

A Novel Multivalent Ligand That Bridges the Allosteric and Orthosteric Binding Sites of the M₂ Muscarinic Receptor

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ABSTRACT

THRX-160209 is a potent antagonist at the M₂ muscarinic acetylcholine (ACh) receptor subtype that was designed using a multivalent strategy, simultaneously targeting the orthosteric site and a nearby site known to bind allosteric ligands. In this report, we describe three characteristics of THRX-160209 binding that are consistent with a multivalent interaction: 1) an apparent affinity of the multivalent ligand for the M₂ receptor subtype (apparent pK_i = 9.51 ± 0.22) that was several orders of magnitude greater than its two monovalent components (apparent pK_i values < 6.0), 2) specificity of THRX-160209 for the M₂ receptor subtype compared with the closely related M₄ (apparent pK_i = 8.78 ± 0.24) and M₁, M₃, and M₅ receptors (apparent pK_i values ≤ 8.0), and 3) acceleration (>10-fold) of the dissociation rate of tritium-labeled THRX-160209 from M₂ receptors by competing monovalent ligands that are known to

interact with either the orthosteric site (e.g., atropine) or a well characterized allosteric site (e.g., obidoxime) on the receptor. In complementary kinetic studies assessing allosteric modulation of the receptor, unlabeled THRX-160209 retarded dissociation of [³H]N-methyl scopolamine (NMS). The effects of THRX-160209 on retardation of [³H]NMS dissociation were competitively inhibited by obidoxime, suggesting that obidoxime and THRX-160209 bind to an overlapping region coincident with other typical muscarinic allosteric agents, such as 3-methyl-5-[7-[4-[(4S)-4-methyl-1,3-oxazolidin-2-yl]phenoxy]heptyl]-1,2-oxazole (W84) and gallamine. Taken together, these data are consistent with the hypothesis that THRX-160209 binds in a multivalent manner to the M₂ receptor, simultaneously occupying the orthosteric site and a spatially distinct allosteric site.

The muscarinic receptor family consists of five subtypes that are found in smooth and cardiac muscle, epithelial and endothelial cells, secretory cells, neurons, and inflammatory cells (Caulfield, 1993; Racke and Matthiesen, 2004). These receptors represent attractive targets for drug design in a variety of therapeutic areas, including overactive bladder, chronic obstructive pulmonary disease, and Parkinson's disease (Eglen et al., 2001; Katzenschlager et al., 2003; Barnes, 2004; Hegde et al., 2004). However, most currently marketed, muscarinic receptor-targeted therapies exhibit significant side effects, some of which are due to potent drug interactions at undesirable muscarinic receptor subtypes outside the intended organ system. High sequence homology within the orthosteric site, across the five receptor subtypes, makes the

synthesis of tissue-specific or subtype-specific drugs challenging (Hulme et al., 1990). A drug with specificity for a given subtype or tissue is predicted to have increased efficacy but decreased side effects relative to a drug with equal affinity for all subtypes (Gainetdinov and Caron, 1999).

Muscarinic receptors are known to be subject to modulation by ligands that do not bind to the ACh binding pocket (the "orthosteric site") (Ellis et al., 1991). Targeting these "secondary" or "allosteric" sites may allow design of subtype-specific ligands, because these regions are less conserved than the orthosteric region among the muscarinic receptor subtypes. However, not all allosteric modulators of muscarinic receptors seem to bind to identical sites on the receptors (Trankle and Mohr, 1997; Birdsall et al., 2001). Obidoxime is a valuable tool with which to define binding to the same region on M₂ receptors to which the typical allosteric modulators gallamine and W84 bind (Ellis and Seidenberg, 1992; Trankle and Mohr, 1997).

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ABBREVIATIONS: THRX-160209, 4-[N-[7-(3-(S)-(1-carbamoyl-1,1-diphenylmethyl)pyrrolidin-1-yl)hept-1-yl]-N-(n-propyl)amino]-1-(2,6-dimethoxybenzyl)piperidine; NMS, N-methyl scopolamine; QNB, quinuclidinyl benzilate; ACh, acetylcholine; W84, 3-methyl-5-[7-[4-[(4S)-4-methyl-1,3-oxazolidin-2-yl]phenoxy]heptyl]-1,2-oxazole; CHO, Chinese hamster ovary; 3-BHP, 3-benzhydryl pyrrolidine; 3-BHP-L, 3-BHP with seven-carbon alkyl chain; 3-BHP-OH, 3-BHP with heptanol chain; 4-ABP, 4-aminobenzylpiperidine; 4-ABP-L, 4-ABP with seven-carbon alkyl chain; BSA, bovine serum albumin; ANOVA, analysis of variance; ATCM, allosteric ternary complex model.

Based upon comparison with the X-ray crystal structure of bovine rhodopsin, the ACh binding pocket of the muscarinic M_2 receptor is believed to be located in a narrow cavity formed by the seven membrane-spanning regions of the receptor (Wheatley et al., 1988; Wess, 1993). The typical² allosteric site is thought to be located on the extracellular lip of the receptor (Ellis et al., 1993; Leppik et al., 1994; Tuček and Proška, 1995). Ligands such as gallamine and obidoxime have been shown to bind to this secondary binding site (Clark and Mitchelson, 1976; Trankle and Mohr, 1997), and this site is estimated to be in close proximity to the orthosteric site (Tuček and Proška, 1995). Based upon this estimation, we believed that a priori design of novel multivalent ligands, designed to span and bind to both sites simultaneously, should be possible.

A multivalent approach to ligand design would be predicted to yield ligands with greater affinity and subtype specificity for one or more of the five muscarinic receptor subtypes among the family of receptors M_1 – M_5 . The valency of a ligand refers to the number of distinct binding interactions it makes with a target protein (Mammen et al., 1998a). Multivalent binding by our definition is the simultaneous interaction of multiple binding motifs on a ligand with adjacent concavities on a target or adjacent targets. The principles of multivalency suggest that higher binding affinities can be achieved when multiple ligands are coupled and simultaneously bind to multiple sites on a single target (Mammen et al., 1998a,c). The energetic basis of this phenomenon is that multivalent interactions allow the free energy gain of each component interaction between the multivalent ligand and its target but with reduced entropic cost relative to that of multiple separate (monovalent) ligands interacting with the same set of sites. The source of this entropy savings is that translational freedom is given up by only a single rather than multiple molecules (Mammen et al., 1998bc). Therefore, the total free energy of interaction of multivalent ligands should be of greater magnitude than the sum of the free energies of interaction of the molecular components.

Furthermore, multivalent ligands have the potential to demonstrate greater target specificity than corresponding monovalent ligands if even one binding site interaction discriminates between related targets. Consequently, potent and selective muscarinic receptor ligands, developed using a multivalent approach, might prove to be valuable therapeutics. Others have used a similar strategy and have reported the advantages of a series of agonist-allosteric bivalent ligands (Disinigrini et al., 2006). Here, we provide evidence for a multivalent interaction between the antagonist THRX-160209 and the muscarinic M_2 receptor. We suggest that this novel multivalent ligand simultaneously occupies the orthosteric site and the “typical” allosteric site of the receptor, resulting in tighter binding and greater subtype specificity than typically described with monovalent ligands.

Materials and Methods

Materials. [³H]N-methyl scopolamine ([³H]NMS; specific activity, 84 Ci/mmol) was obtained from GE Healthcare (Piscataway, NJ). Tritium labeling of THRX-160209 ([³H]THRX-160209; specific activ-

ity, 24 Ci/mmol) was carried out at ViTrax, Inc. (Placentia, CA). Atropine, gallamine, quinuclidinyl benzilate (QNB), and acetylcholine (ACh) were obtained from Sigma Chemical Co. (St. Louis, MO). W84 was purchased from Tocris Cookson, Inc. (Ellisville, MO). Obidoxime chloride was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Cell membrane homogenates from Chinese hamster ovary (CHO) cells expressing the human muscarinic M_5 receptor were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). THRX-160209, 3-benzhydryl pyrrolidine (3-BHP), 3-BHP with seven-carbon alkyl chain (3-BHP-L), 3-BHP with heptanol chain (3-BHP-OH), 4-aminobenzylpiperidine (4-ABP), and 4-ABP with seven-carbon alkyl chain (4-ABP-L) were prepared in-house (Fig. 1).

Cell Culture and Membrane Preparation. CHO cells stably transfected with the human muscarinic receptors (M_1 – M_4) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 250 μ g/ml G-418 at 37°C under 5% CO₂. Cells were grown to confluence and harvested with phosphate-buffered saline containing 2 mM EDTA. Lifted cells were suspended in ice-cold 10 mM HEPES with 10 mM EDTA, pH 7.4, and homogenized using a cell disrupter. The homogenate was then sedimented by centrifugation (500g, 10 min). The supernatant was then centrifuged again (40,000g, 20 min) and the pellets were suspended in 10 mM HEPES buffer with 20 mM NaCl, pH 7.4 and stored at –80°C. In general, this preparation yielded 2.7, 2.5, 2.4, and 2.0 pmol/mg of protein of M_1 , M_2 , M_3 , and M_4 receptors, respectively.

Inhibition of [³H]NMS Radioligand Binding Studies. To estimate muscarinic receptor subtype selectivities, inhibition radioligand binding assays were employed to estimate affinities of test ligands for each of the five subtypes. In these studies, competitive interactions were assumed. Identical conditions were used for studies with each receptor. The assays were conducted with 0.8 nM [³H]NMS in a buffer consisting of 10 mM HEPES, 20 mM NaCl, and 0.025% bovine serum albumin (BSA), pH 7.4, at 20°C. Nonspecific binding was defined in the presence of 10 μ M atropine. Membrane fractions were incubated with radioligand and unlabeled ligands for 1 h at 20°C. After separation by vacuum filtration onto GF/B filter plates presoaked with 0.3% polyethylenimine, the quantity of membrane bound radioligand was measured by scintillation counting. Data (counts per minute) were normalized to percentage of specific binding and analyzed using a four-parameter logistic equation in Prism 3.0 (GraphPad Software, San Diego, CA). Because Hill coefficients did not significantly differ from unity, IC₅₀ values were determined with slopes fixed to 1. Affinity estimates for test compounds (apparent pK₁) were calculated from the IC₅₀ values using the Cheng and Prusoff (1973) correction.

To characterize the interaction between fragments of THRX-160209 when coincubated in inhibition radioligand binding assays, fractional inhibition of [³H]NMS binding data were analyzed according to methods adapted from the Loewe additivity model (Suhnel, 1998). Experimental data were normalized from CPM to fractional inhibition of [³H]NMS binding. Using eq. 1, additive interactions between coincubated ligands can be defined by:

$$10^{(A-AB)} + 10^{(B-AB)} = i \quad (1)$$

with the interaction index $i = 1$. Values for $i > 1$ represent an antagonistic interaction between ligands, and those < 1 represent a synergistic interaction. In this equation, A and B are the negative logarithm of the dissociation constants of the monovalent ligands, and AB represents the values for the combination of agents, A and B . For inhibition radioligand binding studies, we used the apparent pK₁ values for these terms. The apparent pK₁ for a multivalent ligand can be used for AB when calculating the multivalent effect, τ . This term is simply defined as $\tau = 1/i$ and more clearly demonstrates the degree of synergy when ligands are covalently linked.

For visualization of coincubated fragment interactions, data were plotted according to the Bliss model of independence (Suhnel, 1998).

² We refer to the typical allosteric site as the secondary binding site that binds most described muscarinic allosteric ligands, including gallamine, W84, and obidoxime.

According to this model, the fractional response of a combination of two drugs equals the sum of the individual drug fractional responses less their product. Deviation from this model points to synergy or antagonism. For the ligands tested, the combined effect of two ligands was simulated using IC₅₀ values determined for each compound tested alone. These simulations were then compared with experimental results obtained when ligands were coincubated.

Nonequilibrium Radioligand Binding Assay to Determine Cooperativity Factors and Affinity for the Unoccupied M₂ Receptor. To determine cooperativity factors (α) and binding affinities for the free-receptor (K_A), various concentrations of test drug were incubated with membranes expressing the human M₂ receptor and two concentrations (0.2 and 2 nM) of [³H]NMS for 1 h at 20°C. Assays were conducted in 10 mM HEPES buffer with 20 mM NaCl and 0.025% BSA, pH 7.4 at 20°C. Nonspecific binding was defined with 10 μ M atropine. Assays were terminated as described above. In practice, these methods are similar to those described for our inhibition radioligand binding studies, but the data are analyzed differently. CPM data were normalized to controls to yield percentage of maximal specific binding. Data were fit to a kinetic allosteric ternary complex model as defined by Avlani et al. (2004) to estimate the affinity for the free receptor and cooperativity with the radioligand.

Equations were modified to fit our definitions of cooperativity, defined below. The equilibrium allosteric ternary complex model (Ehler, 1988) is defined as:

$$\frac{Y}{Y_{\max}} = \frac{[L]}{[L] + K_{\text{App}}} \quad (2)$$

and

$$K_{\text{App}} = K_L \times \left(\frac{K_A + [A]}{K_A + [A]/\alpha} \right) \quad (3)$$

where Y/Y_{\max} denotes fractional receptor occupancy, $[L]$ and K_L are the concentration and equilibrium dissociation constants, respectively, for the radioligand $[A]$ and K_A are the concentration and equilibrium dissociation constants, respectively, for the modulator, α is the cooperativity factor for the allosteric interaction between L and A [where $\alpha > 1$, $\alpha = 1$, and $\alpha < 1$ indicate negative, neutral, and positive cooperativity, respectively]. K_{App} is defined by eq. 2 and is the midpoint of the occupancy curve for the radioligand in the presence of modulator A.

However, because high concentrations of an allosteric ligand or

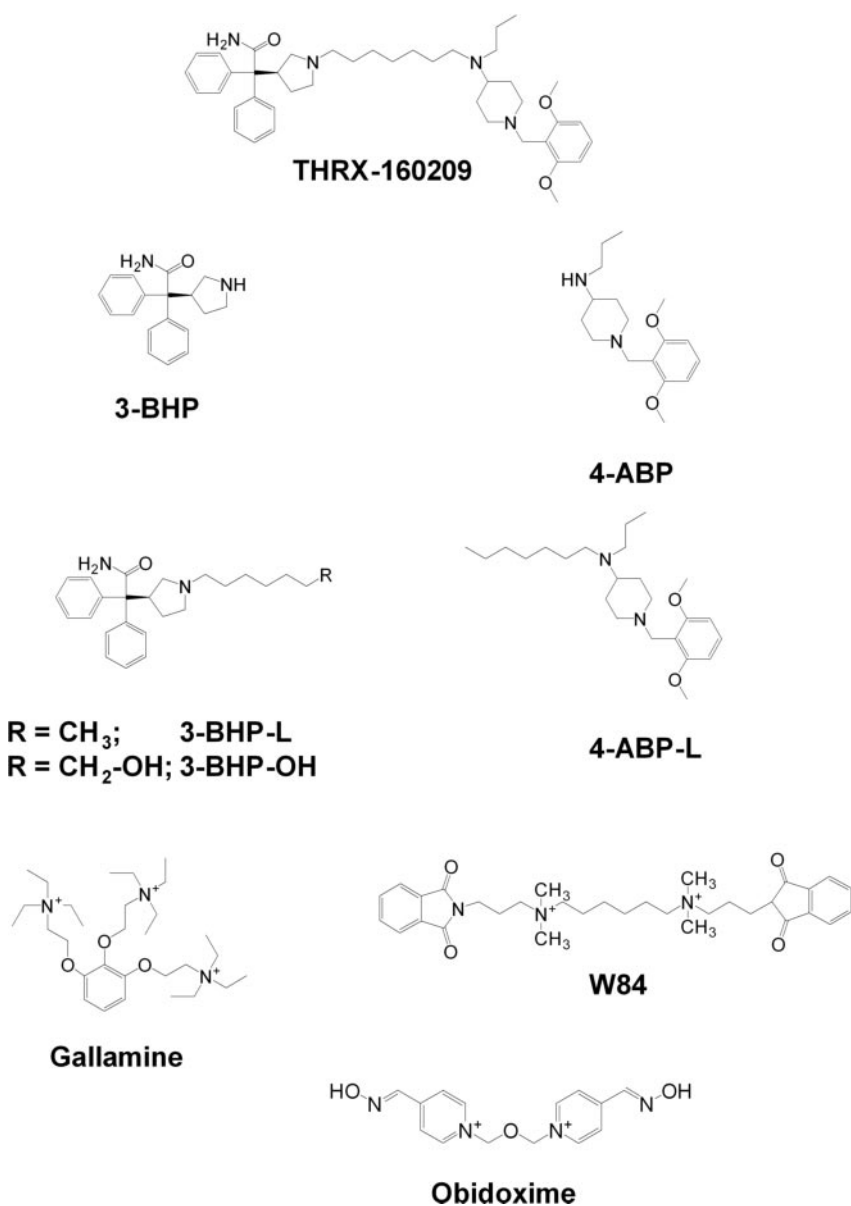


Fig. 1. Chemical structures of THRX-160209, mono-valent fragments of THRX-160209 (3-BHP and 4-ABP), linker conjugated fragments (3-BHP-L, 3BHP-OH, and 4-ABP-L) and the muscarinic allosteric ligands gallamine, W84, and obidoxime.

entry blocker can delay the time required to reach equilibrium, a kinetic model is sometimes more appropriate.

$$B_t = B_{LA} \times [1 - e^{-k_{on,obs} \times t}] \quad (4)$$

Equation 4 is the standard equation for monoexponential association of an orthosteric ligand, A; B_{LA} denotes the specific binding of A in the presence of B, and t denotes time. The term $k_{on,obs}$ is the apparent association rate and is defined by the equation:

$$k_{on,obs} = k_{off,obs} \times (1 + [L]/K_{App}) \quad (5)$$

The term $k_{off,obs}$ is the observed dissociation rate of the orthosteric ligand in the presence of allosteric modulator and is defined by the equation:

$$k_{off,obs} = \frac{k_{off} + ([A] \times k_{off,A}) / (K_A \times \alpha)}{1 + [A] / (K_A \times \alpha)} \quad (6)$$

Equation 6 is the equation for equilibrium binding of the orthosteric ligand in the presence of allosteric modulator, where K_{App} is the parameter as defined in eq. 3.

$$B_{LA} = \frac{B_{max} \times [L] / K_{App}}{1 + [L] / K_{App}} \quad (7)$$

Excess Ligand Dissociation Assay to Determine Occupied Receptor Affinity. Studies were a modification of procedures described by Ellis and colleagues (Ellis and Seidenberg, 1992; Ellis et al., 1993). Membranes expressing the M_2 receptor were labeled with 0.8 nM [3 H]NMS for 1 h at 20°C in a buffer consisting of 10 mM HEPES buffer with 20 mM NaCl and 0.025% BSA, pH 7.4, at 20°C. Experiments were initiated with the addition of 10 μ M atropine in the presence or absence of modulator and with or without obidoxime chloride. Membranes were collected onto polyethylenimine-treated GF/B filter plates, and radioactivity was measured as mentioned above. CPM data were normalized to percentage of total specific binding and analyzed with Prism, using the standard monophasic or biphasic exponential decay function. The process yielded the apparent dissociation rate constant determined in the presence of each concentration of allosteric modulator, $k_{off,obs}$. The apparent rate constant was then expressed as a percentage of the apparent rate constant for the radioligand determined in the absence of allosteric modulator, k_{off} . This was then plotted versus ligand concentration and analyzed by nonlinear regression.³ When present with a test allosteric ligand, obidoxime induced rightward shifts of the concentration-response curves. These shifts were analyzed by nonlinear regression methods described by Lew and Angus (1995) to check for competitive interactions. Fits to these models were compared using a partial F-test:

$$pEC_{50,diss} = -\log([B] + 10^{-pK_B}) - \log C \quad (8)$$

$$pEC_{50,diss} = -\log([B]^n + 10^{-pK_B}) - \log C \quad (9)$$

$$pEC_{50,diss} = -\log[B](1 + n[B]/10^{-pK_B}) + 10^{-pK_B} - \log C \quad (10)$$

where $pEC_{50,diss}$ is the concentration of allosteric modulator required to decelerate the dissociation rate of bound radioligand by 50% in the presence of obidoxime at concentration B. The constant $-\log C$ is the difference between the pK_B of the antagonist (i.e., obidoxime) and the $pEC_{50,diss}$ of the agonist, or in this case, the test allosteric modulator.

³ Concentration-effect curves were best fit using a four-parameter logistic function, except for obidoxime, where a two-site fit was used. The upper plateau was the k_{off} and set at 100%. When checking for competitive interactions with obidoxime, the $k_{off,obs}$ determined in the absence of test allosteric modulator and presence of obidoxime, was the upper plateau, again set at 100%. The lower plateau was fixed at 0%. Hill coefficients were fixed at 1, because they did not differ significantly from unity. The midpoint of the curve is the $EC_{50,diss}$, or the concentration of allosteric modulator required to decelerate the dissociation rate of bound radioligand by 50% and is the affinity of the allosteric ligand for the receptor when orthosteric radioligand is bound.

Equation 8 is the equivalent of a Schild plot with a slope of unity. Equation 9 is equivalent to a Schild plot with a slope $\neq 1$. Equation 10 is equivalent to a nonlinear Schild plot. Results were plotted in a manner analogous to Schild (Arunlakshana and Schild, 1959) for visualization purposes.

"Infinite" Dilution Dissociation Assay to Demonstrate Fragment-Induced Acceleration. Membranes expressing the M_2 receptor were labeled with 0.5 nM [3 H]NMS or [3 H]THR-160209 for 1 h at 20°C in a buffer consisting of 10 mM HEPES, with 20 mM NaCl and 0.025% BSA, pH 7.4, at 20°C. Labeled membranes were diluted 1000-fold in buffer with or without 10 μ M atropine, 3 mM ACh, 1 μ M QNB, 8 mM obidoxime, 7 mM 3-BHP, or 2 mM 4-ABP. After separation of bound from free radioligand at various times, radioactivity was measured using a scintillation counter. Data were analyzed with Prism using the standard monophasic or biphasic exponential decay function.

Statistical Analyses. The data are presented as means \pm S.D. When checking for a competitive interaction between allosteric test ligands and obidoxime, fits to the various equations were compared using an F-test. The statistical significance of differences between obidoxime apparent pK_B values (apparent dissociation constants of obidoxime for the [3 H]NMS occupied receptor) was determined by one-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

Results

Characterization of Ligand Affinities and Selectivities for Muscarinic Receptors Using Conventional Radioligand Inhibition Binding Assays. THR-160209 is a novel molecule containing a benzhydryl (3-BHP) group linked to 4-aminopiperidine motif (4-ABP) by a C7 polymethylene chain (Fig. 1). THR-160209 was demonstrated to be a functional antagonist of all five receptors via cyclic AMP accumulation assays or studies evaluating mobilization of intracellular calcium (data not shown). In typical inhibition radioligand binding studies using CHO cell membrane fractions expressing the human M_1 , M_2 , M_3 , M_4 , or M_5 receptor, THR-160209 completely inhibited the binding of 0.8 nM [3 H]NMS at high concentrations ($\geq 5 \mu$ M). The shapes of the inhibition curves for each receptor were similar, with Hill slopes close to unity. Although it was unclear whether test compounds were acting through a competitive or allosteric mechanism, we assumed a competitive interaction and converted IC_{50} values to apparent K_i values according to Cheng-Prusoff, as summarized in Table 1. THR-160209 bound tightly to all five muscarinic receptor subtypes, with the highest apparent affinity for the M_2 receptor (mean apparent $pK_i = 9.51 \pm 0.22$; Table 1). THR-160209 apparent affinities for the M_1 , M_3 , M_4 , and M_5 receptors were also high (7.11 to 8.78) but still lower than for M_2 . The subtype selectivities ranged from 5-fold (M_4/M_2) to 223-fold (M_5/M_2).

To demonstrate the multivalent nature of THR-160209, various truncated analogs were prepared and tested alone, and in combination, in the M_2 inhibition radioligand binding assay. Fragments lacking the C7 alkyl chain (3-BHP and 4-ABP) bound with micromolar apparent affinities to the muscarinic receptors. The M_2 receptor apparent pK_i values were 5.39 ± 0.33 and 5.70 ± 0.03 , respectively. Adding back the C7 alkyl chain gave the linker-conjugated fragments 3-BHP-L and 4-ABP-L. These compounds bound with an apparent affinity that was several orders of magnitude higher than the unmodified monomers, with M_2 receptor apparent pK_i values of 7.75 ± 0.07 and 8.28 ± 0.07 , respec-

tively (Table 1). The apparent affinity for the hydroxyl terminated linker analog (3-BHP-OH) was much lower with an apparent pK_I of 6.73 ± 0.04 . Coincubating the methyl-terminated linker conjugates with equimolar concentrations of their complementary monovalent fragment counterpart yielded apparent affinities (apparent pK_I values) of 8.30 ± 0.15 for 3-BHP with 4-ABP-L and 7.77 ± 0.03 for 3-BHP-L with 4-ABP, respectively (Fig. 2). Apparent ligand affinities for the M₂ receptor determined in the presence of the respective second fragment were altered in a manner consistent with an additive, not synergistic, model of ligand affinity enhancement. This is as would be expected for two compounds with very different affinities that exhibit additivity and not synergy. Thus, the weaker, unmodified monomer had no effect on the binding of the [³H]NMS at or near IC₅₀ concentrations of the linker-conjugated fragment. The apparent binding affinities for these monovalent fragments and linker conjugates alone (Table 1), and the ligand mixtures were greater than 10-fold lower than the apparent binding affinity of the intact multivalent ligand THRX-160209 (M₂ apparent $pK_I = 9.51 \pm 0.22$). For the M₂ receptor, the synergistic increase in binding affinity, or multivalent effect, achieved by covalently linking compounds 3-BHP and 4-ABP-L can be quantified with the ratio (τ) of 17.0. Likewise, the covalent linkage of the fragments 3-BHP-L and 4-ABP also demonstrated a multivalent effect, $\tau = 57.0$.

Excess Ligand Dissociation Assay to Determine Occupied Receptor Affinity for Allosteric Ligands. Kinetic assays using excess competing ligand were employed to study potential interactions of THRX-160209 with a secondary binding site located proximal to the primary binding site on muscarinic receptors. In general, ligands that bind to defined secondary (allosteric) sites on muscarinic receptors decelerate the dissociation rate of ligands bound to the primary or orthosteric site. In these kinetic assays, THRX-160209 produced a concentration-dependent retardation of [³H]NMS dissociation from membrane fractions expressing the human M₂ receptor in a manner similar to that observed with the known muscarinic allosteric ligands W84 and gallamine (Fig. 3). Dissociation curves were monophasic in the presence and absence of test compounds. In the absence of test ligand, the [³H]NMS dissociation $t_{1/2} = 3.5 \pm 0.16$ min and the apparent rate constant of dissociation, $k_{off} = 0.20 \text{ min}^{-1}$. The $pEC_{50,diss}$ values for gallamine and W84 were determined to be 6.2 ± 0.12 and 7.2 ± 0.19 , respectively. For THRX-160209, the $pEC_{50,diss}$ was determined to be 6.5 ± 0.06 , differing from the apparent pK_I determined in inhibition binding studies by approximately 3 orders of magnitude. Similar to W84, a high concentration of THRX-160209 (100 μM) inhibited the dissociation rate of [³H]NMS from the M₂ receptor by 1000-fold ($t_{1/2} = 280 \pm 30.9$ min; $k_{off,obs} = 0.0025 \pm 0.0003 \text{ min}^{-1}$).

These results are consistent with an allosteric interaction between THRX-160209 and the M₂ receptor.

The effects of fragments of THRX-160209 on the dissocia-

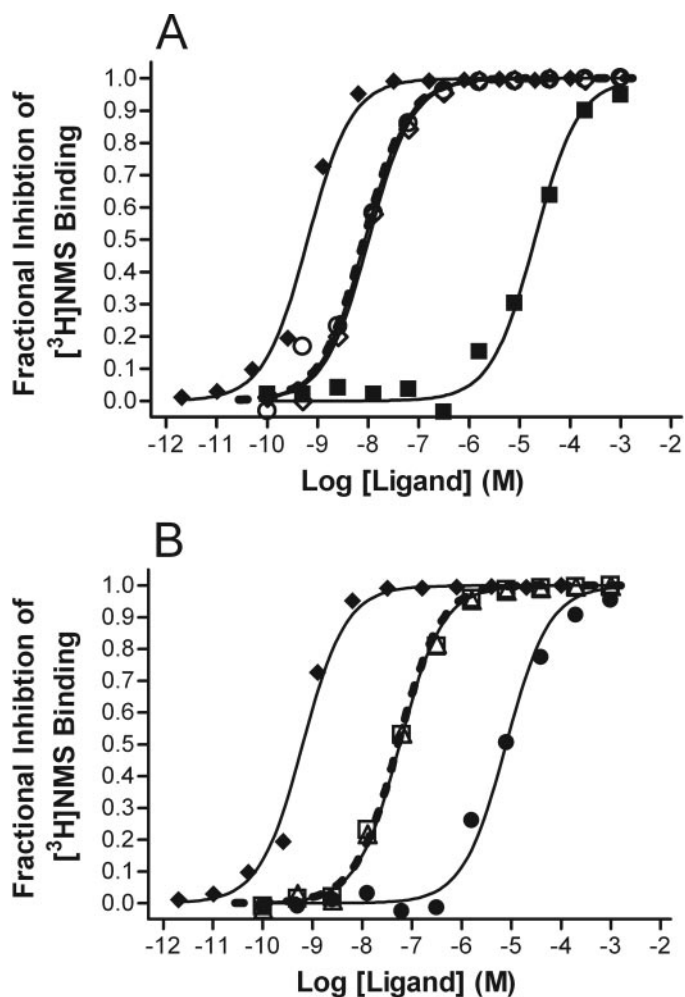


Fig. 2. Coincubation of THRX-160209 fragments to demonstrate synergy of linker connected monomers. A, the binding of 0.8 nM [³H]NMS to M₂ receptors expressed in membrane fractions was inhibited with various concentrations of 3-BHP (■), 4-ABP-L (○), 3-BHP and 4-ABP-L together (◇), or THRX-160209 (◆). [³H]NMS and compounds were allowed to incubate with receptors for 1 h at 20°C. B, 3-BHP-L (□), 4-ABP (●), 3-BHP-L and 4-ABP together (△), or THRX-160209 (◆) were also studied in similar inhibition binding assays. CPM data were normalized to fractional inhibition of [³H]NMS binding. These data were compared with simulations according to the Bliss Independence model (dashed lines), which assumes two ligands interact in an additive manner. According to this model, independence of two agents used in combination can be demonstrated if the sum of their fractional effects minus their product equals the fractional response of the two drugs in combination. Experimental data shown (solid lines) are representative of three separate experiments. Curves were fit using the standard four parameter logistic equation.

TABLE 1

Muscarinic receptor affinities (apparent pK_I) for THRX-160209 and fragments

Values were determined using typical [³H]NMS inhibition radioligand binding assays. Mean values \pm S.D. are from three separate experiments performed in duplicate. Competitive interactions between test ligand and radioligand were assumed, thus IC₅₀ values were converted to apparent pK_I values according to the Cheng-Prusoff equation.

	THRX-160209	3-BHP	3-BHP-L	3-BHP-OH	4-ABP	4-ABP-L
M ₁	8.02 ± 0.17	5.44 ± 0.20	7.06 ± 0.16	6.33 ± 0.07	4.83 ± 0.08	6.94 ± 0.11
M ₂	9.51 ± 0.22	5.39 ± 0.33	7.75 ± 0.07	6.73 ± 0.04	5.70 ± 0.03	8.28 ± 0.07
M ₃	7.81 ± 0.22	5.34 ± 0.21	8.59 ± 0.06	7.09 ± 0.02	5.35 ± 0.03	7.03 ± 0.04
M ₄	8.78 ± 0.24	5.45 ± 0.16	7.18 ± 0.23	6.91 ± 0.05	4.48 ± 0.15	7.20 ± 0.08
M ₅	7.11 ± 0.13	5.46 ± 0.18	7.47 ± 0.21	6.35 ± 0.05	4.18 ± 0.09	6.07 ± 0.12

tion rate of [^3H]NMS were also investigated. Here, the fragment 4-ABP slowed the off-rate of [^3H]NMS from the M_2 receptor at concentrations higher than $10\ \mu\text{M}$. A $\text{pEC}_{50,\text{diss}}$ of 4.6 ± 0.08 was determined for this fragment. In the presence of the 3-BHP fragment, the effects on [^3H]NMS dissociation only occurred at the highest concentrations used ($>100\ \mu\text{M}$); these concentrations were similar to those required for dissociation rate retardation by the orthosteric ligands scopolamine (data not shown).

Excess Ligand Dissociation Assay—Interactions of Allosteric Ligands with Obidoxime. In excess ligand dissociation assays, obidoxime alone retards the dissociation rate of [^3H]NMS (Fig. 4A). Similar to findings presented by Trankle and Mohr (1997), we found the concentration-response curve for obidoxime fit better to a two-site model rather than a single site ($P < 0.05$; F test). Dissociation $\text{pEC}_{50,\text{diss}}$ values of 2.4 ± 0.15 and 4.1 ± 0.10 were determined for obidoxime at the low- and high-affinity sites, respectively (Fig. 4B). At concentrations as high as $10\ \text{mM}$, obidoxime did not fully inhibit the dissociation rate of [^3H]NMS (Fig. 4A). At this concentration, the $k_{\text{off,obs}}$ was $0.04\ \text{min}^{-1}$, 20% of the dissociation rate of [^3H]NMS in the absence of allosteric modulator. The concentration response curve plateaus at $0.026\ \text{min}^{-1}$, 13% of the control dissociation rate. Our findings are in agreement with those of other investigators and indicate that the actions of obidoxime are analogous to those of a partial agonist, in that obidoxime only partially inhibits dissociation of the orthosteric radioligand (Ellis and Seidenberg, 1992).

Test ligands were studied in the excess ligand dissociation assay in the presence or absence of various concentrations of obidoxime to determine whether each ligand competed for the obidoxime (allosteric) site. As described above, obidoxime alone decelerated the rate of [^3H]NMS dissociation; therefore, off-rates were normalized to a control curve (in the absence of test ligand) for each concentration of obidoxime. [^3H]NMS dissociation curves in the presence of THRX-160209 and the absence or presence of $10\ \text{mM}$ obidoxime are shown in Figs. 3A and 5A, respectively. In the presence of $10\ \text{mM}$ obidoxime, the low-to-intermediate concentrations of THRX-160209 ($< 10\ \mu\text{M}$) had no significant effect on the rate of [^3H]NMS dissociation, relative to the control curve (in the absence of THRX-160209). In fact, only the highest concentration of THRX-160209 ($10\ \mu\text{M}$) tested in the presence of $10\ \text{mM}$ obidoxime slowed the dissociation rate of [^3H]NMS to a greater extent than $10\ \text{mM}$ obidoxime alone. This was in contrast to results from studies performed in the absence of obidoxime, where concentrations of THRX-160209 as low as $0.1\ \mu\text{M}$ retarded the off-rate of [^3H]NMS relative to the control. The effects of obidoxime can be seen when comparing the dissociation rate of [^3H]NMS in the presence of $10\ \mu\text{M}$ THRX-160209 without obidoxime ($k_{\text{off,obs}} = 0.0081 \pm 0.0008\ \text{min}^{-1}$) to the rate when $10\ \text{mM}$ obidoxime was present with $10\ \mu\text{M}$ THRX-160209 ($k_{\text{off,obs}} = 0.025 \pm 0.002\ \text{min}^{-1}$). Concentration-response curves for THRX-160209 in the presence of a range of obidoxime concentrations are shown in Fig. 6A. Here, $\text{pEC}_{50,\text{diss}}$ values ranged from 4.5 (when determined in the presence of $10\ \text{mM}$ obidoxime) to 6.5 (determined in the

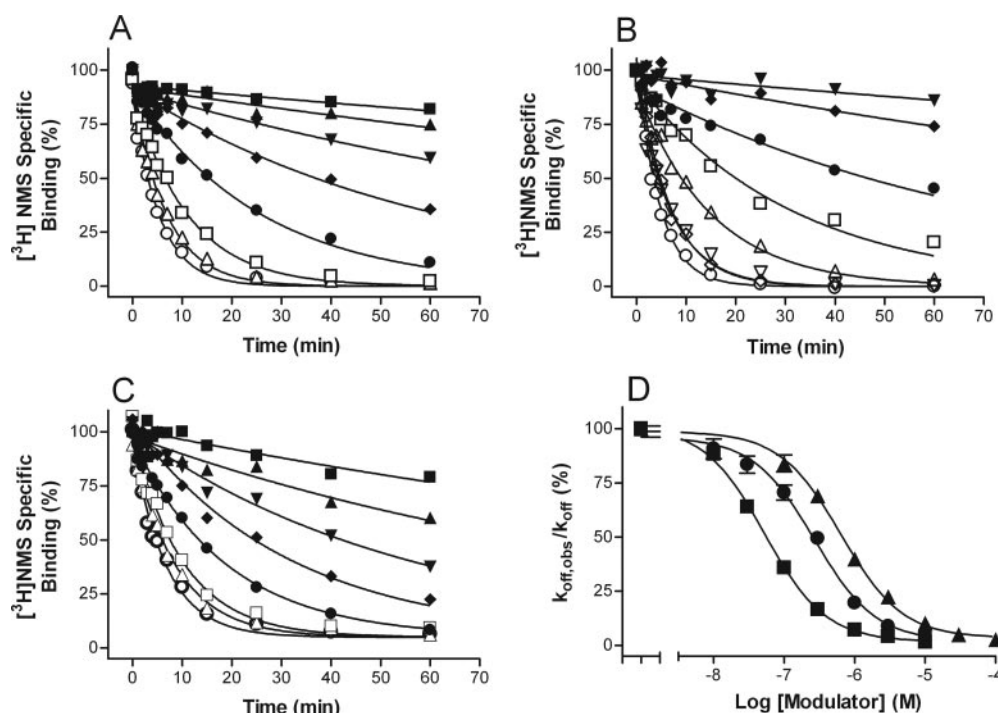


Fig. 3. Concentration-dependent effects of allosteric ligands on observed dissociation rates of [^3H]NMS from M_2 receptors. Dissociation of [^3H]NMS from human M_2 receptors expressed in CHO cell membranes in the absence (\circ) or presence of $100\ \mu\text{M}$ (\blacksquare), $30\ \mu\text{M}$ (\blacktriangle), $10\ \mu\text{M}$ (\blacktriangledown), $3\ \mu\text{M}$ (\blacklozenge), $1\ \mu\text{M}$ (\bullet), $0.3\ \mu\text{M}$ (\square), $0.1\ \mu\text{M}$ (\triangle), $0.03\ \mu\text{M}$ (∇), or $0.01\ \mu\text{M}$ (\diamond) THRX-160209 (A), W84 (B), or gallamine (C). Membrane homogenates were incubated with $0.5\ \text{nM}$ [^3H]NMS for 60 min at 20°C before the addition of $10\ \mu\text{M}$ atropine and allosteric modulator or buffer. Curves represent the best fit of a monoexponential decay model. Data are representative of five separate experiments conducted. Data were normalized to the percentage of specific binding before analysis. D, concentration-effect curves were plotted from dissociation rate retardation studies for THRX-160209 (\bullet), W84 (\blacksquare), and gallamine (\blacktriangle). The observed dissociation rates ($k_{\text{off,obs}}$) were normalized to the percentage of the control dissociation rate (k_{off}) measured in the absence of test ligand. Data are mean \pm S.E. values from five independent experiments and were fit to a four-parameter logistic equation with a Hill coefficient fixed to 1.

absence of obidoxime). Increasing concentrations of obidoxime shifted the curves to the right in a parallel fashion. Similar results were seen when W84 or gallamine were used as test ligands (Figs. 5, B and C, and 6, B and C).

Analysis of these data assumes that the modulators and obidoxime rapidly associate with the allosteric site such that the effect observed on the dissociation rate of the radioligand represents the equilibrium binding of the allosteric modulator. Data were analyzed with the application of eqs. 8 to 10 (Lew and Angus, 1995) to check for competitive interactions. For all compounds tested, the data were best described by eq. 8 (i.e., the equation analogous to a Schild plot with a slope of unity, indicating a competitive interaction) (Fig. 7A). The apparent pK_B (apparent dissociation constants of obidoxime for the [³H]NMS-occupied receptor) values for obidoxime determined in the presence of THRX-160290, W84, and gallamine were 3.97 ± 0.09 , 4.15 ± 0.09 , and 4.12 ± 0.14 , respectively. These values were not significantly different from each other (one-way ANOVA; $P > 0.05$). According to the allosteric ternary complex model (ATCM), these apparent

pK_B values should be equal to the high-affinity $pEC_{50,diss}$ value for obidoxime ($pEC_{50,diss} = 4.1$) determined in the absence of other allosteric modulators. Indeed, there were no significant differences between the obidoxime $pEC_{50,diss}$ and the apparent pK_B values (one-way ANOVA, $P > 0.05$). These curve shifts were also transformed in a manner analogous to that described by Arunlakshana and Schild (1959) to more easily visualize the data (Fig. 7B).

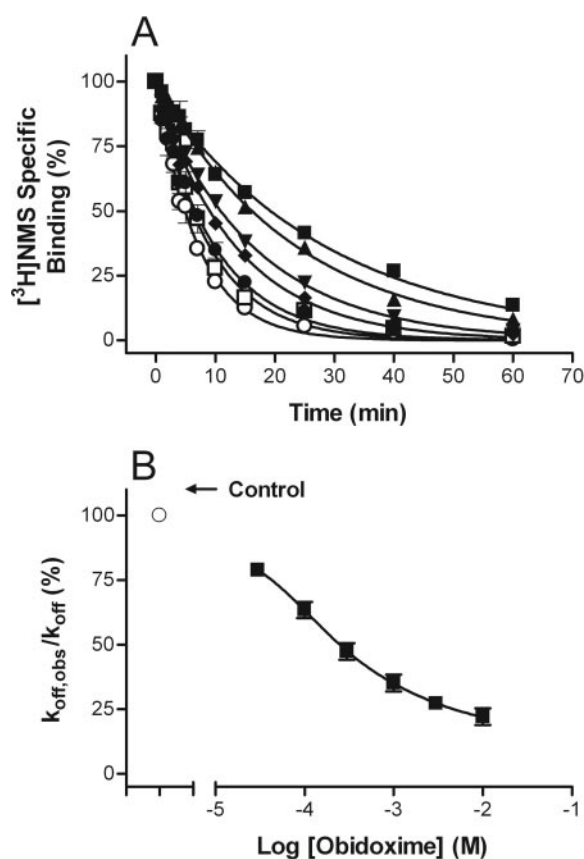


Fig. 4. Effects of obidoxime on [³H]NMS dissociation rates. A, the dissociation of [³H]NMS from human M₂ receptors expressed in CHO cell membranes was studied in the absence (○) or presence of 10 mM (■), 3 mM (▲), 1 mM (▼), 300 μM (◆), 100 μM (●), 30 μM (□), or 10 μM (△) obidoxime. Membrane homogenates were incubated with 0.5 nM [³H]NMS for 1 h at 20°C before the addition of 10 μM atropine and obidoxime or buffer. Curves represent the best fit of a monoexponential decay model. Data representative of three separate experiments conducted in duplicate were normalized to the percentage of specific binding. B, concentration-effect curve for obidoxime in retarding the observed dissociation rate of [³H]NMS at human M₂ receptors expressed in CHO cell membranes. Observed dissociation rates ($k_{off,obs}$) were normalized to the percentage of the control dissociation rate (k_{off}) measured in the absence of obidoxime. Data are mean \pm S.E. values from three independent experiments and were fit to a two-site model.

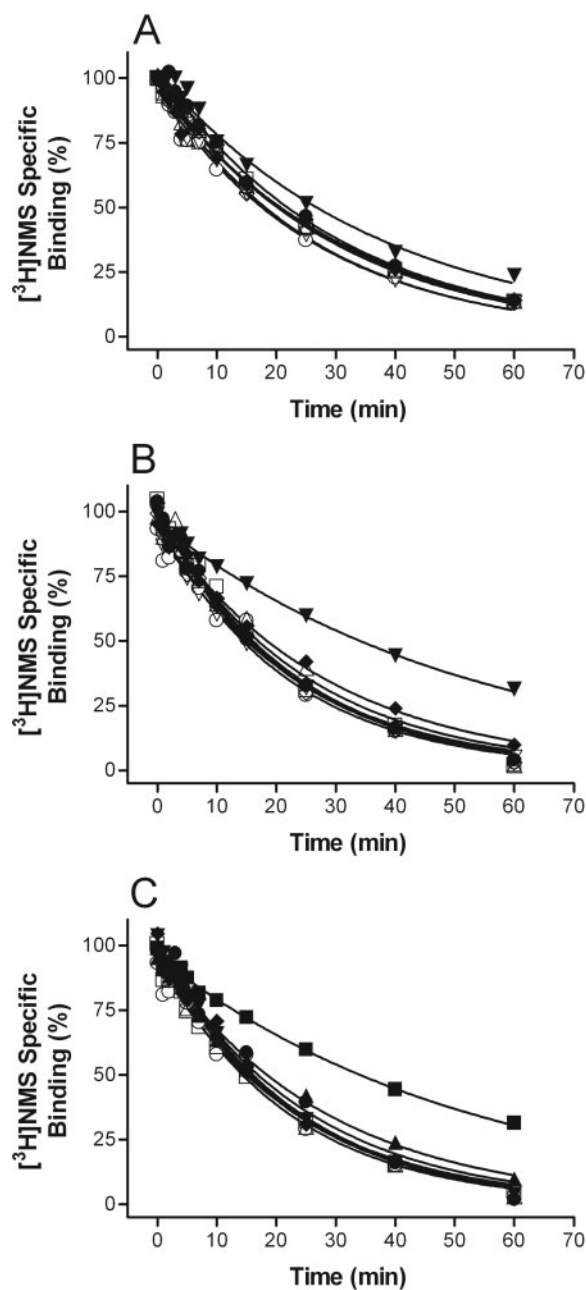


Fig. 5. Effects of obidoxime on activities of allosteric ligands. The dissociation of [³H]NMS from human M₂ receptors expressed in CHO cell membranes in the presence of 10 mM obidoxime and the absence (○) or presence of 100 μM (■), 30 μM (▲), 10 μM (▼), 3 μM (◆), 1 μM (●), 0.3 μM (□), 0.1 μM (△), 0.03 μM (▽), or 0.01 μM (◇) THRX-160290 (A), W84 (B), or gallamine (C) was studied. Membrane homogenates were incubated with 0.5 nM [³H]NMS for 1 h at 20°C before the addition of 10 μM atropine and 10 mM obidoxime and modulator. Data were normalized to the percentage of specific binding. Curves represent the best fit of a monoexponential decay model. Data shown are representative of three separate experiments.

Nonequilibrium Radioligand Binding Assay to Determine Cooperativity Factors and Affinity for the Unoccupied Receptor. Nonequilibrium radioligand binding assays were used to estimate the cooperativity factor (α) and

affinity of allosteric modulators for the free M_2 receptor. The cooperativity factor describes the interaction of the allosteric modulator and the orthosteric ligand. It is defined as the ratio of affinity of a ligand for the free receptor compared with its affinity for the occupied receptor. As defined in the ATCM, $\alpha > 1$ denotes negative cooperativity and $\alpha < 1$ denotes positive cooperativity. Data for THRX-160209, W84 and gallamine using these experiments were fit to eqs. 2 to 7 (Lazareno and Birdsall, 1995; Christopoulos and Kenakin, 2002; Avlani et al., 2004). Figure 8 shows the nonequilibrium concentration-response curves for each compound tested. The curves for W84 and gallamine were biphasic, as would be predicted by the ATCM. The cooperativity factors determined for gallamine and W84 were 45 ± 9.14 ($p\alpha = 1.7$) and 6.9 ± 1.35 ($p\alpha = 0.83$), respectively, whereas the pK_A values (affinity for the free-receptor) were 8.2 ± 0.05 and 8.0 ± 0.07 , respectively. For THRX-160209, the curves were monophasic and yielded a cooperativity factor of 2547 ± 281 ($p\alpha = -3.4$) and a pK_A of 10.2 ± 0.14 . The ratio of the free and [3H]NMS-occupied receptor affinities also provided an estimate of the cooperativity factor. For all compounds tested, the estimated cooperativity factors ($pEC_{50,diss} - pK_A$) are in good agree-

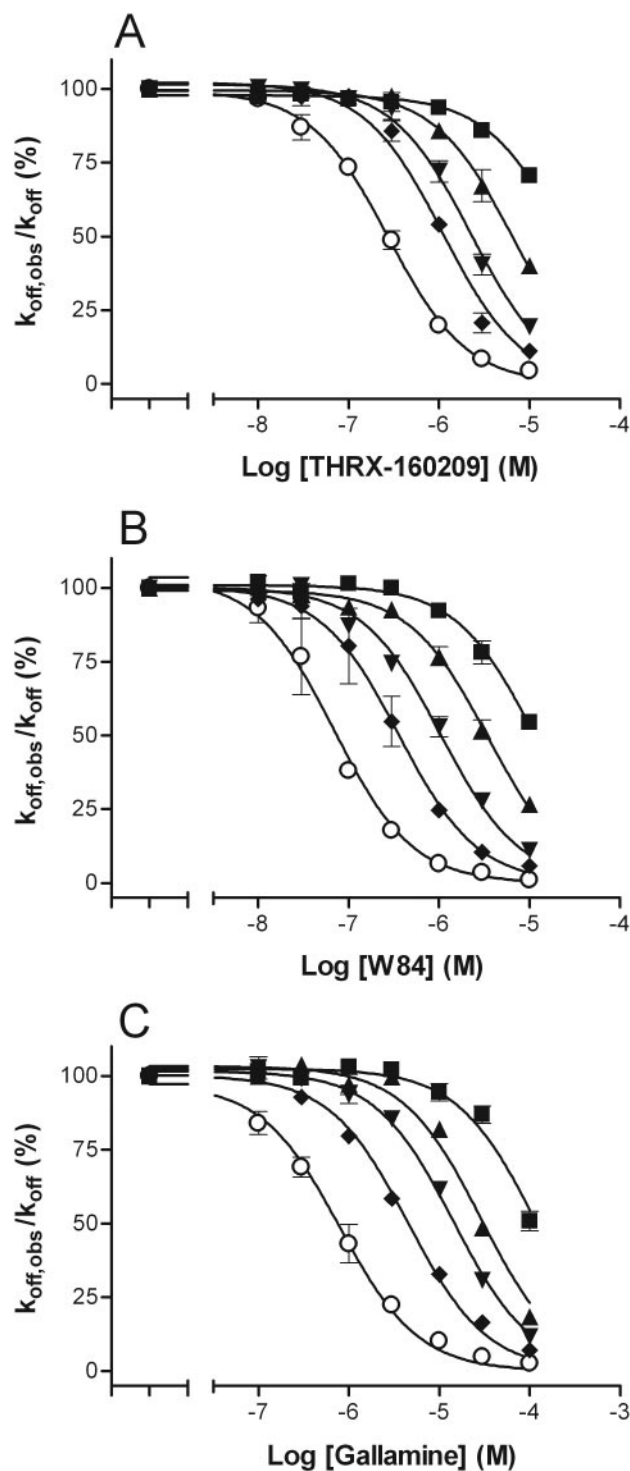


Fig. 6. Concentration-effect curves for the retardation of [3H]NMS dissociation from M_2 receptors by THRX-160209 (A), W84 (B), or gallamine (C) in the absence (\circ) or presence of 10 mM (\blacksquare), 3 mM (\blacktriangle), 1 mM (\blacktriangledown), or 0.3 mM (\blacklozenge) obidoxime. Observed dissociation rates of [3H]NMS ($k_{off,obs}$) were normalized to dissociation rates determined in the absence of test allosteric modulator to account for the effects of obidoxime. Data are mean \pm S.E. values from three independent experiments and were fit to a four-parameter logistic equation.

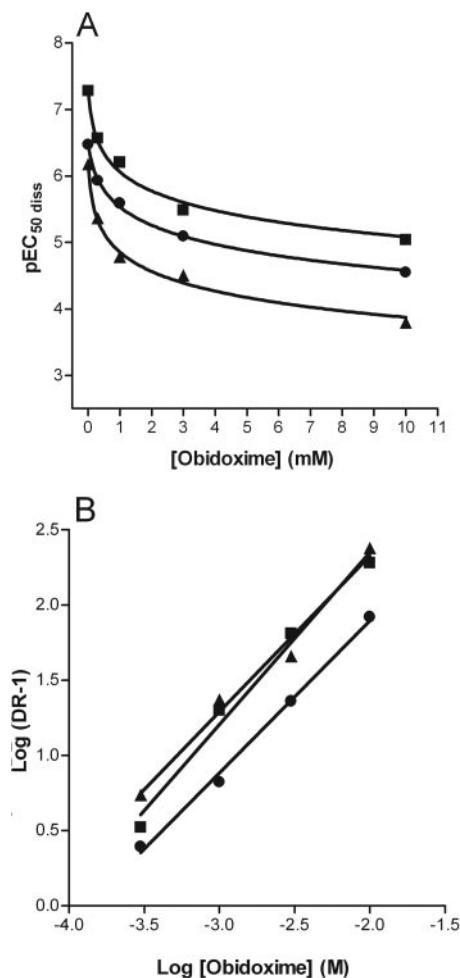


Fig. 7. Analysis of obidoxime-induced shifts in concentration-effect curves for THRX-160209 (\bullet), W84 (\blacksquare), and gallamine (\blacktriangle). A, obidoxime-induced curve shifts were analyzed using nonlinear regression (Lew and Angus, 1995), fitting best to eq. 8. B, curve shifts were expressed as dose ratios (DR) in the form of a Schild plot. Dose ratios were calculated by dividing the $EC_{50,diss}$ determined in the presence of obidoxime by the $EC_{50,diss}$ determined in the absence of obidoxime.

ment with the experimentally determined cooperativity factor (e.g., THRX-160209 estimated $p\alpha = -3.7$; experimental $p\alpha = -3.4$; Table 2), thus these data are in good agreement with the affinity estimates for the occupied receptor as predicted by the ATCM. However, because these values follow the rules of an allosteric interaction, the cooperativity factor for THRX-160209 does not seem practical. A cooperativity factor of this magnitude is indicative of a competitive rather than allosteric interaction. Other concerns regarding the validity of these analysis methods were described recently. May

et al. (2007) reported that the assumptions of the ATCM may not hold for a multivalent ligand. However, as they suggest, we believe that deviation from the model would only be significant for a multivalent ligand with positive cooperativity rather than one with negative cooperativity.

"Infinite" Dilution Assay to Test for Fragment-Induced Acceleration of Multivalent Ligand Dissociation Rates. [³H]NMS is a monovalent ligand that binds to the orthosteric ligand binding site of muscarinic receptors; the dissociation rate from M₂ receptors at 20°C was determined to be $0.20 \pm 0.037 \text{ min}^{-1}$ ($t_{1/2} = 3.46 \pm 0.61 \text{ min}$) by the "infinite" dilution method. In the presence of the competitive antagonist atropine, the dissociation rate was identical ($k_{\text{off}} = 0.22 \pm 0.022 \text{ min}^{-1}$, $t_{1/2} = 3.18 \pm 0.33 \text{ min}$) (Fig. 9A). In addition, dissociation rates for [³H]NMS determined in the presence of other monovalent orthosteric ligands, ACh and QNB, were indistinguishable from rates determined in the presence or absence of atropine (data not shown). Using a similar "infinite" dilution assay, the off rate of [³H]THRX-160209 was rapid and monophasic in the presence of 10 μM atropine ($k_{\text{off}} = 0.82 \pm 0.17 \text{ min}^{-1}$, $t_{1/2} = 0.88 \pm 0.19 \text{ min}$). However, the dissociation rate in the absence of atropine (via "infinite" dilution) revealed a biphasic dissociation curve (Fig. 9B). The initial rapid phase ($k_{\text{off,fast}} = 0.89 \pm 0.26 \text{ min}^{-1}$, $t_{1/2} = 0.84 \pm 0.26 \text{ min}$) accounted for ~50% of the total binding. The $t_{1/2}$ of the slower dissociating species was found to be $13 \pm 2.4 \text{ min}$ ($k_{\text{off,slow}} = 0.054 \pm 0.011 \text{ min}^{-1}$).

Truncated analogs, 3-BHP and 4-ABP also accelerated the dissociation of [³H]THRX-160209 with rates comparable with those seen with atropine ($k_{\text{off}} = 0.93 \pm 0.15 \text{ min}^{-1}$ and $k_{\text{off}} = 0.72 \pm 0.16 \text{ min}^{-1}$, respectively) (Fig. 9C). These results are consistent with a multivalent interaction (Kramer and Karpen, 1998; Rao et al., 1998; Smith et al., 2006). Likewise, the presence of orthosteric or allosteric muscarinic ligands accelerated the rate of dissociation of [³H]THRX-160209 over the rates determined in the absence of competing ligand (Fig. 9D). The agonist ACh and the allosteric ligand obidoxime both induced monophasic dissociation curves, with dissociation rates of $k_{\text{off}} = 0.79 \pm 0.18 \text{ min}^{-1}$ ($t_{1/2} = 0.92 \pm 0.22 \text{ min}$) and $k_{\text{off}} = 0.76 \pm 0.10 \text{ min}^{-1}$ ($t_{1/2} = 0.93 \pm 0.13 \text{ min}$), respectively. These rates are comparable with the rate observed in the presence of the antagonist atropine.

Discussion

The binding interaction of THRX-160209 to muscarinic receptors exhibits three characteristics that are typical of

TABLE 2

Parameters characterizing the allosteric interactions of the test ligands and [³H]NMS at the M₂ receptor

The ratios of affinities ($p\text{EC}_{50,\text{diss}} - pK_A$) were calculated from affinity for the [³H]NMS-occupied receptor ($p\text{EC}_{50,\text{diss}}$) and free receptor (pK_A) and compared with the negative log cooperativity factor ($p\alpha$) as determined in the kinetic allosteric ternary complex model.

	$p\text{EC}_{50,\text{diss}}$	pK_A	$p\text{EC}_{50,\text{diss}} - pK_A$	$p\alpha$	α
THRX-160209	6.5	10.2	-3.7	-3.4	2547
W84	7.2	8.0	-0.8	-0.8	6.9
Gallamine	6.2	8.2	-2.0	-1.7	45

$p\text{EC}_{50,\text{diss}}$, negative log value of the concentration reducing [³H]NMS dissociation by 50% determined using excess ligand dissociation assays; pK_A , negative log value of the equilibrium dissociation constant for test compounds binding to unoccupied receptors determined from nonequilibrium radioligand binding assays; $p\alpha$, negative log value of the cooperativity factor (α) determined from nonequilibrium radioligand binding assays.

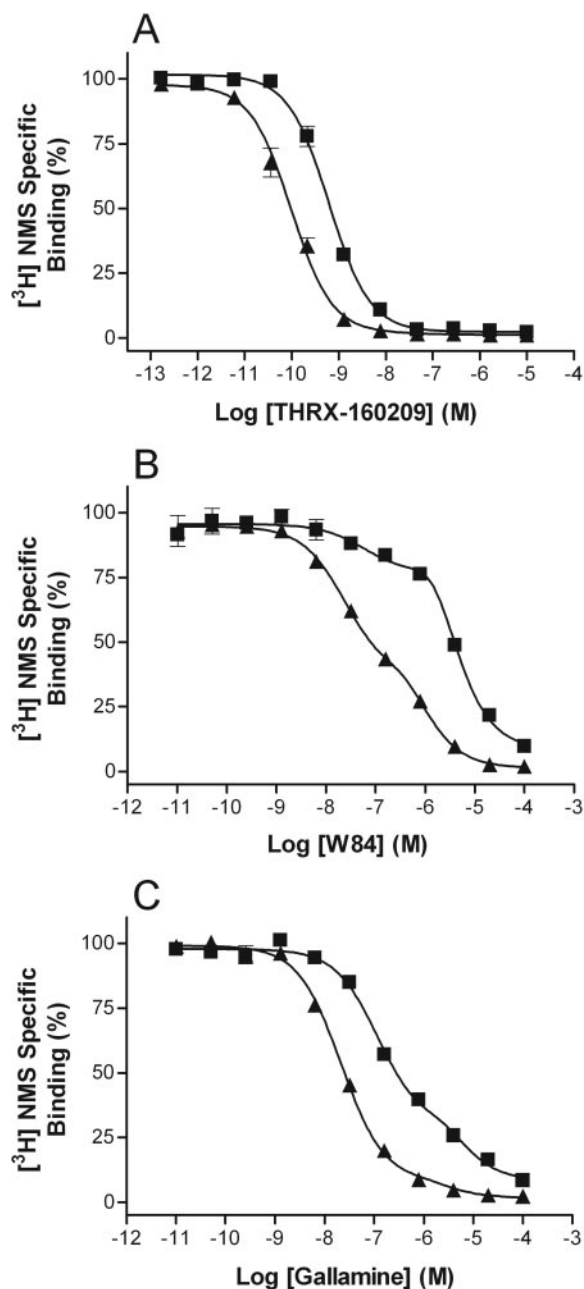


Fig. 8. Effects of THRX-160209 (A), W84 (B), or gallamine (C) on the nonequilibrium binding of 2 nM (■) and 0.2 nM (▲) [³H]NMS at human M₂ receptors expressed in CHO cell membranes. Compounds were incubated for 1 h at 20°C. Nonspecific binding was defined using 10 μM atropine. Data, representative of three separate experiments conducted, were normalized to percent specific binding and fit to the kinetic allosteric ternary complex model. The following parameters were constrained in the analysis: $k_{\text{off}} = 0.20 \text{ min}^{-1}$; $K_L = 0.15 \text{ nM}$.

multivalent systems. The first of these typical characteristics is high affinity. High affinity of THRX-160209 is the result of fusing two ligands into a single multivalent ligand. Unconnected, these monovalent ligands have low (micromolar) apparent affinities for the muscarinic receptors; when connected together with a seven-carbon chain, the affinities toward the M_2 muscarinic receptor subtype were increased by 3 to 4 orders of magnitude. Data for the seven-carbon chain fragments 3-BHP-L and 4-ABP-L described here suggest that the linker itself might play a significant role in receptor binding. However, estimating the linker contribution to THRX-160209 affinity, based on these affinity increases, is problematic. Given that the seven-carbon linker is hydrophobic, it is plausible that this portion of the molecule might bind to hydrophobic regions in the interior of the protein as opposed to interacting with residues on the surface. Such a phenomenon would probably lead to an overestimation of the contribution of the seven-carbon linker to the overall affinity of THRX-160209, because the polar functionality found in the intact multivalent ligand THRX-160209 would probably preclude such a binding mode. Indeed, some evidence in support of this hypothesis can be seen in the comparison with 3-BHP-OH, which displays a terminal polar hydroxyl group. It is noteworthy that this change leads to a 10.5-fold reduction in apparent binding affinity relative to the methyl-terminated linker conjugate 3-BHP-L. To summarize, the actual contribution of the linker to the binding of the multivalent compound remains unknown at this time, but we anticipate it to be less than is suggested by the binding of these linker analogs.

Coincubating THRX-160209 fragments, such as 4-ABP-L with 3-BHP, resulted in only a small increase in apparent M_2 affinity, as predicted by an additive model of ligand affinity enhancement. In contrast, the increase in affinity of THRX-160209 relative to the coincubated ligands demonstrates the synergy possible when ligands are covalently linked. The

gains in affinity exhibited by the intact ligand over the fragments are consistent with a multivalent ligand (Mammen et al., 1998a).

A second feature of some multivalent systems is specificity. The challenge in discovering ligands with specificity for a given muscarinic receptor subtype or group of subtypes is due in part to the highly conserved nature of the orthosteric (ACh) binding pocket. We hypothesized that binding outside of this region of high conservation to a site with lower subtype homology would probably result in subtype selectivity. THRX-160209 binds to M_2 receptors with 26-, 41-, 5-, and 223-fold higher affinity compared with M_1 , M_3 , M_4 , and M_5 receptors, respectively. The lower degree of specificity for the M_2 over the M_4 receptor is not surprising given the high sequence homology reported between these subtypes—higher homology than between the M_2 and the M_1 , M_3 , and M_5 receptors (Bonner et al., 1988). Because the muscarinic ACh receptor primary binding site is thought to be highly conserved across all subtypes, these selectivity ratios suggest that THRX-160209 binds, at least in part, to a region outside of the primary binding pocket. Consistent with this proposal is the observation that THRX-160209 stabilizes the [3 H]NMS- M_2 receptor complex. That is, at high concentrations, THRX-160209 significantly attenuates dissociation of this orthosteric radioligand, a finding consistent with other allosteric ligands.

From mutagenesis studies, amino acid residues essential for binding the well characterized allosteric modulators gallamine and W84 include the M_2 -EDGE sequence, M_2 -Tyr¹⁷⁷ and M_2 -Thr⁴²³ (Leppik et al., 1994; Voigtländer et al., 2003). Of these, only M_2 -D¹⁷³ and M_2 -Y¹⁷⁷ can be found in other subtypes (Bonner et al., 1988). The present study has not examined binding of THRX-160209 to appropriate mutant M_2 receptors to confirm interactions with these specific amino acids; however, we have shown a competitive interaction between obidoxime and THRX-160209. Obidoxime and

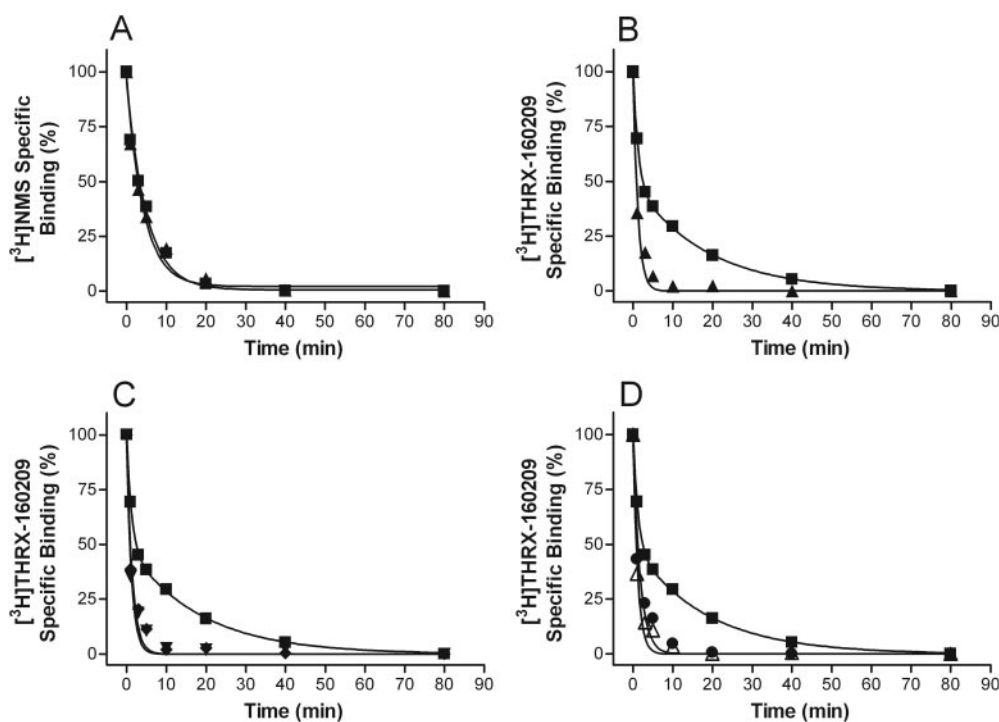


Fig. 9. Dissociation of [3 H]NMS or [3 H]THRX-160209 from M_2 receptors expressed in membrane fractions after “infinite” dilution in the presence or absence of atropine. [3 H]NMS (0.5 nM) or [3 H]THRX-160209 (0.5 nM) was allowed to bind to M_2 receptors at 20°C. After 1 h, 50- μ L aliquots were added to 50 ml of assay buffer (10 mM HEPES buffer with 20 mM NaCl and 0.025% BSA, pH 7.4, at 20°C) with or without 10 μ M atropine. Membranes were harvested at various times, and bound radioactivity was measured. A, [3 H]NMS data were normalized to percentage of specific binding. Data were best fit to monoexponential decay model in the presence (\blacktriangle) or absence (\blacksquare) of atropine. B, normalized [3 H]THRX-160209 data fit best to a monoexponential decay model in the presence of atropine but fit best to a biphasic dissociation model in the absence of atropine or other competing ligand. Other competing ligands tested included 7 mM 3-BHP (\blacklozenge) or 2 mM 4-ABP (\blacktriangledown) (C) or 3 mM acetylcholine (\blacktriangle) or 8 mM obidoxime (\bullet) (D). Data are representative of four separate experiments.

THR-160209 apparently bind to an overlapping region coincident with other typical muscarinic allosteric agents, such as W84 and gallamine. The M₂ receptor specificity of THR-160209 is probably therefore the result of the interaction with this secondary site.

A third feature of some multivalent systems is fragment-induced acceleration of dissociation rates. As described above, dissociation kinetics of radiolabeled ligands are often determined after the addition of excess, unlabeled competing ligand, to prevent reassociation with the receptor. To investigate the multivalent mode of binding of THR-160209, an “infinite” dilution protocol was employed. In these “infinite” dilution studies, dilution of the assay mixture reduces the radioligand concentration such that minimal radioligand reassociation occurs. In general, estimates of ligand dissociation rates should be independent whether determined with the excess competing ligand assay or the “infinite” dilution assay. However, this is not necessarily true for multivalent ligands and differences in dissociation rate would be expected if a competing monovalent ligand alters the affinity of the multivalent ligand for the receptor. Certain systems allow the entry and binding of a fragment to a binding pocket with a partially bound multivalent ligand—this is typical of some, but not necessarily all, multivalent systems (Rao et al., 1998; Smith et al., 2006). From our data, it seems that fragment-

induced acceleration is a feature of the THR-160209 interaction with the M₂ receptor. This acceleration is of particular interest because this phenomenon distinguishes multivalent from monovalent systems (Rao et al., 1998; Smith et al., 2006). It is noteworthy that using the “infinite” dilution protocol, the shape of the [³H]THR-160209 dissociation curve generated in the absence of competing monovalent ligand is biphasic. We hypothesize that this reflects different modes of [³H]THR-160209 binding with the rapid phase representing dissociation of ligand (which was bound to either the orthosteric or allosteric site) and the slower phase representing dissociation of ligand (which was bound simultaneously to both sites). Atropine and other orthosteric ligands had no effect on the dissociation rate of the monovalent ligand [³H]NMS, but atropine did accelerate the slower dissociation phase of [³H]THR-160209, consistent with multivalent interaction. A high concentration of monovalent ligand is required for fragment-induced acceleration because the monovalent ligand must compete with high effective local concentrations of the multivalent ligand (Kramer and Karpen, 1998; Smith et al., 2006). Thus, in the present study, high concentrations of atropine or obidoxime were required to accelerate the dissociation rate of [³H]THR-160209. Likewise, the monovalent ligands 3-BHP and 4-ABP accelerated the “off” rate of [³H]THR-160209, providing strong evidence

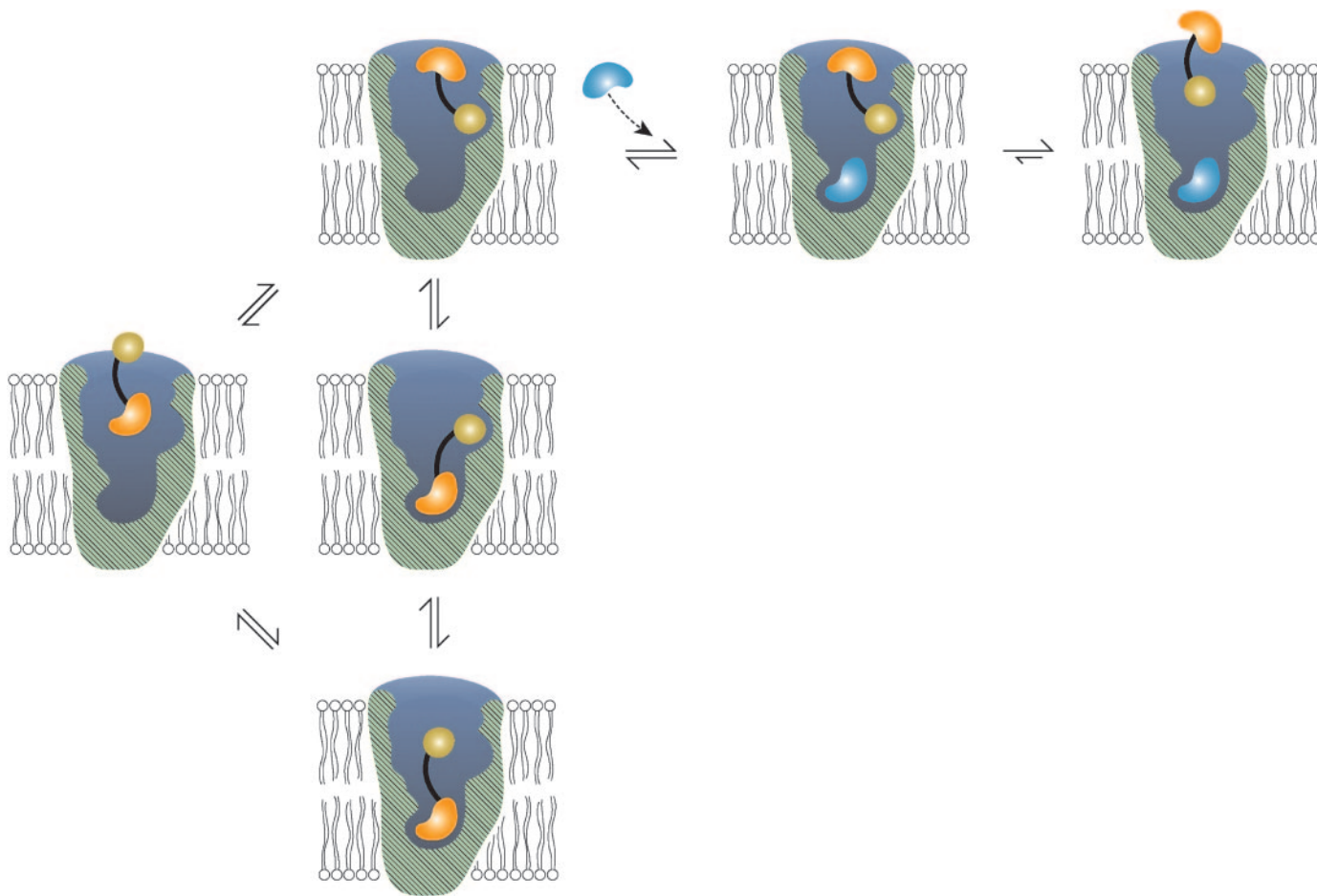


Fig. 10. Model describing multivalent ligand dissociation from a target receptor. A multivalent ligand (orange and yellow object) may bind to its target more tightly in part because one bound pharmacophore maintains a high local concentration of the other pharmacophore, even if the second is unbound. A competing monovalent ligand (blue object) can accelerate the dissociation of the multivalent ligand by occupying one binding site. This model illustrates our proposed “step-wise” mode of multivalent ligand dissociation from a target receptor.

that THRX-160209 can simultaneously bind two distinct sites on the muscarinic receptor.

Although we have no direct evidence demonstrating THRX-160209 binding to the orthosteric site, we can infer from our data an interaction between the ligand and the site that binds ACh. Thus, THRX-160209 exhibited 2500-fold lower affinity for [³H]NMS-occupied receptors than for unoccupied receptors. That is, THRX-160209 cannot bind as tightly to the receptor when an orthosteric ligand is bound, suggesting that THRX-160209 binds at least in part to the orthosteric site putatively bound by [³H]NMS. Further evidence is provided by the acceleration of "off" rates in the presence of orthosteric ligands (atropine and ACh). These data suggest that [³H]THRX-160209 is competitively displaced from one of its binding sites—the orthosteric site on the M₂ receptor. The acceleration by orthosteric ligands can be compared with acceleration by obidoxime, which was shown to compete for the same M₂ allosteric site as THRX-160209. It is not possible from the present data set to definitely state the orientation of THRX-160209 binding to the M₂ receptor. However, we can speculate that the retardation of [³H]NMS dissociation by 4-ABP suggests that this portion of THRX-160209 binds to the allosteric site. In contrast, the actions of the 3-BHP portion were more similar to those of an orthosteric ligand, suggesting that this region of THRX-160209 binds to the orthosteric site.

In conclusion, THRX-160209 is an example of a multivalent ligand that simultaneously occupies two distinct sites on a G-protein coupled receptor, specifically the orthosteric site and typical allosteric site of the muscarinic M₂ receptor. This rationally designed multivalent ligand competitively inhibits receptor activation at the ACh binding pocket with marked subtype specificity as a result of interaction with a low subtype homology secondary site. The high-affinity displayed by THRX-160209 is probably the result of a relatively slow, step-wise dissociation of two tethered pharmacophores (Fig. 10). These results demonstrate that multivalent ligand design can lead to enhanced affinity and target specificity. THRX-160209 illustrates the potential of the multivalent approach to provide high-affinity ligands can discriminate between highly homologous proteins.

Acknowledgments

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