

In Vitro and *Ex Vivo* Evaluation of Cyclic Aminoalkyl Benzilates as Potential Emission Tomography Ligands for the Muscarinic Receptor

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A series of muscarinic antagonists were screened as potential receptor imaging agents. (+)-2 α -tropanyl benzilate (TRB), N-methyl-4-piperidyl benzilate (NMPB) and several analogs amenable to labeling with positron emitting isotopes were evaluated for muscarinic binding to mouse brain tissue *in vitro* and *ex vivo* using [³H]quinuclidinyl benzilate as the probe. The *in vitro* assay directly compared the innate binding affinities of the compounds. The rank order of binding (IC₅₀) was TRB (0.7 nm), QNB (0.8 nm), scopolamine (1.3 nm) and NMPB (1.6 nm). The *ex vivo* assay was used to gain information regarding the pharmacokinetics and brain penetration of the compounds in live animals. *Ex vivo* results demonstrated that TRB was rapidly taken up into the brain and was equipotent with QNB in occupying muscarinic binding sites at early time points, but TRB binding decreased twice as fast over time as QNB binding. The results suggest TRB would be a good candidate for radiolabeling and further study.

Introduction

As part of an effort to develop new muscarinic receptor (mAChR) imaging agents for study of the central muscarinic cholinergic system using positron emission tomography (PET), we sought a method to allow screening of potential ligands in unlabeled form. It would be desirable if the screen yielded information about the penetration and retention of ligands in the brain *in vivo* as well as compare their *in vitro* binding affinities for the mAChR. The screening procedure would be applied to a group of potent, centrally active muscarinic antagonists and analogs that could be labeled with high specific activity carbon-11 or fluorine-18 (Fig. 1). It was hoped that a combination of *in vitro* and *ex vivo* assays (Cho *et al.*, 1986; Sethy *et al.*, 1987) would provide answers to these questions and allow selection of promising candidates without resorting to expensive and time consuming radiosynthesis of each compound under initial consideration.

Materials and Methods

Drugs

Samples of (+)-2 α -tropanol, nor-(+)-2 α -tropanyl benzilate (nor-TRB) and nor-(+)-2 α -tropanyl

diphenylfluoroacetate (TRDFA) (Atkinson *et al.*, 1983) were kindly provided by Dr Edward R. Atkinson. (+)-2 α -Tropanyl benzilate (TRB) (Atkinson *et al.*, 1977), 4-piperidyl benzilate (PB) (Biel *et al.*, 1961), N-methyl-4-piperidyl benzilate (NMPB) (Biel *et al.*, 1961) and (\pm) quinuclidinyl benzilate (QNB) (Meyerhoffer, 1972; Kadin and Cannon, 1962) were prepared by standard literature procedures involving base catalyzed transesterification of the respective aminoalcohols with methyl benzilate. Norscopolamine·HCl was prepared from scopolamine (Schmidt *et al.*, 1965). The following drugs were obtained commercially: (-)atropine sulfate·H₂O (Aldrich), scopolamine·HBr·2H₂O (Sigma) and [³H]QNB (specific activity 33 Ci/mmol, DuPont NEN). N-(2-Fluoroethyl)-4-piperidyl benzilate (FEPB) and N-(2-fluoroethyl)nortropanyl benzilate (FENTRB) were prepared by reacting PB and nor-TRB respectively, with 2-fluoroethyl iodide and K₂CO₃ in refluxing acetonitrile, and purified by flash chromatography (SiO₂, 20:1:1 CH₂Cl₂:MeOH:Et₃N).

In vitro assay

Female CD mice (24-30 g, Charles River) were killed by decapitation under ether anesthesia. Brains were rapidly removed and the cerebellum discarded.

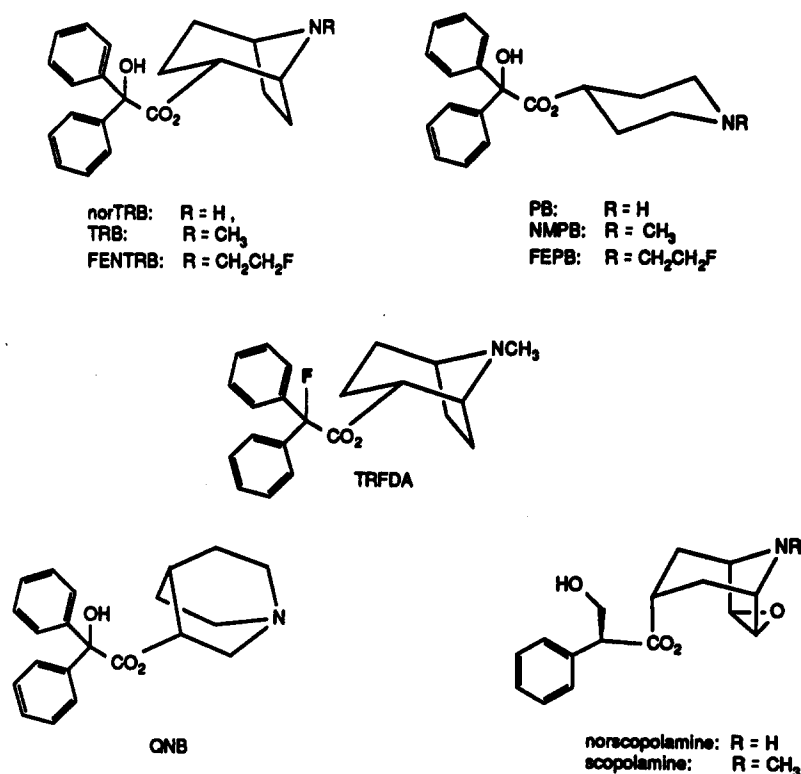


Fig. 1. Structures of ligands.

The remaining brain tissue was stored at -80°C until used. For the assay, each brain was thawed and homogenized in 3 mL of ice-cold 50 mM sodium-potassium (Na-K) phosphate buffer (pH 7.4) containing 10 mM EDTA. The homogenate was kept on ice for 30 min and then centrifuged at 48,000 *g* for 10 min. The pellet was resuspended in 5 mL of Na-K phosphate buffer. Protein concentration was determined using the Bradford assay (standard: bovine serum albumin). Protein concentration of the homogenate was 6–7 $\mu\text{g}/\mu\text{L}$. To assay the inhibition of [^3H]QNB binding to brain tissue, triplicate samples of 50 μL of homogenate in 50 mM Na-K phosphate buffer containing 0.5 nM [^3H]QNB with and without varying concentrations of drugs were used. A minimum of ten different concentrations of each drug were used. Nonspecific binding was also determined in triplicate by including 1 μM atropine in the assay. The entire assay was repeated at least twice with each drug. The assay was initiated by addition of homogenate to yield a final volume of 2.0 mL and incubated at room temperature for 60 min. The assay was terminated by addition of 3.0 mL of ice-cold buffer followed by an immediate vacuum filtration using glass fibre filters (S&S #32). Each filter was washed immediately and rapidly with 3 \times 3.0 mL of ice-cold buffer and transferred to a scintillation vial. After adding cocktail (3.0 mL) the vials were stored in the dark for 12 h and then counted (58% efficiency). The IC_{50} for each compound is the average of at least two separate studies

conducted in triplicate. The IC_{50} values were determined from plots of ligand concentration vs percent inhibition of radiotracer binding and are tabulated in Table 1.

Ex vivo assay

Four mice were injected with drug dissolved in 0.1 mL of 10% EtOH/saline vehicle. Control mice were injected with an equal volume of vehicle alone. Drug dosages ranged from 1×10^{-7} to 1×10^{-5} mol/kg. The dosages of \pm QNB were doubled to compensate for the presence of the inactive enantiomer. At designated times, the mice were killed as above, the brains were rapidly removed, and cerebellum discarded. Each brain was individually frozen at -80°C until used. Assays were conducted

Table 1. IC_{50} values for various mAChR ligands obtained from competitive binding assays using [^3H]QNB in mouse brain homogenates

Drug	IC_{50} (nM)
QNB	0.8 ± 0.2
Scopolamine	1.3 ± 0.3
Nor-scopolamine	6.9 ± 0.9
PB	17.3 ± 2.7
NMPB	1.8 ± 0.1
FEPB	6.4 ± 0.4
TRB	0.7 ± 0.3
nor-TRB	1.3 ± 0.4
TRFDA	8.5 ± 1.0
FENTRB	2.5 ± 0.2

within 2 weeks of sacrifice. Immediately prior to assay, each brain was thawed and homogenized individually in 4 mL of 50 mM Na-K phosphate buffer, pH 7.4. Homogenates were stored on ice (<10 min) until added to the assay. Protein concentration was determined as above; concentrations ranged from 6–11 $\mu\text{g}/\mu\text{L}$. The assay was conducted as above except that homogenate incubation was carried out at 37°C for 30 min and each brain was assayed in quadruplicate with and without 1 μM atropine. The counts were corrected for background and normalized for protein concentration. Binding levels are expressed as cpm/ μg protein.

Results and Discussion

To the best of our knowledge, these *in vitro* and *ex vivo* binding studies of TRB, NMPB and their analogs provide the first direct comparisons between these potent central muscarinic antagonists. No receptor binding studies have been reported for TRB or its analogs. Our attention was drawn to these compounds by live animal pharmacological studies which indicated very high potency (fourfold greater than QNB) and rapid onset of central effects for TRB and some of its analogs (Atkinson *et al.*, 1977). The N-fluoroethyl analogs FENTRB and FEPB have not been examined previously although a structurally similar quaternized N-fluoroethyl 3-tropanyl benzilate is reported to have potent peripheral anticholinergic actions (Bauer and Fugner, 1986). Extensive pharmacological data on NMPB have been collected (Kloog and Sokolovsky, 1977; Sokolovsky *et al.*, 1980) and studies directly comparing *in vitro* binding of NMPB with QNB and scopolamine are available (Rehavi *et al.*, 1978). The inclusion of these reference ligands in our test procedures helped to assess the validity of the results obtained.

In vitro assay: structure-binding relationships

IC_{50} values were determined for each ligand by means of traditional, competitive binding assays. Values for QNB and scopolamine were also obtained for comparison. The results are compiled in Table 1. The values for NMPB, TRB, scopolamine, FENTRB and nor-TRB were similar in magnitude to those of QNB.

Although TRB and NMPB had similar IC_{50} values (0.6 and 1.8 nM respectively), the effects of demethylation were quite different. In the piperidyl series, demethylation decreased the binding affinity by an order of magnitude whereas in the tropanyl series, demethylation only reduced binding affinity by half. Replacement of N-methyl by N-fluoroethyl in TRB and NMPB reduced the binding by a factor of approximately 3 in each case. In other studies, the N-propyl, N-cyclopropylmethyl and N-allyl tropanyl derivatives showed peripheral effects comparable to TRB and nor-TRB (Atkinson *et al.*, 1977). These

Table 2. Percent inhibition of control [^3H]QNB binding 45 min after *in vivo* drug injection. Drug concentration was 1×10^{-5} mol/kg

Drug	Percent inhibition
Control (saline)	0
NMPB	26
Scopolamine	35
nor-TRB	63
TRB	65

results suggest that there is some steric tolerance of the receptor binding site towards N-substitution in the tropanyl series.

The fluoro analog TRFDA had an IC_{50} value of 8.5 nm, however the position of fluorine attachment may be sensitive to hydrolysis to produce TRB. Further studies are necessary to establish whether the measured value represents the true binding affinity of TRFDA or is an overestimation caused by *in situ* formation of TRB from TRFDA.

Ex vivo assay

To assess brain penetration of the compounds, an *ex vivo* assay patterned on that used by Cho *et al.* (1986) and Sethy *et al.* (1987) was conducted. This *ex vivo* assay consisted of injection of unlabeled ligand into an animal followed by sacrifice and use of an *in vitro* binding assay to measure residual [^3H]QNB binding to brain homogenates. Several types of studies were performed: determination of effect of ligand and dose on receptor occupancy, and effect of time on receptor occupancy.

Effect of ligand and dose on receptor occupancy

Several ligands with low IC_{50} values were initially examined in the *ex vivo* assay at a dose of 1×10^{-5} mol/kg, a pharmacologically active dose for NMPB (Sokolovsky *et al.*, 1980). The percent inhibition of control [^3H]QNB binding at 45 min for TRB, nor-TRB, NMPB and scopolamine are summarized in Table 2. TRB occupied the most recep-

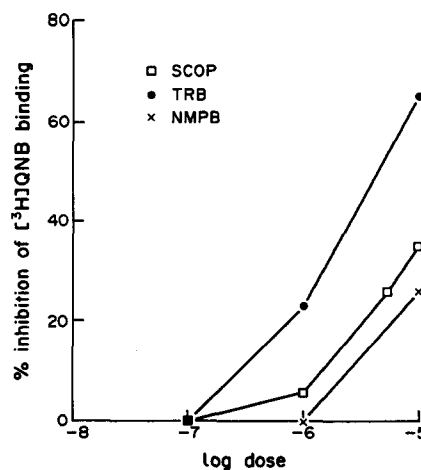


Fig. 2. The percent inhibition of control [^3H]QNB binding vs ligand identity and dose after *i.v.* administration of ligands to mice and sacrifice at 45 min.

tors, NMPB the least and scopolamine binding was intermediate. TRB and nor-TRB binding were nearly identical, suggesting similar brain penetration and retention for these two compounds. This further emphasizes the close biological resemblance between TRB and nor-TRB indicated in this work by the IC_{50} values and by the peripheral pharmacology data previously reported (Atkinson *et al.*, 1977).

The range of doses tested in the *ex vivo* assay was extended from 1×10^{-5} mol/kg to 1×10^{-7} mol/kg for TRB, NMPB and scopolamine. The results are depicted in Fig. 2. TRB inhibited more [3H]QNB binding than either NMPB or scopolamine at doses ranging from 1×10^{-5} to 1×10^{-6} mol/kg. The lowest detectable binding varied with ligand identity and apparently can be as low as 1×10^{-7} mol/kg as observed for TRB. As the assay was conducted at 37°C for 30 min it is probable that a portion of the ligand bound *in vivo* dissociated during the incubation; the measured value of [3H]QNB binding inhibition is therefore a lower limit for ligand receptor occupancy at the time of sacrifice.

Effect of time on receptor occupancy

The effect of time on receptor availability was compared for QNB and TRB. The results, plotted as percent inhibition of control [3H]QNB binding vs time are shown in Fig. 3. In this study, mice were injected either with QNB or TRB (1×10^{-5} mol/kg) and sacrificed at designated times. The binding level for drug is expressed as a fraction of the binding level for controls. Both TRB and QNB demonstrated almost complete receptor occupancy at 2 min post injection. While the percentage of receptor vacancy increased with time for both compounds, the rate of increase was about twice as fast for TRB compared to QNB: $t_{1/2} = 113$ min for TRB and 234 min for QNB (correlation coefficients of $\ln C_0/C_t$ vs time were

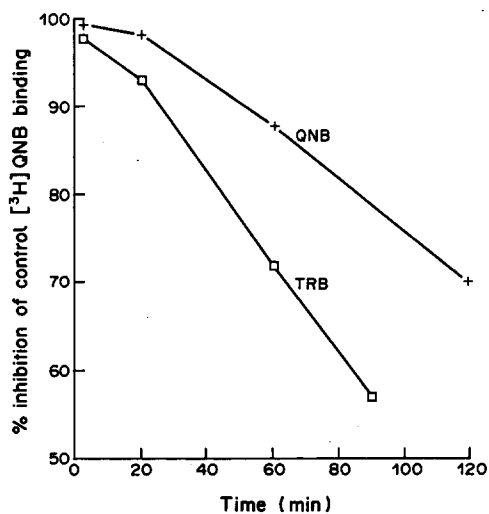


Fig. 3. Inhibition of [3H]QNB binding at various time intervals after i.v. administration of 1×10^{-5} mol/kg doses of TRB or QNB to mice.

0.992 for TRB and 0.986 for QNB). It is noteworthy that these data are similar to the time course of anti-tremorine (central) effects of QNB and TRB previously reported (Atkinson *et al.*, 1977).

Evaluation of receptor-based agents employing only *in vitro* studies suffers because normal physiological compartments and barriers are disrupted and tissue homogenization may expose more binding sites than are accessible *in vivo*. For example, striking differences between [3H]spiperone binding to *in vitro* homogenates, *in vitro* tissue slices and *in vivo* tissue samples have been described in normal and dopamine denervated rat striatal tissue (Bennett and Wooten, 1986). Although the *ex vivo* assay does not distinguish binding of parent drug from binding of active metabolites, it does provide a more realistic assessment of radiopharmaceutical potential than *in vitro* binding assays alone. The combination of *in vitro* competitive binding assays and estimation of *in vivo* receptor occupancy does not generate all the information needed to evaluate a potential receptor imaging agent, but it helps narrow the list of potential receptor-based agents without the need for prior radiosynthesis.

Conclusion

In vitro competitive binding assays have shown that several piperidyl and tropanyl benzilates have binding affinities for the mAChR comparable to QNB and scopolamine. Radiolabeled forms of QNB (Eckelman *et al.*, 1984) and scopolamine (Frey *et al.*, 1987; Mulholland *et al.*, 1988) have been used to image the mAChR in human brain. These piperidyl and tropanyl benzilates may also serve as imaging agents. *Ex vivo* assay results indicate that TRB occupies more receptors than the other ligands examined and appears to be equipotent with QNB while possessing a faster rate of clearance from the brain. The accumulated evidence points to TRB as a likely candidate for radiolabeling and further evaluation as a PET agent for mAChR imaging.

Of considerable importance is that the *ex vivo* assay can be employed to (1) demonstrate CNS localization of a ligand or its active metabolites (2) rank ligands in terms of *in vivo* binding potency and (3) assess receptor clearance rates. Thus, the combination of both *in vitro* and *ex vivo* assays is beneficial in selection of potential imaging agents.

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