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THE N^4 NITROGEN OF PIRENZEPINE IS RESPONSIBLE FOR SELECTIVE BINDING OF THE M1 SUBTYPE HUMAN MUSCARINIC RECEPTOR

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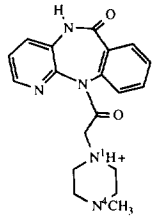
Abstract: An analog (SCH55112) of pirenzepine was prepared in which the N^4 piperazyl nitrogen was replaced with carbon. This structrual change abolished the 55-fold m1/m2 selectivity of pirenzepine due to specific loss of m1 affinity. Mutagenesis mapping and modeling studies suggested this change was due to loss of a hydrogen bond to m1Thr32 (m2Ala30). Copyright © 1996 Elsevier Science Ltd

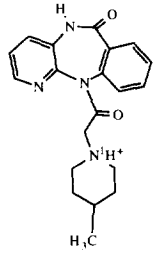
Introduction

The postsynaptic m1 and presynaptic m2 disposition of basal forebrain muscarinic receptors suggests m1 selective agonists or m2 selective antagonists will provide enhancement of cholinergic function. Class selective m1 agonists or m2 antagonists may therefore prove beneficial for therapy in neurodegenerative disorders, including Alzheimer's disease.[1–3] Elucidation of the molecular basis for subtype selective binding for class-specific antagonists will aid design of selective agents. Pirenzepine (1) is the most m1 versus m2 selective antagonist known; its discovery[4] led to the development of class-specific muscarinic ligands and elucidation of the molecular sequences of human muscarinic receptor subtypes.[5,6] The m2 selective pirenzepine analogs AF-DX-116 (3) and AF-DX-384 (4) lack pirenzepine's N^4 piperazyl nitrogen, prompting us to synthesize the corresponding carbon analog of pirenzepine.

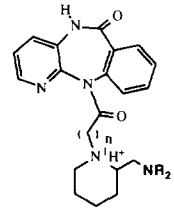
Chemistry



Pirenzepine
1



SCH55112
2

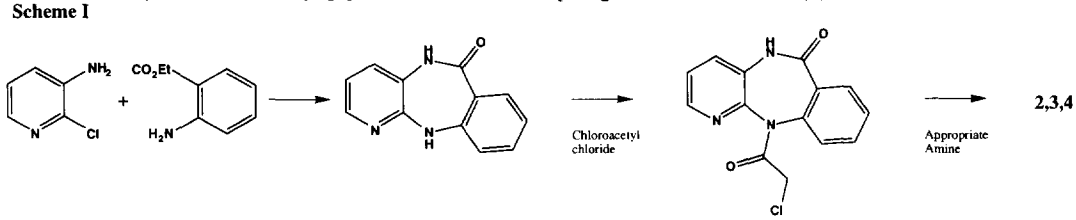


AF-DX-116 n=1,R=Et
3

AF-DX-384 n=2,R=nPr
4

The synthesis of of AF-DX-116 has been described elsewhere.[7] This procedure, outlined in Scheme I, was modified through use of 4-methyl piperidine in the last step to generate SCH55112 (2).

Scheme I



Biological Results

Radioligand binding assays were performed using CHO cells expressing the mutant or wildtype muscarinic receptors. Membranes were obtained by dounce homongenization of cells in 10 mM Na^+, K^+-phosphate buffer, pH 7.4 (assay buffer). Homogenates were centrifuged at 40,000 * g for 1 h and the resultant pellet was resuspended in assay buffer. Protein concentration was determined using the BCA reagent (Pierce). Saturation and competition radioligand binding assays were conducted for 2 h at 25°C in a total volume of 2mL with 1 μM atropine used to define nonspecific binding. ^{3}H-QNB was used for all assays except for m1Asp105 and m1Asp99 mutants. In competitive studies, membrane homogenates were incubated with 100 pM of ^{3}H-QNB in the presence of increasing concentrations of competing drugs . Binding assays were terminated by filtration onto Whatman GF/C filters using a Brandel harvester followed by 10 mL of cold assay buffer.

Due to the difficulty experienced using ^{3}H-QNB with the rm1Asp105 mutant, binding assays were conducted using ^{3}H-NMS. For saturation binding experiments, increasing concentrations of ligand were incubated with 40 μg of membrane protein at 37°C for 1 h in a final volume of 1.0 mL. Binding reactions were terminated by rapid filtration over Whatman GF/C filters soaked in assay buffer with 0.1% PEI. Non specific binding was determined in the presence of 10 μM atropine. Competitive binding experiments were conducted using 40 μg of membrane protein and 1.0 nM ^{3}H-NMS following the above conditions.

Radioactivity was determined using liquid scintillation spectroscopy. Binding data was analyzed using the nonlinear least squares curve-fitting program,LIGAND.[8,9]

Table 1. Displacement of ^{3}H-QNB from Human m1 Receptor in CHO cells

Compound	m1 (K_i (nM))	m2 (K_i (nM))	m2/m1
1	4.6	251.	54.6
2	82.4	71.2	0.86
3	179.	52.	0.29
4	12.4	5.28	0.43

All mutations were introduced into pCD expression vectors containing the entire coding sequence of the human m1 and human m2 muscarinic receptors. Primer-directed mutagenesis was performed following the Pharmacia mutagenesis kit. Oligonucleotides were designed to replace selected amino acids of m2 with the corresponding m1 amino acids unless otherwise stated. The same procedure was performed on the m1 receptor gene. As a means of selection, an oligonucleotide was designed to change a unique restriction enzyme site on the vector.

Mutant receptors were sequenced on an ABI 373 automated sequencer using the PRISM dideoxy terminator kit. The mutant receptor expression vector constructs were stably transfected into CHO cells. The rat m1 muscarinic receptor mutants were graciously provided by Dr. Claire Fraser at The Institute for Genomic Research.[10] Effects of mutations on pirenzepine binding are provided in Table 2.

Table 2. Mutagenesis Mapping of m1 Pirenzepine Binding Site

Site-Directed Mutation(Reference)	Receptor Region	Fold Binding Relative to Wildtype	Position in Molecular Model
rm1Asp105>Asn*	TM3 Helix	1070	Binding Site
rm3Asn382>Asp(11)	TM6 Helix	612	Binding Site
hm1Tyr82>Phe(12)	TM2 Helix	3	Binding Site
hm1Trp101>Ala(13)	TM3 Helix	155	Adjacent
rm1Asp99>Asn*	TM3 Helix	39	Adjacent
hm1Glu397->Asn*	TM7 Helix	1.2	Adjacent
hm1Leu174,Ala175,Gln177>Glu,Asp,Glu*	O2 Loop	0.7	Distal

*This study; r = rat, h = human

Molecular Modeling

Methods for the construction of G-protein coupled receptor homology models from the three-dimensional structure of Bacteriorhodopsin have been reviewed in detail previously.[14,15] Several previous models of Human m1 or m2 receptors, some complexed to pyridobenzodiazepinone antagonists, have been reported.[15-19] Our model included all 7 transmembrane helices and the three extracellular loop regions of the m1 receptor. The m1 model was constructed with the Homology program[20] and a conformational ensemble for pirenzepine was generated with the Catalyst program[21-24] (both from Biosym/MSI, San Diego, CA). The lowest energy pirenzepine conformer was docked to the m1 site in an orinetation consistent with the mutagenesis results (see below) and the resulting model was refined with the Discover program[20] (Biosym/MSI). Mutagenesis mapping of pirenzepine's interactions with muscarinic receptors suggests TM3Asp105 binds a positively charged ligand nitrogen with its sidechain carboxylate (this study); TM2Tyr82 binds a ligand heteroatom with its sidechain hydroxyl[12]; and TM6Asn382 binds a ligand oxygen with its sidechain amide nitrogen[11]. Measurement of receptor interresidue distance and analysis of pirenzepine intramolecular nitrogen-oxygen distances in the Catalyst-generated ensemble showed the only possible hydrogen bonds were between Asn382 and the endocyclic carbonyl and Tyr82 and the exocyclic carbonyl if Asp105 is to pair to the N^1 nitrogen of pirenzepine. All m1 residues contacting pirenzepine in the refined m1/pirenzepine model are conserved between m1 and m2 receptors except m1Thr32 which corresponds to m2 Ala30. We propose the hydroxyl of m1Thr32 hydrogen bonds to the N^4 nitrogen of pirenzepine and the absence of this interaction in SCH55112 results in loss of m1 versus m2 selectivity. This conclusion is supported by the corresponding specific 20-fold loss of m1 affinity.

Figure 1. Schematic of m1/Pirenzepine Interaction

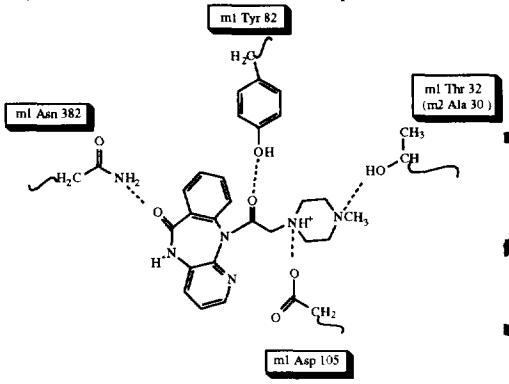
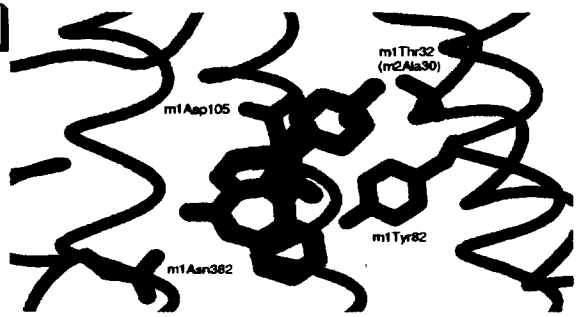


Figure 2. Molecular Model of m1/Pirenzepine Binding Site



This m1/pirenzepine model differs from prior models which predicted the endocyclic carbonyl interacted with TM2m1Tyr82[17] or TM6m1Tyr381.[18] Our model differs from these prior models by a frameshift in the sequence alignment of m1 with Bacteriorhodopsin of 1 residue in helix TM6. Recent molecular dynamics simulations of TM6Asn382 mutations from the former of the prior models suggested that the loss of affinity on mutation is due to structural modification of the antagonist binding domain.[19] The original study on Asn382 mutagenesis noted that mutant receptors were able to bind acetylcholine with high affinity and couple productively with G-proteins in response to carbachol binding.[11] While it is possible that the mutations allosterically block pirenzepine from accessing its binding site, a reasonable explanation for the dramatic 612-

fold loss of pirenzepine binding on mutation of Asn382 is loss of a direct hydrogen bond to pirenzepine. The recent determination of the lumenal orientation of TM1Thr32 at the TM1/TM7 interface is also consistent with our proposed model.[25]

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References and Notes

1. McKinney, M.; Coyle, J. T. *Mayo Clin. Proc.* **1991**, *66*, 1225.
2. Grimm, U.; Moser, U.; Mutschler, E.; Lambrecht, G. *Pharmazie* **1994**, *49*, 711.
3. Doods, H. N.; Quiron, R.; Mihm, G.; Engel, W.; Rudolf, K.; Entzroth, M.; Schiavi, G. B.; Ladinsky, H.; Bechtel, W. D.; Ensinger, H. A.; Mendla, K. D.; Eberlein, W. *Life Sci.* **1993**, *52*, 497.
4. Hammer, R.; Berrie, C. P.; Birdsall, N. J. M.; Burgen, A. S. W.; Hulme, E. C. *Nature* **1980**, *283*, 90.
5. Peralta, E. G.; Ashkenazi, A.; Winslow, J. W.; Smith, D. H.; Ramachandran, J.; Capon, D. J. *EMBO J.*, **1987**, *6*, 3923.
6. Bonner, T. I.; Young, A. C.; Brann, M. R.; Buckley, N. J.; *Neuron* **1988**, *1*, 403.
7. Engel, W. E.; Eberlein, W. G.; Mihm, G.; Hammer, R.; Trummlitz, G. *J. Med. Chem.* **1989**, 32, 1718.
8. Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980**, *107*, 220.
9. McPherson, G. A. *Comput. Programs Biomed.* **1983**, *17*, 107.
10. Fraser, C. M.; Wang, C.-D.; Robinson, D. A.; Gocayne, J. D.; Venter, J. C. *Mol. Pharm.* **1992**, *262*, 173.
11. Bluml, K.; Mutschler, E.; Wess, J. *J. Biol. Chem.* **1994**, *269*, 18870.
12. Drubbish, V.; Lameh, J.; Philip, M.; Sharma, Y. K.; Sadee, W. *Pharm. Res.* **1992**, *9*, 1644.
13. Matsui, H.; Lazareno, S.; Birdsall, N. J. M. *Molec. Pharm.* **1995**, *47*, 88.
14. Kontoyianni, M.; Lybrand, T. P.; *Perspectives Drug Disc. Des.* **1993**, *1*, 291.
15. Trumpp-Kallmeyer, S.; Hoflack, J.; Bruinvels, A.; Hibert, M.; *J. Med. Chem.* **1992**, *35*, 3448.
16. Nordvall, G.; Hacksell, U. *J. Med. Chem.* **1993**, *36*, 967.
17. Fanelli, F.; Menziana, M. C.; Carotti, A.; DeBenedetti, P. G. *Bioorg. Med. Chem.* **1994**, *2*, 195.
18. Kerckhoff, H.; Holtje, H.-D. *Pharmazie* **1994**, *49*, 566.
19. Fanelli, F.; Menziana, M. C.; DeBenedetti, P. G. *Protein Eng.* **1995**, *8*, 557.
20. Homology v. 2.3 was used for model generation. After ligand docking, models were refined for 500 steps by Molecular Mechanics Minimization with Discover v. 2.95.
21. Catalyst v. 2.1 was used to generate the conformational ensemble using the following conditions: 255 maximum number of conformers, a 20 Kcal/mol energy window, the CHARMM forcefield, the BEST conformer generation algorithm. Under these conditions an ensemble of 57 conformers was generated.
22. Smellie, A.; Teig, S. L.; Towbin, P. *J. Comp. Chem.* **1995**, *16*, 171.
23. Smellie, A., Teig, S. L.; Towbin, P. *J. Chem. Inf. Comput. Sci.* **1995**, *35*, 285.
24. Smellie, A.; Kahn, S. D.; Teig, S. *J. Chem. Inf. Comput. Sci.* **1995**, *35*, 295.
25. Liu, J.; Schoneberg, T.; vanRhee, M.; Wess, J. *J. Biol. Chem.* **1995**, *270*, 19532.

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