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PROGRESS REPORT

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DEVELOPMENT OF GAMMA-EMITTING, RECEPTOR BINDING
RADIOTRACERS FOR IMAGING THE BRAIN AND PANCREAS

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PROGRESS REPORT. This progress report covers period from Nov. 1, 1989 to Aug. 31, 1990.

The long term objective was to develop receptor-binding radiotracers for SPECT or PET imaging of CNS or peripheral nervous system. The specific chemistry aims, as understood on the basis of past findings, were: 1) to synthesize and develop a more polar analogs of 4IQNB, possessing similar binding characteristics but eliminated more rapidly from the surrounding tissues and the target organ, 2) to design a method of introducing a technetium chelating group onto a molecule of cholinergic agent without drastic lowering of its apparent affinity, 3) to synthesize and develop radiotracers based on m-AChR antagonists selective for one of the subtypes of the receptor. The chemistry service aims were to prepare and characterize (R,R)- and (R,S)-4IQNB and derivatives, to provide the triazene intermediate to other investigators, and to provide [¹²³I]4IQNB for in vivo imaging. The biochemistry aims were to characterize the in vitro and in vivo properties of novel compounds and to perform the pharmacokinetic studies.

Results obtained in the reported period indicating progress towards achieving previous application's specific aims:

CHEMISTRY

In the reported period chemistry completed or pursued the following projects:

1. **Synthesis of 3-quinuclidinyl α -alkoxyalkyl- α -hydroxyphenylacetates.** The starting acids were synthesized either by treating diazoacetophenone with corresponding alcohol, followed by reaction with trimethylsilyl cyanide and hydrolysis of the resulting trimethylsilyl cyanohydrines to acids or by treating chloromethylmethyl- or ethyl ethers and styrene derivatives with anhydrous zinc chloride in carbon tetrachloride. The chlorides were converted to alcohols with 20% aqueous KOH, and the alcohols converted to ketones by a Jones oxidation. The ketones were reacted with TMSCN in the presence of ZnI₂ and the hydrolysis of resulting cyanohydrines provided the required acids. The ethyl esters were prepared by adding Tf₂O to a solution of the desired acid in anhydrous EtOH, and heating under reflux. Transesterification of the ethyl esters with 3-quinuclidinol in the presence of sodium metal provided 3-quinuclidinyl derivatives. Further synthetic details are described in the enclosed manuscript. Their apparent affinity toward m-AChR is reported in the pharmacology section.

2. **Synthesis of 3-quinuclidinyl α -hydroxy- α -heteroaryl- α -phenylacetates.** The synthesis of diarylglycolic esters was accomplished by reacting 2- or 3-thienyl, 2- or 3-furyl, or

4-bromophenyl magnesium bromide with methyl or ethyl 2- or 3-thienyl, 2- or 3-furyl or phenyl or 4-bromophenyl-glycolates. The respective 3-quinclidinyl derivatives were prepared in transesterification reaction of 3-quinuclidinol and methyl or ethyl ester of the desired acid. Further synthetic details are described in the enclosed manuscript.

The results of binding studies of the above compounds for their affinity to mAChR subtypes are reported in pharmacology section.

3. Synthesis of lipophilic, m2-AChR-selective antagonists. The synthesis of AQ-RA 741, some of its halogenated analogs (13), and some of its hybrids with UH-AH 37 (14) was accomplished together with some of the QNB hybrids (15).

We have not been able to find the synthetic methods for preparation of m2-selectivity vesting side chains 4-N,N-dimethyl- and 4-N,N-diethylaminobutyl-piperidines and have developed one of our own. Namely, to a solution of 4-lithiomethylpyridine (16) in ether was added N,N-dimethyl- or N,N-diethylaminopropyl bromide (17) at 0°C. The resulting solution was then stirred for 1 h. Thus obtained 4-alkylpyridines (18) were hydrogenated in acidic EtOH using PtO₂ as a catalyst to give the desired piperidines (19).

Synthesis of 5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-11-one (20) was accomplished as described in literature:^{30,33} to a

solution of 2-chloro-3-aminopyridine (21) in 1,2,4-trichlorobenzene were added potassium t-butoxide followed by methyl 2-amino-benzoate (22) and the mixture heated for 1h at 50°C. After dilution of the reaction mixture with additional 1,2,4-trichlorobenzene, concentrated sulfuric acid was added, t-butyl alcohol distilled off in vacuum, and the mixture stirred at 115°C for 24 h and at 160°C for another 2 h. Purified product (20) was chloroacetylated by refluxing the its solution with chloroacetyl chloride and N,N-dimethylaniline for 5h in dry THF. Obtained chloroacetyl derivative (23) was refluxed in dry acetonitrile in the presence of Na₂CO₃ with the desired 4-alkylpiperidine (19) to give the final product (24).

Synthesis of 11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepine (25) was accomplished as described in literature:⁵⁶ a suspension of 2-chlorobenzoic acid (26), o-phenylenediamine (27) and powdered copper in C₆H₅Cl was heated for 8h and thus obtained product (25) recrystallized from n-BuOH. Chloroacetylation of (25) carried as described above gave (28) and its condensation with 4-alkylpiperidines gave the expected products (29).

Synthesis of QNB hybrids was carried as follows: dissolved in methylene chloride benzoylformic acid (30) and BPO-Cl (bis(2-oxo-3-oxazolidinyl)phosphinic chloride) reacted at room temperature with the desired 4-alkylpiperidine (19) to give the expected benzoylformamide (31). Grignard reaction with phenylmagnesium bromide gave the hybrids (32). The results of binding studies are reported in pharmacology section.

4. Synthesis of 3-(2'-aminobenzhydryloxy)tropane (ABT) conjugate with Rhodamine B.

Rhodamine B (33) was dissolved in 1,4-butanediol diglycidyl (34) and the solution stirred at 24h at 37°C. The

reaction mixture was then loaded on flash chromatography column and eluted with a $\text{CHCl}_3/\text{MeOH}$ gradient. The eluted epoxide (35) was added to a solution of ABT in EtOH and the mixture refluxed for 14h. The reaction mixture was evaporated to dryness, dissolved in small amount of MeOH and loaded on flash chromatography column. The product (36) was eluted by triethylamine gradient in $\text{CHCl}_3/\text{MeOH}$ (50/50). Pharmacological studies (see below) indicate very small loss of affinity when compared with ABT.

PHARMACOLOGY

1. Evaluation of 3-quinuclidinyl α -alkoxyalkyl- α -hydroxy- α -phenylacetates.

Seven alkoxy analogs of QNB were prepared and their affinities for m-AChR from rat brain determined. The results of binding studies are summarized in Table I.

TABLE I. Receptor Affinities of α -Alkoxyalkyl-analogs.

Compd	R	n	m	K_A^a	C.V. ^b	RBI ^c
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1	H	1	1	$1.47 \times 10^6 \text{M}^{-1}$	6.11%	4.00%
2	H	1	2	$3.26 \times 10^6 \text{M}^{-1}$	9.63%	7.95%
3	H	1	3	$2.66 \times 10^6 \text{M}^{-1}$	10.82%	6.11%
4	H	2	1	$4.27 \times 10^6 \text{M}^{-1}$	10.54%	87.30%
5	H	2	2	$8.64 \times 10^6 \text{M}^{-1}$	6.98%	17.35%
6	3-Br	2	1	$1.29 \times 10^6 \text{M}^{-1}$	5.15%	2.66%
7	4-Br	2	1	$1.73 \times 10^6 \text{M}^{-1}$	6.47%	3.45%
QNB				$4.50 \times 10^6 \text{M}^{-1}$	100.00%	

^aAffinity constant from LIGAND program, ^bCoefficient of variation between each analog determinations, ^cRelative binding index $\text{RBI} = K_A/K_{A(+/-)-\text{QNB}}$

2. Evaluation of 3-quinuclidinyl α -hydroxy- α -heteroaryl- α -phenylacetates.

The results of screening of novel compounds are summarized in Table II.

TABLE II. Inhibition Constants (K_i) of Heterocyclic Analogs of QNB

Compd	R	Ar	m1	m2	m3	m4
1	H	phenyl	44+/-10	60+/-14	241+/-94	117+/-24
2	Br	phenyl	88+/-6	1080+/-100	237+/-70	497+/-64
3	Br	2-furyl	186+/-57	1920+/-930	468+/-20	1520+/-249
4	Br	3-furyl	193+/-102	751+/-82	349+/-21	561+/-23
5	Br	2-thienyl	38+/-4	261+/-39	118+/-28	185+/-37
6	Br	3-thienyl	15+/-1	108+/-11	33+/-3	104+/-31

7	H 2-(5-Bromo- thienyl)	46+/-18	264+/-26	173+/-54	322/-118
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Competition experiments were performed by incubating membranes with 0.5nM [³H]NMS and varying concentrations of compound in PBS pH 7.2 for 60 min at 37°C. The mixture was then rapidly filtered over glass-fiber filters, washed and processed for scintillation counting. Data was analyzed by the LIGAND program using the following experimentally determined values for the K_d of [³H]NMS: 0.235, 0.425, 0.164 and 0.144 nM for m1, m2, m3 and m4-AChR, respectively. Data is expressed in pM and is the mean+/-S.D. of three determinations.

To locate a compound with similar to QNB binding characteristics but with lower relative lipophilicity we determined the retention times of some of the above compounds on a reverse-phase HPLC column. The results are listed in Table III.

TABLE III. Relative Lipophilicity of Compounds Listed in Table II

Compd	R	Ar	Retention Time (min)
1 (QNB)	H	phenyl	7.6+/-0.1
2 (4BrQNB)	Br	phenyl	14.3+/-0.5
3	Br	2-furyl	8.6+/-0.1
5	Br	2-thienyl	11.9+/-0.1
7	H	2-(5-bromothienyl)	13.9+/-0.4

Compounds were analysed on a Waters C-18 Radial Pak column eluted at 2 mL/min with MeOH:water:acetonitrile (50:40:10) containing 1g/L of 1-octanesulfonate and 1.2 mL of formic acid. Compounds 3 and 5 have significantly shorter retention times than 4BrQNB.

As a result of screening the novel compounds listed above, we found a heterocyclic derivative of QNB that may surpass IQNB as a CNS receptor radioligand. This novel compound (compd 3) binds to muscarinic receptors with an affinity that is greater than that of IQNB, is selective for the m1 subtype, and is considerably less lipophilic than IQNB. This last characteristic should reduce the serum protein binding as compared with IQNB, and would permit a reduction in the administered dose. The triazene precursor of this compound is currently being synthesized to enable us to radioiodinate it and perform biodistribution and pharmacokinetic studies with it.

3. Evaluation of lipophilic, m2-AChR-selective antagonists.

Synthesized by us tricyclic antagonists were tested for their apparent affinity at each of four m-AChRs. The following K_i values (means $\pm$ S.D.) are from displacement binding studies of [3 H]NMS binding to membranes from transfected cells or heart tissue (for m2-AChR), as described in experimental part of our proposal.

TABLE IV. Apparent Affinities of Novel Tricyclic Antagonists.

Compd	AFFINITY (K_i , nM, (S.D. (N)))			
	m1-AChR	m2-AChR	m3-AChR	m4-AChR
1	740 $\pm$ 87(3)	73.4 $\pm$ 2(3)	786 $\pm$ 51(3)	545 $\pm$ 26(2)
2	34.4 $\pm$ 5.5(3)	3.71 $\pm$ 0.42(3)	86.1 $\pm$ 12.2(3)	14.8 $\pm$ 0.05(3)
3	11.2 $\pm$ 1.0(3)	3.7 $\pm$ 2.1(3)	38.2 $\pm$ 10(2)	7.6 $\pm$ 1.8(3)
4	3.73 $\pm$ 1.0(4)	0.64 $\pm$ 0.64(3)	11.27 $\pm$ 1.6(3)	1.85 $\pm$ 0.63(4)

These data show that the diethyl hybrid (compd 4) is potent the most potent in the group and is selective for m2-receptors over m1-receptors (the most abundant in brain) by almost 6-fold. Since very few m4-receptors are present in the brain, its high affinity for m4-receptors is of little concern.

These results also demonstrate that we designed and synthesized a compound that is approximately 5 times more potent at m2-receptors than previously known compound AQ-RA 741. Although the selectivity of this novel compound is not yet adequate for imaging purposes, these data prove our growing expertise in designing and synthesizing increasingly selective and potent compounds.

Using Rekker's tables of fragmental partition coefficients we have calculated that the diethyl hybrid compound should be

approximately 15 times more lipophilic than AQ-RA 741. Furthermore, we **determined the CNS penetrability** of this compound using the ex-vivo binding protocol described in our proposal. For these studies, mice were injected (IP) with this compound and, after 3 hours, their brains were homogenized and the number of unoccupied receptors was determined by [3 H]NMS binding. In preliminary studies (N=2), we found that 3 hours following injection of 0.05 mg/mous of this compound, 0.042% of the dose penetrated the brain whereas the injection of 0.26 mg/mouse gave 0.014% of dose penetrated. We have reason to believe that a greater percentage of the dose will penetrate the brain at lower injected doses. In contrast, upon injections of the same doses, no detectable amount of AQ-RA 741 penetrated the brain. This data again demonstrates our growing expertise in designing potent m2-selective compounds that penetrate the blood-brain barrier.

4. Evaluation of 3-(2'-aminobenzhydryloxy)tropane (ABT) conjugate with Rhodamine B.

The apparent binding affinity of the ABT-Rhodamine b conjugate was compared to pure ABT as follows. Membranes from rat brain were prepared and incubated in phosphate-buffered saline containing 0.5 nM [3 H]NMS and various concentrations of either ABT or ABT-Rhodamine B conjugate. These mixtures were incubated at 37°C for 60 min, then rapidly filtered and the filters were processed for scintillation counting. The results showed that ABT inhibited [3 H]NMS binding with an IC_{50} of 0.9 - 1 nM and ABT-Rhodamine B conjugate inhibited [3 H]NMS binding with an IC_{50} of 1.5 - 2 nM, thereby demonstrating that the conjugate was almost as potent as the pure ABT.

5. Determination of the blood-brain barrier penetrability by hemicholinium-3 (HC-3).

The following studies were undertaken in order to determine how well [3 H]HC-3 crosses the blood-brain barrier of rats. Rats were injected either with 5 microCi of [3 H]HC-3 (for total binding, T), or with 5 microCi of [3 H]HC-3 and 4 nanomoles of unlabeled HC-3 (for non-specific binding, N). The compounds were administered by injection into tail veins of Sprague Dawley female rats (about 250 g). After the indicated times, the rats were sacrificed and the amount of radioactivity in the forebrain, mid-brain and cerebellum was determined. (Table V)

TABLE V. Penetration of the Blood-brain Barrier by HC-3

		% dose/ g tissue			
TIME	INJECTION	FOREBRAIN	MIDBRAIN	CBL	
15 min	T	.0123 +/- .0026	.0074 +/- .0013	.0195 +/- .0015	
	N	.0117 +/- .0044	.0053 +/- .0012	.0122 +/- .0039	

1 h	T	.0101 +/- .0006	.0034 +/- .0012*	.0098 +/- .0031
	N	.0097 +/- .0017	.0007 +/- .0002	.0087 +/- .0030
4 h	T	.0090 +/- .0021	.0050 +/- .0022*	.0096 +/- .0029
	N	.0054 +/- .0010	.0029 +/- .0020	.0066 +/- .0024

The data are expressed as percent dose per gram tissue. They are means +/- S.D. from three animals, each determined in triplicate. * Indicates a statistically significant difference between total (T) and non-specific (N) binding at the $p < 0.05$ level.

These data show that a small amount of [^3H]HC-3 does cross the blood-brain barrier. Of that small amount a statistically significant difference between total (T) and nonspecific (N) binding was found in the mid-brain. The difference between total and nonspecific binding at other times and in other brain areas was not statistically significant. These data demonstrate that [^3H]HC-3 is not a good compound for use as an imaging ligand, since so little crosses the blood-brain barrier and results in specific binding. However, these data also demonstrate that hemicholinium-3 is a suitable lead compound for developing such an agent. It should be possible to synthesize derivatives of HC-3 that are up to seven orders of magnitude better at crossing the blood-brain barrier while maintaining a high affinity for the HACU site.⁵

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