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Affinity, Efficacy, and Stereoselectivity of Oxotremorine Analogues for Muscarinic Receptors in the Isolated Guinea Pig Ileum

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SUMMARY

The seven possible tertiary structural isomers obtained by the introduction of a single methyl group in the muscarinic agent oxotremorine [*N*-(4-pyrrolidino-2-butynyl)-2-pyrrolidone], and some related compounds, were investigated for muscarinic and antimuscarinic activity in the isolated guinea pig ileum. The compounds were agonists, partial agonists, or competitive antagonists. For agonists (including oxotremorine) and partial agonists, the muscarinic potency was separated into affinity and efficacy components using the method of partial irreversible blockade of spare receptors with dibenamine. From these experiments, a dissociation constant of 1.09×10^{-6} M was obtained for oxotremorine. The presence of a methyl group at position 1 of the butynyl chain or at position 5 of the pyrrolidone ring of oxotremorine increases the affinity 21 and 12 times, respectively. A methyl group at positions 2 and 3 of the pyrrolidine ring of oxotremorine has no significant effect on its affinity. In contrast, at position 3 or 4 of the pyrrolidone ring and at position 4 of the butynyl chain, a methyl group markedly decreases affinity. The structural requirements for achieving high affinity appear to be independent of those leading to high efficacy. Some compounds showed a pronounced stereoselectivity of action, whereas others exhibited little or no stereoselectivity. The degree of stereoselectivity is well correlated with the affinity of the more potent member of each enantiomeric pair. A good correlation was observed between parasympatholytic potency *in vitro* and previously reported tremorolytic potencies in mice of 24 structurally related oxotremorine analogues.

INTRODUCTION

Classical antimuscarinic agents may be derived formally from muscarinic agonists by the introduction of hydrophobic ring systems which are responsible for a very significant fraction of the over-all binding energy of the antagonists. It has been suggested that the hydrophobic moieties of antagonists interact with accessory binding sites located close to the agonist binding region of the receptor (1). A high degree of complimentarity between antagonists and the agonist binding site does not seem necessary for high antimuscarinic potency. Furthermore, the stereochemical requirements for muscarinic and antimuscarinic activity are different (2), supporting the view that the critical moieties involved in the binding of classical muscarinic agonists and antagonists are not identical.

It therefore appears that studies of the affinities of classical antimuscarinic agents would give information primarily on the nature of the hydrophobic interactions at the accessory receptor areas, but contribute less to an understanding of the interactions occurring at the agonist binding region of the receptor. In light of these concepts, pharmacological studies of antagonists produced by minor structural modification in an agonist molecule, and for which binding to accessory receptor areas is not likely to be important, should provide more information on the relationship between agonist and antagonist binding at the muscarinic receptor.

We previously suggested that agonists and antagonists related to the specific muscarinic agent oxotremorine, *N*-(4-pyrrolidino-2-butynyl)-2-pyrrolidone, interact with a common receptor site (3). In this paper we report a further investigation of structure-activity relationships of antagonists related to oxotremorine.

We have shown that only one of the seven possible tertiary structural isomers obtained by the introduction of a single methyl group in oxotremorine has oxotremorine-like properties *in vivo*. The remaining six isomers antagonize the central effects of oxotremorine in mice (4–7). We now have determined the affinities of these compounds (*1–7*, Table 2), some resolved into enantiomeric pairs (Table 3), for muscarine-sensitive receptors in the isolated guinea pig ileum. Their affinities have been compared with that of oxotremorine obtained after irreversible blockade of a fraction of the receptors with

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dibenamine (8). The four optical isomers of two oxotremorine analogues containing two alkyl groups as well as the enantiomers of an ethyl- and a propyl-substituted oxotremorine (*8–11*, Table 3) were also investigated.

MATERIALS AND METHODS

Drugs. Compounds *1–3* (6), *4* (9, 10), *5* (7), *6* and *7* (5), *8* and *11* (11), and *9* and *10* (12) were prepared as previously described. Other drugs and their sources were the following: oxotremorine sesquioxalate (prepared in the Department of Pharmacology, University of California, Los Angeles), carbamylcholine chloride (Aldrich Chemical Company, Milwaukee, Wisc.), hexamethonium chloride (K&K Laboratories, Plainview, N. Y.), atropine sulfate (Mallinckrodt Inc., Paris, Ky.), and dibenamine (*N,N*-dibenzyl-2-chloroethylamine) hydrochloride (gift of Dr. P. T. Ridley, Smith Kline & French Laboratories, Philadelphia, Pa.).

The Tyrode's solution had the following composition (millimolar): NaCl, 137; $NaHCO_3$, 12; glucose, 5.0; KCl, 2.7; $MgSO_4$, 1.0; NaH_2PO_4, 0.4; $CaCl_2$, 1.8; and pH 7.4.

Isolated guinea pig ileum. Guinea pigs (male, English short hair, 350–400 g) were killed by a blow to the head and bled. Segments of the ileum (2–3 cm long) were removed and suspended in a 10-ml organ bath containing Tyrode's solution at 37° and aerated with O_2 containing 5% CO_2. Contractions were recorded isotonically at 1 g of tension, using an electromechanical displacement transducer and a potentiometric recorder.

Dissociation constants of antagonists. Antagonistic activity was measured against carbachol, which was added cumulatively. The preparation was allowed to equilibrate with each concentration of antagonist for 15 min before dose-response curves to carbachol were obtained. pA_2 values (negative logarithm of the dissociation constant) were calculated according to the method of Arunlakshana and Schild (13).

Dissociation constants and relative efficacies of agonists. Dissociation constants and relative efficacies of oxotremorine and Compound *1* at muscarinic receptors of guinea pig isolated ileum were determined according to the method of Furchgott and Bursztyn (8), using cumulative additions of the two agonists. After the determination of the control dose-response curves, from which the ED_{50} values were obtained, the preparation was treated with an adequate amount (successive 15-min incubations with 1.5×10^{-5} M, 5×10^{-5} M, and in some cases 5×10^{-6} M, respectively) of dibenamine to occlude a fraction of receptors. The tissue was washed several times, allowed to rest for 20 min, and challenged with a submaximal dose of oxotremorine until constant responses were obtained. The dose-response curves of oxotremorine and Compound *1* were then obtained in the dibenamine-treated tissue. Several equipotent doses of each agonist before (A) and after (A') dibenamine treatment were determined graphically. $1/(A)$ was plotted versus $1/(A')$, and a straight line was fitted to the data by linear regression analysis. From the slope and the intercept on the ordinate, the dissociation constant (K_A) of the agonist-receptor complex and the fraction (q) of receptors still active were calculated. The K_A values, q, and the control dose-response curves were used to generate theoretical dose-response curves after dibenamine treatment (8).

The efficacy of Compound *1* (ϵ_1) relative to that of oxotremorine (ϵ_{OT}) was determined using Relationship 1 (8):

$$\frac{\epsilon_1}{\epsilon_{OT}} \equiv \frac{\dfrac{A_{OT}}{K_{A_{OT}} + A_{OT}}}{\dfrac{A_1}{K_{A_1} + A_1}} \qquad (1)$$

where A_{OT} and A_1 are the concentrations of oxotremorine and Compound *1*, respectively, eliciting 50% response. $K_{A_{OT}}$ and K_{A_1} are the dissociation constants of oxotremorine and Compound *1*, respectively.

Dissociation constants and relative efficacies of partial agonists. Dose-response curves for carbachol and the partial agonist (±)-*6*, *R-6*, or *S-6* were obtained, and the ileum was treated with dibenamine (three successive 15-min incubations with 5×10^{-6} M). This treatment completely abolished the response to the partial agonists, while still permitting maximal response to carbachol. Dissociation constants of the partial agonists were then determined in the dibenamine-treated tissue as described above for antagonists. The efficacies of Compounds *R-6* and *S-6* relative to that of oxotremorine were obtained from Relationship 1.

RESULTS

Compound *1* was an agonist at muscarinic receptors in the guinea pig ileum (Table 1). Hexamethonium (3×10^{-4} M) had no appreciable effect on the dose-response curve of Compound *1*, whereas atropine (10^{-8} M) caused a parallel shift to the right of the curve. Dibenamine (1.5×10^{-5} M for 15 min) produced a shift to the right of the dose-response curves to oxotremorine and Compound *1* without much decrease in the maximal response obtainable (Figs. 1 and 2). After another incubation with dibenamine (5×10^{-6} M for 15 min), a decrease in the maximal response to both oxotremorine (Fig. 1) and Compound *1* (Fig. 2) was normally observed. The results from the estimation of dissociation constants (K_A) and relative efficacies of oxotremorine and Compound *1* are summarized in Table 1. There was a good agreement between the experimental dose-response curves (which could be drawn through the *solid triangles* but are not shown in Figs. 1 and 2) and the theoretical dose-response curves after dibenamine treatment.

The racemate and enantiomers of Compound *6*, substituted at position α of the pyrrolidine ring, behaved in a manner typical of partial agonists. At low concentrations their effects were additive with that of carbachol, whereas at higher levels the action of carbachol was competitively antagonized (Fig. 3). Compound *S-6* was about twice as active as Compound *R-6* in causing contractions of the ileum. However, the maximal response obtained with the *R*-enantiomer was consistently higher than that of the *S*-enantiomer (Table 1). Treatment of the ileum with dibenamine (5×10^{-6} M for 15 min) caused a decrease in the maximal response of Compound *6*. Another two identical incubations completely abolished the response to Compound *6* and its enantiomers, but did

TABLE 1

Parameters characterizing the muscarinic activity of oxotremorine, Compounds 1, R-6, and S-6 in the isolated guinea pig ileum

The ED_{50} and K_A values represent the mean ± standard error of the mean. The number of estimates is given in parentheses. The dissociation constants (K_A) were determined after partial irreversible blockade of spare receptors with dibenamine. Relative efficacies were calculated from the ED_{50} and K_A values. Relative maximal effects were measured as the height of the contractile responses.

Compound[a]	ED_{50}	K_A	Relative efficacy	Relative maximal effect
	moles/liter			
Oxotremorine	$3.90 \pm 0.27 \times 10^{-8}$ (5)	$1.09 \pm 0.36 \times 10^{-6}$ (6)	1.0	1.0
Compound *1*	$4.99 \pm 0.30 \times 10^{-7}$ (7)	$1.15 \pm 0.26 \times 10^{-5}$ (6)	0.83	1.0
Compound *R-6*	$6.04 \pm 0.61 \times 10^{-6}$ (4)	$6.76 \pm 1.41 \times 10^{-6}$ (4)	0.073	0.5–0.8
Compound *S-6*	$2.93 \pm 0.49 \times 10^{-6}$ (4)	$1.29 \pm 0.24 \times 10^{-6}$ (4)	0.050	0.3–0.5

[a] For structures see Table 2.

not affect the maximal response to carbachol. With the ileum so treated, the racemate and enantiomers of Compound *6* were used as competitive antagonists to carbachol (Fig. 3), and their pA_2 values were estimated from their respective Schild plots. The *S*-enantiomer was about 5 times more potent than the *R*-enantiomer in antagonizing carbachol-induced contractions (Table 3), in agreement with previously reported tremorolytic potencies of the enantiomers (5). The relative efficacies of Compounds *R-6* and *S-6* are given in Table 1.

The β-substituted pyrrolidine *7* was a pure competitive antagonist to carbachol with an affinity similar to that of Compound *6* (Table 2). As previously found *in vivo* (5), the enantiomers of Compound *7* exhibited no difference in potency (Table 3).

Although the introduction of a methyl group at position 3 of the lactam ring of oxotremorine (Compound *1*) has only a small effect on the efficacy, methyl substitution at positions 4 and 5 of the lactam ring abolished efficacy since the resulting compounds (*2* and *3*) were antagonists.

As previously reported (3), Compound *4* is a rather potent competitive antagonist. The *R*-enantiomer was 257 times more potent than the *S*-enantiomer (Table 3). Compound *5*, a weak antagonist, had the lowest affinity of the compounds studied.

The four optical isomers of Compound *8* (Table 3),

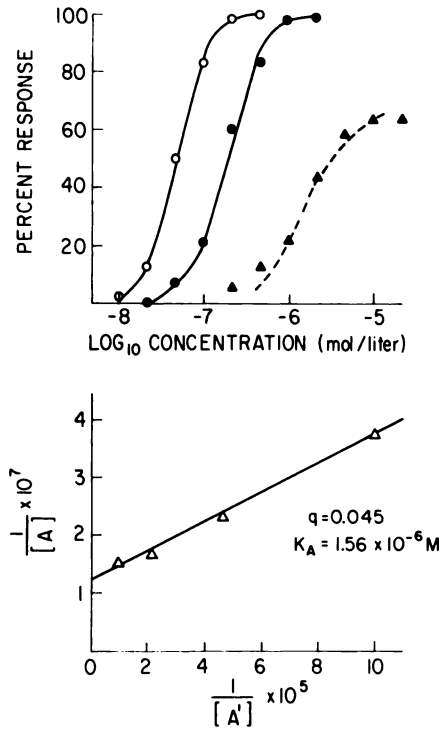


FIG. 1. *Dose-response curves for oxotremorine in the isolated guinea pig ileum (upper graph) and double-reciprocal plot of A versus A' (lower graph)*

The curve to the *left* (○) is the dose-response curve of oxotremorine before dibenamine treatment (control curve). To the *right* are shown the experimental dose-response curves of oxotremorine after two successive 15-min incubations with dibenamine at 1.5×10^{-5} M and 5×10^{-6} M, respectively (● and ▲). Values for *A* and *A'* were obtained from the control dose-response curve and the plotted points (▲) after the second dibenamine incubation, respectively. From the K_A, *q*, and the control curve, the theoretical dose-response curve (– – –) after dibenamine treatment was computed.

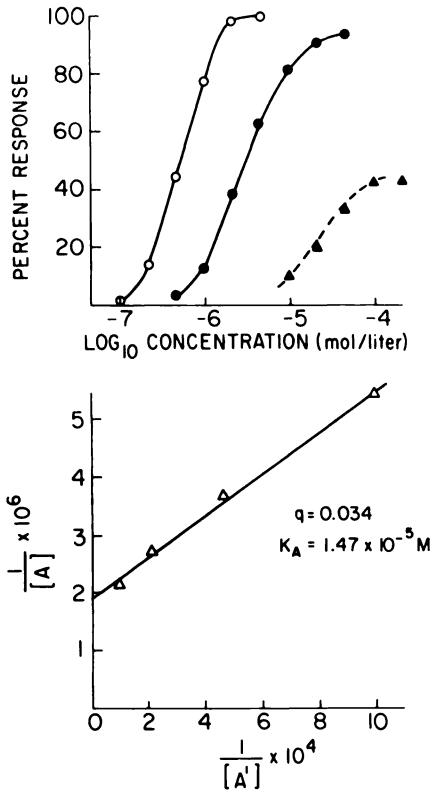


FIG. 2. *Dose-response curves for Compound 1 in the isolated guinea pig ileum (upper graph) and double-reciprocal plot of A versus A' (lower graph)*

For plotting procedure and experimental conditions see Fig. 1.

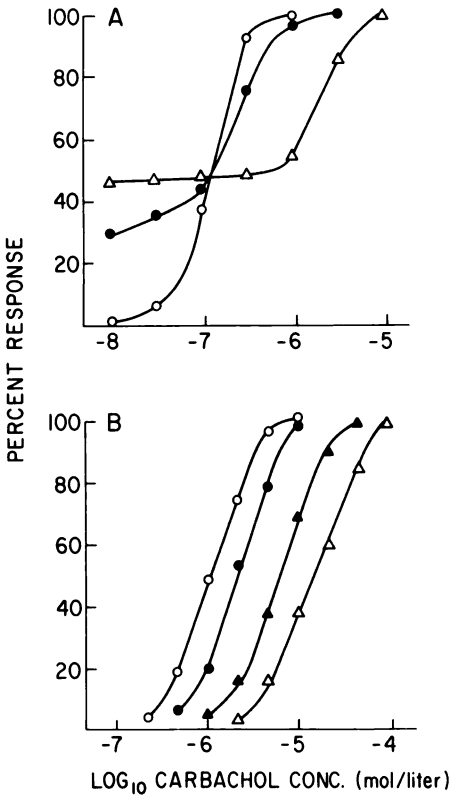


FIG. 3. *Dose-response curves for carbachol in the isolated guinea pig ileum*

A, Dose-response curves of carbachol alone (○) and in the presence of *R-6*, 10^{-5} M (●) and 10^{-4} M (△), showing the partial agonist properties of *R-6*. B, Dose-response curves of carbachol alone (○) and in the presence of *R-6*, 10^{-5} M (●), 3.2×10^{-5} M (▲), and 10^{-4} M (△), after treatment of the ileum with dibenamine (three successive 15-min incubations with 5×10^{-6} M). Note the disappearance of any spasmogenic action of *R-6*, which behaves like a competitive antagonist after the irreversible blockade of reserve receptors.

containing two methyl groups, were competitive antagonists to carbachol. As for all other antagonists studied, their Schild plots were linear, with slopes not significantly different from unity (Fig. 4). The potency difference between the isomers of Compound *8* was somewhat larger *in vitro* than previously reported *in vivo* (11), although the rank of potencies was the same. As expected from the results obtained with Compounds *4* and *6*, the *RS*-isomer of Compound *8* was the most potent, being 646 times more potent than its enantiomer. When comparison is made between epimers of Compound *8* having the opposite configuration in the butynyl chain but the same configuration in the pyrrolidine ring, i.e., *RS-8* versus *SS-8* or *RR-8* versus *SR-8*, the potency ratios are 219 and 221, respectively. The potency ratios of epimers of Compound *8*, having the same configuration in the butynyl chain but the opposite configuration in the pyrrolidine ring, i.e., *RS-8* versus *RR-8* or *SS-8* versus *SR-8*, are much smaller (ratios 2.8 and 3). These epimeric potency ratios agree well with the enantiomeric potency ratios observed for Compounds *4* and *6* and show that the contributions to the potency made by the two chiral centers in Compound *8* are virtually independent of one another.

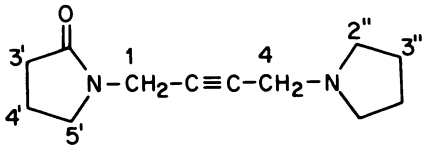
Since a methyl group at position 1 of the butynyl chain (Compound *4*) increases the affinity more than it does at any other position in the oxotremorine molecule, we also investigated the enantiomers of the corresponding ethyl- and propyl-substituted Compounds *9* and *10*. As seen in Table 3, the potency and the stereoselectivity decrease with increasing size of an alkyl group at position 1 of the butynyl chain.

The four optical isomers of Compound *11* were less potent than the corresponding isomers of Compound *8*. Although smaller, the enantiomeric and epimeric potency ratios follow a pattern similar to that observed for the isomers of Compound *8*.

TABLE 2

Affinity for muscarinic receptors in the isolated guinea pig ileum and tremorolytic potency in mice of some methyl-substituted oxotremorine analogues

The pA_2 values represent the mean ± standard error of the mean. The number of estimates is given in parentheses. The relative affinities of Compounds *1-7* for muscarinic receptors in the guinea pig ileum are expressed as ratios between their dissociation constants in the denominator and the dissociation constant of oxotremorine in the numerator. Data for the tremorolytic effects were obtained from refs. 4–7.



Compound	Position of CH_3	*In vitro* parasympatholytic potency (pA_2)	Relative affinity	Tremorolytic potency in mice[a]
				μmoles/kg
Oxotremorine		5.99 ± 0.17 (6)[b]	1.0	
Compound *1*	3′	4.94 ± 0.11 (6)[b]	0.10	
Compound *2*	4′	5.00 ± 0.04 (4)	0.11	37
Compound *3*	5′	7.03 ± 0.05 (4)	12	0.4
Compound *4*	1	7.28 ± 0.02 (4)	21	0.5
Compound *5*	4	4.43 ± 0.03 (3)	0.03	11
Compound *6*	2″	5.61 ± 0.05 (3)	0.45	5.0
Compound *7*	3″	5.68 ± 0.04 (4)	0.52	5.2

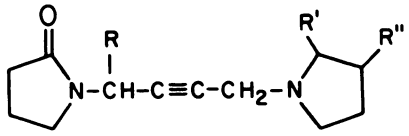
[a] Dose required to double the dose of oxotremorine inducing a predetermined tremor intensity in 50% of the mice.

[b] $-\text{Log } K_A$ as given in Table 1.

TABLE 3

Affinity for muscarinic receptors in the isolated guinea pig ileum and tremorolytic potency in mice of some optically active alkyl-substituted oxotremorine analogues

The pA_2 values represent the mean ± standard error of the mean. The number of estimates is given in parentheses. Enantiomeric potency ratios refer to *in vitro* parasympatholytic potency. Relative affinities for muscarinic receptors in the isolated guinea pig ileum were obtained as in Table 2. Data for tremorolytic effects were obtained from refs. 4, 5, 11, and 12.



Compound[a]	*R*	*R'*	*R"*	*In vitro* parasympatholytic potency (pA_2)	Enantiomeric potency ratio	Relative affinity	Tremorolytic potency in mice[b]
							μmoles/kg
R-4	CH_3	H	H	7.55 ± 0.02 (5)	257	39	0.26
S-4				5.14 ± 0.01 (6)		0.15	20
S-6	H	CH_3	H	5.89 ± 0.08 (4)	5.2	0.85	2.6
R-6				5.17 ± 0.09 (4)		0.16	56
S-7	H	H	CH_3	5.68 ± 0.05 (4)	1.0	0.52	5.8
R-7				5.69 ± 0.06 (4)		0.54	6.5
RS-8	CH_3	CH_3	H	7.80 ± 0.06 (4)	646	69	0.1
SR-8				4.99 ± 0.04 (3)		0.11	20
RR-8	CH_3	CH_3	H	7.35 ± 0.02 (4)	78	25	0.5
SS-8				5.46 ± 0.05 (3)		0.32	12
R-9	C_2H_5	H	H	7.09 ± 0.03 (3)	47	13	0.52
S-9				5.42 ± 0.05 (3)		0.29	27
R-10	C_3H_7	H	H	6.79 ± 0.05 (3)	14	6.8	3.5
S-10				5.63 ± 0.05 (3)		0.47	51
RS-11	C_3H_7	CH_3	H	6.96 ± 0.03 (3)	17	10	1.5
SR-11				5.73 ± 0.04 (3)		0.59	24
RR-11	C_3H_7	CH_3	H	6.68 ± 0.05 (4)	5.8	5.2	4.2
SS-11				5.92 ± 0.06 (4)		0.91	8.9

[a] For Compounds *8* and *11*, the first configurational symbol refers to the configuration of the chiral center in the butynyl chain and the second symbol to the configuration of the chiral center in the pyrrolidine ring.

[b] See Footnote *a*, Table 2.

The relative affinity of each of the compounds studied for muscarinic receptors in the guinea pig ileum was expressed as a ratio between its dissociation constant in the denominator and the dissociation constant of oxotremorine in the numerator. From the relative affinities given in Table 2, it is obvious that the introduction of a

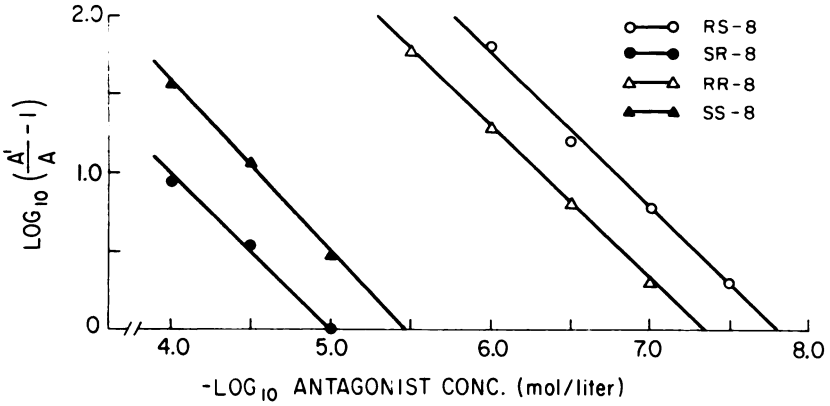


FIG. 4. *Schild plots of the antagonism between carbachol and the four optical isomers of Compound 8 in the isolated guinea pig ileum* The slopes of the regression lines are close to unity, indicating competitive antagonism. The intercept on the abscissa equals the pA_2 value.

methyl group at position 5 of the lactam ring (Compound *3*) and at position 1 of the butynyl chain (Compound *4*) of oxotremorine enhances the affinity. With a methyl group present at positions 3 and 4 of the lactam ring (Compounds *1* and *2*) or at position 4 of the butynyl chain (Compound *5*), the affinity is decreased as compared with oxotremorine. The affinities of the two compounds (*6* and *7*) having a methyl group in the pyrrolidine ring are not significantly ($p > 0.1$) different from that of oxotremorine. The relative affinities of the optical isomers are summarized in Table 3.

The tremorolytic doses listed in Tables 2 and 3 (4–7, 11, 12) were obtained by estimating the ED_{50} value of oxotremorine as a function of antagonist dose and calculating the dose of antagonist which doubles the ED_{50} of oxotremorine (6, 14). Since the antagonism of oxotremorine-induced tremor by these and previously studied analogues (14, 15) generally is of a competitive nature, the negative logarithms of the tremorolytic doses (molar concentrations) are equal to *in vivo* pA_2 values.

There is a highly significant ($r = 0.894$; $p < 0.001$) correlation between the affinities of the 24 antagonists studied for muscarinic receptors in the guinea pig ileum and their abilities to antagonize oxotremorine-induced tremors in mice (Fig. 5). The calculations were made by using *in vitro* pA_2 values and negative logarithms of tremorolytic doses (*in vivo* pA_2 values).

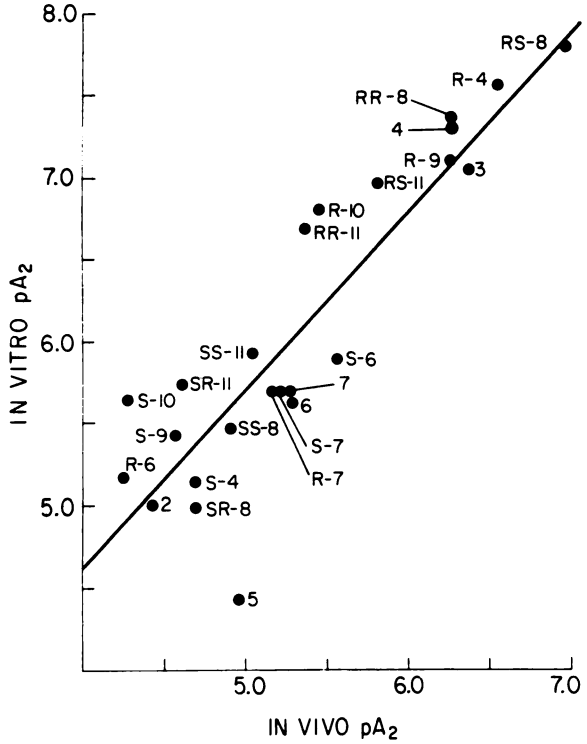


FIG. 5. *Relationship between in vitro and in vivo pA_2 for oxotremorine analogues*

In vitro pA_2 values were obtained from antagonism of carbachol-induced contractions of the isolated guinea pig ileum (Tables 2 and 3) and *in vivo* pA_2 values from antagonism of oxotremorine-induced tremor in mice (4–7, 11, 12). For explanation of symbols see Tables 2 and 3. The regression line is described by: *in vitro* $pA_2 = 1.08 \times$ *in vivo* $pA_2 + 0.31$ ($n = 24$; $r = 0.894$; $t = 9.36$).

Figure 6 shows a correlation of the enantiomeric potency ratio and the affinity of the more potent member of each enantiomeric pair for Compounds *4* and *6–11*. The degree of stereoselectivity is highly correlated with affinity for muscarinic receptors in the isolated guinea pig ileum ($r = 0.947$; $p < 0.001$) and with tremorolytic potency in mice, expressed as *in vivo* pA_2 values ($r = 0.896$; $p < 0.001$).

DISCUSSION

The estimated K_A of 1.09×10^{-6} M for oxotremorine is about 28 times greater than the concentration required to give a half-maximal contractile response (ED_{50}) prior to dibenamine treatment. This observation indicates that in the guinea pig ileum there is a fairly large receptor reserve for oxotremorine. With this K_A value, it may be calculated (8) that, prior to receptor inactivation, a maximal response was obtained when about 10–15% of the total active receptors were occupied by oxotremorine.

Our K_A value of oxotremorine is not significantly ($p > 0.1$) different from that (5×10^{-7} M) determined pharmacologically by Takeyasu *et al.* (16) on the isolated guinea pig ileum. Our estimate of the K_A of oxotremorine also agrees rather well with the concentration of oxotremorine ($5–8 \times 10^{-7}$ M) required to inhibit by 50% the binding of ^{3}H-labeled 3-QNB[1] to a membrane fraction of guinea pig ileal smooth muscle (17). This concentration should approximate the dissociation constant of oxotremorine, since the concentration of [^{3}H]QNB used was well below its own apparent dissociation constant. Our K_A value of oxotremorine agrees with its low-affinity dissociation constant (K_L) determined from inhibition of [^{3}H]QNB binding to muscarinic receptors in the rat brain and in the longitudinal muscle of the rat ileum, but is 130–215 times greater than its similarly determined high-affinity dissociation constant (K_H) (18, 19).

The receptor reserve for Compound *1* is similar to that for oxotremorine. On the other hand, Compound *6* has only a negligible receptor reserve. Thus a concentration of dibenamine (1.5×10^{-5} M for 15 min) that produced a shift of the dose-response curves to oxotremorine (Fig. 1), Compound *1* (Fig. 2), and carbachol (Fig. 3), without any noticeable decrease in the maximal response, completely *abolished* the contractile response to Compound *6* and its enantiomers, which then behaved as competitive antagonists.

Compounds *1–7* represent a unique series, since all are derived from a potent agonist by the introduction of a single methyl group. Moreover, the type of activity observed in the ileum ranges from agonists (Compound *1*) to partial agonists (Compound *6*) and competitive antagonists (Compounds *2–5* and *7*), the latter with a potency range of almost 3 log units (Table 2). The appearance of rather potent antagonists (Compounds *3* and *4*) by the addition of a single methyl group to oxotremorine is an unusual phenomenon among muscarinic compounds. Thus in acetylcholine, the effect of methyl substitution in different positions is always to preserve at least some of the muscarinic activity. Potent antagonists emerge only when relatively bulky groups are introduced in

[1] The abbreviation used is: QNB, 3-quinuclidinyl benzilate.

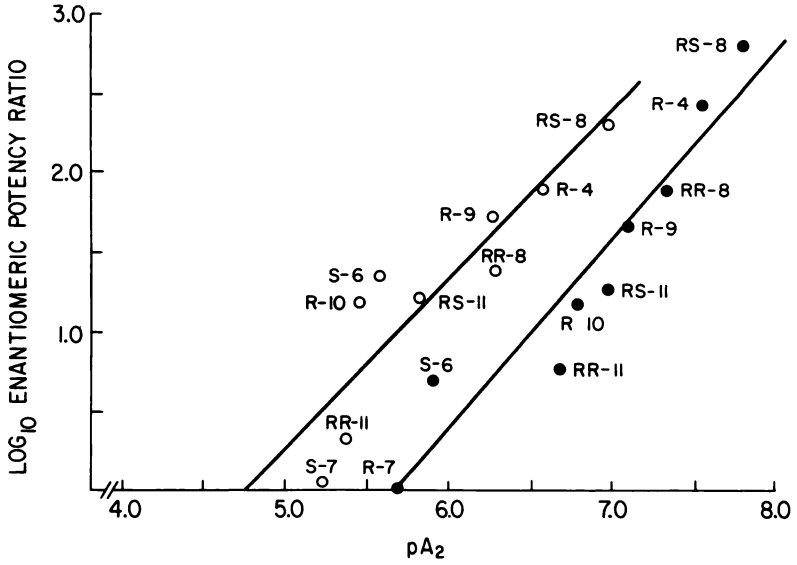


FIG. 6. *Relationship between stereoselectivity and affinity of some oxotremorine analogues*

The logarithm of the enantiomeric potency ratio (EPR) is plotted against the affinity for muscarinic receptors in the isolated guinea pig ileum (*in vitro* pA_2) (●) and against the tremorolytic potency in mice (*in vivo* pA_2) (○) of the more potent enantiomers. For explanation of symbols, which denote the more potent member of each enantiomeric pair, see Table 3. The regression lines are described by: log EPR = 1.18 × *in vitro* pA_2 − 6.71 ($n = 9$; $r = 0.947$; $t = 7.80$) and log EPR = 1.05 × *in vivo* pA_2 − 4.98 ($n = 9$; $r = 0.896$; $t = 5.33$).

acetylcholine (1). For example, diphenylacetylcholine has a pA_2 value against carbachol of 7.16 in the guinea pig ileum (20), a value comparable to those observed for Compounds *3* and *4* (Table 2).

When considering the effect of methyl substitution on the affinity of oxotremorine, at least three factors must be taken into account (21): (a) additional binding to the receptor by the substituent, (b) disturbance of the binding of the rest of the molecule caused by the substituent, and (c) substituent-induced alterations of the preferred solution conformation of oxotremorine. Factor a makes a positive, Factor b a negative, and Factor c a positive or negative contribution to the affinity.

The molecular structure of oxotremorine is known from X-ray structure determinations (22, 23). Inspection of molecular models of oxotremorine suggests that methyl substitution into positions 3 and 4 of the lactam ring (Compounds *1* and *2*) and into position 3 of the pyrrolidine ring (Compound *7*) should have only minor effects on its preferred conformation. At the remaining positions, a methyl group is more likely to affect the conformation of oxotremorine through steric interactions. For enantiomeric pairs, any deviation from the preferred conformation of oxotremorine must be of the same magnitude for both enantiomers.

The low affinities observed for Compounds *1* and *2* should then result mainly from a disturbance of existing binding caused by the substituent. Since Compound *7* has about the same affinity as oxotremorine, it appears that the methyl group at position 3 of the pyrrolidine ring neither provides additional binding nor disturbs existing binding. The absence of any difference in affinity between the enantiomers of Compound *7* further emphasizes the relative unimportance of this methyl group in the binding to the receptor. These results suggest that oxotremorine and compound *7* bind in a virtually identical manner to the receptor, which is in agreement with our previous suggestion that antagonists related to oxotremorine bind primarily at the agonist binding site, in contrast to classical muscarinic antagonists, which bind strongly at accessory receptor areas (3). For Compound *5*, the very low affinity probably results from a combination of Factors b and c. Although the affinity of Compound *6* is not significantly ($p > 0.1$) different from that of oxotremorine, the affinity difference between its enantiomers indicates that the methyl group at position 2 of the pyrrolidine ring somehow participates in the binding process.

The relatively high affinities of Compounds *3* and *4* suggest that in these compounds the methyl group provides an additional point for attachment to the receptor. The large difference in affinity between *R-4* and *S-4* is in agreement with such a specific interaction. Since the replacement of the methyl group in Compound *4* by progressively larger alkyl groups (Compounds *9* and *10*) is accompanied by an attenuation of affinity as well as stereoselectivity, there appears to be a rather specific binding locus on the receptor for a methyl group at position α to the lactam nitrogen in oxotremorine. The observed affinities of the four optical isomers of Compound *8* confirm that such a methyl group contributes much more to the affinity than a methyl group in the pyrrolidine ring.

Stephenson (24) showed that high affinity for acetylcholine receptors in the guinea pig ileum generally is incompatible with high efficacy. This phenomenon is readily explained by the rate theory of drug action (25) and may account for the absence of stimulatory actions of some of the compounds studied herein, especially those in which the alkyl substituent appears to provide additional binding to the receptor (Compounds *3* and *4* and *8–11*). However, Compounds *2* and *5* are also devoid of stimulatory action, in spite of their low affinities. A low affinity per se does not seem to be responsible for

loss of efficacy, since Compound *1*, which has only one-tenth of the affinity of oxotremorine, retains most of its efficacy (Table 1). The complex relationship between affinity, efficacy, and structure is further emphasized by the results obtained with Compounds *6* and *7*. In the case of the partial agonist Compound *6*, the *S*-enantiomer has the highest affinity, whereas the *R*-enantiomer has the highest efficacy (Table 1). The methyl group in Compound *7* apparently is located in a part of the molecule that is not involved in the binding to the receptor, but still its presence abolishes efficacy. These results suggest that the structural and steric requirements which optimize affinity are different from those that optimize efficacy.

The good correlation between *in vitro* parasympatholytic potency and oxotremorine blockade in mice (Fig. 5) shows that differences among the compounds studied in ability to penetrate to a central site of action are relatively unimportant. This is not unexpected in view of the structural similarity of the compounds and, as a consequence, similar lipid solubility. The base strength, the other major factor determining the rate of penetration of tertiary amines into the central nervous system (26, 27), of the compounds also should be similar. Only Compounds *5* and *6* would be expected to have pK_a values that differ significantly from that of oxotremorine (pK_a 7.91) (28). The pK_a values of 1-methyl- and 1,2-dimethylpyrrolidine differ by only 0.1 unit (29), suggesting only a small influence of a methyl group α to the pyrrolidine nitrogen atom (Compounds *5* and *6*) on the base strength of oxotremorine. The correlation also provides support for the view (15) that oxotremorine antagonism *in vivo* basically is due to antimuscarinic properties. *In vitro* pA_2 values are regarded as equal to the logarithm of the affinity constant. The excellent correlation between *in vitro* and *in vivo* pA_2 values (Fig. 5) and the fact that the antagonism in both cases is competitive suggest that the *in vivo* pA_2 values are a measure of the affinity of the antagonists for central muscarinic receptors.

Lehmann *et al.* (30) have shown that stereoselectivity does not occur randomly but is in general a linear function of the logarithm of potency or affinity. The observed correlation between degree of stereoselectivity and affinity (Fig. 6) shows that this is true indeed for the closely related oxotremorine analogues studied. Correlations such as that in Fig. 6 may be useful in predicting stereoselectivity of compounds with a similar mode of action to those included in the correlation. The intercept on the abscissa ($pA_2 = 5.68$) of the regression line for the *in vitro* affinities (Fig. 6) agrees well with the pA_2 value of oxotremorine. It therefore appears that the achiral lower homologue (oxotremorine) of the analogues studied also may be included in the correlation of affinity and stereoselectivity.

In the compounds substituted at position 1 of the butynyl chain (Compounds *4* and *8–11*), it may be assumed as a first approximation that, in the more potent isomer, the alkyl substituent interacts with one of the loci at the receptor (*vide supra*), whereas in the less potent isomer it does not. The affinities of the less potent isomers of Compounds *4* and *8–11* then should be similar and approximate the affinity of the achiral lower homologue oxotremorine. These expectations are generally fulfilled, since the affinities of five of seven "inactive" isomers are not significantly ($p > 0.05$) different from that of oxotremorine (Table 3). The affinities of the more potent isomers diminish about 2.5 times for each additional methylene group attached to position 1 of the butynyl chain (Compounds *R-4* versus *R-9* and *R-10*; *RS-8* versus *RS-11*; *RR-8* versus *RR-11*), presumably reflecting a progressively less efficient interaction with the receptor sublocus with increasing length of the alkyl group. By extrapolation, it may be estimated that with an *n*-hexyl substituent, the affinity becomes similar to those of the "inactive" isomers; i.e., no stereoselectivity will be observed. Thus, a 6-carbon chain would not interact at all with the receptor sublocus and the affinity, corresponding to the intercept on the abscissa in Fig. 6, should ideally equal that of the lower achiral homologue, i.e., oxotremorine.

The correlation in Fig. 6 suggests that, with chiral compounds having lower affinity than oxotremorine, the affinity of the *R*-isomer becomes lower than that of the *S*-isomer. Although we have no experimental results from the present study to substantiate this suggestion, it is interesting to note that in a series of muscarinic 1,3-dioxolanes, an inversion of stereoselectivity was observed when the number of carbon atoms attached to position 2 of the dioxolane nucleus was increased above 4 (31).

From the correlation in Fig. 6, an *in vivo* pA_2 value of 4.76 for oxotremorine may be estimated using the intercept on the abscissa of the regression line (*vide supra*). This pA_2 value corresponds to an *in vivo* dissociation constant of 1.74×10^{-5} moles/kg, which exceeds the *in vivo* ED_{50} value (6.3×10^{-7} moles/kg) of oxotremorine (6) by a factor of 28. These results indicate a similar receptor reserve for oxotremorine with respect to tremorogenic effect in mice and with respect to contractile response in the isolated guinea pig ileum (*vide supra*).

The conclusions reached regarding the relationship between stereoselectivity and affinity, of course, rely heavily on the optical purity of the compounds studied. All of the optically active compounds were synthesized from resolved starting materials by chemical reactions not expected to cause any racemization. Therefore, the compounds should be at least as optically pure as the starting materials used. These have been shown to be essentially optically pure by nuclear magnetic resonance spectroscopy and by chemical transformations to compounds of known high optical purity (5, 10, 32). A further indication, although not a proof, of the high optical purity of the compounds is the excellent agreement between the optical rotations of enantiomeric pairs (5, 10–12). Barlow *et al.* (33) have shown that enantiomeric potency ratios can be used to calculate a lower limit for the degree of resolution. Values as high as 257 for *R-4/S-4* or 646 for *RS-8/SR-8* (Table 3) can only be obtained if the samples are 99.6% and 99.8%, respectively, optically pure. Since it is unlikely that one isomer is completely inactive, as is assumed in these calculations, the optical purity of the two compounds should actually be even higher. Compounds *RR-8* and *SS-8* were prepared from the *same* starting materials as *RS-8* and *SR-8* and must also be

close to 100% optically pure. Thus the 8-fold difference in enantiomeric potency ratios observed for the two enantiomeric pairs of Compound *8* (Table 3) cannot be due to differences in optical purity. The same reasoning can be applied to show that the different potency ratios observed for the two enantiomeric pairs of Compound *11* are not due to different degrees of resolution.

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