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Neuromuscular blocking and antimuscarinic activity of a series of bis-atropinium and *N*-n-alkyl atropinium compounds

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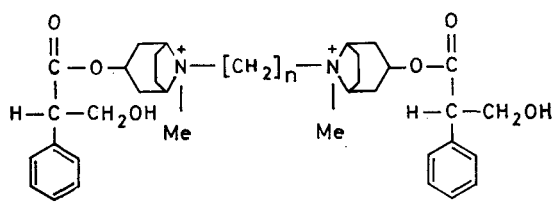
Measurements of neuromuscular blocking and antimuscarinic activity have been made in a series of bis-atropinium (BA) and *N*-n-alkyl atropinium (N-AA) compounds. That the second atropinium group contributed to the neuromuscular blocking activity of BA compounds was shown by the relative lack of such activity in the N-AA series of compounds. Peak neuromuscular blocking activity occurred when two atropinium groups were separated by a chain of either 10 or 11 methylene groups. Members of both series of compounds displayed antimuscarinic properties but estimates of activity differed according to whether they were obtained by determination of affinity constants on guinea-pig isolated ileum or production of mydriasis in mice. From measurements of affinity constant on the guinea-pig ileum it is concluded that BA compounds interact with only one muscarinic receptor. However, the high activity of the deca- and undecamethylene BA compounds in producing mydriasis suggest that these compounds possibly interact with two receptors at once.

It has been demonstrated (Kimura & Unna, 1950; Eckfield, 1959) that in a series of polymethylene bis-atropinium (BA) compounds (general formula I) there was an ascending order of neuromuscular blocking or 'curare-like' activity with increase in polymethylene chain length. To date, only compounds containing up to ten methylene groups in the chain have been tested and we have extended previous investigations by examining BA compounds containing longer polymethylene chains to determine where neuromuscular blocking activity reaches a maximum.



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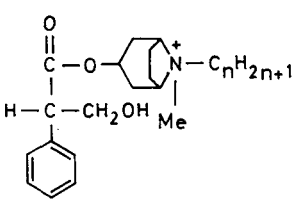

II

Kimura & Unna (1950) found that the decamethylene bis-atropinium compound (referred to as BA10) besides possessing approximately three times the activity of (+)-tubocurarine, also showed approximately twice the antimuscarinic activity of methyl atropine. The antimuscarinic activity of BA compounds was discussed by Barlow (1955) who thought it possible that BA compounds with a polymethylene chain of suitable length might interact with two receptors at once. This author inferred that such pharmacologically 'bivalent' compounds should be adsorbed

much more than twice as strongly as atropine at the biological surface for two reasons. Firstly, when one end of a pharmacologically bivalent molecule becomes detached the compound may still be held to the biological surface at the other end, and it is very likely that the detached end will become reattached. Secondly, if the receptor sites are some distance apart there may be considerable Van de Waal's attraction between the polymethylene chain and non-polar areas on the biological surface that separates the receptors. If such non-polar interactions make a major contribution to affinity it may be possible to obtain potent antimuscarinic compounds from atropine quaternized only with long-chain alkyl halides and the presence of a second atropinium grouping may not be essential. This point has been investigated by comparing the antimuscarinic activities of a series of *N*-n-alkyl atropinium (N-AA) compounds (general formula II) with those of BA compounds. A comparison between the neuromuscular blocking activities of BA and N-AA compounds was also made in order to determine the importance of the second atropinium group in contributing to neuromuscular blocking activity.

METHODS

Toxicity

All the compounds were tested for their intravenous toxicity in male albino mice. Groups containing 5 mice were used and LD50 values were calculated using the method of probit analysis (Finney 1947).

Neuromuscular blocking activity

Antagonism of carbachol-induced contractions on frog isolated rectus abdominus muscle. The method for determining the affinity constants of the compounds was similar to that of Barlow, Scott & Stephenson (1967) except that carbachol replaced acetylcholine as agonist. The affinity constant using the four point assay procedure was calculated according to the Edinburgh Staff (1970a). Dose ratios ranged from 5 to 20 and the logarithm of the affinity constant (log K) was expressed as a mean of four determinations using two concentrations of antagonist; one preparation was used for one concentration of antagonist. Compounds were classified as competitive antagonists when plots of dose ratio against concentration were linear in accordance with the Gaddum equation (1957).

Antagonism of suxamethonium-induced contractions on isolated semispinalis muscle of chick. The muscle was set up according to Child & Zaimis (1960) using Tyrode gassed with 5% carbon dioxide in oxygen. Compounds were assayed for neuromuscular blocking activity by assessing their ability to inhibit contractions induced by suxamethonium (10^{-6}M) kept in contact with the muscle for 1 min before being washed out; the antagonist was added 1 min before the agonist; a total time cycle of 5 min was used. A latin square 2×2 point assay was carried out using (+)-tubocurarine as standard (10^{-6} and $2{\cdot}5 \times 10^{-7}$M) and the relative activity of the test compound to (+)-tubocurarine was calculated. The chick muscle was preferred to the frog rectus muscle because a faster time cycle could be used.

Effects on anterior tibialis muscle of the cat. The preparation was set up according to the Edinburgh Staff (1970b). Twitches were elicited every 10 s by rectangular pulses of 200 μs and of twice the strength to evoke a maximal twitch. A frequency of 50 Hz for 5 s was used to elicit a fused tetanic contraction. The compounds were dissolved in physiological saline and injected through the femoral vein.

Antimuscarinic activity

Antagonism of carbachol-induced contractions of the guinea-pig ileum. Affinity constants of the compounds were determined by the method of Brimblecombe, Green & others (1971). Dose ratios ranged from 100 to 400 and log K was expressed as a mean of 4–6 determinations using 2–3 concentrations of antagonist. One preparation was used for one concentration of antagonist.

Production of mydriasis in mice. Mydriatic activity was assayed according to Brimblecombe & others (1971) with the exception that methyl atropine was used as standard instead of atropine.

Compounds

(+)-Tubocurarine chloride was purchased from Koch Light Laboratories Ltd., and atropine methyl nitrate from Evans Medical Ltd. All the other compounds were synthesized in these laboratories and were prepared as bromide salts with the exception of *N*-ethyl, *N*-propyl, *N*-hexyl and *N*-octyl atropinium compounds which were prepared as iodides. Infrared spectroscopic and nmr data were consistent with the assigned structure of the compounds. In all experimental procedures relative activities were expressed in terms of the base.

Table 1. *Toxicity and neuromuscular blocking activity of a series of bis-atropinium (BA) and* N*-n-alkyl atropinium (N-AA) compounds.*

	Toxicity		Neuromuscular blocking activity	
Compound	Mouse LD50 μmol/kg i.v. (95% confidence limits)	Relative potency to (+)-tubocurarine	Chick semispinalis. Relative potency (+)-tubocurarine (95% confidence limits)	Antagonism of carbachol-induced contractions on frog rectus abdominis LogK ± s.e. (No. of estimates)
(+)-Tubocurarine	0·28 (0·23–0·36)	1·0	1·0	6·33 ± 0·05 (4)
BA series chain length (n)				
4	3·02 (2·35–3·08)	0·09	0·20 (0·14–0·29)	—
6	0·55 (0·47–0·63)	0·52	1·06 (0·61–1·68)	n.c.*
7	0·44 (0·38–0·51)	0·64	2·76 (1·83–4·36)	n.c.
8	0·42 (0·35–0·59)	0·68	2·45 (1·66–3·99)	6·83 ± 0·03 (4)
9	1·04 (0·95–1·24)	0·26	2·53 (2·12–3·02)	6·90 ± 0·04 (4)
10	0·50 (0·42–0·71)	0·57	3·60 (2·83–4·55)	6·94 ± 0·03 (4)
11	0·53 (0·46–0·61)	0·53	3·37 (2·67–4·34)	6·94 ± 0·03 (4)
12	0·63 (0·57–0·69)	0·45	3·05 (1·75–4·78)	6·83 ± 0·02 (4)
13	1·11 (1·02–1·21)	0·28	1·53 (1·06–2·34)	n.c.
14	1·41 (1·05–2·56)	0·20	1·66 (1·20–2·45)	n.c.
16	3·34 (3·03–3·70)	0·08	0·61 (0·38–0·90)	n.c.
N-AA series chain length (n)				
1 (methyl atropine)	20·9 (15·6–25·9)	0·013	<0·1	—
2	25·9 (21·0–32·0)	0·011	<0·1	—
3	24·1 (19·5–29·7)	0·011	<0·1	—
4	21·7 (19·1–24·7)	0·013	<0·1	—
5	13·7 (11·2–17·4)	0·020	<0·1	—
6	15·4 (11·8–18·3)	0·018	<0·1	—
8	25·3 (20·6–31·2)	0·011	<0·1	—
10	25·9 (21·9–30·5)	0·011	<0·1	—
11	>50	—	<0·1	—
12	>50	—	<0·1	—

* n.c. non-competitive block.

RESULTS AND DISCUSSION

Toxicity and neuromuscular blocking activity

The LD50 values of the compounds are shown in Table 1 together with estimates of neuromuscular blocking activity obtained on the frog isolated rectus and chick semispinalis muscle preparations.

None of the BA compounds produced a contracture of the frog rectus or chick semispinalis muscles, thus demonstrating that the neuromuscular blocking action of these compounds is not due to a depolarization effect at the neuromuscular junction. Compounds BA 6, 10 and 16 tested on the cat anterior tibialis muscle were found to produce a typical non-depolarizing curare-like block. High frequency indirect stimulation produced a poorly maintained tetanus with antagonism of the block; in addition the block was antagonized by neostigmine. An 80% reduction in twitch tension was obtained with BA 10, 0·3 mg/kg, and this compound was approximately equipotent with (+)-tubocurarine. A comparable 80% block was obtained with BA 6 and 16 at 0·75 mg/kg. These results and those obtained on the chick and frog muscle preparations confirm the findings of Kimura & Unna (1950) and Eckfield (1959) that with BA compounds containing up to 10 methylene groups there is an ascending order of neuromuscular blocking activity with increase in polymethylene chain length. From measurements of neuromuscular blocking activity of longer chain members in the BA series we have shown that activity reached a maximum with BA 10 on the chick semispinalis muscle and with compounds BA 10 and 11 on frog rectus muscle.

Compounds ranging from BA 8 to 12 were competitive curare-like antagonists on frog rectus muscle but all the other BA compounds appeared to be non-competitive. Thesleff & Unna (1954) and van Rossum & Ariëns (1959) found in a series of derivatives of the depolarizing neuromuscular blocking compound decamethylene bis-trimethyl ammonium (decamethonium, C10), that if one or more of the methyl groups on the quaternary nitrogen were substituted by bulkier groups e.g. pentyl, then the type of blocking action was altered. Thesleff & Unna (1954) described these compounds as having curare-like action and van Rossum & Ariëns (1959) described their antagonistic action on frog rectus muscle as non-competitive. In view of these findings it is not surprising that BA compounds possess marked non-depolarizing 'curare-like' properties as they consist of two bulky atropine groups separated by a polymethylene chain between the nitrogen atoms. However, no explanation can be given for the finding that some BA compounds exhibit competitive and others non-competitive modes of action.

The best estimate of effectiveness of interaction between drug and receptor is probably provided by measurements of affinity constants. In such measurements sufficient time is allowed for the compound to penetrate and reach equilibrium with the receptor and has advantages over *in vivo* methods of assay (see later) and over other *in vitro* methods in which a fixed contact time for the antagonist is used. In the latter procedures activity may be related or partly related to different diffusion rates to the receptor and not to true affinity for the receptor. The chick semispinalis assay procedure we used has this disadvantage, but because affinity constants could not be determined with compounds BA 4 to 8 and BA 13 to 16 (as they exhibited a non-competitive action) the chick assay procedure was adopted to provide a quantitative estimate of the neuromuscular blocking activity. The results appear to be of value since with competitive BA compounds there was reasonable agreement between estimates of activity made in the chick and frog muscle preparations.

There was a significant correlation ($P < 0{\cdot}01$ coeff. of correlation $r = 0{\cdot}71$) of neuromuscular blocking activity of BA compounds, as assayed on the chick semispinalis muscle, and the intravenous toxicity in mice, indicating that the lethal action of these compounds is due to an action at the neuromuscular junction. The

relative lower toxicity and weaker action at the neuromuscular junction of the N-AA compounds demonstrates that a second atropinium group is required for potent neuromuscular blocking activity. This is in accord with the two point attachment theory for potent neuromuscular blockade (Barlow & Ing, 1948; Paton & Zaimis 1949). The recent finding by Everett, Lowe & Wilkinson (1970) that (+)-tubocurarine is a mono and not a bis-quaternary compound does not necessarily contradict the two point attachment theory because under physiological conditions the tertiary nitrogen may be active in its protonated form.

Although in the BA series peak activity occurred with BA 10 and 11, it is not possible from our findings to reach any conclusions about the inter-onium distance required for maximum activity. Conductimetric work on polymethylene bis-onium compounds has shown that there is much flexing of the chain with consequent shortening of the interonium distance (Elworthy, 1963, 1964). The polymethylene chains of BA compounds would also be likely to behave similarly, optimum activity depending on the conformation of the chain as well as its length. For example, Barlow & Zoller (1964) found that peak neuromuscular blocking activity was reached in a series of bis-triethyl ammonium compounds (BTE) with polymethylene chains possessing 15, 16 and 17 methylene groups and this difference between the BA and BTE series may be explained if the conformations of the polymethylene chains are different.

Table 2. *Antimuscarinic activity.*

Compound	Antagonism of carbachol-induced contractions on guinea-pig ileum. Log K ± s.e. (no. of estimates)	Mydriasis in mice, relative potency to methyl atropine (95% confidence limits)
BA series chain length (n)		
4	8·30 ± 0·03 (4)	1·52 (1·25–1·80)
6	7·84 ± 0·06 (4)	0·46 (0·41–0·52)
7	7·77 ± 0·03 (4)	0·49 (0·40–0·59)
8	8·47 ± 0·08 (4)	0·49 (0·41–0·58)
9	8·59 ± 0·04 (4)	0·28 (0·24–0·31)
10	8·89 ± 0·06 (4)	1·36 (1·22–1·56)
11	9·00 ± 0·09 (4)	1·60 (1·38–1·81)
12	8·93 ± 0·06 (6)	0·67 (0·59–0·77)
13	9·01 ± 0·06 (6)	0·32 (0·28–0·36)
14	8·82 ± 0·06 (4)	0·17 (0·14–0·19)
16	8·76 ± 0·04 (5)	0·039 (0·035–0·043)
N-AA series Chain length (n)		
1 (methyl atropine)	9·53 ± 0·04 (4)	1·0
2	8·82 ± 0·04 (4)	0·41 (0·35–0·51)
3	7·88 ± 0·04 (4)	0·06 (0·05–0·07)
4	7·45 ± 0·04 (4)	0·04 (0·02–0·05)
5	7·38 ± 0·02 (4)	0·07 (0·05–0·09)
6	7·93 ± 0·06 (4)	0·15 (0·12–0·19)
8	8·20 ± 0·07 (4)	0·12 (0·09–0·16)
10	8·51 ± 0·03 (4)	0·09 (0·07–0·13)
11	8·54 ± 0·03 (4)	0·16 (0·13–0·19)
12	8·35 ± 0·05 (6)	0·02 (0·01–0·03)
(+)-tubocurarine*	—	Inactive

* This compound was tested up to its lethal dose.

Antimuscarinic activity

The results of antimuscarinic activity as measured by affinity for the muscarinic receptor in the guinea-pig ileum and production of mydriasis in mice are listed in Table 2.

In the BA and N-AA series there was an initial decline in affinity for the muscarinic receptors in the guinea-pig ileum with increase in chain length of the grouping on the nitrogen atom of the atropine moiety. This decline may be explained by the chains being of sufficient size to reduce the accessibility of the positive charge on the nitrogen atom to the negatively charged site of the muscarinic receptor. After minimum affinity had been reached with BA 7 and N-AA 5, further increase in chain length gradually increased affinity similarly in *both* series (Fig. 1). This increase may possibly be explained on the basis of an increase in interaction between the polymethylene chain and lipophilic areas in the receptor region. From our results it may be inferred that in the BA series the second atropine group does not make any marked contribution to affinity and it is thus unlikely that such compounds interact with two muscarinic receptors at once.

The increase in affinity with chain length in the BA series reached a limit in the region of BA 11, 12 and 13, further increase in chain length to BA 16 reduced affinity only slightly. Similarly, no marked decline in affinity for the muscarinic receptor was found in the series of BTE compounds (Barlow & Zoller, 1964) with up to 21 methylene groups in the chain. Those results were in contrast to the marked decline in neuromuscular and ganglion blocking activity with BTE compounds possessing

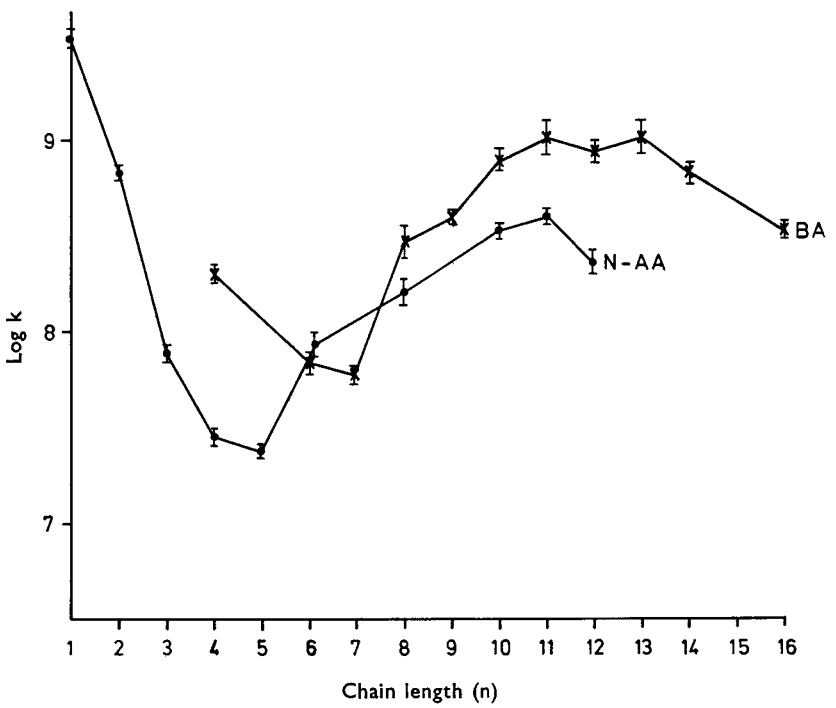

FIG. 1. Guinea-pig isolated ileum. Graph of log affinity constant with standard errors, against chain length.

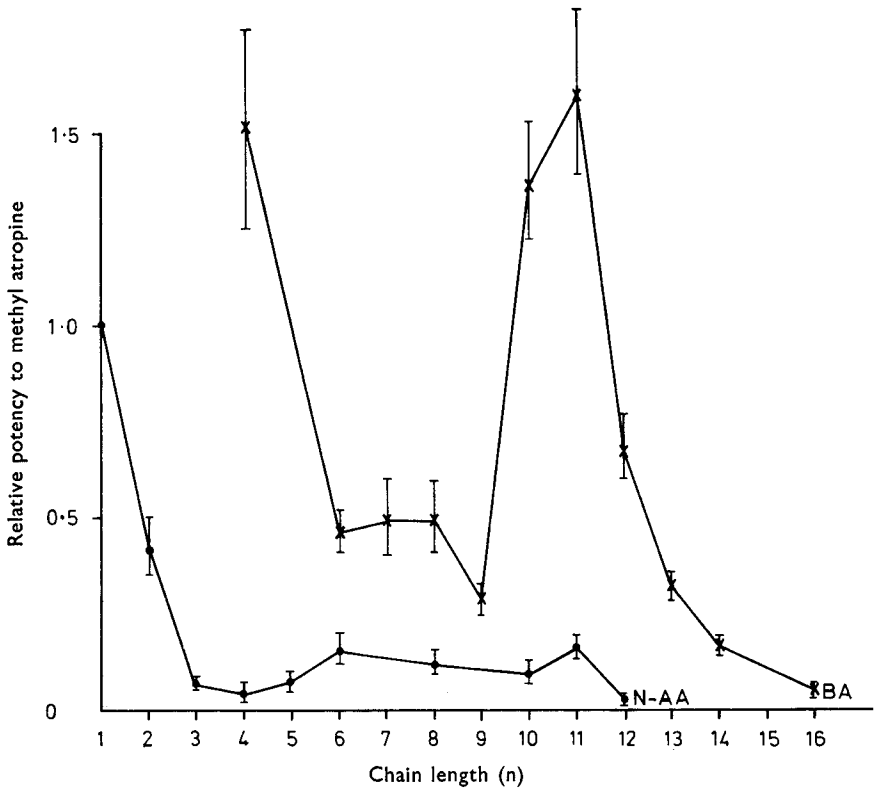

FIG. 2. Mouse mydriasis assay. Graph of relative potency to methyl atropine with 95% confidence limits, against chain length.

more than 17 or 18 methylene groups, and we found a marked decline in neuromuscular blocking activity of BA compounds with more than 12 methylene groups.

Comparison of results *in vitro* on the guinea-pig ileum with those obtained *in vivo* in the mouse mydriasis test show differences where peak and minimal antimuscarinic activity occurs. For example, after the initial decline in activity with increase in polymethylene chain length in the BA series, minimal mydriatic acitvity was found with BA 9 whereas BA 7 possessed the least affinity for the muscarinic receptor in the ileum. There was also a large difference between relative activities obtained by the two different test procedures; for example BA 10 was 1·36 times more active than methyl atropine in producing myriasis whereas BA 10 (log K 8.89) had only 0·24 times the affinity of methyl atropine (log K 9·53). The finding that in the mydriasis test there was a sharp peak in the region of BA 10 to 11 with no such comparable rise in activity in the N-AA series (Fig. 2) is at variance with the inference, based on the results on the guinea-pig ileum, that it is unlikely that BA compounds with polymethylene chains of a certain length could interact with two muscarinic receptors at once. As mentioned previously measurement of affinity constants, most probably provides the best estimate of the interaction of the drug with the receptor since such results are relatively little affected by such factors as diffusion, distribution absorption and metabolism (Brimblecombe, Green, & others, 1971).

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