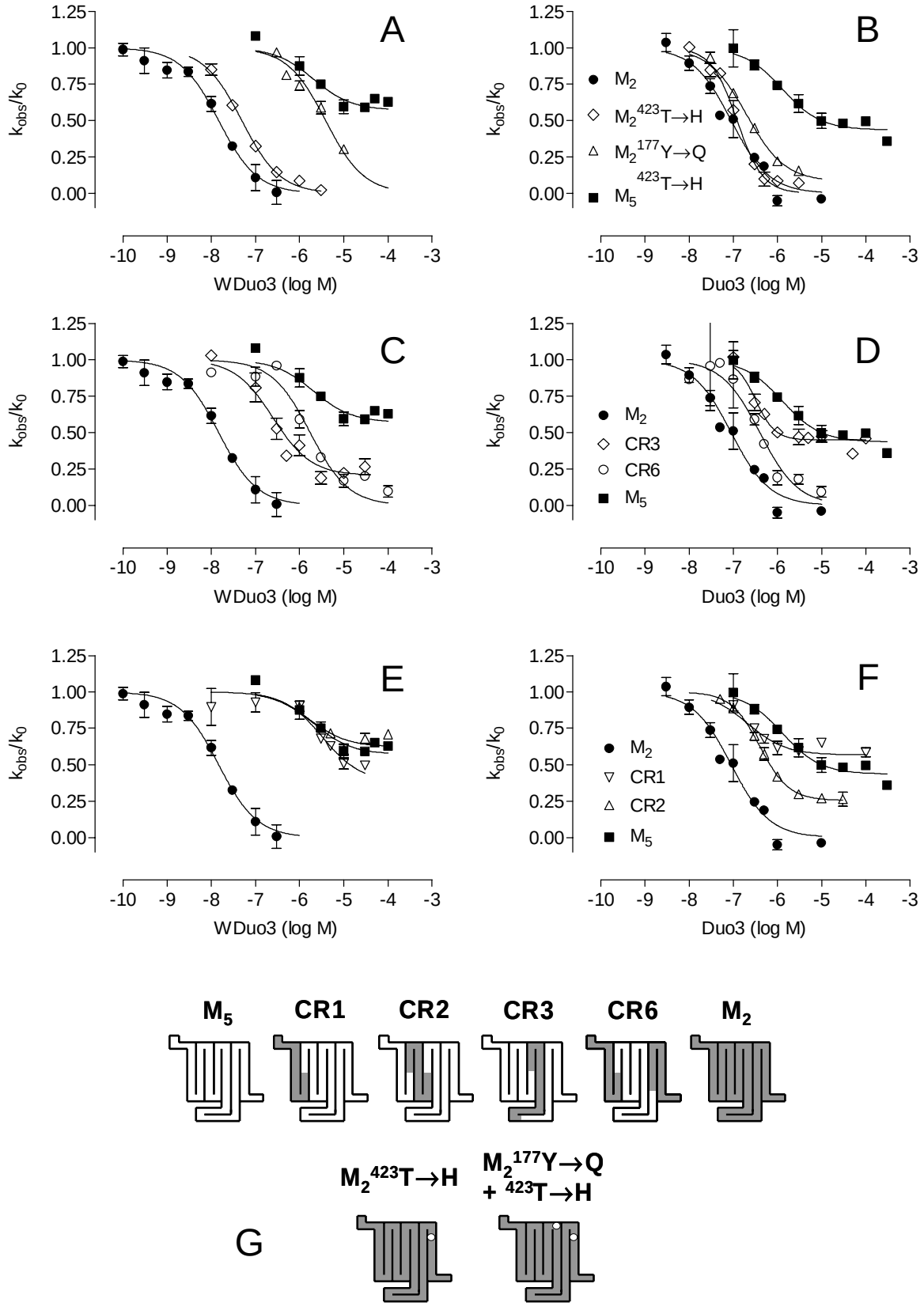

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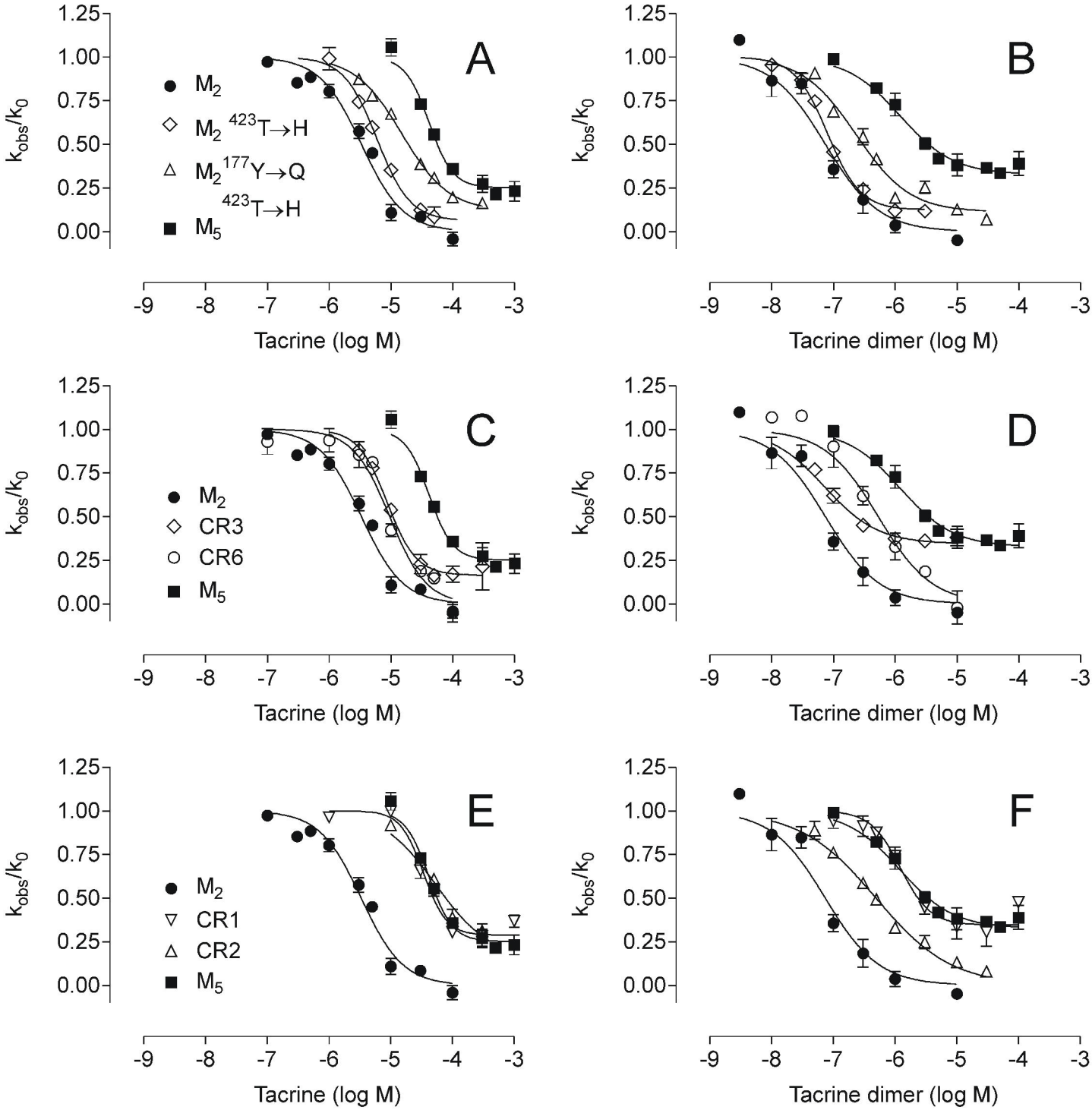

Figure 7 MOLPHARM/2005/017707

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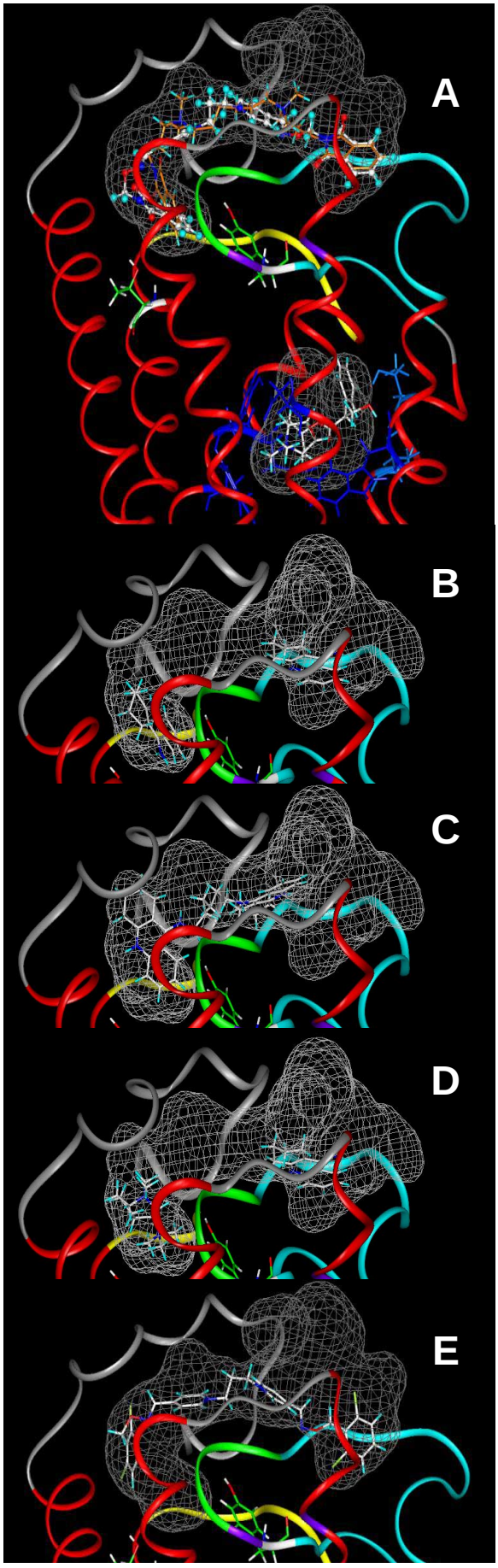

Figure 8 MOLPHARM/
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