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Functional identification of rat atypical β-adrenoceptors by the first β_3-selective antagonists, aryloxypropanolaminotetralins

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1 We have assessed the relative abilities of compounds belonging to the new aryloxypropanolaminotetralin (APAT) class and of the reference β-adrenoceptor-blocking agent, alprenolol, to antagonize functional responses *in vitro* and *in vivo* involving atypical (β_3) or conventional (β_1 and β_2) β-adrenoceptors.

2 The range of pA_2 values for three representative APATs against inhibition of spontaneous motility in the rat isolated colon by the selective β_3-adrenoceptor agonist, SR 58611A (8.1–8.8), was well above similarly calculated values for non-competitive antagonism of guinea-pig trachea relaxation by salbutamol (β_2, 6.5–6.9) and the atrial chronotropic response by isoprenaline (β_1, 6.7–7.3). Alprenolol, however, was substantially more potent in antagonizing atrial (pA_2, 8.2) and tracheal (pA_2, 8.9) responses than SR 58611A mediated inhibition of colonic motility (pA_2, 6.8).

3 Several APAT isomers with different configurations at the chiral carbons, when tested on isolated organs, presented stringent stereochemical requirements for β_3-selectivity, including high antagonist potency-ratios between active and inactive enantiomers.

4 *In vivo*, the inhibition of colonic motility and the thermogenic response of brown adipose tissue elicited in rats by the selective β_3-adrenoceptor agonists SR 58611A and BRL 37344 respectively were substantially diminished by the representative APAT, SR 59230A, at oral doses ($\leqslant 5$ mg kg^{-1}) well below those half maximally effective (ID_{50}) for preventing β_1-(isoprenaline tachycardia $\geqslant 80$ mg kg^{-1}) or β_2-(salbutamol bronchodilatation, 44 mg kg^{-1}) mediated responses. Alprenolol, as expected, was a less potent and nonselective antagonist of the putative β_3-responses.

5 These findings support APATs as the first potent, orally effective selective antagonists at β_3-adrenoceptors, and provide final unambiguous evidence that β_3-adrenoceptors underlie inhibition of colonic motility and brown adipose tissue thermogenesis in rats

Keywords: Aryloxypropanolaminotetralins; β_3-adrenoceptors; selective β_3-adrenoceptor antagonists; rat BAT thermogenesis; atypical β-adrenoceptors; rat isolated colon

Introduction

The first distinction of adrenoceptors into the α and β types on the basis of different sensitivity to natural and artificial catecholamines (Ahlquist, 1948) was substantiated by the discovery of selective β-antagonists (Powell & Slater, 1958), soon developed as life-saving major cardiovascular drugs. Later subdivision into β_1- and β_2-subtypes (Lands *et al.*, 1967) likewise relied on appropriate agonists and antagonists, β_2-selective agonists being recognized as the most useful agents for the management of obstructive lung disease. Interest is currently growing in the further heterogeneity of β-adrenoceptors, bearing in mind the additional potential for therapeutic advancement.

Comprehensive pharmacological data suggest the existence of an atypical (non β_1, non β_2) form in several tissues, as discussed in several reviews (Zaagsma & Nahorski, 1990; Arch & Kaumann, 1993; Manara *et al.*, 1995b). Genes reportedly coding for human and rodent 'β_3-adrenoceptors' have now been isolated (Emorine *et al.*, 1989; Granneman *et al.*, 1991; Muzzin *et al.*, 1991; Bensaid *et al.*, 1993; Lelias *et al.*, 1993). Newly synthesized agonists, reviewed by Howe (1993), selectively activate atypical β-adrenoceptors, but their precise identity remains elusive in the absence of potent and selective antagonists providing unambiguous evidence of their distinctive functional features.

The aryloxypropanolaminotetralins (APATs) are the first representatives of such antagonists that we have tested *in vitro* and *in vivo* for their potency and β_3-selectivity in preventing β-adrenoceptor agonist-mediated responses of rat colon and adipose tissue with a view to identifying conclusively the underlying receptors on functional grounds. This report sets out in detail the evidence supporting the unprecedented subtype-selective properties of APATs for β_3-adrenoceptors, assessed in the above experimental setting.

Methods

Assays in isolated tissues

Animals from which tissues were used to set up the following *in vitro* preparations were humanely killed: rats, by cervical dislocation, and guinea-pigs, by stunning and exanguination.

The first 3 cm segment of the rat (male Crl:CD BR) proximal colon, starting from the ileo-caecal junction, was mounted longitudinally in a 20 ml organ bath containing Krebs-Ringer solution maintained at 37°C and aerated with 5% CO_2 in O_2. Spontaneous motility was recorded isotonically under a constant load of 1 g in the presence of 10 μM phentolamine, 0.5 μM desmethylimipramine and 30 μM hydrocortisone. Quantitative computer analysis was based on a motility index (expressed as the percentage difference) established by comparing the area under the spontaneous contrac-

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tion waves (computed as a time integral of the region between the waves and the baseline tonus, sampled every 15 s) over 10 min periods, before and after addition of cumulative concentrations of the agonist.

Spontaneously beating right atria from guinea-pigs (male DH IFFA-CREDO) were set up in Krebs solution maintained at 32°C, aerated with 5% CO_2 in O_2; after about 30 min, they were exposed to a 'priming' concentration (1 μM) of isoprenaline (for 2.5 min followed by washing with fresh Krebs solution) and 60 min later they were incubated with 10 μM phenoxybenzamine for 30 min, then washed again. Sixty min later, isoprenaline cumulative concentration-response curves were established (2.5 min contact time for each concentration), first without antagonist added, then in the presence of increasing concentrations of antagonist (30 min preincubation). Chronotropic responses, recorded isometrically under a constant load of 0.25 g, were expressed as a percentage of maximal agonist effect.

Tracheal chains from guinea-pigs (male DH IFFA-CREDO) mounted in pairs (two pairs from the same animal) were set up in Krebs solution; after equilibrating for about 30 min, the preparation was completely relaxed with a maximally effective concentration of isoprenaline (0.1 μM for 30 min: 100% relaxation), washed by fresh Krebs, then 60 min later, incubated 30 min with 10 μM phenoxybenzamine. After washing again (30 min), carbachol at a submaximally effective concentration (1 μM) was added to the incubation medium and 90 min later salbutamol cumulative concentration-response curves were established either in the presence or absence of increasing concentrations of antagonist (30 min preincubation). Salbutamol-induced relaxation (contact time for each concentration about 1 min or longer until the effect reached a steady state) was recorded isotonically under a constant load of 0.5 g and expressed as a percentage of the maximal agonist (isoprenaline) response. Only one concentration-response curve was obtained in each preparation.

In vivo *assays*

All the following experiments were performed in Crl:CD BR (Charles River Italia) male rats, fasted overnight (except for thermogenesis tests), weighing 160 ± 30 g (thermogenesis tests) or 230 ± 20 g (other tests) when tested. They were housed four per cage at an environmental temperature of 22 ± 1°C and humidity of 55 ± 10%, fed standard laboratory chow (4RF25 certificate, Mucedola, Milan, Italy) and received tap water *ad libitum* until 08 h 00 min on the day of the test. Rats were handled according to internationally accepted guidelines for the care of laboratory animals (E.E.C. Council directive 86/609 OJL 358, 1 Dec. 12, 1987). Alprenolol and SR 59230A were compared for their ability to antagonize responses elicited by β-adrenoceptor agonists in the following test systems.

Colonic motility was myoelectrically assessed and recorded by chronically implanted wire electrodes from non-anaesthetized animals in the form of long spike burst (LSB) action potentials (Croci *et al.*, 1991) with a basal frequency (i.e. the frequency recorded in the 120 min before the scheduled time of administration of the agonist, SR 58611A) of (LSB $min^{-1} \pm$ s.e.): 1.71 ± 0.07, 1.80 ± 0.04 and 1.78 ± 0.03 respectively in drug-free and in animals receiving only alprenolol or SR 59230A. The colonic potentials spike frequency recorded for 120 min from the scheduled time of agonist administration in drug-free rats and rats given only either antagonist, did not differ significantly (LSB $min^{-1} \pm$ s.e.: drug-free 1.77 ± 0.10; alprenolol 1.66 ± 0.07; SR 59230A, 1.83 ± 0.06), whereas this frequency was reduced to 0.53 ± 0.08 in the 60 min after administration of SR 58611A, in the absence of antagonists.

Heart rate was recorded indirectly in non-anaesthetized animals with an air sphygmomanometer, with a piezoelectric ring transducer to measure pulse, connected to the rat's tail (Croci *et al.*, 1991), and the chronotropic effect of isoprenaline, with or without antagonist, was scored for 15 min from injection of the agonist. Basal heart rate (beats $min^{-1} \pm$ s.e.) was 380 ± 7 and increased by 50.2 ± 3.9% 15 min after isoprenaline, in the absence of antagonists; neither antagonist by itself, at the highest tested dose, produced any significant change (% change alprenolol -0.7 ± 4.2; SR 59230A $+3.4 \pm 3.4$).

Changes in specific airway resistance (SAR) were assessed according to Pennock *et al.* (1979) with a two-chamber plethysmograph connected to a respiratory analyzer (Buxco Electronics Inc., NY, U.S.A.), in non-anaesthetized rats selected seven days before on the basis of methacholine responses. Three minutes after salbutamol rats received nebulized methacholine (6% in distilled water for 10 s) and the former's bronchodilator action was monitored for 10 min in control rats and in rats pretreated with antagonists. Basal SAR was 4.19 ± 0.31 ($cmH_2O\ s^{-1} \pm$ s.e.); after methacholine, SAR became 3 to 4 times higher and salbutamol, given 10 min before methacholine, largely prevented its effect (SAR 140 to 150% of basal). Neither antagonist by itself, at the highest tested dose, influenced basal SAR, or the metacholine response (alprenolol, 100, SR 59230A, 125% of SAR of methacholine without antagonist).

Brown adipose tissue (BAT) thermogenesis was evaluated in urethane-anaesthetized (1.25 g kg^{-1}, i.p.) rats. Antagonists were given orally before anaesthesia. A 'miniature' thermistor (TM-36/S1, L.S.I. Settala, Milan, Italy) was placed under the interscapular brown fat pad to monitor its temperature (T-BAT) and a similar (TM-35/S1) probe was inserted into the rectum (T-core monitor). After anaesthesia animals were placed in a thermoregulated box (8500, Basile, Varese, Italy) at 33 ± 0.3°C. The agonist (BRL 37344) was injected i.v., 1 h after administration of the antagonists. Rats that did not receive either the antagonist or the agonist, or both, were treated with the vehicle (saline) as appropriate. T-BAT and T-core were recorded for about 100 min (40 min basal plus 60 min after agonist or vehicle treatment) from four animals simultaneously by a multi-channel digital recorder (BABUC/A.C.S.I., Settala, Milan, Italy).

Calculation and statistical analysis

In the assays on isolated tissues, the agonist concentrations producing 50% maximal response and the antagonist concentrations causing 50% inhibition of agonist response were calculated by use of a four-parameter logistic model according to Ratkovsky & Reedy (1986); adjustment was made by non-linear regression using the Levenberg-Marquardt algorithm in RS/1 software (BBM, Cambridge Ma., U.S.A.).

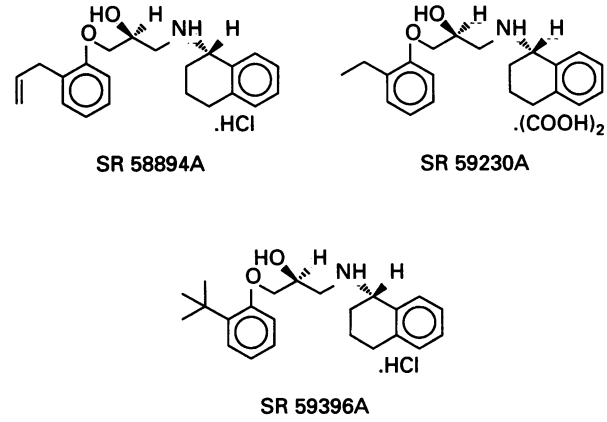

Figure 1 Structures and code numbers of representative aryloxypropanolaminotetralins.

The pA_2 values for antagonists, as defined by Arunlakshana & Schild (1959), were obtained from linear regression of mean values of the log (DR − 1) vs negative log of the antagonist concentration. Computer analysis was done as described by Tallarida & Murray (1987) using three or more concentrations of antagonist. Schild slopes were analysed by the *t* test and were considered significantly different from unity at confidence intervals of 95% or greater (MacKay, 1978).

In vivo, the antagonist doses causing 50% inhibition of agonist responses (ID_{50}) were calculated from log-dose response curves obtained from linear regression by the least squares method. Statistical analysis was based on ANOVA plus Duncan's test (Kramer, 1956).

Chemicals

The aryloxypropanolaminotetralins SR 58894A, 3-(2-allylphenoxy)-1-[(**1S**)-1,2,3,4-tetrahydronaphth-1-ylamino]-(**2S**)-2-propanol hydrochloride, SR 59230A, 3-(2-ethylphenoxy)-1-[(**1S**)-1,2,3,4-tetrahydronaphth-1-ylamino]-**2S**-2-propanol oxalate, SR 59396A, 3-(2-*tert*-butylphenoxy)-1-[(**1S**)-1,2,3,4-tetrahydronaphth-1-ylamino]-(**2S**)-2-propanol hydrochloride (see also Figure 1), the stereoisomers of SR 58894A (SR 58893A, SR 58895A, SR 58892A) and SR 59230A (SR 59483A, SR 59231A), the β_3-selective agonist BRL 37344 (Arch *et al.*, 1984), (**RR**+**SS**)[4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino]propyl]phenoxy]acetic acid, the phenylethanolaminote-

Table 1 *In vitro* quantitative antagonism of β-adrenoceptor responses mediated by atypical (β_3) or previously recognized β-adrenoceptor subtypes

	Rat proximal colon (β_3)		*Guinea-pig atrium* (β_1)		*Guinea-pig trachea* (β_2)	
	pA_2	*slope*	pA_2	*slope*	pA_2	*slope*
	mean ± s.e.mean					
Alprenolol	6.82 ± 0.15	1.14 ± 0.14	8.17 ± 0.02	1.05 ± 0.03	8.88 ± 0.09	0.92 ± 0.09
SR 58894A	8.06 ± 0.43	1.06 ± 0.40	6.71 ± 0.24	1.22 ± 0.37	6.51 ± 0.10	1.06 ± 0.17
SR 59230A	8.76 ± 0.17	0.93 ± 0.09	7.31 ± 0.97	0.54 ± 0.38*	6.63 ± 0.06	0.81 ± 0.06
SR 59396A	8.36 ± 0.18	1.20 ± 0.15	6.13 ± 0.15	0.96 ± 0.22	6.87 ± 0.08	1.16 ± 0.11

Values are mean ± s.e.mean.
The pA_2 was obtained from the concentration-response curves shown in Figures 2 and 3. Computer analysis was done as described in Methods. Neither alprenolol, nor the three tested APAT had any intrinsic agonist activity (% difference from control < 10) up to the highest concentrations shown in Figures 2 and 3.
*Significantly different from unity.

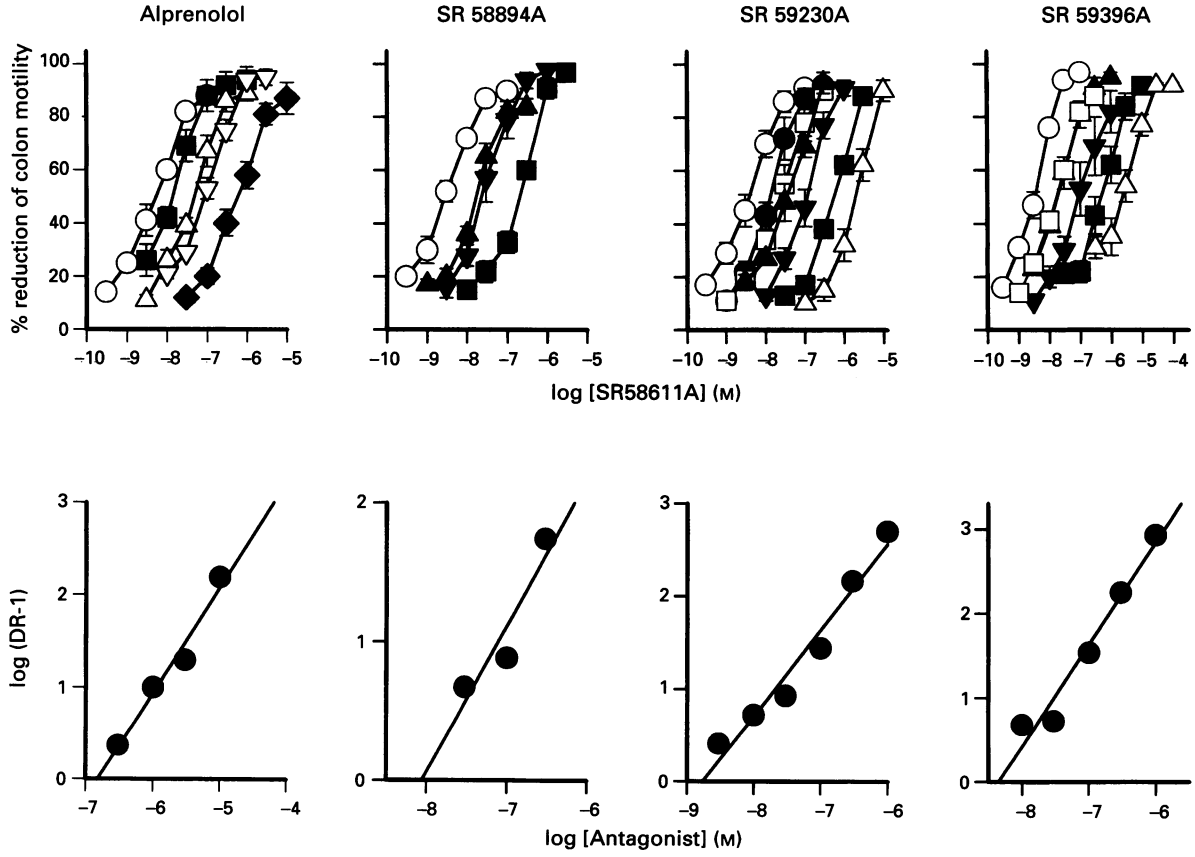

Figure 2 *In vitro* antagonism of the inhibition of rat colon motility by the selective β_3-agonist SR 58611A: agonist concentration-response curves and Schild plots. Colonic motility was scored by quantitative computer analysis as indicated in Methods. The following concentrations (μM) of alprenolol, SR 58894A, SR 59230A and SR 59396A, were added 30 min before the agonist, each giving one cumulative agonist concentration-response curve (○) control; (●) 0.003; (□) 0.01; (▲) 0.03; (▼) 0.1; (■) 0.3; (△) 1; (▽) 3; (◆) 10. Points are averages (with s.e.) from at least 7 replications.

tralin β_3-selective agonists SR 58375A (Bianchetti & Manara, 1990), (7**R**)-7-[(2**R**)-2-hydroxy-2-phenyl-ethylamino]-5,6,7,8-tetrahydro-naphthalen-2-ol hydrochloride, and SR 58611A (Bianchetti & Manara, 1990), ethyl{(7**S**)-7-[(2**R**)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphthalen-2-yloxy}acetate hydrochloride, were synthesized in the Chemistry Department of the SANOFI MIDY Research Centre.

Other chemicals were from commercial sources as indicated: Sigma-Aldrich Corp., St. Louis, Missouri, U.S.A.: (±)-alprenolol HCl, (±)-isoprenaline HCl, methacholine, carbachol, phentolamine HCl, salbutamol hemisulphate; Ciba-Geigy, Varese, Italy: desmethylimipramine HCl; Richter-Lepetit, Milan, Italy: hydrocortisone sodium hemisuccinate (Flebocortid ampoules); Ricerchimica, Milan, Italy: phenoxybenzamine HCl.

Results

The reference nonselective β-adrenoceptor antagonist, alprenolol, and the representative APATs, SR 58894A, SR 59230A and SR 59396A (Figure 1), concentration-dependently inhibited *in vitro* responses mediated by different β-adrenoceptor subtypes (Figures 2 and 3). Quantitative antagonism scores (pA_2), shown in Table 1, clearly indicated that the potency of all the APATs in antagonizing responses elicited by the β_3-selective agonist SR 58611A in the rat colon (pA_2 range 8.1–8.8) greatly exceeded that of alprenolol (pA_2 6.8), as well as their own potency in antagonizing β_1- or β_2-adrenoceptor-mediated responses to isoprenaline or salbutamol, in guinea-pig atrium and trachea respectively (apparent pA_2 ranges 6.7–7.3 and 6.5–6.9, as listed). Alprenolol, however, was a more potent antagonist of responses mediated by β_1 and β_2-adrenoceptors (pA_2 8.2 and 8.9 respectively) than of those mediated by β_3-adrenoceptors.

As shown by the slope values in Table 1 or the concentration-response curves in Figures 2 and 3, antagonism of the β_3-response in the colon always appeared competitive, irrespective of whether alprenolol or the APATs were tested; the β_1- and the β_2-responses were also antagonized apparently competitively by alprenolol, whereas the antagonism of these responses by APATs was apparently non-competitive: slope significantly ($P<0.05$) less than 1 and/or reduction in the maximal agonist effect.

Further *in vitro* tests designed to compare the ability of several APAT stereoisomers to antagonize β-adrenoceptor-mediated responses at different receptor subtypes are summarized in Table 2. Both SR 58894A and SR 59230A antagonized the β_3-agonist response elicited by SR 58375A at lower concentrations than their respective stereoisomers and, unlike the latter, did not affect β_1 or β_2-adrenoceptor-mediated responses except at substantially higher concentrations. Thus the **SS** configuration, which is present in SR 58894A as well as SR 59396A and SR 59230A, is the preferred one for β_3-adrenoceptor antagonist potency and selectivity.

Animals were set up for assessing β_3-adrenoceptor antagonist potency and subtype selectivity by the oral route *in vivo*. Table 3 compares the 50% effective antagonist doses (ID_{50}) of alprenolol and the representative APAT, SR 59230A. As in the *in vitro* experiments, alprenolol was less potent (ID_{50} 11 mg kg^{-1}) than SR 59230A (ID_{50} 3.6 mg kg^{-1}) in preventing the inhibition of colonic motility by the β_3-selective agonist, SR 58611A; alprenolol was also less potent in antagonizing the putative β_3-colonic response to SR 58611A than isoprenaline-induced tachycardia, presumably a β_1-mediated response. SR 59230A, however, proved unequivocally selective

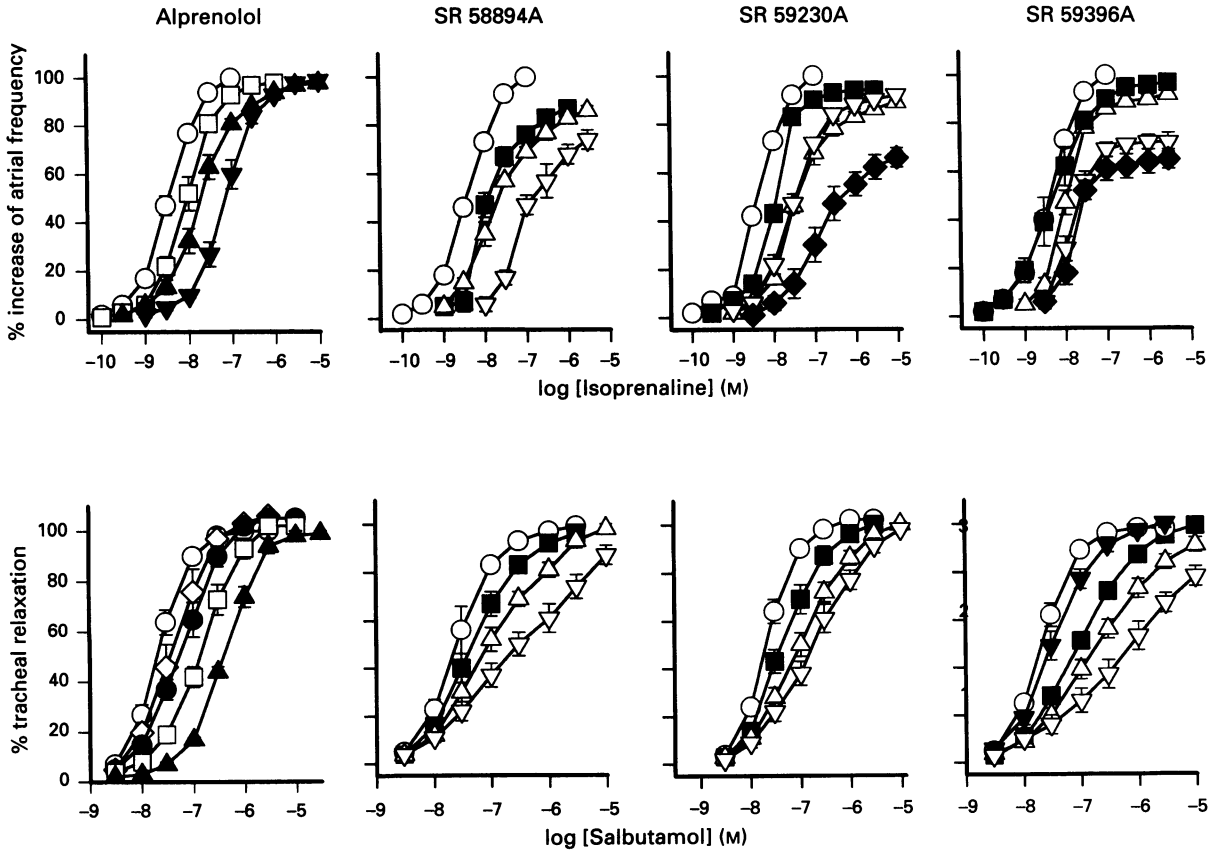

Figure 3 *In vitro* antagonism of chronotropic effect of isoprenaline on guinea-pig atria and salbutamol relaxation of guinea-pig trachea: agonist concentration-response curves. Guinea-pig atrial contraction frequency and tracheal relaxation were monitored as described in Methods. The following concentrations (μM) of alprenolol, SR 58894A, SR 59230A and SR 59396A, were added 30 min before the agonist, each giving one cumulative agonist concentration-response curve: (○) control; (◇) 0.001; (●) 0.003; (□) 0.01; (▲) 0.03; (▼) 0.1; (■) 0.3; (△) 1; (▽) 3; (◆) 10. Points are averages (with s.e.) from at least 7 replications.

for β_3-adrenoceptors, being 20 and 12 times more potent against the colonic response than against cardiac (β_1, $ID_{50} \geqslant 80$ mg kg^{-1}) and bronchial (β_2, ID_{50} 44 mg kg^{-1}) responses, respectively.

Additional *in vivo* tests compared the potency of alprenolol and SR 59230A in preventing the BAT thermogenic response to a submaximally effective dose (Figure 4a) of the β_3-selective agonist, BRL 37344. As shown in Figure 4c, the time course of the presumably β_3-adrenoceptor-mediated thermogenic response to BRL 37344 was significantly reduced by SR 59230A at a dose ($\leqslant 5$ mg kg^{-1}) within the confidence limits of its ID_{50} for preventing colonic motility inhibition by the β_3-selective agonist SR 58611A, and lower than the doses of SR 59230A with 50% effect for antagonism of the putative β_1 and β_2 responses in Table 3. Unlike SR 59230A, alprenolol at 20 mg kg^{-1}, i.e. higher than the dose at which it showed 50% effect in preventing the putative β_1 and β_2 responses in Table 3, hardly affected the time course of the BAT thermogenic response, though this was definitely reduced by the highest dose (80 mg kg^{-1}) (Figure 4b). Both SR 59230A and alprenolol, at the lower doses tested, lowered the time course of the T-BAT/T-core difference, compared to rats receiving only saline.

Discussion

The Lands classification (Lands *et al.*, 1967), identifying only two β-adrenoceptor subtypes (β_1 and β_2), was soon challenged

Table 2 Antagonism by SR 58894A, SR 59230A and their stereoisomers of *in vitro* responses mediated by atypical (β_3) or previously recognized β-adrenoceptor subtypes

		Rat proximal colon (β_3) IC_{50} (nM)	*Guinea-pig atrium* (β_1) IC_{50} (nM)	*Guinea-pig trachea* (β_2) IC_{50} (nM)
SR 58894A	**(SS)**	90 (70–117)	715 (401–1,030)	1,110 (817–1,510)
SR 58893A	**(RR)**	>10,000§	⩾3,000	~3,000
SR 58895A	**(SR)**	>3,000§	~1,600	370 (240–570)
SR 58892A	**(RS)**	1,740 (1,300–2,330)	>3,000§	~3,000
SR 59230A	**(SS)**	40 (33–48)	408 (287–528)	648 (535–786)
SR 59483A	**(RR)**	>3,000§	>3,000	~4,000
SR 59231A	**(SR)**	375 (304–461)	587 (473–701)	~500

The letters in parentheses after each compound's identification code indicate the configuration of the stereogenic carbons of the aryloxypropanolamine and of the tetralin part of the moiety, in that order. The enantiomers **(RR)**, **(SS)** and **(RS)**, **(SR)** were prepared by independent synthesis and showed opposite optical rotation of identical magnitude. The concentrations causing 50% inhibition of agonist response (IC_{50}, in parentheses 95% confidence limits) in guinea-pig atria and trachea, were determined as described in Figure 3, from concentration-response curves using cumulative concentrations of agonist; in rat colon, cumulative concentrations of the antagonist (one every 10 min) were added to the bath 10 min after the selective β_3-adrenoceptor agonist, SR 58375A (Bianchetti & Manara, 1990) at a concentration (0.3 μM) reducing spontaneous motility by 80 to 90%.
§ 20 to 30% inhibition at the stated concentration.
~ = approximately.

Table 3 *In vivo* selectivity of antagonists for β-adrenoceptor subtype mediated responses in rats

	Colonic motility inhibition by SR 58611A (β_3) ID_{50} (mg kg^{-1})	*Isoprenaline chronotropic effect* (β_1) ID_{50} (mg kg^{-1})	*Salbutamol bronchodilator action* (β_2) ID_{50} (mg kg^{-1})
Alprenolol	11 (7–18)	2.8 (1.2–3.7)	~8*
SR 59230A	3.6 (2–6.5)	⩾80§	44 (39–52)

Antagonists were administered orally 60 min before a submaximally effective (about 80%) dose of the appropriate agonist: SR 58611A (45 μg kg^{-1}, i.v.), isoprenaline (5 μg kg^{-1}, s.c.), salbutamol (40 μg kg^{-1}, i.v.). The dose of antagonist causing 50% inhibition of the agonist response of control (antagonist-free) animals (ID_{50}, in parentheses 95% confidence limits) was established from the dose response curve.
For colonic motility experiments, the doses of antagonists were: alprenolol 2.5, 5, 10, 20, 40, 80; SR 59230A 0.31, 1.25, 5, 20, mg kg^{-1}. For preventing isoprenaline or salbutamol chronotropic or bronchodilator responses, the doses of antagonists tested were: alprenolol 1, 2, 5, 20; SR 59230A 20, 40, 80, mg kg^{-1}. For scored endpoints in drug-free control rats and rats receiving different treatments, and other details see Methods.
*With 5 mg alprenolol, salbutamol was still 93% effective, but 20 mg alprenolol completely prevented salbutamol's action; ~ = approximately.
§At 40 mg kg^{-1}, SR 59230A inhibited isoprenaline response by 15% (not significant) and at 80 mg kg^{-1} halved isoprenaline response ($P<0.01$ from isoprenaline, Duncan's test).

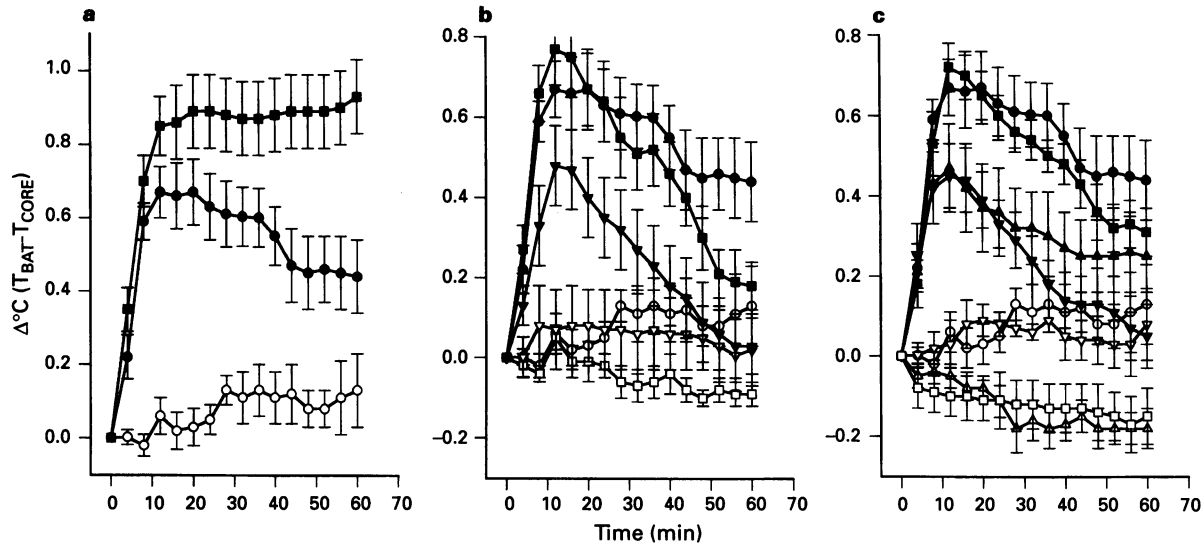

Figure 4 Antagonism of rat brown adipose tissue thermogenic responses by alprenolol and SR 59230A. Antagonists were administered orally; BRL 37344 was administered i.v. at time 0, 60 min after the scheduled time of administration of antagonists with fairly constant baseline T-BAT minus T-core values. Each point indicates the average of individual values from 8 to 20 animals and vertical bars indicate s.e.: in (a), (b) and (c) (○) rats receiving only vehicle(s); in (a), (●) BRL 37344, 1 $\mu g\,kg^{-1}$, (■), BRL 37344, 10 $\mu g\,kg^{-1}$ (in (b) and (c) the dose of BRL 37344 was always 1 $\mu g\,kg^{-1}$); in (b) (●) BRL 37344, (■) alprenolol, 20 $mg\,kg^{-1}$ and BRL 37344, (▼) alprenolol, 80 $mg\,kg^{-1}$ and BRL 37344, (□) alprenolol, 20 $mg\,kg^{-1}$, (▽) alprenolol 80 $mg\,kg^{-1}$; in (c) (●) BRL 37344, (■) SR 59230A, 1.25 $mg\,kg^{-1}$ and BRL 37344, (▲) SR 59230A, 5 $mg\,kg^{-1}$ and BRL 37344, (▼) SR 59230A, 20 $mg\,kg^{-1}$ and BRL 37344, (□) SR 59230A, 1.25 $mg\,kg^{-1}$, (△) SR 59230A, 5 $mg\,kg^{-1}$, (▽) SR 59230A 20 $mg\,kg^{-1}$.

as additional experimental findings suggested further β-adrenoceptor heterogeneity. The main anomalies applied to lipolysis and intestinal relaxation, since independent investigators had shown that β-adrenoceptor-mediated responses in these tissues were much less sensitive to β-blocking agents (up to 1,000 times) than anywhere else (Bristow *et al.*, 1970; Furchgott, 1972).

This early and previously disputed (Buckner & Christopherson, 1974; Raper, 1987) evidence of atypical (non-β_1, non-β_2) β-adrenoceptors is now recognized as one of the main functionally distinctive features of the generally accepted β_3-subtype (Arch & Kaumann, 1993). Indeed, we have recently tested over 40 conventional β-adrenoceptor-blocking agents for their ability to prevent β_3-subtype-mediated agonist responses in the rat isolated proximal colon: only a few were effective in the submicromolar range, but they all showed far more potent antagonism in preparations where responses were mediated by either β_1- or β_2-adrenoceptors (Manara *et al.*, 1995a). Since the resistance of β_3-adrenoceptors to currently available β-blockers is thereby well established, the availability of potent antagonists with inverse subtype selectivity, i.e. with substantially lower affinity for the β_1- and β_2-sites than for the putative β_3-sites, is vital for the latter's unquestionable functional identification.

The direction of our search for such antagonists was based on our previous experience (Bianchetti & Manara, 1990; Cecchi *et al.*, 1994) with phenylethylaminotetralins (PEATs). The PEATs are β_3-adrenoceptor agonists, which *in vitro* and *in vivo* showed greater subtype selectivity (very little β_2-stimulating action, if any, and virtual absence of activity at β_1-adrenoceptors) than previously reported β_3-adrenoceptor agonists (Bianchetti & Manara, 1990; Manara *et al.*, 1990), and have been found in clinical trials to be remarkably free from the troublesome sympathomimetic side-effects (cardiovascular and tremors) experienced in human studies with these earlier β_3-adrenoceptor selective agonists (Landi *et al.*, 1993).

Our reasoning implied that the aminotetralin moiety of PEAT could confer similar subtype recognition properties on the aryloxypropanolamines, the β-adrenoceptor blocking action of which is well known (Ruffolo, 1991). Indeed APATs such as SR 58894A, SR 59230A and SR 59396A, tested *in vitro*, were at least 40 times more potent antagonists of β-adrenoceptor mediated responses in the rat colon (β_3) than in either guinea-pig atrium or trachea (β_1 and β_2 respectively).

Although the present paper is not the place for a full structure-activity analysis, some molecular characteristics seem worth mentioning that are apparently critical for antagonist potency and selectivity at the β_3-adrenoceptor. SR 58894A, SR 59230A and SR 59396A, like similarly active aryloxypropanolaminotetralin compounds prepared by us and not discussed here, but unlike our phenylethanolamine agonists (Bianchetti & Manara, 1990), are all 1- rather than 2-aminotetralins. Additional molecular features of importance relate to the two asymmetric carbon atoms and attest to the stringent stereochemical requirements for receptor targeting.

We prepared and tested all three possible stereoisomers of SR 58894A plus the enantiomer of SR 59230A and its diastereoisomer with the hydroxyl in the S configuration: the SS configuration at the chiral carbons, that occurs in both SR 58894A and SR 59230A, as well as in SR 59396A, proved essential for the intended receptor subtype antagonist potency and selectivity. This was not completely unexpected as regards the stereogenic carbon atom of the aryloxypropanolamine, since the most active enantiomers of conventional β-antagonists with this structure have an S configuration for the hydroxyl group (Ruffolo, 1991).

We have presented elsewhere the results of *in vitro* competition binding assay with the representative APAT, SR 59230A, that support its selectivity in a more general context, since it had scant potency in displacing several radiolabelled ligands, each indicative of non-β-adrenoceptor sites for given neurotransmitters or autacoids, including 5-hydroxytryptamine, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT_2, 5-HT_3, 5-HT-uptake, sites; dopamine, D_1 and D_2; adrenoceptor, α_1 and α_2; and histamine, H_1 (Manara *et al.*, 1995a).

We further assessed the potency and selectivity of APATs for β_3-adrenoceptors *in vivo* in animal models. Thus the selectivity of action in conscious rats against responses believed to be mediated by β_3-adrenoceptors were similar to those in isolated organs. SR 59230A *in vivo* proved a potent, orally effective and, unlike alprenolol, β_3-selective antagonist. While these results support APATs as the first β-adrenoceptor

blocking agents selective for the β_3-subtype *in vitro* and *in vivo*, they may also be taken as providing final unambiguous evidence of the distinctive functional features of those abundant in the rat proximal colon (Bianchetti & Manara, 1990).

The *in vivo* selectivity of SR 59230A for β_3-adrenoceptors was also tested by monitoring the BAT-thermogenic response in anaesthetized rats. These experiments, like those in which we recorded myoelectrically assessed β_3-selective agonist-induced inhibition of colonic motility, were designed also with a view to functional identification of the underlying receptor subtype. The overall outcome of our BAT-thermogenesis study, including the results with the reference compound, alprenolol, strongly suggests that β_3-adrenoceptors are involved and provides additional evidence of the *in vivo* β_3-selectivity of SR 59230A. Interestingly enough, in no other *in vitro* or *in vivo* tests did the APATs, including SR 59230A, have any significant effects on the experimental endpoints in the absence of the appropriate agonists, whereas SR 59230A (and alprenolol), at the lower doses tested, apparently lowered the time course of the T-BAT/T-core difference, compared to rats receiving only saline. Precise interpretation of this incidental finding is beyond the scope of our present study, though it might relate to an interaction at the BAT β_3-adrenoceptor with catecholamines released as a consequence of anaesthesia. However, it does not affect our conclusions on the antagonists' selectivity and on the identity of the β-adrenoceptor subtype involved.

We also attempted *in vitro* lipolysis tests with APATs, but they were not successful. The β_3-adrenoceptor agonist-elicited free fatty acid release was weakly antagonized by the three APATs studied, only when the incubating medium, containing albumin, was saturated with high concentrations of the appropriate inactive APAT stereoisomer. Thus, in all probability high protein binding prevented APATs from exerting their β_3-selective adrenoceptor antagonist action under these experimental conditions.

The work described here has already produced an orally-effective antagonist selective for $\beta_3$adrenoceptors and the results encourage further efforts, including ongoing tests on human tissues, to ascertain the therapeutic potential of this new class of compounds, as suggested by the variety of physiological roles for which these novel receptors are candidates (Howe, 1993). In any case, we trust that the APATs will fill a major gap as regards conclusive identification of β-adrenoceptors not conforming to the previously recognized β_1- and β_2-subtypes as distinct functional entities.

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