# Allosterism at Muscarinic Receptors: Ligands and Mechanisms

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**Abstract:** The evaluation of allosteric ligands at muscarinic receptors is discussed in terms of the ability of the experimental data to be interpreted by the allosteric ternary complex model. The compilation of useful SAR information of allosteric ligands is not simple, especially for muscarinic receptors, where there are multiple allosteric sites and complex interactions.

**Keywords:** Muscarinic receptors, allosterism, cooperativity, enhancers, receptor models, structure-activity relationships, interaction studies, receptor subtype selectivity.

## INTRODUCTION

### Acetylcholine and Muscarinic Receptors

Acetylcholine (ACh) has a venerable position in the history of our understanding of neurotransmission, perhaps because it is the neurotransmitter utilised by the motor nervous system, the presynaptic nerves of the autonomic nervous system and postsynaptic nerves of the parasympathetic nervous system, as well as being widely distributed in the central nervous system.

This ubiquitous distribution and importance gave rise both to amenable physiological preparations and to the discovery and use of a range of poisons which target the various molecular components of the cholinergic neurotransmission system. Claude Bernard [1] found that curare caused paralysis by interfering with the signal from nerves to muscles. Langley [2] extended these studies by showing that nicotine could stimulate a frog's muscle even after it had been denervated, and the stimulation was also blocked by curare. These findings led him to suggest that both compounds acted on a common 'receptive substance'. There had also been a number of earlier reports that muscarine and atropine had actions on vagal nerve endings, salivation, pupillary constriction and on other systems, (e.g. [3,4]).

In 1914 Dale was the first to clearly provide the pharmacological dissection of the actions of choline esters, including ACh, into two classes; those acted on by the agonist-antagonist pair, nicotine-curare (nicotinic receptors) or by the muscarine-atropine pair (muscarinic receptors) [5]. This was the first definition of receptor subtypes even though Dale, at that time, was not familiar with the receptor concept. It took the brilliant work of Loewi and Navratil [6] and Feldberg and Krayer [7] to establish the chemical basis of neurotransmission and identify ACh as the endogenous neurotransmitter at nicotinic and muscarinic receptors.

### Muscarinic Receptor Subtypes

The widespread distribution of muscarinic receptors and the different responses generated by activation of the receptors in different tissues begged the question of whether there were different receptor subtypes mediating the different responses. Progress was hindered by the lack of selective ligands, although there are hints in the early studies of the selective actions of gallamine on the heart [8] and of the agonist, McN-A-343 (4-*N*-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammonium chloride, on sympathetic ganglia [9]. The advent of careful and accurate quantitation of $pK_B$ values by Barlow and coworkers (e.g. [10]), the accurate measurement of antagonist affinity constants in receptor binding studies (e.g. [11,12]) and the discovery of antagonists, whose therapeutic effectiveness was dependent on their subtype selectivity (e.g. pirenzepine [13]) pointed the way to the presence of multiple muscarinic subtypes.

The exact number of subtypes has only been known for 15 years. Cloning identified the five molecular subtypes of muscarinic receptors, termed $M_1$-$M_5$. They are members of the G-protein coupled receptor (GPCR) superfamily and the pharmacological and functional properties of the subtypes are now well established (for reviews, see e.g. [14-16]).

### First Indications of Allosterism at Muscarinic Receptors

Muscarinic receptors were the first GPCRs to provide evidence for an allosteric modulatory site on the receptor. The first indications that some antagonists might be acting allosterically, rather than competitively, came from whole tissue studies of the interactions of muscarinic agonists, such as ACh, with hexamethonium analogues in ileum [17] and gallamine (**1**, Fig. (**1**)) in heart [18]. The main observations leading these authors to suggest an allosteric mechanism are shown in idealised form in Fig. (**2**). Agonist concentration/response (cr) curves are shifted to the right in the presence of a fixed concentration of antagonist and, if the antagonist is competitive, the rightward shift increases with increasing concentrations of antagonist without limit, leading to straight-line 'Schild plots' {log(dose-ratio -1) vs. log [antagonist], where dose-ratio is the ratio of equieffective agonist concentrations in the presence and absence of antagonist}. What was observed by Lüllmann *et al.* [17] and by Clark and Mitchelson [18] was that low concentrations of the antagonist shifted the agonist cr curve as expected, but the degree of shift was limited, with higher concentrations of antagonist having no further effect, leading to Schild plots which curved over at the highest antagonist concentrations.

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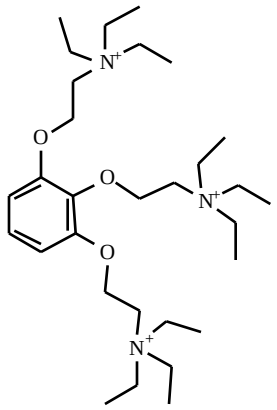


**1:** Gallamine

**Fig. (1).** Structure of gallamine.

Additionally, these authors found that the dose-ratios of single concentrations of test agent and atropine (a competitive antagonist) were not additive, as would be expected if the test agent were also acting competitively. Both sets of authors recognised that their observations were consistent with earlier theoretical models of ligand interaction at receptors, referred to as allosteric or metaffinoid interactions [19].

Subsequent radioligand binding studies confirmed and extended the nature of the allosteric interaction of gallamine at muscarinic receptors [20], and an idealised form of the observations is shown in Fig. (**3**). The limited inhibitory effect of gallamine was demonstrated by constructing inhibition curves of gallamine in the presence of increasing concentrations of the radioligand [$^3$H]-N-methyl scopolamine ([$^3$H]-NMS). Gallamine did not completely inhibit [$^3$H]-NMS binding and this effect became larger with increasing radioligand concentrations. In addition, the rightward shift in the gallamine inhibition curves in the presence of increasing concentrations of [$^3$H]-NMS became limiting.

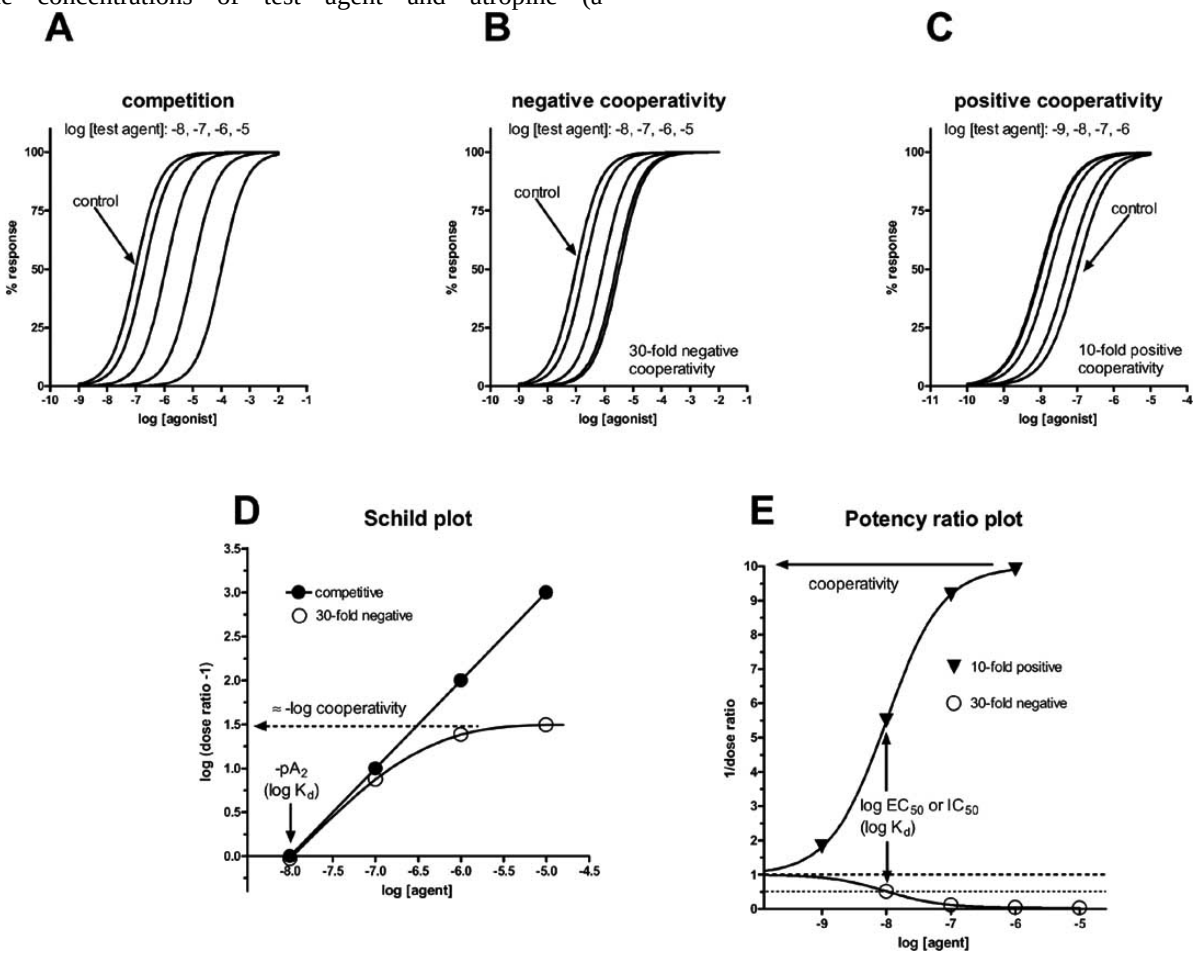



**Fig. (2).** Analysis of concentration-response curves for a competitive inhibitor, an allosteric inhibitor and an allosteric enhancer.

Theoretical dose-response curves are shown for an agonist ($EC_{50}$ $10^{-7}$M) in the presence increasing concentrations of a test agent with a $K_d$ of $10^{-8}$M, which is either (A) a competitive inhibitor, (B) an allosteric inhibitor, or (C) an allosteric enhancer. The parallel rightward shift of the agonist curve in the presence of inhibitor is expressed as a dose-ratio (ratio of equieffective agonist concentrations in the presence and absence of inhibitor) and the Schild plot (D) shows the log(dose-ratio –1) plotted against the log concentration of inhibitor. The Schild plot of the competitive inhibitor is a straight line, whereas the Schild plot of the allosteric inhibitor curves over and eventually reaches a asymptotic value, which is approximately equal to –log cooperativity (where negative cooperativity has values <1). For both competitive and allosteric inhibitors the log concentration of inhibitor associated with zero on the Y axis (i.e causing a 2-fold increase in the agonist $EC_{50}$) is the log $K_d$ of the inhibitor. In the affinity ratio (or potency ratio) plot (E) 1/dose-ratio is plotted against the log concentration of allosteric agent: the curves start at 1 and reach an asymptotic value equivalent to the cooperativity. For both positive and negative allosteric agents the log $EC_{50}$ or log $IC_{50}$ values correspond to the log $K_d$ of the allosteric agent.

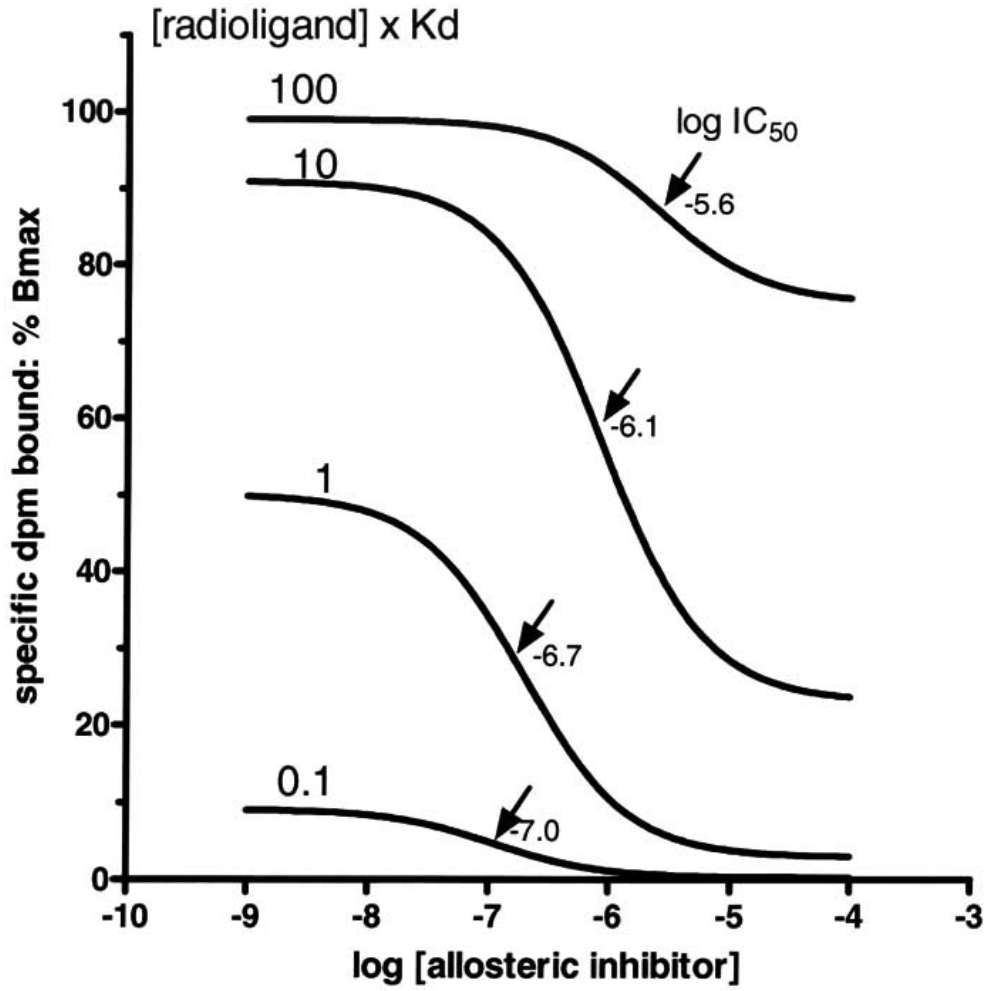



**Fig. (3).** A binding assay to distinguish between competitive and allosteric inhibition.

Theoretical curves show inhibition by an allosteric inhibitor (cooperativity = 0.033, or 30-fold negative cooperativity, Kd = $10^{-7}$M) of various concentrations of a radioligand. At radioligand concentrations much less than its Kd, the inhibition of binding seems almost complete and the log $IC_{50}$ of the inhibitor approximates to its log Kd. With increasing radioligand concentrations the inhibitor becomes less potent, as would also be seen with a competitive inhibitor, but crucially, the allosteric inhibitor becomes increasingly unable to inhibit all the radioligand binding because of formation of the ternary complex. A competitive inhibitor, in contrast, will completely inhibit radioligand binding, regardless of the radioligand concentration (unless there are other issues, e.g. insolubility at high inhibitor concentrations).

An additional crucial observation was that gallamine inhibited the dissociation of [$^3$H]-NMS [20] and (-)-[$^3$H]-3-quinuclidinylbenzilate ([$^3$H]-QNB) [21], an effect that must by its nature be mediated by a site which is different from the orthosteric site to which the radioligand is bound.

Gallamine was shown to interact allosterically, but with different affinities at muscarinic receptors in different rat tissues considered to express different subtypes [20] and later the presence of an allosteric site for gallamine on all the cloned muscarinic receptor subtypes was demonstrated [22,23].

## The Meaning of 'Allosterism'

The word 'allosteric' has been used in somewhat different ways in the past [24,25]. In the current context, and for the purposes of this review, an allosteric site is defined as a domain of the receptor that is spatially distinct from the site to which the endogenous ligand binds (which is referred to as the 'orthosteric' site). This spatial separation allows allosteric and orthosteric ligands to bind simultaneously and reversibly with the receptor. The two sites are also conformationally linked such that the binding of a ligand to one site changes the conformation, or the balance between different conformational states, at the second site. Of course, other allosteric sites on the receptor are also engaged in numerous binding events with components of the endogenous signal transduction system, such as G-proteins, receptor kinases and sodium, but this review will concentrate on *exogenous* allosteric ligands and the site or sites to which they bind.

There have been a number of reviews of allosteric interactions at muscarinic receptors [26-33] and allosteric interactions at GPCRs in general have been reviewed recently [34,35]. The aim of this review is to discuss how allosteric ligands have been evaluated at muscarinic receptors, and in particular, how well the experimental data have been capable of being interpreted in terms of the

allosteric ternary complex model. The complexities of the determination of useful SAR information of allosteric ligands are also discussed. These are especially in evidence in the case of muscarinic receptors, where there are multiple allosteric sites and complex interactions. In this review structural inferences resulting from published mutagenesis studies are not discussed in detail.

## THE ALLOSTERIC TERNARY COMPLEX MODEL

### Description

The binding of the allosteric ligand may alter any property of the receptor, such as its preferred set of conformations, its affinity for orthosteric ligands, its affinity for other membrane constituents etc. so a large variety of allosteric phenomena are conceivable. Fortunately, with a few exceptions, the effects of allosteric agents at muscarinic receptors can be accounted for with the simplest allosteric model, in which binding of the allosteric ligand alters only the affinity of the orthosteric ligand. The allosteric interaction between two ligands is also referred to as a 'cooperative' effect.

The simplest possible ternary complex allosteric model is shown in Fig. (**4**). The receptor exists in a single state which can bind simultaneously the orthosteric and allosteric ligands with affinity constants $K_A$ and $K_X$ respectively and the sole consequence of the binding of one type of ligand is to alter the affinity of the other type of ligand for the receptor [20,36,37]. The change in affinity of the orthosteric ligand for the free and allosterically-liganded receptor is the cooperativity of the system. *This relationship is reciprocal*, i.e. if the affinity of the orthosteric ligand for the free and allosteric-liganded receptor differs by a factor $\alpha$, then the affinity of the allosteric ligand for the free receptor is changed by the same factor $\alpha$ when the orthosteric ligand is bound.

The value of the cooperativity factor $\alpha$ depends on the specific identities of the three components of the system - the orthosteric and allosteric ligands and the receptor subtype, so that a particular allosteric agent may have different cooperativities with different orthosteric ligands at the same receptor, and different cooperativities with the same orthosteric ligand at different subtypes. This model does not distinguish between agonists and antagonists and does not predict any change in either the efficacy of agonists or the total concentration of receptors (the Bmax measure from radioligand saturation studies). An analysis of the equilibrium effects of an allosteric ligand on the binding of an orthosteric ligand provides independent estimates of $K_X$ and $\alpha$ (and hence $\alpha.K_X$) [20,37].

### Relationship to the TCM

The allosteric ternary complex model shown in Fig. (**4**), is very similar to the ternary complex model of ligand-receptor-G protein interaction (TCM), where the allosteric ligand is a G-protein [38]. There are two main differences:

(1) The TCM 'recognises' the efficacy of the ligand, i.e. agonists show positive cooperativity, neutral antagonists show neutral cooperativity, and inverse agonists show negative cooperativity, whereas in the allosteric ternary complex model the efficacy of the ligand does not determine the cooperativity of the system.

(2) The allosteric ternary complex model assumes that the allosteric agent is present in excess, i.e. the free concentration of allosteric ligand is always the same as the added concentration, whereas in the TCM the free concentration of G-protein is reduced as receptors and G-proteins bind together: it is this depletion of free G-protein which, in theory [39], accounts for the 'flat' inhibition curves often seen in radioligand binding assays with unlabelled agonists [40], but not with unlabelled antagonists [11]: in contrast, the allosteric ternary complex model always generates curves with slope factors of 1.

### More Complex Models

The allosteric ternary complex model is an extension of the simplest mass action model of receptor-ligand interaction. More elaborate models have been developed to

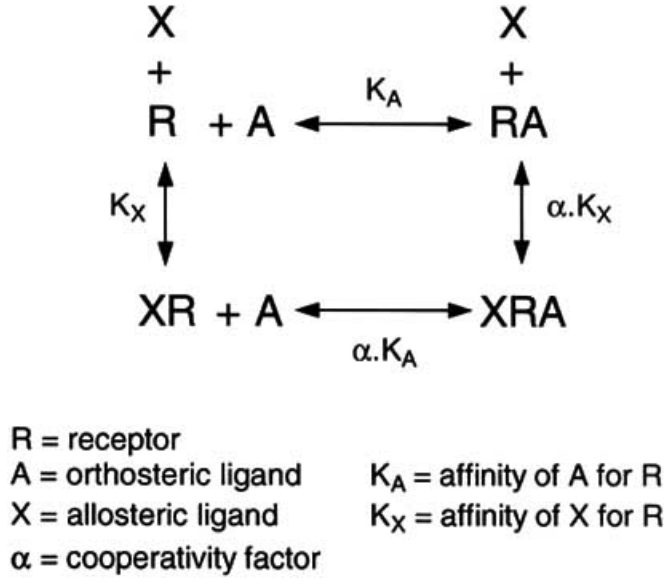



**Fig. (4).** The allosteric ternary complex model.

take account of complexities of receptor-ligand interactions found in binding and functional studies, and of increasing understanding of receptor regulation. Thus an allosteric site has been added to the TCM to yield a receptor which simultaneously binds orthosteric and allosteric ligands and a G-protein [41], and to the two-state receptor model [42] to yield a receptor which simultaneously binds orthosteric and allosteric ligands and which is in an inactive or active state [20,43]. A two-state model containing both allosteric ligand- and G-protein- binding sites has also been described [34].

These are 'cubic' models (i.e. the different receptor states and the constants linking them can be depicted as a cube) or even more complex models which contain a large number of parameters and interactions, many of which are not individually observable. In these instances, experimental data are related to the model by simulation, rather than by non-linear regression analysis. The allosteric ternary complex model itself can be used for non-linear regression analysis, as can extended versions containing two allosteric sites [44,45] or allowing a ligand to bind to both the orthosteric and allosteric sites [44,46].

Other complex models of GPCR-ligand interaction, such as the model incorporating effects of the βγ G-protein subunits [47], have not yet been modified by incorporating an allosteric ligand site. A general model of orthosteric and allosteric interactions of ligands with receptors and G-proteins which can themselves form oligomeric arrays has been provided by Wells [48].

## EVALUATION OF ALLOSTERISM AT MUSCARINIC RECEPTORS

This section deals with the methods that have been devised to measure and quantitate allosteric interactions at muscarinic receptors. However the methodology is applicable to the study of allosterism at other GPCRs.

### Radioligand Kinetic Assays

Allosteric agents often modify the kinetics of radioligand binding, both association and dissociation. The effects on radioligand dissociation, in particular, provide a clear indication of an allosteric, as opposed to competitive, interaction. If the radioligand dissociates monoexponentially and only its dissociation rate constant is modified by the allosteric agent, and if it can be assumed that the allosteric agent equilibrates rapidly with the radioligand-occupied receptor, then the concentration-effect curve of the allosteric agent for modifying the radioligand dissociation rate constant corresponds to the occupancy curve of the allosteric agent for the radioligand-occupied receptor [37,49]. Most allosteric agents cause strong or complete inhibition of [$^3$H]NMS dissociation, but have different effects on other muscarinic radioligands (for example [$^3$H]QNB and [$^3$H]ACh) [50,51]. Even with [$^3$H]NMS, allosteric agents can cause partial inhibition, no effect, or even stimulation of dissociation [45,50,52,53].

### Radioligand Equilibrium Assays

The ability to detect allosteric effects on equilibrium radioligand binding depends on the direction of the cooperativity and the radioligand concentration. If a single concentration of radioligand is used, positive cooperativity is best determined with low radioligand concentrations, below the Kd if practicable, and negative cooperativity, which is detected as a non-zero asymptote of radioligand inhibition, is best determined at high radioligand concentrations [see Fig. (**3**)]. Preferably a number of radioligand concentrations are used: the data can be considered both as a set of allosteric agent titrations and as a set of radioligand saturation curves.

Allosteric effects on an unlabelled ligand such as ACh are determined by measuring the effects of the allosteric agent on radioligand binding in the absence and presence of the unlabelled ligand. Typically this involves constructing inhibition curves of the unlabelled ligand in the absence and presence of various concentrations of the allosteric agent and analysing the data with non-linear regression analysis, but a more efficient design, which is suitable for medium-throughput screening, measures effects of a range of concentrations of the allosteric agent in the absence and presence of a single concentration of unlabelled ligand: this design can be analysed both by non-linear regression analysis and by visual inspection of the data transformed into 'affinity ratios' (Fig. (**2e**)) [37,49,54].

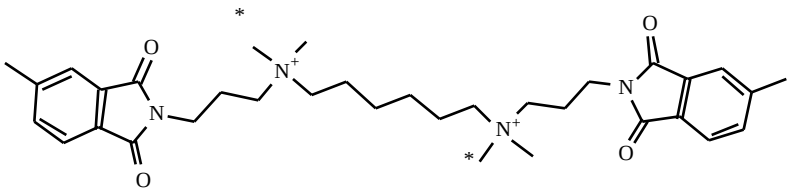
It is always wise to consider the results from equilibrium assays in the context of effects on radioligand dissociation. A slowing of dissociation will often lead to a slowing of the observed association rate, so if the allosteric agent slows radioligand dissociation within the same concentration range as it affects 'equilibrium' binding then the binding may not, in fact, have reached equilibrium. This will result in inhibition of radioligand binding that may be misinterpreted as allosteric or competitive inhibition, rather than the kinetic artefact it really is. Kinetic artefacts are most likely to be seen with allosteric agents showing positive, neutral or low negative cooperativity with the radioligand. Equations for handling such kinetic effects have been described [37,49].

### Checks on the Validity of the Allosteric Ternary Complex Model

The effects of allosteric ligands on the dissociation rate of radiolabelled orthosteric ligands (described above) provide a means of confirming the validity of the allosteric ternary complex model. The concentration-dependence of an allosteric ligand for changing the dissociation rate constant is governed by the affinity of the allosteric ligand for the radiolabelled ligand-occupied receptor ($\alpha.K_X$). This estimate can be compared with the product of the estimates of $\alpha$ and $K_X$ obtained from the equilibrium studies; if the allosteric ternary complex model is applicable, these two estimates of $\alpha.K_X$ should be identical.

The labelling of the allosteric site on $M_2$ receptors by the radioligand [$^3$H]Dimethyl-W84 (**2**, *N*,*N*'-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2*H*-isoindol-2-yl)propyl]-*N*,*N*,*N*',*N*'-tetramethyl-1,6-hexanediaminium diiodide, Fig. (**5**)) has also provided independent estimates of $K_A$, $K_X$ and $\alpha.K_A$ which were the same as the equivalent values obtained using [$^3$H]NMS as a label of the orthosteric site [55,56].

It is a general finding in muscarinic receptor studies (and studies of allosterism at other GPCRs) that the allosteric ternary complex model provides a satisfactory description of allosterism in radioligand binding studies. However there are many ligands, including atropine and the selective



**2:** Dimethyl-W84

**Fig. (5).** Structure of the allosteric radioligand, [$^3$H]Dimethyl-W84. The asterisks show the positions of the $^3$H labels.

antagonists AF-DX 116 ((±)-11-([2-[(diethylamino)methyl]-1-piperidinyl]acetyl)-5,11-dihydro-6-pyrido[2,3-b] [1,4]benzodiazepin-6-one), AF-DX 384 ((±)-5,11-dihydro-11-[2-[2-[*N*,*N*-dipropylaminomethyl)piperidin-1-yl]ethyl-amino]carbonyl]-6*H*-pyrido[2,3-b][1,4] benzodiazepin-6-one), and pirenzepine, that inhibit [$^3$H]NMS dissociation at high (mM) concentrations (with α values predicted to be less than $10^{-3}$ and sometimes as low as $10^{-6}$) [57,58]. It is not yet known whether these compounds are binding to an allosteric site or affect radioligand dissociation through a non-specific mechanism, for example by binding to the carbohydrate residues of the glycosylated receptor and thereby providing a positively charged 'haze' that the positively charged radioligand has to permeate in order to enter or exit the binding site [14].

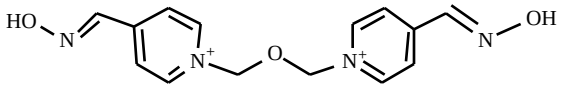
## Functional Assays

Functional assays allow the detection of allosteric effects on the efficacy as well as the potency of agonists. Agonist concentration-effect curves are shifted to the left by positively cooperative allosteric agents and to the right by negatively cooperative agents. Negative cooperativity is manifested by a limit to the degree of rightward shift, and may require high concentrations of allosteric agent and agonist. The allosteric ternary complex model predicts that an allosteric agent will affect only the potency of the agonist, and not the shape of its concentration-effect curve (e.g. slope factor) or maximal effect. For a negatively cooperative interaction the data may be visualised as Schild plots of log (dose-ratio –1) versus log [allosteric agent], where dose-ratio is the ratio of equi-effective agonist concentrations in the presence and absence of a particular concentration of allosteric agent (Fig. (**2a)**), and can be analysed according to Ehlert [59]. For a positively cooperative interaction the plot of the potency ratio (the reciprocal of the dose-ratio) versus log [allosteric agent] should have a slope of unity, with $EC_{50}$ corresponding to the Kd of the allosteric agent and Emax corresponding to the allosteric constant (Fig. (**2a)**). Alternatively, either sort of cooperative interaction may be analysed by fitting the raw data directly to the allosteric model [37,49].

Functional assays may also identify an allosteric inhibitor (Y) by its interactions with a known orthosteric competitor (Z). If Y were an orthosteric inhibitor then the dose-ratio of a combination of Y and Z would be the sum of the dose-ratios in the presence of either ligand alone. If Y and Z have non-additive effects this argues for an allosteric mechanism for Y. Non-additivity may arise through differences in the cooperativity of Y with Z and the agonist [35] but also through Z (or the agonist) not reaching binding equilibrium in the presence of Y as a consequence of its slowing down the kinetics of Z (or the agonist).

## Competition Between Allosteric Ligands – Multiple Allosteric Sites?

Although interaction studies with two allosteric agents in equilibrium and kinetic radioligand assays can sometimes yield clear conclusions, the task of deciding whether two allosteric ligands act competitively with respect to each other (implying action at the same site) or with negative cooperativity (implying action at different sites) can be difficult or impossible. This is because the allosteric effect on radioligand binding to the dually liganded receptor, and the effect on dissociation of the radioligand from the dually liganded receptor, may in principle be quite different from the effect of each allosteric ligand alone. Also, high concentrations of allosteric ligands must be used to detect and quantify the interactions, but these high concentrations may cause a profound prolongation of the time taken for binding to come to equilibrium as well as small nonspecific effects. On the one hand, allosteric ligands which appear to bind competitively might be found to bind noncompetitively if sufficiently stringent analyses were employed; on the other hand, small but significant deviations from a purely competitive interaction, as we have found with obidoxime (**3**, Fig. (**6**)) and brucine at [$^3$H]-NMS-occupied $M_2$ receptors (Lazareno & Birdsall, unpublished observations), may reflect nonspecific effects of high ligand concentrations rather than binding to two distinct sites.



**3:** Obidoxime

**Fig. (6).** Structure of obidoxime.

If two allosteric agents have different maximum effects then it is possible to test whether they are acting at the same site or allosterically with each other *via* different binding sites. This approach has been used in equilibrium assays [44,45] and radioligand dissociation studies [45,49,53,54] and the specific methodologies are described in these references. The results of some of these studies are described in the later sections of this review that deal with SARs and the pharmacology of the two well characterised allosteric sites on muscarinic receptors.

## Agonist and Inverse Agonist Actions of Allosteric Ligands

The allosteric ternary complex model predicts that allosteric agents will not show agonist actions themselves, or modify the efficacy of orthosteric agonists, and this is what is usually observed [59-61]. However there are some

reports that indicate that some agonists may activate muscarinic receptors from an allosteric site [62-64] and that allosteric ligands *alone* can increase or decrease constitutive activity [65-67] and change agonist efficacy [65]. These results cannot be explained by the allosteric ternary complex model, though one of the more complex models described above, or a variant thereof, may in due course be found to accommodate some of these data.

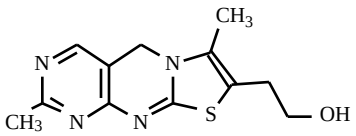
## SELECTIVITY ASSOCIATED WITH ALLOSTERISM

It has been difficult to develop subtype-selective muscarinic agonists and antagonists, perhaps because the structure of the binding region for ACh in the receptor (the orthosteric site) is strongly conserved across the muscarinic receptor subtypes [14,15,68,69]. It might be anticipated that an allosteric site on a receptor would be less strongly conserved across subtypes, provided the site is not the binding site for another endogenous ligand or is part of a domain of the receptor that performs another important function.

Allosteric ligands may therefore have a greater potential for showing subtype-selective affinity than orthosteric agonists and competitive antagonists. In fact the first two $M_2$-selective antagonists to be discovered, gallamine and methoctramine, act wholly or in part through an allosteric mechanism [44]. However, the idea that allosteric agents have a tendency for selective affinity at $M_2$ receptors is not always supported by our experience. For example, strychnine and brucine analogues show equal affinity for $M_1$, $M_2$ and $M_4$ receptors [54,70], staurosporine and some other indolocarbazoles have a tendency towards $M_1$ selectivity [45], and a series of analogues of WIN 62,577 (17-β-hydroxy-17-α-ethynyl-$\Delta^4$-androstano[3,2-*b*]pyrimido[1,2-*a*]-benzimidazole) have a degree of $M_4$ selectivity [53]. The most selective ligand, the muscarinic MT7 toxin from the venom of the Eastern Green mamba, is an extremely potent allosteric ligand with > 3,000 fold selectivity for $M_1$ receptors over all the other subtypes [71-74]. It differsfrom other muscarinic toxins both in terms of its potency and its pseudo-irreversible action on $M_1$ receptors.

In addition to the possibility of selective affinity, allosteric agents can also achieve selectivity through cooperativity. For example, brucine shows positive cooperativity with ACh only at $M_1$ receptors, with some degree of negative cooperativity at the other subtypes, while N-chloromethyl brucine is positively cooperative with ACh only at $M_3$ receptors, exhibiting neutral cooperativity at $M_4$ receptors, weak negative cooperativity at $M_1$ and $M_5$ receptors and strong negative cooperativity at $M_2$ receptors [54,61]. Clearly, allosteric agents have the potential to display many varied patterns of cooperativity with ACh or other orthosteric ligands across the muscarinic receptor subtypes. The enhancement of the actions of submaximal concentrations of ACh by brucine and N-chloromethyl brucine are diagnostic of activation of $M_1$ and $M_3$ receptors respectively.

The existence of neutral cooperativity, where the allosteric ligand binds to the receptor but does not alter the affinity of an orthosteric ligand (in particular the endogenous ligand, ACh), gives rise to the possibility of a particularly powerful and novel form of selectivity which we refer to as 'absolute subtype selectivity'. If an allosteric agent shows positive or negative cooperativity with ACh at one receptor subtype, A, and neutral cooperativity with ACh at all the other subtypes, then application of the allosteric agent will only affect ACh action at subtype A, regardless of the concentration or dose of the allosteric agent and regardless of its relative affinities for the receptor subtypes [37,54,75]. The first example of 'absolute subtype selectivity' at $M_1$-$M_4$ receptors is thiochrome (**4**, 2,7-dimethyl-5*H*-thiachromine-8-ethanol, Fig. (**7**)), an oxidation product and metabolite of thiamine. Thiochrome enhances ACh affinity at $M_4$ receptors and has neutral cooperativity at the other subtypes [76]. It appears to have a selectively low affinity for $M_5$ receptors so, whether through selective affinity or absolute subtype selectivity, an enhancement of ACh action by thiochrome is diagnostic of an effect at $M_4$ receptors [76].




**4:** Thiochrome

**Fig. (7).** Structure of thiochrome.

## REFLECTIONS ON STRUCTURE-ACTIVITY RELATIONSHIPS OF ALLOSTERIC LIGANDS

### Multiple SARs

The simplest parameter that describes the binding of an allosteric ligand, X, is its affinity for the unliganded receptor, $K_X$. However, as presented in an earlier section, the properties of an allosteric ligand are also characterised by its affinities for receptor-orthosteric ligand complexes, $\alpha.K_X$. As any given $\alpha$ depends on the nature of the orthosteric ligand, A, there is a *set* of affinity constants $\{\alpha_A.K_X\}$, that describes how the binding of different A has changed the structure of the allosteric site, as reflected in the modified binding of X. Equally the binding of X changes the conformation (or conformational preferences) of the orthosteric site and hence the SAR of different A for that site. The first demonstration of the latter phenomenon was that gallamine changed the SAR of muscarinic agonists and antagonists at the cardiac ($M_2$) muscarinic receptor [20]. Detailed studies have examined the cooperativities between a number of additional allosteric ligands and a range of antagonists [77] and agonists [78] at $M_2$ receptors.

For a set of allosteric ligands, {X}, interacting with n orthosteric ligands ($A_1 \ldots A_n$), the binding properties of a given allosteric site on a single receptor subtype are described by (n + 1) SARs, i.e. one for binding to the unliganded receptor and one for the binding to each liganded receptor. The use of these SARs to *predict* the cooperativity between {X} and a given $A_i$ relies on the evaluation of the *difference* of the two SARs. The intrinsic uncertainty associated with each SAR makes the prediction of cooperativity difficult, especially where the cooperativity values are close to 1 and it is important to be able to predict whether a given allosteric ligand will be an allosteric enhancer or inhibitor.

A further complication is that, in the case where an agonist is the orthosteric ligand, one has to consider the SAR of the allosteric site at both the high affinity agonist-receptor-G protein complex and at the receptor uncoupled from the G protein. In this instance, the G protein has a positively cooperative allosteric effect on agonist affinity and may well also have an allosteric effect on the binding of the allosteric ligand as well as changing the observed cooperativity between the agonist and the allosteric ligand.

In the case of muscarinic receptors much of the earlier published SAR data is limited in that it only described the potencies of allosteric ligands to modulate (mostly slow down) the dissociation rate of an orthosteric radioligand (generally [$^3$H]-NMS). Values of $\alpha_{NMS}.K_X$ were reported but there was no information regarding $K_X$ or $\alpha_{NMS}$. This was because most ligands were exhibiting strong negative cooperativity with the radioligand, and the authors considered that only the effects on radioligand dissociation were a valid reflection of allosterism; it was not possible to exclude the possibility that a given ligand occupied the orthosteric site at low concentrations, only occupying the allosteric site at much higher concentrations when the orthosteric site was occupied by the radioligand, with this latter interaction being what was detected in the dissociation experiments. This is a valid argument and indeed, in the limit, it is difficult to exclude this possibility by equilibrium binding studies where both $\alpha_A$ and $K_X$ can be estimated, even when there is positive or neutral cooperativity.

Only structural or detailed biophysical and kinetic studies can resolve the nature of the site on the unliganded receptor to which an allosteric ligand binds. Suggestive, but not conclusive, evidence may be provided by mutagenesis studies in which the mutation of a specific residue on the receptor modifies the binding of an allosteric ligand to the unliganded receptor and to the liganded receptor in the same direction, but possibly to different extents, and that furthermore this mutation does not affect the binding of a range of orthosteric ligands. One example is the mutation of W400 of the $M_1$ receptor to alanine that decreases the affinity of gallamine to the unoccupied and NMS-occupied receptor to similar extents whilst having much smaller effects on the binding of orthosteric ligands [79]. A converse example is provided by the suggestion that the cardioselective antagonist UH-AH 37 (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11*H*-dibenzo[*b,e*][1,4]diazepine-11-one hydrochloride) utilises different epitopes when it binds to the unoccupied and NMS occupied $M_2$ receptors [80].

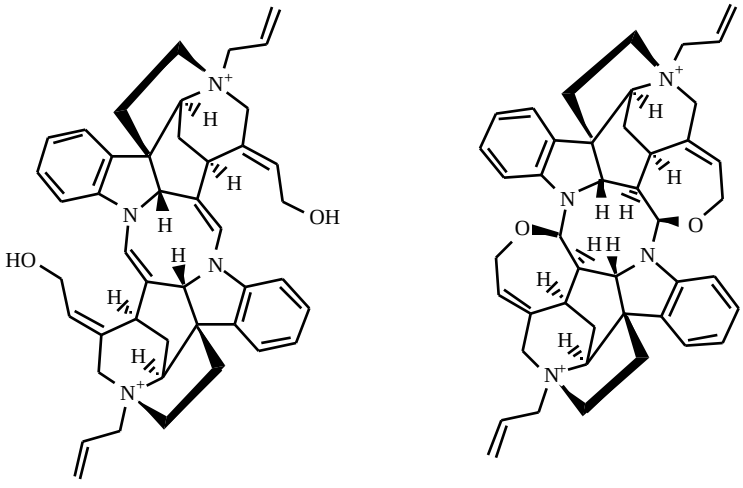
Another indication that an allosteric ligand is binding to the allosteric site on the unliganded receptor is that the two SARs of a series of allosteric ligands, one for the unliganded and the other for the liganded receptor, occupied by a given orthosteric ligand (e.g. NMS), should be similar. The two SARs would not be expected to be identical, because the orthosteric ligand can modify the structure of the allosteric site, and vice versa, and thus change the cooperativity. This argument assumes that the allosteric ligand is interacting with a set of conserved residues that constitute the allosteric site and that the relative disposition of these residues is only modestly perturbed by the binding of the orthosteric ligand.

## Different Modes of Binding to a Single Site

Even when it can be determined that two ligands are likely to be interacting at the same site, sensitive approaches can sometimes provide evidence that they are probably binding in different modes. If that is so, these ligands will not be part of the same SAR series. For example, mutagenesis studies have suggested that the structurally related ligands, alcuronium (**5**) and an N-allyl caracurine V analogue (**6**) utilise different binding epitopes on the $M_2$ receptor (Fig. (**8**)) [81]. This conclusion is in accord with NMR evidence that these two molecules have somewhat different conformations in solution [82].

## Experimental Conditions can Profoundly Modify SARs

The binding of allosteric ligands to muscarinic receptors can be very sensitive to the incubation conditions of the binding assay. Binding is sensitive to both ionic strength as well as to the concentration of divalent cations and to a lesser extent to temperature. (e.g. [22, 57, 83-89]. The binding of gallamine can change by up to 1000-fold under conditions where the binding of [$^3$H]-NMS is essentially unchanged. The change in affinity of allosteric ligands is



**5:** Alcuronium **6:** Diallylcaracurine V

**Fig. (8).** Two closely related allosteric ligands that use different binding epitopes on the $M_2$ receptor.

found at both the unoccupied and [$^3$H]-NMS occupied receptors [84,89] and seems to be particularly pronounced for ligands that are di- or trivalent cations. The most potent allosteric actions are generally found in a 5 mM phosphate buffer in the absence of divalent cations [86-89]. For gallamine these potencies can be up to 1000 fold higher than are found in functional studies. The different effects of buffer conditions on the potencies of allosteric ligands of different classes has also been used to infer different modes of binding of allosteric ligands [89].

In this article we mention when binding studies were carried out at low ionic strength. These results cannot be directly compared to those carried out in higher ionic strength buffers that contain divalent cations (where the potencies of allosteric ligands are in reasonable agreement with those found in functional studies).

An example of a profound change in SAR produced by performing the binding assays under different experimental conditions was provided by Pedder and coworkers [84]. The affinity of AF-DX 116 (a ligand which had been reported to have allosteric actions, [23,90,91]) for the unoccupied $M_1$ receptor *decreased* 10 fold when a 100mM NaCl/10mM $Mg^{2+}$/20mM Hepes buffer at 30$^o$ was changed to a 1mM $Mg^{2+}$/20mM Hepes buffer. This change increased the affinity of gallamine 400 fold whereas the affinities of a number of 'conventional' orthosteric antagonists were increased by less than 2-fold. The effect of these changes is that, depending on the conditions, the ratio of the gallamine/AF-DX 116 affinities can vary from 60:1 to 0.02:1. Clearly these two ligands are binding to the unliganded receptor in different ways from the orthosteric antagonists examined.

## Multiple Allosteric Sites

Any SAR analysis requires the knowledge of the identity of the binding site to which specific ligands bind. As discussed above, this is often not as simple as might first appear because many ligand-ligand interactions may appear to be competitive when in reality they are high negatively cooperative [22]. This difficulty is amplified when one examines interactions between allosteric ligands as these interactions can, in general, only be observed indirectly by their effects on the binding of a radiolabelled orthosteric ligand.

In the case of muscarinic receptors, most allosteric ligands have such profound effects on the dissociation of the favoured radioligand, [$^3$H]-NMS, that there is no measurable dissociation from the ternary complex. This means that it is effectively not possible to determine negative cooperative interactions between two such allosteric ligands ($\alpha < 0.2$) in equilibrium studies as the high concentrations of both allosteric ligands required to form the receptor complex with the two allosteric ligands bound results in immeasurably slow [$^3$H]-NMS dissociation rates. Positive or neutral cooperativity between the two allosteric ligands ($\alpha \geq 1$) can however be measured.

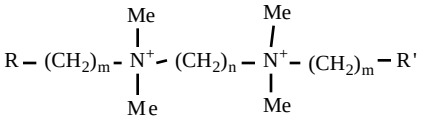
Ellis and co-workers advanced the field when they discovered that obidoxime (**3**) only causes a small inhibition of the [$^3$H]-NMS dissociation rate constant at $M_2$ receptors [50]. This allowed interaction experiments to be performed as only one ligand was producing a profound kinetic slowing. It was found in off-rate assays that gallamine shifted the concentration-response curve for obidoxime to the right in a manner which was consistent with both ligands acting at the same site. However, in this study, a negative cooperativity of 0.1 would not be distinguishable from competition because obidoxime only binds weakly to muscarinic receptors, even at low ionic strengths, and sufficiently high concentrations of obidoxime cannot be used. There are now a number of more potent allosteric muscarinic ligands that either have small or no effects on radioligand dissociation or even enhance radioligand dissociation and interaction studies have been reported [44,45,51-53].

In a complementary approach Waelbroeck [44] found that *d*-tubocurarine caused only a small (3-4 fold) reduction in the affinity of [$^3$H]-NMS, which allowed interaction experiments to be performed in equilibrium studies. The potency of gallamine for inhibiting $^3$H-NMS binding was reduced in the presence of *d*-tubocurarine to a degree which was consistent with a competitive interaction between the allosteric ligands, while tubocurarine did not affect the potency of AF-DX 116 or telenzepine, demonstrating that these ligands must bind to a site (possibly the orthosteric site or another allosteric site) which is different from the gallamine/tubocurarine site. In the case of the gallamine/tubocurarine interaction the kinetic slowing effects limited the concentrations of gallamine and *d*-tubocurarine that could be used and so a moderately strong negatively cooperative interaction between these two allosteric ligands could not be excluded.

Studies of the interactions between pairs of allosteric ligands using the equilibrium and kinetic approaches is strongly favoured if one of the allosteric ligands has small effects on radioligand dissociation *and* is not strongly negatively cooperative with the radioligand. Examples of the small number of such ligands that are commercially available are obidoxime, KT5720 (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*] [1,6]benzodiazocine-10-carboxylic acid hexyl ester), WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole) and WIN 62,577. The latter three ligands are relatively potent and have been used to demonstrate that they to bind to a different site from that to which gallamine, strychnine and probably obidoxime bind [45,53]. The small number of suitable ligands limits the number of allosteric pairs that can be investigated and one has to build up a consistency argument in the attempt to decide whether a given allosteric ligand is binding to one of the two known allosteric sites on muscarinic receptors or to another, as yet uncharacterised, site. At the present time the SARs of the two allosteric sites are being determined.

## Summary

Much of the published quantitative data that report the allosteric interactions of any given ligand, or series of ligands, with muscarinic receptors describe their potency to inhibit [$^3$H]-NMS dissociation from $M_2$ receptors under low ionic strength conditions. There is a much less extensive literature on the potency at the unliganded or ACh-occupied $M_2$ receptor, at other muscarinic receptor subtypes, or on the effects of these allosteric interactions on muscarinic receptor function. This lack of data relevant to drug design has been

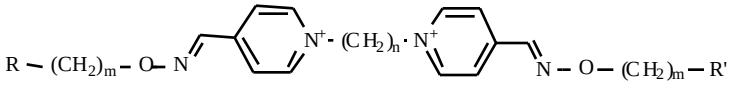


**7**

R = R' = H, m = 1, n = 6, Hexamethonium

R = R' = Phth, m = 3, n = 6, W84

R = R' = Phth, m = 3, n = 7, C7/3'Phth



**8**

**Fig. (9).** Bis-onium ligands related to hexamethonium [Phth = N-phthalimido- ].

due to an emphasis on understanding the molecular aspects of allosterism at muscarinic receptors rather than attempting to develop the allosteric molecules into lead structures for drug development.

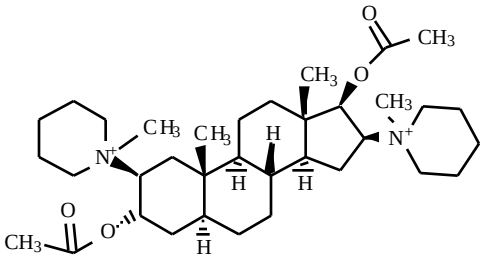
For all investigations it is crucial to determine whether the phenomena observed are compatible with the predictions of the allosteric ternary complex model (or one of the more complex models). Where this has been investigated, there has been excellent agreement between the data and theory [see e.g. 38,45,53,54,69,75,92-98], including recent reports when the radiolabelled allosteric ligand (**2**) was used to directly monitor the competitive binding of ligands to the allosteric site on the $M_2$ receptor and the allosteric effects of orthosteric ligands on its binding [55,56].

It is clear from the above comments that SAR approaches to study and analyse the binding of muscarinic allosteric ligands are complex and are at an early stage. In the following sections we consider ligands that have been reported to have allosteric actions on muscarinic receptors.

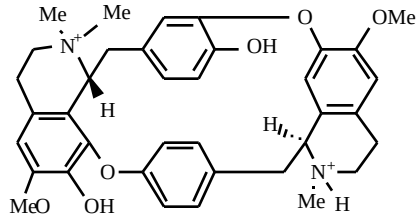
## ALLOSTERIC MUSCARINIC LIGANDS

### Molecules Related to Neuromuscular Blockers – Bis-Onium Ligands

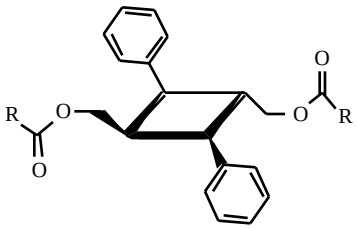
The first reported muscarinic allosteric ligands were the polycationic neuromuscular blockers, gallamine (**1**) [18,20,21] and many molecules illustrated by the generic formula (**7**, Fig. (**9**) [e.g. 17,99]. The latter compounds are related to hexamethonium (**7**, R = R' = H, m = 1, n = 6) and include W84 (**7**, R = R' = *N*-phthalimido, m = 3, n = 6, *N*,*N*'-bis[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)propyl]-*N*,*N*,*N*',*N*'-tetramethyl-1,6-hexanediaminium diiodide).



**9:** Pancuronium



**10:** *d*-Tubocurarine



**11**

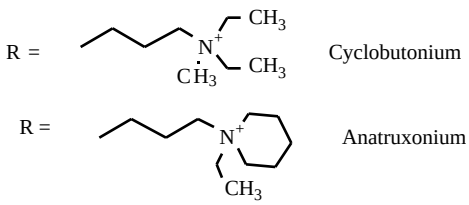



**Fig. (10).** Some neuromuscular blockers that are allosteric muscarinic ligands.

Other neuromuscular blockers reported many years ago to have allosteric muscarinic actions include, pancuronium (**9**, Fig (**10**)) [21,85] and stercuronium [100], followed by cyclobutonium and benzoquinonium [57], atracurium [101], tercuronium [85], ritebronium [102] and *d*-tubocurarine (**10**) [44,85,103]. In addition, acetylcholinesterase inhibitors and reactivators, of general structure **8** (Fig. (**9**)) and also bis-oniums related to obidoxime, were also shown to have an allosteric action at muscarinic receptors [50,103-107]. All these molecules, where investigated, appeared to exhibit negative cooperativity with [$^3$H]NMS or [$^3$H]QNB at $M_2$ receptors.

A breakthrough was the demonstration that another neuromuscular blocker, alcuronium (**5**) exhibited positive cooperativity with [$^3$H]-NMS at $M_2$ receptors [108]. At low ionic strengths, alcuronium had a very potent enhancing action at this subtype but had weaker actions and was negatively cooperative with [$^3$H]-NMS at the other subtypes at both low and high ionic strengths [70,109,110]. This indicated that positive cooperativity was attainable and that the cooperativity differed between subtypes. The enhancing action of alcuronium and its kinetic effects on [$^3$H]-NMS binding were also present in solubilized $M_2$ receptors, indicating that the alcuronium binding site was on the receptor molecule [111]. Alcuronium also enhanced the antagonist action of NMS on agonist-stimulated cardiac ($M_2$) receptors [112].

In the initial studies an apparent inhibitory effect of high concentrations of alcuronium on [$^3$H]-NMS binding to $M_2$ receptors was observed. This was interpreted in terms of a second molecule of alcuronium binding with low affinity to the orthosteric site. However the inhibition was later shown to be a kinetic artefact caused by the profound slowing by alcuronium of the association rate of [$^3$H]-NMS [29,37,113]. The positive cooperative action of alcuronium has only been reported at $M_2$ receptors and only for certain antagonists [77,114]. In the case of agonists, alcuronium has been shown to be negatively cooperative with ACh and oxotremorine-M at the $M_1$-$M_4$ subtypes [70,78,115]. At $M_2$ receptors, detailed binding and functional studies have shown that alcuronium is neutrally cooperative with pilocarpine [65], whereas the initial binding studies suggested a 3-fold positive cooperativity [78].

The most substantial SAR studies in this field have investigated the allosteric action of substituted *symmetrical* bis-ammonio alkanes (**7**) at $M_2$ receptors. Considerable elements of the SAR are embedded in the findings reported in the original paper of Lüllman *et al*. in 1970 [17] where the data relate to the potencies at the unoccupied receptor. A recent excellent review contains a summary of the more recent extensive findings in this area [33]. Most of these studies have involved the interaction of the molecules with the NMS-occupied $M_2$ receptor but some recent papers have also reported their potencies at the unoccupied $M_2$ receptor and hence have quantitated the cooperativity with NMS. There have been very few reports on the magnitude the cooperativity of the allosteric ligands with ACh or other agonists; to our knowledge there have been no reports that these molecules in this series are allosteric enhancers of ACh binding and actions at muscarinic receptors.

The generic molecule can be considered as two quaternary ammonium residues, separated from each other by a methylene chain of length n, with each quaternary residue also joined to a lateral substituent *via* an m-carbon chain (Fig. (**9**)). Molecules lacking a N-methyl substituent, i.e. the bis-tertiary N-methylamines, show little change in potency [116] but this preliminary report has not been followed up. The optimum length of the methylene chains between the onium groups is reported to be n = 8 at the unoccupied receptor [17] and n ≥ 7 carbons at the NMS-occupied receptor [117]. The effect of change in the value of m has not been systematically explored, the only evidence being that m = 2-4 carbon spacers gave molecules with comparable potencies at the unoccupied receptor [17]. Most analogues have m = 3. Alkylation of this chain has been reported in preliminary studies to increase affinity and favour positive cooperativity with [$^3$H]-NMS [96,118].

There have been extensive investigations of the effects of modifying the lateral phthalimido groups of W84 or the homologue C7/3´Phth (**7**, R = R' = *N*-phthalimido, m = 3, n = 7, *N*,*N*'-bis[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)propyl]-*N*,*N*,*N*',*N*'-tetramethyl-1,6-heptanediaminium diiodide). Initial studies indicated that replacement of the phthalimido ring of W84 by a benzene ring only resulted in a small decrease in potency [17]. However, removal of the aromatic ring or its reduction in the W84 analogue C7/3´Phth results in loss of affinity [119]. One of the keto groups of the phthalimido group does not seem to be important in that replacement by a methylene group does not affect the affinity [120]. However the affinity can be increased by introduction of a benzylidene group at this position [120], or by the presence of a methyl group on the aromatic ring [121] and by replacement of the phthalimido group by the larger 1,8-naphthalimido group [95,122]. In fact a number of large volume substituents can be tolerated at this position [95,123,124]. A tritiated bis-4-methyl-phthalimido analogue of W84 (**2**, Dimethyl-W84) has been developed as a radioligand for its allosteric site on $M_2$ receptors [55,56,121].

Extensive studies have also been carried out by the Bonn/Würzburg group on a series of symmetrical O-alkylated bispyridinium oximes (**8**) closely related to obidoxime [97,117,125,126]. These compounds tend to have a three-carbon methylene spacer between the positively charged nitrogens of the pyridine rings (n = 3). However n can be increased to 6 without affecting potency [117]. This contrasts to the preferred n > 6 carbon spacer in the bis-ammonio alkane series related to W84 (**7**). The properties of asymmetrical analogs of the bispyridinium oximes and the (2,6-dichlorobenzyl)ether derivatives (R ≠ R') will be considered in later sections.

A number of α-truxillic esters (**11**), including the neuromuscular blockers cyclobutonium and anatruxonium (Fig. (**10**)), are allosteric agents at $M_1$-$M_4$ receptors, exhibiting an element of $M_2$ selectivity [57,88,127]. Under low ionic strength conditions, the bis-3-piperidylpropyl ester of α-truxillic acid was reported to have a dissociation constant of less than 0.1 nM at the unliganded $M_2$ receptors but this value is increased 400 fold under the higher ionic strength conditions where function can be measured [88]. The compounds in this series were, in general, negatively cooperative with [$^3$H]-NMS except for bisquaternary analogs of anatruxonium bearing bulky N-substituents [127]. It appears that the effect of the increased bulk is to increase

affinity at the NMS-occupied $M_2$ receptor whilst having little effect on their affinity for the unoccupied receptor and increasing their muscarinic/peripheral nicotinic receptor selectivity.

So far symmetrical bis-oniums have been discussed. Most of these exhibit a considerable degree of conformational flexibility as regards the spatial separation of the cationic nitrogens. However pancuronium, (**9**), and other neuromuscular blockers (including alcuronium, **5**) have their positive charges separated from each other by a relatively fixed distance on a rigid backbone. Indeed pancuronium and related compounds are moderately potent allosteric ligands at $M_2$ receptors [21,57,85,88,100,127,128]. These results may provide constraints on any pharmacophore model of the conformation of the bound bis-oniums, assuming that a single model can describe these binding interactions.

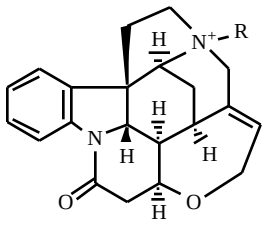
It has been concluded that the minimum separation between the two positive charges in the bis-oniums for high affinity binding to $M_2$ receptors is 10Å [117]. At first sight this is contradicted by the fact that a spacing of three carbons between the two nitrogens in the O-alkylated bispyridinium oximes, (**8**), is sufficient for optimum affinity. However, this paradox may be explained by the semi-empirical calculations of the charge distribution over the pyridinium rings that suggest the positive charges are located over the *para* carbon atoms and not over the nitrogen atoms [129].

In the specific area of the bis-onium ligands, there has been a strong emphasis on the synthesis of symmetrical bis-oniums. Where the binding properties of non-symmetrical ligands have been investigated [95,97,126,130,131] it appears, to a first level of approximation, that a lateral substituent which makes a major contribution to high affinity binding in the symmetrical bis-oniums also determines the binding affinity if it is present in a asymmetrical bis-onium. That is, there is a single major binding site on the receptor for only one of the lateral substituents, say R (Fig. (**9**)), of a given bis-onium with the second lateral substituent, R', only making a minor contribution to the binding affinity.

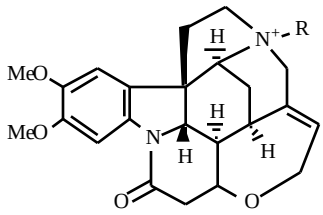
It has been perceived that bis-oniums are considerably more potent than the equivalent mono-quaternary species. In part, this assumption has been based on many assays of potency being carried out under low ionic strength conditions where the bis-quaternaries are much more potent than mono-quaternaries. It appears that one of the quaternary moieties of the bis-oniums interacts with a site on the receptor which either binds monovalent and divalent cations or whose conformation is changed by the binding of cations to another site. Indeed W84 has been reported to interact competitively with $Mg^{++}$ [87]. When the binding of bis-oniums is measured under higher ionic strength conditions, their potency may not to be very different from the potency of the equivalent monomer. However it is difficult to decide what structure to use as an 'equivalent monomer' and also it is important to remember a statistical factor of 2 in comparing the affinity of a symmetrical bis-onium to that of a monomer. The evidence is incomplete at present, but it appears that the site on the receptor that is sensitive to ionic strength may only interact with the quaternary ammonium moiety distal to the lateral substituent that is making the major contribution to binding affinity. The replacement of one of the quaternary nitrogens in W84 by silicon does not produce a decrease in affinity and in fact generates positive cooperativity at $M_2$ receptors whereas replacement of both cationic nitrogens by silicon results in a much weaker derivative [132]. It is not known whether the binding of this latter compound is sensitive to ionic strength; this fact could give an indication of its mode of binding, as could the synthesis of the two asymmetric silicon analogues of W84 lacking one lateral substituent.

## Mono-Quaternaries and Tertiary Amines Related to Alkaloids

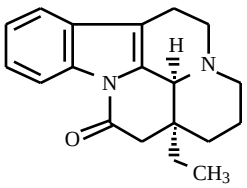
Alcuronium may be viewed as a functionalised dimer of strychnine and indeed strychnine (**12**, R = H, Fig. (**11**)) is the starting material for its synthesis. We and others found that strychnine itself is an allosteric ligand at $M_1$-$M_4$ muscarinic receptors [37,133]. The binding of strychnine satisfies the predictions of the allosteric ternary complex model and it has a comparable potency at 'physiological' ionic strengths to that of alcuronium at $M_1$, $M_3$ and $M_4$ receptors [70]. Alkylation of strychnine to give the quaternary derivatives (**12**) produces modest increases in



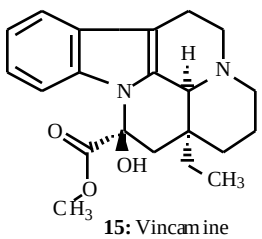
**12:** Strychnine, R = H



**13:** Brucine, R = H



**14:** (-)-Eburnamonine



**15:** Vincamine

**Fig. (11).** Some alkaloids and derivatives that are allosteric muscarinic ligands.

affinity, with the N-benzyl, N-2-naphthylmethyl and N-4-biphenylylmethyl derivatives having up to 60 fold higher affinities than alcuronium at both the unoccupied and NMS-occupied muscarinic receptor subtypes and comparable affinities to alcuronium at $M_2$ receptors [70]. Even N-allyl strychnine (**12**, R = allyl), with the same N-substitution as alcuronium has a higher affinity than alcuronium at $M_3$ and $M_4$ receptors, comparable affinity at $M_1$ receptors and only 4-5 fold lower affinity at $M_2$ receptors [70]. This demonstrates that it is not necessary to have a bis-onium structure in order to develop high affinity allosteric ligands.

Strychnine and all the reported quaternary derivatives are positively cooperative with [$^3$H]-NMS at $M_2$ receptors and at least one other subtype but were negatively cooperative with ACh [37,70]. In contrast, brucine (**13**, R = H) is positively cooperative with ACh at $M_1$ receptors [54,61,75], a result that has been confirmed [64,78,134]. Minor modifications to the structure of brucine generates ACh enhancers at $M_3$ (e.g. N-chloromethylbrucine, **13**, R = $CH_2Cl$) and $M_4$ receptors (brucine-N-oxide, **13**, R = $O^-$) but the positive cooperativity with ACh at $M_1$ receptors is lost [54,61,75]. This indicates the potential to develop selective allosteric enhancers of ACh binding and function at different muscarinic receptor subtypes by minor modification of a lead structure. Equally it demonstrates the potential difficulty in understanding the SAR associated with retention of positive cooperativity at a given subtype.

Other structurally related alkaloids, e.g. (-)-eburnamonine (**14**) and vincamine (**15**) have also been reported to be allosteric muscarinic ligands [78,135]. The former compound was reported to be an allosteric enhancer of ACh binding at $M_2$-$M_4$ receptors but we find it to be an allosteric inhibitor at $M_1$-$M_4$ receptors [93].

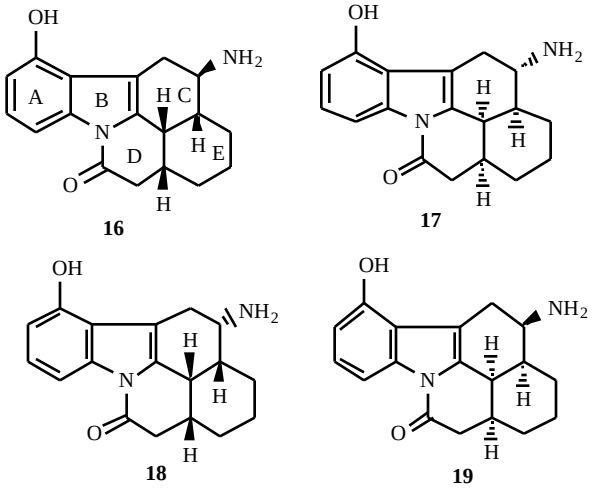



**Fig. (12).** Isomers of an aminotetrahydroisoquinolinocarbazole with different allosteric properties.

We have described the properties of four optical isomers of an aminotetrahydroisoquinolinocarbazole (**16-19**, Fig. (**12**)) [93]. The ring system represents a relatively rigid conformationally constrained structure that is related to that of brucine and in which the importance of the relative orientations of different groups in the molecule to affinity and cooperativity could be examined. NMR studies show that the ring system ABCD in these compounds forms an almost planar structure with ring E perpendicular to this plane. In the enantiomers (**16**) and (**17**), the amino group is axial and out of plane of the ABCD rings whereas in (**18**) and (**19**) the amino group is equatorial and almost in the same plane as the ABCD rings. All four isomers are allosteric muscarinic ligands but differ up to 100 fold in their affinities for a given subtype and in their cooperativities with NMS and ACh. (**16**) had a potency of ca 100 nM at $M_1$ receptors but was strongly negatively cooperative with ACh and NMS at all subtypes. In contrast, (**19**), was a selective and moderately potent allosteric enhancer of both ACh and NMS at $M_2$ receptors. It was postulated that ring E of (**19**) was making a specific interaction with the ACh-occupied $M_2$ receptor.

Bisquaternary dimers of strychnine and brucine have recently been synthesised [136]. These analogues have 6-8 methylene unit spacers between the alkaloids and, like strychnine, were positively cooperative with [$^3$H]-NMS at $M_2$ receptors but ca. 100 times more potent (under low ionic strength conditions). The strychnine dimers approached the potency of alcuronium under these conditions. The authors interpreted their data in terms of their pharmacophore model for potent ligands at NMS-occupied $M_2$ receptors [137,138] in which the two positively charged nitrogens are separated by ca 10 Å and these are flanked by two aromatic ring systems. However it is unclear whether a substantial part of the increase in affinity is due to the larger effects of low ionic strength conditions on the binding of bis-quaternaries, relative to mono-quaternaries, combined with the effects of N-alkylation of strychnine and brucine [70] and a statistical factor of 2 associated with the symmetry. That is, one strychnine moiety may bind to the 'strychnine' site but the second moiety, separated by the polymethylene chain, may not be making a major binding interaction.

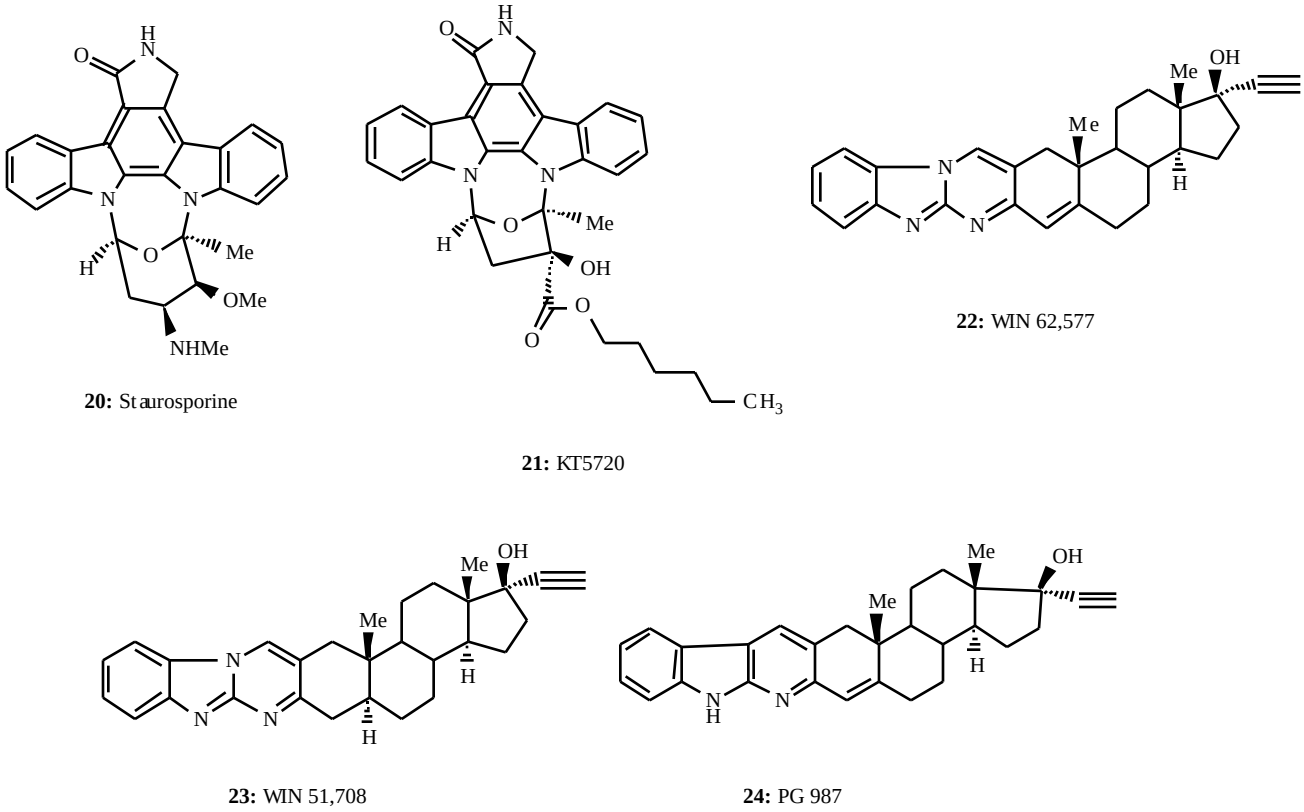
## Staurosporine/WIN 62,577 Analogues

A number of indolocarbazoles, typified by staurosporine, (**13**), and KT5720, (**14**, Fig. (**13**)), have been shown to be moderately potent allosteric muscarinic ligands at $M_1$-$M_4$ receptors, and exhibited a range of positive, neutral and negative cooperativities with NMS and ACh at the different subtypes [45]. KT5720 is an allosteric enhancer of ACh at $M_1$ receptors. Small changes in the structure of the indolocarbazoles produce significant changes in affinity and cooperativity.

These ligands differ in their allosteric behaviour from most other allosteric ligands in a number of ways. Firstly they have a tendency to be $M_1$ selective, in contrast to the $M_2$ selectivity exhibited by quaternary and bis-quaternary ligands. Secondly, some of these molecules *do not have a positively charged nitrogen* under the assay conditions. Indeed the neutral KT5720 is more potent than the positively charged staurosporine. Thirdly, some of these allosteric ligands have very small or no (in the case of KT5720 at $M_2$ receptors) effect on [$^3$H]-NMS dissociation.

These small but potent effects on equilibrium binding and [$^3$H]-NMS dissociation allowed the interactions of KT5720 with other allosteric ligands to be quantitated [45]. Gallamine exhibited neutral cooperativity with KT5720 at $M_1$ receptors indicating that these molecules bound to spatially separated allosteric sites on the receptor. Similarly brucine exhibited neutral cooperativity with KT5720.

We have also reported on the allosteric properties of another series of molecules related to the commercially




**20:** Staurosporine

**21:** KT5720

**22:** WIN 62,577

**23:** WIN 51,708

**24:** PG 987

**Fig. (13).** Allosteric ligands that bind to the second allosteric site on muscarinic receptors.

available molecules WIN 62,577, (**22**, Fig. (**13**)) and its close analogue WIN 51,708, (**23**) [53]. WIN 62,577 is an allosteric enhancer of ACh at $M_3$ receptors and is ca. 15 times weaker than (**23**) at the NMS-occupied $M_4$ receptors, indicating small structural changes associated with the 4-5 bond of the steroid moiety can have substantial effects on binding to certain receptor-ligand complexes. In common with the indolocarbazoles, (**22**) and (**23**) have small or zero effects on slowing the dissociation rate of [$^3$H]-NMS and this therefore opens the possibility of carrying out interaction studies. Even more useful is a deaza analogue of WIN 62,577 (**24**, PG987, 17-β-hydroxy-$\Delta^4$-androstano[3,2-*b*]pyrido[2,3-*b*]indole) that uniquely *speeds up* the dissociation rate of [$^3$H]-NMS. It is therefore possible to readily monitor the kinetic interactions between PG987 and allosteric molecules that slow [$^3$H]-NMS dissociation. It seems that PG987 binds to the same site as KT5720 and staurosporine on $M_3$ receptors but not to the site to which gallamine and strychnine bind. Using this approach it was possible to demonstrate that a ligand, WIN 51,708, that did not affect [$^3$H]-NMS dissociation at $M_3$ receptors nevertheless was binding to the same site as PG987. Ellis and Seidenberg [52] also utilised this approach when they exploited the finding that obidoxime, (**3**), acts allosterically with [$^3$H]QNB but has no effect on its dissociation rate at low ionic strength.

Small changes to the structure of the WIN 62,577 produce considerable changes in affinity and cooperativity [53]. Some analogues are active at sub-nanomolar concentrations but are not allosteric enhancers [139]. Interestingly *both* steroid and heterocycle fragments of WIN 62,577 are individually capable of interacting allosterically with [$^3$H]-NMS and with comparable affinities [53]. This suggests that these fragments interact with different but contiguous or partially overlapping subdomains of the same pharmacophore of this new allosteric site.

## Ligands with Slope Factors Greater Than 1

The acetylcholinesterase inhibitor tacrine (**25**, Fig. (**14**)) was reported a number of years ago to inhibit the binding of [$^3$H]-NMS to muscarinic receptors in equilibrium and kinetic experiments with slope factors greater than 1 [140]. This behaviour, at least in principle, is compatible with positive homotropic cooperativity. The authors suggested on the basis of this and other evidence that one explanation for the data was that muscarinic receptors were present as dimers. These steep slope factors have also been reported in the antagonism by tacrine of agonist activation of $M_1$-$M_4$ receptors [141] and by tacrine inhibition of the binding of [$^3$H]Dimethyl-W84 to its allosteric site [56]. Tacrine cannot be binding to both the allosteric and orthosteric sites as the steep slopes are seen in dissociation studies when the orthosteric site is occupied by [$^3$H]-NMS [56,140].

In the literature there has been an apparent discrepancy between the equilibrium and kinetic binding data of tacrine and the predictions of the allosteric ternary complex model. The equilibrium data suggested it had strong negative cooperativity with [$^3$H]-NMS and yet tacrine inhibited [$^3$H]-NMS binding in the 'equilibrium' studies at concentrations comparable to those at which it inhibited the off-rate, suggestive of low negative cooperativity. The paradox was resolved recently [56] as a further example of the kinetic artifact of the 'equilibrium' assay described earlier in this

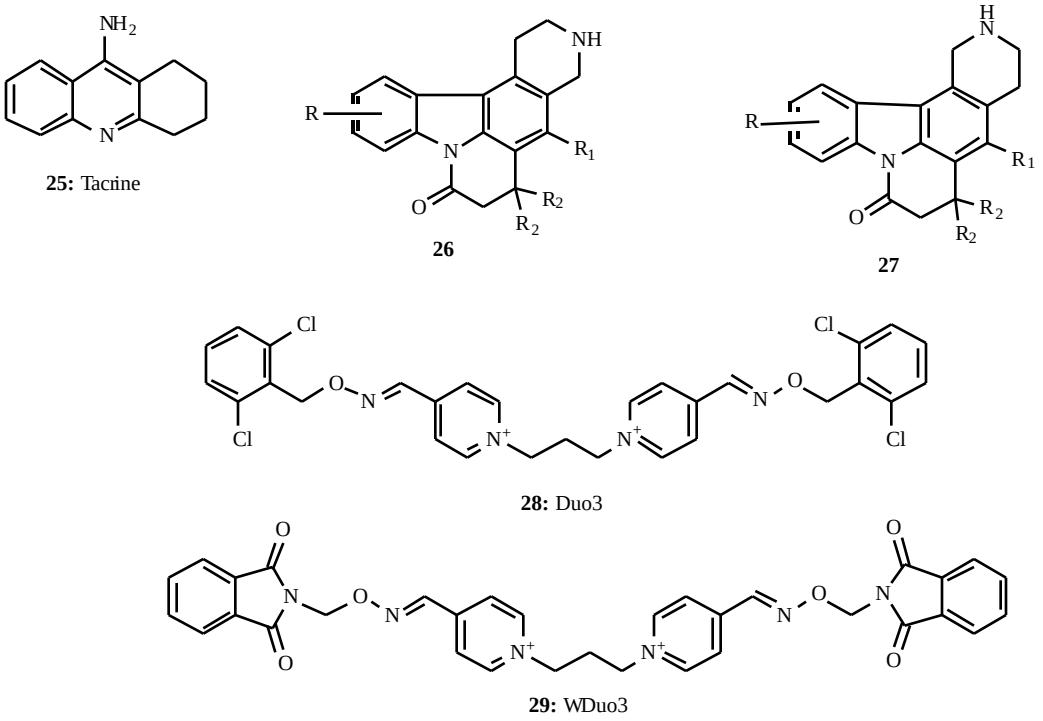



**Fig. (14).** Allosteric ligands giving steep slope factors in binding and functional assays of muscarinic receptors, and related compounds.

article. This artifact is more pronounced because it is associated with the very strong concentration dependence of tacrine to inhibit [$^3$H]-NMS dissociation and association that resulted from its steep slope factor – a 10 fold increase in tacrine concentration could result in up to a 60 fold decrease in the [$^3$H]-NMS kinetics. When the duration of the experiment was extended to 15 hours, true equilibrium was reached and the kinetic and equilibrium data were both compatible with a negative cooperativity of ca. 0.1 between tacrine and [$^3$H]-NMS [56]. Therefore tacrine does not seem to be producing its allosteric effects by a non-specific perturbation of the receptor or membrane.

Two series of pentacyclic carbazolones, (**26,27**, Fig. (**14**)) exhibit similar properties to those of tacrine [92]. Molecules in these series are relatively potent with dissociation constants as low as 30 - 100 nM. They can exhibit positive, neutral or negative cooperativity with [$^3$H]-NMS at $M_1$-$M_4$ receptors, but only negative cooperativity with ACh. The slope factors in both the equilibrium and kinetic assays are greater than 1 (ca 1.7 at $M_1$, $M_2$, $M_4$ but with lower values, ca 1.4 at $M_3$ receptors) [92].

The bis 2,6-dichlorobenzyl ether of the bis-pyridinium oxime with a three carbon spacer (**28**, Duo3, 4,4'-bis-[(2,6-dichloro-benzyloxy-imino)-methyl]-1,1'-propane-1,3-diyl-bis-pyridinium dibromide ) exhibits the same steep slopes as tacrine and the carbazolones in equilibrium and kinetic assays at $M_2$ receptors [89,142]. Interestingly the bis-phthalimido analogue (**29**, WDuo3, 1,3-bis[4-(phthalimidomethoxyimino-methyl)-pyridinium-1-yl]propane dibromide) exhibits a slope factor of ca 1 and obidoxime has a slope factor of less than 1 in their abilities to slow down [$^3$H]-NMS dissociation under low ionic strength conditions [89,130,142]. It is possible that WDuo3 and obidoxime (**3**) have neutral and negative cooperativity respectively as regards their homotropic interactions at $M_2$ receptors. It appears surprising that the presence of a single 2,6-dichlorobenzyl group without a second bulky lateral substituent does not result in steep slope factors [126,130] and yet, when there is a phthalimidomethyl group as the second substituent, positive homotropic cooperativity is restored [130].

The interactions of WDuo3, (**29**), and Duo3, (**28**), with obidoxime are different [142]. This has been interpreted in terms of the presence of more than one allosteric site on $M_2$ receptors; one site binding WDuo3 and obidoxime and one that binds Duo3. Additional evidence for the different mode of binding of Duo3 is that its binding, in contrast to that of WDuo3, gallamine, and alcuronium, is not very sensitive to changes in the incubation conditions, i.e. buffer and temperature [89]. The binding of these ligands therefore presents a very complex picture. At present we do not know about the interactions of obidoxime with other ligands that exhibit steep slope factors or bind to the KT5720/WIN site nor how the binding of these ligands is sensitive to incubation conditions.

A number of 'three-fingered' mamba toxins are potent inhibitors of muscarinic receptors and can exhibit a high selectivity for different muscarinic receptor subtypes [71-73,143,144]. There are toxins that are selective for $M_1$ [71,145,146], $M_2$ [147] and $M_4$ [148] receptors. One toxin, MT7, is active at subnanomolar concentrations at $M_1$ receptors and exhibits the highest subtype selectivity of any known muscarinic ligand. MT7 is reported to bind pseudo-irreversibly to $M_1$ receptors with slope factors greater than 1 [71,74] and also inhibits the dissociation of [$^3$H]-NMS from $M_1$ receptors with a similar high slope factor [71]. The reported activity of both purified and recombinant MT7 at $M_1$ receptors vary by factors of over 100-fold, the highest

estimate of its inhibitory potency being ca.10pM [145]. The activity of chemically synthesised MT7 has been reported to be the same as the recombinant toxin [146]. It is not known whether the discrepancies in the potency are due to a variable (and sometimes very small) fraction of the toxin used in the assays being active or to undetected adsorption of the toxin to surfaces. It also remains to be determined whether the steep slope factors are the consequence of the pseudoirreversible nature of the interaction, an artefact due to depletion and/or adsorption of the active species, or to a similar interaction to that exhibited by the above low molecular weight compounds such as tacrine.

What gives rise to the steep slope factors/positive homotropic cooperativity? Despite the fact that two different allosteric sites have been described on muscarinic receptors, it is intrinsically unlikely that both would have a comparable pharmacology and affinities for the different ligands and also interact in a positively cooperative manner. A more plausible interpretation is that muscarinic receptors can exist as dimers or a higher oligomer. The binding of these ligands to a *single* allosteric site in a given mode on a receptor molecule could change the conformation of the putative receptor-receptor interface such that a positively cooperative interaction between the sites in the dimer or higher oligomer is generated. Equally it may be possible to have molecules that exhibit a negatively cooperative interaction at the dimer and give slope factors less than one in their allosteric interactions. The only ligand that might exhibit this property at present is obidoxime [142].

In this context it is of interest that certain amiloride analogues (but not others) inhibit the dissociation of radiolabelled antagonists from $\alpha_{1A}$ but not $\alpha_{2A}$ adrenoceptors with slope factors greater than one. This suggests that a similar positively homotropic cooperative interaction may occur at other GPCRs [149,150]. An analogous, but not identical complex positively cooperative interaction of certain amilorides with dopamine receptors has also been reported [46,151].

## Additional Ligands that have been Reported to Show Allosteric Actions

A large number of additional ligands have been reported historically to interact allosterically with muscarinic receptors. In general these are ligands which have a pharmacological action at other targets. These include verapamil [22,23,152-154], quinidine [57,154,155], lidocaine [155], quinacrine [57], secoverine [156], lobeline [57], DPI 201-106 (4-[3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile) [157], TMB-8 (8-(*N*,*N*-diethylamino)-n-octyl-3,4,5-trimethoxybenzoate) [50,52,158], a number of antiarrhythmic drugs [159,160], cocaine [161], isomers of 2α-(2'-phenyl-2'-cyclopentyl-2'-hydroxy-ethoxy)tropane [162,163], tetrandrine [109] and ebeinone [128]. The criterion for the ligands being allosteric was, in most instances, an observed slowing of the dissociation rate of a radiolabelled antagonist. In general the kinetic data were not compared to the predictions of the analysis of equilibrium binding data by the allosteric ternary complex model.

Batrachotoxin has been reported to enhance the binding of some agonists but does not affect antagonist binding to $M_2$ receptors [164,165]. However its effects may be *via* intracellular components as its actions are sensitive to guanyl nucleotides. In contrast, gallamine and alcuronium and other putative impermeant ligands act on whole cells and their binding and actions are not dependent on G protein function [22,23,111] nor on the G-protein selectivity [166].

Some high molecular weight polycations, including polylysine, polyarginine, polyethyleneimine and protamine inhibit the binding of [$^3$H]-NMS [29,167]. The specificity of these actions have not been determined but they do not seem to interact with obidoxime. The major basic protein of eosinophils also has an allosteric inhibitory action on $M_2$ muscarinic receptors that is thought to be of possible importance in the enhancement of bronchoconstriction in asthma [168]: its site of action remains to be determined. Class I histocompatibility antigens [169] and myelin basic protein [170] have also been reported to interact allosterically with $M_2$ receptors.

A cautionary example of a ligand that appears to be allosteric (but isn't) is provided by heparin that has been reported to increase the affinity of [$^3$H]-NMS at $M_2$ receptors and to slow down its dissociation rate [171,172]. However these actions are only found if the assay is carried out on membranes in a low ionic strength medium containing $Mg^{2+}$, the enhancing effects being abolished or attenuated in the presence of 100mM NaCl, GTP, or in pertussis-treated membranes and in whole cells. The explanation is that, under those special assay conditions, the $M_2$ receptor-G protein precoupling that is present [173] is disrupted by heparin, possibly by its binding to the G protein.

A number of other ligands have been claimed to be allosteric but the binding of these ligands to muscarinic receptors is associated with a real or apparent decrease in the observed Bmax of a radiolabelled antagonist. Such ligands include 4-aminopyridine [174], amiodarone [175] and clomiphene [176]. Another ligand, SCH-202676 (*N*-2,3-diphenyl-1,2,4-thiadiazol-5-(2*H*)-ylidene)methanamine), has been postulated to be an allosteric regulator at a number of GPCRs, including muscarinic receptors [177]. For many GPCRs, the binding of SCH-202676 is associated with a decrease in Bmax of the relevant radiolabelled antagonist. However a more detailed recent study [178] has also highlighted the very steep slope factor (≥ 2.5) that the ligand exhibited in a competition experiments versus [$^3$H]NMS and the lack of effect on the dissociation rate of [$^3$H]NMS. It also appears that the ligand behaves differently in whole cell assays relative to what is observed in radioligand binding assays on membranes.

## Molecules Reported to Allosterically Regulate the Activity of Muscarinic Receptors

There have been reports of the activation of $M_1$-$M_4$ receptors by gallamine, strychnine and alcuronium in whole cells [66] and at $M_2$ receptors in a reconstituted system [67]. The activating effect has only been found at low receptor expression levels or at specific R:G stoichiometries; at different receptor and G protein levels, alcuronium switched from being an agonist to an inverse agonist [67]. Such findings have not been reported in other whole cell or membrane functional studies. We cannot explain the mechanism of how these ligands might activate muscarinic

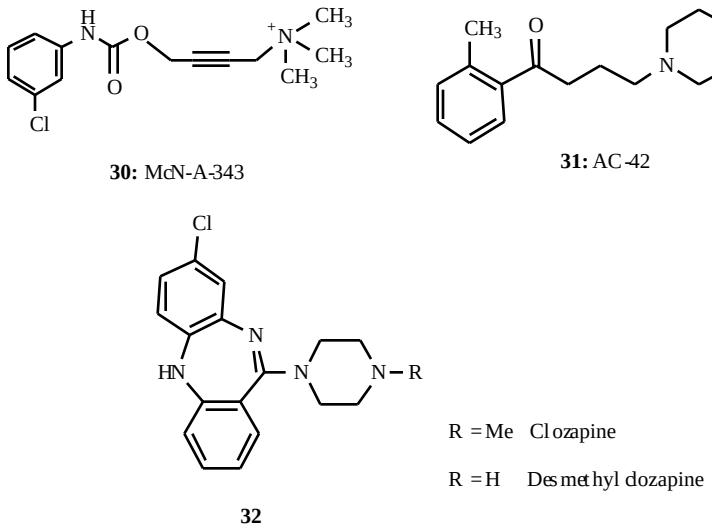



**Fig. (15).** Putative allosteric muscarinic agonists.

receptors; the effects of gallamine and strychnine occur at up to 100 fold *lower* concentrations than those at which these ligands have been reported to act on muscarinic receptors under similar conditions. These effects were almost insensitive to the potent antagonist QNB which the authors explained by the existence of low negative cooperativity between QNB and gallamine (and strychnine). However this interpretation implies the QNB-receptor-gallamine complex, for example, can activate G-proteins.

The inverse agonism of alcuronium (but not agonism) has also been described by Zahn *et al.* [65] who, as might be expected from this result, found that pilocarpine switched from a low efficacy agonist to an antagonist in the presence of alcuronium.

Recently an agonist AC42 (**31**, 4-*n*-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride, (Fig. (**15**)) has been reported to have a greater element of functional selectivity (for $M_1$ receptors) than that found for any other muscarinic agonist [63]. However this ligand exhibits no binding selectivity and appears to interact competitively with orthosteric muscarinic antagonists and with carbachol. The special feature of its interaction with muscarinic receptors is that it does not appear to utilise the same binding epitopes on the receptor as ligands such as ACh. It is postulated that it binds to an 'ectopic' site. It may be that the 'ectopic' site is one of the allosteric sites that have been characterised or a different mode of binding to the orthosteric site.

McN-A-343, (**30**), the prototypical selective muscarinic agonist [9], is also of interest because, as stated earlier in this article, it has appears to interact allosterically with [$^3$H]NMS at $M_2$ receptors [62], with the equilibrium binding data exhibiting the behaviour shown in Fig. (**3**) and conforming to the allosteric ternary complex model. The interactions between [$^3$H]NMS and *d*-tubocurarine at $M_2$ receptors (at low ionic strength and in the absence of GTP) were examined by Waelbroeck [44]. The McN-A-343-[$^3$H]NMS interactions in equilibrium studies were qualitatively similar to those reported [62], suggesting an allosteric interaction. A slowing effect of McN-A-343 on [$^3$H]NMS dissociation was also observed. However it was not possible to simply confirm whether there was agreement with the equilibrium and kinetic estimates of the affinity of McN-A-343 at the [$^3$H]NMS-occupied receptor according to the allosteric ternary complex model. This results from the equilibrium curves being 'flat' [44,62] and GTP sensitive [62], indicating the presence of the complicating factor of McN-A-343-receptor-G protein complexes, detected in the equilibrium experiments, that do not contribute to the picture in the [$^3$H]NMS dissociation experiments.

The data are broadly compatible with a 25-fold negative cooperativity between McN-A-343 and [$^3$H]NMS at the *uncoupled* $M_2$ receptor. However the presence of a high concentration of *d*-tubocurarine did not dramatically affectthe inhibitory potency of McN-A-343 on [$^3$H]NMS binding. The evidence was that McN-A-343 is binding to the unoccupied receptor and having 5-15 fold negative cooperativity with *d*-tubocurarine. This result was interpreted in terms of McN-A-343 binding to the orthosteric site on the unoccupied receptor; at somewhat higher concentrations it binds to the *d*-tubocurarine-occupied receptor and to its allosteric site when the orthosteric site is occupied by NMS. The fact that carbachol and McN-A-343 appear to interact competitively in functional studies at $M_2$ receptors [179] would agree with the interpretation of McN-A-343 primarily to the orthosteric site and activating the receptor from that site.

However there are interesting similarities between McN-A-343 and AC-42. Not only is there an element of structural similarity between the two compounds but their agonist actions are both competitive (or highly negatively cooperative) with carbachol. It may be that McN-A-343 is binding to the same allosteric or 'ectopic' site as AC-42 (which is a different site from the allosteric site to which tubocurarine binds) and activates the receptor from that site.

Another twist is provided by the unanticipated finding that clozapine (**32**, R = Me) and some other atypical neuroleptics are potent muscarinic agonists at $M_4$ receptors [180-183]. Like 'conventional' muscarinic agonists, the binding of clozapine is sensitive to GTP and no evidence has been found of its acting allosterically [184]. In addition, it has been reported recently that a major metabolite of

clozapine, N-desmethylclozapine (**32**, R = H) is a potent muscarinic agonist with some $M_1$ selectivity [64]. In initial mechanistic and structural studies, the interaction of desmethylclozapine and clozapine with the Y381A mutant of the human $M_1$ receptor has been investigated [64]. This tyrosine residue is considered to be very important for ACh binding and agonism, as the Y381A mutant exhibits strongly decreased ACh potency (up to 3,000 fold) [64,185,186]. In contrast to the deleterious effects of this mutation on ACh actions, the efficacy of both N-desmethylclozapine and clozapine was *increased* considerably at the Y318A mutant, with their potencies being unchanged or increased. This result indicates a different mode of binding between both N-desmethylclozapine (and clozapine) and ACh. Because the potency and efficacy of AC-42 are also increased or unchanged in the Y381A mutant [64], it is possible that N-desmethylclozapine and clozapine could be binding to the same site as AC-42 (and possibly McN-A-343) at an allosteric site, and are not just binding in a different mode to the orthosteric site. Structural and further mutagenesis studies should resolve the issue.

## ALLOSTERIC LIGANDS AS THERAPEUTIC AGENTS

Allosteric sites on muscarinic receptors have many attractions as therapeutic targets:

(a) they may be less well conserved across receptor subtypes than the orthosteric site, allowing a greater potential for ligands with a subtype selectivity based on affinity;

(b) allosteric ligands allow subtype selectivity based on cooperativity as well as affinity: they have the potential for 'absolute subtype selectivity', a new and powerful type of receptor subtype selectivity.

(c) allosteric ligands may confer useful properties to relatively non-selective exogenous orthosteric agonists or antagonists to generate a desired subtype selectivity: they may increase subtype-selectivity of orthosteric ligands, or modify their kinetics orefficacy and thereby 'tune' their effects as drugs;

(d) allosteric ligands may act as 'allosteric enhancers' to increase the affinity of the endogenous ligand, ACh, thus providing a use-dependent amplification of the endogenous signal, much as benzodiazepine tranquillisers such as diazepam act by allosterically enhancing the affinity for certain $GABA_A$ receptor subtypes for GABA [187].

(e) the actions of allosteric ligands have a 'ceiling'; i.e. their maximum effect is limited by their cooperativity, $\alpha$. This is not just applicable to allosteric enhancers but also to allosteric inhibitors, which 'tune down' but do not abolish the signalling of a receptor molecule in the way that an orthosteric antagonist does.

(f) allosteric ligands, provided that they do not modulate constitutive activity, do not have an action on muscarinic receptor in the absence of ACh.

(g) as most allosteric muscarinic ligands do not increase the maximum response to ACh, it is possible to envisage that an enhancer will have a selective action on tissues that are not being optimally stimulated because of a local ACh deficit.

In terms of therapeutics, we see allosteric agents as stand-alone drugs which selectively enhance (or inhibit) the affinity of the endogenous ligand, ACh, at the desired muscarinic receptor subtype(s). They have the potential to provide a spatial and temporal selective action at a given receptor subtype that is not possible with orthosteric ligands. In addition they have the capability to make an exogenous non subtype-selective orthosteric agonist or antagonist selective. Basically any muscarinic subtype or disease that is or has been a target for a selective muscarinic antagonist or agonist (e.g. [188,189]), but where the efficacy of the orthosteric ligands has been limited by its muscarinic side effects, is fair game for an allosteric approach. One example is that allosteric enhancers with selectivity for the $M_1$ receptor could be of use in the treatment of the cognitive decline in the earlier stages of Alzheimer's disease, where they would compensate for the effects of the localised ACh deficit.

## THE FUTURE IS BRIGHT

There is now a very large body of experimental data dealing with the allosteric ligands and molecular pharmacology of their interactions at muscarinic receptors. Despite this, until recently there has been a lack of highly potent allosteric ligands with varying selectivities and cooperativities that could provide the basis of the development of potential drugs. In part this may be due to the difficulty in quantitating allosteric interactions. There has also been a past reluctance on the part of the pharmaceutical industry to initiate programs for allosteric ligands, in particular allosteric enhancers, in the absence of a clear demonstration of their *in vivo* efficacy. However the number of reports from industrial groups, dealing with the discovery of allosteric sites on other GPCRs are testament to an ever-increasing interest from the pharmaceutical industry in this general area of research.

Below are listed a number of questions that, once answered, should dramatically increase our understanding of the interactions of allosteric ligands at muscarinic (and other) receptors. The answers to these questions will provide a series of relevant SARs of allosteric ligands for their binding to the unoccupied- and ACh-occupied muscarinic receptors and will facilitate the development of new drugs.

1. How many allosteric sites are there on muscarinic receptors?
2. Which ligands bind to which site?
3. Do ligands from different structural classes bind in different modes to a given allosteric site?
4. Can muscarinic allosteric ligands activate the receptors from the allosteric site?
5. What is the molecular basis of the positive homotropic cooperative interaction of allosteric ligands with muscarinic receptors?
6. And the Holy Grail – what are the x-ray structures of unliganded and liganded muscarinic receptor complexes?

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