Development of a New Type of Allosteric Modulator of Muscarinic Receptors: Hybrids of the Antagonist AF-DX 384 and the Hexamethonio Derivative W84

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Various fragments of the hexamethonio-type allosteric agent W84 were linked to the secondary amino group of the muscarinic M_2 acetylcholine receptor-preferring antagonist AF-DX 384 to increase the area of attachment with the allosteric site. Addition of only the phthalimido moiety of W84 gave an allosteric enhancer of NMS binding. Thus, a new lead structure for the development of allosteric enhancers of NMS binding has been discovered.

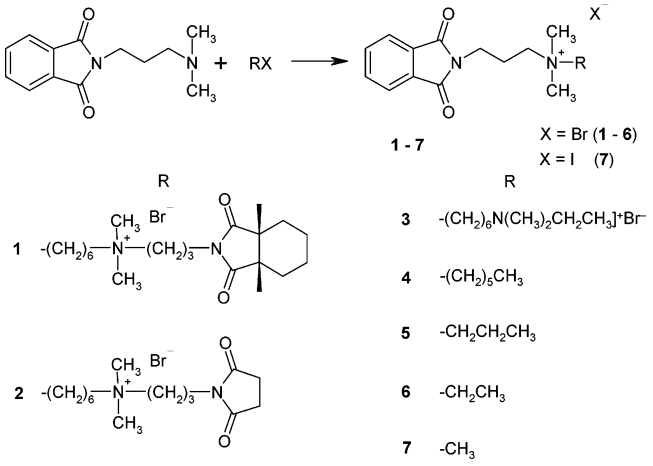
Introduction

Allosteric modulators are able to enhance the binding of orthosteric ligands, indicating positive cooperativity, or to diminish the ligand binding, reflecting negative cooperativity, or to leave the binding unchanged (neutral cooperativity). Especially, the increase of the equilibrium binding of an orthosteric ligand is attractive for novel therapeutic purposes. Recently, the hexamethonio-type allosteric modulator W84[1−4] was studied for its ability to influence allosterically the binding of various antagonists including the M_2-preferring AF-DX 384 and the nonselective *N*-methylscopolamine (NMS). The affinity of W84 to the NMS-occupied receptor was found to be far higher than for the AF-DX 384 liganded receptor.[5] In the NMS-bound receptor, AF-DX 384 revealed a moderate affinity for the allosteric site. These findings were interpreted to suggest that the receptor binding domain used by AF-DX 384 in NMS-free receptors overlaps partially with the allosteric binding site. To test this hypothesis, we applied stepwise shortened derivatives of W84 in order to probe the allosteric site in M_2 receptors either occupied by AF-DX 384 or NMS and to compare the respective structure−activity-relationships. Subsequently, fragments of W84 were linked with AF-DX 384 to increase its overlap with the allosteric site. Thus, a hybrid molecule was found that strongly enhanced the binding of the orthosteric ligand NMS. The SAR findings were compatible with the hypothesis that AF-DX 384 occupies part of the M_2 receptor allosteric site.

Chemistry

The synthesis of the W84 derivatives characterized by an unilateral succinimide **2** was already reported by Bender et al.[2] The synthesis of an unilateral trans-substituted cyclohexanedicarbonic acid imide compound **1** was performed in analogy to Staudt et al.[1] by alkylation of *N*,*N*-dimethylaminopropylphthalimide with dibromohexane and connecting the monobromo compound with the corresponding dimethylaminopropyl-cyclohexanedicarbonic acid imide. Compound **3** missing the second imide moiety was obtained by alkylation of dimethylaminopropylphthalimide with the corresponding amino-substituted bromohexane which can be achieved be refluxing dibromohexane and *N*-ethyldimethylamine in ethanol for 20 h. The monoquaternary compounds **4**−**7** can be easily obtained by alkylation of dimethylaminopropylphthalimide with the corresponding iodo- or bromoalkane (Scheme 1).

Scheme 1. Synthesis Pathway of Shortened W84 Molecules



The multistep synthesis of the AF-DX 384 analogue **7** starting from pipecolic acid has been recently reported by our group.[6] Compound **9**−**11** can be achieved by conversion of **8** with the corresponding bromoalkylimide, and the monoquaternary compound **12** was obtained by using the W84 fragment **13** (cf. refs 2, 6, 13). AF-DX 384 and its hybrids **9**−**12** were synthezised and tested as racemates (Scheme 2).

Pharmacology

The nonselective orthosteric antagonist [^{3}H]NMS and the M_2-preferring antagonist [^{3}H]AF-DX 384 were applied to measure the effects of allosteric test compounds on radioligand dissociation kinetics and equilibrium binding. Effects were characterized by the following parameters. $EC_{0.5,diss.}$ is the concentration of allosteric agent at which the monophasic orthosteric radioligand dissociation is half-maximally reduced; $EC_{0.5,diss.}$ reflects the equilibrium dissociation constant for allosteric agent

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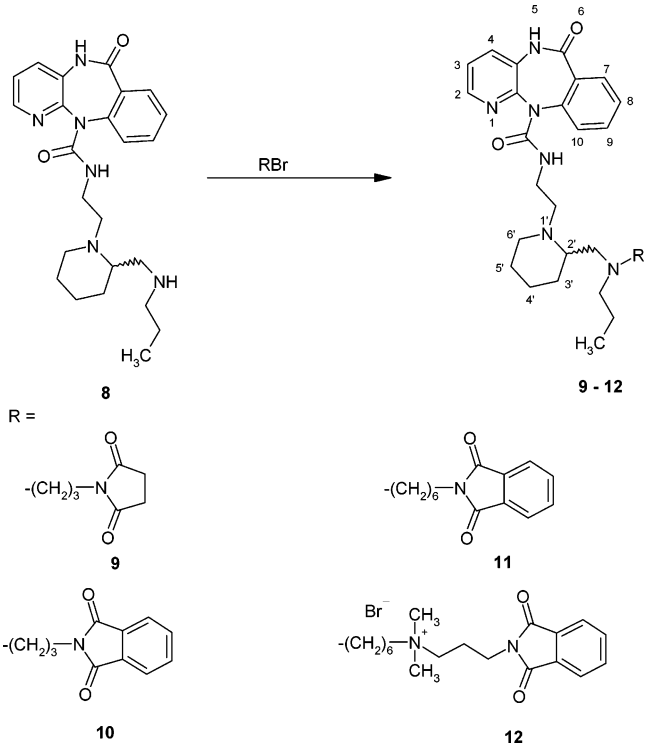
Table 1. Parameters Characterizing the Allosteric Interactions of W84 and Test Compounds **1**−**7** with the Binding of [^{3}H]*N*-Methylscopolamine and [^{3}H]AF-DX 384 at Guinea Pig M_2 Receptors (mean values ± SEM from Three to Four Independent Experiments or Mean Value with Single Values from Two Independent Experiments)[a]

compd	$pEC_{0.5,diss.}$ [^{3}H]NMS	$E_{max,diss}$ [%]	$pEC_{0.5,diss.}$ [^{3}H]AF-DX 384	$E_{max,diss}$ [%]	pK_A [^{3}H]NMS	pK_A [^{3}H]AF-DX 384	pα [^{3}H]NMS	pα [^{3}H]AF-DX 384
W84	5.89 ± 0.05	100	4.51 ± 0.09	78 ± 4.6	6.00 ± 0.13	6.53 ± 0.05	−0.26 ± 0.09	−1.15 ± 0.09
1	5.30 ± 0.04	100	4.52 ± 0.09	68 ± 3.2	5.66 (5.59; 5.73)	6.09 (6.03; 6.16)	−0.48 (−0.48; −0.48)	−1.22 (−0.83; −1.60)
2	4.82 ± 0.03	100	3.67 ± 0.14	68 ± 6.0	5.24 (5.27; 5.20)	5.93 (5.87; 5.99)	−0.49 (−0.53; −0.45)	−1.56 (−1.57; −1.55)
3	4.85 ± 0.05	100	3.86 ± 0.11	56 ± 4.1	5.14 (5.20; 5.07)	6.02 ± 0.04	−0.72 (−0.72; −0.72)	−1.78 ± 0.17
4	4.06 ± 0.03	100	4.11 ± 0.05	82 ± 2.8	5.08 (4.96; 5.20)	5.80 ± 0.07	−1.55 (−1.27; −1.83)	−1.35 ± 0.15
5	3.76 ± 0.05	90 ± 2.5	2.98 ± 0.13	73 ± 7.4	3.55 (3.50; 3.60)	4.10 ± 0.07	−0.78 (−0.81; −0.74)	<−2
6	3.35 ± 0.04	100	3.33 ± 0.49	34 ± 15.7	4.12 (4.11; 4.13)	4.44 (4.46; 4.42)	−1.03 (−0.93; −1.13)	<−2
7	3.21 ± 0.03	100	3.00 ± 0.65	25 ± 12.4	3.95 (3.93; 3.97)	4.51 (4.67; 4.35)	−1.21 (−1.11; −1.30)	<−2

[a] $pEC_{0.5,diss.}$ reflects test compounds' affinity to radioligand-occupied receptors, pK_A reflects the affinity to free receptors.

Scheme 2. Synthesis Pathway of the Hybrid Molecules



binding to radioligand-occupied M_2 receptors. The action of allosteric agents on radioligand equilibrium binding was analyzed according to the ternary complex model of allosteric actions[18,7] and yielded the following parameters: K_A is the equilibrium dissociation constant for binding to ligand-free M_2 receptors; α is the cooperativity factor for the interaction between the allosteric agent and the respective radioligand.

Results and Discussion

All compounds synthesized were subjected to the pharmacological evaluation. The $pEC_{0.5diss.}$ values, pK_A values and the minus log cooperativity factors pα in relation to the substitution pattern are summarized in Tables 1 and 2. Cutting off elements of W84 step-by-step resulted in an almost continuous loss of affinity for the free receptor (pK_A in Table 1), independent of which radioligand was used as a probe. However, the fall of affinity levels off at compounds **5**−**7**. As to be expected, the variations of pK_A are running in parallel independent of the radioligand used as a probe. The difference of about 0.5 pK_A units between both series is probably due to the divergent incubation temperatures used with the two radioligands: in contrast to [^{3}H]NMS

Table 2. Parameters Characterizing the Allosteric Interactions of W84, AF-DX 384, and Test Compounds **9**−**12** with the Binding of [^{3}H]NMS at Porcine Heart M_2 Receptors (mean values ± SEM from three to four Independent Experiments

compd	$pEC_{0.5,diss.}$	$E_{max,diss}$ [%]	pK_A	pα
W84	5.87 ± 0.10	100	6.43 ± 0.16	−0.45 ± 0.04
AF-DX 384	4.65 ± 0.07	100	8.14 ± 0.09	<−2
9	4.95 ±0.11	76 ± 6.2	8.01 ±0.06	−1.80 ± 0.05
10	6.87 ± 0.05	100	5.89 ± 0.05	1.07 ± 0.13
11	6.75 ± 0.05	100	6.71 ± 0.02[a]	0.05 ± 0.01
12	6.78 ± 0.04	100	6.83 ± 0.10	−0.58 ± 0.04

[a] Deduced from $\alpha K_A = EC_{0.5,diss}$.

whose binding was measured at 37 °C, experiments with [^{3}H]AF-DX 384 were conducted at 23 °C to obtain similar dissociation half-times and similar incubation periods with the two radioligands. A control experiment carried out with compound **4** at 37 °C for both radioligands revealed identical pK_A values for **4** (data not shown). The affinity for liganded receptors ($pEC_{0.5,diss.}$) has been previously found hardly to depend on the temperature applied.[8] The variations in binding affinity to [^{3}H]NMS- and [^{3}H]AF-DX 384-occupied receptors, respectively, are divergent ($pEC_{0.5,diss.}$, Table 1): whereas stepwise shortening of the W84 molecule reduced the affinity to the [^{3}H]NMS-liganded muscarinic receptors by almost 3 orders of magnitude, the affinity for the [^{3}H]-AF-DX 384-occupied receptor started at a lower level and fell by only 1.5 orders of magnitude. Notably, removal of one charged nitrogen of W84 (**4** versus **3**) reduced the affinity for NMS-liganded receptors by almost 10-fold whereas the affinity for AF-DX 384-liganded receptors was not affected by this modification. Equilibrium binding of both orthosteric ligands was reduced by W84 and its derivatives corresponding to a negative cooperativity (pα < 0). From the SAR findings we tentatively concluded that the allosteric site in the [^{3}H]AF-DX 384-occupied receptor, as compared to the [^{3}H]NMS-occupied receptor, leaves less room for the W84-type agents. This might be caused by an overlap of receptor-bound AF-DX 384 with the allosteric site of the M_2 receptor. Previously, Kerckhoff and Hoeltje[9] concluded from modeling studies that M_2–preferring AF-DX compounds attach to the ligand binding cavity of the receptor with the side chain directed upward to the receptor surface, i.e., to the location of the allosteric site. The same affinity of the monoquaternary **4** for AF-DX 384- and NMS-liganded receptors was taken to suggest that the allosteric site in the AF-DX 384-occupied receptor leaves room for about half of the W84 molecule. Compounds smaller than **4** should likewise not differ in their affinities for both states of receptor occupancy, as was the case with **6** and **7**.

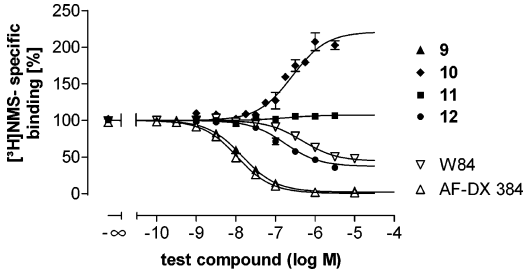


Figure 1. Effects of the indicated test compounds on [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) equilibrium binding to porcine heart M_2 receptors. Ordinate: specific [^{3}H]NMS binding in percent of the value in the absence of test compound. Abscissa: log concentration of the test compounds. Indicated are mean values ± SEM of three to four independent experiments. Error bars are not visible when smaller than the symbols; curve fitting is based on the ternary complex model of allosteric interactions[17] (see text for details).

In an attempt to increase the area of attachment of AF-DX 384 with the allosteric site, hybrids were constructed from AF-DX 384 and fragments of the W84 molecule which corresponded to **4** and smaller derivatives thereof. The corresponding compounds **9**−**12** characterized by a linker of varying length between the AF-DX 384 part and the phthalimide fragment were tested in porcine cardiac membranes using [^{3}H]NMS as a probe to evaluate agents' affinities to free receptors and cooperativity factors (Figure 1) and, second, using [^{3}H]-NMS dissociation experiments to determine affinities to [^{3}H]NMS-occupied receptors. Compound **9** missing the annelated aromatic ring at the imide moiety reduced [^{3}H]NMS equilibrium binding reflecting a low affinity of **9** to the NMS-liganded receptor ($pEC_{0.5,diss.}$) whereas the affinity to the free receptor is high (resulting in a high negative cooperativity between the hybrid and NMS). Since the respective affinities of AF-DX 384 and compound **9** were almost the same (Table 2) it can be concluded that the propylsuccinimide skeleton does not participate in the receptor binding and, thus, does not affect the AF-DX 384/receptor-interaction to a relevant extent. Compound **10** having the annelated benzene ring at the imide strikingly enhanced [^{3}H]NMS binding. It shows a more than 100-fold higher affinity to the NMS-occupied M_2 receptor and a more than 100-fold lower affinity to the free receptor compared with AF-DX 384. Thus, the aromatic ring is pivotal for the binding to the NMS-occupied receptor and, vice versa, extinguishes the high affinity to the free receptor. Compound **11** having a hexane linker between the phthalimide and the AF-DX 384 moiety and compound **12** having an additional quaternary nitrogen not only show high affinities for NMS-occupied receptors but also for free receptors (Table 2), resulting in a neutral and negative cooperativity with NMS, respectively (Figure 1). The observation that hybrids **10**−**12** bind to free M_2 receptors with a rather low affinity compared with AF-DX 384 suggests that hybridization hinders the AF-DX 384 part of the molecules to attach to the receptor in the location that is normally used by AF-DX 384. In contrast, the hybrids **10**−**12** have a greatly increased affinity, compared with W84 and AF-DX 384, to M_2 receptors whose orthosteric site is blocked by NMS ($pEC_{0.5diss.}$, Table 1). This suggests that the AF-DX 384 part of the hybrids contributes to a major extent to the binding at the allosteric site.

The allosteric site of M_2 receptors is located in a cleft at the entrance of the receptor protein and connected through a corridor with the orthosteric site which is situated deep in the ligand binding cavity of the M_2 receptor. According to the model of Voigtländer et al.,[10] orthosteric ligands would have to pass the allosteric site on their way down to the orthosteric location. It is tempting to speculate that the phthalimide fragment of W84 anchors the hybrids to the allosteric site and hinders the AF-DX 384 part to pass down to the orthosteric site. In line with this, the succinimide moiety, which is known to be an insufficient substitute for phthalimide,[1] was found here to hardly affect the binding characteristics of AF-DX 384. Interestingly, the length of the linker between AF-DX 384 and the phthalimide fragment does not influence the affinity to the NMS-liganded receptor but governs the affinity to the free receptor and, thus, the cooperativity. In any case, the AF-DX 384/W84-hybrids provide a new lead for the development of allosteric enhancers of muscarinic ligand binding.

Conclusion

For the last 10 years, much effort has been spent on the development of allosteric modulators which are characterized by a high affinity and selectivity to orthosteric ligand-occupied muscarinic receptors in connection with an elevation of the ligand binding and, thus, a positive cooperativity.[3,4] Within the frame of this study, we found an AF-DX 384/W84-hybrid-type allosteric modulator with high affinity to the NMS-occupied M_2 receptor and a smaller affinity to the free M_2 receptor. Thus, a new type of allosteric enhancer of the orthosteric ligand binding has been developed.

Experimental Section

Analytical instruments see ref 4. W84 was synthesized according to ref 11, **2** according to ref 2, **12** and **8** according to ref 6, the 3,3-dimethylaminopropylphthtalimide according to refs 12 and 14, (*ω*-bromohexyl)dimethylethylammonium bromide according to ref 13, and **13** according to ref 14.

Synthesis of 2-(3a*R*,7a*S*)-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-propyl]ammonio}-hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}octahydroisoindole-1,3-dione Dibromide 1. (6-Bromohexyl)dimethyl-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)propyl]ammonium bromide (0.95 g, 2 mmol) and 3,3-dimethylaminopropylphthtalimide (0.48 g, 2 mmol) dissolved in acetonitrile (20 mL) were refluxed for 4.5 h. After cooling, white crystals were collected by filtration and washed with petroleum ether to give 2. Yield 81%, mp. 231 °C (dec).

General Procedure for Synthesis of Compounds 3−7. Equimolar amounts of 3,3-dimethylaminopropylphthtalimide and the corresponding alkyl bromide were dissolved in ethanol and refluxed for several hours. The solvent was evaporated, and the residue was stirred with petroleum ether. The solvent was filtered off, and the residue was recrystallized from dichloromethane/diethyl ether to get colorless solids.

2-{3-[1-(6-{1,1-Dimethyl-1-ethylammonio}hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}-1,3-dihydroisoindole-1,3-dione Dibromide 3. Reaction time: 20 h. Yield: 16%, mp 213−215 °C. **[3-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)propyl]hexyldimethylammonium Bromide 4**. Reaction time: 20 h. Yield: 59%, mp 187−189 °C. **[3-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)propyl]dimethylpropylammonium Bromide 5.** Reaction time: 16 h. Yield: 56%, mp 199−200 °C. **[3-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)propyl]ethyldimethylammonium Bromide 6**. Reaction time: 16 h. Yield: 45%, mp 209−210 °C. **[3-(1,3-Dioxo-1,3-dihydroisoin-**

dol-2-yl)propyl]trimethylammonium Iodide 7. Reaction time: 16 h. Yield: 45%, mp 209−210 °C (cf. [15]).

General Procedure for Synthesis of Compounds 9−12. 6-Oxo-5,6-dihydrobenzo[*e*]pyrido[3,2-*b*][1,4]diazepine-11-carboxylic acid [2-(2-propyl-aminomethylpiperidin-1-yl)ethyl]-amide **8** (0.44 g, 1.0 mmol), the corresponding bromoalkylisoindole (1.0 mmol), and Hünig's base (0.7 mL, 4.0 mmol) were dissolved in chloroform (30 mL) and heated to 60 °C in a sealed tube for several days. The progress of the reaction was monitored by TLC (Al_2O_3, $CHCl_3$/MeOH 6:1). When the reaction was finished, the solution was diluted with 40 mL of 2.0 M Na_2CO_3 and the layers were separated. The aqueous layer was extracted with chloroform (3 × 80 mL), and the combined organic layers were dried and evaporated. The residue was purified by column chromatography (Al_2O_3, $CHCl_3$/MeOH 6:1) and recrystallized from CH_2Cl_2/petroleum ether to get a pale yellow solid.

6-Oxo-5,6-dihydrobenzo[*e*]pyrido[3,2-*b*][1,4]diazepine-11-carboxylic Acid {2-[2-({[3-(2,5-dioxopyrrolidin-1-yl]-propyl]-propyl-amino}methylpiperidin-1-yl]ethyl}-amide 9. Reaction time: 7 d. Yield: 0.23 g (40%), mp 143−144°C. **6-Oxo-5,6-dihydrobenzo[*e*]pyrido[3,2-*b*][1,4]diazepine-11-carboxylic Acid {2-[2-({[3-(2,5-dioxoisoindol-2-yl]-propyl]-propyl-amino}methylpiperidin-1-yl]ethyl}-amide 10.** Yield: 0.15 g (24%), mp: 126−127 °C. **6-Oxo-5,6-dihydrobenzo[*e*]pyrido[3,2-*b*][1,4]diazepine-11-carboxylic Acid {2-[2-({[3-(2,5-dioxoisoindol-2-yl]-hexyl]-propyl-amino}methylpiperidin-1-yl]ethyl}amide 11.** Yield: 0.11 g (16%), mp: 127−129 °C.

Radioligand Binding Studies. Membrane preparations from hearts of Dunkin-Hartley guinea pigs and of domestic pigs were carried out as described previously.[16,17] Porcine cardiac membranes were incubated at 37 °C with the radioligand [^{3}H]NMS (0.2 nM) in a buffer composed of 3 mM $MgHPO_4$ and 50 mM TrisHCl, pH 7.3. Guinea pig cardiac membranes were incubated in the same buffer either at 37 °C with [^{3}H]NMS (0.2 nM) or at 23 °C with [^{3}H]AF-DX 384 (0.5 nM). Nonspecific binding was measured in the presence of 1 μM atropine and amounted to 5% ([^{3}H]NMS) or 15% ([^{3}H]AF-DX 384) of total radioligand binding. NMS binding affinity (pK_D) and concentration of receptors in the cardiac membranes (B_{max}) was as follows (mean values ± SEM): $pK_D = 9.32 \pm 0.04$ and $B_{max} = 154 \pm 27$ fmol/mg membrane protein for guinea pig heart membranes; $pK_D = 9.46 \pm 0.03$ and $B_{max} = 64 \pm 5.7$ fmol/mg membrane protein for porcine cardiac membranes. AF-DX 384 binding to guinea pig cardiac membranes was characterized by $pK_D = 7.94 \pm 0.05$ and $B_{max} = 318 \pm 37$ fmol/mg membrane protein. To study the effect of test compounds on radioligand dissociation kinetics, membranes were preincubated with [^{3}H]NMS for 30 min or with [^{3}H]AF-DX for 60 min before addition of 1 μM atropine alone or in combination with the test compound to reveal radioligand dissociation. Half-life times of radioligand dissociation in the absence of a test compound amounted to $t_{1/2} = 3.50$ min ± 0.10 ([^{3}H]NMS, guinea pig cardiac membranes, 37 °C, n=36), $t_{1/2} = 2.83$ min ± 0.11 ([^{3}H]NMS, porcine heart membranes, 37°C, n=32) and $t_{1/2} = 7.60$ min ± 0.10 ([^{3}H]AF-DX 384, guinea pig cardiac membranes, 23 °C, $n = 62$). Radioligand dissociation was followed for 120 min. Bound radioligand was separated by vacuum filtration through glass fiber filters which were presoaked in 0.2% polyethylenimine-solution for at least 24 h to reduce nonspecific [^{3}H]AF-DX 384 binding. The membrane-bound radioactivity was determined by liquid scintillation counting. Binding data were subjected to computer-aided nonlinear regression analysis using the Prism software (Ver.3.0 Graph Pad). Radioligand dissociation was characterized by the apparent rate constant of dissociation k_{-1}. To obtain concentration-effect curves for the allosteric delay of radioligand dissociation, the k_{-1} values were plotted over the log concentration of test compound and curve fitting was based on a four parameter logistic function. The incubation time required to obtain binding equilibrium in the presence of allosteric agents was calculated using eq 31 in the study of Lazareno and Birdsall[9] and amounted to 120−360 min. Data were analyzed by nonlinear regression according to the ternary complex model of allosteric interactions[18] using eq 3 in ref 5. Each concentration−effect curve was based on 5−11 different concentrations of a test compound.

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Supporting Information Available: Analytical data, ^{1}H NMR, ^{13}C NMR, IR, MS data, and pharmacological data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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