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MUSCARINIC RECEPTOR PROBES BASED ON AMINE CONGENERS OF PIRENZEPINE AND TELENZEPINE

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Abstract

The N-methyl groups of pirenzepine and telenzepine (M_1-antagonists) were modified to produce chemically functionalized N-alkyl analogs using a "functionalized congener" approach. The derivatives were tested in binding assays vs. [^{3}H]N-methylscopolamine in rat forebrain and cardiac membranes. The potency/selectivity were highly dependent on substitutions of the N-methyl group. The affinity in a series of *n*-alkyl amino derivatives progressively increased with the number of methylene groups. The amines were acylated with various reporter groups resulting in molecular probes of nanomolar affinity. The effect of chain length on aryl isothiocyanate affinity labels is explored.

Introduction

Acetylcholine acts as a neurotransmitter in the brain through activation of receptors of the nicotinic and muscarinic types.[1,2] Five subtypes of muscarinic receptors occur in the brain (m1 - m5) and the anatomical distribution of the RNA coding for these proteins has been described.[3] Alzheimer's disease is associated with a deficit of presynaptic m2 receptors as a result of neurodegeneration of nucleus basalis cells.[4]

To develop agents that can be used to characterize muscarinic receptors at the molecular level, we have synthesized novel agonist and antagonist ligands to complement site-directed mutagenesis studies[2,5-7] of the receptor proteins that are currently underway in several laboratories. In addition to introducing new receptor probes we hope to define the parameters of the binding of antagonists to the receptors.

The strategy employed has been to design new analogs using a "functionalized congener approach",[8,9] by which positions suitable for attachment of chains on a pharmacophore have been empirically identified through synthesis and binding assays. The site of attachment must correspond to a region of relaxed steric requirements at or near the receptor binding site. This strategy has allowed us to target accessory sites of favorable interaction on various receptors, and actually to enhance the affinity of the ligands.[8-10] We have used such congeners to make fluorescent probes, biotin conjugates, affinity labels, etc.[11]

Results and Discussion

Pirenzepine, *1*, is the prototypical m1 antagonist, being 20-fold selective for m1 versus m2 receptors. It consists of a tricyclic moiety (a pyridobenzodiazepine derivative) that contains a lactam in the central ring and a diamino side chain, derived from N-methyl piperazine. The structure of pirenzepine was modified at each of these sites in search of positions at which the receptor would tolerate a functionalized chain.[9] The general findings are summarized in Figure 1. It was found that modifications of the tricyclic ring were not well tolerated, and modifications of the piperazine ring led to derivatives of enhanced potency versus pirenzepine, although this was highly dependent on the chain length.

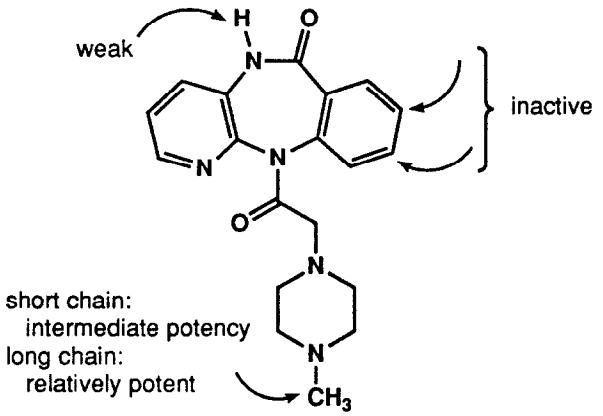
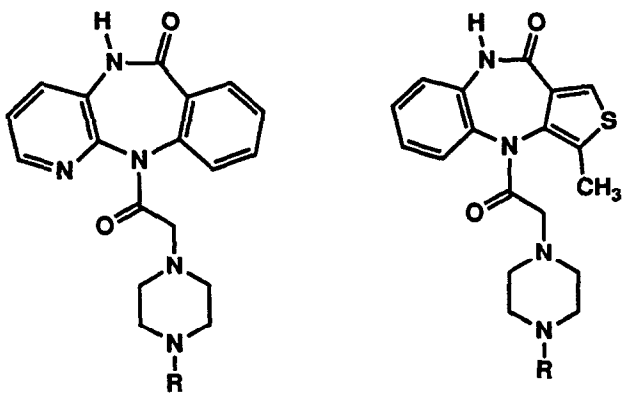


Figure 1. Summary of effects of incorporating a functionalized chain in pirenzepine.

A series of alkyl amine derivatives, *2*, of pirenzepine (Figure 2) were prepared as probes of the receptor environment and as synthetic intermediates. In the case of primary amines (R' = H) and various acylated derivatives the length of the alkyl chain was systematically varied in order to arrive at an optimal affinity, which was achieved at a length of ten methylene groups. This dependence is illustrated in Figure 3. The most potent member of the series (R' = H) containing an N-decyl group has been designated "PAC", a pirenzepine amine congener. Although the moderate selectivity for m1 receptors of pirenzepine was diminished in most of the derivatives, modest m1 selectivity was restored in certain derivatives. PAC was non-selective, although some alkylated derivatives of this intermediate, such as the o-methoxybenzyl derivative (Figure 3) were somewhat m1 selective.

There is a rough dependence of binding on hydrophobicity, with a substantial rise in affinity between 6 and 7 methylene groups. An appealing hypothesis to explain this effect follows the proposal of Herbette and coworkers.[12] They have proposed that hydrophobic and amphipathic drugs approach the receptor binding site predominantly via the phospholipid bilayer, reaching a preferred depth of the pharmacophore within the membrane for optimal binding to the receptor. Thus, the amino group might align with the polar head groups of the phospholipids, in effect orienting the uncharged pharmacophore, at the most extended conformation of the alkyl chain, nearly midway within the bilayer.



1 R = CH_3
2 R = $(CH)_nNHR'$

3 R = CH_3
4 R = $(CH)_nNHR'$

Figure 2. Structures of amine congeners (*2* and *4*) of pirenzepine, *1*, and telenzepine, *3*.

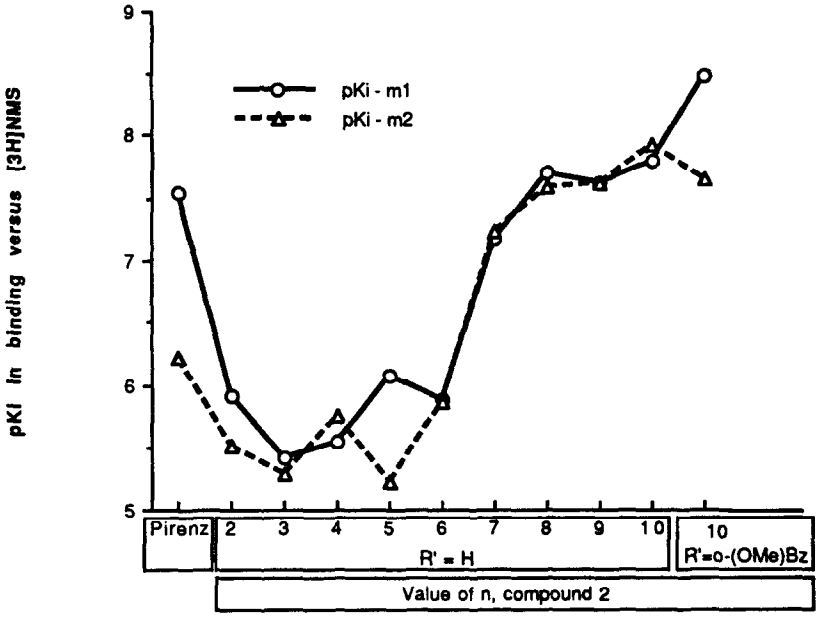


Figure 3. Affinity of pirenzepine functionalized congeners at rat m1 receptors (transfected A9L cells) and at rat heart m2 receptors as a function of chain length ([^{3}H]NMS binding).*9*

We extended this design strategy to telenzepine, *3*,[13] a more potent and slightly more m1-selective antagonist, with a large degree of structural similarity to pirenzepine. The analogous side chain modification resulted in "TAC",[11] a telenzepine amine congener, *4* (n=10, R' = H). K_i values for inhibition of [^{3}H]NMS 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.

Derivatives of pirenzepine containing electrophilic groups designed for irreversible binding to the receptor were prepared.[11,12] Curiously, a highly reactive group (aziridinium) placed on the terminal nitrogen of the piperazine ring[9] was ineffective as an affinity label of the receptor. Only in the longer chain isothiocyanates was affinity labeling achieved. The dependence on the chain length of the affinity and irreversible nature of the NCS derivatives was studied. Primary amines of structure *2* (R' = H), in which n=8, 9, or 10, were coupled to the crosslinker *m*-phenylene diisothiocyanate,[14] resulting in conjugates *5*, *6*, or *7*, respectively (R' = NHCSNH-ϕ-*m*-NCS). These chain lengths were associated with considerable binding potency (Figure 3) in the amines. Preincubation of rat forebrain membranes with one of these isothiocyanates irreversibly inhibited [^{3}H]N-methylscopolamine binding at muscarinic receptors in a dose-dependent fashion (diminished B_{max} value, K_d value not affected).[15] The receptor binding site was not restored upon repeated washing, indicating that irreversible inhibition had occurred. Following a 30 min preincubation of rat forebrain membranes with an isothiocyanate at a concentration of 0.5 μM, the percent reduction in specific binding of 0.5 nM [^{3}H]NMS was: 51.1±4.7% (*5*, n = 8), 48.7±1.7% (*6*, n = 9), and 76±1% (*7*, n = 10). This corresponds to the relative potencies observed for the parent amines.

In order to estimate the kinetics of labeling, we incubated membranes with 0.25 μM of *7* (*m*-DITC-PAC) for various periods of time, then assayed the specific [^{3}H]NMS binding. As shown in Fig. 4, there was a rapid loss in receptor binding within the first minute of incubation, followed by a slower decrease which continued throughout an incubation of one hour. IC_{50} values for the irreversible inhibition at rat forebrain muscarinic receptors were 0.15 nM for *7* and 0.19 nM for the analogous derivative of telenzepine, *8* (*m*-DITC-TAC, Fig. 5). *7* was non-selective as an irreversible muscarinic inhibitor, and *8* was 7-fold selective for forebrain receptors versus cardiac receptors in this assay.

Other probes prepared in the telenzepine series contained prosthetic groups, such as biotin for avidin complexation, and groups for radioiodination such as *p*-hydroxyphenylpropionyl (BH-) and *p*-aminophenylacetyl (PAPA-), photoaffinity labeling (ASA-), and fluorescent labeling (not shown). The affinities for muscarinic receptors in rat forebrain (mainly m1 subtype) were determined in competitive binding assays vs. [^{3}H]N-methylscopolamine, and many of the derivatives were found to be extremely potent.[11] The biotin conjugate of TAC, *10* displayed a K_i value of 0.60 nM at m2-receptors and 5-fold selectivity versus forebrain. The *p*-aminophenylacetyl group, *12*, following radioiodination and conversion of the amine to an azide, is potentially useful for photoaffinity labeling of the receptor, by analogy with use of this group in purines that are high affinity ligands for adenosine receptors.[16] The ASA- (4-azido-2-hydroxybenzoyl-) derivative of TAC, *13*, had a K_i value of 1.25 nM at forebrain muscarinic receptors.

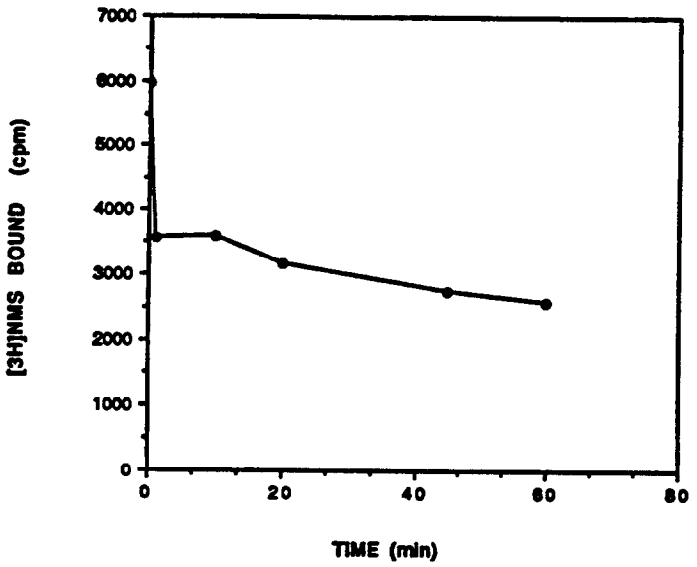
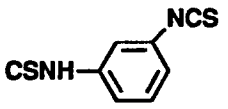


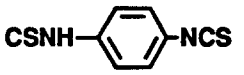
Figure 4. Time course for affinity labeling of rat forebrain muscarinic receptors with 0.25 μM m-DITC-TAC. Incubation was carried out for the indicated times at room temperature, then washed twice with 25 ml of buffer before being assayed for [^{3}H]NMS binding (0.5 nM).

R' =



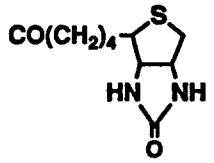
m-DITC-TAC, *8*

K_i = 42.5 nM



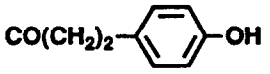
p-DITC-TAC, *9*

1.21 nM



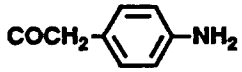
Biotinyl-TAC, *10*

3.20 nM



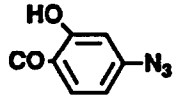
BH-TAC, *11*

K_i = 2.22 nM



PAPA-TAC, *12*

0.29 nM



ASA-TAC, *13*

1.25 nM

Figure 5. Structures of prosthetic groups incorporated into molecular probes related to telenzepine. (R' refers to structure*4* in Figure 2). Affinity at rat forebrain muscarinic receptors (displacement of [^{3}H]NMS) is shown (values from ref. 11).

In conclusion, we have introduced new tools for use in characterizing the nature of muscarinic receptors in binding and histological studies and at the molecular level, for defining the receptor regions that play a role in ligand binding. By probing the site of attachment of *m*-DITC-PAC and *m*-DITC-TAC to muscarinic receptors, through chemical modification or peptide mapping of the inhibited receptor or through site-directed mutagenesis, one may gather further structural or conformational knowledge of muscarinic receptors with the eventual goal of designing more selective agents.

Acknowledgement

We thank Prof. Leo Herbette for helpful discussions.

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14. The appropriate amine (prepared as in ref. 9) and 8 equivalents of *m*-phenylenediisothiocyanate (prepared as in ref. 17) were combined as solids and dissolved in a small volume of $CHCl_3$ or $CHCl_3$/dimethylformamide with agitation. After 5 min, the solution was examined by TLC (silica, $CHCl_3$/MeOH/HOAc, 85/10/5, by volume, product R_f ~0.3) and no trace of the amine remained. Most of the solvent was evaporated under a stream of N_2, and the residue was washed with pet. ether (4X with vortex mixing) and ether (2X). The resulting solid was dried under vacuum at rm. temp. Yields of homogeneous mono-isothiocyanate product ranged from 51-82%. H-NMR and MS (CI-NH_3) were consistent with the assigned structures. High resolution MS (FAB using glycerol matrix in the pos. ion mode) determined parent ion mass: 657.2778 (calcd. 657.2794) for *5* and 671.2938 (calcd. 671.2950) for *6*. Preparation of compound *7* is described in ref. 11.
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