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# Selective blockade of $M_2$ and $M_3$ muscarinic receptors by hexahydrobenzyl- fourdapine and a comparison with zamifenacin

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**1** 4-Diphenylacetoxy-N-*cyclo*hexylmethyl-piperidine HCl (hexahydro-benz-4DAP) is more active as an antagonist of carbachol at receptors in guinea-pig isolated ileum, log $K$ ($pA_2$) = 6.64 ± 0.14 (s.e. 7 results), than at receptors in guinea-pig isolated atria, log $K$ = 5.43 ± 0.14 (7). In the presence of neostigmine bromide (0.2 μM) the value for atria was 5.62 ± 0.19 (4), so the lower activity on atria cannot be attributed to hydrolysis of the compound by cholinesterases present in this tissue.

**2** The limit of solubility of the free base in Krebs solution (pH 7.6) is about 50 μM for both hexahydro-benz-4DAP and benzyl-fourdapine (benz-4DAP).

**3** In experiments on guinea-pig isolated ileum with hexahydro-benz-4DAP given together with 4-DAMP methobromide, the combined dose-ratio was consistent with competition: similar results were obtained with benz-4DAP.

**4** In rats anaesthetized with pentobarbitone, hexahydro-benz-4DAP antagonized the effects of bethanechol on blood-pressure in doses that had little effect on heart rate or airflow. There was a limit to the effect which could be obtained, however, and the slopes of the Schild plots were less than one. The effects on rat blood-pressure had a half-life of at least 30 min.

**5** In similar experiments with zamifenacin the slopes of the Schild plots were close to 1 and the compound was 10 to 20 times as active on blood-pressure at it was on heart-rate.

**6** The limited solubility of the base probably accounts for the flat Schild plots obtained with hexahydro-benz-4DAP, which had about 10 fold selectivity for effects on blood-pressure and was more active than expected from tests on isolated ileum.

**Keywords:** $M_2$ muscarinic receptors; $M_3$ muscarinic receptors; selective anti-muscarinic drugs; affinity; benzylfourdapine; hexahydro-benzyl- fourdapine; zamifenacin; atropine

## Introduction

From tests on a range of atropine-like compounds Barlow *et al.* (1976) found that 4-diphenylacetoxy-*N*-methyl piperidine (4-DAMP) methiodide (or methobromide) had higher affinity for $M_3$ muscarinic receptors in guinea-pig ileum (log $K$ = 9.0) than for $M_2$ receptors in guinea-pig atria (log $K$ = 7.7). A search was made for more selective antagonists (for references see Barlow *et al.*, 1990) and it appeared that a chemical intermediate in the synthesis of 4-DAMP metho-salts, 4-diphenylacetoxy-N-benzyl piperidine (benz-4DAP) HBr, had greater selectivity though it was less active. Barlow *et al.* (1992) obtained log $K$ (guinea-pig atria: effects on rate) = 6.0 compared with log $K$ (guinea-pig ileum) = 7.65. The compound appeared to be competitive in concentrations up to 10 μM. In the anaesthetized rat it antagonized the actions of bethanechol on blood-pressure in doses which did not antagonize its effects on heart rate but higher doses did not produce higher dose-ratios, suggesting that the blockade might not be competitive.

In binding and functional studies with benz-4DAP, however, Caulfield *et al.* (1993) concluded that it was not selective. For $M_3$ receptors the values of log $K$ were 7.8 (binding: rat lachrimal gland), 7.3 (binding: $Hm_3$ cloned cells) and 7.0 (functional tests with rat ileum). For $M_2$ receptors the values were 7.0 (binding: rat heart), 6.9 (binding: $Hm_2$ cloned cells) and 6.7 (functional tests with rat atria) and with guinea-pig atria (binding) the value was 7.0. Although the values for $M_3$ receptors and ileum were similar (7.8 and 7.65), those for atria are very different (7.0 and 6.0). Caulfield *et al.* (1993) suggested that some of the variability encountered may be due to its low solubility.

Another possibility is that fourdapines, which are esters, might be weaker on isolated atria than on ileum because they are hydrolysed by cholinesterases present in heart muscle and the extent to which this occurs might vary, depending on the experimental conditions.

The apparent selectivity of benzyl-fourdapine (Benz-4DAP) was found with similar compounds (Barlow & Veale, 1990), particularly with its *cyclo*hexyl analogue, hexahydrobenzyl-fourdapine (hexahydro-benz-4DAP: Barlow & Veale, 1991), the structure of which is shown in Figure 1 (Barlow *et al.*, 1993). This was prepared as the hydrochloride which might be more soluble than benz-4DAP HBr. This paper describes experiments made with the hexahydro-benz-4DAP to find out whether the lower activity on isolated atria than on ileum is an artefact by checking the solubility and observing the effects of an anticholinesterase (neostigmine bromide) on the activity on isolated atria. The validity of the *in vivo* experiments with anaesthetized rats has been tested by comparing the activity of hexahydro-benz-4DAP with that of zamifenacin (Wallis *et al.*, 1993; Quinn *et al.*, 1993; McRitchie *et al.*, 1993; Wallis, 1995), a substance which is known to be ileoselective and with a chemical structure that is not unrelated (Figure 1). In addition the nature of the antagonism produced by hexahydro-benz-4DAP has been checked in experiments with guinea-pig isolated ileum in which it was given along with 4-DAMP methobromide, which is known to act competitively (Barlow *et al.*, 1976): similar experiments were also carried out with benz-4DAP.

## Methods

### *Solubility*

Solutions (10 mM) of benz-4DAP HBr and hexahydro-benz-4DAP HCl, were prepared in ethanol: this concentration is

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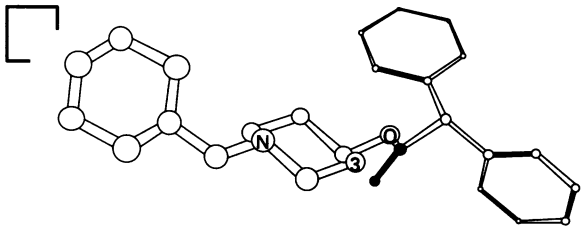
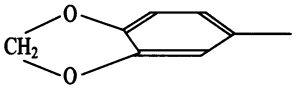


**Figure 1** One form of hexahydro- benz-4DAP HCl found in the crystal (Barlow *et al.*, 1993). The scale shows 100 picometres. In zamifenacin the *cyclo*hexylmethyl group is replaced by 3:4-methylenedioxy phenyl (as seen below), the carbonyl (filled atoms) in the ester group is absent and the attachment is at the adjacent (3-) carbon atom of the piperidine ring.



close to the limit of solubility for both compounds in this solvent at room temperature. When this solution of hexahydro-benz-4DAP was diluted with saline (0.9% sodium chloride) to given concentrations in the range 0.1–1.6 mM, there was slight cloudiness at 1.6 mM: with benz-4DAP there was slight cloudiness at 0.8 mM and definite cloudiness at 1.6 mM. When the stock solutions were diluted with Krebs solution (which had been aerated with oxygen + 5% carbon dioxide), both compounds appeared clear up to 0.04 mM but were slightly cloudy at 0.06 mM. The absorbance was scanned from 250 to 280 nm with a Phillips PU9700 spectrophotometer and the values for the aromatic peak (258 nm for saline, 260 nm for Krebs solution) were plotted against concentration to see if they fitted the Lambert-Beer law. Although the lines declined slightly at higher concentrations (1.6 mM in saline, 0.1 mM in Krebs solution) there was no abrupt change, suggesting that the cloudiness was caused by the base coming out of solution while much of the ionised form remained dissolved. It seems that at physiological pH (that of Krebs solution) the limit of solubility is around 50 μM for both compounds.

### *Guinea-pig isolated ileum*

Short lengths of ileum (about 15 mm) were set up as described by Edinburgh Staff (1974) in Krebs solution containing hexamethonium bromide (100 μM), aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The volume of the bath was about 8 ml and contractions of the longitudinal muscle were recorded isotonically with a load of about 0.5 g. Initially experiments were carried out at 30°C (for comparison with atria) and at 37°C but the experiments to test for competition were done only at 37°C.

The agonist, carbachol, was allowed to act for 30 s and added once every 25 min by computer-operated relays. The dose-ratios were calculated exactly as in previous work (Barlow *et al.*, 1990; 1992).

### *Guinea-pig isolated atria*

The atria were set up as described by Edinburgh Staff (1974) in Krebs solution containing norphenylephrine (5 μM: Barlow & Shephard, 1986; Barlow *et al.*, 1988), aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The volume of the bath was about 21 ml and the contractions were recorded isotonically with a load of about 0.5 g. The temperature was 30°C. The agonist, carbachol, was allowed to act for 5 min and added once every 15 min by computer-operated relays. The dose-ratios were calculated as in previous work (Barlow *et al.*, 1990).

For experiments on ileum and on atria, stock solutions (10 mM) of hexahydro-benz-4DAP HCl were prepared in dimethylsulphoxide or ethanol and diluted with Krebs solution to the required concentration (1–2 μM for ileum, 5–100 μM for atria).

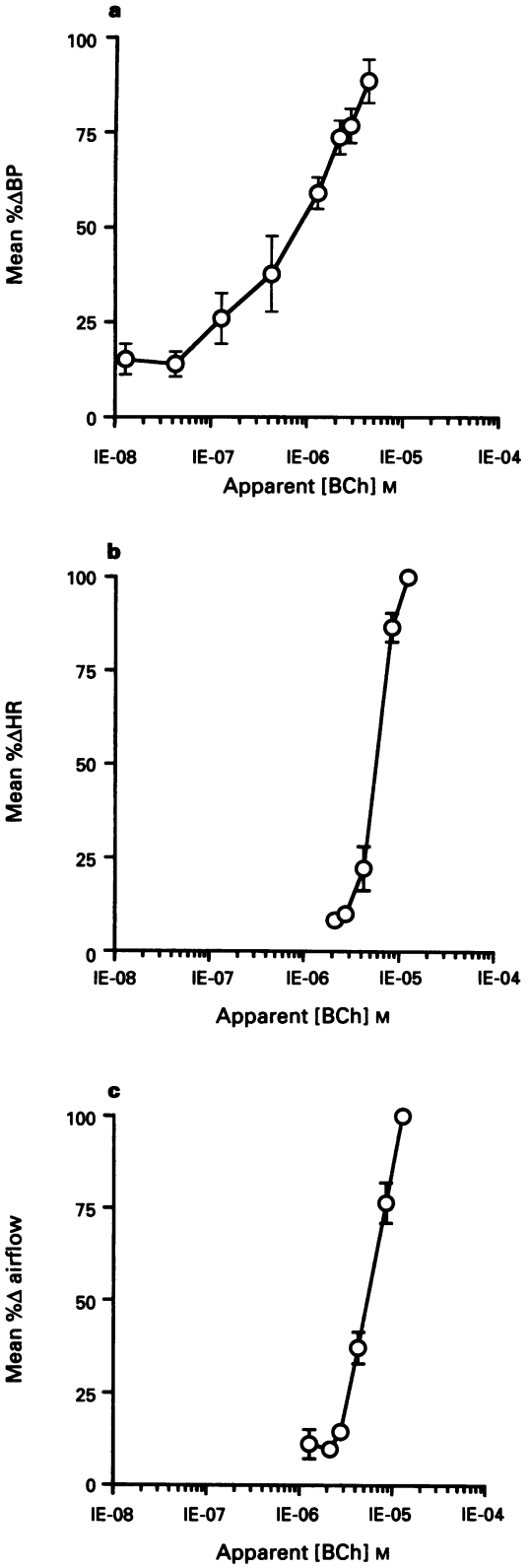



**Figure 2** Graphs of response against expected concentration of bethanechol in anaesthetized rats (assuming the dose is distributed in a volume which is 55% of the body mass): (a) shows the effects on blood-pressure, with the highest response which did not produce bradycardia taken as 100%: (b) shows the effects on heart rate and (c) shows effects on air-flow; with these the maximum effect is a reduction to zero. Note the flatness of the curve for blood-pressure.

### *Anaesthetized rat*

Rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, intraperitoneally), with further injections (3–6 mg, i.v.) at intervals of 1 h used where necessary to maintain anaesthesia. The trachea was cannulated and connected to a pneumotachograph (Model CS5, Mercury Electronics Ltd., Newton Mearns, Glasgow, UK), which was reset every 6 s, and recorded respiratory rate, tracheal air flow and ventilatory volume.

The femoral artery and vein in one hind limb were cannulated, with the cannulae containing heparinized saline (50 units $ml^{-1}$), and the arterial pressure recorded with a Statham transducer (model P23Dc) and recorded, together with the airflow signal, on an Apple-Macintosh LC II computer using a MacLab 4 charting system and Chart 3.3 software. Heart-rate was measured from the blood-pressure record. Blood samples were taken from the arterial cannula and analysed for the partial pressures of oxygen (70–90 mmHg) and carbon dioxide (30–36 mmHg) and for pH (mean pH 7.33; $n=39$). Body-temperature was monitored by a rectal thermometer and kept constant (38°C) with a servo-controlled blanket. When artificial ventilation was necessary, a respiratory pump (C.F. Palmer Ltd.) was used, set to 3 ml at 55 strokes $min^{-1}$.

Drugs were injected through the intravenous cannula. The animals were all treated with propranolol (1 mg $kg^{-1}$, i.v.) administered every hour, to block reflex tachycardia and any effects on α-adrenoceptors (e.g. by actions of muscarinic agonists on sympathetic ganglia). The agonist used was bethanechol and stock solutions were made up in 0.9% saline: the volume injected was 0.1 ml, washed in with 0.2 ml of 0.9% saline, and the concentrations were usually in the range 30 μM to 3 M, so the lowest dose was 3 nanomoles. It was usually only concentrations above 5 mM that slowed the heart.

After control responses had been obtained with bethanechol, the antagonist was given in a dose calculated in proportion to the weight of the animal, allowing 1 ml for each kg (e.g. 0.25 ml for a 250 g rat), and washed in with 0.2 ml saline (0.9%). After 5 min the doses of bethanechol were re-tested, using higher concentrations where necessary.

Stock solutions of hexahydro-benz-4DAP HCl were prepared in aqueous ethanol (50% by volume) and diluted with saline (0.9%) before injection. The vehicle produced only transient hypotension and apnoea.

### *Compounds*

Carbachol, hexamethonium bromide, neostigmine bromide and propranolol HCl were obtained from Sigma: norphenylephrine HCl was obtained from Aldrich: bethanechol was obtained from Koch-Light Laboratories: sodium pentobarbitone was obtained from May & Baker. The samples of benz-4DAP HBr and of hexyhydro-benz-4DAP HCl were those used in previous work (Barlow *et al.*, 1992; 1993), with a fresh supply of the latter provided by the same source (Aston Molecules, Birmingham). Zamifenacin was kindly donated by Pfizer Ltd., Sandwich, Kent.

## Results

### *Isolated preparations*

The mean dose-ratios (±s.e.) produced by hexahydro-benz-4DAP on ileum were: 5.37±2.0 (4) at 1 μM and 16.0±2.3 (3) at 2 μM. The mean estimates of log $K$ ($pA_2$) were 6.475 (±0.21) and 6.850 (±0.085), respectively. An analysis of variance gives the variance ratio, $F=2.09$ compared with 5.99 for $P=0.05$: using Fisher's randomization test $P=0.114$. The results are not inconsistent with competition and the pooled estimate of log $K$ is 6.64±0.14 (7).

In these concentrations the compound did not produce any detectable antagonism on guinea-pig atria: concentrations in the range 10–100 μM were needed (i.e. approaching the limit of solubility of the base). The mean estimate of log $K$ ($pA_2$) for effects on force was 5.43±0.14 (7): the value was 5.62±0.19 (4) in experiments made in the presence of 0.2 μM neostigmine bromide. This concentration is known to potentiate markedly the effects of acetylcholine and methacholine on atria (Barlow & Weston-Smith, 1985), so it seems unlikely that hydrolysis of hexahydro-benz-4DAP can contribute much to its selectivity.

In separate experiments (at 37°C made more than a year later) to test the nature of the antagonism on guinea-pig ileum the mean dose-ratio obtained with 2 μM hexahydro-benz-4DAP ($DR_1$) was 8.93 (±1.06, s.e., 9 experiments), with 100 nM 4-DAMP MeBr ($DR_2$) it was 138.3 (±16.0, 11) and with the two together ($DR_{12}$) it was 158.6 (±44.1, 3). If both compounds are competing for the same receptors the combined dose-ratio, $DR_{12}=DR_1+DR_2-1$ (Ariens *et al.*, 1964; Paton & Rang, 1965; Abramson *et al.*, 1969), rather than $DR_1 \times DR_2$, i.e. 146.2 rather than 1235. In those experiments

**Table 1** Mean dose-ratios obtained for doses of antagonist on the effects of bethanechol in anaesthetized rats on blood-pressure, heart-rate and airflow, together with the slope of the graphs of log (dose-ratio −1) against log dose of antagonist

| *Hexahydro-benz-4DAP* Dose (μmoles $kg^{-1}$) | *Blood pressure* | *Heart rate* | *Airflow* | *SR* |
|---|---|---|---|---|
| 0.01 | 7.66±2.82 (4) | 1.66±0.29 (5) | 1.16±0.24 (2) | 10.1 |
| 0.1 | 11.9±4.05 (5) | 2.25±0.33 (6) | 1.92±0.46 (5) | 8.7 |
| 1.0 | 20.7±5.72 (5) | 2.67±0.44 (6) | 1.63±0.38 (4) | 11.8 |
| 10 | 78.2±20.9 (4) | 6.72±2.43 (5) | 1.79±0.45 (3) | 13.5 |
| Slope±s.e. | 0.36±0.08 | 0.30±0.06 | 0.18±0.19 | |
| *Zamifenacin* | 4.59±1.40 (3) | 1.27±0.08 (4) | 1.64±0.47 (3) | 13.3 |
| 0.01 | 8.62±2.57 (4) | 1.66±0.30 (4) | 2.85±0.99 (4) | 11.5 |
| 0.1 | 52.7±11.3 (4) | 3.47±0.36 (4) | 23.5±7.89 (4) | 20.9 |
| 1.0 | 2362±831 (4) | 121.8±26.5 (4) | 485.1±69.1 (4) | 19.5 |
| 10 | 0.88±0.13 | 0.88±0.10 | 1.05±0.11 | |
| Slope±s.e. | | | | |
| *Atropine (one experiment)* | | | | |
| 0.00288 | 2.09 | 1.41 | 1.09 | 2.66 |
| 0.0288 | 8.30 | 4.10 | 2.63 | 2.35 |
| 0.288 | 69.3 | 47.4 | 57.8 | 1.47 |
| Slope | 0.90 | 1.03 | 1.34 | |

Values are mean±s.e. with number of result in parentheses. SR indicates the selectivity for blood-pressure compared with heart-rate, calculated from the ratio of the values of (dose-ratio −1).

where the effects of hexahydro-benz-4DAP on the responses in the presence of 4-DAMP MeBr were measured directly the mean dose-ratio was 1.60 (±0.62, 3), compared with 1.06 (competitive) or 8.93 (noncompetitive). These results confirm the competitive nature of the antagonism and the dose-ratios obtained with hexahydro-benz-4DAP give log $K = 6.57 \pm 0.05$ (9 results), very close to that obtained earlier in this work (pooled estimate $6.64 \pm 0.14$, 7 results: see above).

In experiments with benz-4DAP (0.5 μM) the mean dose-ratio was 49.2 (±15.5, 6), and with 100 nM 4-DAMP MeBr the combined dose-ratio was 205.2 (±36.9, 5): the value expected for competition is $49.2 + 138.3 - 1 = 186.5$. The mean dose-ratio obtained where the effects of benz-4ADP were measured directly on the responses in the presence of 4DAMP MeBr was 1.48 (±0.29, 5) compared with 1.35 (competitive) or 49.2 (noncompetitive). The dose-ratios obtained with benz-4ADP give log $K = 7.87$ (±0.14, 6), close to the value (7.74), obtained by Barlow *et al.* (1992).

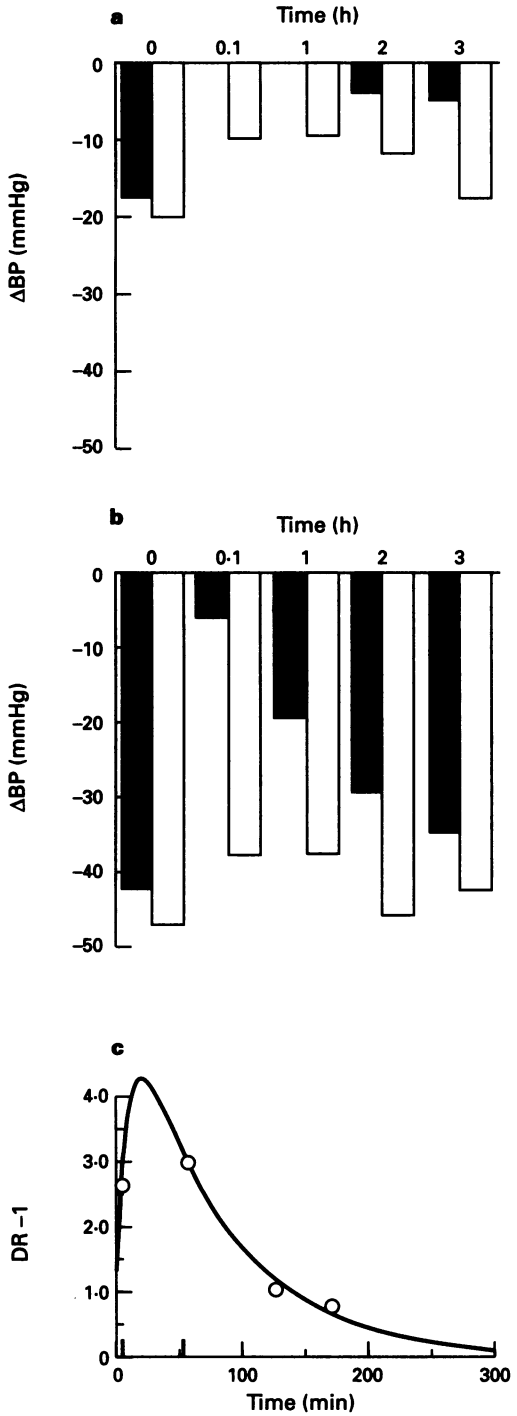



**Figure 3** Changes in the effects of doses of bethanechol (a, 10 nanomoles: b, 300 nanomoles) with time after doses of the antagonists zamifenacin (solid columns) and hexahydro-benz-4DAP (open columns). These were converted into dose-ratios and in (c), the values of mean (dose-ratio −1) for hexahydro-benz-4DAP are plotted against time and fitted to an exponential uptake (half-time 6.1 min) and decay (half-time 53.7 min).

### *Anaesthetized rats*

The log dose-response curve for the hypotensive/depressor effects of bethanechol is flat compared with the curve for bradycardia or for the reduction in tracheal airflow, and starts with much lower doses (Figure 2). After an antagonist, the line is moved towards higher concentrations and the dose-ratio can be measured roughly by eye. A more precise measure was obtained by fitting the responses in the linear portion of the control curve to a straight line by least-squares and using the fitted line to calculate the matching concentration for each response in the presence of the antagonist, giving an estimate of the dose-ratio. The average dose-ratio was calculated for all the responses obtained after a particular dose of antagonist had been given. Because the blood-pressure is affected by changes in heart-rate, responses where there was also bradycardia were excluded. Results obtained with hexahydro-benz-4DAP and zamifenacin, and from one experiment with atropine, are summarized in Table 1. With hexahydro-benz-4DAP (as had already been found with benz-4DAP: Jackson & Smith unpublished) the dose-ratio did not increase with concentration as would be expected if the compound behaved competitively. This can be seen from the slopes of the Schild plots of log (dose-ratio −1) against log (antagonist dose), which are much less than one. With zamifenacin the results are consistent with competition, as is the result with atropine.

The selectivity, i.e the relative activity of the compound on two systems, can be calculated from the ratio of the values of (dose-ratio −1). Values for blood pressure compared with heart rate are included in Table 1 and show that the selectivity of hexahydro-benz-4DAP and zamifenacin is maintained over the range of doses tested.

The duration of the effects of the compounds were studied by following the responses to doses of bethanechol (10 and 300 nanomol) for 3 h (Figure 3). From the control values for bethanechol it is possible to convert the responses into dose-ratios and the values of (dose-ratio −1) and time indicate a half-life of about 1 min for zamifenacin, compared with 110 min for hexahydro-benz-4DAP. The results can, in fact, be fitted to a rising phase with a half-life of 7 min and a decay with a half-life of 54 min (Figure 3c). The results for zamifenacin are consistent with the observations of Beaumont *et al.* (1994) that the compound is, in the rat, rapidly and extensively bound to plasma proteins.

## Discussion

This work confirms the selectivity of the effects of hexahydro-benz-4DAP. The results obtained on guinea-pig atria with and without neostigmine make it unlikely that the low activity on this preparation is due to hydrolysis of the compound. Such a rapid breakdown is improbable, anyway, in view of the persistence of its effects *in vivo*.

The suggestion by Caulfield *et al.* (1993) that some of the variation observed in the estimates of affinity of benzyl-4DAP for different types of receptor might be due to its low solubility seems likely to be correct when it is appreciated that it is the solubility of the free base which is limited. In experiments *in vitro* it may be possible to keep the base in solution by making the pH slightly more acid or by using solvents such as dimethylsulphoxide or ethanol: this must lead to different results from experiments *in vivo*. The limited solubility of the base, however, does not explain the greater selectivity seen *in vivo*.

**Table 2** Mean estimates of apparent log $K$ obtained for antagonism of the effects of bethanechol in anaesthetized rats

| *Zamifenacin* *Dose* ($\mu$moles kg$^{-1}$) | *Blood pressure* | *Heart rate* | *Airflow* | *Sel* |
|---|---|---|---|---|
| 0.01 | 8.41 ± 0.08 (3) | 7.08 ± 0.19 (4) | 7.39 ± 0.37 (3) | 1.33 |
| 0.1 | 7.48 ± 0.24 (4) | 6.42 ± 0.20 (4) | 6.99 ± 0.16 (4) | 1.06 |
| 1.0 | 7.42 ± 0.09 (4) | 6.12 ± 0.06 (4) | 7.00 ± 0.17 (4) | 1.30 |
| 10 | 7.88 ± 0.29 (4) | 6.79 ± 0.10 (4) | 7.41 ± 0.07 (4) | 1.09 |
| log $K$ (Wallis *et al.*, 1993) | *Ileum* 9.0, 9.3 | *Atria* 7.1 | | |
| *Atropine* | | | | |
| 0.00288 | 8.32 | 7.89 | 7.24 | 0.43 |
| 0.0288 | 8.14 | 7.77 | 7.49 | 0.37 |
| 0.288 | 8.12 | 7.95 | 8.04 | 0.17 |
| log $K$ (Barlow *et al.*, 1976) | *Ileum* 9.3 | *Atria* 9.1 | | |
| *Hexahydro-benz-4DAP* | | | | |
| 0.01 | 8.41 ± 0.22 (4) | 7.41 ± 0.17 (5) | 7.43 ± 0.17 (2) | 1.00 |
| 0.1 | 7.65 ± 0.17 (5) | 6.76 ± 0.11 (6) | 6.16 ± 0.49 (5) | 0.99 |
| 1.0 | 6.92 ± 0.18 (5) | 5.89 ± 0.11 (6) | 5.55 ± 0.20 (4) | 1.03 |
| 10 | 6.54 ± 0.18 (4) | 5.37 ± 0.16 (5) | 4.78 ± 0.11 (3) | 1.17 |
| log $K$ (this work) | *Ileum* 6.6 | *Atria* 5.4 | | |

Values are mean ± s.e. with number of results in parentheses. Sel is the difference between the values for blood-pressure and heart rate and indicates the selectivity on a logarithmic scale. Values of log $K$ ($pA_2$) for receptors in guinea-pig isolated ileum and atria are shown for comparison.

Although only small effects can be produced on atria because solutions which should produce large effects are saturated, the selectivity of a saturated solution, expressed (as in Table 1) as the ratio of (dose-ratio $-1$), is exactly the same as that at lower concentrations.

The experiments on guinea-pig ileum with an antagonist given together with 4-DAMP MeBr confirm that both hexahydro-benz-4DAP and benz-4DAP act competitively. The results obtained in tests on anaesthetized rats which give flat Schild plots, therefore, probably do not indicate a change in the nature of the antagonism but simply that the limited solubility of the free base makes it impossible to obtain large dose-ratios. The slopes of the Schild plots for zamifenacin and atropine are close to one.

A comparison of the results obtained on anaesthetized rats with the *in vitro* activity of the compounds can be made (Table 2) by converting the dose-ratios obtained into estimates of an apparent log $K$ ($pA_2$), assuming that the volume of distribution is 55% of the body mass. This is not an equilibrium constant because it ignores loss though metabolism and excretion but it indicates what might be expected if this is negligible. The values of the apparent log $K$ of zamifenacin for effects on heart rate, for example, are slightly less than the $pA_2$ value, 7.1, for guinea-pig atria *in vitro* obtained by Wallis *et al.* (1993) with carbachol as agonist at 32°C. The values on blood-pressure, however, are considerably lower than the $pA_2$ values for ($M_3$) receptors in guinea-pig ileum (9.27 with acetylcholine as agonist, 9.00 with carbachol). Lower activity *in vivo*, indicates that the volume of dilution has been under-estimated or that there is appreciable loss of the drug during the time the observations were made, and it is known that in rats zamifenacin is extensively bound to plasma proteins (Beaumont *et al.*, 1994). With atropine, too, the apparent log $K$ is much lower than would be expected indicating that the volume of dilution has been under-estimated.

Hexahydro-benz-4DAP, however, is more active *in vivo* than would be expected from the *in vitro* results. The values for effects on heart rate in Table 2 range from 7.4 to 5.4, compared with the value 5.4 obtained *in vitro*, and the values for blood-pressure range from 8.4 to 6.5, compared with 6.6 for ileum *in vitro*. This might indicate that there are different types of $M_3$ receptors, as has been suggested by Carter *et al.* (1991), Kaiser *et al.* (1993) and Wallis (1995).

Those involved in airflow and blood-pressure, for instance, do not appear to be identical. In this work the antagonism of effects on airflow by hexahydro-benz-4DAP fourdapine was even less than that on heart-rate: with zamifenacin the antagonism of effects on airflow was intermediate between that on heart-rate and that on blood-pressure. The extremely flat log dose-response curve for the effects of bethanechol on blood-pressure (Figure 2a), in fact, suggests that more than one type of $M_3$ receptor is involved. To increase the effect from 25% to 75% it is necessary to multiply the dose by roughly 100, which indicates a Hill coefficient of less than 0.5 (to increase receptor occupancy from 25% to 75% it is only necessary to multiply the concentration by 9 if binding follows the mass-action isotherm). It is unlikely that the second process involves effects on the heart because results where hypotension was accompanied by bradycardia have been excluded. The log dose-response curve for bethanechol on the heart *in vivo*, on the other hand, is very steep (Figure 2b), which suggests an action at only one type of receptor. The relatively high activity observed with low doses of hexahydro-benzyl-4DAP might indicate that the volume of distribution has been greatly overestimated and that there is a high local concentration in the heart after the intravenous injection.

This difference between the apparent log $K$ values for effects on heart and on blood-pressure (the column, Sel, in Table 2) is a measure of selectivity on a logarithmic scale and should be similar to the logarithm of the selectivity ratio (SR in Table 1). The discrepancy seen with the estimates for the highest concentration of hexahydro-benzyl-4DAP arises from the difference between arithmetic and logarithmic means with large dose-ratios. With zamifenacin the selectivity is around 20 fold: with atropine there is little selectivity. With hexahydro-benz-4DAP it is around 10 fold, even though the estimates of apparent log $K$ depend upon the concentration and there is a limit to the size of effect which can be produced. Interestingly it was possible to obtain higher effects on blood-pressure with hexahydro-benz-4DAP than with benz-4DAP, for which the highest dose-

ratio obtainable was about 10 (Jackson & Smith, unpublished), even though the hexahydro- compound was less active on ileum *in vitro* (log $K$ 6.6 and 7.9, respectively in this work).

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