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SYNTHESIS, BIOCHEMICAL ACTIVITY AND BEHAVIORAL EFFECTS OF A SERIES OF 1,4,5,6-TETRAHYDROPYRIMIDINES AS NOVEL LIGANDS FOR M_1 RECEPTORS

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Abstract

A series of novel tetrahydropyrimidines was synthesized and examined for M_1 muscarinic receptor activity. 1,4,5,6-Tetrahydro-5-methoxycarbonyl-pyrimidine hydrobromide (**1a**; CDD-0034-C) displayed a high affinity for muscarinic receptors in rat brain and stimulated PI metabolism in rat hippocampus. Compound **1a** ameliorated memory deficits associated with lesions of the septohippocampal cholinergic system in rats.

Introduction

Recent work has focused on the development of M_1-selective agonists for Alzheimer's disease[1,2] based on the preferential localization of M_1 receptors in the cerebral cortex and hippocampus,[3-9] and the finding that M_1 antagonists such as pirenzepine produce memory impairments in experimental animals.[10-13] An M_1 agonist is expected to bind selectively to M_1 muscarinic receptors and stimulate phosphoinositide (PI) turnover in the hippocampus.[14,15] It is predicted that a centrally-active, M_1-selective agonist would reverse the cognitive and memory deficits associated with a loss of cholinergic neurons as found in Alzheimer's disease.

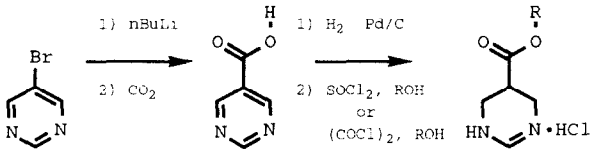
A key factor in the development of centrally active muscarinic agonists is the ability to incorporate a suitable replacement for the ammonium group in acetylcholine, while still affording penetration into the central nervous system. The imidazole system in pilocarpine suggested the utility of amidines in general as suitable ammonium bioisosteres. A series of compounds incorporating the amidine moiety has been synthesized in efforts to develop centrally active muscarinic agonists. The data presented here describe the synthesis and biological testing of a series of novel muscarinic ligands.

Materials and Methods

Chemical synthesis of 1,4,5,6-tetrahydropyrimidines

A series of reduced pyrimidine esters was synthesized by esterification of reduced pyrimidine-5-carboxylic acid (see Scheme 1). Halogen metal exchange using n-butyl lithium at -100° C followed by carboxylation with dry ice was used to form pyrimidine-5-carboxylic acid.[16] Reduction of pyrimidine carboxylic acid was accomplished readily in aqueous acid over 10 % Pd-on-carbon.[17] The 1,4,5,6-tetrahydropyrimidine-5-carboxylic acid then was esterified by refluxing in the desired alcohol (e.g., methanol to produce 1,4,5,6-tetrahydro-5-methoxycarbonyl-pyrimidine hydrobromide [**1a**; CDD-0034-C]) with a dehydrating agent, thionyl bromide. In the case of a reactive alcohol (e.g., propargyl alcohol), an acid chloride was formed first using oxalyl chloride, then reacted further with the alcohol.

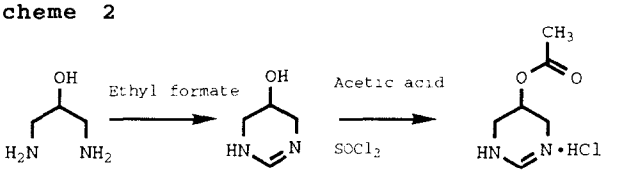
Scheme 1



R	#
Methyl	**1a**
Ethyl	**1b**
nPropyl	**1c**
Isopropyl	**1d**
Propargyl	**1e**
Benzyl	**1f**

The reverse ester **2a** was synthesized by refluxing 1,3-diamino-2-propanol in ethyl formate,[18] followed by esterification with acetic acid catalyzed by thionyl chloride (see Scheme 2).

Scheme 2



2a

Receptor binding

Binding to muscarinic receptors was carried out essentially as described previously.[19] Binding was determined indirectly by the ability of compounds to compete with 50 pM [^{3}H]-(R)-quinuclidinyl benzilate ([^{3}H]-QNB) in a suspension of brain membranes. Nonspecific binding was evaluated by the inclusion of 1000-fold excess atropine in a separate set of samples. IC_{50} values were determined from Hill plots of the inhibition data and are reported as means ± s.e.m. of three independent experiments each performed in triplicate.

Phosphoinositide metabolism

The methods were modified from those described by Brown and associates[20] as reported previously.[21,22] The cerebral cortex and hippocampus were dissected according to the method of Glowinski and Iversen.[23] In these studies, [^{3}H]-inositol was purified prior to use by passing over a Dowex AG1-X8 anion-exchange column to remove charged degradation products of [^{3}H]-inositol. The amount of [^{3}H]-inositol phosphates formed in the assay was determined essentially according to Wreggett and Irvine[24] except that the separation of inositol phosphates was accomplished using an Amersham Super Separator Manifold.

Behavioral studies

The ability of **1a** to improve cognitive function was assessed using a representational memory task in a T-maze. The behavioral methods were modified from procedures outlined by Thomas and colleagues[25,26] and have been used in previous studies to demonstrate representational memory deficits following injections of selective muscarinic antagonists[13] or lesions of the cholinergic system by intrahippocampal injections of AF64A.[27] Similar procedures also have documented improvement of memory function following injections of muscarinic agonists.[27]

Rats were trained to perform the paired-run alternation task and then administered either vehicle or 5.0 nmoles of AF64A to each hippocampus via

stereotaxic technique. Lesions of the septohippocampal cholinergic system were induced under general anesthesia. With bregma and lambda in the same vertical plane, the injection cannulae were inserted at AP-4.8 mm behind bregma, ± 3.7 mm (re midline), and 4.0 mm below the skull surface for hippocampal injections. AF64A was purchased from Sigma, synthesized using established methods,[27-30] and used fresh to assure lesion specificity.

One week following surgery, animals were retested in the maze following i.p. injections of either 0.9 % saline or 1.0 mg/kg of **1a** in saline. Following the conclusion of the injection and behavioral schedules, rats were sacrificed for histological verification of lesions through cresyl violet staining.

Results

The binding affinity of each ligand was determined indirectly by assessing the inhibition of specific [^{3}H]-(R)-QNB binding to rat brain membranes. Compound **1a** displayed a moderate affinity for muscarinic receptors in the central nervous system (see Table 1). Increasing the alkyl substituent yielded compounds with slightly higher affinity for muscarinic receptors in rat brain. The reverse ester, 5-acetoxy-1,4,5,6-tetrahydropyrimidine hydrochloride (**2a**), displayed approximately three-fold lower affinity. The affinity of other muscarinic agonists also are presented for comparison.

Table 1. The inhibition of [^{3}H]-(R)-QNB binding to rat brain membranes by several muscarinic ligands. Also shown is the stimulation of PI metabolism in rat cortical slices. Data represent the mean (± s.e.m. where indicated) from three assays each performed in triplicate.

Ligand	IC_{50}	Hill slope	PI at 100 µM
Carbachol	5.5 ± 1.0 µM	0.32 ± 0.02	580
Arecoline	1.0 ± 0.25 µM	0.76 ± 0.16	-
Pilocarpine	7.6 ± 4.4 µM	0.74 ± 0.03	-
Aceclidine	0.51± 0.10 µM	0.64 ± 0.05	-
1a	9.2 ± 1.9 µM	0.52 ± 0.03	130
1b	2.2 ± 0.28 µM	0.69 ± 0.03	150
1c	2.4 ± 0.44 µM	0.69 ± 0.04	7.1
1d	3.3 ± 0.12 µM	0.60 ± 0.03	7.2
1e	3.3 ± 0.80 µM	0.51 ± 0.04	230
1f	2.6 ± 1.0 µM	0.89 ± 0.11	3.5
2a	32 ± 8.1 µM	0.55 ± 0.09	45

The ability of each ligand to stimulate PI metabolism was tested initially in the cerebral cortex at a single concentration (see Table 1). Compound **1a** stimulated PI turnover in the rat cerebral cortex to 130 % above basal levels at 100 µM. The ethyl (**1b**) and propargyl (**1e**) esters also stimulated PI metabolism to 150 % and 230 % above basal levels respectively at 100 µM. The n-propyl, isopropyl and benzyl esters were inactive in the cerebral cortex with stimulation less than 10 % above basal levels at 100 µM.

Compound **1a** also stimulated PI turnover in hippocampal slices (see Table 2). A maximal stimulation of 240 % above basal levels was observed at 1.0 mM, and the EC_{50} was 55 ± 4 µM. The reversed ester **2a** exhibited much lower activity (roughly one-third the response) in the hippocampus. A reliable estimate of the EC_{50} could not be obtained due to the low response. The

response elicited by **1a** was blocked by atropine, indicating the activation of muscarinic cholinergic receptors.

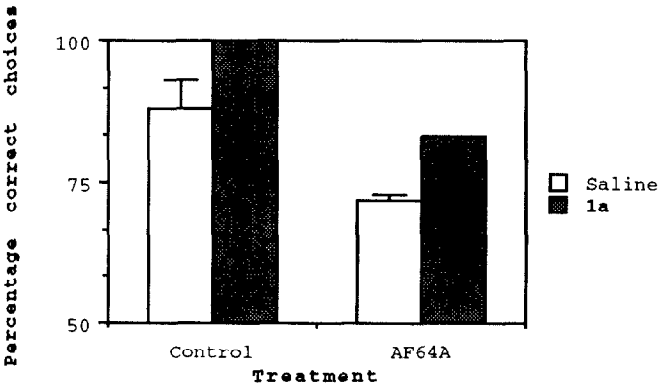
Table 2. Stimulation of PI metabolism in rat hippocampal slices by **1a**, **2a** *and aceclidine. Also shown is the inhibition of the response to 100 μM* **1a** *and 50 μM aceclidine by the selective muscarinic antagonists pirenzepine and AF-DX 116.*

Ligand	EC_{50}	Maximal Stimulation	IC_{50} Pirenzepine	IC_{50} AF-DX 116
Aceclidine	21 μM	300 %	120 nM	280 nM
1a	55 μM	240 %	44 nM	750 nM
2a	n.d.	70 %	n.d.	n.d.

The selectivity of the PI response in rat hippocampus was assessed using the selective antagonists pirenzepine and AF-DX 116. The M_1 antagonist pirenzepine was more effective than the M_2 antagonist AF-DX 116 in blocking the response elicited by 100 μM **1a**. The data can be compared with the response elicited by aceclidine, which was similar in magnitude and potency to that elicited by **1a**. The inhibition of the aceclidine response by pirenzepine and AF-DX 116 is approximately equal.

In the behavioral studies, animals that had received AF64A were impaired relative to control animals one week following surgery. All animals received i.p. injections of either saline or **1a**, 15 min. prior to testing. Control animals (N=3) were not impaired significantly, while animals previously injected with AF64A (N=3) achieved only 72 ± 1.0 % correct choices. Following i.p. injection of **1a** (1.0 mg/kg i.p.) into the AF64A-treated animals, performances improved to 83 ± 0.0 % correct choices, and were significantly above chance levels ($p < 0.05$, by binomial expansion). The data suggest that **1a** can ameliorate the memory deficits produced by lesions of the septohippocampal cholinergic system.

Figure 1. Effects of i.p. saline and 1.0 mg/kg **1a** *on performance of a representational memory task one week following bilateral intrahippocampal injections of either 5 nmoles AF64A or vehicle. Data represent the mean (± s.e.m.) for one session of twelve trials from three animals in each treatment group.*



Discussion

The data described above indicate the utility of the tetrahydropyrimidine as an ammonium bioisostere for muscarinic agonists. The binding affinity of **1a** and other 5-carboxylic esters (e.g., ethyl and propargyl) for muscarinic receptors is comparable to muscarinic agonists such as pilocarpine and carbachol. The reversed ester **2a** exhibits slightly lower affinity.

Several derivatives of 1,4,5,6-tetrahydropyrimidine displayed activity in functional assays of muscarinic agonist activity. The activity of **1a** was higher than that of the reversed ester **2a**, though somewhat lower than for the ethyl (**1b**) and propargyl (**1e**) esters. Longer alkyl substituents produced compounds with reasonable affinity for muscarinic receptors, yet reduced agonist activity. These data are in agreement with studies of other muscarinic ligands that indicate decreased agonist efficacy with longer alkyl substituents. In general, agonist activity is limited to methyl, ethyl and propargyl derivatives in a similar fashion to that found with arecoline and arecaidine propargyl ester.[31] The propargyl derivative **1e** displayed a relatively high affinity and potency, may have improved central nervous system bioavailability and warrants further investigation in functional assays of activity and selectivity.

Receptor selectivity is important issue for the development of muscarinic agonists for cognitive disorders such as Alzheimer's disease. Nonselective ligands are likely to produce unwanted peripheral side effects. Future studies will assess the affinity of amidine derivatives for each muscarinic receptor subtype and also address selective activation of each subtype in greater detail. The selective activation of M_1 receptors by **1a** was examined in preliminary fashion using the selective muscarinic antagonists pirenzepine and AF-DX 116.

The magnitude of the hippocampal PI response elicited by **1a** was comparable to that of aceclidine and that found previously for arecoline and pilocarpine.[22] The higher potency of pirenzepine relative to AF-DX 116 against **1a** was consistent with activation of M_1 receptors coupled to PI turnover in the hippocampus. In contrast, the comparable potency for both pirenzepine and AF-DX 116 against aceclidine matched the profile expected for activation of M_3 receptors.

The effects of **1a** on performance of the representational memory task is consistent with the hypothesis that M_1 agonists can ameliorate memory deficits associated with a loss in cholinergic activity. It should be noted that control animals were not impaired in performance after injections of either saline or **1a** when tested one week following the sham lesion. Animals treated with AF64A were impaired following saline injections yet were significantly above chance levels after injections of **1a**. Similar behavioral results have been observed with pilocarpine.[27]

The goal for future work will be to examine the functional selectivity of 1,4,5,6-tetrahydropyrimidines through further chemical modification. Building in selectivity for M_1 receptors will be an important consideration for the further development of muscarinic agonists for the treatment of cognitive disorders such as Alzheimer's disease.

Acknowledgments

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