Allosteric modulators of ligand binding to muscarinic acetylcholine receptors

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Allosteric modulators of muscarinic receptors act at a site apart from the common ligand binding site of the receptor protein. These compounds affect both of the molecular events that determine the level of equilibrium binding, namely ligand association and dissociation. As a consequence, ligand binding can be elevated, reduced or remain unchanged, depending on the receptor subtype, the type of ligand and the type of allosteric modulator. Thus, allosterically acting drugs represent a novel approach for affecting muscarinic receptors. Notably, they may act as subtype-selective enhancers of acetylcholine binding. This might be exploited therapeutically, to improve cognition or to promote antinociception, for example.

Ligand-gated ion channels are known to be sensitive to allosteric modulation. In the case of $GABA_A$ receptors, this has been therapeutically utilized with the benzodiazepines, which amplify the action of the endogenous transmitter γ-aminobutyric acid (GABA). The risk/benefit ratio of this therapeutic principle is excellent. This may, in part, be because of the lack of effect at $GABA_B$ receptors and the fact that an action on $GABA_A$ receptors depends on the presence of an endogenous agonist; so an effect is induced mainly at the synapses where GABA is released for neurotransmission. It would therefore be of interest to investigate whether G-protein-coupled receptors are similarly a target for allosteric modulation of ligand binding. Reports about allosteric actions have been published for adenosine A_1 receptors[1], α_2-adrenoceptors[2] and dopamine D_2 receptors[3]. Intensive research efforts have been, and still are, directed at the allosteric modulation of muscarinic acetylcholine receptors (for reviews see Refs 4–6).

The first evidence of allosteric interactions with muscarinic receptors came from animal and organ-bath experiments. Lüllmann and coworkers[7] observed in mice that combinations of atropine with alkane bis-ammonium compounds such as W84 (Figure 1) induced an unexpectedly powerful protection against organophosphate poisoning. In beating atria isolated from guinea pig hearts, these compounds antagonized the action of the muscarinic agonist carbachol. By contrast to conventional antagonists, the shift of the agonist curve did not steadily increase with increasing concentration of the compounds; instead, the shift approached an upper limit. Furthermore, combinations of atropine and W84 had a more than additive antimuscarinic action[8]. It was postulated that W84 is an allosteric antagonist. In 1976, Clark and Mitchelson[9] demonstrated a saturating antimuscarinic action for the neuromuscular blocking agent gallamine (Figure 1) in guinea pig atria but a less than additive action of combinations of gallamine and atropine. With the advent of radiolabelled ligands for muscarinic receptors, the hypothesis of an allosteric action could be tested at the receptor level. Stockton and coworkers[10]

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  1359-6446/98/$19.00. PII: S1359-6446(97)01161-6

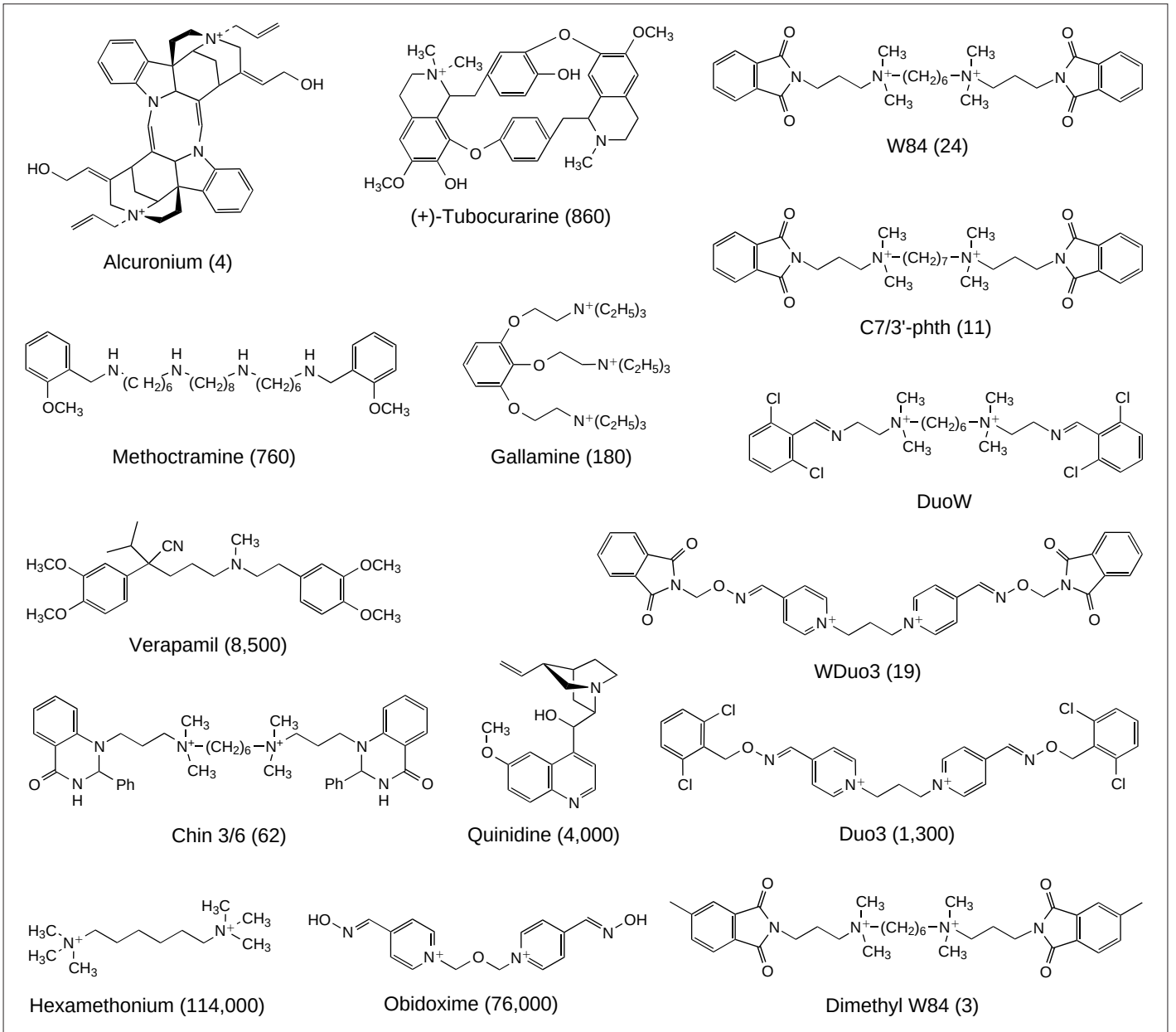


Figure 1. *Structural formulae of the allosteric modulators discussed in the text, and their potencies in allosterically retarding the dissociation of [^{3}H]NMS from M_2 receptors[25]. The EC_{50} (nM) values measured in Na–K–P_i buffer (4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4, 23°C) are given in parentheses.*

demonstrated the allosteric interaction between gallamine and *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) in equilibrium binding and dissociation experiments. Alteration of the dissociation characteristics of a ligand–receptor complex requires binding to a site apart from the ligand binding site; this effect is thus indicative of an allosteric action. Jepsen and coworkers[11] demonstrated allosteric activity for W84, showing a retarding effect on [^{3}H]NMS dissociation in guinea pig cardiac homogenates. Furthermore, it was verified in beating guinea pig atria that the allosteric action is also present under organ-bath conditions. The equilibrium binding of [^{3}H]NMS is reduced by gallamine and W84. Tuček and Proška[12,13] opened a new perspective when they reported that the neuromuscular blocking agent alcuronium is capable of elevating the equilibrium binding of [^{3}H]NMS at cardiac muscarinic receptors.

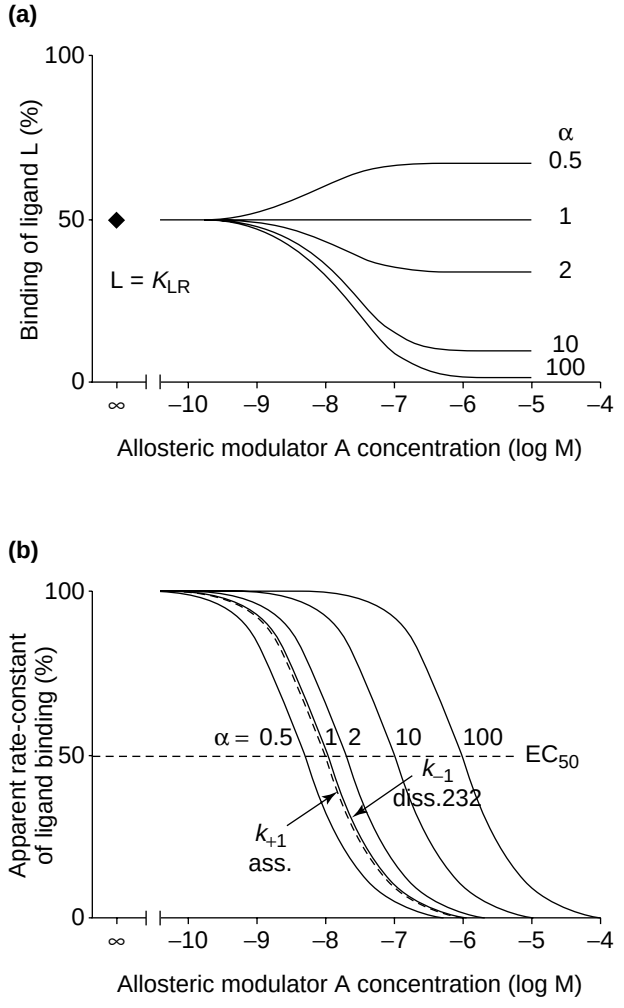


Figure 2. *Allosteric effects on the equilibrium binding of (radio)ligand L (a) are dependent on the action of allosteric modulator A on ligand association and dissociation expressed as percentage of control (b). In (b) the concentration–effect curve for the allosteric reduction of the apparent rate constant of ligand association* k_{+1} *is indicated by a dashed line. A set of curves (full lines) reflecting various potencies is shown for the decrease in the apparent rate constant of ligand dissociation* (k_{-1}). *The concentrations for half-maximum effects (i.e.* $EC_{50,ass.}$ *and* $EC_{50,diss.}$), *indicated by the horizontal dashed line, are taken to indicate the equilibrium dissociation constants of the alloster at the free and the ligand-occupied receptor, respectively. With this prerequisite, the ratio* $EC_{50,diss}/EC_{50,ass.}$ *equals the cooperativity factor* α *of the ternary model of allosteric interaction*[19]. *The binding curves shown in (a) were calculated using these* α *values on the basis of this model. Curves were generated for a (radio)ligand concentration (L) leading in the absence of allosteric modulator A to half-maximum receptor occupancy;* K_{LR} *is the equilibrium dissociation constant of the reaction* $L + R \rightleftharpoons LR$. $\alpha < 1$, $\alpha = 1$, $\alpha > 1$ *signify positive, neutral and negative cooperativity, respectively (C. Tränkle, unpublished).*

Actions of allosteric modulators at the molecular level

Allosteric modulators affect both events underlying (radio)ligand binding at the receptor level – ligand association and dissociation. The balance between these actions determines the effect an allosteric modulator ('alloster') has on the equilibrium binding of the ligand.

Effect on ligand dissociation

Generally, allosteric modulators reduce the probability of (radio)ligand dissociation (Refs 4,14; but see Refs 15,16). If certain conditions are met, the concentration–effect curve for the delay of ligand dissociation probably reflects the binding curve of the alloster at the radioligand–receptor complex[6]. The inflection point of the curve ($EC_{50,diss.}$ in Figure 2) would then indicate the equilibrium dissociation constant of alloster binding to the ligand-occupied receptor.

The molecular events underlying the delay of ligand dissociation have yet to be clarified. The allosteric agent might alter the conformation of the ligand–receptor complex. Alternatively, the modulator might attach to the ligand-occupied receptor at the entrance of the ligand binding pocket of the receptor protein, thus imposing a steric hindrance for ligand dissociation (for review see Ref. 5). Furthermore, a bound ligand might interfere with binding of the allosteric agent. For instance, the M_2-preferring antagonist AF-DX384 appears to attach partially to the allosteric site of M_2 receptors and thus hinders binding of the modulator W84 (Ref. 17). However, it can also be speculated that a bound ligand molecule might provide points of attachment for an allosteric agent; this is one possible explanation for the finding that some modulators have a higher affinity for ligand-occupied receptors than for free receptors (see below).

Effect on ligand association

To the best of our knowledge, all allosteric modulators known to date inhibit ligand

association. In principle, this action may result from binding to the allosteric site, to the 'orthosteric' ligand binding site or to another site that is available in the absence of a bound ligand. Phenomenologically, the decreased probability of ligand association is an action that is shared by allosteric modulators and conventional competitive agents.

The concentration–effect curve for the inhibition of ligand association (Figure 2) is likely to reflect occupation of the free receptor by the alloster[18]. The inflection point of the effect curve ($EC_{50,ass.}$) at which the probability of ligand association is reduced by one-half may well indicate the concentration of the alloster at which half of the receptors are occupied.

Effect on equilibrium binding of the ligand

Retardation of ligand dissociation *per se* is an action promoting receptor occupancy by the ligand. By contrast, inhibition of ligand association tends to reduce equilibrium binding of the ligand. It depends on the balance between these actions whether or not equilibrium binding of the ligand is affected by the alloster and in which direction[18]. This is illustrated in Figure 2.

The ternary complex model of allosteric interactions[6,10,19] allows the results of equilibrium binding experiments to be evaluated in terms of binding constants (provided that radioligand equilibrium binding is affected by the alloster – i.e. the cooperativity factor α is different from unity[18]). The model describes a cooperative interplay between the ligand and the alloster, so the shift of alloster-binding affinity induced by the ligand equals the shift of the ligand-binding affinity induced by the alloster.

Receptor specificity of allosteric modulation

In a comparative study with guinea pig, rat and porcine muscarinic M_2 receptors, no evidence was found for a species-dependent action of the modulator W84 (Ref. 20).

Interaction with other receptors

To check for specificity of the allosteric interaction with muscarinic receptors, the effects of W84, gallamine and alcuronium on radioligand binding at several other G-protein-coupled receptors was investigated. These compounds hardly affected the dissociation characteristics and equilibrium binding of (−)-[^{125}I]iodocyanopindolol at cardiac β-receptors, of [^{3}H]prazosine at cardiac α_1-receptors, of [^{3}H]cyclopentyldipropylxanthine at cerebral adenosine A_1 receptors[21] or, of [^{3}H]mepyramine at histamine H_1 receptors[22].

With regard to nicotinic acetylcholine receptors, it was found that W84 competitively inhibits the binding of (−)-[^{3}H]nicotine in brain homogenates with an IC_{50} of 30 μM (Ref. 23). Neuromuscular transmission in electrically stimulated rat phrenic nerve–diaphragm preparations was inhibited at the same concentration of W84 (Ref. 24). In comparison, the concentrations of W84 that interfere allosterically with [^{3}H]NMS binding to muscarinic M_2 receptors under comparable conditions are more than ten-times lower[17]. Furthermore, the SARs for the interaction with nicotinic acetylcholine receptors and muscarinic receptors appear to differ: compared with hexamethonium, the binding affinity of W84 at rat brain nicotinic sites was only seven-times higher[23], whereas the potency to retard allosterically the dissociation of *N*-[^{3}H]methylscopolamine from cardiac membranes (in Na–K–P_i buffer – 4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4, at 23°C) was about 2,000-times higher with W84 than with hexamethonium[25].

Interaction with muscarinic receptor subtypes

The action of allosteric modulators at muscarinic receptors is subtype dependent. As regards interaction with the free receptor (inhibition of ligand association), many of the allosteric modulators have their highest affinity at M_2 receptors. At [^{3}H]NMS-occupied receptors, gallamine affects radioligand dissociation in a rank order of potency of $M_2 > M_4 > M_1 > M_3 > M_5$ (Ref. 16). The same order of affinities to [^{3}H]NMS-occupied receptors has been reported for alcuronium[26].

The ratio between the affinities of a modulator at the free and the ligand-occupied receptor depends on the receptor subtype under investigation. This is reflected by the observation that alcuronium elevated [^{3}H]NMS binding only at M_2 and M_4 receptors, whereas it reduced [^{3}H]NMS binding at M_1, M_3 and M_5 receptors (Ref. 26). In terms of the ternary complex model, this means that the direction and extent of cooperativity of binding are subtype specific.

Ligand specificity of allosteric modulation

To the best of our knowledge, the inhibitory effect of allosteric modulators on ligand association does not depend on the type of ligand. For instance, Hejnová and coworkers[27] investigated the interaction of alcuronium with the equilibrium binding of various radioligands. Data evaluation based on the ternary complex model revealed similar values for the affinity of alcuronium to bind to free receptors (to inhibit the association of the radioligands). By

contrast, the affinity of alcuronium at the radioligand-occupied receptors (the potency of alcuronium to inhibit radioligand dissociation) varied considerably, depending on the type of ligand.

The dependence of the allosteric interaction on the type of ligand implies that it is indispensable to define the target ligand in the search for novel allosteric modulators.

Type of allosteric modulator

The direction and the extent of the effect on ligand equilibrium binding depends on the type of allosteric modulator (see, for example, Ref. 5). Because the effect is also determined by the type of receptor subtype and the type of ligand, the field is wide open for the development of modulators with various patterns of action.

Experimental conditions

The potency of allosteric modulators to interact with ligand binding is influenced by the ionic composition of the incubation medium[25]. Recently, it has been reported that divalent cations such as Mg^{2+} might interact with the allosteric site and competitively interfere with allosteric agents[28,29]. Some compounds, such as the dichlorobenzyl-substituted bispyridinium Duo3 (Figure 1), have a rather weak sensitivity to the ionic composition of the medium[25]. This is in line with the notion that the molecular interaction with the receptor protein may not be uniform, even among structurally related cationic modulators[30]. For drug development, the divergent sensitivity to the incubation conditions implies that SARs evaluated under one buffer condition may not be valid under another condition.

With regard to signal transduction, allosteric agents generally behave as antagonists; for example, W84, its heptamethonium analogue C7/3′-phth, gallamine and alcuronium under organ-bath conditions in contracting atria[8,9,17,31–33]. In cardiac membranes in the absence of agonist, M_2-receptor–G-protein interaction was inhibited by W84 in an inverse agonist fashion as found with other antagonists[34]. With muscarinic receptors expressed in Chinese hamster ovary cells, however, Jakubík and coworkers[35] encountered an intrinsic agonist-like activity of the allosteric agents alcuronium, gallamine and strychnine, although in isolated cardiomyocytes they found a negative effect on G-protein activation.

In conclusion, drug development with therapeutic perspectives should include testing in a physiological medium, such as an organ bath, at an early stage.

Allosteric recognition site

There is some evidence to support the hypothesis of a distinct allosteric site, at least on M_2 receptors. First, the activity may sensitively depend on seemingly minor structural modifications (see below). Second, competitive interaction between various allosteric modulators have been found at muscarinic M_2 receptors in homogenates[36–39] and isolated heart preparations[33]. As an exception, evidence was recently found for another molecular mode of action of the agent Duo3 when checking its sensitivity to the antagonist action of the alloster obidoxime[39]. Third, by using the first available radiolabelled allosteric ligand, [^{3}H]dimethyl-W84, it was possible to demonstrate directly at NMS-occupied M_2 receptors a preferential and specific binding of the novel alloster, and similarly for W84, gallamine and alcuronium (C. Tränkle and coworkers, unpublished).

Receptor mutagenesis studies have been carried out to identify the binding site of allosteric modulators[40–42]. Pivotal amino acids, the replacement of which would lead to a total loss of allosteric potency, were not found. Nevertheless, the results are compatible with the notion that the allosteric site is probably located at the entrance of the ligand binding pocket of the muscarinic receptors (for review see Refs 5,43).

There is no experimental evidence that the allosteric effects of cationic modulators result from an action on receptor–G-protein coupling (for review see Ref. 43). However, an effect on G-protein coupling has been reported for some molecules, such as the polyanionic heparin[44]. By contrast to the cationic modulators mentioned above, allosteric effects were not seen with heparin in intact cells[45].

Therapeutic perspectives

Use as antagonists

An allosteric modulator with an antagonistic action saturating at higher concentrations would be safe in the event of an overdose (see introductory section).

Allosteric modulators might have the potential for greater subtype selectivity of antagonistic action. ‘Classical’ antagonists, which act by receptor occupation and inhibition of ligand association, depend on differences in the pattern of affinity to the free receptor to obtain subtype selectivity; furthermore, selectivity will diminish with increasing antagonist concentrations. Allosteric antagonists have, in principle, an additional means of gaining subtype specificity: they may interact with the acetylcholine-occupied receptor and affect agonist dissociation from the receptor differently. Ideally, the alloster would be negatively cooperative with acetylcholine

at the target receptor and neutrally cooperative with acetylcholine at the receptors not to be antagonized. In this case, the subtype selectivity would be maintained even at high antagonist concentrations[46].

In combination with a conventional antagonist, allosteric modulators may prove beneficial in organophosphate poisoning (see introductory section).

Use as subtype-selective enhancers of acetylcholine action

The alloster would elevate acetylcholine binding at the target receptor by a positively cooperative interaction with the agonist, whereas other receptor subtypes would not be influenced or affected in a neutrally cooperative manner. The amplifying action would become relevant upon neuronal release of acetylcholine[46]. Enhancers of acetylcholine action might be beneficial, for instance, in the treatment of dementia[47] or pain[48].

To date, these considerations appear rather speculative. Nevertheless, derivatives of the compound brucine have recently been reported to enhance the binding and action of acetylcholine in a subtype-specific fashion[46]. Radioligand binding experiments have shown that appropriate modulators may induce an elevation of acetylcholine binding in the muscarinic receptor subtypes M_1–M_4 (Ref. 49).

Strategies for the development of new allosteric modulators

Many of the compounds used to study allosteric interactions have another significant pharmacological action, for instance, neuromuscular blockade. Yet, the alkane bis-ammonium compound W84 was tolerated in mice[7] in doses that would probably result in allosterically relevant concentrations[25]. This encourages the development of novel modulators and the assessment of their therapeutic usefulness.

SARs of allosteric modulators differ in their inhibitory effects on ligand association and dissociation[30,50–52]. Compared with competitive antagonists, allosteric compounds are distinct because of their ability to affect ligand dissociation. Therefore, attention was initially directed to this aspect of the action of allosteric modulators. As [^{3}H]NMS has typically been used to study the action of allosteric modulators, this ligand will be focused on here.

Structural requirements for stabilizing NMS binding

Because no X-ray analysis of the muscarinic receptors has been performed, the structure of the allosteric binding site is unknown, and it is not possible, therefore, to design a highly potent and selective allosteric modulator using the structure of the receptor protein. An alternative way to optimize the alloster is to derive structure–potency relationships from the allosteric compounds known so far.

First, the pharmacophore was pinpointed by comparing allosteric modulators that are likely to interact with a common allosteric site on the receptor protein (see above) – that is, alcuronium, W84, gallamine and (+)-tubocurarine. The most potent and almost rigid compound, alcuronium, was used as a template. An approximate inspection of the molecules revealed as common elements at least two positively charged nitrogens and two aromatic skeletons (with the exception of gallamine, which has only one phenyl ring). Using these elements, gallamine and (+)-tubocurarine [built up using CORINA (Ref. 53) and semi-empirical geometry optimization] could be directly aligned onto alcuronium, whereas for W84 an S-shaped conformation (of low energy; see Figure 3) had to be created for suitable superimposition. The neural network driven Kohonen maps developed by Gasteiger and coworkers[54,55] were used to compare the molecular shape, lipophilic potential and electrostatic potential (MEP) of the compounds. This approach revealed similarities between alcuronium and W84 with regard to the MEP, but significant differences between gallamine and (+)-tubocurarine and between these compounds and alcuronium. These findings parallel the rank order of allosteric potency: alcuronium > W84 > gallamine > (+)-tubocurarine (Na–K–P_i buffer)[25]. Taken together, the pharmacophore of allosteric stabilizers of [^{3}H]NMS binding consists of two centres of positive charge and two lateral aromatic skeletons in the spatial arrangement, as found in alcuronium and in the S-shaped conformation of W84 (Ref. 56).

Second, an extended series of symmetrical allosteric model compounds was synthesized and tested for their ability to adopt a similar spatial arrangement. Bispyridinium-type (WDuo, Duo and IWDuo derivatives[57]) and hexamethonium-type (W84, DuoW) molecules were aligned onto alcuronium using corresponding structural elements. By means of the torsional flexible fit option in QUANTA and a molecular mechanics calculation for geometry optimization, the S-shaped conformation of the entire molecule appeared to be the active conformation in all cases. A subsequent molecular shape analysis employed the overlap and non-overlap volume (i.e. the volume not shared by the two molecules), as well as electrostatic and lipophilic field potentials as QSAR descriptors[58]. In this case, the allosteric potency to

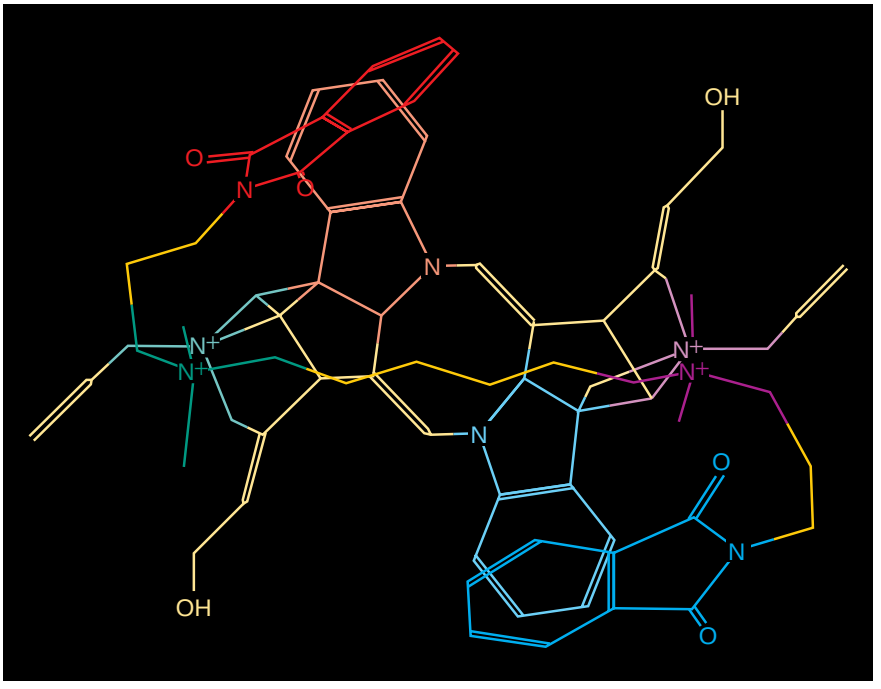


Figure 3. *Superposition of W84 (using the S-shaped conformation) onto alcuronium. Colours indicate corresponding moieties in the two molecules.*

retard [^{3}H]NMS dissociation in Na–K–P_i buffer was found to be related to electrostatic and spatial parameters, especially the non-overlap volume[57], indicating that the compounds studied occupy a space apart from alcuronium. This is in good agreement with the hypothesis of the pharmacophore described above.

Various chemical modifications have been performed to test this hypothesis:

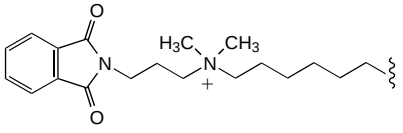
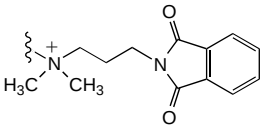
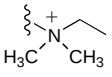
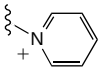
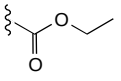
- Systematic variation of the central chain in the hexamethonium series revealed the strong dependence of the allosteric effect on [^{3}H]NMS dissociation on the length of the chain. The optimum length was found to be equivalent to six or seven methylene groups (~10 Å)[59]. This result is compatible with the postulation of two positively charged centres being necessary in the centre of the molecules.
- Theoretical 'removal' of both lateral aromatic (phthalimido) skeletons in W84 and WDuo results, respectively, in hexamethonium and TMB-4, an obidoxime analogous derivative. Both compounds are very weak stabilizers of [^{3}H]NMS binding[25,30]. Thus, the lateral substituents are critical for binding to the allosteric site, but the question remains as to whether both aromatic rings are of the same importance. Unilateral shortening in the W84 series diminished the potency significantly. Thus, cutting off the phthalimido substituent resulted in a tenfold decrease in potency, and additional removal of the positively charged nitrogen resulted in a 50-fold decrease in potency[51] (Table 1); corresponding variations in the WDuo assembly resemble the hexamethonium results[30]. These findings strongly support the hypothesis that the pharmacophore consists of two lateral cyclic skeletons.

On the basis of our pharmacophore model, the potency of some compounds that are described in the literature as allosteric modulators can qualitatively be explained by the lack of essential elements, or a different spatial arrangement of the pharmacophore groups. Gallamine lacks one phenyl ring. DL-Verapamil[60] contains two lateral aromatic rings but only one protonated, cationic nitrogen at physiological pH. Furthermore, the distances between these elements do not match the distances in the S-shaped W84. Tacrine[61] and quinidine[62] resemble only one-half of the pharmacophore. (+)-Tubocurarine contains all the pharmacophore elements, but the spatial arrangement is different[56]. Strychnine[6,38], being a half of alcuronium, and the structurally similar eburnamonine[63] consequently have a weaker potency to retard the dissociation of [^{3}H]NMS from M_2 receptors. Methoctramine[37] contains four positively charged nitrogens in the middle chain and lateral aromatic rings, and has a considerable potency to affect [^{3}H]NMS dissociation [cf. the similar structure of DuoW (Ref. 64); see Figure 1].

The pharmacophore model is also in accordance with results of Waelbroeck[37], who tested a series of single, double and triple positively charged molecules, such as pentamethylene bis-(4-DAMP), for their ability to slow the [^{3}H]NMS dissociation and found that compounds with single positive charges are far less active than compounds with two or more positive charges.

In addition, the high potency of some compounds studied by our group can also be explained. Since the allosteric potency is governed by the non-overlap volume (compared with alcuronium), apart from electrostatic descriptors, highly

Table 1. Allosteric potency of hexamethonium derivatives of the lead compound W84 to inhibit the dissociation of [^{3}H]NMS from M_2 receptors

Hexamethonium derivative	EC_{50} (μM)[a]
	
	1.3
	10.0
	7.2
	24.0
	54.0
	111.0

[a]Measured in 3 mM $MgHPO_4$, 50 mM Tris-HCl, pH 7.3 at 37°C.

substituted compounds appear to be highly potent. These include: Chin3/6 (Ref. 65), which consists of lateral quinazolinone rings with protruding phenyl rings; M85, which is a hexamethonium derivative with benzylidene-phthalimido groups at both ends of the molecule (Ref. 66); and tetra-W84, which contains four phthalimidopropyl moieties. Moreover, increasing the non-overlap volume by addition of methyl groups in position 4 of the phthalimido moiety of W84 led to the most potent allosteric modulator known to date, dimethyl-W84, which has been used to develop the radioligand mentioned above (C. Tränkle and coworkers, unpublished).

Taken together, the pharmacophore hypothesis derived from various molecular modelling investigations agrees well with the experimental findings described so far.

Possible modes of attachment

Because the pharmacophore is complementary to the binding site on the receptor protein, the structural requirements of the receptor can be derived.

Lateral substituents. Comparison of derivatives with aromatic and corresponding saturated moieties[67] indicates that the interaction of these moieties with the receptor is hydrophobic. A face-to-face orientation of either aromatic ring of the alloster and aromatic amino acid residues seems likely. The receptor binding pockets for these moieties must be rather big because they can even accommodate substituents with large volumes; see, for instance, Chin3/6 (Ref. 65) or M85 (Ref. 66). Furthermore, the potent modulator alcuronium is a rigid molecule. Taken together, these structural properties are in line with the notion that the allosteric site is located at the entrance of the ligand binding pocket of the M_2 receptor.

Positive charges. The two positive charges of the allosteric modulators can be involved in ion–ion interactions or π–charge interactions; the latter is believed to be stronger[68,69]. Interestingly, in a Mg–Tris–Cl–P_i medium (3 mM $MgHPO_4$, 50 mM Tris-HCl, pH 7.3, 37°C) a protonated tertiary W84 shows a fivefold higher potency than the quaternary W84. The finding that the N^+-hydrogen of the tertiary W84 can form a strong hydrogen bond with an acidic amino acid argues for an acidic amino acid rather than a π–charge interaction with an aromatic ring of tyrosine or tryptophan. Additionally, in the series of unilaterally shortened W84 derivatives (Table 1), a monocationic compound with a lateral alcohol shows an unexpected high potency; this can also be explained by the formation of a hydrogen bond with a corresponding amino acid. An acidic amino acid is suitable to form hydrogen bonds and ion–ion interactions leading to high affinity. Two aspartate residues and one glutamate assigned to the second extracellular loop of the M_2-receptor subtype by homology modelling (Ref. 70 and references therein) support the hypothesis of the involvement of an acidic amino acid and explain the M_2 selectivity of the allosteric modulators considered. Jakubík and Tuček[71] masked the carboxylic group of the aspartic and glutaminic acids by treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Loss of the carboxyl function prevented the binding of both alcuronium and [^{3}H]NMS, thus supporting the location hypothesis.

Outlook

Molecular modelling and SAR analysis have focused on the interaction of allosters with the NMS–M_2-receptor complex. But these considerations may serve as a paradigm showing that it is possible to identify the pharmacophoric elements

shared by various, seemingly heterogeneous, allosteric modulators. Analogously, SARs should be evaluated for the interaction of allosters with other ligands; for example, the endogenous transmitter acetylcholine if the aim is to develop enhancers of acetylcholine action.

Furthermore, SARs for the interaction of allosteric modulators with the free M_2 receptor need to be defined. Because the effect of allosteric modulators on ligand association does not parallel the effect on ligand dissociation, the respective SARs should be different. By comparison, structural elements may be recognized that are pivotal for either positive or negative, or even neutral, cooperativity with ligand binding.

Obviously, SARs of allosteric modulators are rather complex, but this complexity offers the perspective for a novel and subtype-specific means of 'fine-tuning' the ligand-binding properties of muscarinic receptors.

Acknowledgements

This work is supported by the Deutsche Forschungsgemeinschaft. We thank Dr. Christian Tränkle (Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn) for generating Figure 2 and for stimulating discussions, and also Wiebke Bender and Rainer Pick (Department of Pharmaceutical Chemistry) for drawing Figure 1 and the structures in Table 1.

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