DOE/ER/60528--4

DE92 008735

RADIOLABELLED D2 AGONISTS AS PROLACTINOMA IMAGING AGENTS

Final Technical Report

for Period January 31, 1990 - August 31, 1991

Charlotte A. Otto

University of Michigan - Dearborn
Dearborn, Michigan 48128

DISCLAIMER

December 31, 1991

Prepared for

THE U.S. DEPARTMENT OF ENERGY
AGREEMENT NO. DE-FG02-87ER69528

Abstract

Research conducted in this terminal year of support centered on three distinct areas: mAChR ligand localization in pancreas and the effect of Ca^{+2} on localization, continuation of assessment of quaternized and neutral mAChR ligands for possible use as PET myocardial imaging agents, and initiation of a study to determine the relationship of the nAChR receptor to the cellular receptor for measles virus. Several tables and figures illustrating the results are included.

The completion of research funded by DOE included assessment of pancreatic uptake of muscarinic receptor (mAChR) ligands and the effect of Ca^{+2} on that uptake, assessment of structure and charge on in vivo myocardial localization of mAChR ligands and initiation of the relationship of nicotinic receptors (nAChR) to the cellular receptor for measles virus (MV). In addition, assistance in characterization of ^{13}C-N-methyl tropanyl benzilate (^{13}C-MTRB) and its metabolites in rats and monkey via tlc analysis was given. Each project is summarized below.

Binding of mAChR ligands to pancreatic tissue

A major cause of death in diabetics is heart failure due to cardiomyopathies. Evidence in the literature indicates that changes in mAChR density (1,2) might serve as a more valid indicator of potential heart failure than the current use of Tl-201 scintigraphy (3-5). In addition to myocardial changes of mAChR function and/or density, it is possible that pancreatic mAChRs undergo changes in density and/or function prior to or coincident with onset of diabetes. In order to begin this project, it was necessary to assess the presence of mAChR in pancreatic tissue (6) and to determine which of the various ligands used in characterization of the mAChR would be the most useful probe. A second phase of the initial study was to determine the effect of Ca^{+2} on localization.

Materials and Methods

The pancreatic tissue obtained from mice was stored in a liquid N_2 cooler until use. Each pancreas was thawed and homogenized in 8 ml of a 300 mM sucrose buffer, containing 0.2 mg/ml bacitracin and 500 kallikrein inhibitor units/ml aprotinin, for approximately 5 seconds. The resulting homogenate was then poured through two offset layers of medical gauze and immediately diluted with 88 ml of the incubation buffer. In each reaction tube, 1.0 ml of the homogenate was mixed with 50 µl ddH_2O and 50 µl of an atropine solution, or 100 µl of ddH_2O. Atropine, at a concentration of 2.4×10^{-5} M, was employed to determine non-specific binding. The radioactive ligand to be evaluated was added in varying concentrations. For assessment of the effect of Ca^{+2}, 1.9 M $CaCl_2$ was included in the incubation mixture; controls included EDTA to complex any Ca^{+2} present. Final concentrations of other constituents in the assay were 50 mM sodium phosphate, 2 mM $MgCl_2$, 1% bovine serum albumin, 0.2 mg/ml bacitracin, and 500 kallikrein inhibitor units/ml aprotinin. The tubes were vortexed briefly and allowed to incubate for 4 hours at room temperature. Incubation was stopped by the addition of 2-3 ml of ice-cold 50 mM sodium phosphate buffer at a pH of 7.4, and each assay was immediately filtered through glass-fiber filters (Whatman #30) presoaked 30 minutes in 0.05% polyethylenimine. Each assay tube was rinsed twice and the rinses filtered on the same filters. The filters were transferred to glass scintillation vials and 3 ml of Cytoscint fluid added. After equilibrating overnight, the vials

were counted on a Beckman LS5801 liquid scintillation counter.

Results and Discussion

To date the only ligand evaluated completely is ^{3}H-N-methylscopolamine (^{3}H-NMS). The K_D and B_{max} values are comparable to those reported in the literature (7) thus verifying the accuracy of the assay technique. The presence or absence of Ca^{+2} had little or no effect on either K_D or B_{max} values. Additional ligands to be evaluated include MTRB (a quaternized mAChR ligand), TRB (a neutral mAChR ligand) and QNB (the traditional standard for mAChR characterization). In addition, pancreatic tissue was obtained from mice treated in vivo with various mAChR ligands and this tissue will be analyzed to determine if mAChR ligands distribute to the pancreas under in vivo conditions.

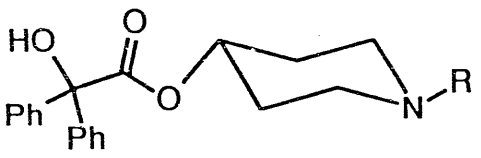
Assessment mAChR ligand structure for myocardial imaging

As indicated in a prior progress report (dated 8/1/89), assessment of neutral vs quaternized mAChR ligands as possible myocardial imaging agents was undertaken. Previous studies by Dr. G.K. Mulholland of the University of Michigan - Ann Arbor in conjunction with this laboratory indicated that tropanyl benzilate was an excellent ligand for detecting mAChR in brain (data recently accepted for publication, ref 8). Unfortunately, TRB, a neutral ligand, has high localization in the lung preventing its use as a myocardial imaging agent. Quaternization of TRB to provide MTRB, in a manner similar to that employed for the conversion of neutral QNB to quaternized MQNB, eliminated the lung localization and yielded an excellent myocardial agent. Further work (9-11) indicated that several quaternized mAChR ligands were potential ligands for the heart. The objective of this phase of research was to characterize the K_D and B_{max} values of some of these ligands and to assess their potential via an ex vivo assay to determine their in vivo localization.

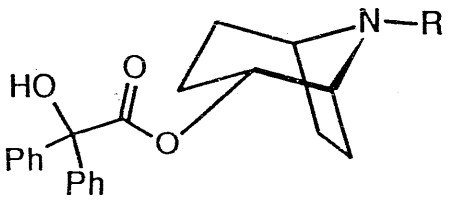
The ligands selected for further study included both neutral and quaternized compounds. Additional selection criteria placed an emphasis on compounds that were likely to be easily synthesized with ^{13}C-CH_3I or with ^{18}F-alkylating agents and possessed varying cyclic aminoalkyl groups (see Figure 1 for structures and abbreviations of compounds chosen for study).

In the first phase, the effect of quaternization of neutral mAChR ligands with CH_3I on IC_{50} values was determined. Table 1 is a compilation of the results. Generally, quaternization with CH_3I had minimal effect on IC_{50} values. Included in this table is a comparison of IC_{50} values for DAMP and DMPB. The structural difference between these ligands is the replacement of the 2-OH group in DMPB with 2-H in DAMP. Clearly, the 2-OH is significant for effective binding to the receptor - an effect previously described in the literature.

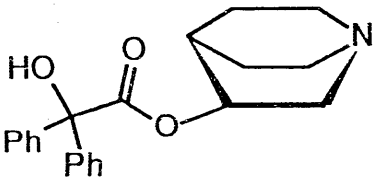
Quaternization with various benzyl halides comprised the second phase of the study. The increased steric bulk of the benzyl analogues prepared reduced the binding affinity as measured by IC_{50} values by at least an order of magnitude as illustrated in Table 2.



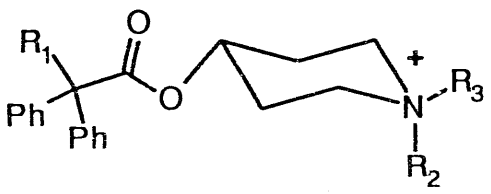
R	LIGAND
methyl	NMPB
fluoroethyl	NFePB
benzyl	NBzPB



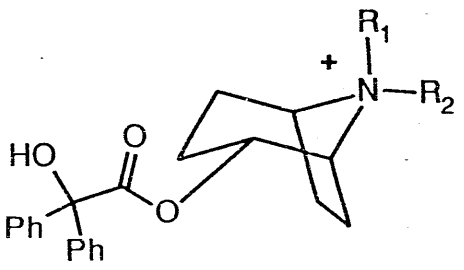
R	LIGAND
methyl	TRB
fluoroethyl	FeNTRB



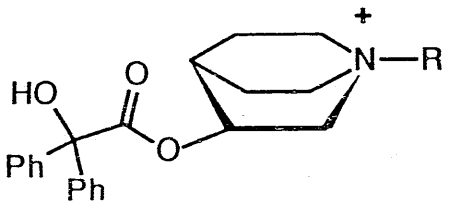
QNB



R1	R2	R3	LIGAND
OH	methyl	methyl	DMPB
H	methyl	methyl	DAMP



R1	R2	LIGAND
methyl	methyl	MTRB
methyl	fluoroethyl	NFeTRB



R	LIGAND
methyl	MQNB
2-F-benzyl	2FBzQNB
4-F-benzyl	4FBzQNB

FIGURE 1

TABLE 1

Neutral and Quaternary Methyl mAChR Ligands

Comparison of IC50 Values (nM)

Neutral Ligand	IC50	N-CH3 Ligand	IC50
NMPB	1.4	DMPB	1.9
		DAMP	37
TRB	1.7	MTRB	2.1
QNB	.46	MQNB	1.6

Quaternization of these neutral mAChR ligands with CH3X has minimal effect on IC50 values.

Replacement of 2-OH by H (DAMP vs. DMPB) significantly decreased binding affinity.

TABLE 2

Quaternary Methyl and Benzyl Ligands

Comparison of IC50 Values (nM)

N-Methyl Ligand	IC50	N-Benzyl Ligand	IC50
DMPB	1.9	NBzNMPB	11.6
MQNB	1.6	N2FBzQNB	26.1
		N4FBzQNB	17.5

IC50 values increased for all N-benzyl ligands relative to their N-methyl analogs. The larger steric bulk of the benzyl group apparently reduces binding affinity.

Use of quaternizing agents potentially capable of producing ^{18}F-labelled ligands was studied in the third phase. Results as shown in Table 3 were mixed. The best ligand appears to be the neutral FENTRB which had an IC_{50} comparable to TRB. Because FENTRB is neutral, it would be of use only for brain imaging. Of interest is that replacement of one of two methyl groups in MTRB by FCH_2CH_2- produced a ligand with relatively poor binding affinity. Coupled with the data from quaternization with benzyl halides, this data suggests limited ability to quaternize with alkylating agents larger than CH_3X.

Once in vitro characterization was complete, ex vivo studies were undertaken. In these studies, cold mAChR ligand was injected at varying doses into mice. Sacrifice followed by binding studies with ^{3}H-QNB to determine the number of available mAChR binding sites provided information on the effectiveness of the best ligands to bind to myocardial mAChR in vivo. Figure 2 shows the dose response study data for MQNB, MTRB and DMPB. Apparently, MTRB and MQNB are capable of binding more mAChR sites in vivo than DMPB. MTRB appears to be slightly better than MQNB at higher injected doses but the difference is not significant.

The last study undertaken in this area was a time course of inhibition as determined by the ex vivo method. Data contained in Figure 3 shows that MTRB binds to myocardial mAChR in a similar fashion to MQNB. Both DMPB and the benzylated derivative N4FBzQNB dissociated from myocardial mAChR faster than either MQNB or MTRB.

Part of these results were presented at various meetings (12,13). The ex vivo results have not yet been submitted for publication or presentation.

Relationship of nAChR to the cellular receptor for Measles virus

Viruses utilize cell surface determinants which serve other normal functions for cells as attachment sites for the initiation of infection. We thought it might be interesting to test the hypothesis that measles virus binds to a receptor belonging to the nicotinic subclass of acetylcholinergic receptors. The virus infects cells of the central nervous system as indicated by the presence of MV nucleocapsid inclusions in brain tissue from individuals with SSPE and the fact that the virus can also cause encephalitis and EEG changes as a result of normal infection. Another virus which infects nerve cells and which is distantly related to measles virus is rabies virus. The cellular binding site for rabies virus has been identified as being a nicotinergic receptor.

Since α-bungarotoxin is a powerful reagent useful in identifying nAChR, sequence homology studies were done to determine whether there existed any sequence homology between α-bungarotoxin and the measles H protein. Comparison of the sequence of the toxic loop (binding portion) of α-Bgtx with the antigenic regions of the measles virus H protein (as determined by the IBI-Pustell Sequence Analysis Program) indicated that the nucleotide sequence encompassing amino acid residues 292 to 319 of the H protein has >60% homology with the nucleotide sequence toxic loop. Although there is considerable homology on the nucleic acid level, amino

TABLE 3

Neutral and Quaternary Fluorinated Ligands

Comparison of IC50 Values

Neutral Ligand	IC50	Charged Ligand	IC50
NMPB	1.4		
NFEPB	14.8		
TRB	1.7		
FENTRB	2.0		
MTRB	1.2	NFETRB	87*
QNB	0.46	N2FBzQNB	26.1
		N4FBzQNB	17.5

Replacement of N-CH_3 with N-CH_2CH_2F generally decreased binding affinity by at least one order of magnitude. Only FENTRB had an affinity similar to the parent ligand.

*Preliminary Data

FIGURE 2

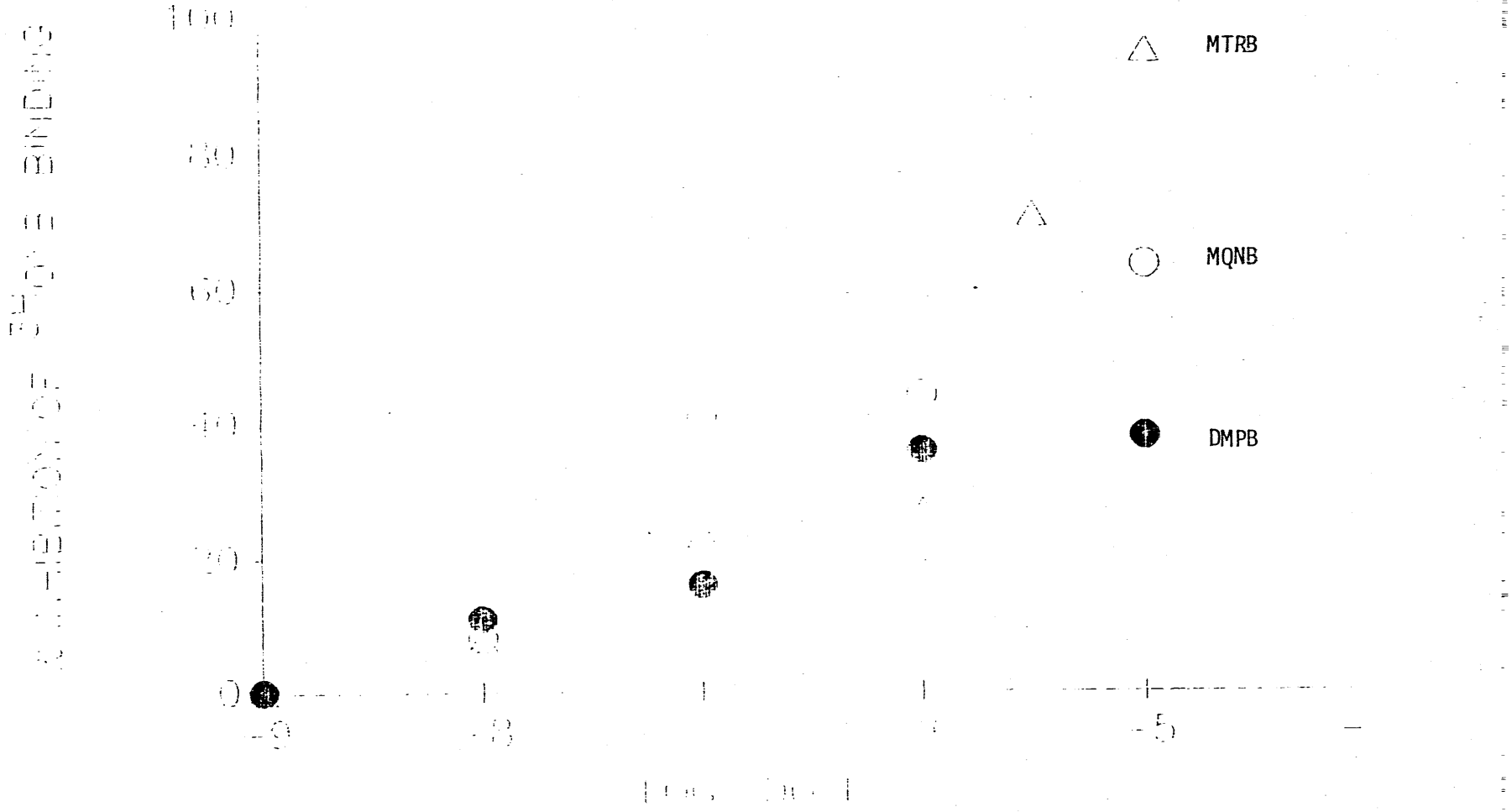
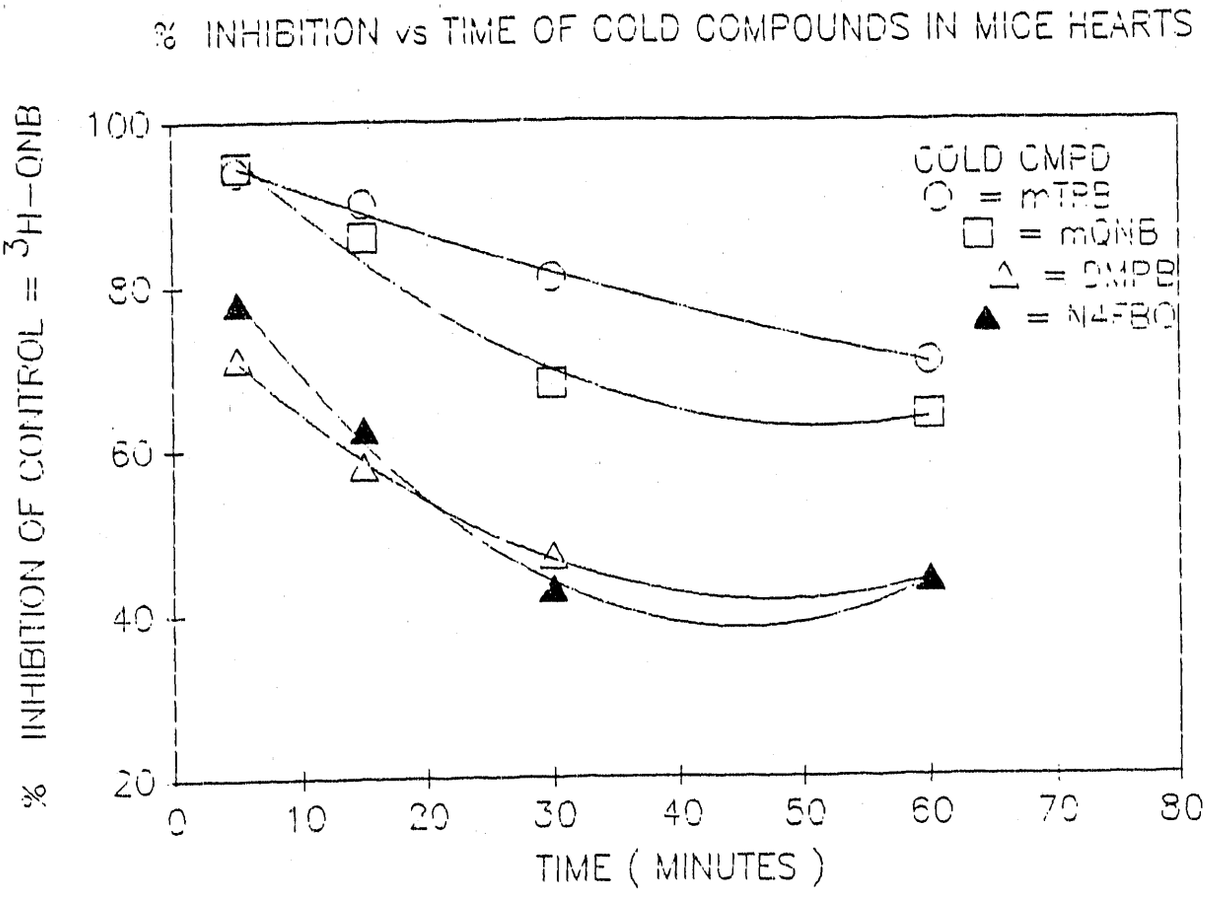


FIGURE 3


% INHIBITION vs TIME OF COLD COMPOUNDS IN MICE HEARTS
% INHIBITION OF CONTROL = ^{3}H-ONB
100
80
60
40
20
0
10
20
30
40
50
60
70
80
TIME (MINUTES)
COLD CMPD
○ = mTPS
□ = mQNB
△ = CMPD
▲ = NEFBO

acid homology is limited. Four point mutations in the measles virus sequence would increase the amino acid homology of the sequences.

a) R K M W C D A F C S SRG K V V E L G C

b) G E L K L A A L C H G E D S I T I P Y Q G S G K G V S F
a A f C s S G K **v v e l**

The amino acid sequence of the α-Bgtx toxic loop is shown in a). The underlined residues are highly conserved in neurotoxins. The measles virus H amino acids 292 to 319 are shown in b). Point mutations shown in boldface would increase amino acid sequence homology. As indicated above, there is >60% homology at the nucleotide level but only about 30% at the amino acid level.

Vero cells were tested to confirm that they contain αBgtx binding sites. Statistical analysis of the binding of ^{125}I-αBgtx to Vero cell homogenates suggests one site binding together with non-specific binding. The results of such an analysis are shown below.

The nm range of K_D for α-bgtx binding indicates that α-bgtx binds to Vero cells with exceptionally high affinity. The relatively poor affinity demonstrated by the competitive agonists is typical of binding observed with other nAChR receptors in the brain and periphery. In general, antagonists bind with higher affinity and are more stable than agonist binding. These data confirm that a receptor site recognizing α-bgtx is present on the Vero cells and suggest that this receptor site may belong to a subclass of nAChR. Further definitive studies to identify the nAChR site as muscular or neuronal and identifying the α subunit (see below) are required. Molecular characterization is also necessary for accurate identification.

Binding of known nAChR ligands to Vero cell

Ligand	K_D(moles)	B_{max} (moles/liter)
competitive antagonists		
^{125}I-αBgtx	$0.29 \pm 0.0 \times 10^{-9}$	0.20×10^{-12}
D-tubocurarine	$1.13 \pm 0.11 \times 10^{-5}$	9.45×10^{-8}
competitive agonists		
carbamoylcholine	$1.33 \pm 0.18 \times 10^{-3}$	3.67×10^{-11}
nicotine	$6.84 \pm 0.13 \times 10^{-6}$	6.19×10^{-11}

Competitive binding studies were carried out using unlabeled

α-bgtx to compete with measles virus for binding to Vero cells. The amount of measles virus added or remaining unbound to cells was assayed by hemagglutination titration. One million Vero cells were treated with 1×10^{-9} M unlabeled α-bgtx or with phosphate buffered saline, before adding 1×10^{6} PFU/mL. After a 1 hour adsorption period, the cells were washed three times with buffer and the washes were collected to determine the amount of virus bound and unbound. The results of such an experiment is shown below.

	PFU/mL	
	virus bound	virus unbound
αBgtx treated	3×10^{4}	1.97×10^{6}
control	5×10^{5}	1.5×10^{6}

Additional studies are currently underway to verify the nature of the MV - Vero cell interaction.

References

1) Latifpour J and McNeill JH: Cardiac autonomic receptors: effect of long-term experimental diabetes. J Pharmacol Exp Ther 230:242, 1984.

2) Carrier GO and Aronstam RS: Altered muscarinic receptor properties and function in the heart in diabetes. J Pharmacol Exp Ther 242:531, 1987.

3) Mizuno S, Genda A, Nakayama A, et al: Myocardial involvement in diabetic patients evaluated by exercise thallium-201 scintigraphy and cardiac catheterization. J Cardiogr 15:427, 1985.

4) Rubler S and Fisher VJ: The significance of repeated exercise testing with thallium-201 scanning in asymptomatic diabetic males. Clin Cardiol 8:621, 1985.

5) Koistinen MJ, Huikuri HV, Pirttiaho H, et al: Evaluation of exercise electrocardiography and thallium tomographic imaging in detecting asymptomatic coronary artery disease in diabetic patients. Br Heart J 63:7, 1990.

6) Clark WG, et al. Goth's Medical Pharmacology Twelfth Edition. St. Louis, The C.V. Mosby Co., 1988.

7) Waelbrock M, Gillard M, Robberecht P, and Christophe J: Life Sci 41:2235-2240, 1987.

8) Mulholland GK, Otto CA, Jewett DM et al: Synthesis, rodent biodistribution, dosimetry, metabolism, and monkey images of carbon-11 labeled (+)-2α-tropanyl benzilate, a central muscarinic receptor imaging agent. Accepted for publication by J Nucl Med, 1992.

9) Mulholland GK, Otto CA, Kilbourn MR et al: Synthesis and regional brain distribution of [C-11]N-methyl-4-piperidyl benzilate ([C-11]NMPB) in the rat. J Nucl Med 29:P768, 1988.

10) Mulholland GK, Otto CA, Jewett DM, et al: Radiosynthesis and comparisons in the biodistribution of carbon-11 labeled muscarinic antagonists: (+)-2α-tropanyl benzilate and N-methyl-4-piperidyl benzilate. J Labelled Cmpd Radiopharn 26:202-203, 1988.

11) Mulholland GK, Schwaiger M, Otto CA, et al: Synthesis and animal studies of C-11 tropanyl benzilate methiodide (MTRB). J Nucl Med 30:P930, 1989.

12) Otto CA, DeMattos, Mulholland GK et al: Evaluation of neutral and quaternized mAChR ligands in heart homogenates. J Nucl Med 31:P897, 1990.

13) Otto CA, Mulholland GK, DeMattos SB, et al: Evaluation of neutral and charged mAChR ligands as potential PET agents for myocardial imaging. J Labelled Cmpd Radiopharm 30: 248-249, 1991.

END

DATE
FILMED
5/07/92