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CHARACTERISATION OF [^{3}H]-DARIFENACIN AS A NOVEL RADIOLIGAND FOR THE STUDY OF MUSCARINIC M_3 RECEPTORS

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ABSTRACT

Darifenacin, (S)-2-[1-[2,3-dihydrobenzofuran-5-yl]-3-pyrrolidinyl]-2,2-diphenylacetamide, is a novel muscarinic M_3 antagonist. In this study we have compared the binding of [^{3}H]-darifenacin to the five cloned human muscarinic receptors (m_1 - m_5) expressed in CHO cells. [^{3}H]-darifenacin binds with 6 fold higher affinity to m_3 (K_D = 0.33 nmol/l) over m_1 (K_D = 1.6 nmol/l) receptors. There was no specific binding of [^{3}H]-darifenacin to m_2 receptors and specific binding to m_4 and m_5 receptors was insufficient to determine a K_D. Binding of [^{3}H]-darifenacin to m_1 and m_3 was displaced by atropine (m_1 pK_i = 9.36, m_3 pK_i = 9.4), 4-DAMP (m_1 pK_i = 9.04, m_3 pK_i = 9.19), pirenzepine (m_1 pK_i = 8.63, m_3 pK_i = 6.85), methoctramine (m_1 pK_i = 7.28, m_3 pK_i = 6.63), and darifenacin (m_1 pK_i = 8.36, m_3 pK_i = 9.14), demonstrating that [^{3}H]-darifenacin represents the first selective m_3 radioligand.

INTRODUCTION

Five subtypes of human muscarinic receptor, designated as m_1, m_2, m_3, m_4 and m_5, have been identified and cloned (1). The pharmacologically defined M_1, M_2, M_3 and M_4 receptors correspond to the cloned m_1, m_2, m_3 and m_4 receptors, however, the pharmacological M_5 receptor corresponding to the m_5 gene product has yet to be reported. Although a few muscarinic antagonists have proved useful in characterising muscarinic receptor subtypes, definitive pharmacological characterisation has been hampered by the lack of subtype-specific compounds. Most commonly used standard antagonists include pirenzepine (M_1 selective), AF-DX-116(11[[2-[dimethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-6][1,4] benzodiazepine-6-)one) and methoctramine (M_2 selective), 4-diphenylacetoxy-N-

methylpiperadine methobromide (4-DAMP), hexahydrosiladifenidol (HHSiD) and p-fluoro-HHSiD (M_1/M_3 selective). Darifenacin is a novel muscarinic antagonist shown to be potent and selective for the M_3 receptor both in isolated tissue studies (2) and in radioligand binding studies to the cloned human muscarinic subtypes (3). In this study we have compared the binding of [^{3}H]-darifenacin to the five cloned human muscarinic receptors expressed in CHO cells.

MATERIALS

[^{3}H]-Darifenacin (Specific Activity 22Ci/mmol) was prepared by tritiation of the benzofuran ring to give 2,3-ditritiobenzofuran by Amersham International (Buckinghamshire, UK). Atropine, 4-DAMP, methoctramine and pirenzepine, were purchased from RBI (Natick, MA). Darifenacin was synthesised in our laboratories at Pfizer Central Research.

METHODS

Membrane Preparation.

The CHO-K1 cells stably expressing the human m_1 - m_5 receptors have been described previously (4) and were obtained from Dr. Tom Bonner (National Institute of Neurological Disorders and Stroke, Bethesda, MD). Transfected cells were grown to 80% confluency and washed twice in phosphate buffered saline (PBS) prior to harvesting by scraping. Scraped cells were resuspended in PBS, pelleted at 1000 xg for 10 min. and stored at -80°C until use. Thawed membranes were homogenised in ice cold HEPES buffer (20 mM pH 7.4) and washed twice by centrifugation at 38,000 x g for 20 min. The protein concentration of the resuspended membranes was determined using a Sigma protein assay kit. Membranes were stored in aliquots at -80°C before use.

Radioligand Binding Studies.

All membranes, drugs and radioligand solutions were made up in 20 mM HEPES buffer (pH 7.4 at 25°C). Final protein concentrations were 120 µg/ml (m_1), 60 µg/ml (m_3) and 400 µg/ml (m_2, m_4, m_5). Assays were performed in a total volume of 500 µl. Saturation analysis for [^{3}H]-darifenacin binding to m_3 receptors was performed over the concentration range 0.1 - 10 nmol/l and for m_1, m_2, m_4 and m_5 over the range 0.1 - 25 nmol/l. For competition experiments, pK_is were determined by displacement of [^{3}H]-darifenacin (0.4 nmol/l for m_3 and

1 nmol/l for m_1) using 12 concentrations of antagonist. Time course experiments with the m_3 receptor were performed using 0.4nmol/l [^{3}H]-darifenacin at various incubation times. Non specific binding (NSB) was defined using 1μM atropine. Incubations were initiated by the addition of [^{3}H]-darifenacin and carried out at 25°C for 2 hr. Binding was terminated by rapid filtration through a Brandell cell harvester onto Whatman GF/B filters followed by 3 washings with ice-cold HEPES buffer. Each filter was dried and trapped radioactivity was measured by liquid scintillation counting using Meltilex solid scintillant and a Wallac 1204 Beta counter.

Data Analysis.

Results are expressed as mean values ± the standard deviation of *n* experiments. The maximal number of binding sites (B_{max}) and the equilibrium dissociation constant (K_D) were determined from direct analysis of the saturation binding data using Grafit. Hill coefficients and IC_{50} values were obtained from competition experiments by the method of Hill (5) using an in-house data fitting programme. K_i values were derived from IC_{50} values using the Cheng-Prussoff IC_{50} correction (6).

RESULTS

Kinetics of [^{3}H]-Darifenacin Binding.

Specific binding of [^{3}H]-darifenacin to m_1 and m_3 receptors (which was >80% of total binding) was time-dependent and reached equilibrium at 25°C after 2 h. A representative association time-curve for [^{3}H]-darifenacin binding to the m_3 receptor is shown in Figure 1.

Representative saturation curves for specific binding of [^{3}H]-darifenacin to m_1 and m_3 receptors are shown in Figure 2. The linear representation of the data following Scatchard transformation indicates the presence of a single homogeneous population of sites in each cell line. The equilibrium binding parameters K_D and B_{max} for m_1 and m_3 are shown in Table 1.

It was not possible to determine K_D and B_{max} for [^{3}H]-darifenacin binding to the m_2 receptor as there was no specific binding of [^{3}H]-darifenacin even at concentrations as high as 25 nmol/l. Similarly, binding of [^{3}H]-darifenacin to m_4 and m_5 receptors was not sufficient to allow accurate determination of K_D and B_{max}. When the same membrane preparations of m_2, m_4 and m_5 used to investigate [^{3}H]-darifenacin binding were incubated with the non-selective antagonist [^{3}H]- N-methyl scopolamine ([^{3}H]-NMS) (0.1nmol/l) , high levels of specific binding (> 80% of total binding) were observed (data not shown) indicating that muscarinic receptors were present in these preparations.

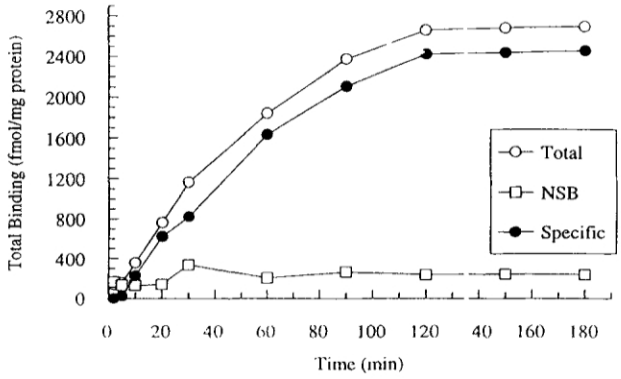

FIG. 1 Time course of association of [^{3}H]-darifenacin binding to the cloned human m_3 receptor. Membranes expressing m_3 receptor (60 µg protein / ml) were incubated at 25 °C with 0.4 nmol/l [^{3}H]-darifenacin for the times indicated. Each data point is the mean of 6 determinations from 1 representative experiment. NSB = non-specific binding

[^{3}H]-Darifenacin Displacement Experiments.

Table 2 shows the selectivity of standard muscarinic antagonists at displacing [^{3}H]-darifenacin from m_1 and m_3 receptors. None of the Hill coefficients were significantly different from unity (range 0.8 - 1.1), consistent with the presence of a single population of receptors in each cell line. Representative binding curves to show displacement of [^{3}H]-darifenacin from the m_3 receptor are shown in Figure 3.

Darifenacin was 6-fold selective for m_3 over m_1. Atropine and 4-DAMP had similar affinities towards m_1 and m_3. Pirenzepine was 60-fold more seletive for m_1 over m_3 and methoctramine was 4-fold more selective for m_1 over m_3.

DISCUSSION

The number of m_1 and m_3 binding sites labelled by [^{3}H]-darifenacin was consistent with those we have obtained previously using the non-selective muscarinic antagonists [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) (3) and [^{3}H]-NMS (*data not shown*). Similarly, the binding affinity and receptor selectivities of standard muscarinic antagonists (atropine, 4-DAMP, methoctramine and pirenzepine) at m_1 and m_3 receptors were similar to that reported for [^{3}H]-NMS (4,7). However, the potencies of compounds in this study were consistently 0.5 - 1 log unit greater than we have previous reported for the same compounds against [^{3}H]-QNB. Our

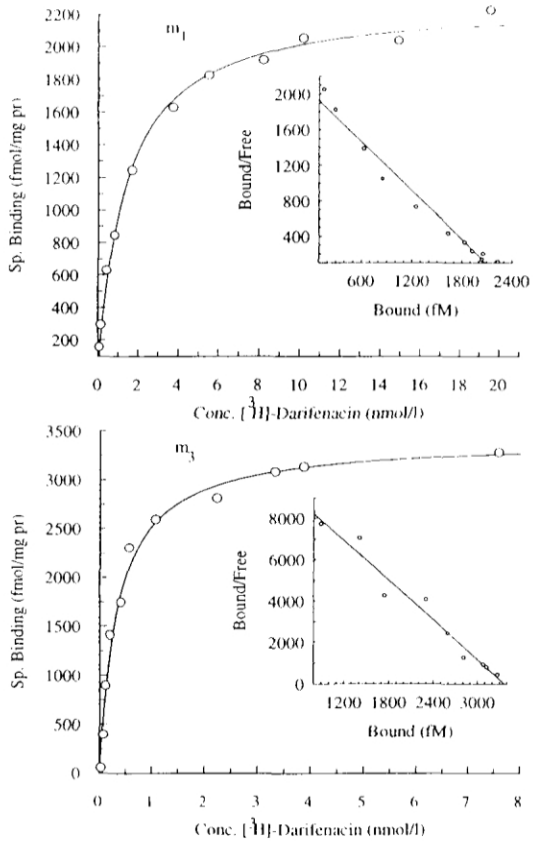

FIG. 2 Saturation isotherms of specific [^{3}H]-darifenacin binding to the cloned human m_1 and m_3 receptors. Membranes expressing m_1 (120 µg protein / ml) (*upper figure*) and m_3 receptor (60 µg protein / ml) (*lower figure*) were incubated at 25 °C for 2 h over the range of [^{3}H]-darifenacin concentrations indicated. Data shown are taken from representative experiments carried out in duplicate. Scatchard analysis of the data is shown in the insets. Values for K_D and B_{MAX} are given in Table 1.

TABLE 1.

Equilibrium Binding Parameters (K_D and B_{MAX},) for [^{3}H]-Darifenacin Binding to the Cloned Human Muscarinic Receptor Subtypes

Parameter	m_1	m_2	m_3	m_4	m_5
K_D (nmol/l)	1.6 ± 0.8	insufficient specific binding to determine K_D	0.33 ± 0.1	insufficient specific binding to determine K_D	insufficient specific binding to determine K_D
B_{MAX} (fmol/mg protein)	1980 ± 500		2780 ± 590		

The estimates of K_D and B_{max} were obtained from saturation experiments as shown in Fig. 2. Data are expressed as the mean ± S.D. from 4-6 experiments.

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