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IN VITRO CHARACTERIZATION OF A NOVEL, POTENT AND SELECTIVE M_3 ANTAGONIST

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Summary

The pharmacological profile of the competitive muscarinic antagonist (2S, 3'R) 3-quinuclidinyl tropate, abbreviated (-)-2a, was evaluated on rabbit vas deferens (M_1/M_4-like; pA_2=9.10), guinea-pig left atrium (M_2; pA_2=9.30), guinea-pig ileum (M_3; pA_2=10.33) and guinea-pig uterus (M_4 putative; pA_2=9.70) muscarinic receptors and on the five subtypes of muscarinic receptors expressed individually in CHO-K1 cells. The drug shows an affinity for the M_3 receptor subtype at least 10-fold higher than 4-DAMP, p-HHSiD and zamifenacin, used as reference drugs. These results suggest (-)-2a as a novel, potent and selective M_3 antagonist that may have therapeutic potential in the treatment of conditions associated with increased smooth muscle contractility.

Key Words: **M_1 receptors, M_2 receptors, M_3 receptors, M_4 receptors, muscarinic antagonist**

Molecular biological techniques have identified five distinct, but related, genes encoding for muscarinic receptors. Four of these genes m_1-m_4 have been shown to encode for the four pharmacologically characterized M_1-M_4 receptors (1, 2). Pharmacological characterization of muscarinic receptors is hampered by a lack of ligands selective for any one receptor subtype over all others (3). Several antagonists have been identified with high apparent affinity for M_3 receptors and relatively low affinities for muscarinic M_2 receptors, including 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) (4), DAC 5945 (5), *p*-F-HHSiD (*para*-fluorohexahydroxyladiphenidol) (6), UH-AH 37 (7) and zamifenacin ((3*R*)-(+)-diphenylmethoxy-1-(3,4)-methylenedioxyphenethyl piperidine) (8).

In the present paper, the selectivity of a novel muscarinic receptor antagonist (-)-2a ((2S, 3'R) 3-quinuclidinyl tropate), for muscarinic M_3 receptors over muscarinic M_1, M_2 and putative M_4 receptors was evaluated by using in vitro functional studies. Experiments to determine the (-)-2a pharmacological profile were carried out on rabbit vas deferens M_1/M_4-like (9), guinea-pig atrial M_2 (10), guinea-pig ileal M_3 (11) and guinea-pig uterus putative M_4 receptors (12). Furthermore, the binding affinities of (-)-2a at the five muscarinic receptor subtypes expressed in Chinese hamster ovary cells (CHO-K1) (3) were evaluated.

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Methods

In vitro functional studies

Isolated rabbit vas deferens: Experiments on isolated rabbit vas deferens were performed according to the method described by Eltze (9) and modified by Dei et al. (13). The preparations were maintained at 32°C and tissues were stimulated through platinum electrodes by square-wave pulses (2 ms, 0.1 Hz, 10-30 V). Contractions were measured isometrically after tissues had been equilibrated for 1 h, then a cumulative dose-response curve for the inhibitory effect of McN-A-343 was plotted.

Isolated guinea-pig left atria: Isolated left atria were prepared according to the method described by Eltze et al. (10) and modified by Dei et al. (14). Bath fluid temperature was maintained at 30°C. Atria were electrically stimulated (1 Hz, 1 ms, 4-10 V) by means of two platinum electrodes. Carbachol negative inotropic effects on isometric atria contractions were recorded before and 1 h after perfusion with antagonists.

Isolated guinea-pig ileum: Isolated ileum fragments were prepared according to Eltze and Figala (11). Bath fluid temperature was maintained at 37 °C. Isotonic ileum contractions induced by acetylcholine (ACh) were recorded before and 1 h after perfusion with antagonists.

Isolated guinea-pig uterus: Experiments on isolated immature guinea-pig uterus were performed according to Dörje et al. (12). The preparations were maintained at 30 °C and after a 1 h equilibration period isotonic contractions to carbachol were recorded. Initially the tissues were exposed to a single concentration of carbachol (3 nmol l^{-1}) to check their responsiveness to the agonist; subsequently a dose response curve for carbachol was obtained.

Determination of antagonist affinities: After a stabilization time of 30-60 min, agonist concentration-response curves were plotted before and after equilibration with antagonists. In separate control experiments no significant changes in tissue sensitivity to the agonist were observed over the period required for the determination of two concentration-response curves. Three different concentrations of each antagonist were used (log conc. interval 0.5; n= 4-6 for each conc.). The antagonists were allowed to equilibrate for 60 min. No more than two concentrations of antagonist were tested in the same preparation. Agonist EC_{50} values in the absence and presence of antagonists were determined graphically for the calculation of dose ratios. The slopes of Arunlakshana-Schild plots (15) were determined by linear regression, according to the least-squares method. pA_2 values were estimated by fitting the best straight line to the data with a slope of unity as required by the theory (16).

Binding studies

Chinese hamster ovary cells (CHO-K1), obtained from the American Type Culture Collection, were used. Cell culture, membrane preparation and radioligand binding studies were performed according to the method of Dörje et al. (3). Cells were transfected according to the method of Chen and Okayama (17) using a modified calcium phosphate procedure involving the use of cotransfected pcDneo as a selectable marker. Selection with neomycin analog G 418 (600 μg/ml) was started 72 h after transfection and continued for two to three weeks. Media were changed every three days. Clonal cell lines were obtained by single-cell cloning. Cells were grown to about 80% confluence, washed, scraped into ice-cold binding buffer and homogenized for 30 s using a Brinkmann Homogenizer (setting 5). Membranes were pelleted at 16.000 x *g* for 15 min and rehomogenized. In [^{3}H]N-methylscopolamine ([^{3}H]NMS) saturation experiments, 8 to 10 different

concentrations of the radioligand (2-1400 pM) were employed. For displacement experiments, the concentration of [^{3}H]NMS was 150 pM and 10 different concentrations of the cold displacers were used. Incubation was carried out at 22°C for 3 h. Assays were terminated by filtration through a Brandel cell harvester onto Whatman GF/C filters. Membranes were washed three times with 5 ml of ice-cold binding buffer before being dried, transferred to 10 ml of scintillant (New England Nuclear Aquasol) and counted in a LKB-ß counter.

The following drugs were used: (-)-2a, (2S, 3'R) 3-quinuclidinyl tropate; prepared in the Department of Pharmaceutical Sciences of Florence, Italy as previously described by Dei et al. (13); the compound has an optical purity greater than 99%; acetylcholine chloride (Merck); McN-A-343 4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride; 4-DAMP 4-diphenylacetoxy-*N*-methylpiperidine methiodide (R.B.I.); carbamylcholine chloride and yohimbine hydrochloride (Sigma); [^{3}H]-scopolamine methyl chloride (71 Ci/mmol) (New England Nuclear, Boston), fetal calf serum and G418 (Gibco, Grand Island, NY), penicillin G, streptomycin and glutamine (M. A. Bioproducts, Walkersville, MD). Other chemicals were of the highest quality commercially available. All the drugs were dissolved in the nutritive solutions immediately before use. The concentrations given in the text are expressed as salts.

Results

In vitro functional studies

(-)-2a concentration-dependently blocked the McN-A-343-induced inhibition of twitch contractions of rabbit vas deferens (Fig. 2) and antagonized the negative inotropic carbachol-induced effect in guinea-pig left atrium (Fig. 3) as well as contractile responses to acetylcholine in guinea-pig ileum (Fig. 4) and the carbachol-induced contractions in immature guinea-pig uterus (Fig. 5). Increasing concentrations of (-)-2a produced parallel shifts of the agonist concentration-response curves progressively to the right. No appreciable change in basal tension or maximum agonist response was observed. pA_2 values of (-)-2a, 4-DAMP, p-F-HHSiD and zamifenacin, used as reference drugs, are shown in Table 1. The slopes of Arunlakshana-Schild regression lines for (-)-2a were not significantly different from unity (Fig. 6). The subtype selectivity ratios for (-)-2a, 4-DAMP, p-F-HHSiD and zamifenacin, obtained as differences between pA_2 values, are reported in Table 1.

Binding studies

Table 2 shows the pKi values and Hill numbers determined by competition for the binding between (-)-2a and [^{3}H]-NMS on the five human muscarinic receptor subtypes expressed in Chinese hamster ovary cell lines (CHO-K1). Binding isotherms of (-)-2a were characterized by Hill numbers not significantly different from unity and are consistent with interaction at a homogeneous population of receptors. In these binding studies, as in the functional ones described above, selectivity of (-)-2a for the m_3 receptor subtype was observed. The rank order of affinities was $m_3>m_5>m_4>m_1>m_2$. These data showed up to 23-fold selectivity of (-)-2a for m_3 receptors over m_2 and 17-fold over m_1.

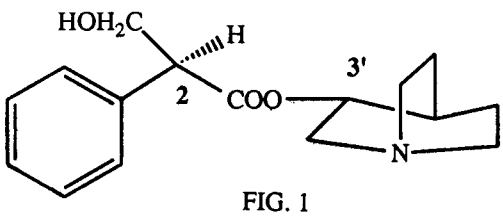

FIG. 1
Chemical structure of (2S,3'R) 3-quinuclidinyl tropate, abbreviated (-)-2a.

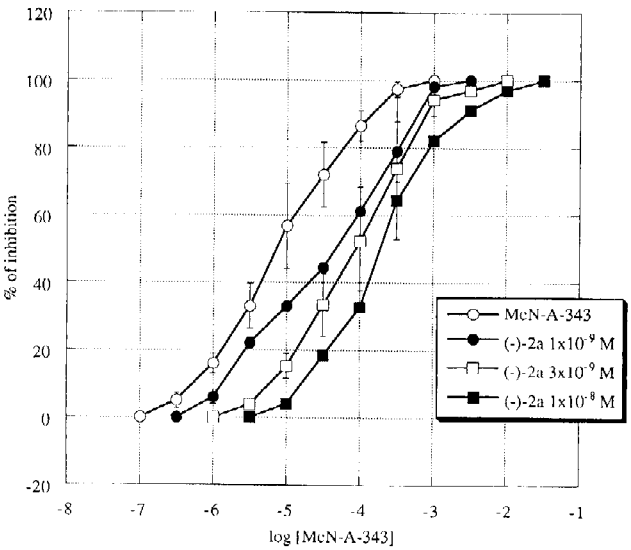

FIG. 2

Antagonism by (-)-2a of muscarinic receptors mediating inhibition of rabbit vas deferens. Concentration-response curves for McN-A-343 in the absence (○) or in the presence of increasing concentrations (● $1x10^{-9}$; □ $3x10^{-9}$; ■ $1x10^{-8}$M) of (-)-2a.
Each point represents the mean ± s.e.m. of at least 4 experiments.

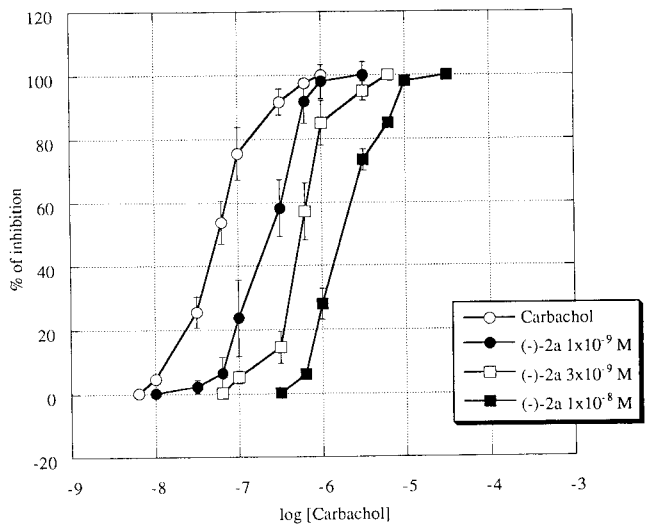

FIG. 3

Antagonistic effect of (-)-2a on muscarinic receptors mediating inhibition of guinea-pig left atrium. Concentration-response curves for carbachol in the absence (○) or in the presence of increasing concentrations (● $1x10^{-9}$; □ $3x10^{-9}$; ■ $1x10^{-8}$ M) of (-)-2a.
Each point represents the mean ± s.e.m. of at least 4 experiments.

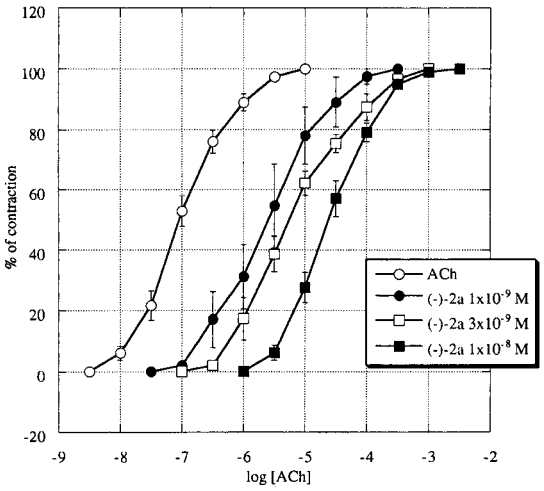

FIG. 4

Antagonistic effect of (-)-2a on muscarinic receptors mediating contraction of guinea-pig ileum. Concentration-response curves for acetylcholine in the absence (○) or in the presence of increasing concentrations (● $1x10^{-9}$; □ $3x10^{-9}$; ■ $1x10^{-8}$ M) of (-)-2a. Each point represents the mean ± s.e.m. of at least 5 experiments.

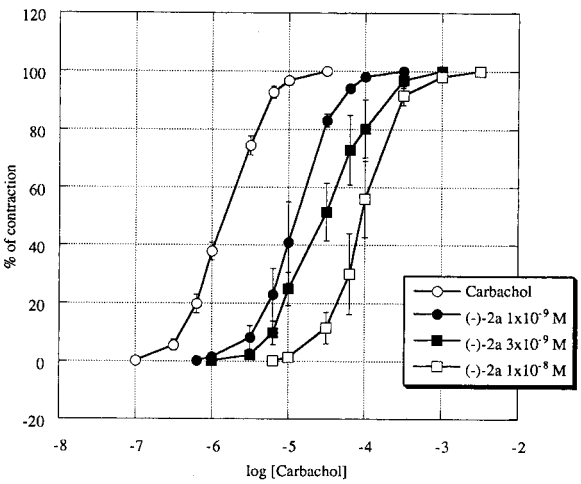

FIG. 5

Antagonistic effect of (-)-2a on muscarinic receptors mediating inhibition of guinea-pig uterus. Concentration-response curves for carbachol in the absence (○) or in the presence of increasing concentrations (● $1x10^{-9}$; □ $3x10^{-9}$; ■ $1x10^{-8}$ M) of (-)-2a.
Each point represents the mean ± s.e.m. of at least 4 experiments.

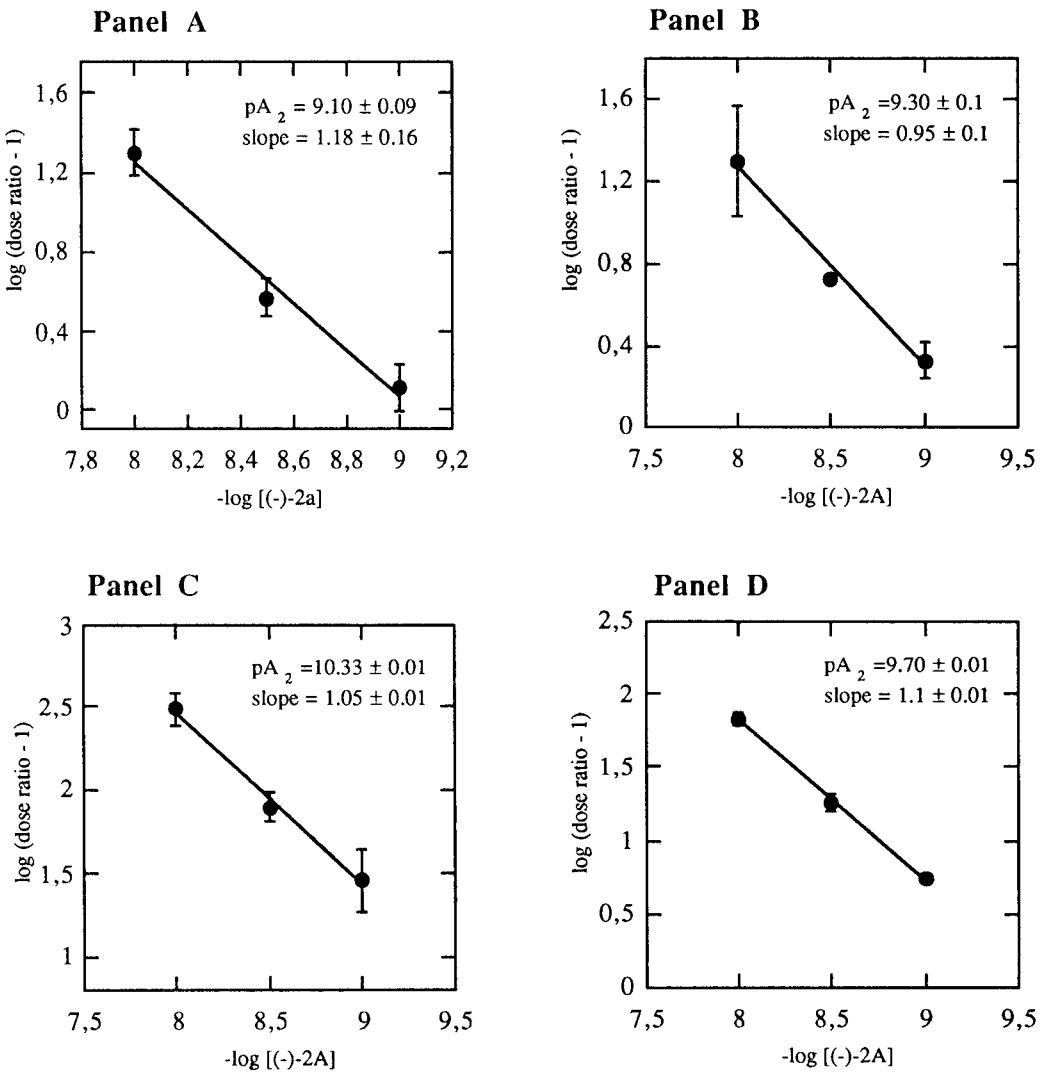

FIG. 6

Schild analysis of antagonism by (-)-2a of (panel A) McN-A-343 induced inhibitory effect on rabbit vas deferens, (panel B) carbachol-induced negative inotropic effect on guinea-pig left atrium, (panel C) ACh-induced contractions on guinea-pig ileum and (panel D) carbachol-induced contractions on immature guinea-pig uterus.

TABLE 1

Affinity profiles of (-)-2a, 4-DAMP, p-F-HHSiD and zamifenacin in rabbit vas deferens (M_1-/M_4 like), guinea-pig left atrium (M_2), guinea-pig ileum (M_3) and immature guinea-pig uterus (putative M_4). The ratios of affinity constants are given as a measure of receptor selectivity.

Muscarinic Antagonists	pA_2 values				Selectivity ratios		
	rabbit vas deferens (M_1/M_4-like)	guinea-pig left atrium (M_2)	guinea-pig ileum (M_3)	guinea-pig uterus (putative M_4)	M_3/M_1	M_3/M_2	M_3/M_4
(-)-2a	9.10 ± 0.09	9.30 ± 0.1	10.33 ± 0.02	9.70 ± 0.01	17	11	4
	(1.18 ± 0.16)	(0.95 ± 0.1)	(1.05 ± 0.01)	(1.10 ± 0.01)			
	[9.20 ± 0.07]	[9.30 ± 0.03]	[10.39 ± 0.01]	[9.80 ± 0.03]			
4-DAMP	9.39 ± 0.06[a]	8.40 ± 0.04[a]	9.34 ± 0.05[a]	8.87 ± 0.01	0.9	9	3
p-F-HHSiD	6.95 ± 0.03[b]	6.01 ± 0.06[b]	7.84 ± 0.03[b]		8	68	
zamifenacin	7.40 ± 0.04[c]	6.60 ± 0.04[d]	9.31 ± 0.06[d]		81	631	

pA_2 values are the mean ± SEM of 12-16 preparations. In parentheses is the slope of the Arunlakshana-Schild linear regressions. In square parentheses are the pA_2 values obtained by constraining the slope of the linear regressions to the unity. Selectivity ratios were calculated as antilogs of the difference between pA_2 values.

[a]: Dorje et al. Naunyn-Schmiedeberg's Arch. Pharmacol. 342: 248-289; 1990. [b]: Lambrecht et al. Eur. J. Pharmacol. 152: 193-194; 1988. [c]: Wallis et al. Br. J. Pharmacol. 109: 36P, 1993. [d]: Watson et al. Eur.J.Pharmacol. 285: 135-42; 1995.

TABLE 2

Binding affinities and Hill coefficients of the compound (-)-2a for the five muscarinic receptor subtypes expressed in Chinese hamster ovary cells (CHO-K1).

pKi ± S.E.M.				
m_1	m_2	m_3	m_4	m_5
8.88±0.06	8.75±0.12	10.11±0.06	9.05±0.10	9.32±0.09
[0.95±0.09]	[0.91±0.14]	[0.89±0.05]	[0.87±0.07]	[1.03±0.12]

The values represent mean±S.E.M. from four individual experiments. In brackets are the Hill coefficients.

Discussion

The affinity profile of (-)-2a versus rabbit vas deferens (M_1/M_4-like), guinea-pig atrium (M_2), guinea-pig ileum (M_3) and prepuberal guinea-pig uterus (putative M_4) was evaluated by in vitro functional studies in comparison with the reference drugs 4-DAMP, p-F-HHSiD and zamifenacin. The muscarinic receptor subtype present in the rabbit vas deferens has been defined M_1/M_4-like since the presence of only m1 and a smaller amount of m4 receptors have been demonstrated in this preparation (2). The prepuberal guinea-pig uterus muscarinic receptor subtype has been defined as M_4 putative since it has not been confirmed that the mRNA coding for M_4 is expressed in the prepuberal uterine tissue. However, pharmacological and biochemical studies show that the M_4 putative receptor of prepuberal guinea-pig uterus has a pharmacological and biochemical profile identical to that of the muscarinic m_4 receptor subtype expressed in the rat striatum (18, 19) and in NG 108-15 cells (20, 21). The affinity profile of (-)-2a versus the five muscarinic receptor subtypes m_1, m_2, m_3, m_4 and m_5 expressed in Chinese hamster oocytes (CHO-K1), was also evaluated by using binding studies. As shown in functional studies, (-)-2a interacts with the muscarinic receptor as a competitive antagonist. In fact, agonist concentration-response curves were displaced to the right in a parallel manner without depression of maximum responses. Moreover, the slopes of the Schild regression lines were not significantly different from unity. These studies revealed that (-)-2a has an affinity for M_3 receptors in guinea-pig ileum (pA_2 = 10.33) that is respectively about 10-, 300- and 10-fold higher than those obtained for 4-DAMP (pA_2 = 9.34), p-F-HHSiD (pA_2 = 7.84) and zamifenacin (pA_2 = 9.31). On the other hand, (-)-2a has a higher affinity for M_1 (pA_2 =9.10), M_2 (pA_2 = 9.30) and putative M_4 (pA_2 = 9.70) receptor subtypes than the reference drugs used. Regarding its ability to discriminate among the different muscarinic receptor subtypes, (-)-2a is able to discriminate between M_3/M_1, M_3/M_2 and M_3/M_4 receptors better than 4-DAMP. In fact, (-)-2a has a selectivity ratio of 17 for M_3/M_1 and 11 for M_3/M_2 whereas 4-DAMP has selectivity ratios of 0.9 and 9, respectively. In comparison with p-F-HHSiD, (-)-2a descriminates better between M_3/M_1, but not between M_3/M_2. By contrast, (-)-2a shows about a 5 and 48 times lower ability to discriminate between M_3/M_1 and M_3/M_2 receptors than zamifenacin. However, the degree of subtype selectivity of zamifenacin is variable since its affinity for M_3 receptors appears to be tissue-dependent. In fact, the pA_2 values of zamifenacin evaluated in guinea-pig trachea, oesophageal muscularis mucosae and urinary bladder preparations that, like guinea-pig ileum, express M_3 receptors, are respectively 8.16, 8.84 and 7.57. These values are at least 10 times lower than the pA_2 value calculated in guinea-pig ileum (22).

The absolute pKi value obtained at m_3 receptors in the binding studies (10.11±0.06) was in agreement with the affinity value extimated functionally in the guinea-pig ileum (10.33±0.02). Similarly to the functional data, binding studies revealed an affinity of (-)-2a at the five muscarinic receptor subtypes about 20-fold lower for m_3/m_1 and m_3/m_2 and 10-fold lower for m_3/m_4.

In conclusion, we have shown by functional and binding studies that (-)-2a is a potent and selective M_3 antagonist. This compound displays a higher affinity for M_3 muscarinic receptors than 4-DAMP, p-F-HHSiD and zamifenacin. The unique profile of (-)-2a may confer a therapeutic advantage to the treatment of smooth muscle disorders associated with increased cholinergic drive.

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