Exploration of the Orthosteric/Allosteric Interface in Human M1 Muscarinic Receptors by Bitopic Fluorescent Ligands

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ABSTRACT

Bitopic binding properties apply to a variety of muscarinic compounds that span and simultaneously bind to both the orthosteric and allosteric receptor sites. We provide evidence that fluorescent pirenzepine derivatives, with the M1 antagonist fused to the boron-dipyrromethene [Bodipy (558/568)] fluorophore via spacers of varying lengths, exhibit orthosteric/allosteric binding properties at muscarinic M1 receptors. This behavior was inferred from a combination of functional, radioligand, and fluorescence resonance energy transfer binding experiments performed under equilibrium and kinetic conditions on enhanced green fluorescent protein–fused M1 receptors. Although displaying a common orthosteric component, the fluorescent compounds inherit bitopic properties from a linker-guided positioning of their Bodipy moiety within the M1 allosteric vestibule. Depending on linker length, the fluorophore is allowed to reach neighboring allosteric domains, overlapping or not with the classic gallamine site, but distinct from the allosteric indolocarbazole “WIN” site. Site-directed mutagenesis, as well as molecular modeling and ligand docking studies based on recently solved muscarinic receptor structures, further support the definition of two groups of Bodipy-pirenzepine derivatives exhibiting distinct allosteric binding poses. Thus, the linker may dictate pharmacological outcomes for bitopic molecules that are hardly predictable from the properties of individual orthosteric and allosteric building blocks. Our findings also demonstrate that the fusion of a fluorophore to an orthosteric ligand is not neutral, as it may confer, unless carefully controlled, unexpected properties to the resultant fluorescent tracer. Altogether, this study illustrates the importance of a “multifacet” experimental approach to unravel and validate bitopic ligand binding mechanisms.

Introduction

The five muscarinic cholinergic receptor subtypes (mAChRs; M1–M5) play prominent roles in the central and parasympathetic nervous systems, and are potential therapeutic targets (Eglen, 2012). Decades of research have enriched a wide collection of high-affinity and efficacious molecules competing with the neurotransmitter acetylcholine (ACh) for binding to muscarinic receptors. Unfortunately, most of these agonists and antagonists lack true subtype selectivity, a drawback related to the conservation of the orthosteric binding site across mAChRs. Thus, drug discovery programs shifted to the development of allosteric compounds targeting topographically distinct and less conserved binding sites (May et al., 2007; De Amici et al., 2010).

A number of allosteric modulators and agonists now provide useful tools to finely tune orthosteric ligand affinity and efficacy at muscarinic receptors, to select subsets of signaling or regulatory pathways, and to mediate subtype-selective functional outcomes (Birdsall and Lazareno, 2005; Gregory et al., 2007; Conn et al., 2009; Digby et al., 2010). The molecular interpretation of functional versatility and its exploitation in drug discovery are still very challenging, as one has to consider the ability of every compound, or combination of allosteric partners, to stabilize discrete conformational and functional receptor states within a given cell context (Deupi and Kobilka, 2010; Kenakin and Miller, 2010; Gregory et al., 2012). The recent resolution of M2 (Haga et al., 2012) and M3 (Kruse et al., 2012) receptor structures confirmed the close proximity of allosteric and orthosteric sites and the possibility for orthosteric antagonists to pause at an allosteric site while associating with and dissociating from the receptors (Jakubik et al., 2000; Redka et al., 2008; Ilien et al., 2009). Such features may explain the difficulty in defining the molecular

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ABBREVIATIONS: ACh, acetylcholine; Bo, Bodipy; Bodipy, boron-dipyrromethene; BoPz, Bodipy pirenzepine; ecl, extracellular loop; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; hM1, human M1 muscarinic receptor; FRET, fluorescence resonance energy transfer; mAChR, muscarinic cholinergic receptor; NMS, N-methylscopolamine; PDB, Protein Data Bank; QNB, 3-quinuclidinyl benzilate; TM, transmembrane domain; WIN 51,708, 17-β-hydroxy-17-α-ethynyl-5-α-androstan-3,2-b[pyrimido][1,2-a]benzimidazole.

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mechanisms (allosteric transition versus steric hindrance) underlying the effects of muscarinic allosteric compounds (Proksa and Tucek, 1994; Canals et al., 2012) and the persistent observation of hybrid properties for several molecules. Indeed, orthosteric antagonists, such as pirenzepine derivatives (Tränkle et al., 1998; Ellis and Seidenberg, 1999; Lanzafame et al., 2001) and methoctramine (Giraldo et al., 1988), as well as “allosteric” agonists such as McN-A-343 (Valant et al., 2008), xanomeline (Jakubik et al., 2002), and AC-42 derivatives (Avlani et al., 2010; Gregory et al., 2010; Daval et al., 2012) most probably inherit subtype-dependent affinity or efficacy patterns from mixed orthosteric/allosteric binding properties.

Hybrid, dualsteric, or bitopic compounds form an emerging class of molecules aimed at major improvements in affinity, efficacy, and functional selectivity through the combination (via a spacer arm) of orthosteric and allosteric building blocks (Mohr et al., 2010; Valant et al., 2012). Proof of concept of the bitopic strategy has been done by dissecting the partial muscarinic agonist McN-A-343 into an orthosteric and an allosteric fragment whose combination allowed the recapitulation of the pharmacology of the parent molecule (Valant et al., 2008). Pioneering de novo design of dualsteric molecules has been accomplished on the M2 receptor, too, with the description of molecules combining a nonselective orthosteric antagonist (Steinfeld et al., 2007) or agonist (Disingrini et al., 2006; Antony et al., 2009; Bock et al., 2012) with M2-prefering allosteric moieties. Binding and functional experiments indicated that such hybrids may indeed display better subtype selectivity, improved affinity (agonist hybrids), or stimulus-biased (agonist hybrids) signaling properties.

We previously reported on the synthesis and similar nancellar affinity at muscarinic M1 receptors of a family (BoPz) of Bodipy pirenzepine derivatives, differing in length (10–22 atoms) of the linker connecting the Bodipy (558/568) fluorophore to the M1-selective antagonist (Tahtaoui et al., 2004). Fluorescence resonance energy transfer (FRET) studies performed on enhanced green fluorescent protein (EGFP)–fused M1 muscarinic receptors suggested the existence of two groups of molecules on the basis of linker length, donor (EGFP)–acceptor (Bodipy) distance in ligand-receptor complexes, and allosteric modulation. We proposed a bitopic binding mode for the Bo15–22Pz series of ligands, with their fluorophore and pirenzepine moieties exploring allosteric and orthosteric receptor domains, respectively.

In the present work, we undertook a systematic characterization of all BoPz members to gain a better knowledge on the orthosteric and allosteric partners (receptor sites and ligand moieties) involved in their interaction with M1 receptors. To validate true bitopic binding mechanisms, these ligands were taken as “competitors” (functional and [3H]NMS methscopolamine [NMS] assays) and as fluorescent tracers (FRET studies) and compared for their behavior under equilibrium and kinetic conditions. Topographical information on the receptor domains recruited by these fluorescent derivatives was essentially from the examination of M1W400 (7.35) and M1W405 (7.40) receptor mutants and molecular modeling.

**Materials and Methods**

**Materials.** [3H]NMS (65 Ci/μmol) was from PerkinElmer Life and Analytical Sciences (Courtaboeuf, France). Atropine sulfate, NMS bromide, pirenzepine dihydrochloride, acetylcholine chloride, carbamoyl chloride, gallamine triethiodide, brucine sulfate, staurosporine, and WIN 51,708 [17-β-hydroxy-17-α-ethynyl-5-α-androstan-3,2-b]pyrido[1,2-a]benzimidazole hydrate were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bodipy (558/568) pirenzepine hydrochloride [referred to as Bo10Pz in this paper] and Indo-1 acetoxyethyl ester were from Molecular Probes (Invitrogen, Cergy Pontoise, France).

**Chemistry.** Bo12Pz, Bo15Pz, and Bo22Pz fluorescent pirenzepine derivatives, together with the Bo5 compound, were synthesized as reported (Tahtaoui et al., 2004). Their purity was checked by analytical reversed-phase-high-performance liquid chromatography. Absorbance and fluorescence spectroscopic properties were examined using a Varian Cary 1E (SpectraLab Scientific, Markham, ON, Canada) photometer and a Spex Fluorolog 2 (Horiba Jobin-Yvon, Longjumeau, France) fluorimeter. The chemical structures of fluorescent derivatives and of other compounds used throughout this study are presented in Fig. 1.

**EGFP-Fused Muscarinic M1 Receptors, Mutagenesis, and Cell Expression.** The human M1 muscarinic receptor (hM1) with a truncated N terminus (deletion of 17 amino acids) fused to EGFP is the construct of reference, defined as EGFP(Δ17)hM1 (Ilien et al., 2003). The corresponding cDNA served as a template in polymerase chain reaction to get single-point-mutated M1 receptors at tryptophan residues 400 and 405. Using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands), Ala or Phe residues were inserted in place of Trp by addition of synthetic oligonucleotide primers containing the required triplet changes. Following subcloning into the pCEP4 expression vector (Invitrogen) and extraction of the plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France), the mutations were finally confirmed by sequencing.

Human embryonic kidney (HEK) cells were transfected with wild-type [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W400A/P) hM1 receptors. The corresponding cDNA served as a template in polymerase chain reaction to get single-point-mutated M1 receptors at tryptophan residues 400 and 405. Using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands), Ala or Phe residues were inserted in place of Trp by addition of synthetic oligonucleotide primers containing the required triplet changes. Following subcloning into the pCEP4 expression vector (Invitrogen) and extraction of the plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France), the mutations were finally confirmed by sequencing.
EGFP-W405Af) plasmids by calcium phosphate precipitation, and selected with 2 mg/ml hygromycin-B (Eurobio, Courtabeouf, France) for stable receptor expression.

**Cell Culture.** IMR 32 human neuroblastoma cells and HEK293 cells (American Type Culture Collection, Manassas, VA; LGC Promochem, Molshaim, France) were grown at 37°C in a 5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium and in minimal essential medium (with 2 mM glutamine), respectively. Both media (Gibco/Fisher Scientific, Illkirch, France) were supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Nearly confluent cells were harvested by mild 0.05% trypsin/0.02% EDTA (w/v) or Versene (phosphate-buffered saline with 5 mM EDTA) treatment, counted, and washed twice by centrifugation before final resuspension in physiologic Hepes buffer (10 mM Hepes, 0.4 mM NaH₂PO₄, 137.5 mM NaCl, 1.25 mM MgCl₂, 1.25 mM CaCl₂, 6 mM KCl, 10 mM glucose, and 1 mg/ml bovine serum albumin, pH 7.4). Cell suspensions were kept on ice until further use in binding assays.

**Calcium Mobilization Assays.** Adherent cells (either nontransfected or stably expressing various M1 receptor constructs) were loaded with 5 μM Indo-1 acetoxymethyl ester for 40 minutes at 37°C, harvested by rapid trypsin/EDTA treatment, washed, and finally suspended (10⁶ cells/ml) in Hepes buffer. Agonist-evoked increases in intracellular calcium were recorded over time at 20°C through fluorescence emission at 400 nm (excitation at 338 nm). Cells were preincubated for 10 minutes with antagonists before agonist challenge. Peak amplitudes were normalized to basal and maximal (cells permeabilized with 20 μM digitonin) fluorescence levels and expressed as a percentage of the maximal control response.

**Radioligand Binding Assays.** [³H]NMS binding assays were conducted in Hepes buffer using 30,000 (EGFP-W405F), 60,000 (EGFP-A17hM1, EGFP-W400F, and EGFP-W405A), or 100,000 (EGFP-W400A) HEK cells per assay. Equilibrium binding experiments proceeded in the presence of 0.1 nM [³H]NMS (unless otherwise stated) and of various concentrations of unlabeled ligands, in a 1-ml final volume. Incubation lasted for 22 hours at 20°C and was terminated by filtration through Whatman GF/B glass fiber filters (presoaked in 0.2% polyethyleneimine) positioned on a Brandel cell harvester (Alpha Biotech, London, UK). Tubes and filters were rinsed three times with 3-ml ice-cold 25 mM Tris-HCl buffer (pH 7.4). Radioactivity on filters was counted by liquid scintillation spectrometry. Specific [³H]NMS binding was defined as the difference between total and nonspecific binding measured in the presence of 4 μM atropine.

Off-rate assays were performed according to a “three time point dilution and blocking” procedure (adapted from Lazareno and Birdsell, 1995). A 100-fold concentrated cell suspension was preincubated for 30 minutes at 25°C with 3 nM [³H]NMS, in the absence (total binding) or presence (nonspecific binding) of 10 μM atropine, then kept on ice. Ten-microliter aliquots of both prelabeled cell batches were added to a series of test tubes filled with 1 ml of Hepes buffer (at 25°C) containing 10 μM atropine alone or combined with other drugs at various concentrations. At three given dissociation time points, the reaction was stopped by rapid filtration as described earlier. Specific [³H]NMS binding at equilibrium was assessed in parallel through direct filtration of undiluted 10-μl labeled cell samples and radioactivity counting.

**FRET Monitoring of BoPz Binding to EGFP-Fused M1 Receptors.** Fluorescence data were acquired from living cells suspended in Hepes buffer (1–3 10⁶ cells/ml, depending on receptor expression) and kept at 20°C in a thermostated quartz cuvette under magnetic stirring. The interaction of BoPz tracers with chimeric M1 receptors was followed as a variation in cell fluorescence intensity (recorded at 510 nm) due to resonance energy transfer from the EGFP donor (excited at 470 nm) toward the ligand Bodipy acceptor species (Ilien et al., 2003, 2009).

Equilibrium binding studies, which require the parallel treatment of large series of samples, were conducted in a manner very similar to radioligand binding experiments. Cells were incubated in test tubes for 22 hours at 20°C with the fluorescent tracer and unlabeled drugs (1-ml final volume). As FRET assays do not require separation of free from bound tracer, binding levels were quantified through simple fluorescence readouts at 510 nm (excitation at 470 nm).

Association and dissociation kinetics were followed in real time as described (Tahtaoui et al., 2004; Ilien et al., 2009). Briefly, recordings of fluorescence intensity started with the addition of 4 μl of fluorescent ligand (250-fold–concentrated dimethylsulfoxide stock) to the 1-ml cell suspension (preincubated or not at 20°C with various compounds) and lasted until binding equilibrium was confirmed by visual inspection of the stability of the fluorescence trace. Thereafter, dissociation of ligand-receptor complexes was initiated by adding 10 μM atropine (or any combination of unlabeled drugs; “isotopic” dilution protocol) to the incubation medium, and fluorescence was recorded over time until full recovery. An alternate “volumetric” dilution protocol was applied as follows: the 1-ml incubation was cooled down at 4°C and centrifuged for 20 seconds at 1500g, and the supernatant was carefully removed. The tiny cell pellet was rapidly resuspended in 1 ml of Hepes buffer (20°C; supplemented or not with 10 μM atropine) and immediately monitored for fluorescence recovery over time.

Specific binding of BoPz derivatives to EGFP-fused hM1 receptors was defined, under equilibrium binding conditions, as the difference in fluorescence intensity of cells incubated with the tracer, in the absence or presence of a saturating concentration of atropine. Under kinetic conditions, specific binding was derived from the amplitude of tracer-induced fluorescence extinction (association step) or of atropine-promoted fluorescence recovery (dissociation step). Theoretically, both determinations should define an identical FRET signal amplitude. When using high tracer concentrations or colored compounds, samples were systematically checked (and corrected) for possible interferences with cell fluorescence measurements.

**Homology Modeling.** The structure of the human muscarinic M1 receptor (UNIPROT database identifier: ACMI_HUMAN) was obtained by homology to the rat muscarinic M3 receptor [Protein Data Bank (PDB) ID 4daj, chain A]. Sequence alignment and homology modeling were performed using the Molecular Operating Environment MOE 2011 program (Chemical Computing Group Inc., Montreal, QC, Canada). The disulfide bonds were automatically detected. For each receptor, a maximum of 10 models were constructed independently for the main chain, and a single set of side chains was then built for each main chain model. The models were refined by tethered minimization and ranked according to Coulomb and generalized Born interaction energies. The top-ranked model was further refined by energy minimization. The AMBER99 force field was used for all energy calculations.

**Docking of Ligands into the M1 Receptor.** The three-dimensional structures of brucine, gallamine, pirenzepine, and its fluorescent BoPz derivatives were generated using Corina 3.1 (Molecular Network GmbH, Erlangen, Germany). An ensemble of 63 conformers was generated for pirenzepine using Omega 2.4.3 (OpenEye, Inc., Santa Fe, CA). Each conformer was compared with the tiotropium-bound M3 structure (PDB ID 4daj, chain A) using ROCS 3.2.1 (OpenEye, Inc.). The best matched conformer, which yielded aCombo score of 1.27, was pasted into the M1 model, and the resulting complex was refined by energy minimization using MOE.

Brucine, gallamine, and BoPz compounds were individually docked into the M1 receptor using GOLD 5.1 (Cambridge Crystallographic Data Centre, Cambridge, UK). The boron atom of Bodipy was replaced with a carbon atom, because boron is not supported by the docking program. The M1 binding pocket was defined as all protein residues located in a 16 Å-radius sphere centered on the C2 atom of Tyr404. Docking of brucine and gallamine was performed with the default settings of the program. Docking of each BoPz derivative was performed with the parameters of the genetic algorithm preset for 10,000 operations, and was biased with constraints on the pirenzepine moiety (constraints on donors, acceptors, and shape, with weight set to 10) and on the linker (hydrophobic atoms preferentially placed in...
a 2.5 Å sphere centered on the mass center defined by the 6-membered rings of Tyr82, Trp101, and Tyr404. BoPz does not reproducing the reference binding mode of pirenzepine were filtered using the in-house Interaction Fingerprint program (Marcou and Rognan, 2007). Bodipy positioning in the M1 T7 transmembrane domain (TTM) core was then explored by manually modifying the rotatable bonds in the linker using Sybyl-X1.3 (Tripos, Inc., St. Louis, MO).

Data Analyses. Nonlinear regression analyses of functional and binding data were performed using Kaleidagraph 4.0 (Synergy Software, Reading, PA).

Occupancy curves were generated by plotting the signal amplitude, Y, as a function of agonist or tracer concentration, X, and analyzed according to the empirical Hill equation:

\[ Y = Y_{\text{max}} / \left( 1 + \left( L_{0.5}/[X]^{n_H} \right) \right) \]  

where \( Y_{\text{max}} \) is the maximal response (\( E_{\text{max}} \)) for an agonist, the maximal number (\( B_{\text{max}} \)) of binding sites for a radioactive tracer, or the maximal amplitude of fluorescence extinction (\( F_{\text{max}} \)) at saturating concentrations of a fluorescent tracer; \( L_{0.5} \) is the agonist concentration (EC50) leading to half-maximal response or the apparent equilibrium dissociation constant (Kd) for tracer binding; and \( n_H \) is the midpoint slope.

Antagonist-induced rightward shifts of occupancy curves were checked for competitive interaction using the Lew and Angus (1995) equation:

\[ pL_{0.5} = - \log([B] + \alpha_{\text{t}} \times pK) - \log[c] \]  

where \( pL_{0.5} \) is the negative logarithm of agonist EC50,obs or tracer Ka, \( K_{\text{app}} \) measured at each antagonist concentration \( [B] \), \( pK \) and log c are fitting constants, and \( s \) is the Schild slope.

Curve shifts were also analyzed according to the Arunlakshana and Schild (1959) equation:

\[ \log(DR-1) = s \times \log[B] - \log[K] \]  

where dose-ratios (DR) (EC50,obs/EC50,control or Ka/Kd,control) serve to quantify midpoint shifts at each antagonist concentration \( [B] \). K is an estimate of antagonist potency, and \( s \) is the Schild slope. If not significantly deviating from unity, the \( s \) value was constrained as such in eqs. 2 and 3, and K represented the affinity constant Kd for a competitive antagonist. Otherwise, a \( pA_2 \) value was estimated via the \( pA_2 = pK'K \) relationship. Functional antagonism associated with depression of maximal responses was also analyzed, as reported (Christopoulos et al., 1999), using eqs definitive agonist EC50 (leading to a constant level of response equal to 25% of the maximal control response) as the dependent variable in eqs. 2 or 3.

Data from competition-type binding experiments were expressed as \( B/B_0 \) ratios, with \( B_0 \) and \( B \) referring to specific tracer binding at equilibrium, in the absence and presence of an unlabeled competitor, respectively. Fractional receptor occupancy \( B/B_0 \) was plotted against the concentration of competitor \( [X] \) and analyzed using the mass-action equation:

\[ B/B_0 = \text{Bottom} + \left( \left( \text{Top} - \text{Bottom} \right) / \left( 1 + \left( IC_{50}/[X] \right)^{n_H} \right) \right) \]  

where “Top” and “Bottom” parameters refer, respectively, to the upper and lower plateau values of the curve; \( IC_{50} \) denotes the \( X \) value at the inflection point; and \( n_H \) is the slope factor.

\( IC_{50} \) values of compounds that led to “full displacement” at high concentrations (bottom and slope values nonsignificantly different from 0 and 1, respectively) whatever the tracer concentration, were converted into equilibrium dissociation constants Kd using the Cheng and Prusoff relationship for a competitive interaction. In case of submaximal inhibition (bottom > 0; \( B/B_0 \approx 1 \)) or binding potentiation (bottom = 1; \( B/B_0 \geq 1 \)), data were fitted to the allosteric ternary complex model (Ehliert 1988; Lazareno and Birdsall, 1995):

\[ B/B_0 = (L + K_0) / (L + K_0 + \left( 1 + [X]/(K_a) / (1 + [X]/(\alpha \times K_a)) \right) \]  

where \( [L] \) and \( [X] \) are the concentrations of tracer and allosteric agent, respectively. \( K_0 \) and \( K_a \) denote the equilibrium dissociation constants of the tracer and the alloster at the free receptor, respectively. The cooperativity factor \( \alpha \) denotes the magnitude by which the equilibrium dissociation constants of either ligand to its site is modified (\( \alpha > 1 \): negative cooperativity; \( \alpha < 1 \): positive cooperativity) by the concomitant presence of the other ligand. In case of nearly neutral cooperativity (\( \alpha \) close to 1), an allosteric ligand weakly impacts tracer binding at equilibrium, and curve fitting using eq. 5 does not work. Assuming the equivalence of \( \alpha \) to \( K_a \) and EC50,dis, (see eq. 8) parameters to define the affinity of an allosteric agent for tracer-occupied receptors (Rains et al., 2002), \( \alpha \) was replaced by EC50,dis/Kd in eq. 5, and the remaining variable \( K_a \) was obtained from curve fit. The cooperativity factor \( \alpha \) was then derived from the EC50,dis/Kd ratio.

Biphasic binding kinetics, as observed from real-time monitoring of the association of fluorescent ligands to EGEF-fused M1 receptors or of the dissociation of Bo10/Pz from the EGF-P405A mutant, were analyzed by fitting individual traces to a two-exponential model:

\[ p(t) = A_0 + A_1 \times \exp(-k_1 t) + A_2 \times \exp(-k_2 t) \]  

where \( A_1 \) and \( A_2 \) are the amplitudes of fluorescence changes associated with the rapid and slow components, and \( A_0 \) is the fluorescence intensity at infinite time \( t \). Depending on the experimental paradigm, \( k_1 \) and \( k_2 \) denote either apparent association rate constants or off-rate constants measured in the fast and slow events, respectively.

Unless otherwise stated, all dissociation kinetics for [3H]NMS or BoPz tracers from wild-type or mutant receptors, when examined in the absence or presence of allosteric agents, followed a monoexponential time course reaction:

\[ B_t = B_0 \times \exp(-k_{\text{off}} t) \]  

where \( B_0 \) and \( B_t \) represent specific binding at equilibrium and at dissociation time \( t \), respectively, and \( k_{\text{off}} \) denotes the tracer dissociation rate constant.

Concentration-effect curves for the allosteric delay of tracer dissociation were generated by plotting \( k_{\text{off},\text{obs}}/k_{\text{off},\text{control}} \) ratios (i.e., off-rate constants measured in the presence or absence of an allosteric agent) as a function of \( [X] \), the alloster concentration. Fitting to eq. 8:

\[ k_{\text{off},\text{obs}}/k_{\text{off},\text{control}} = \text{Bottom} + \left( 1 - \text{Bottom} \right) / \left( 1 + \left( \text{EC50,dis}/[X] \right)^{n_B} \right) \]  

allowed the determination of the alloster concentration EC50,dis, leading to half-maximal reduction of control tracer off rate, the amplitude \( E_{\text{max},\text{dis}} \) (1 - bottom value) of the retardation effect, and the slope factor \( n_B \) of the curve. If not significantly different from unity, \( n_B \) was constrained as such, and the EC50,dis parameter was taken as an estimate of the equilibrium affinity constant of the allosteric modulator at tracer-occupied receptors.

## Results

The members of the BoPz family (Fig. 1) display two typical building blocks, i.e., the Pz pharmacophore and the Bo (558/568) fluorophore, connected via spacers of varying length (10–22 atoms) and nature (isopropylid or polyethylene glycol type). The Bo(5) compound, with a short aliphatic chain, lacks the pirenzepine moiety.

Along with their first description (Tahtaoui et al., 2004), BoPz derivatives have been suspected to divide into two separate Bo(10,12)/Pz and Bo(15,22)/Pz groups. To further explore the properties of all these ligands, a number of
experiments have been conducted and will be illustrated with select members of each putative group.

**Functional Antagonism by BoPz Derivatives of M1-Mediated Calcium Mobilization.** We first examined the ability of EGFPΔ17hM1-expressing HEK cells to adequately report on allosteric modulation of M1-mediated responses to ACh. Brucine and gallamine were taken as compounds of reference (Fig. 1), as they are known to positively and negatively tune, respectively, ACh affinity and potency at M1 receptors (Birdsall et al., 1999; Birdsall and Lazareno, 2005). The presence of endogenous muscarinic M3 receptors in HEK cells was found to introduce bias in the modulation of ACh-induced calcium signals in EGFPΔ17hM1-expressing HEK cells (Supplemental Fig. 1). In contrast, IMR 32 neuroblastoma cells, which are deprived of M3 sites and elicit a clear M1-mediated calcium response to muscarinic agonists (Heikkilä et al., 1991), allowed the observation of brucine and gallamine properties in agreement with their modulatory roles in M1 receptor function. Therefore, IMR 32 cells were selected as a useful companion system to EGFPΔ17hM1 cells to examine, from a functional point of view, the nature of Bo(10)Pz, Bo(15)Pz, and pirenzepine interaction with M1 receptors.

As shown in Fig. 2, increasing concentrations (nanomolar to micromolar range) of all three compounds promotes a dextral shift of dose-response curves together with a profound depression in agonist maximal response, both in EGFPΔ17hM1 (top panel) and in IMR 32 (lower panel) cells. Often taken as a hallmark for noncompetitive inhibitors, insurmountable antagonism for orthosteric antagonists is widespread across a range of receptor systems and assays (Kenakin et al., 2006), including pirenzepine effects on muscarinic responses in various cell lines (Christopoulos et al., 1999 and references therein). A common explanation is to consider that a slowly dissociating antagonist, the agonist, and the receptor cannot come to proper equilibrium in fast responding systems, thereby limiting maximal response amplitude. Such kinetic artifacts most probably apply to pirenzepine, Bo(10)Pz, and Bo(15)Pz inhibition of transient calcium responses in both cell lines. Indeed, they slowly dissociate from M1 receptors with off-rate constants (sec$^{-1} \times 10^{-4}$) close to 5 for pirenzepine (Potter et al., 1989; Mohr and Trankle, 1994; Christopoulos et al., 1999) and in the range of 5–15 for BoPz derivatives (Table 5). One should also add that BoPz compounds, at the lowest concentrations which were tested, probably did not reach true equilibrium when preincubated with cells for 10 minutes.

Potency estimates for insurmountable antagonists can be obtained with no prior knowledge of molecular mechanism through pA2 measurements (Kenakin et al., 2006). Lew and Angus (1995) as well as Arunlakshana and Schild (1959) analyses (Fig. 2, right) favored the hypothesis of a competitive interplay of the antagonists with ACh (slope factors not significantly different from 1). The pA2 values for Bo(10)Pz (7.98 ± 0.29 and 8.15 ± 0.22), Bo(15)Pz (8.10 ± 0.21 and 7.95 ± 0.23), and pirenzepine (7.89 ± 0.10 and 7.77 ± 0.25) antagonism in EGFPΔ17hM1 and IMR 32 cells, respectively, were consistent with their binding affinity constants determined from FRET and [$\text{H}$]NMS studies (Tables 1 and 2). Regression analyses using equieffective agonist concentrations (EC$_{25}$ values) instead of standard EC$_{50}$ values provided similar potency estimates (not shown). Thus, these functional experiments aimed
at defining the competitive or noncompetitive interplay of BoPz antagonists with ACh remained nondecisive.

**FRET Studies: Kinetic Insights into BoPz Binding at EGFP(Δ17)hM1 Receptors.** Given the ideal acceptor property of the Bodipy (558/568) fluorophore for energy transfer from excited EGFP, BoPz compounds have proven valuable FRET probes to dissect ligand binding mechanisms at EGFP(Δ17)hM1 receptors (Ilien et al., 2003; 2009; Tahtaoui et al., 2004). Figure 3 depicts real-time recordings of association and dissociation processes for BoPz tracers and the impact of allosteric modulators (brucine and gallamine) on their kinetics. Top and bottom panels refer to BoPz probes with short and long linkers, respectively.

Preincubation of EGFP(Δ17)hM1 cells with increasing concentrations of brucine (Fig. 3A) led to a dose-dependent slowing down of Bo(12)Pz association (kapp values for the fast binding step vary from 0.034, 0.016 to 0.0096 seconds⁻¹ in the absence and presence of 100 or 500 μM brucine, respectively). Interestingly, brucine slightly increased the amplitude of EGFP fluorescence extinction at Bo(12)Pz binding equilibrium. Such a small potentiation might be related to the use of an almost saturating tracer concentration (200 nM). Brucine modulated Bo(10)Pz binding very similarly, whereas gallamine reduced both the association rate and the amplitude of Bo(10)Pz and Bo(12)Pz binding at equilibrium (not shown).

As shown in Fig. 3B, and in agreement with previous findings on Bo(10)Pz binding properties (Tahtaoui et al., 2004), brucine (in the presence of a saturating concentration of atropine) induced a profound deceleration of Bo(12)Pz dissociation. Brucine alone (up to 2.5 mM) did not promote any dissociation of ligand-receptor complexes (not shown). Brucine had a greater impact on dissociation than on association kinetics (at 100 μM, on and off rates were reduced by 2- and 5-fold, respectively), another indication in favor of a positive modulation of Bo(12)Pz affinity. Bo(10)Pz and Bo(12)Pz dissociation from EGFP(Δ17)hM1 receptors were dose dependently retarded by brucine (Fig. 3C) and by gallamine (Fig. 3D), with half-maximal on and off rates being defined the EC50,diss values reported in Table 1.

Identical experimental conditions were applied to the second series of fluorescent derivatives. Bo(15)Pz association rate (kapp values for the fast binding step are 0.040, 0.011, or 0.004 seconds⁻¹ in the absence and presence of 100 or 500 μM brucine, respectively) and binding amplitude at equilibrium (Fig. 3E) were both reduced by brucine, indicating either a competitive or a negatively cooperative mode of interaction. The first hypothesis was privileged as the dissociation of Bo(15)Pz-receptor complexes (Fig. 3F), and Bo(22)Pz ones (not shown), followed superimposable kinetics when using saturating concentrations of atropine or brucine, alone or in combination, to prevent tracer reassociation.

Control Bo(10)Pz and Bo(22)Pz off-rate constants (sec⁻¹ 10⁻⁶; mean values for two separate determinations) did not significantly vary whether determined using the classic isotopic [Bo(10)Pz: 6 ± 1; Bo(22)Pz: 14 ± 1] or the volumetric [Bo(10)Pz: 5 ± 1; Bo(22)Pz: 13 ± 1; plus or minus atropine] dilution protocol. Finally, the possibility for BoPz tracers to recruit the indolocarbazole WIN allosteric site (Lazareno et al., 2000, 2002) was ruled out, as staurosporine (10 μM) and WIN 51,708 (100 μM) slowed down, although to various extents, Bo(10,12)Pz and Bo(15,22)Pz dissociation rates (not shown).

**FRET Studies: Equilibrium Binding Properties of BoPz Derivatives.** To further explore the origin of their distinct behavior, BoPz ligands were examined for their equilibrium binding properties under FRET conditions. EGFP(Δ17)hM1 cells were incubated with the fluorescent tracers in physiologic buffer, at 20°C to minimize receptor and ligand internalization, and for 22 hours to ensure equilibrium even in the presence of allosteric ligands (Lazareno and Birdsal, 1995). Saturation studies provided very similar equilibrium dissociation constants for all tracers (Table 1), as previously reported (Tahtaoui et al., 2004).

Competition-type experiments were undertaken using typical orthosteric (atropine and pirenzepine) and allosteric (gallamine and brucine) muscarinic receptor ligands (Fig. 4A). Atropine and pirenzepine fully displaced Bo(12)Pz binding according to a competitive mode of interaction at the orthosteric receptor site. Submaximal inhibition by gallamine was consistent with saturable negative binding cooperativity. Brucine clearly potentiated Bo(12)Pz binding (taken here at a concentration below its Kd value). Analyses according to the allosteric ternary complex model (Ehrlert, 1988) yielded estimates of their equilibrium dissociation constant Kd for free EGFP(Δ17)hM1 receptors and of the cooperativity factor α as a measure of their impact on Bo(12)Pz affinity (Table 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>Tracer</th>
<th>pKd, Pz</th>
<th>pEC50_diss, %</th>
<th>pKd, Pz</th>
<th>pEC50_diss, %</th>
<th>pKd, Pz</th>
<th>pKd, Pz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bo(10)Pz</td>
<td>7.91 ± 0.04</td>
<td>6.77 ± 0.01</td>
<td>7.97 ± 0.01</td>
<td>7.92 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td>8.89 ± 0.06</td>
<td>9.02 ± 0.04</td>
<td>9.10 ± 0.03</td>
<td>8.92 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.70 ± 0.06</td>
<td>7.96 ± 0.06</td>
<td>8.25 ± 0.04</td>
<td>7.96 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallamine</td>
<td>4.79 ± 0.03</td>
<td>4.82 ± 0.05</td>
<td>3.47 ± 0.04</td>
<td>4.68 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucine</td>
<td>-1.04 ± 0.06</td>
<td>-1.09 ± 0.07</td>
<td>(92 ± 5)</td>
<td>4.68 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>4.25 ± 0.05</td>
<td>4.32 ± 0.08</td>
<td>5.04 ± 0.04</td>
<td>4.27 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20 ± 0.03^2</td>
<td>0.68 ± 0.05^2</td>
<td>(94 ± 2)</td>
<td>4.21 ± 0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α Values are from Ilien et al., 2003.
β Cooperativity factor α (negative logarithm).
γ Because of nearly neutral cooperativity, EC50_diss was used for curve fitting according to eq. 5 as described in Data Analyses.

Data Analyses; Materials and Methods; Data Analyses.
When using Bo(15)Pz as the tracer (Fig. 4B), all compounds, including brucine and gallamine, displayed a competitive-like binding behavior. Bo(22)Pz, again, shared Bo(15)Pz properties. Figure 4C illustrates Bo(22)Pz saturation experiments performed in the absence or presence of brucine (50, 200, and 500 \text{mM}). Brucine promoted a homogenous dextral shift of occupancy curves, with a decrease in Bo(22)Pz apparent affinity (\(K_{\text{app}}\) values) which was submitted to Lew and Angus analysis (Fig. 4C inset). With a Schild slope factor not significantly different from 1 (1.03 \pm 0.04), the hypothesis for a competitive interplay between brucine and Bo(22)Pz binding was clearly privileged. Moreover, the affinity constant for brucine (40.7 \pm 2.9 \text{mM}) determined here was comparable to that (44.9 \pm 6.1 \text{mM}) defined from similar saturation experiments performed with Bo(15)Pz, and was in overall agreement with \(K_x\) and \(K_i\) values derived from competition experiments (Table 1).

**TABLE 2**

Summary of drug binding parameters at EGFP(\(\Delta 17\))hM1 receptors as assessed from equilibrium and off-rate \([3H]\)NMS binding assays

| Affinity constants (\(\log M\); mean \pm S.E. values for 3–8 independent determinations) are from analyses of saturation (\(K_d\), eq. 1), competition (\(K_c\), eq. 4; \(K_x\) and \(a\), eq. 5), and \([3H]\)NMS off-rate (\(EC_{50,\text{diss}}\), \(E_{\text{max,\text{diss}}}\), eq. 8) experiments (Materials and Methods; Data Analyses).

<table>
<thead>
<tr>
<th>(pK_d)</th>
<th>(pK_i)</th>
<th>(pK_x)</th>
<th>(a)</th>
<th>(EC_{50,\text{diss}})</th>
<th>(E_{\text{max,\text{diss}}}) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>10.02 \pm 0.03</td>
<td>9.96 \pm 0.04</td>
<td>9.19 \pm 0.03</td>
<td>4.03 \pm 0.03</td>
<td>9.96 \pm 0.04</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.19 \pm 0.03</td>
<td>\n</td>
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<td>\n</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8.03 \pm 0.08</td>
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<td>\n</td>
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<td>\n</td>
</tr>
<tr>
<td>Carbachol</td>
<td>4.89 \pm 0.14</td>
<td>\n</td>
<td>\n</td>
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<td>\n</td>
</tr>
<tr>
<td>Gallamine</td>
<td>5.04 \pm 0.06</td>
<td>-1.12 \pm 0.02</td>
<td>3.94 \pm 0.07</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Brucine</td>
<td>4.59 \pm 0.02a</td>
<td>-0.10 \pm 0.02</td>
<td>4.49 \pm 0.02</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Bo(10)Pz</td>
<td>6.40 \pm 0.05</td>
<td>-0.40 \pm 0.02</td>
<td>\n</td>
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<td></td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td>8.02 \pm 0.04</td>
<td>\n</td>
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<td></td>
</tr>
<tr>
<td>Bo(15)Pz</td>
<td>7.51 \pm 0.01</td>
<td>\n</td>
<td>\n</td>
<td>\n</td>
<td></td>
</tr>
<tr>
<td>Bo(17)Pz</td>
<td>7.77 \pm 0.05</td>
<td>\n</td>
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<td></td>
</tr>
<tr>
<td>Bo(22)Pz</td>
<td>7.74 \pm 0.07</td>
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</table>

N.A., not applicable because of insufficient data points (Fig. 5D); N.D., not determined.

\(a\) \(EC_{50,\text{diss}}\) was used for curve fitting according to eq. 5 because of nearly neutral cooperativity, as described in Data Analyses.

Fig. 3. Real-time FRET monitoring of BoPz binding to EGFP(\(\Delta 17\))hM1 receptors: impact of brucine and gallamine. Association step (A and E): EGFP(\(\Delta 17\))hM1 cells were preincubated for 10 minutes at 20°C with vehicle (black) or brucine (100 \text{mM}: light gray; 500 \text{mM}: dark gray). Following the addition of Bo(12)Pz (A) or Bo(15)Pz (E) (time 0; 200 \text{nM final concentration), association was monitored over time as a decrease in fluorescence intensity. Amplitudes for fluorescence extinction (percentage) at binding equilibrium in control, 100 and 500 \text{mM}-brucine treated cells were, respectively, 36, 40, and 45 [Bo(12)Pz] and 34, 32, and 27 [Bo(15)Pz]. Dissociation step (B and F): Cells were first equilibrated for 20 minutes at 20°C with 200 \text{nM} Bo(12)Pz or Bo(15)Pz. Bo(12)Pz dissociation (B) started with the addition (time 0) of 5 \text{mM} atropine alone (black) or combined with various concentrations of brucine (3, 10, 30, 100, and 200 \text{mM}). Bo(15)Pz dissociation (F) proceeded similarly except that it was initiated either with 5 \text{mM} atropine alone (black) or combined with 500 \text{mM} brucine (light gray), or with 2 \text{mM} brucine alone (dark gray). Tracer dissociation was monitored as a recovery in fluorescence intensity over time and expressed as a percentage of the fluorescence extinction amplitude at binding equilibrium. All traces followed a monoexponential decay for fluorescence, and dissociation rate constants were derived from fitting to eq. 7. Off-rate values (sec\(^{-1}\) \cdot \text{M}^{-1}) for Bo(15)Pz ranged from 9 (atropine) and 11 (brucine) to 12 (atropine plus brucine). Dose dependency for the allosteric delay of Bo(10)Pz (\(\bullet\)) and Bo(12)Pz (\(\square\)) dissociation (C and D): Off-rate constants for the tracers in the presence of modulator (\(k_{\text{off,obs}}\), relative to control [5.8 and 15.8 \text{sec}^{-1}\] for Bo(10)Pz and Bo(12)Pz, respectively), were plotted as a function of brucine (C) or gallamine (D) concentration. Data are the means \pm S.E. for three independent experiments. Application of eq. 8 allowed the determination of \(EC_{50,\text{diss}}\) and \(E_{\text{max,\text{diss}}}\) parameters listed in Table 1.
Table 1 summarizes drug binding parameters at EGFP(Δ17)hM1 receptors as afforded from FRET experiments using each of the four fluorescent pirenzepine derivatives as an individual tracer. As expected, drug affinity constants at the free receptor do not significantly vary with tracer type or underlying binding mechanism. They are in overall agreement with affinity values reported in the literature ([3H]NMS binding, M1-enriched membrane preparations) for atropine and pirenzepine (Caulfield and Birdsall, 1998), gallamine (Matsui et al., 1995; Lazareno et al., 2000; Fruchart-Gaillard et al., 2006), and brucine (Gharagozloo et al., 1999; Fruchart-Gaillard et al., 2006). The equivalence of EC_{50,dis} and α · K_i parameters for gallamine and brucine suggests that variations in their affinity for free and Bo(10,12)Pz-occupied receptors are driven by binding cooperativity within a ternary allosteric complex. Thus, kinetic and equilibrium FRET studies provided robust information on BoPz binding properties at M1 receptors, with a clear-cut distinction between Bo(10,12)Pz and Bo(15,22)Pz series of ligands regarding the recruitment of the gallamine allosteric site.

An Orthosteric [3H]NMS Point of View on BoPz Binding Properties. A series of experiments were performed using EGFP(Δ17)hM1 cells, [3H]NMS as the radioactive orthosteric tracer, and unchanged incubation conditions (physiologic buffer, 22-hour incubation time at 20°C to ensure equilibrium). Displacement experiments were performed at two [3H]NMS concentrations (1 × K_d and 10 × K_d) to better distinguish competitive from negatively cooperative modes of interaction (Birdsall and Lazareno, 2005; May et al., 2007). Slope factors for all inhibition curves did not statistically differ from 1.

As shown in Fig. 5A, atropine fully displaced specific [3H]NMS binding at low and high tracer concentrations, as expected for competition at the orthosteric receptor site. Gallamine displayed a typical submaximal inhibition (with plateau levels depending on [3H]NMS concentration) consistent with negative binding cooperativity. Brucine was a very partial displacer, featuring almost neutral binding cooperativity with [3H]NMS. Analyses according to the allosteric ternary complex model yielded affinity (K_a) and cooperativity factor (α) estimates (Table 2) in agreement with literature reports for gallamine (Matsui et al., 1995; Lazareno et al., 2000; Fruchart-Gaillard et al., 2006) and brucine (Gharagozloo et al., 1999; Fruchart-Gaillard et al., 2006).

Bo(10)Pz and Bo(15)Pz displayed an apparent competitive mode of interaction (Fig. 5B), as did the two other derivatives. Their K_a values listed in Table 2, closely match K_d values determined under FRET conditions (Table 1). Interesting information (Fig. 5B) was provided by the Bo(5) compound, which lacks the pirenzepine moiety (Fig. 1) and exhibits negative binding cooperativity with [3H]NMS in the low micromolar range. Thus, the Bodipy fluorophore may be able, per se, to interact with an M1 allosteric site.

This prompted us to test Bo(5), Bo(10)Pz, and Bo(15)Pz derivatives (in the presence of 10 μM atropine to prevent [3H]NMS reassociation) for their ability to alter [3H]NMS off rate from EGFP(Δ17)hM1 receptors (Fig. 5C). All kinetics followed a monoeponential time course. As expected for a competitive interplay at the orthosteric site, the addition of atropine or pirenzepine (alone or in combination) led to superimposable dissociation traces (control k_{off} = 0.043 ± 0.001 minutes⁻¹; n = 6). Gallamine and brucine, taken here as references at 200 μM, strongly impacted the [3H]NMS dissociation rate. Bo(5) and Bo(10,15)Pz compounds, at 10 μM (i.e., 25- and 1000-fold their respective equilibrium affinity constants), significantly retarded [3H]NMS dissociation. The possibility for Bo(10)Pz to bind to [3H]NMS—occupied receptors was totally unexpected, as previous FRET (Table 1) and equilibrium [3H]NMS (Table 2) studies privileged a conventional orthosteric nature for this derivative.

Figure 5D depicts the concentration dependence of the retardation effects of the allosteric modulators gallamine and...


Fig. 5. Impact of unlabeled drugs and fluorescent compounds on equilibrium binding and dissociation kinetics of [3H]NMS at EGFP(Δ17)hM1 receptors. (A) Competition experiments were performed at low (dashed lines) or high (solid lines) [3H]NMS concentrations with atropine (○), 90 or 770 pM [3H]NMS, gallamine (□), 90 or 925 pM [3H]NMS, or brucine (■), 140 pM [3H]NMS. Fractional occupancy (B/F) values are the means ± S.E. from three independent experiments. Curve fitting is based on eqs. 4 (atropine) and 5 (gallamine, brucine; Kᵦ parameter for brucine constrained to EC₅₀,off/α). (B) Competition experiments were performed at low (dashed lines) or high (solid lines) [3H]NMS concentrations with Bo(10)Pz (●), 52 or 650 pM [3H]NMS, Bo(15)Pz (▲), 52 or 735 pM [3H]NMS, or Bo(5) (■), 87 pM [3H]NMS. Data from these typical experiments were fitted according to eq. 4 [Bo(10)Pz and Bo(15)Pz] or eq. 5 [Bo(5) derivative]. (C) Dissociation of [3H]NMS from EGFP(Δ17)hM1 receptors was followed at 25°C after addition of 10 pM atropine alone (control; ○) or combined with 10 μM of pirenzipine (▲), Bo(5) (■), Bo(10)Pz (●), or Bo(15)Pz (▲), or with 200 μM gallamine (□) or brucine (■). Data are normalized to Bo₅₀, the specific [3H]NMS binding at equilibrium. Control 0.043 ± 0.001 minutes⁻¹ and observed (kₐff,off) off-rate constants are from data fitting to eq. 7. (D) The concentration-dependent retardation of [3H]NMS dissociation exerted by Bo(5) (■), Bo(10)Pz (●), Bo(15)Pz (▲), brucine (□), or gallamine (○) is presented as normalized kₐff,off/kₐff,off,control ratio plots as a function of drug concentration. Data are the means ± S.E. from 3–4 independent experiments; symbols without error bars are from a single determination. Best fits to eq. 8 are shown for Bo(15)Pz (slope factor constrained to 1), brucine, and gallamine. All derived parameters are listed in Table 2.

Rationale Behind the Selection of M1 W400 and W405 Receptor Mutants. Accumulating evidence supports the bitopic character of the BoPz derivatives: they recognize epitopes within both orthosteric (competitive character when tested against orthosteric ligands in functional and equilibrium binding assays) and allosteric (retardation of [3H]NMS dissociation) sites. There is no doubt that the Bo(15,22)Pz curves were almost superimposable, and the Bo(15)Pz effect (EC₅₀,diss 3.5 μM because of stock and solubility limitations. Nevertheless, their retardation effect on [3H]NMS dissociation manifested a net concentration dependency. Bo(10)Pz and Bo(15)Pz curves were almost superimposable, and the Bo(15)Pz effect (EC₅₀,diss 3.5 ± 0.4 μM; n = 3) was probably partial (E₅₀,diss 72.5 ± 2.5%; n = 3).

Brucine and of the three fluorescent derivatives on [3H]NMS dissociation from EGFP(Δ17)hM1 receptors. In agreement with M1 literature, brucine (Gharagozloo et al., 1999) and gallamine (Matsui et al., 1995) fully inhibited [3H]NMS dissociation, with EC₅₀,diss values close to 30 and 110 μM, respectively, reflecting their affinity for [3H]NMS–occupied receptors (Table 2). Fluorescent derivatives could not be tested at concentrations above 30 μM because of stock and solubility limitations. Moreover, their retardation effect on [3H]NMS dissociation manifested a net concentration dependency. Bo(10)Pz and Bo(15)Pz curves were almost superimposable, and the Bo(15)Pz effect (EC₅₀,diss 3.5 ± 0.4 μM; n = 3) was probably partial (E₅₀,diss 72.5 ± 2.5%; n = 3).

Impact of Mutations on Orthosteric and Allosteric Ligand Binding Properties. The properties of the EGFP(Δ17)hM1 receptor, with W400 or W405 residues replaced by Ala or Phe (to preserve the aromatic character), were examined through [3H]NMS binding experiments. Results are shown in Fig. 6A and summarized in Table 3.

[3H]NMS saturation studies indicated a modest impact of the mutations on receptor expression levels, with maximal 2-fold variations in Bₘₐₓ values (W405F, increase; W404A, decrease) as compared with wild type. High [3H]NMS affinity was preserved, as reported for the M1 W400 mutant (Matsui et al., 1995) and for homologous W400 (M2W422, M5W477) and W405 (M2W427, M5W482) mutations in M2 and M5 receptors (Prilla et al., 2006).

Competition studies pointed to an unchanged atropine affinity, a discrete alteration in pirenzipine affinity related to the suppression of the aromatic nature of the residues (as shown for the M1 W400 mutant; Matsui et al., 1995), and an increase in carbachol affinity for the EGFP-W405 construct. An elevation in carbachol affinity and constitutive activity has already been noticed for this M1 mutant (Matsui et al., 1995; Lu et al., 2001) and interpreted as a consequence of the destabilization of a ground-state intramolecular contact network.

[3H]NMS equilibrium and kinetic studies pointed to a 2-fold reduction in gallamine affinity at free EGFP-W405A/F receptors, and to a slight reduction in its binding cooperativity with NMS. In contrast, the W404A/F mutations led to a more than 10-fold decrease in gallamine affinity at free- and [3H]NMS–occupied receptors, with little impact on binding cooperativity. These data are in agreement with previous reports on gallamine properties at the W404A/F M1 mutants (Matsui et al., 1995) and at the alanine mutants of the homologous W400 and W405 residues in M2 and M5 receptors.
(Prilla et al., 2006). Alterations in brucine modulation were more difficult to ascertain given its neutral cooperativity with [3H]NMS binding. The impact of the two series of mutations was thus best apprehended from dissociation kinetics, which again pointed to the crucial importance of the W400 residue. At variance with gallamine, the affinity of brucine (EC50,diss value) for [3H]NMS–occupied EGFP-W405A/F receptors was slightly but significantly increased, as compared with wild type.

All of these controls confirm that the fusion of EGFP to an hM1 receptor with a truncated N terminus does not modify mutation binding phenotypes and extend available information on the role of the W405 residue in M1 orthosteric and allosteric ligand binding.

**Differential Alterations of BoPz Binding Properties at W400 and W405 M1 Mutants.** The binding affinity profiles of BoPz ligands at EGFP-W400A/F and EGFP-W405A/F receptor mutants were examined through competition (taking [3H]NMS as the tracer) and FRET saturation (taking each BoPz derivative as a fluorescent tracer) studies. As shown in Table 4, Kp and Kd values are in remarkable agreement, with the exception of Bo(12)Pz and Bo(15)Pz affinities at the EGFP-W400F mutant, which differ by a factor of 2–3. Another general observation is that substitution of Trp for Phe is less well tolerated than Ala insertion [Bo(15)Pz –3. Another general observation is that substitution of Trp for Phe is less well tolerated than Ala insertion [Bo(15)Pz –

Figure 6B illustrates the impact of mutations on equilibrium binding properties of the fluorescent derivatives. Bo(10,12)Pz compounds displayed a similar and moderate sensitivity to the mutation of the W405 residue but did not discriminate between EGFP-400A/F mutants and wild-type receptors, at variance with the prominent role of the W400

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**TABLE 3**

| Drug binding properties at the EGFP-A17 hM1 receptor and its mutants as assessed from equilibrium and kinetic [3H]NMS binding (A) or from Table 4 and Supplemental Table 1 (FRET data) (B). |
|------------------|------------------|------------------|
| Drug            | EGFP-W400A       | EGFP-W405F       |
|                 | pKd (M)          | pKd (M)          |
| Atropine        | 10.02 ± 0.03     | 9.70 ± 0.02     |
| Pirenzepine     | 9.00 ± 0.02      | 8.60 ± 0.01     |
| Carbachol       | 4.90 ± 0.08      | 4.56 ± 0.04     |
| Gallamine       | 8.00 ± 0.08      | 7.74 ± 0.03     |
| Brucine         | 4.90 ± 0.08      | 4.56 ± 0.04     |

**Data Analyses**

1. All of these controls confirm that the fusion of EGFP to an hM1 receptor with a truncated N terminus does not modify mutation binding phenotypes and extend available information on the role of the W405 residue in M1 orthosteric and allosteric ligand binding.

2. Another general observation is that substitution of Trp for Phe is less well tolerated than Ala insertion [Bo(15)Pz –3. Another general observation is that substitution of Trp for Phe is less well tolerated than Ala insertion [Bo(15)Pz –

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**Fig. 6.** Mutation-induced changes in the affinities of EGFP-fused hM1 receptors for orthosteric and allosteric ligands and for fluorescent tracers. Histograms represent variations of log-affinity constants ΔpK (or ΔEC50,diss, values marked with an asterisk) of drugs at each mutant, relative to wild type, as inferred from Table 3 ([3H]NMS binding) (A) or from Table 4 and Supplemental Table 1 (FRET data) (B).
residue in Bo(15,22)Pz binding. Bo(15)Pz was actually severely impacted by all mutations (with a 10-fold decrease in affinity at the W405F and W400A mutants). Bo(22)Pz displayed an intermediate profile, with a weaker contribution of the W405 residue (especially when replaced by Ala).

Altogether, these findings provide additional support to the delineation of two subgroups of BoPz ligands with differential sensitivity to W405 (10–12 series) or W400 (15–22 series) mutations. They also reinforce the idea that their Bodipy moiety, depending on linker length, may explore distinct (or partially overlapping) allosteric epitopes, including (15–22 series) or not (10–12 series) the key W400 gallamine residue.

To get deeper insight into the role of the two tryptophan residues, kinetic studies were carried out to compare [3H]NMS and BoPz dissociation rates at the four mutants (Table 5).

[3H]NMS dissociation was slightly retarded in all mutants, with a more significant impact of the W405 substitution. These findings corroborate previous reports on [3H]NMS dissociation from M1 W400A/F (Matsui et al., 1995) and M2 homologous W400A and W405A (Prilla et al., 2006) mutants. As none of these mutations have relevant effects on [3H]NMS affinity, association and dissociation steps are probably retarded to the same extent. Thus, W405, and to a minor degree W400, may have a structural role in maintaining proper access of [3H]NMS to and egress from the M1 orthosteric pocket.

In contrast with [3H]NMS, the dissociation of BoPz tracers was accelerated in all instances, most often very similarly at Ala and Phe mutants (Table 5). Bo(15)Pz and Bo(22)Pz took some benefit from an aromatic Phe residue to compensate for kinetic alterations. Obviously, increases in off rates of Bo(10)Pz from W405F and W400A/F receptors, and of all other derivatives from all mutants, account for the decreases in affinity observed under equilibrium conditions (Table 4). More attention was paid to Bo(10)Pz dissociation from the W405A mutant as, in marked contrast with all other BoPz derivatives, it proceeded according to a biphasic process. An empirical two-exponential model was adopted to fit its dissociation traces: a fast ($k_{off}$ 0.19 ± 0.01 seconds$^{-1}$) and a slow ($k_{off}$ 0.0065 ± 0.0001 seconds$^{-1}$) component, contributing by 40% and 60%, respectively, to Bo(10)Pz binding reversal amplitude, best described its dissociation when promoted by 10 μM atropine. Gallamine and brucine revealed striking differences in their ability to modulate rates and relative amplitudes of both phases (preliminary observation). Underlying mechanisms merit further investigation.

We finally investigated, through FRET, the impact of the four point mutations on gallamine and brucine affinity parameters at free (equilibrium studies) and BoPz-occupied (dissociation studies) receptors (Supplemental Table 1). This allowed us to verify that the determination of gallamine affinity for each free receptor mutant is rather robust: affinity constants are independent of the nature of the BoPz tracer and similar to those afforded from [3H]NMS equilibrium studies (Table 3). The same observation applied to brucine, although the comparison was limited by the neutral cooperativity behavior it manifested in many instances: for example, its weakly positive cooperativity with Bo(10,12)Pz binding on the wild-type receptor shifted to neutral cooperativity on the W400A/F mutants. Bo(10,12)Pz equilibrium studies also revealed a stronger negative binding cooperativity with gallamine on the W400F mutant. An important point, based on equilibrium and kinetic data from all mutants, is that gallamine and brucine preserved their competitive character with Bo(15,22)Pz binding, as illustrated several ways on the wild-type EGFP(Δ17)hM1 receptor. Last, it is worth mentioning the pronounced loss of efficacy of gallamine to inhibit Bo(10,12)Pz dissociation from W400A/F mutants, reflected in an up to 30-fold increase in its $EC_{50,diss}$ value and in a reduction in maximal retardation effect (especially at the W400A mutant).

**Validation of an M1 Receptor Homology Model and Ligand Docking.** The recent resolution of the crystal structures of the human M2 (Haga et al., 2012) and rat M3 (Kruse et al., 2012) muscarinic receptors, both in an inactive state and in complex with nonselective antagonists, revealed their great similarity. Structural conservation includes the organization of their transmembrane domains (T7M), an identical fold of their extracellular loops (despite a low sequence conservation), and a deeply buried orthosteric binding pocket (delimited by residues absolutely conserved among the five mACHRs) separated from a solvent accessible vestibule by a narrow gate surrounded by three conserved tyrosine (Tyr3.34, Tyr6.51, and Tyr7.39) residues. On the basis of these observations and of overall sequence conservation (49.7% similarity to the human M2 structure), we finally modeled the Bitopic Poses of Fluorescent BoPz Ligands on M1 Receptors.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EGFP-wt hM1</th>
<th>EGFP-W405A</th>
<th>EGFP-W405F</th>
<th>EGFP-W400A</th>
<th>EGFP-W400F</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]NMS</td>
<td>8.02 ± 0.04</td>
<td>7.77 ± 0.05</td>
<td>7.42 ± 0.05</td>
<td>8.25 ± 0.02</td>
<td>8.00 ± 0.01</td>
</tr>
<tr>
<td>FRET</td>
<td>7.91 ± 0.04</td>
<td>7.60 ± 0.10</td>
<td>7.42 ± 0.06</td>
<td>8.12 ± 0.08</td>
<td>8.01 ± 0.01</td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td>[3H]NMS</td>
<td>7.51 ± 0.01</td>
<td>7.20 ± 0.01</td>
<td>6.99 ± 0.09</td>
<td>7.49 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>FRET</td>
<td>7.67 ± 0.01</td>
<td>7.35 ± 0.06</td>
<td>7.08 ± 0.04</td>
<td>7.66 ± 0.09</td>
</tr>
<tr>
<td>Bo(15)Pz</td>
<td>[3H]NMS</td>
<td>7.77 ± 0.05</td>
<td>7.06 ± 0.04</td>
<td>6.80 ± 0.06</td>
<td>6.60 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>FRET</td>
<td>7.97 ± 0.01</td>
<td>7.19 ± 0.07</td>
<td>6.94 ± 0.08</td>
<td>6.88 ± 0.02</td>
</tr>
<tr>
<td>Bo(22)Pz</td>
<td>[3H]NMS</td>
<td>7.74 ± 0.07</td>
<td>7.60 ± 0.06</td>
<td>7.18 ± 0.04</td>
<td>7.17 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>FRET</td>
<td>7.92 ± 0.09</td>
<td>7.77 ± 0.07</td>
<td>7.15 ± 0.12</td>
<td>7.19 ± 0.01</td>
</tr>
</tbody>
</table>
sequence identity between M1 and M3; 45.0% between M1 and M2), the M3 receptor was selected as the template to model M1 structure.

3-Quinuclidinyl benzilate (QNB) and tiotropium bind in a remarkably similar pose to M2 and M3 receptors (Kruse et al., 2012) that is likely to represent a conserved binding mode for structurally close anticholinergics. We were therefore interested in examining whether this could also apply to pirenzepine binding in our M1 model (Supplemental Fig. 2). This was indeed the case, as all three antagonists are enclosed in a highly conserved aromatic cage, establish an ionic bond with Asp3.33, and fit a common three-dimensional pharmacophore defined by a positively charged nitrogen atom in an aliphatic 6-membered ring, a carbonyl group, and two aromatic rings. This allowed us to build the M1-pirenzepine complex, to show (Fig. 7A) that pirenzepine binding pose well mimicks that of the two other antagonists in M2 and M3 crystal structures, and to validate our M1 model, at least from the orthosteric site point of view.

A more global image of the M1 receptor model is provided in Fig. 7B, which shows that the cavity in the 7TM M1 receptor core displays a highly hydrophobic region at the junction between the orthosteric site and the allosteric vestibule, in the vicinity of the side chain of Trp101 in TM3. The two residues which were mutated in this study, W400 and W405, sit, respectively, in the vestibule and at the TM7/lipid bilayer interface. They form the ends of a network of aromatic residues bridging TM helices (including the Tyr106-Tyr381-Tyr404 triad but also Tyr82, Tyr85, Trp91, Trp101, Trp157, and Tyr408) and may lock its conformation. This M1 model provides the structural foundations for further discussion of our experimental data on fluorescent pirenzepine derivatives.

**Discussion**

We investigated in much detail the binding properties of a series of fluorescent Bodipy-pirenzepine derivatives at EGFP-fused M1 receptors. A combination of radioligand and FRET binding experiments and of mutagenesis and molecular modeling studies was necessary to demonstrate their bitopic nature: they bind, through their pirenzepine pharmacophore, to the orthosteric site and simultaneously occupy, through their Bodipy moiety, the M1 allosteric vestibule (Fig. 7). Whereas long (15–22 atoms) linkers allow the fluorophore to access the gallamine/brucine site, shorter (10–12 atoms) linkers confine it within a neighboring but separate allosteric subdomain.

Pirenzepine and BoPz compounds display similar affinities (10 nM range), suggesting a weak contribution of the fluorophore to overall binding energy of the derivatives. Such a view is supported by the observation of monophasic and unchanged dissociation kinetics for all BoPz derivatives, whatever the protocol which is applied. Thus, the fluorescent ligands display a homogenous binding mode, and probably firmly anchor to the receptor through binding of their pirenzepine moiety within the orthosteric site. One should mention, however, that the Bo(5) fragment has allosteric affinity in the low micromolar range, and that Bo(15,22)Pz binding is clearly impacted by W400A/F mutations, whereas that of pirenzepine is much less affected. These latter findings suggest that both the linker and the pirenzepine pharmacophore help the Bodipy moiety reach a local concentration high enough to interact with allosteric residues.

When examined from the orthosteric point of view, all BoPz derivatives exhibit either competitive-like (when tested against [3H]NMS and other orthosteric compounds under equilibrium conditions) or allosteric (as afforded from their ability to alter [3H]NMS dissociation kinetics) properties. Such a mixed orthosteric/allosteric behavior is often observed for bitopic ligands (Mohr et al., 2010; Daval et al., 2012; Valant et al., 2012). Other explanations include a strong negative binding cooperativity with [3H]NMS (if one considers BoPz affinity ratios at free and [3H]NMS-occupied receptors) or a differential positioning of these derivatives, depending on orthosteric site occupancy. Although retardation of [3H]NMS dissociation occurs at high BoPz concentrations, fluorophore-driven occupation and occupancy of the allosteric vestibule by the bulky BoPz derivatives are unlikely. An alternate possibility is to consider an interaction of their pirenzepine moiety with a peripheral site that sterically hinders [3H]NMS egress from the orthosteric pocket. Indeed, real-time FRET monitoring of Bo(10)Pz binding kinetics at EGFP-fused M1 receptors indicated that an initial fast and low affinity binding event, occurring at the receptor surface close to EGFP, precedes ligand translocation within the transmembrane core (Ilien et al., 2009). Molecular dynamic simulations of tiotropium binding to M2 and M3 receptors also revealed the possibility for an orthosteric ligand to pause within the allosteric vestibule while entering or dissociating from the orthosteric pocket (Kruse et al., 2012).

FRET experiments provided further information on BoPz properties, now regarded as individual tracers. Indeed, equilibrium and kinetic FRET data indicated that 1) all BoPz

---

**Table 5**

Effect of point mutations in the EGFP(Δ17)hM1 receptor on dissociation kinetics of [3H]NMS and BoPz tracers

<table>
<thead>
<tr>
<th>Tracer</th>
<th>EGFP- wt hM1</th>
<th>EGFP- W405A</th>
<th>EGFP- W405F</th>
<th>EGFP- W400A</th>
<th>EGFP- W400F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decay Rate</td>
<td>sec⁻¹ · 10⁻⁴</td>
<td>sec⁻¹ · 10⁻⁴</td>
<td>sec⁻¹ · 10⁻⁴</td>
<td>sec⁻¹ · 10⁻⁴</td>
<td>sec⁻¹ · 10⁻⁴</td>
</tr>
<tr>
<td>[3H]NMS</td>
<td>6.9 ± 0.3</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>3.6 ± 0.3</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Bo10Pz</td>
<td>5.9 ± 0.5</td>
<td>Biphase</td>
<td>24 ± 1</td>
<td>9.0 ± 0.6</td>
<td>9.4 ± 0.5</td>
</tr>
<tr>
<td>Bo12Pz</td>
<td>15.4 ± 0.3</td>
<td>47 ± 4</td>
<td>39 ± 6</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Bo15Pz</td>
<td>13.7 ± 1.6</td>
<td>97 ± 7</td>
<td>75 ± 6</td>
<td>368 ± 15</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Bo22Pz</td>
<td>15.5 ± 0.5</td>
<td>28 ± 2</td>
<td>48 ± 2</td>
<td>152 ± 8</td>
<td>62 ± 1</td>
</tr>
</tbody>
</table>
derivatives involve an orthosteric binding component; 2) Bo(10,12)Pz affinity is allosterically modulated by brucine (potentiation) and gallamine (inhibition), and thus, their capacity to alter [3H]NMS dissociation rate probably originates from the recruitment of allosteric epitopes that do not overlap with the classic allosteric domain; 3) Bo(15,22)Pz, in marked contrast with the former ligands, inherits allosteric properties from occupancy of the gallamine/brucine site; and 4) Bo(15,22)Pz dissociation proceeds similarly, whatever the agent (atropine, brucine, or gallamine) selected to prevent tracer reassociation to the receptor, as expected for concomitant binding of the fluorescent pirenzepine derivative and either allosteric modulator. Thus, these data highlight the prominent role of linker length in the definition of the bitopic nature and the selection of different allosteric binding poses for BoPz compounds. Previous reports already indicated that the fusion of bulky groups (including fluorophores) to pirenzepine was well tolerated in terms of affinity, provided the linker was long enough (at least 6–10 methylenes) to relax the constraints imposed by pirenzepine binding to the M1 orthosteric site (Karton et al., 2007; our modeling studies).
1991; Tahtaoui et al., 2004). Although the nature (polyethylene glycol or isopeptidic) of the linker seems here of marginal importance, the examples of Bo(5) and of similar derivatives (Daval et al., 2012) clearly indicate that the linker conveys affinity and negative binding cooperativity to these molecules.

Valuable insights into linker-guided positioning of the fluorophore of Bo(10,12)Pz and Bo(15,22)Pz compounds were gained from M1 modeling and ligand docking studies. The structures of all ligand-receptor complexes were built assuming a fixed pose for pirenzepine (substituted or not) we found to perfectly fit a three-dimensional pharmacophore (Fig. 7A) common to QNB and tiotropium in M2 and M3 (Kruse et al., 2012) receptors, respectively. Interestingly, the distal N-methyl group of pirenzepine (the anchor point for the linker) is not oriented toward the larger opening between the orthosteric and allosteric modules (Fig. 7B). Instead, it faces a narrow channel (between Aasp105, Tyr404, and Tyr408 residues), filled with a water molecule in the M2 structure (Haga et al., 2012). In BoPz-M1 complexes (Fig. 7, C and D), the linker passes through this channel, hence replacing the water molecule, to reach the most hydrophobic region of the cavity (Fig. 7B; brown shaded area), near Trp101. This configuration fixes the position of the first seven atoms of the linker (close to pirenzepine), thereby restricting possibilities for positioning the fluorophore, especially if the linker is short. Experimental support of this view is provided by the measurement of a 10-fold-faster off rate of the Bo(10)Pz compound from the W101 AM1 mutant (unpublished data). In Bo(10)Pz-M1 complexes (Fig. 7C), the Bodipy group locates at the entrance of the vestibule, in an opening between TM2 and TM7, yet in a position compatible with concomitant binding of brucine or gallamine. When presented as volumes, these allosteric modulators best evidence plugs over the orthosteric pocket (Supplemental Fig. 3). Long linkers, such as in Bo(22)Pz (Fig. 7D), fold into the allosteric cavity near W400 and allow the fluorophore to locate between extracellular loops 2 and 3, so that the binding of brucine and gallamine is prevented.

These models nicely account for most of our experimental observations, including the estimation of a distance (10 Å) between the Bodipy boron atoms in Bo(10)Pz- and Bo(22)Pz-receptor complexes, which rather well coincides with the average difference in EGFP-Bodipy separation (5 Å) determined from FRET efficacy using the same derivatives (Tahtaoui et al., 2004). One should add here that docking solutions are consistent with multiple placement of the fluorophore within a large and well-accessible receptor vestibule that does not define an enclosed “lock” suitable for tight binding of Bodipy, viewed as a “key.” As the fluorophore, according to linker length, is allowed to explore topographically distinct and nonconserved allosteric domains, including extracellular loops, it would be interesting to test BoPz ligands for their binding selectivity at M1–M5 receptor subtypes.

In conclusion, this work describes a step-by-step strategy to define the bitopic binding properties of a family of fluorescent pirenzepine derivatives at muscarinic M1 receptors. It highlights the pivotal role of the linker which connects orthosteric and allosteric building blocks within a bitopic ligand. Indeed, it imposes structural constraints to and dictates pharmacological outcomes for the hybrid molecule (including biased signaling as evidenced recently for dualsteric agonists on M2 receptors; Bock et al., 2012) that are not readily predictable from the properties of unconnected partners. Our findings also demonstrate that a fluorophore fused to an orthosteric compound is not an innocent bystander, as it may confer an allosteric texture to the resultant tracer. Unless carefully controlled, this may lead to data misinterpretation, especially when working with bioamine receptors which contain vicinal orthosteric and allosteric modules. Thus, the rational design and in-depth study of bitopic molecules may provide, in addition to advantages expected from the combination of orthosteric and allosteric moieties within a single molecule, valuable guidelines to decipher “hidden” dual drug binding properties.

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Authorship Contributions

Participated in research design: Daval, Galzi, Ilien.
Conducted experiments: Daval, Kellenberger, Bonnet, Utard, Ilien.
Contributed new reagents or analytic tools: Bonnet.
Performed data analysis: Daval, Kellenberger, Ilien.
Wrote or contributed to writing of the manuscript: Kellenberger, Galzi, Ilien.

References


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