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Antinociceptive Profile of $3-\alpha$ -tropanyl-(2-Cl)-acid phenoxybutyrate (SM-21): A Novel Analgesic with a Presynaptic Cholinergic Mechanism of Action¹

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ABSTRACT

The antinociceptive effect of (\pm) -3- α -tropanyl-(2-Cl)-acid phenoxybutyrate (SM-21) (10–40 mg kg⁻¹ s.c., 10–30 mg kg⁻¹ i.p., 20–60 mg kg⁻¹ p.o., 3–20 mg kg⁻¹ i.v. and 5–20 μ g per mouse i.c.v.) was examined in rodents and guinea pigs by using the hot-plate, abdominal constriction, tail-flick and paw-pressure tests. The antinociception produced by (\pm)-SM-21 was prevented by atropine, pirenzepine and hemicholinium-3 but not by quinpirole, R-(α)-methylhistamine, [1-[2(methylsufonyl)amino]ethyl]-4-piperidinyl]methyl-5-floro-2-methoxy-1H-indole-3-carboxylate hydrochloride, N⁶-cyclopentyladenosine, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide, naloxone, 3-aminopropyl-diethoxy-methyl-phos-

Ghelardini *et al.* (1990) have reported that the antimuscarinic compound atropine, at very low doses, was able to induce a central cholinergic antinociception in rodents regardless of the route of administration and the noxious stimulus applied. Furthermore, this antinociceptive activity was not accompanied by the typical cholinergic symptomatology. The atropine-induced increase in the pain threshold was attributable to the R-(+)-enantiomer of atropine, R-(+)-hyoscyamine, because S-(-)-hyoscyamine was ineffective in all antinociceptive tests used (Ghelardini *et al.*, 1992). More recently, Bartolini *et al.* (1994), investigating the antinociceptive effect of atropine, demonstrated, using microdialysis phinic acid or reserpine. On the basis of the above data, it can be postulated that (±)-SM-21 exerted an antinociceptive effect mediated by a central potentiation of cholinergic transmission. Affinity profiles of (±)-SM-21 for muscarinic receptor subtypes, determined by functional studies (rabbit vas deferens for M₁, guinea pig atrium for M₂, guinea pig ileum for M₃ and immature guinea pig uterus for putative M₄) have shown a selectivity ratio M₂/M₁ of 4.6 that, although very low, might be responsible for the antinociception induced by (±)-SM-21 through an increase in ACh extracellular levels. In the antinociceptive dose range, (±)-SM-21 did not impair mouse performance evaluated by the rota-rod and hole-board tests.

techniques, that R-(+)-hyoscyamine, at analgesic doses, produced an increase in the release of ACh from the rat cerebral cortex *in vivo*. On the bases of the above-mentioned results, the racemate (Gualtieri *et al.*, 1994) and the enantiomers (Romanelli *et al.*, 1995) of the compound labeled SM-21 (fig. 1), which is structurally related to atropine, have been synthesized in order to obtain a new cholinergic amplifier endowed with more intensive antinociceptive activity than atropine but as lacking as atropine in cholinergic side effects. To this end, we investigated (\pm)-SM-21 antinociceptive properties by using the hot-plate, abdominal constriction, pawpressure and tail-flick tests, and the incidence of behavioral side effects was detected by the rota-rod and hole-board tests.

Materials and Methods

Animals. Male Swiss albino mice (23–30 g), Wistar rats (200–300 g) from Morini (San Polo d'Enza, Italy), Fisher 344 rats (200–300) from Charles River (Calco, Italy) and guinea pigs (150–200 g) from Rodentia (Bergamo, Italy) breeding farms were used. Fifteen mice

ABBREVIATIONS: SM-21, 3-α-tropanyl-(2-Cl)-acid phenoxybutyrate; HC-3, hemicholinium-3; NAN 190, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide; McN-A-343, 4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride; GR 125487, [1-[2(methylsufonyl)amino]ethyl]-4-piperidinyl] methyl-5-fluoro-2-methoxy-1H-indole-3-carboxylate hydrochloride; AFDX-116, 11,2-(diethylamino)methyl-1-piperidinil acetyl-5,11-dihydro-6H-pyrido 2,3-b 1,4 benzodiazepine-6-one; CGP 35348, 3-aminopropyl-diethoxy-methyl-phosphinic acid; RAMH, (R)-α-methylhistamine.

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Fig. 1. Chemical structure of SM-21 racemate and its enantiomers.

and four rats or guinea pigs were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were kept at $23 \pm 1^{\circ}$ C with a 12-h light/dark cycle, light on at 7 A.M., with food and water *ad libitum*. All experiments were carried out according to the guidelines of the European Community Council.

Analgesic Tests

PHARMACOLOGY AND EXPERIMENTAL THERAPEU

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Hot-plate test. The method adopted was described by O'Callaghan and Holtzman (1975). Mice were placed inside a stainless steel container thermostatically set at $52.5 \pm 0.1^{\circ}$ C in a precision water bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stopwatch before treatment and at regular intervals up to a maximum of 60 min after treatment. The endpoint used was the licking of the fore or hind paws. Those mice scoring below 12 and over 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

Abdominal constriction test. Mice were injected i.p. with a 0.6% solution of acetic acid (10 ml kg⁻¹), according to Koster *et al.* (1959). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Paw-pressure test. The nociceptive threshold in the rat and guinea-pig was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton *et al.* (1988). Threshold pressure was measured before treatment and 15, 30 and 45 min after treatment. Rats and guinea-pigs scoring below 30 g or over 85 g during the test before drug administration were rejected (25%). An arbitrary cutoff value of 250 g was adopted.

Tail-flick test. An analgesimeter from Ugo Basile (Varese, Italy) was used to perform the tail-flick test described by D'Amour and Smith (1941). The light from a project bulb situated beneath the platform where the animal was placed was focused through a small hole on the ventral part of the tail at a point about 4 cm from the tip. Withdrawal of the tail exposed a photocell to the light, which turned off the thermal stimulus and automatically stopped the clock. The intensity was regulated so that the reaction time varied between 2 and 4 s. The analgesia was tested before treatment and 15, 30 and 45 min after treatment. Each value was derived from the mean of three consecutive readings in which the light was focused on three adjacent points of the tail.

Anti-inflammatory Test

Carrageenan-induced paw edema. Rats paw volumes were measured using a plethysmometer (Ugo Basile, Varese, Italy). Rats received (\pm)-SM 21, indomethacin or saline 90 min after a 0.1-ml injection of 0.5% carrageenan in the right hind paw. Two hours after the injection of carrageenan, the paw volume of the right hind paw was measured and compared with saline-treated controls.

Additional Behavioural Tests

Hole-board test. The hole-board test consists of a 40-cm-square plane with 16 flush-mounted cylindrical holes (diameter 3 cm) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and left to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animals on the surface of the plane. Miniature photoelectric cells in each of the 16 holes recorded the exploration of the holes (head plunging activity) by the mice.

Rota-rod test. The apparatus consisted of a base platform and a rotating rod 3 cm in diameter with a nonslippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught *et al.* (1985). The performance time was measured before treatment and 15, 30 and 45 min after treatment.

In Vitro Functional Studies

Isolated rabbit vas deferens. Experiments on isolated rabbit vas deferens were performed according to the method described by Eltze (1988) and modified by Dei *et al.* (1995). 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, and 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.* (1985) and modified by Dei *et al.* (1995). 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.

Guinea pig isolated ileum. Isolated ileum fragments were prepared according to Eltze and Figala (1988). Bath fluid temperature was maintained at 37°C. Isotonic ileum contractions induced by ACh were recorded before and 1 h after perfusion with antagonists.

Guinea pig isolated uterus. Experiments on isolated immature guinea pig uterus were performed according to Dörje *et al.* (1990). 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 the responsiveness to the agonist, and a dose-response curve for carbachol was obtained.

Determination of antagonist affinities. After a stabilization time of 30 to 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. The antagonists were allowed to equilibrate for 60 min. No more than two concentrations of antagonist were tested in the same preparation. Agonist EC₅₀ values in the absence and presence of antagonists were determined graphically for the calculation of dose ratios.



Fig. 2. Dose-response curves of (±)-SM-21 administered s.c. (panel A), i.c.v. (panel B), p.o. (panel C) and i.v. (panel D) in the mouse hot-plate test. The doses are expressed as mg kg⁻¹ s.c., p.o. and i.v. and as μ g per mouse i.c.v. Vertical lines show S.E.M. ^ P < .05; * P < .01 in comparison with saline controls. Each point represents the mean of at least 10 mice.

AChE activity. AChE activity was assayed according to Ellman et al. (1961), using 0.5 mM acetylthiocholine iodide as substrate. The (\pm)-SM-21 inhibitory effect was tested at various concentrations on a purified preparation of AChE from the electric eel.

Drugs. SM-21 racemate was prepared according to Gualtieri et al. (1994); R-(+)-SM-21 and S-(-)-SM-21 were prepared according to Romanelli et al. (1995); R-(+)-hyoscyamine was prepared according to Gualtieri et al. (1991). Also used were altropine sulfate, carbamylcholine chloride, carrageenan, physostigmine hemisulfate and yohimbine hydrochloride (Sigma, Milan, Italy), HC-3, pirenzepine dihydrochloride, naloxone hydrochloride, quinpirole hydrochloride, (R)- α -methylhistamine dihydrochloride, indomethacin, N⁶-cyclopentyladenosine, NAN 190, McN-A-343 (R.B.I., Milan, Italy); ACh chloride (Merck, Florence, Italy); GR 125487 (Boehringer Ingelheim, Milan, Italy); morphine hydrochloride (U.S.L. 10/D, Florence, Italy), diphenhydramine hydrochloride and AFDX-116 (De Angeli, Milan, Italy); clomipramine hydrochloride (anafranil), CGP 35348 and reserpine (Ciba Geigy, Basel, Switzerland); (+)-amphetamine sulfate (Recordati, Rome, Italy). Other chemicals were of the highest quality commercially available. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution or dispersed in sodium carboxymethylcellulose 1% immediately before use, except reserpine, which was dissolved in a 20% solution of ascorbic acid, and R-(+)-hyoscyamine, which was dissolved in 0.1 M HCl and then diluted with saline (1:10). Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by s.c., i.p., and p.o. route or 5 ml kg⁻¹ by i.v. route. Intracerebroventricular administration was performed under ether anaesthesia using isotonic saline as solvent, according to the method described by Haley and McCormick (1957) for mice and that we adapted for rats. Briefly, during anaesthesia, mice and rats were grasped firmly by the loose skin behind the head. A hypodermic needle 0.4 mm in external diameter, attached to a $10-\mu$ l syringe, was inserted perpendicularly through the skull at a depth of no more than 2 mm into the brain of the mouse and 4 mm into the brain of the rat, where 5 μ l (mice) or 10 μ l (rats) were then administered. The injection site was 1.5 mm (mice) or 2.5 mm (rats) from either side of the midline on a line drawn through to the anterior base of the ears. To ensure that the drugs were administered exactly into the cerebral ventricle, some mice and rats were injected i.c.v. with 5 to 10 μ l of diluted 1:10 Indian ink and their brains examined macroscopically after sectioning. Intraplantar injections of carrageenan were performed by injecting 100 μ l of a suspension in sterile saline solution of 0.5% carrageenan in the rat hind paw.

Statistical analysis. Results are given as the mean \pm S.E.M.; analysis of variance (ANOVA), followed by Fisher's PLSD procedure for *post-hoc* comparison, was used to verify the significance of the difference between two means. P values of less than .05 were con-

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Vol. 282

Gspet PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

sidered significant. Data were analyzed with the StatView for the Macintosh computer program (1992).

Results

Antinociceptive activity of SM-21. (±)-SM-21, as shown in figure 2, produced a dose-dependent increase in the pain threshold in the mouse hot-plate test after s.c. (10–40 mg kg⁻¹; panel A), i.c.v. (5–20 μ g per mouse; panel B), p.o. (20–60 mg kg⁻¹; panel C) and i.v. (3–20 mg kg⁻¹; panel D) administration. The antinociceptive effect of (±)-SM-21 peaked 15 min after s.c. and i.c.v. administration and then slowly diminished. (±)-SM-21, after p.o. and i.v. administration, reached its maximum analgesic effect respectively 30 and 10 min after injection. Figure 3 (panels A and B) illustrates the analgesic effect of (±)-SM-21 in the mouse acetic acid abdominal constriction test. (±)-SM-21 induced an increase in the pain threshold in a dose-dependent manner starting from the dose of 10 mg kg⁻¹ s.c. (fig. 3, panel A). (±)-SM-21 showed antinociceptive properties also after the injection of 1 and 5 μ g per mouse i.c.v., reaching its maxi-



Fig. 3. A) Dose-response curves of (±)-SM-21 administered s.c. and effect of both HC-3 (1 µg per mouse i.c.v.) and reserpine (2 mg kg⁻¹ i.p.) pretreatments on antinociception induced by (±)-SM-21 (10 mg kg⁻¹ s.c.) in the mouse abdominal constriction test induced by acetic acid 0.6%. The nociceptive responses were recorded 15 min after (±)-SM-21 administration. HC-3 was injected 5 h and reserpine 48 and 24 h before testing. (B) Time course of (±)-SM-21 administered i.c.v. The nociceptive responses were recorded 15, 30, 45 and 60 min after (±)-SM-21 administration. Vertical lines show S.E.M. ^ P < .05; * P < .01 in comparison with saline controls. ° P < .01 in comparison with (±)-SM-21 (10 mg kg⁻¹ s.c.). Numbers inside the columns indicate the number of mice.

mum effect between 15 and 30 min after administration (fig. 3, panel B).

(±)-SM-21 was able to produce an increase in the pain threshold not only in mice but also in rats and guinea pigs. In the paw pressure test (±)-SM-21 administered i.p. at the dose of 20–30 mg kg⁻¹ in the rat, and 20 mg kg⁻¹ in guinea pigs, induced antinociception starting 15 min after injection, reaching a maximum after 30 min and persisting up to 45 min (table 1). The analgesic profile of (±)-SM-21 was also investigated in Wistar and Fisher 344 rat strains by using the tail flick test (fig. 4). In both rat strains used (±)-SM-21 exhibited a similar antinociceptive activity 30 min after i.p. injection of 20–30 mg kg⁻¹ (fig. 4).

The analgesic effect of the two enantiomers of (\pm) -SM-21, R-(+)-SM-21 and S-(-)-SM-21, was evaluated in the mouse hot-plate test (fig. 5, panels A and C) and in the acetic acid abdominal constriction test (fig. 5, panels B and D). Both enantiomers dose-dependently were able to increase the pain threshold, even if R-(+)-SM-21 was slightly more effective than S-(-)-SM-21.

The areas under the curve of the antinociception induced by (±)-SM-21 (30 mg kg⁻¹ i.p.), R-(+)-hyoscyamine (5 μ g kg⁻¹ i.p.), morphine (8 mg kg⁻¹ i.p.), diphenhydramine (20 mg kg⁻¹ i.p.) and clomipramine (25 mg kg⁻¹ i.p.) are reported in figure 6. The doses of the analgesic drugs chosen were the highest that did not impair rota-rod performance.

The potentiating effect on antinociception exerted by nonanalgesic doses of physostigmine (0.05 mg kg⁻¹ i.p.) on AFDX-116 (6.3 ng per mouse i.c.v.) and R-(+)-hyoscyamine (5 μ g kg⁻¹ s.c.) in the mouse hot-plate test is illustrated in table 2. In the same experimental conditions physostigmine was not able to potentiate (±)-SM-21 (30 mg kg⁻¹ i.p.) antinociception (Table 2).

Antagonism of the (±)-SM-21 induced antinociception. In the mouse hot-plate test, the antinociceptive effect of (\pm) -SM-21 (30 mg kg⁻¹ s.c.) was not antagonized by naloxone $(1 \text{ mg kg}^{-1} \text{ i.p.}), \text{ CGP-35348} (2.5 \ \mu\text{g per mouse i.c.v.}), (R)-\alpha$ methylhistamine (10 mg kg⁻¹ i.p.), quinpirole (0.1 mg kg⁻¹ i.p.), GR-48125 (20 mg kg⁻¹ i.p.), N⁶-cyclopentyladenosine (5 μ g per mouse i.c.v.), NAN 190 (0.5 μ g per mouse i.c.v.) (table 3) and, in the abdominal constriction test, by reserpine (2 mg kg^{-1} i.p.) (fig. 3). Conversely, atropine (5 mg kg^{-1} i.p.), pirenzepine (0.1 μ g per mouse i.c.v.) and hemicolinium-3 (1 μ g per mouse or rat i.c.v.) were able to completely prevent (\pm) -SM-21 antinociception in the mouse hot-plate (table 3), abdominal constriction (fig. 3) and the rat paw-pressure tests (table 1). All antagonists were injected 15 min before (\pm) -SM-21, with the exception of reserpine, injected twice 48 and 24 h before the test, N⁶-cyclopentyladenosine, administered simultaneously with (\pm) -SM-21 and CGP 35348, injected 5 min before (\pm) -SM-21.

Evaluation of the (±)-SM-21 effect in the carrageenan-induced paw edema test. (±)-SM-21 failed to suppress paw edema in response to carrageenan administration at the dose of 20 and 30 mg kg⁻¹ i.p. The positive control, indomethacin at the dose of 1 mg kg⁻¹ i.p., produced a significant inhibition over saline + carrageenan treated control animals (data not shown).

Evaluation of the SM-21 effect on spontaneous activity and motor coordination. The motor coordination of mice treated with (\pm) -SM-21, R-(+)-SM-21 and S-(-)-SM-21 was evaluated by using the rota-rod test (table 4) while their

433

TABLE 1

Antinociception exerted by (\pm) -SM-21 in the paw-pressure test: in the rat (A) and in the guinea pig (B) and antagonism by atropine and HC-3 on (\pm) -SM-21 antinociception in the rat

				Paw Pressure (g)			
	Pretreatment	Treatment, i.p.	Dose (mg kg ⁻¹)	Refore Pretreatment	After Treatment		
				Boloro Protoatmont	15 min	30 min	45 min
A)	Saline	Saline		58.3 ± 2.2	61.8 ± 2.8	60.2 ± 3.4	61.2 ± 2.6
	10 ml kg ⁻¹ i.p.			(18)	(18)	(18)	(18)
	Saline	(±)-SM-21	10	60.3 ± 4.0	62.8 ± 4.4	59.4 ± 4.2	57.8 ± 4.0
				(5)	(5)	(5)	(5)
		(±)-SM-21	20	60.4 ± 3.2	97.2 ± 4.2*	$100.4 \pm 4.6^{*}$	$86.6 \pm 3.8^{*}$
				(16)	(16)	(16)	(16)
		(±)-SM-21	30	62.0 ± 3.6	$93.2 \pm 5.2^{*}$	$95.4 \pm 3.8^{*}$	$84.5 \pm 3.6^{*}$
				(8)	(8)	(8)	(8)
	Atropine	Saline		58.8 ± 3.2	60.4 ± 4.2	59.7 ± 3.2	58.7 ± 3.0
	5 mg kg ⁻¹			(10)	(10)	(10)	(10)
	i.p.	(±)-SM-21	20	62.7 ± 2.8	$63.2 \pm 4.6^{\circ}$	$64.5 \pm 4.0^{\circ}$	61.1 ± 4.8°
		-		(5)	(5)	(5)	(5)
	HC-3	Saline		56.8 ± 3.4	62.4 ± 4.0	65.3 ± 4.2	62.3 ± 3.6
	1 μ g per rat			(10)	(10)	(10)	(10)
	i.c.v.	(±)-SM-21	20	60.6 ± 2.6	$66.8 \pm 4.4^{\circ}$	$68.5 \pm 4.4^{\circ}$	$60.4 \pm 4.5^{\circ}$
D)		0.1		(5)	(5)	(5)	(5)
B)		Saline		44.6 ± 3.6	41.4 ± 2.8	43.6 ± 3.8	42.0 ± 2.4
		(.) 011 01	~~	(10)	(10)	(10)	(10)
		(±)-SM-21	20	40.0 ± 2.2	$92.0 \pm 4.0^{\circ}$	$110.0 \pm 6.6^{\circ}$	64.0 ± 6.2
				(5)	(5)	(5)	(5)

The number of rats and guinea pigs is shown in parentheses.

Atropine and HC-3 were injected 15 min and 5 h before (±)-SM-21, respectively.

* P < .01 in comparison with saline-saline; ° P < .01 vs. saline-(\pm)-SM-21.



Fig. 4. Antinociceptive effect of (±)-SM-21 in the rat tail-flick test. Nociceptive responses were recorded 30 min after drug administration. Vertical lines show S.E.M. * P < .01 in comparison with saline controls. Numbers inside the columns indicate the number of mice.

spontaneous activity was investigated by using the holeboard test. The rota-rod performance of mice treated with (\pm) -SM-21 at the dose of 30 and 40 mg kg⁻¹ s.c. and both enantiomers at the dose of 20 and 30 mg kg⁻¹ s.c. was not impaired in comparison with controls (table 4). On the contrary, (\pm) -SM-21 administered at higher doses (50 and 60 mg kg⁻¹ s.c.) produced a significant impairment of the rota-rod performance (table 4). The number of falls by control animals progressively decreased at every measurement since the mice learnt how to balance on the rotating rod. The spontaneous motility and exploratory behavior of mice was not modified by treatment with (\pm) -SM-21 (20 and 40 mg kg⁻¹ s.c.) as revealed by the hole-board test (data not shown).

In vitro functional studies. (\pm) -SM-21 blocked the McN-A-343-induced inhibition of twitch contractions of the rabbit vas deferens (pK_B = 5.97 \pm 0.11), antagonized the negative inotropic carbachol-induced effect in guinea-pig left atrium (pK_{\rm B} = 6.63 \pm 0.10), the contractile responses to acetylcholine in guinea-pig ileum (pK_{\rm B} = 6.35 \pm 0.04) and to carbachol in immature guinea-pig uterus (pK_{\rm B} = 6.26 \pm 0.05) as shown in table 5. Increasing concentrations of (±)-SM-21 produced parallel shifts of the agonist concentration-response curves progressively to the right and no appreciable change in basal tension or maximum agonist response was observed (data not shown). pA₂ values of R-(+)-hyoscyamine and AFDX-116, used as reference drugs, are shown in table 5. The selectivity ratios for (±)-SM-21, R-(+)-hyoscyamine and AFDX-116, obtained as differences between respectively $pK_{\rm B}$ or pA_2 values, are reported in table 5.

Finally, (±)-SM-21 was shown to be endowed with a weak antiacetylcholinesterase activity, its $\rm IC_{50}$ value on electrical eel acetylcholinesterase being $1.1\cdot 10^{-4}$ M (data not shown).

Discussion

(±)-SM-21 was able to induce antinociception in mice, rats and guinea-pigs. Antinociception was elicited regardless of which noxious stimulus was used: thermal (hot-plate and tail flick tests), chemical (abdominal constriction test) and mechanical (paw pressure test). (±)-SM-21 antinociception was obtained without producing any visible modification of animal gross behavior. Moreover, (±)-SM-21 treated mice showed a complete integrity of motor coordination on the rota-rod test, normal spontaneous motility, as well as exploratory behavior as revealed by the hole-board test. 1997





(±)-SM-21 exerted its antinociceptive effect by acting centrally. It was, in fact, possible to reach the same intensity of analgesia by injecting directly into the cerebral ventricles doses (5–20 μ g per mouse) of (±)-SM-21 which were one thousand times lower than those needed parenterally. That the antinociception depends on a retrodiffusion of the drug from the cerebral ventricles to the periphery can thus be ruled out.

(±)-SM-21 antinociception was found to be dependent on central cholinergic activation since it was prevented by the non-selective muscarinic antagonist atropine, the M₁-antagonist pirenzepine and the ACh depletor HC-3. Taking into account that HC-3 and pirenzepine were able to antagonize (±)-SM-21 antinociception after i.c.v. injection, this supports the hypothesis that the analgesic site of action of (±)-SM-21 is localized in the CNS. A presynaptic mechanism facilitating cholinergic transmission is involved in (±)-SM-21 antinociception as revealed by the antagonism by HC-3. A postsynaptic mechanism of action can be ruled out since, as reported by Bartolini et al. (1987; 1992), HC-3 was not able to antagonize antinociception induced by agonists of postsynaptic muscarinic receptors such as oxotremorine, McN-A-343 and AF-102B.

The hypothesis of a presynaptic cholinergic mechanism for (\pm) -SM-21 is in agreement with previous results demonstrating, by microdialysis studies, an increase in ACh release from rat cerebral cortex induced by (\pm) -SM-21 administration (Bartolini et al., 1994). This effect occurred in the same range of doses (10 and 20 mg kg⁻¹ i.p.) in which the above-mentioned compound exerted its antinociceptive activity.

(±)-SM-21, like R-(+)-hyoscyamine (see introduction), demonstrated antinociceptive properties underlying a presynaptic cholinergic mechanism, but with greater efficacy than that exerted by R-(+)-hyoscyamine. The analgesic effect of (±)-SM-21 was also compared with the analgesia induced by some analgesic drugs such as morphine, diphenhydramine and clomipramine at the highest doses that did not impair the rota-rod performances. By comparing the areas under the curve, the antinociceptive efficacy of (±)-SM-21 (30 mg kg^{-1} s.c.) resulted almost equal to that exerted by morphine (8 mg kg^{-1} s.c.), but greater than those induced by diphenhydramine (20 mg kg^{-1} s.c.) and clomipramine (25 mg kg^{-1} s.c.).

(±)-SM-21 and R-(+)-hyoscyamine have been reported to increase the extracellular levels of ACh in cortical microdialysis studies (Bartolini *et al.*, 1994; Romanelli *et al.*, 1995). Since ACh release can be increased by blocking M_2/M_4 mus-



Fig. 6. Area under the curve of the analgesic effect of (\pm) -SM-21 in comparison with R-(+)-hyoscyamine, morphine, diphenhydramine and clomipramine in hot-plate test. The nociceptive responses were recorded from 15 min after drug injection to 90 min after drug administration. Numbers inside the columns indicate the number of mice.

carinic autoreceptors (Lapchak *et al.*, 1989; Töröcsik and Vizi, 1991; McKinney *et al.*, 1993; Stillman *et al.*, 1993) and R-(+)-hyoscyamine showed a very high affinity for the prepuberal guinea-pig uterus putative M_4 receptors (Ghelardini *et al.*, 1993), the (±)-SM-21 affinity profile towards muscarinic receptor subtypes was investigated in vitro. The affinity profile of (±)-SM-21 versus M_1 (rabbit vas deferens), M_2 (guinea-pig atrium), M_3 (guinea-pig ileum) and putative M_4 receptors (prepuberal guinea-pig uterus) was evaluated by in vitro functional studies. The M_4 muscarinic receptor subtype has been defined as putative since it has not been confirmed that the mRNA codifying M_4 is expressed in the prepuberal

uterus tissue. However, pharmacological and biochemical studies show that the M₄ putative receptor of prepuberal guinea-pig uterus has a pharmacological and biochemical profile identical to that of the muscarinic m₄ receptor subtype expressed in the rat striatum (McKinnev et al., 1991; Waelbroeck et al., 1992) and in NG 108-15 cells (Leiber et al., 1984; Marc et al., 1986). (±)-SM-21 showed, unlike R-(+)-hyoscyamine, a very low M₄/M₁ muscarinic receptor subtype selectivity ratio (twice), but higher M₂/M₁ selectivity ratio (4.6 times). By comparing the affinity profile of the M₂ muscarinic antagonists: AFDX-116 (Giachetti et al., 1986), methoctramine (Melchiorre et al., 1987) and AQRA-741 (Doods et al., 1991), a selectivity ratio M_2/M_1 lower than that showed by (\pm) -SM-21 can be observed. Moreover, all the above-mentioned M_2 antagonists, like (±)-SM-21, are also endowed with cholinergic presynaptic antinociceptive properties (Bartolini et al., 1989; Gualtieri et al., 1989; Ghelardini et al., 1991) and AFDX-116 and methoctramine are able to increase the ACh release (Lapchak et al., 1989; Töröcsik and Vizi, 1991). It seems, therefore, reasonable to suppose that a selectivity ratio of 4.6, even if small, may be high enough to enhance the pain threshold as a consequence of ACh release. The antinociception induced by (\pm) -SM-21 may be due to the antagonism of the M₂ muscarinic autoreceptor. The selectivity on blocking the M2/M4 towards M1 was evaluated since Bartolini et al., (1992) have demonstrated that the muscarinic postsynaptic receptor responsible for central cholinergic antinociception belongs to the M_1 subtype. The antinociceptive efficacy of (\pm) -SM-21 was greater than that of R-(+)-hyoscyamine, AFDX-116, methoctramine or AQRA-741. Therefore, we cannot exclude that other mechanisms able to potentiate the endogenous cholinergic system may be involved in the antinociception induced by (\pm) -SM-21.

Both enantiomers of SM-21, R-(+) and S-(-), contrary to atropine in which the analgesic activity resides only in the R-(+) isomer (Ghelardini *et al.*, 1992), showed very similar antinociceptive properties in the presence of either a thermal or chemical stimulus. However, in both analgesic tests used,

TABLE 2

Effect of physostigmine on (±)-SM-21, AFDX-116 and R-(+)-hyoscyamine antinociception in the mouse hot-plate test

		Licking L	atency(s)			
Treatment	Before		After Treatment			
	Pretreatment	15 min	30 min	45 min		
Saline	13.9 ± 0.9	14.3 ± 1.6	14.6 ± 1.2	13.8 ± 1.5		
	(8)	(8)	(8)	(8)		
Physostigmine	15.7 [`] ± 1.1	16.2 ± 1.5	14.1 ± 1.2	13.6 ± 1.4		
0.05 mg kg^{-1} i.p.	(15)	(15)	(15)	(15)		
(±)-SM-21	14.1 ± 1.0	$33.8 \pm 1.6^{*}$	$27.5 \pm 1.3^{*}$	$25.7 \pm 1.5^{*}$		
$30 \text{ mg kg}^{-1} \text{ s.c.}$	(18)	(18)	(18)	(18)		
AFDX-116	13.9 ± 1.3	22.1 ± 1.3*	$18.3 \pm 1.2^{\circ}$	15.2 ± 1.2		
6.3 ng/mouse i.c.v.	(12)	(12)	(12)	(12)		
R-(+)-hyoscyamine	14.2 ± 0.9	$25.2 \pm 0.7^{*}$	$20.4 \pm 0.8^{*}$	15.1 ± 1.1		
5 μ g kg ⁻¹ s.c.	(25)	(25)	(25)	(25)		
Physostigmine +	14.5 ± 0.8	$32.7 \pm 1.3^{*}$	$28.4 \pm 1.6^{*}$	$23.8 \pm 1.3^{*}$		
(±)-SM-21	(10)	(10)	(10)	(10)		
Physostigmine +	14.4 ± 0.5	31.6 ± 2.1*°	29.8 ± 2.7*°	22.4 ± 1.9*°		
AFDX-116	(10)	(10)	(10)	(10)		
Physostigmine +	13.8 ± 0.9	$35.2 \pm 1.4^{*\circ}$	32.1 ± 2.2*°	23.3 ± 1.3*°		
R-(+)-hyoscyamine	(16)	(16)	(16)	(16)		

The number of mice is shown in parentheses. Physostigmine was injected 5 min before the other drugs. P < .05; * P < .01 in comparison with saline controls. $^{\circ}$ P < .05 vs. the corresponding group treated with the same analgesic drug.

TABLE 3

Effects of atropine, pirenzepine, HC-3, naloxone, CGP-35348, RAMH, quinpirole, GR-48125, N⁶-cyclopentyladenosine and NAN-190 on antinociception induced by (±)-SM-21 in the mouse hot-plate test

			п	Licking Latency(s)			
Pretreatment	Treatment	$\mathrm{mg}\cdot\mathrm{kg}^{-1}$ s.c.		Before Pretreatment	After Treatment		
				Boloro Protocalmont	15 min	30 min	45 min
Saline 10 ml kg ⁻¹ i.p.	Saline		44	14.4 ± 0.6	14.1 ± 1.1	13.6 ± 1.2	14.0 ± 0.9
Saline 5 µl i.c.v.	Saline		28	13.7 ± 0.7	14.3 ± 1.0	14.5 ± 0.8	14.3 ± 0.7
Saline i.p. or i.c.v.	(±)-SM-21	30	41	15.8 ± 0.7	$33.7 \pm 0.2^{*}$	$28.1 \pm 0.7^{*}$	$24.3\pm0.6^{*}$
Atropine 5 mg kg ⁻¹ i.p.	Saline		15	14.3 ± 0.6	13.8 ± 0.8	14.1 ± 1.2	14.4 ± 1.6
	(±)-SM-21	30	18	14.9 ± 0.9	18.8 ± 1.7°	$18.0 \pm 1.5^{\circ}$	$14.5 \pm 1.6^{\circ}$
Pirenzepine 0.1 μ g mouse	Saline		10	13.9 ± 1.3	14.5 ± 1.7	14.2 ± 2.0	15.0 ± 1.6
i.c.v.	(±)-SM-21	30	10	14.0 ± 1.0	$18.3 \pm 2.1^{\circ}$	18.2 ± 2.3°	$17.6 \pm 1.8^{\circ}$
HC-3 1 μ g per mouse	Saline		14	14.8 ± 1.0	14.1 ± 1.7	13.8 ± 1.2	14.1 ± 2.1
i.c.v.	(±)-SM-21	30	11	13.6 ± 0.8	18.7 ± 2.2°	18.1 ± 1.9°	$16.7 \pm 1.6^{\circ}$
Naloxone 1 mg kg ⁻¹	Saline		13	15.0 ± 0.8	13.9 ± 1.3	14.8 ± 1.3	15.0 ± 1.4
i.p.	(±)-SM-21	30	8	15.2 ± 1.2	$30.8 \pm 2.2^{*}$	$27.6 \pm 2.3^{*}$	$26.1 \pm 2.3^{*}$
CGP 35348 2.5 µg mouse	Saline		10	14.1 ± 0.7	$12.6 \pm 1.8^{\circ}$	13.1 ± 2.1	12.8 ± 1.6
i.c.v.	(±)-SM-21	30	8	14.5 ± 1.0	$35.1 \pm 2.0^{*}$	$31.2 \pm 2.4^{*}$	$26.0 \pm 1.9^{*}$
RAMH 10 mg kg ⁻¹	Saline		10	14.0 ± 0.7	12.8 ± 0.6	13.7 ± 1.1	15.8 ± 1.7
i.p.	(±)-SM-21	30	10	15.4 ± 0.6	$33.5 \pm 2.8^{*}$	$28.6 \pm 3.6^{*}$	$25.7 \pm 2.8^{*}$
Quinpirole 0.1 mg kg ⁻¹	Saline		10	15.0 ± 1.0	14.2 ± 2.0	14.3 ± 1.8	14.1 ± 1.6
i.p.	(±)-SM-21	30	10	13.7 ± 1.2	$34.1 \pm 2.2^{*}$	27.1 ± 1.7*	$25.6 \pm 2.0^{*}$
GR 48125 20 mg kg ⁻¹	Saline		10	13.7 ± 1.1	13.2 ± 1.9	14.1 ± 1.7	14.4 ± 1.5
i.p.	(±)-SM-21	30	10	14.6 ± 0.6	$31.5 \pm 2.3^{*}$	$26.5 \pm 1.9^{*}$	$22.7 \pm 2.2^{*}$
N ⁶ -CPA 5 μg mouse	Saline		12	13.9 ± 0.7	15.0 ± 0.9	15.7 ± 1.1	16.4 ± 1.7
i.c.v.	(±)-SM-21	30	8	15.2 ± 0.9	$30.7 \pm 3.0^{*}$	$33.1 \pm 3.0^{*}$	$26.7 \pm 2.1^{*}$
NAN-190 0.5 μ g mouse	Saline		12	15.2 ± 1.3	14.7 ± 1.5	13.2 ± 1.5	14.9 ± 1.7
i.c.v.	(±)-SM-21	30	9	14.4 ± 0.8	$34.7 \pm 2.1^{*}$	$29.5 \pm 1.7^{\star}$	$22.1 \pm 1.5^{*}$

* P < .01; ^ P < 0.05 in comparison with saline-saline; °P < .01 vs. saline-(±)-SM-21-treated mice. N⁶-cyclopentyladenosine = N⁶ CPA.

ABLE 4		
Effect of (±)-SM-21, R-(+)-SM-2	21 and S-(-)-SM-21 in the	mouse rota-rod test

		Number of Falls in 30 s					
Treatment	Dose	Defere Dretreatment		After Treatment			
		Delote Pretreatment	15 min	30 min	45 min		
Saline	10 mg kg ⁻¹ s.c.	3.2 ± 0.3	1.8 ± 0.3	1.4 ± 0.2	0.8 ± 0.2		
(±)-SM-21	$30 \text{ mg kg}^{-1} \text{ s.c.}$	2.8 ± 0.2	1.9 ± 0.3	1.5 ± 0.5	1.2 ± 0.5		
(±)-SM-21	40 mg kg ^{-1} s.c.	3.3 ± 0.4	1.6 ± 0.6	1.2 ± 0.4	1.2 ± 0.4		
(±)-SM-21	50 mg kg ^{-1} s.c.	2.8 ± 0.6	2.2 ± 0.4	$2.6\pm0.5^{\star}$	$2.4 \pm 0.5^{*}$		
(±)-SM-21	60 mg kg ^{-1} s.c.	2.7 ± 0.4	$3.5\pm0.5^{\star}$	$4.3 \pm 0.4^{\star}$	$4.4 \pm 0.6^{*}$		
R-(+)-SM-21	30 mg kg ^{-1} s.c.	2.9 ± 0.3	2.0 ± 0.4	1.5 ± 0.3	1.4 ± 0.4		
R-(+)-SM-21	40 mg kg ^{-1} s.c.	3.1 ± 0.4	$3.6\pm0.5^{\star}$	$4.1 \pm 0.4^{*}$	$3.5 \pm 0.5^{*}$		
S-(–)-SM-21	30 mg kg ^{-1} s.c.	3.0 ± 0.3	2.1 ± 0.4	1.6 ± 0.2	0.8 ± 0.4		
S-(–)-SM-21	40 mg kg ^{-1} s.c.	2.8 ± 0.4	$3.4\pm0.5^{\star}$	$3.4 \pm 0.4^{*}$	$2.5 \pm 0.6^{*}$		
Saline	5 μl i.c.v.	2.5 ± 0.4	1.8 ± 0.3	1.1 ± 0.3	0.7 ± 0.2		
(±)-SM-21	20 μg i.c.v.	2.9 ± 0.5	2.2 ± 0.4	1.2 ± 0.3	1.0 ± 0.2		
(±)-SM-21	40 µg i.c.v.	2.4 ± 0.4	$3.5\pm0.3^{\star}$	$3.3\pm0.4^{\star}$	$2.2 \pm 0.4^{*}$		
Saline	10 ml kg ⁻¹ p.o.	2.8 ± 0.3	2.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.4		
(±)-SM-21	60 mg kg^{-1} p.o.	2.6 ± 0.4	1.5 ± 0.4	1.3 ± 0.4	0.6 ± 0.2		
(±)-SM-21	90 mg kg ⁻¹ p.o.	3.0 ± 0.4	$3.2\pm0.5^{\star}$	$2.9\pm0.3^{*}$	$2.4 \pm 0.4^{\star}$		
Saline	10 ml kg ^{-1} i.v.	3.1 ± 0.3	2.8 ± 0.3	2.3 ± 0.4	1.2 ± 0.2		
(±)-SM-21	20 ml kg ^{-1} i.v.	2.8 ± 0.4	2.4 ± 0.3	1.3 ± 0.3	1.1 ± 0.3		
(±)-SM-21	30 ml kg ⁻¹ i.v.	2.6 ± 0.3	$3.9\pm0.4^{\star}$	$3.3\pm0.3^{\star}$	$2.4 \pm 0.3^{*}$		

Each value represents the mean of 5 to 10 mice.

* P < .01 in comparison with saline controls.

R-(+)-SM-21 resulted weakly more effective than S-(-)-SM-21. Since R-(+) and S-(-)-SM-21 were not endowed with a different analgesic profile, their in vitro selectivity towards the muscarinic receptor subtypes was not considered worth investigating.

It has been demonstrated that D_2 dopaminergic (Gorell and Czarnecki, 1986; Wedzony *et al.*, 1988; Scatton, 1992; Imperato *et al.*, 1993), A_1 adenosinergic (Jackisch *et al.*, 1984; Carter *et al.*, 1995), H_3 histaminegic (Clapham and Kilpatrick, 1992), 5-HT₄ serotoninergic heteroreceptors (Consolo *et al.*, 1994), all located on central cholinergic neurones, increase ACh release. The involvement of the above-mentioned heteroreceptors was, therefore, investigated. Quinpirole (D₂ agonist), N⁶-cyclopentyladenosine (A₁ agonist), R-(α)-metylhistamine (H₃ agonist), and GR-48125 (5-HT₄ antagonist), at doses able to prevent the antinociception in-

Affinity profiles of (±)-SM-21, R-(+)-hyoscyamine and AFDX-116 at muscarinic M_1 receptors in rabbit vas deferens, M_2 receptors in guinea pig left atrium, M_3 receptors in guinea pig ileum and M_4 putative receptors in guinea pig uterus. The ratios of affinity constants are given as a measure of receptor selectivity

	pA ₂ Values					Selectivity Ratios	
Drug	M ₁ Rabbit Vas Deferens	M ₂ Rat Left Atrium	M ₃ Rat Ileum	M ₄ -putative Guinea Pig Uterus	M ₂ /M ₁	M ₄ /M ₁	
(±)-SM-21	5.97 ± 0.11*	6.63 ± 0.10*	$6.35 \pm 0.04^{*}$	$6.26 \pm 0.05^{*}$	4.6	1.9	
R-(+)-hyoscyamine AFDX-116	$\begin{array}{l} 7.05\pm0.05^{a} \\ 6.84\pm0.14^{b} \end{array}$	7.25 ± 0.04 ^a 7.12 ± 0.11 ^b	$\begin{array}{l} 6.88 \pm 0.05^{a} \\ 6.34 \pm 0.13^{c} \end{array}$	$\begin{array}{l} 9.56 \pm 0.01^{a} \\ 6.70 \pm 0.06 \end{array}$	1.6 1.9	323 0.7	

* pK_B values are obtained with (±)-SM-21 1 μ M. Each value is the mean of at least five experiments. pA_2 values are the mean ± S.E.M. of 12 to 16 preparations. Selectivity ratios were calculated as antilogs of the difference between pK_B or pA_2 values.

^a Ghelardini et al., (1993); ^b Eltze (1988); ^c Eltze and Figala (1988).

duced respectively by haloperidol, caffeine (Ghelardini et al., 1992), thioperamide (Malmberg-Aiello et al., 1994), BIMU 1 and BIMU 8 (Ghelardini *et al.*, 1996), failed to prevent (\pm) -SM-21 antinociception. Previous data have shown that antinociception induced by (\pm) -SM-21 and its enantiomers was partially prevented by the 5-HT₄ antagonist SDZ 205–557 (Romanelli et al., 1995). Since GR-48125 was more selective than SDZ 205–557 towards 5-HT₄ receptors and was not able to antagonize SM-21 antinociception, the prevention of the SM-21 effect produced by SDZ 205-557 was probably not related to an antagonism of 5-HT₄ receptors. It has also been observed that the activation of the serotoninergic autoreceptor 5-HT_{1A} enhances ACh release from the guinea-pig cortex (Bianchi et al., 1990). Pretreatment with the 5-HT_{1A} selective antagonist NAN 190 at doses which block the antinociception induced by 5-HT_{1A} agonists (Ghelardini *et al.*, 1994), did not prevent the enhancement of the pain threshold produced by (±)-SM-21 administration. The present data suggest that the above-mentioned receptors, even though they are able to increase ACh release, are not involved in (\pm) -SM-21 mechanism of analgesic action.

The antinociception induced by antagonists of the muscarinic autoreceptors, such as R-(+)-hyoscyamine and AFDX-116, was significantly potentiated by pretreatment with a subliminary non-analgesic dose of physostigmine. By contrast, in the same experimental conditions, (±)-SM-21 antinociception was not modified by physostigmine pretreatment. These observations may suggest that (±)-SM-21 is endowed with very low anticholinesterase activity, a hypothesis that seems to be in agreement with the in vitro evaluation of the IC₅₀ value of (±)-SM-21 (IC₅₀ = $1.1 \cdot 10^{-4}$ M). It is possible that (±)-SM-21 is able to amplify cholinergic neurotransmission through the antagonism of the muscarinic autoreceptor and that this effect is in its turn potentiated by its low cholinesterase inhibitory activity.

Other neurotransmitter systems are not involved in (\pm) -SM-21 antinociception since the opioid antagonist naloxone, the GABA_B antagonist CGP-35348 and the polyamine depletor reserpine, were all unable to prevent the effect of (\pm) -SM-21. The doses and administration schedules of the abovementioned drugs were ideal for preventing antinociceptions induced respectively by morphine (Ghelardini *et al.*, 1992), the GABA_B agonist baclofen (Malcangio *et al.*, 1991) and the antidepressant drugs clomipramine and amitriptyline (Galeotti *et al.*, 1995).

 (\pm) -SM-21 at analgesic doses failed to suppress paw edema in response to carrageenan administration, suggesting that its antinociception is not due to an antiinflammatory action. In summary, our results have shown that (\pm) -SM-21 is able to produce dose-dependent antinociception in rodents and guinea pigs, without impairing motor coordination, by potentiating endogenous cholinergic activity.

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