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MOLECULAR PROBES FOR MUSCARINIC RECEPTORS: FUNCTIONALIZED CONGENERS OF SELECTIVE MUSCARINIC ANTAGONISTS

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Summary

The muscarinic agonist oxotremorine and the tricyclic muscarinic antagonists pirenzepine and telenzepine have been derivatized using a functionalized congener approach for the purpose of synthesizing high affinity ligand probes that are suitable for conjugation with prosthetic groups, for receptor cross-linking, fluorescent and radioactive detection, etc. A novel fluorescent conjugate of TAC (telenzepine amine congener), an *n*-decylamino derivative of the m1-selective antagonist, with the fluorescent trisulfonated pyrene dye Cascade Blue may be useful for assaying the receptor as an alternative to radiotracers. In a rat m3 receptor mutant containing a single amino acid substitution in the sixth transmembrane domain (Asn507 to Ala) the parent telenzepine lost 636-fold in affinity, while TAC lost only 27-fold. Thus, the decylamino group of TAC stabilizes the bound state and thus enhances potency by acting as a distal anchor in the receptor binding site. We have built a computer-assisted molecular model of the transmembrane regions of muscarinic receptors based on homology with the G-protein coupled receptor rhodopsin, for which a low resolution structure is known. We have coordinated the antagonist pharmacophore (tricyclic and piperazine moieties) with residues of the third and seventh helices of the rat m3 receptor. Although the decylamino chain of TAC is likely to be highly flexible and may adopt many conformations, we located one possible site for a salt bridge formation with the positively charged $-NH_3^+$ group, i.e. Asp113 in helix II.

Keywords

telenzepine; molecular modeling; fluorescence; G-protein coupled receptors

An important strategy in our investigation of the structure and function of G-protein coupled receptors has been the synthesis of new ligands using a functionalized congener approach (1). By this approach, positions for attachment of chains on a pharmacophore are empirically probed, leading to knowledge of structure activity relationships at distal sites on a ligand. The site of attachment must correspond to a region of relaxed steric requirements for the ligand at or near the receptor binding site. This strategy has allowed us to target accessory sites of favorable interaction on the receptor, and actually enhance the affinity of the ligands (2). Potential applications include therapeutic agents (1) as well as ligand probes.

By analogy, this design approach has been extensively explored by our laboratory at NIH for adenosine and ATP receptor ligands (1,3–5). Primary alkylamino congeners of adenosine and xanthine derivatives selective for A_1 , A_{2a} , or A_3 subtypes of adenosine receptors have been synthesized and shown to accommodate in the receptor binding site a wide range of distal, attached structures. New probes for adenosine receptors, including radioactive agonists and antagonists (1), selective chemical affinity labels (1), fluorescent probes (5), and photoaffinity labels have been introduced. The first photoaffinity experiment

demonstrating that adenosine receptor subtypes are distinct molecular entities was accomplished using this methodology (6). Trifunctional affinity labels that direct a reporter group to the receptor binding site and then cause it to be covalently cross-linked to the protein have also been developed (3). It appears that the functionalized congener approach is a generally applicable approach, potentially useful with many receptors.

Functionalized congener approach to muscarinic ligands

Functionalized congeners related to the muscarinic agonist oxotremorine

In an effort to improve the affinity and/or selectivity of muscarinic ligands we have adopted the functionalized congener approach. Initial studies focussed on the agonist oxotremorine (**1**, Fig. 1), which was probed for insensitive sites in receptor binding (7,8). Lengthening of the alkyl chain of **2** (attached in the region of the pyrrolidine moiety of oxotremorine) resulted in a gradual transformation of weak agonists into relatively potent antagonists, e.g. **2**, $n > 6$. Incorporating polar groups within the chain, e.g. amides, diminished affinity (7).

Functionalized congeners related to tricyclic muscarinic antagonists

Another goal was to develop potent and/or selective muscarinic antagonists using the functionalized congener approach (2,9,10) with derivatives of the m1 antagonists pirenzepine (14), **3** and telenzepine, **5** (Fig. 1). The attachment of a spacer chain to the distal piperazinyl nitrogen was based on extensive structure-activity studies (9) to probe the steric constraints in the antagonist binding site. Lengthening the N-methyl group of pirenzepine into a series of long chain *n*-alkyl amines, **4**, resulted in a gradual transformation of the potent antagonist into a weak ($n = 2-6$) and then again potent ($n > 6$) antagonist. A substantial rise in affinity occurred between 6 and 7 methylene groups. As with the oxotremorine derivatives incorporation of non-alkyl groups, e.g. amides, within the chain were found to be detrimental to affinity (9).

In the case of primary amines derived from pirenzepine and various acylated derivatives the length of the alkyl chain was systematically varied to arrive at an optimal affinity (2). The most potent member of the series, containing an *n*-decyl group, has been designated "TAC" (telenzepine amine congener) in the series related to **5**. K_i values for inhibition by TAC of [3 H]NMS (N-methylscopolamine) binding to rat m1 receptors (transfected A9L cells) and to rat heart m2 receptors were 2.4 and 3.7 nM, respectively. In general, a higher affinity was maintained in the telenzepine versus the pirenzepine derivatives, and the versatility of substitution at a terminal amino group enabled us to introduce chemically diverse reporter groups. Although the moderate selectivity for m1 receptors of **3** and **5** was diminished in most of the derivatives, modest m1 or m2 selectivity was restored in certain derivatives (9,10). Curiously, the selectivity could be modulated through distal structural changes. Thus, the potential for achieving selectivity remains.

TAC was acylated with various reporter groups resulting in molecular probes of nanomolar affinity, and containing prosthetic groups for radioiodination, protein cross-linking, photoaffinity labeling, fluorescent labeling, and biotin for avidin complexation (10). Derivatives, in which TAC was conjugated to protein cross-linking reagents containing electrophilic groups designed for irreversible binding to the receptor, were prepared. Two such groups that proved to be effective for chemical affinity labeling of adenosine receptors, as well, were *m*- and *p*-phenylenediisothiocyanate (DITC). DITC-TAC isomers and other substituted aryl isothiocyanate derivatives were very effective irreversible inhibitors of muscarinic receptors (2, 10,11). A *p*-aminophenylacetyl derivative of TAC designed for radioiodination and photoaffinity labeling of the receptor had a K_i value of 0.29 nM at rat forebrain muscarinic receptors (16-fold higher affinity than telenzepine). A biotin conjugate

displayed a K_i value of 0.60 nM at m2-receptors and a 5-fold selectivity versus forebrain. The high affinity of these derivatives makes them suitable for the characterization of muscarinic receptors in pharmacological and spectroscopic studies, for peptide mapping, and for histochemical studies.

A conjugate with the cross-linking reagent "SBED" was synthesized by treating TAC in dimethylformamide with sulfosuccinimidyl-2-[6-(biotinylamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate, an L-Lys derivative (Pierce Chemical Co., Rockford, IL) and purifying the product by thin layer chromatography. Compound **7** was found to display a K_i value of 8.0 nM at cloned rat m3 receptors (binding assay as in ref. 28). Thus it had nearly the same affinity as the precursor, TAC. The conjugate, **7**, is a trifunctional reagent (3) that incorporates sites for potential photoaffinity labeling of the receptor and complexation with avidin, for purposes of histochemical detection or immobilization on a solid support for affinity chromatography. In principle, after cross-linking to the receptor, the pharmacophore moiety is removable under reducing conditions, and the functionalized handle will remain on the receptor. There is a precedent for a receptor to be stable to mild reducing conditions while preserving the receptor structure and its native disulfide bridges: We have found that A₁-adenosine receptors withstand thiol treatment sufficient for cleavage of a disulfide-containing affinity label, e.g. 50 mM dithiothreitol (12).

Fluorescent tracers for muscarinic receptors

The feasibility of using fluorescent techniques to measure ligand-receptor interactions has now been demonstrated with central benzodiazepine (13), A_{2a}-adenosine (5), muscarinic (10,25) and other receptors (16). Such fluorescent probes are potentially of use in histochemical studies and cell sorting, and complement existing radioreceptor assays. Fluorescent probes are economical and free of the potential health hazards and disposal problems associated with radioactive materials.

A novel fluorescent conjugate of TAC with the fluorescent dye Cascade blue (Compound **8**, Fig. 2) was synthesized by treatment of TAC with Cascade Blue acetyl azide (Molecular Probes, Eugene OR) in dimethylformamide and a final purification using reverse phase high pressure liquid chromatography. This dye emits outside the range of autofluorescence (~420 nm) and is not rapidly photobleached. The conjugate **8**, which retained high affinity for M₁ receptors in rat brain, has proven useful for assaying the receptor by direct fluorescence measurement as an alternative to use of radiotracers. The pharmacological properties of Cascade Blue-TAC, **8**, were examined in rat brain membranes. A radioligand binding assay at m1 receptors using [³H]pirenzepine (15) demonstrated the K_i value of **8** to be 215 nM. Other fluorescent moieties conjugated to TAC resulted in even higher receptor affinity (10).

Functionalized congeners as a tool in molecular modeling of G-protein coupled receptors

Construction of muscarinic receptor model

Modeling of G-protein coupled receptors (GPCRs) has become an important tool in understanding drug-receptor interactions and in the development of new ligands for these receptors. The first widely accepted method was the homology modeling method by Hibert et al (17). This method involved the alignment of the receptor sequence with the sequence of bacteriorhodopsin and the subsequent mapping of the sequence onto the structure of bacteriorhodopsin that was determined by Henderson et al (18). This procedure was based on the assumption that even though GPCRs and bacteriorhodopsin, a proton pump in the outer membrane of *Halobacterium halobium*, lacked any functional or sequence homology,

there would be considerable structural homology. This structural homology was inferred by the extraordinary similarity in the hydrophobicity plots, or Kyte-Doolittle plots, of the biogenic amine subfamily of GPCRs and bacteriorhodopsin. Recently, the low resolution electron density map of rhodopsin, a true member of the GPCR superfamily, was published (19). The low sequence homology with bacteriorhodopsin, the structural differences that must arise from the different placement of proline residues in bacteriorhodopsin- and GPCR-sequences, and the availability of an electron density map of a true member of the GPCR superfamily prompted us (20) to adapt a new method to build models of GPCRs (27) that is based on a computational approach rather than strict compliance with the atomic coordinates of a distantly related protein, albeit with higher resolution. The model was built and optimized using the Discover program (BIOSYM Technologies, San Diego CA, Version 2.90) employing the Amber force field, running on a Silicon Graphics Indigo XZ4000 workstation (Silicon Graphics Inc., Mountain View CA).

Models of the muscarinic m2 (17) and the m1 receptor (21) have been published using the original or a slightly modified method. We present here a model for the rat m3 muscarinic receptor that incorporates results from point mutation studies and structure-activity studies of a specific group of antagonists, the functionalized congeners of telenzepine (10). The commonality among the three models is the Asp147 in helix III, generally regarded as the anchoring point of biogenic amines. Unlike the model of Hibert et al. (17), our model features Tyr533 in close proximity to this Asp residue, as argued by Hulme et al. (29). The conserved Trp503 in helix VI, which was indicated by Hibert et al. (17) to be involved in agonist binding in the m2 receptor, is facing the binding cleft in our model, but is ~11 Å removed from the tricyclic moiety of the antagonist. Thr234, according to Nordvall and Hacksell (21) directly involved in agonist binding, is not pointing into the cleft in our model.

Hypothesis for the location of the tricyclic binding site in muscarinic receptors

To probe the antagonist binding site, TAC was docked into the receptor model in such a fashion that the distance between the piperazine ring (Fig. 3) and Asp147 was ~4 Å, since this residue is essential for antagonist binding (22). Systematic searches (rotation of TAC and conformers of TAC) were carried out to eliminate high energy contacts. The complex that had most favorable interactions was then fully minimized. The tricyclic binding region (Fig. 3B) seems to be formed by Asn526 and two other residues that were demonstrated in point mutation studies to be involved in acetylcholine binding (Tyr148 and Tyr529). Tyr529 corresponds to Asn385 in the 5-HT_{1A} receptor sequence, which is involved in binding of adrenergic antagonists (26). The piperazine ring tentatively coordinates the Asp147 at about 3.9 Å from either aspartate oxygen to either piperazine nitrogen. Both tyrosines seem to be involved in aromatic-aromatic interactions (approximately 4.2 Å center-to-center distance). Trp525 is positioned near these Tyr residues. Trp530 is in approximately 4 Å proximity to the benzyl group of the tricyclic moiety. Both Trp residues are conserved among muscarinic receptor subtypes. Asn526 in helix VII is in proximity to the sulfur atom at 3.2 Å, although an interaction with the carbonyl oxygen (currently at 6.8 Å) in the central ring of TAC remains a possibility. The most surprising interaction, however, is the salt bridge formation between Asp 113 in the second transmembrane domain, which is conserved in most GPCRs as a putative sodium binding site (23), and the primary amino group at the end of the aliphatic chain of TAC. The bridging distance between the oppositely charged groups is 3.5 Å. Chain truncation to 7–9 methylene groups would still allow the amino group to interact with an adjacent hydrophilic area, consisting of Thr536, Ser120, and Asn85, but further truncation (3–6 methylenes) would place the amino group in an unfavorable hydrophobic environment, containing Leu123 and Phe124 side chains. Thus, applying the functionalized congener approach suggests an accessory binding site, *i.e.* in addition to the previously

established binding domain for antagonists of this class. Furthermore this m3 receptor model provides a testable hypothesis for the enhanced binding of TAC.

Correlation of binding hypothesis with receptor mutations

Site-directed mutagenesis has been an invaluable technique for identifying residues involved in ligand recognition by muscarinic receptors and activation by agonists (22,24). A single residue in the sixth transmembrane helix of the m3 receptor, Asn507, was found to be essential for the high affinity binding of certain antagonists (e.g. pirenzepine), while not required for acetylcholine binding (24). Substitution of this amino acid by Ala (24,28), resulted in a dramatic loss of affinity of the parent telenzepine (636-fold, from 1.59 ± 0.45 nM to 1010–170 nM), while TAC lost only 27-fold (from 9.84 ± 1.43 nM to 259 ± 11 nM) in affinity (Fig. 4, Wess and Jacobson, unpublished, methods as in 28). The Hill coefficients were ~1, except for the binding of TAC to w.t. m3 receptors ($n_H = 1.63 \pm 0.16$). Thus, the primary amino group of TAC appears to stabilize the bound state and thus enhances potency by acting as a distal anchor of the antagonist in the receptor.

Although the decylamino chain of TAC is highly flexible and may adopt many conformations, we located one possible site for favorable electrostatic interaction of the positively charged $-NH_3^+$ group, i.e. Asp113 in the second transmembrane helix. In order to accommodate bulky groups present in other conjugates of TAC that bind to the receptor, the chain might be oriented in the extracellular direction, which would allow for even greater freedom of substitution.

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Fig. 1.
Muscarinic ligands oxotremorine, **1**; pirenzepine, **3**; and telenzepine, **5**; and their amine functionalized congeners, **2**, **4**, and **6**. R' = acyl or alkyl.

Fig. 2. Conjugates of telenzepine amine congener (TAC), compound **6**. The primary amino group has been acylated with: **7**, "SBED", a prosthetic groups for chemical cross-linking to the receptor, photochemical cross-linking to the receptor and subsequent cleavage of the TAC unit, and **8**, Cascade Blue, a fluorescent moiety for detection and characterization of the receptor.

Fig. 3. Hypothetical molecular model of the binding of TAC to the rat m3 receptor, showing the energetically favorable conformer, in which the primary amino group is coordinated to the Asp113 residue of helix II. A) shows a view of the receptor in the plane of the membrane and B) shows the mutation site (Asn507 of helix VI) and residues in the vicinity of the thienyl ring (Asn526), the benzene ring (Tyr148 and Tyr529), the piperazine ring (Asp147), and the decylamino group (Asp113). Distances shown are in Å.

Fig. 4. Binding of telenzepine (closed symbols), **5**, and the telenzepine amine congener (TAC, open symbols), **6**, to wild type (circles) and to mutant (diamonds) m3 receptors, expressed in Cos-7 cells. The mutant contained a single amino acid replacement, Asn507->Ala (28).