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THE BINDING OF SOME ANTIDEPRESSANT DRUGS TO BRAIN MUSCARINIC ACETYLCHOLINE RECEPTORS

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1 The binding of some antidepressant drugs, including some new drugs of atypical structure (flupenthixol, iprindole, maprotiline, mianserin, nomifensine, tofenacine and viloxazine) to muscarinic acetylcholine receptors in the brain has been studied by displacement of [^{3}H]-atropine.

2 Many of the drugs are potent muscarinic antagonists.

3 Some correlation can be made between the affinity for binding to the muscarinic acetylcholine receptor and the incidence of anticholinergic side effects in clinical usage.

Introduction

Radio-ligand binding assays have proved very useful for studying the interaction of neurotransmitters and drugs with putative receptor sites in neuronal and non-neuronal tissues (see for example Birdsall & Hulme, 1976; Snyder & Bennett, 1976). In the case of antidepressant drugs of the tricyclic class e.g. amitryptiline, imipramine, the therapeutic effects may be mediated via blockade of the nerve terminal noradrenaline and 5-hydroxytryptamine (5-HT) reuptake sites (Carlson, Corrodi, Fuxe & Hökfelt, 1969a, b; Iversen, 1974); ligand binding experiments have shown, however, that the drugs are also muscarinic acetylcholine receptor antagonists (see for example Snyder & Yamamura, 1977) and α-adrenoceptor antagonists (U'Prichard, Greenberg, Sheehan & Snyder, 1978). These studies offer a rationalization for the anti-muscarinic side effects seen with the drugs as well as the ability of the drugs to cause sedation and hypotension (α-adrenoceptor blockade). These interactions may also contribute to the antidepressant effects of these drugs (see Janowsky, El-Yousef, Davis & Sekerke, 1973; U'Prichard *et al.*, 1978). In addition many antidepressant drugs have been shown to block histamine receptors in studies on histamine-sensitive adenylate and guanylate cyclases (Green & Maayani, 1977; Kanof & Greengard, 1978; Richelson, 1978).

The experiments on α-adrenoceptors, muscarinic, and histamine receptors mentioned above were carried out mainly with antidepressant drugs of the tricyclic class or those which are monoamine oxidase inhibitors, but several antidepressant drugs of different structural classes are now in clinical use e.g. flupenthixol, iprindole, maprotiline, mianserin, nomifensine, tofenacine, viloxazine. Although these drugs are successful antidepressants they are reported to possess varying abilities to block noradrenaline and 5-HT reuptake (Pinder, Brogden, Speight & Avery, 1977a, b; Sulser, Vetulani & Mobley, 1978) and so may elicit their therapeutic effects through other mechanisms. They are also reported to show varying degrees of anticholinergic side effects (El-Deiry, Forrest & Littmann, 1967; Pinder *et al.*, 1977a, b; Kopera, 1978).

In the present paper we describe a study of the binding of these atypical antidepressant drugs to rat brain muscarinic acetylcholine receptors using [^{3}H]-atropine as a radio-ligand. The results indicate that the ability of a drug to block [^{3}H]-atropine binding *in vitro* can be used as a guide for the production of anticholinergic side effects *in vivo*.

Methods

Preparation of brain subcellular fraction (P_2)

Whole rat brains, obtained from male Wistar rats (250 to 300 g) killed by a sharp blow to the neck, were homogenized in ice cold sucrose solution (0.32 M, 9 ml/g wet wt. rat brain) with a teflon-glass homogenizer (0.18 mm radial clearance, 20 strokes). The homogenate was centrifuged (1000 *g*, 12 min) and the supernatant taken. This supernatant was centrifuged (10,000 *g*, 30 min) to give a pellet (P_2) which was resuspended for use in assays in an ice cold HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid)-phosphate-saline buffer (6 ml/g original tissue wet wt.; protein approx. 5 mg/ml). The buffer contained sodium chloride (110 mM), potassium chloride

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(5.3 mM), calcium chloride (1.8 mM), magnesium sulphate (0.8 mM), sodium dihydrogen orthophosphate (0.9 mM) glucose (25 mM), sucrose (50 mM) and HEPES (20 mM) adjusted to pH 7.4 with 1 M sodium hydroxide solution; the osmolality was approx. 340 mosm. In some experiments a modified buffer was used which had no HEPES; the composition was otherwise as stated above except that the sucrose was increased to 70 mM to give an osmolality of approx. 320 mosm. The resuspended P_2 preparation was stored at −20°C until required; storage for up to a week did not result in significant loss of [^{3}H]-atropine binding activity. The protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard and for experimental samples employing a precipitation step with ice cold trichloroethanoic acid (100 g/l final).

Binding studies

Ligand binding was measured by a centrifugation assay similar to that used by Terenius (1974). (±)[G-^{3}H]-atropine (0.2 nM to 50 nM) or [^{3}H]-imipramine (3 nM approx.) was incubated at 30°C for 30 min in triplicate or quadruplicate with rat brain P_2 preparation (1.0 mg/ml approx. final protein concentration) in a final volume of 1 ml in the above HEPES-phosphate-saline buffer in capped polypropylene centrifuge tubes (1.5 ml, Sarstedt, Leicester, Leics.). Known concentrations of competing drugs were added at the beginning of the incubation where appropriate. The bound radioactivity was determined by rapid centrifugation (14,000 *g*, 30 s) in a microcentrifuge (Quickfit, supplied by Northern Media Supplies, Hull, Yorkshire) followed by superficial washing of the pellet with a solution containing sodium chloride (150 mM) and sodium dihydrogen orthophosphate (10 mM) at pH 7.4. The ends of the tubes containing the pellet were cut off and placed in 10 ml of scintillant (Triton X-100/xylene, 1:2 v/v with 0.6 g 2,5-diphenyloxazole and 0.012 g 1,4 bis(5-phenyloxazol-2-yl)benzene per 100 ml) in a counting vial. The pellets were dispersed by vortex mixing and the radioactivity determined on Packard 3255 or 3375 liquid scintillation spectrometers in the external standard mode.

Two types of experiment were performed using [^{3}H]-atropine: direct binding experiments where specific (−)-[^{3}H]-atropine binding was assessed at various free (−)-[^{3}H]-atropine concentrations and displacement experiments where the binding due to a fixed concentration of (−)-[^{3}H]-atropine was displaced by varying concentrations of an added drug. In these experiments with atropine a relatively high receptor site concentration (1 nM) was used so that significant depletion of added (−)-[^{3}H]-atropine occurred for added concentrations of 2.5 nM or less. Consequently the free (−)-[^{3}H]-atropine concentration varied between assays with and without added competing drug. Therefore in displacement experiments, high concentrations (20 nM to 50 nM) of (±)[^{3}H]-atropine were added to each assay so that as the drugs displaced the bound (−)-[^{3}H]-atropine the free (−)-[^{3}H]-atropine changed by less than 15%; an average value for the free (−)-[^{3}H]-atropine concentration was used for calculations in displacement experiments.

Therefore a typical displacement experiment consisted of three sets of assays: (i) (±)-[^{3}H]-atropine (20 nM to 50 nM) was incubated with P_2 preparation, with no further addition to define the sum of specifically and non-specifically bound radioactivities. (ii) as in (i) but with the addition of 20 μM (±)-atropine (non-radioactive) to define the non-specifically bound radioactivity (normally between 30% and 60% of total binding). It is assumed that non-specific binding is the same in all the assays. (iii) as in (i) but with a known concentration of competing drug to determine the degree of displacement of (−)-[^{3}H]-atropine binding.

For these displacement experiments the results were calculated assuming that only the (−)-isomer of atropine was pharmacologically active (see for example Paton & Rang, 1965) and that this isomer represented half the added radioactivity in each assay, half the non-specifically bound radioactivity and all the specificially bound radioactivity. The free (−)-[^{3}H]-atropine concentration was calculated by subtraction for assays with and without excess displacing ligand and an average taken. The concentrations of drugs required to displace 50% of the specifically bound (−)-[^{3}H]-atropine were determined from double reciprocal plots and Hill plots and the two values averaged. Dissociation constants were then calculated assuming simple competitive inhibition (using the average free (−)-[^{3}H]-atropine concentration and a value for its dissociation constant of 0.455 nM).

In direct binding experiments two sets of assays were performed: (i) varying concentrations of (±)-[^{3}H]-atropine (0.2 nM to 50 nM) were incubated with P_2 preparation to determine the sum of specifically and non-specifically bound radioactivities. (ii) As in (i) but with the addition of non radioactive (±)-atropine (20 μM); in this assay only non-specifically bound radioactivity was measured.

Particularly at low added [^{3}H]-atropine concentrations the free (−)-[^{3}H]-atropine concentrations were different in sets (i) and (ii) because of the displacement of bound radioactivity in set (ii). Since non-specific binding is dependent on the free ligand concentration, the non specific binding measured in set (ii) was not an accurate measure of the non specific binding in set (i) so that the specific binding was not

measured accurately by the difference between sets (i) and (ii) (see also Somoza & de Feudis, 1978). This problem was overcome in displacement experiments by using a high added [^{3}H]-atropine concentration but for direct binding experiments this was not possible so a more laborious method was used to calculate the specific binding in these studies. This is outlined in the appendix. Data for specifically bound and free (−)-[^{3}H]-atropine were plotted as Scatchard plots, Hill plots and double reciprocal plots and the average value for the binding parameters was taken.

Stability of [^{3}H]-atropine by thin layer chromatography

(±)-[^{3}H]-atropine (54 nM) was incubated with rat brain P_2 preparation as described above and the bound radioactivity was recovered by centrifugation (ten separate tubes were used). The pellets were suspended in 0.5 ml water per tube, amalgamated and non radiolabelled (±)-atropine (10 µl, 30 mM) added and mixed. The pH was adjusted to 9.0 approximately with sodium hydroxide solution and the solution was extracted with chloroform (3 × 2.5 ml). Where emulsions formed, the mixture could be separated into two phases by centrifugation (1000 *g*, 5 min). The amalgamated chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen gas. The extract was dissolved in chloroform and applied to a silica gel (Merck Kieselgel GF_{254}, 0.3 mm) plate and chromatographed in acetone/water/ammonia (8.75%):90/7/3 v/v. The spots were located under ultra violet light and the plate was divided into sections approximately 2 cm × 2 cm along the track of the spot. These sections were scraped off into counting vials containing 1 ml of 0.2 M sodium chloride solution in 1 M HCl and left for 10 min; 10 ml of scintillant (see above) was added to each vial and the radioactivity determined. Of the bound radioactivity, 88% was recovered from the thin layer plate and 95% of this chromatographed with authentic atropine. Therefore, no significant metabolism of the bound radioactive atropine appeared to be occurring during the experiment. Although this experiment was concerned only with bound radioactivity, part of this bound [^{3}H]-atropine was bound non-specifically. Since non-specific binding is contributed to by entrapment of liquid in the pellet this shows that free atropine is also undergoing insignificant metabolism.

Materials (±)[G-^{3}H]-atropine (426 mCi/mmol) and [^{3}H]-imipramine (21 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Atropine sulphate, bovine serum albumin and N-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset. Other chemicals were of the highest purity available. The drugs used were generous gifts from the following companies: Beecham Pharmaceuticals Ltd., Brentford, Middlesex (mianserin hydrochloride), Brocades Ltd., Weybridge, Surrey (tofenacine hydrochloride), Ciba Laboratories, Horsham, W. Sussex (maprotiline hydrochloride), Geigy pharmaceuticals, Macclesfield, Cheshire (imipramine hydrochloride, desipramine hydrochloride), Hoechst U.K. Ltd., Hounslow, Middlesex (nomifensine hydrogen maleate), I.C.I. Ltd., Macclesfield, Cheshire (viloxazine hydrochloride), Lundbeck Ltd., Luton, Beds. (*cis* flupenthixol hydrochloride), Pfizer Ltd., Sandwich, Kent (doxepin hydrochloride), Roche Ltd., Welwyn Garden City, Herts. (amitryptiline) and Wyeth Laboratories, Maidenhead, Berks. (iprindole hydrochloride). Drugs were generally dissolved at 10^{-2} M in assay buffer and diluted appropriately for assays. Mianserin hydrochloride was dissolved at 10^{-4} M in assay buffer containing 0.5% ethanol and diluted appropriately. The concentration of ethanol used did not affect binding of [^{3}H]-atropine.

Results

Specific (−)-[^{3}H]-atropine binding was found to be at equilibrium within 5 min ((±)-[^{3}H]-atropine 7 nM, results not shown) and assays were carried out for 30 min to ensure equilibration. No significant metabolism of the radio-ligand occurred during this period. From direct binding experiments a dissociation constant of 4.55×10^{-10} M ± 1.12×10^{-10} M (mean ± s.d., 4 experiments) and a Hill coefficient of 1.04 ± 0.12 (mean ± s.d., 4 experiments) were determined. This is in agreement with the data of Hulme, Birdsall, Burgen & Mehta (1978) on rat brain (K_d 6.25×10^{-10} M; Hill coefficient 0.92). It is possible that the HEPES buffer used in the present experiments might bind to the muscarinic receptor (see for example, Richelson, Prendergast & Divinetz-Romero, 1978). Accordingly, parallel P_2 preparations were made in the standard HEPES-phosphate-saline buffer and in a buffer containing identical concentrations of ions but lacking HEPES and containing slightly more sucrose to maintain the osmolarity. Direct binding experiments with atropine gave the following binding parameters in the HEPES containing buffer: K_d 5.39×10^{-10} M, Hill coefficient 1.0, number of sites 1.69 nmol/g protein and in the buffer lacking HEPES: K_d 3.59×10^{-10} M, Hill coefficient 1.2, number of sites 1.46 nmol/g protein. Clearly HEPES has no profound effect on muscarinic binding but if the differences between dissociation constants are significant then this indicates a dissociation constant at the muscarinic receptor for HEPES of approx. 40 mM. This would not affect the dissociation constants for ligands

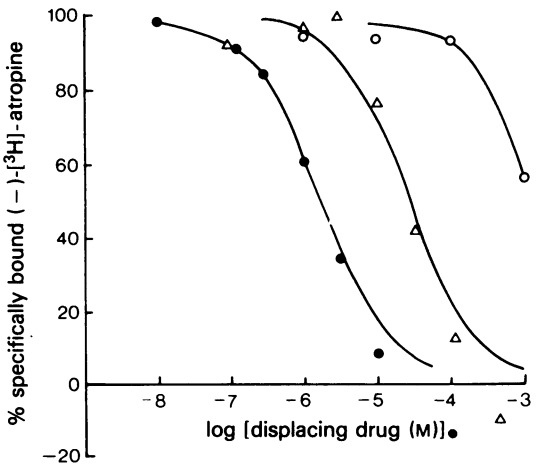

Figure 1 Displacement of specifically bound (−)-[^{3}H]-atropine from rat brain membranes by amitryptiline (●), maprotiline (△) and viloxazine (○), was assayed as described in the experimental section. Zero % specific binding was defined by the addition of 20 μM (±)-atropine so that negative values of specific binding refer to displacement of more bound [^{3}H]-atropine than that produced by 20 μM (±)-atropine. All displacements are expressed as percentages of the total specific binding. The average free (−)-[^{3}H]-atropine concentrations were 24.73 nM (amitryptiline); 12.47 nM (maprotiline) and 12.60 nM (viloxazine). The curves drawn for amitryptiline and maprotiline are theoretical curves obtained by applying the Law of Mass Action to the formation of a bimolecular complex (half maximal inhibition given by 1.9×10^{-6} M amitryptiline and 2.6×10^{-5} M maprotiline).

measured by displacement (see below) since all data were obtained in the same buffer systems.

Various drugs were assayed for inhibition of [^{3}H]-atropine binding and representative displacement curves are shown in Figure 1. Most of the drugs used displaced [^{3}H]-atropine and dissociation constants are given in Table 1; there is good agreement with published data for amitryptiline and imipramine (Rehavi, Maayani & Sokolovsky, 1977) and for flupenthixol (Miller & Hiley, 1974). Hill coefficients were determined where possible and these were mostly close to unity, indicating that the drugs were probably muscarinic antagonists according to the classification of Birdsall & Hulme (1976). In some cases Hill slopes of greater than unity were observed; this may be because at the higher concentrations of drug used, the drugs were displacing some non specifically bound [^{3}H]-atropine as discussed below.

The data of Figure 1 illustrate that some of the drugs tested displaced more than 100% of the specifically bound [^{3}H]-atropine as defined by 20 μM non-radioactive (±)-atropine, suggesting that they are displacing some non-specifically bound, non-receptor associated radioactivity. For example 10^{-3} M imipramine displaced 26% of the non specifically bound (±)-[^{3}H]-atropine whereas 10^{-3} M atropine displaced only 10%. This may be because some of the drugs tested e.g. imipramine and other tricyclic antidepressants are very lipophilic and partition strongly into membrane or organic phases (see for example Leo, Hansch & Elkins, 1971). The inhibition of non-specific binding was tested by an experiment where [^{3}H]-atropine binding in the absence (specific and non-specific binding) and in the presence (non-specific binding) of non-radioactive (±)-atropine (20 μM) was displaced by increasing concentrations of imipramine (Figure 2). In the absence of 20 μM (±)-atropine the displacement data for imipramine concentrations up to 3×10^{-5} M conform well to the theoretical curve for combination of a ligand with a single class of receptor sites: this presumably represents binding to the muscarinic receptor. In the presence of atropine no displacement occurs until imipramine concentrations of greater than 10^{-5} M are used and in this range the data with and without 20 μM (±)-atropine are in good agreement. This shows that in this range displacement of non-specifically bound [^{3}H]-atropine is occurring. Therefore the imipramine displacement curve (Figure 2) may be adequately described as a sum of displacement at muscarinic receptor sites (imipramine $\leqslant 10^{-5}$ M) and displacement of non-specifically bound atropine (imipramine $\geqslant 10^{-4}$ M).

The binding of imipramine to brain membranes has been studied by Hunt, Kannengiesser & Raynaud (1975) and Weinstein, Varon & Roberts (1971) who demonstrated low affinity binding sites. We have confirmed this and could find no reproducible high affinity ($K_d < 3 \times 10^{-8}$ M approx.) saturable binding sites for imipramine. [^{3}H]-imipramine (3 nM approx.) binding was not significantly inhibited by atropine (10 μM); significant binding to the muscarinic receptor would not be expected under these conditions. High concentrations of non-radiolabelled imipramine (10 μM to 1 mM) and other antidepressant drugs did, however, show inhibition (Table 2). This is the same concentration range in which imipramine inhibited non-specific atropine binding as well as showing effects on various membrane bound enzymes e.g. adenylate cyclase (Palmer, 1976); ATPase (Roufogalis, 1975). Therefore this inhibition of imipramine binding is likely to be due to non-specific drug-membrane interactions. The total binding of [^{3}H]-imipramine is quite high, however, considering that the binding is primarily low affinity; for example, at a protein concentration of 1 mg/ml and a total imipramine concentration of 3.2 nM, 41% of the [^{3}H]-imipramine was bound to the membrane pellet at equilibrium. Therefore, although displacement of [^{3}H]-imipramine by

high concentrations (10 μM to 10 mM) of competing drugs may not be physiologically relevant, the high tissue binding of imipramine even at low concentrations may affect the uptake and elimination characteristics of the drug. Imipramine seems, then, to bind to a large number of low affinity membrane sites; this is probably associated with the lipophilicity of the drug and contributes to the high tissue binding of the drug. Inhibition of [^{3}H]-imipramine binding by high imipramine concentrations is also probably due to interaction at these sites. A small number of the sites may also be occupied non-specifically by low concentrations of [^{3}H]-atropine. This contributes to non specific atropine binding and competition for these sites by high concentrations of imipramine leads to the observed inhibition of non specific atropine binding.

Burgermeister, Klein, Nirenberg & Witkop (1978) have shown that local anaesthetics inhibit muscarinic-receptor ligand-binding in a non-competitive manner. Although it has been assumed that the tricyclic antidepressants are competitive muscarinic antagonists because of the lipophilic nature of the drugs, it was necessary to establish this. Therefore direct [^{3}H]-atropine binding was assayed in the presence of different concentrations of imipramine (at concentrations where it shows antimuscarinic effects only). The results (Figure 3) show that imipramine does not alter the maximum number of [^{3}H]-atropine binding sites; a non-competitive model where imipramine decreases the maximum number of receptor sites may be ruled out so that the inhibition is most likely to be a simple competitive one. Assuming competitive inhibition, a value of 1.68×10^{-7} M was calculated for the dissociation constant of imipramine using the data of Figure 3. This is in very good agreement with the data of Table 1. An alternative model was considered where imipramine inhibition was non-competitive but led to an increased disssociation constant for [^{3}H]-atropine in the presence of imipramine and an unchanged total number of binding sites. However, when this model was applied to the direct binding data of Figure 3 the dissociation constants derived would not generate the data observed in displacement experiments (e.g. Figure 2) unless binding of atropine and imipramine are mutually exclusive. Consequently imipramine and the other drugs tested are most likely to be competitive antagonists.

Discussion

Most of the newer atypical antidepressant drugs tested in this radio-ligand binding assay were muscarinic receptor antagonists. When the dissociation constants (K_d) for all the drugs tested (Table 1) were compared they were found to fall approximately into four groups: (i) amitryptiline with the lowest K_d and therefore highest affinity of the drugs tested for the muscarinic receptor; (ii) drugs with dissociation constants in the 1×10^{-7} M to 7×10^{-7} M range: desipramine, doxepin, imipramine, maprotiline, mianserin, tofena-

Table 1 Binding of drugs to the muscarinic acetylcholine receptor

Drug	*Dissociation constant* (M)	*Hill coefficient*	*Incidence of anticholinergic side effects* in vivo
Amitryptiline	3.43×10^{-8}	1.03	+++(a)
Desipramine	3.76×10^{-7}	0.84	++(a)
Doxepin	1.79×10^{-7}	1.35	++(b)
cis-Flupenthixol	4.80×10^{-6}	1.23	−(c)
Imipramine	1.82×10^{-7}	1.18	++(a)
Iprindole	5.52×10^{-6}	1.23	+(d)
Maprotiline	6.25×10^{-7}	1.23	++(e)
Mianserin	4.69×10^{-7}	0.90	−(f)
Nomifensine	$>1.6 \times 10^{-5}$	—	+(c)
Tofenacine	6.84×10^{-7}	1.11	+(g)
Viloxazine	$>3.5 \times 10^{-5}$	—	−(h)

Dissociation constants for binding to the muscarinic acetylcholine receptor were determined as described in the text. Where lower limits are expressed this is an estimate based on the inhibition of [^{3}H]-atropine binding produced by the highest drug concentration used. References to anti-cholinergic side effects: (a) Snyder & Yamamura (1977), (b) Martindale (1977), (c) Herrington (1978), (d) El-Deiry *et al.* (1967), (e) Pinder *et al.* (1977a), (f) Kopera (1978), (g) Data Sheet Compendium (1977), (h) Pinder *et al.* (1977b). +++ Strong anticholinergic side effects; ++ some anticholinergic side effects; + weak anticholinergic side effects; − no anticholinergic side effects.

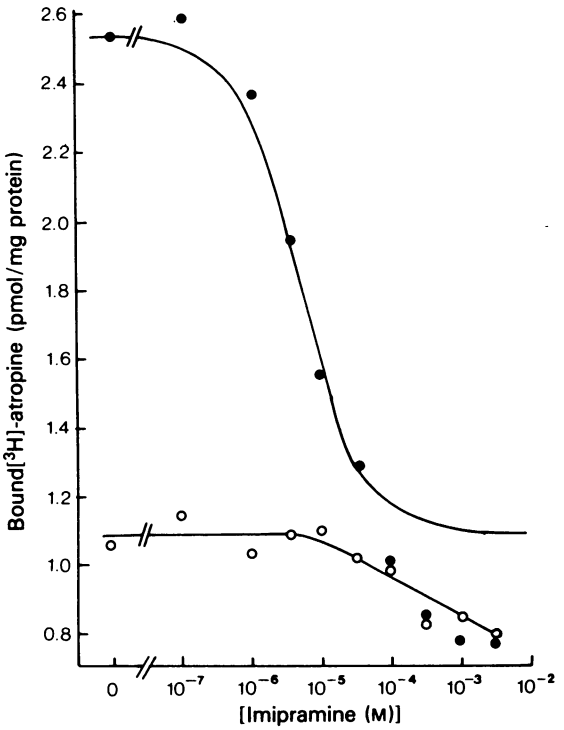

Figure 2 Displacement of specifically and non-specifically bound [^{3}H]-atropine by imipramine: binding of [^{3}H]-atropine was assayed as described in the experimental section in the absence (●) or presence of 20 μM non-radioactive (±)-atropine (○) and with different concentrations of imipramine. Total binding of [^{3}H]-atropine is plotted as a function of the imipramine concentration. In the absence of 20 μM (±)-atropine the data represent the sum of specific and non-specific binding and the curve drawn for imipramine $< 3 \times 10^{-5}$ M is the theoretical one for binding at a single class of sites (half maximal inhibition of [^{3}H]-atropine binding given by 4.6×10^{-6} M imipramine). In the presence of 20 μM (±)-atropine the data represent non-specific binding only. Average free (−)-[^{3}H]-atropine concentration, 12.56 nM.

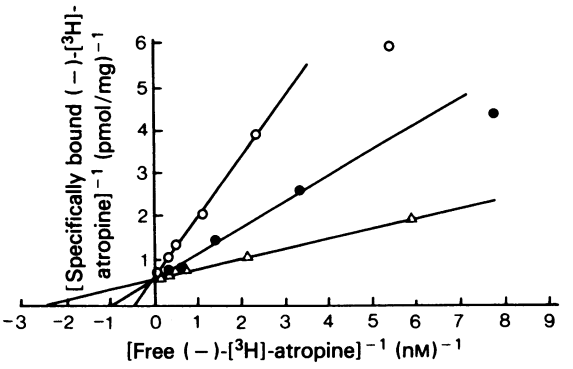

Figure 3 Specific binding of [^{3}H]-atropine to brain membranes in the presence of different concentrations of imipramine: direct specific (−)-[^{3}H]-atropine binding was assayed as described in the experimental section with no added drug (△), and in the presence of 3×10^{-7} M imipramine (●) and 10^{-6} M imipramine (○). Binding data are presented as double-reciprocal plots.

cine. These drugs are 5 to 20 times less potent than amitryptiline for binding to the muscarinic receptor, (iii) iprindole and flupenthixol (K_d: 5×10^{-6} M approx.); approximately 200 times less potent than amitryptiline; (iv) nomifensine and viloxazine ($K_d > 10^{-5}$ M); very poor anti-muscarinic agents.

These values for dissociation constants are in qualitative and quantitative agreement with available published data for tricyclic antidepressant drugs and antipsychotic drugs (Miller & Hiley, 1974; Fjalland, Christensen & Hyttel, 1977; Rehavi *et al.*, 1977; Snyder & Yamamura, 1977).

Some of the drugs tested displaced non-specifically bound [^{3}H]-atropine and this was probably due to their lipophilic nature. At high concentrations the drugs form loose associations with membrane components and this may lead to displacement of non-receptor associated [^{3}H]-atropine from similar sites. Therefore, for imipramine the displacement curve (Figure 2) may be completely described as the sum of two components: muscarinic acetylcholine receptor binding (imipramine $\leqslant 10^{-5}$ M) and membrane associated displacement of non specific [^{3}H]-atropine binding ($\geqslant 10^{-4}$ M imipramine).

There is good agreement between the dissociation constants obtained here in a broken cell preparation of brain muscarinic acetylcholine receptors and those obtained by Shein & Smith (1978) for inhibition of smooth muscle contraction by some of the drugs. This confirms the findings of Snyder & Yamamura (1977) that the receptors from brain and smooth muscle show very similar ligand binding characteristics. Therefore binding of the drugs to brain muscarinic receptors can be used to assess binding in other tissues where anticholinergic side effects occur. Shein & Smith (1978) also observed apparent non-competitive behaviour in some of the drugs at concentrations above 10^{-5} M: this may have been due to the non-specific membrane effects observed in the present studies.

There seems to be broad agreement between the anti-muscarinic potencies of drugs determined from the ability to inhibit [^{3}H]-atropine binding and the ability of drugs to produce anti-cholinergic side effects *in vivo* (Table 1). Drugs with very low dissociation constants e.g. amitryptiline show a high incidence of

anticholinergic side effects whereas drugs which are poor muscarinic ligands e.g. viloxazine show no anticholinergic side effects in clinical use. Although production of side-effects depends on many factors like the dosage, plasma levels of drug, tissue levels of drug, drug accessibility, it appears that the *in vitro* radioligand binding assay can be used broadly to predict the incidence of anticholinergic side effects *in vivo*. Therefore, it may be used, as suggested by Snyder & Yamamura (1977) to judge which drug to prescribe in certain situations where anticholinergic side effects would be undesirable e.g. glaucoma; furthermore it is a very simple test and could be used as a screening technique for assessing anticholinergic potencies in new drugs. From this standpoint the newer drugs tested all showed reduced anticholinergic potency relative to tricyclic antidepressants and so may be preferable where anticholinergic side effects are undesirable. Mianserin is an exception to this correlation; it is moderately potent in the [^{3}H]-atropine displacement test but appears to show no anticholinergic side effects in clinical use. This may be due to poor accessibility of the drug to the relevant area of tissue.

Lastly, it has been suggested that the anticholinergic interaction of antidepressant drugs may contribute to their clinical potency (Janowsky *et al.*, 1973; Snyder & Yamamura, 1977). Some of the drugs tested here e.g. viloxazine, nomifensine as well as the monoamine oxidase inhibitors tested by Snyder & Yamamura (1977) are successful antidepressants but virtually devoid of anticholinergic interactions so that binding to muscarinic receptors is not a prerequisite for antidepressant activity.

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Appendix

Calculation of specifically and non-specifically bound radioactivities in direct binding assays

In direct binding assays, different concentrations of (±)-[^{3}H]-atropine were assayed with (series a) and without (series b) 20 μM (±)-atropine and the results were calculated as follows:

bound radioactivity in series a,

$$B_a = N_a^+ + N_a^- \quad (1)$$

bound radioactivity in series b,

$$B_b = N_b^+ + N_b^- + S^- \quad (2)$$

free (−)-[^{3}H]-atropine in series a,

$$F_a^- = T^- - N_a^- \quad (3)$$

free (−)-[^{3}H]-atropine in series b,

$$F_b^- = T^- - (N_b^- + S^-) \quad (4)$$

N_a^+ = non-specifically bound (+)-[^{3}H]-atropine in series a; N_a^- = non-specifically bound (−)-[^{3}H]-atropine in series a; N_b^+ = non-specifically bound (+)-[^{3}H]-atropine in series b; N_b^- = non-specifically bound (−)-[^{3}H]-atropine in series b; S^- = specifically bound (−)-[^{3}H]-atropine in series b; T^- = total (−)-[^{3}H]-atropine in an assay. B_a, B_b and T^- are measured experimental quantities.

N_a^+ and N_a^- must be equal because series a contains only non-specific binding and the free concentrations of both isomers must be the same; therefore

Table 2 Inhibition of [^{3}H]-imipramine binding by antidepressant drugs

Drug	IC_{50} (M)
Amitryptiline	1.6×10^{-4}
Desipramine	1.9×10^{-4}
Doxepin	2.0×10^{-4}
Imipramine	2.5×10^{-4}
Iprindole	1.2×10^{-4}
Maprotiline	1.1×10^{-4}
Mianserin	$>10^{-4}$
Viloxazine	$>10^{-3}$

[^{3}H]-imipramine binding was assayed as described in the text. The IC_{50} is the concentration of drug which gave 50% inhibition of specific [^{3}H]-imipramine binding as defined by the difference between assays with and without 3×10^{-3} M non-radiolabelled imipramine. Where lower limits are given this is an estimate based upon the inhibition given by the highest drug concentration used.

$N_a^- = B_a/2$ and this enables F_a^- to be calculated (eqn. 3). Since the (+)-isomer is pharmacologically inactive N_b^+ and N_a^+ must be equal, allowing $(N_b^- + S^-)$ to be calculated from eqn. 2 and B_b; hence F_b^- can be calculated from eqn. 4.

Because non-specific binding is proportional to the free ligand concentration we may write

$$\frac{F_a^-}{F_b^-} = \frac{N_a^-}{N_b^-} \quad (5)$$

N_b^- was calculated using eqn. 5 and then S^- was calculated using eqn. 2.

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