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RS-45041-190: a selective, high-affinity ligand for I_2 imidazoline receptors

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1 RS-45041-190 (4-chloro-2-(imidazolin-2-yl)isoindoline) showed high affinity for I_2 imidazoline receptors labelled by [^{3}H]-idazoxan in rat (pK_i = 8.66 ± 0.09), rabbit (pK_i = 9.37 ± 0.07), dog (pK_i = 9.32 ± 0.18) and baboon kidney (pK_i = 8.85 ± 0.12), but had very low affinity for α_2-adrenoceptors in rat cerebral cortex (pK_i = 5.7 ± 0.09).

2 RS-45041-190 showed low affinity for other adrenoceptors, dopamine, 5-hydroxytryptamine, and muscarinic receptors and dihydropyridine binding sites (selectivity ratio > 1000).

3 RS-45041-190 showed moderate potency for the inhibition of monoamine oxidase A *in vitro* (pIC$_{50}$ = 6.12), but had much lower potency for monoamine oxidase B (pIC$_{50}$ = 4.47), neither of which equated with its affinity for I_2 receptors.

4 RS-45041-190 (0.001 to 3 mg kg^{-1}, i.v. and 1 ng–50 μg i.c.v.) had only small, transient effects on blood pressure and heart rate in anaesthetized rats. In conscious rats, RS-45041-190 had no effect on body core temperature or tail skin temperature (1 mg kg^{-1}, s.c.) or on activity or rotarod performance (10 mg kg^{-1}, i.p.). There were also no effects on barbiturate sleeping time in mice after doses of 1–10 mg kg^{-1}, i.p.

5 RS-45041-190 (10 and 25 mg kg^{-1}, i.p.) significantly increased food consumption in rats for up to 4 h after dosing, but unlike idazoxan (10 mg kg^{-1}, i.p.) did not increase water consumption.

6 RS-45041-190 is therefore a selective, high-affinity ligand at I_2 imidazoline receptors and its hyperphagic effect may suggest a role for I_2 imidazoline receptors in the modulation of appetite. However, in the absence of a selective agonist it is unclear whether this ligand is an agonist or an antagonist at I_2 receptors.

Keywords: RS-45041-190; I_2 imidazoline receptors; kidney; [^{3}H]-idazoxan; food intake; appetite

Introduction

Non-adrenoceptor imidazoline receptors have been subdivided into I_1 (high affinity for clonidine and rilmenidine, low affinity for guanabenz) and I_2 (high affinity for idazoxan and guanabenz, low affinity for clonidine) receptor subtypes (Ernsberger, 1992). The I_1 subtype appears to be involved in the central control of blood pressure (Ernsberger *et al.*, 1990), but the role of the I_2 subtype remains unclear. Some roles have been suggested following *in vitro* studies which have shown an effect of idazoxan which could not adequately be explained by α_2-adrenoceptor blockade, including the blockade of non-adrenergic non-cholinergic (NANC) relaxations of the rat anococcygeus muscle (Ramagopal & Leighton, 1989), the inhibition of Na^+/H^+ exchange in renal proximal tubule cells (Bidet *et al.*, 1990) and the inhibition of noradrenaline release in rabbit aorta and pulmonary artery (Göhert & Molderings, 1991). In addition, *in vivo* studies with idazoxan have suggested a role for I_2 receptors in neurodegeneration (Maiese *et al.*, 1992), the inhibition of prolactin release (Krulich *et al.*, 1989) and food consumption (Jackson *et al.*, 1991). Recent studies have suggested a functional interaction of I_2 receptors with monoamine oxidase, in that chronic treatment with inhibitors of this enzyme results in down-regulation of non-adrenoceptor idazoxan binding sites (Olmos *et al.*, 1993). Although suggestive of imidazoline receptor-mediated events, in most of these studies an interaction with 5-hydroxytryptamine (5-HT) receptors, α_1-adrenoceptors or indeed a subtype of α_2-adrenoceptors could not be completely ruled out. This is at least partly due to the lack of selective agents at the I_2 receptor, as most show either agonist or antagonist activity at α_1 and/or α_2-adrenoceptors. The identification of a high affinity I_2 ligand with low affinity for other receptor types would greatly assist our understanding of the functional role of imidazoline binding sites.

In this study we describe the binding characteristics, monoamine oxidase interactions and *in vivo* pharmacology of a high affinity I_2 receptor ligand, RS-45041-190 (4-chloro-2-(imidazolin-2-yl)isoindoline; Figure 1), which has > 1000 fold selectivity for I_2 receptors over a wide variety of other receptor types.

Preliminary accounts of part of this work have been presented to the British Pharmacological Society (MacKinnon *et al.*, 1995a,b).

Methods

In vitro *binding studies*

Membrane preparation Rat kidney membranes were prepared as previously described (MacKinnon *et al.*, 1993). In other binding studies, tissues from male Sprague-Dawley rats, male New Zealand white rabbits, male Beagle dogs, male and female Anubis baboons and female Dunkin Hartley guinea-pigs were used (Table 1). The tissues were rapidly removed, carefully dissected on ice and homogenized in 25 vol. (w/v) ice cold 50 nM Tris HCl, containing 5 mM EDTA (pH 8.0), with a Polytron PT10 tissue disrupter. The homogenate was cen-

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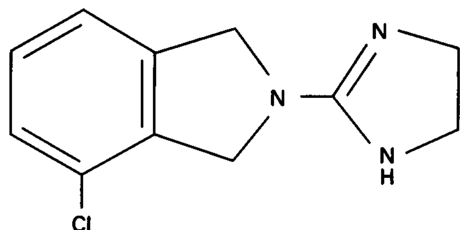

Figure 1 Structure of RS-45041-190.

trifuged at 48,000 *g* in a refrigerated centrifuge (Sorvall RC-RB) at 4°C for 15 min. The resultant pellet was washed a further three times by resuspension and centrifugation in 50 mM Tris HCl (pH 8.0) at 4°C containing 0.5 mM EDTA and the final pellet was resuspended in 3 ml 50 mM Tris HCl (pH 8.0) at 4°C and stored under liquid nitrogen until required.

Binding assays [^{3}H]-idazoxan binding to I_2 receptors on kidney or brain membranes was carried out as previously described (MacKinnon *et al.*, 1993). Rat kidney membranes (300–500 μg protein) were incubated with 1.0 nM [^{3}H]-idazoxan (Amersham, U.K., 40–50 Ci mmol^{-1}) for 90 min at 25°C in the presence of the selective α_2-adrenoceptor antagonist, delequamine (RS-15385-197 ; 0.1 μM; Brown *et al.*, 1993), and various concentrations of drugs in a final assay volume of 0.5 ml assay buffer (50 mM Tris HCl, pH 7.4 containing 0.5 mM EDTA). Non-specific binding was determined in the presence of the selective α_1-adrenoceptor antagonist, cirazoline (1 μM). In all other binding assays, membranes (0.05–0.5 mg protein) were incubated to equilibrium with radiolabelled drug in the presence of 8–15 concentrations of RS-45041-190 or a highly specific drug to define non-specific binding. Assay conditions for measuring affinity at α-adrenoceptors, β-adrenoceptors, 5-HT, dopamine and muscarinic receptors and calcium channels are summarized in Table 1. Separation of bound from free ligand was achieved by filtration over Whatman GF/B filters using a Brandel M24 cell harvester at a constant vacuum pressure of 22 mmHg. Bound radioactivity was determined by liquid scintillation spectrometry.

Competition analysis The inhibition of binding of the radioligands by competing ligands was analysed graphically to estimate the IC_{50} (concentration of competitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness. When Hill coefficients were not significantly different from unity the IC_{50} was converted to an affinity constant (K_i) using the expression derived by Cheng & Prusoff (1973).

Assay of monoamine oxidase

Monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B) activity in rat kidney homogenate was determined as described by Li *et al.* (1992). Kidneys were dissected from male Sprague-Dawley rats and frozen at −80°C until required for use. Each kidney was thawed and homogenized for 2 × 10 s in 25 ml ice cold homogenizing buffer (50 mM Tris HCl; pH 7.4 at 25°C), with a Polytron tissue homogenizer. The homogenate was centrifuged at 49,000 *g* for 15 min at 4°C and the resulting pellet resuspended in the original volume of ice cold homogenizing buffer and recentrifuged. The pellet was suspended in 25 ml ice cold phosphate buffer (KCl 120 mM, Tris HCl 20 mM, KH_2PO_4 5 mM, adjusted to pH 7.4 at 25°C with KOH) and centrifuged once more at 49,000 *g* for 15 min at 4°C. The final pellet was suspended in 10 ml ice cold phosphate buffer (3–4 mg ml^{-1} protein) and kept on ice.

Aliquots of homogenate (50 μl) were incubated in 0.2 ml final volume of phosphate buffer containing substrate and various concentrations of test compound for 30 min at 37°C. For MAO-A activity, the substrate was prepared as a stock solution of 1 μCi [^{14}C]-5-HT (50–60 mCi mmol^{-1}) in 0.4 mM unlabelled 5-HT to give a final assay concentration of 0.1 mM 5-HT and 0.05 μCi [^{14}C]-5-HT. For MAO-B activity, the substrate was made up as a stock solution of 1 μCi [^{14}C]-phenethylamine (50–60 mCi mmol^{-1}) in 0.1 mM phenethylamine to give a final assay concentration of 0.025 mM phenethylamine and 0.05 μCi [^{14}C]-phenethylamine. The reaction was stopped by the rapid addition of 0.25 ml 2 M citric acid followed by 1 ml of a 1:1 toluene:ethylacetate mixture. The tubes were vortexed and stored at 4°C for at least 30 min before centrifuging for 1 min at 400 *g*. An aliquot (0.5 ml) of the upper organic layer was removed and counted for ^{14}C in a Beckman scintillation counter.

The potency of compounds is expressed as the −log of the concentration causing a 50% inhibition of enzyme activity (pIC_{50}).

Cardiovascular effects in anaesthetized rats

Intravenous injections Male Sprague-Dawley rats (250–350 g) and male spontaneously hypertensive rats (SHR; 250–350 g) were anaesthetized with sodium pentobarbitone

Table 1 Receptor binding assay methods

Receptor	*^{3}H-ligand*	*Tissue*	*Incubation*	*Non-specific binding*
I_2	1 nM [^{3}H]-idazoxan	rat kidney	90 min 25°C	1 μM cirazoline
I_2	1 nM [^{3}H]-idazoxan	rabbit kidney	90 min 25°C	1 μM cirazoline
I_2	1 nM [^{3}H]-idazoxan	dog kidney	90 min 25°C	1 μM cirazoline
I_2	1 nM [^{3}H]-idazoxan	baboon kidney	90 min 25°C	1 μM cirazoline
α_1	0.5 nM [^{3}H]-prazosin	rat cortex	30 min 25°C	10 μM phentolamine
α_2	2 nM [^{3}H]-yohimbine	rat cortex	30 min 25°C	10 μM phentolamine
β_1	1 nM [^{3}H]-DHA	guinea-pig heart	30 min 25°C	100 μM isoprenaline
β_2	1 nM [^{3}H]-DHA	rat lung	30 min 25°C	100 μM isoprenaline
5-HT_{1A}	1 nM [^{3}H]-8-OH-DPAT	rat cortex	15 min 37°C	3 μM buspirone
5-HT_2	1 nM [^{3}H]-ketanserin	rat frontal cortex	10 min 37°C	2 μM methysergide
D_1	0.3 nM [^{3}H]-SCH23390	rat striatum	30 min 25°C	0.1 μM SCH23390
D_2	0.1 nM [^{3}H]-spiperone	rat striatum	30 min 37°C	1 μM (+) butaclamol
M_1	0.2 nM [^{3}H]-N-methylscopolamine	rat cortex	180 min 32°C	1 μM atropine
M_2	0.2 nM [^{3}H]-N-methylscopolamine	rat heart	180 min 32°C	1 μM atropine
CEB	0.2 nM [^{3}H]-PN 200-110	rat striatum	180 min 32°C	1 μM atropine

All assays were carried out in 50 mM Tris HCl assay buffer pH 7.4 (at assay temperature) containing 0.5 mM EDTA with the following additions: I_2, 0.1 μM RS-15385-197; D_1, 100 nM ketanserin; D_2, 120 mM NaCl, 1mM $MgSO_4$, 2mM $CaCl_2$, 1 mM EDTA, 100 nM ketanserin; 5-HT_{1A}, 5 mM $MgSO_4$; 5-HT_2, 4 mM $CaCl_2$ 5.7 mM ascorbate, 10 μM pargyline (no EDTA).

(60 mg kg^{-1}, i.p.). Core temperature was monitored with a rectal thermocouple and maintained at 37–38°C with a heating lamp. The trachea was intubated to facilitate respiration, a jugular vein was cannulated to permit injections of drug and anaesthetic, and a carotid artery was cannulated to record arterial blood pressure. Recordings of blood pressure and heart rate were displayed on a Lectromed Multitrace 4 chart recorder. RS-45051-190 was injected in ascending doses (0.01–10 mg kg^{-1}, i.v.) at 10 min intervals.

Intracerebroventricular injections In a separate study to examine centrally-mediated cardiovascular effects, male Sprague-Dawley rats (260–330 g) were prepared as above, then the head was fixed in a stereotaxic frame in the orientation of Paxinos & Watson's (1982) atlas. The ventricular injection system consisted of a 30G steel injection cannula connected via a length of polythene cannula to a 50 μl Hamilton syringe; the injection cannula was held inside a 22G guide cannula mounted in a micromanipulator, such that the tip of the injection cannula protruded 1.5 mm beyond the tip of the guide cannula. The injection assembly was lowered into the brain via a small burr hole (1.6 mm lateral to the mid-line and 0.8 mm caudal to Bregma) so that the tip of the injection cannula was 3 mm below the brain surface. All injections were made in a volume of 10 μl injected over 60 s, with 30 min between injections. When the records had stabilized, saline was first injected as a control; RS-45051-190 was then injected in ascending doses ($\times$10) from 5 ng to 50 μg; after completing the drug injections the location of the cannula tip in the ventricle was confirmed by injection of 10 μl India ink. In each case, the ink was distributed throughout the ventricular system as far as the spinal canal.

Effects on core and tail skin temperature in unanaesthetized rats

The methods used have been described previously (Redfern *et al.* 1995). Male Sprague-Dawley rats weighing 200–330 g were used. The experiments were conducted at an ambient temperature (T_a) of 18.5–20.0°C. The rats were lightly restrained in well-ventilated perspex cages (length 18 cm, width 12 cm, height 10 cm). Core temperature was monitored by a rectal thermocouple, and tail skin temperature (T_{ts}) was measured with a flat disc thermocouple (diameter 7 mm) taped to the dorsal skin of the tail, 7 cm from the tip. Readings of T_a, T_c and T_{ts} were taken at 5 min intervals, beginning 60 min after preparing the animals. Immediately after the fourth reading the rats were injected subcutaneously with drug or vehicle (1 ml kg^{-1}, s.c.; $n=6$ per group), and the responses followed over 1 h.

General CNS effects

Barbiturate sleeping time Male CD1 mice (18–30 g) were injected with hexobarbitone (60 mg kg^{-1}, i.p.) 10 min after injection of RS-45041-190 (10 mg kg^{-1}, s.c.) or saline (5 ml kg^{-1}, s.c.). Body temperature was maintained with a heating blanket. The time to regain the righting reflex was noted.

Activity Male Sprague-Dawley rats (250–300 g) were injected with either RS-45041-190 (10 mg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.) and then placed in transparent plastic cages (22$\times$38 cm base, 15 cm high) positioned between infrared beam activity meters (Opto-Varimex Mini, Columbus Instruments, U.S.A.). Total activity, ambulatory activity and rears were measured over a period of 9 min.

Motor co-ordination Following a pre-trial to eliminate poor performers, male Sprague-Dawley rats (250–300 g) were injected with either RS-45041-190 (10 mg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.) and then placed on an accelerating rotarod (Ugo Basile Instruments, Italy) 30 min later. The latency to fall off was noted.

Effects on food and water consumption

Male Sprague Dawley rats (10 rats per group) were singly housed with free access to pellet diet (RM1(E)SQC) and water. The food and water were weighed immediately prior to dosing. The rats received a single dose of RS-45041-190 (1, 10 and 25 mg kg^{-1}), idazoxan (10 mg kg^{-1}) or distilled water (5 ml kg^{-1}) at 10 h 00 min on the day of the study and the food and water were re-weighed at 1, 2, 4 and 24 h post dose.

Chemicals and drugs

Reagents and chemicals used were of the highest analytical grade available. Compounds were kindly donated by their manufacturers, synthesized at the Institute of Organic Chemistry, Syntex, (Palo Alto) or purchased. The following compounds were used: (-)-adrenaline bitartrate, (-)-noradrenaline bitartrate, isoprenaline HCl, naphazoline HCl, yohimbine HCl, clonidine HCl (Sigma); *p*-aminoclonidine HCl, clorgyline HCl, selegiline HCl and (+)-butaclamol (Research Biochemicals Incorporated); atropine sulphate (BDH); cirazoline HCl (Synthelabo); phentolamine mesylate (Ciba Geigy); cimetidine (Smith, Kline & Beecham); guanabenz (Wyeth); delequamine HCl (RS-15385-197), buspirone HCl, nifedipine HCl and idazoxan HCl were synthesized by Dr R. Clark, Syntex Palo Alto, California, U.S.A., methysergide maleate (Sandoz); SCH 23390 maleate (R(+)7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5 tetrahydro-1H-3 benzazepine hydrochloride; Schering); prazosin HCl (Pfizer); [^{3}H]-idazoxan (40 Ci $mmol^{-1}$), [^{3}H]-5-HT (40 Ci $mmol^{-1}$), [^{3}H]-8-OH-DPAT (220 Ci $mmol^{-1}$) and [^{3}H]-prazosin (86 Ci $mmol^{-1}$) from Amersham, U.K.; [^{3}H]-yohimbine (89 Ci $mmol^{-1}$), [^{3}H]-ketanserin (76 Ci $mmol^{-1}$), [^{3}H]-spiperone (80 Ci $mmol^{-1}$), [^{3}H]-SCH23390 (80.4 Ci $mmol^{-1}$), [^{3}H]-n-methyl scopolamine (71.3 Ci $mmol^{-1}$), [^{3}H]-PN200 110 (isopropyl 4-[2,1,3-benzoxadiazol-4-yl]-1,4-dihydro-2,6-dimethyl-pyridine-3,5-dicarboxylic acid methyl 1-methyl ethyl ester; 71.5 Ci $mmol^{-1}$) and [^{3}H]-dihydroalprenolol (100 Ci $mmol^{-1}$) from DuPont, U.K.

Results

Binding studies

Previous studies have shown that inclusion of 0.1 μM delequamine (RS-15385-197; Brown *et al.*, 1993) in binding assays

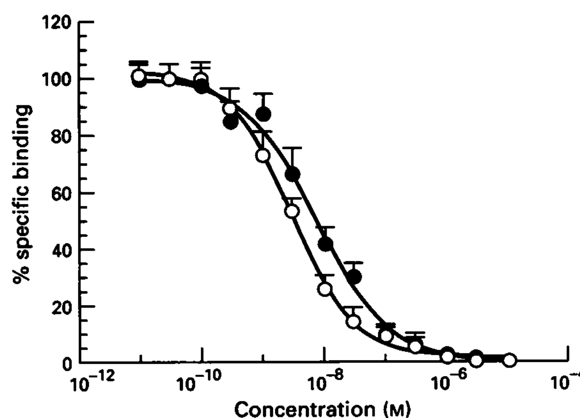

Figure 2 [^{3}H]-idazoxan binding to I_2 receptors on rat kidney membranes. The results represent the mean$\pm$s.e.mean of 3 experiments performed in duplicate: (●) idazoxan; (○) RS-45041-190. RS-45041-190 and idazoxan displaced 100% of the specific binding with high affinity (RS-45041-190 $pK_i=8.66\pm0.09$, $n_H=0.92\pm0.08$; idazoxan $pK_i=8.06\pm0.16$, $n_H=1.00\pm0.01$).

Table 2 Affinity values (pK_i) of RS-45041-190 for a variety of receptor subtypes

Receptor	*Ligand*	*Tissue*	pK_i
I_2	[^{3}H]-idazoxan	rat kidney	8.66±0.09
I_2	[^{3}H]-idazoxan	rabbit kidney	9.37±0.07
I_2	[^{3}H]-idazoxan	dog kidney	9.32±0.18
I_2	[^{3}H]-idazoxan	baboon kidney	8.85±0.12
I_2	[^{3}H]-idazoxan	rat brain	8.61±0.10
α_1	[^{3}H]-prazosin	rat cortex	<5
α_2	[^{3}H]-yohimbine	rat cortex	5.70±0.09
β_1	[^{3}H]-dihydroalprenolol	guinea-pig heart	<5
β_2	[^{3}H]-dihydroalprenolol	rat lung	<5
5-HT_{1A}	[^{3}H]-8-OH-DPAT	rat cortex	5.03±0.09
5-HT_2	[^{3}H]-ketanserin	rat frontal cortex	<5
D_1	[^{3}H]-SCH23390	rat striatum	<5
D_2	[^{3}H]-spiperone	rat striatum	<5
M_1	[^{3}H]-N-methylscopolamine	rat cortex	<5
M_2	[^{3}H]-N-methylscopolamine	rat heart	<5
CEB	[^{3}H]-PN 200-110	rat striatum	<5

Assays were performed as described in Table 1. The results are expressed as the pK_i mean±s.e.mean of 3 experiments performed in duplicate.

with [^{3}H]-idazoxan is sufficient to preclude binding to α_2-adrenoceptors (MacKinnon *et al.*, 1993), so that [^{3}H]-idazoxan will then exclusively label I_2 imidazoline receptors. The specificity of I_2 binding was defined as that displaceable with 1 μM cirazoline, and represented >85% of the total binding in rat, rabbit, dog and baboon kidney. RS-45041-190 had high affinity for I_2 receptors in kidney tissue from rats (pK_i=8.66±0.09; Figure 2), rabbits (pK_i=9.37±0.07), dogs (pK_i=9.32±0.18) and baboons (pK_i=8.85±0.12), and in rat brain (pK_i=8.61±0.10; Table 2).

The selectivity of RS-45041-190 for I_2 receptors over α_2-adrenoceptors was evaluated in binding studies with [^{3}H]-yohimbine in rat cerebral cortical membranes where RS-45041-190 was found to have low affinity (pK_i=5.70±0.09; Table 2). RS-45041-190 was devoid of affinity at α_1, β_1, β_2, 5-HT_{1A}, 5-HT_2, D_1, D_2, M_1 and M_2 receptors and the dihydropyridine binding site (Table 2).

Effects of monoamine oxidase

The potencies of compounds for the inhibition of MAO-A and MAO-B activity in rat kidney homogenates are shown in Table 3. The selective MAO-A inhibitor, clorgyline, inhibited MAO-A activity (pIC_{50}=7.75) but had much lower potency as an inhibitor of MAO-B (pIC_{50}=4.64), whereas the selective MAO-B inhibitor, selegiline, inhibited MAO-B (pIC_{50}=6.79), with lower potency at MAO-A (pIC_{50}=5.19). Clonidine and idazoxan had no effect on the inhibition of MAO-A or MAO-B activity. RS-45041-190 and guanabenz had moderate potency for inhibition of MAO-A (pIC_{50}=6.12 and 6.59 respectively) and lower potency for MAO-B (pIC_{50}=4.47 and 4.42 respectively). The low potency of idazoxan, clonidine and RS-45041-190 suggests that affinity for I_2 imidazoline receptors does not relate to functional inhibition of MAO-A or MAO-B in the rat kidney.

Table 3 Inhibition of monoamine oxidase-A (MOA-A) and MAO-B activity in rat kidney

	MAO-A (pIC_{50})	*MAO-B* (pIC_{50})
Clorgyline	7.75±0.06	4.64
Selegiline	5.19	6.79±0.06
RS-45041-190	6.12±0.10	4.47±0.06
Idazoxan	<4	<4
Guanabenz	6.59±0.26	4.42±0.07
Clonidine	<4	<4

Monoamine oxidase activity was determined as described in Methods. The results represent the mean±s.e.mean of at least 3 experiments performed in duplicate.

Cardiovascular effects

Bolus intravenous injections of RS-45041-190 (1–10,000 μg kg^{-1}) elicited only slight, transient decreases in blood pressure in both normotensive and spontaneously hypertensive rats anaesthetized with pentobarbitone (data not shown). There were no obvious effects on heart rate. Following injection into a lateral cerebral ventricle, RS-45041-190 had no definite effects on blood pressure or heart rate in doses up to

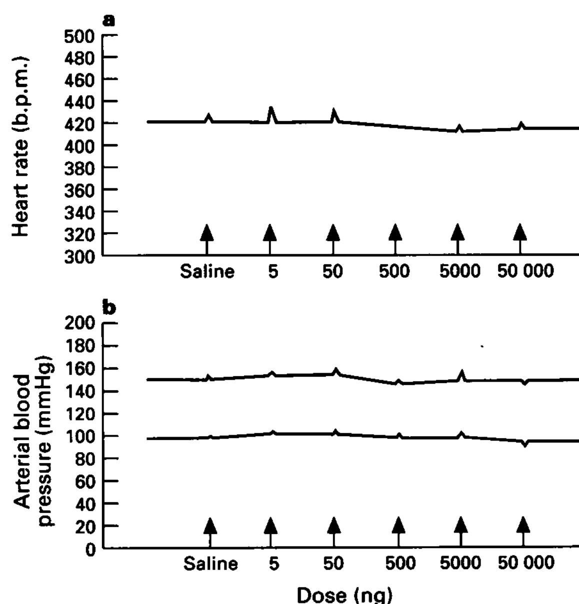

Figure 3 Effects of centrally-administered RS-45041-190 on (a) heart rate and (b) blood pressure in pentobarbitone-anaesthetized male Sprague-Dawley rats (n=5). These graphs show schematically the initial short-lived response followed by the change in baseline before the next dose administered 30 min later. Standard error bars omitted for clarity; maximum standard errors: 10 mmHg and 26 b.p.m.

50 μg i.c.v. (i.e. a 5 mg ml^{-1} solution, the limit of solubility; Figure 3).

Effects on core and tail skin temperature

RS-45041-190 (1 mg kg^{-1}, s.c.) had no effect on core (rectal) or tail skin temperature when compared to a saline-treated control group ($n=6$ per group), measured for up to 1 h after injection (Figure 4).

General CNS effects

RS-45041-190 had no effect on barbiturate sleeping time in mice (1, 3 or 10 mg kg^{-1}, i.p.; Figure 5). Although there was a trend towards a reduction in spontaneous activity in rats following administration of RS-45041-190 (10 mg kg^{-1}, s.c.; Figure 6), this was not significant. The same dose did not affect rotarod performance in rats.

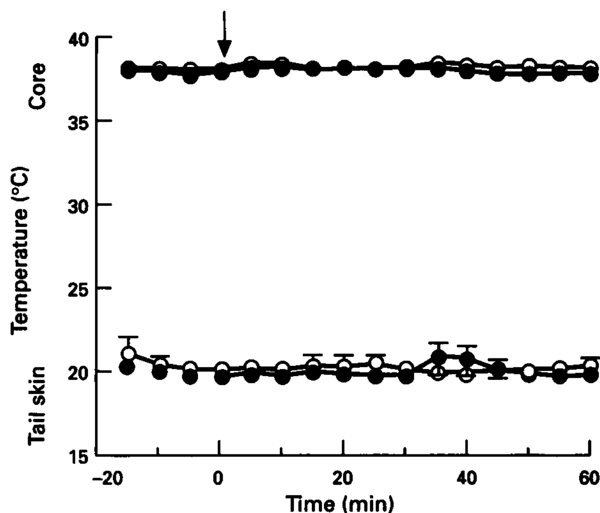

Figure 4 Effects of RS-45041-190 (1 mg kg^{-1}, s.c.) on core (rectal) temperature and dorsal tail skin temperature (measured 7 cm from the tip) in conscious, lightly restrained rats ($n=6$ per group). (○) Saline 1 ml kg^{-1}, s.c.; (●) RS-45041-190. Injection at arrow. Values are means with s.e.mean, $n=6$. The drug had no effects compared to the saline control group.

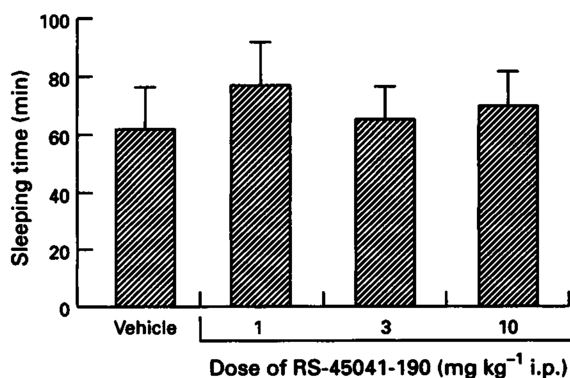

Figure 5 Effects of pretreatment with RS-45041-190 on barbiturate sleeping time in mice. Groups of 6 mice were given an injection of sodium hexobarbitone (60 mg kg^{-1}, i.p.) 10 min after dosing with 1, 3 or 10 mg kg^{-1} RS-45041-190 or saline (1 ml kg^{-1}, i.p.). Body temperature was maintained with a heating lamp. The sleeping time was taken as the duration of the loss of the righting reflex. The drug had no effects compared to the saline control group.

Effects on food and water consumption

As a first step, we compared the effect of a single dose of RS-45041-190 with idazoxan. The effect of 10 mg kg^{-1} idazoxan and 25 mg kg^{-1} RS-45041-190 on food and water consumption in male rats is shown in Figure 7. Idazoxan and RS-45041-190 significantly increased food consumption 1 h post-dose ($P<0.05$) and RS-45041-190 was effective 2 h post-dose ($P<0.05$). Neither compound increased food consumption significantly at 4 h post-dose. Idazoxan produced a significant increase in water consumption 1, 2 and 4 h post-dose ($P<0.05$). RS-45041-190 had no effect on water consumption at any time point tested. Neither idazoxan nor RS-45041-190 had any effect on food or water consumption at 24 h post dose.

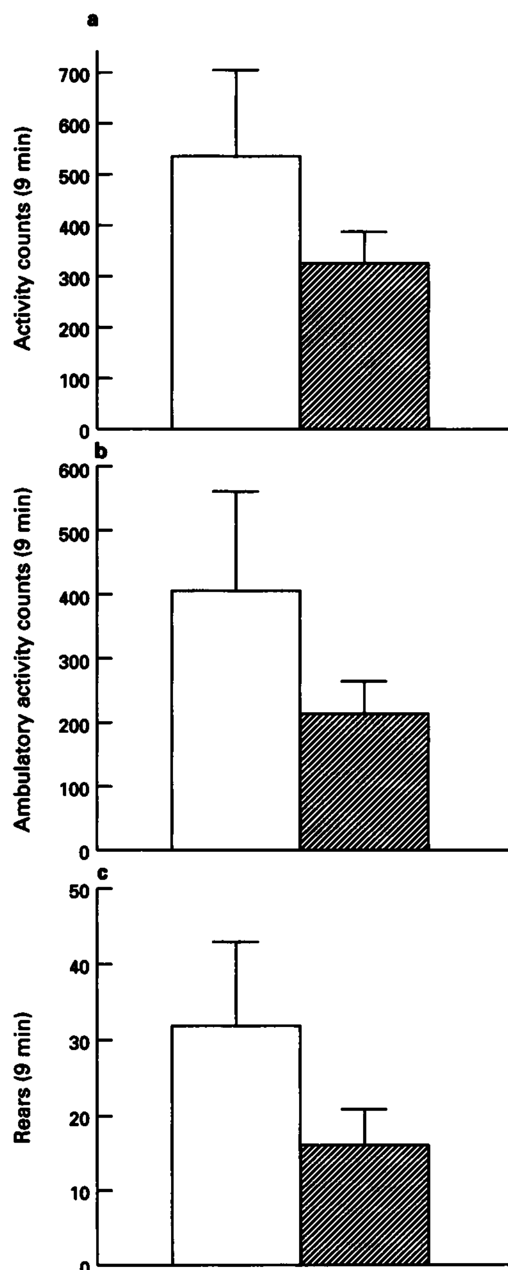

Figure 6 Effect of RS-45041-190 on (a) total activity, (b) ambulatory activity and (c) rearing in male Sprague-Dawley rats placed in an unfamiliar cage. The measurements were made with infrared photocell activity meters, over a 9 min period beginning 30 min after dosing. Open columns: saline (1 ml kg^{-1}, s.c.; $n=6$); hatched columns; RS-45041-190 (10 mg kg^{-1}, s.c.; $n=6$). Although there appears to be a trend towards a reduction in activity in the drug-treated group, this did not reach significance.

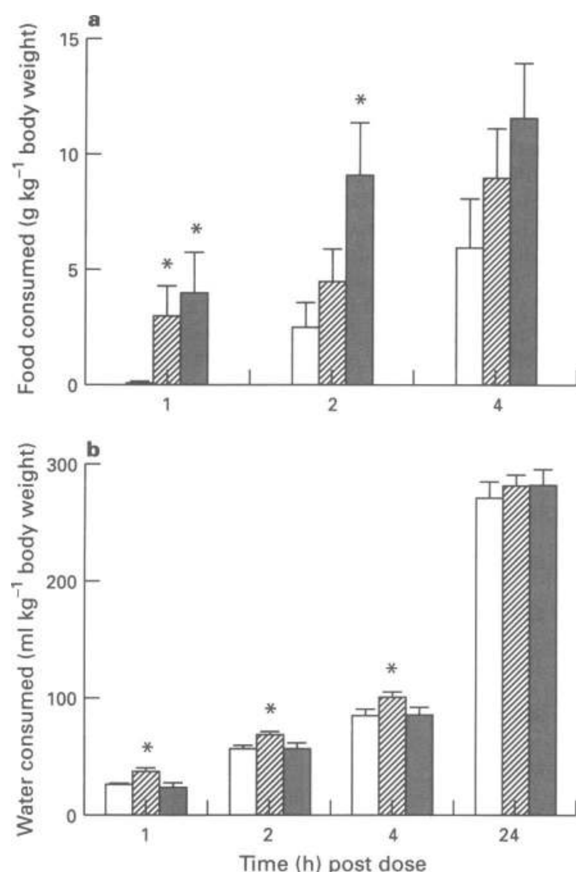

Figure 7 Effect of idazoxan and RS-45041-190 on cumulative food and water consumption in male Sprague-Dawley rats (weight range 190-195 g). The data represent the mean ± s.e.mean of 10 animals per treatment group: (a) food consumption; (b) water consumption. Data for the 24 h food consumption have been omitted for clarity. At each time point, columns are (from left to right) water (5 ml kg^{-1}, i.p.), idazoxan (10 mg kg^{-1}, i.p.) and RS-45041-190 (25 mg kg^{-1}, i.p.). Statistical significance from corresponding time control estimated by two way ANOVA. *Significantly different from control ($P<0.05$).

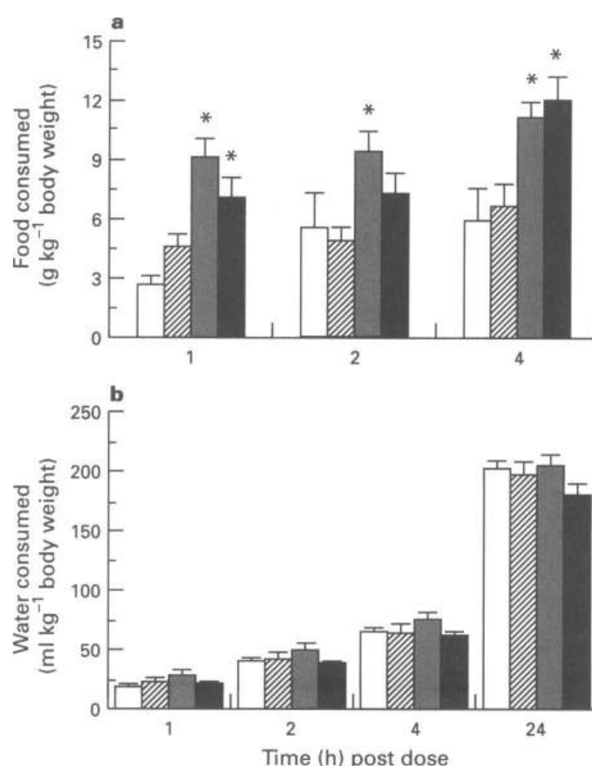

Figure 8 Effect of RS-45041-190 (1, 10 and 25 mg kg^{-1}, i.p.) on cumulative food and water consumption in male Sprague-Dawley rats (weight range 265-315 g). The data represent the mean ± s.e.mean of 10 animals/group: (a) food consumption; (b) water consumption. Data for the 24 h food consumption have been omitted for clarity. At each time point, columns are (from left to right) water (5 ml kg^{-1}, i.p.) and RS-45041-190 (1, 10 and 25 mg kg^{-1}, i.p.). Statistical significance from corresponding time control estimated by two way ANOVA. *Significantly different from control ($P<0.05$).

We then examined the effects of a range of doses of RS-45041-190. The effect of 1, 10 and 25 mg kg^{-1} RS-45041-190 on food and water consumption is shown in Figure 8. RS-45041-190 at 10 and 25 mg kg^{-1} increased food consumption 1, 2 and 4 h post-dose, although in this study, significance was not reached at 2 h with the 25 mg kg^{-1} group. The effect of RS-45041-190 was maximal at the 10 mg kg^{-1} dose. Following a dose of 1 mg kg^{-1} RS-45041-190 there was a trend towards an increased food consumption at 1 h post-dose, but this did not reach significance. There was no significant effect on food consumption at any dose after 24 h. RS-45041-190 had no effect on water consumption at any dose or time point tested.

Discussion

In the mid-1980s, following extensive structure-activity studies with analogues of idazoxan, it was suggested that three major binding regions exists for this type of compound at the α_2-adrenoceptor (Stillings *et al.*, 1985). These were a planar hydrophobic area that interacts with the benzene ring, a site that binds one or both of the benzodioxan oxygens and an imidazoline binding region. Subsequently, we showed, using a series of substituted 2-(tetrahydroisoquinolin-2-yl methyl) and 2-(isoindolin-2-yl methyl) imidazolines, that α_2-adrenoceptor antagonist potency could be maintained with the proviso that an addition methylene spacer was introduced between the planar and imidazoline rings and that the nitrogen of the isoquinoline or isoindoline group bound to the benzodioxan site (Clark *et al.*, 1990).

Zonnenschein *et al.* (1990) investigated a series of substituted imidazoline and guanido structures and suggested that shortening the link between the aromatic group and the imidazoline/guanido group, whilst decreasing α_2-adrenoceptor affinity, increased affinity for imidazoline sites labelled by [^{3}H]-idazoxan in rat liver. In this case the guanido group presumably occupied the imidazoline binding region of the α_2-adrenoceptor. In addition, substitution of halogen groups on the aromatic moeity facilitated binding to the imidazoline binding site whilst α_2-adrenoceptor affinity was unaffected. Thus, it appears that shortening the link between the planar and imidazoline ring and halogen substitution on the former ring would facilitate imidazoline site *vs* α_2-adrenoceptor selectivity. RS-45041-190 (Figure 1) is a 5-chloroisoindoline imidazoline compound with a single link bond between the indoline and imidazoline groups and therefore fits these criteria. The present study showed that RS-45041-190 was a high affinity ligand at I_2 imidazoline receptors on kidney membranes from a variety of species (pK_i 8.66–9.37), but had very low affinity for α_2-adrenoceptors (pK_i = 5.70), giving selectivity ratios for I_2 receptors *vs* α_2-adrenoceptors ranging from 912 (rat) to 4677 (rabbit). Other I_2-selective agents have been described including 2-(1,3-benzodioxanyl)-2-imidazoline (RX 821029; 161 fold selective for I_2 over α_2-adrenoceptors in rabbit brain; Hudson *et al.*, 1995), and 2-(-2-benzo-furanyl)-2-imidazoline (2-BFI; 2874 fold selective; Hudson *et al.*, 1995), although it is not yet clear how selective these agents are for I_2 receptors over other receptors. RS-45041-190 had very low affinity for a number of other receptor subtypes (pK_i < 5.03) suggesting that it is the most selective I_2 imidazoline receptor ligand so far described.

It has been suggested that I_2 imidazoline receptors are somehow linked to monoamine oxidase as some monoamine

oxidase inhibitors displace a component of I_2 receptor binding (Olmos *et al.*, 1993; MacKinnon *et al.*, 1995c) and studies *in vivo* have shown that I_2 receptors are down-regulated by the selective MAO-A inhibitor clorgyline (Olmos *et al.*, 1993). In addition, like MAO, I_2 receptors appear to be enriched in membranes prepared from mitochondria in liver (Tesson *et al.*, 1991), chromaffin cells (Reis *et al.*, 1992) and glial cells (Ruiz *et al.*, 1993) and show a similar autoradiographical distribution to MAO-B (Saura *et al.*, 1992, and see MacKinnon *et al.*, 1995c). A recent study has shown that the I_2-selective compound, 2-BFI, increased noradrenaline release in rat frontal cortex and hippocampus, an effect that it was suggested might be due to inhibition of MAO (Lalies & Nutt, 1995). However, in the present study, a large discrepancy was found between affinity for I_2 receptors and inhibition of MAO activity in rat kidney, as RS-45041-190 had only modest potency ($pIC_{50}=6.12$ and 4.47 for MAO-A and MAO-B respectively) and idazoxan was completely ineffective. Although RS-45041-190 is structurally-unrelated to clorgyline, pargyline and selegiline, its structure does slightly resemble that of the MAO-A inhibitor, brofaromine, which has a benzofuran ring linked to a piperidine ring (Hollister & Claghorn, 1993). Although I_2 receptor ligands were only weak inhibitors of MAO activity *per se*, the possibility remains that they are involved in a more regulatory role of enzyme function.

Our choice of *in vivo* studies was based mainly on the discrete brain loci at which [^{3}H]-RS-45041-190 showed high density binding (MacKinnon *et al.*, 1995c). Three points should be borne in mind when attempting to relate the functional data to the autoradiographic findings. Firstly, the binding sites are not necessarily neuronal, and even if they are, they may not necessarily be located in the plasma membrane, and therefore might not influence neuronal firing. An intermediate step between receptor autoradiography and functional *in vivo* testing could be provided by analysis of neuronal activation by using the techniques of [^{14}C]-2-deoxyglucose utilization and induction of immediate-early genes (Sharp *et al.*, 1993). Secondly, assuming the binding sites are membrane-located receptors, we do not know if RS-45041-190 is an agonist or an antagonist. If it is an agonist at I_2 receptors, then it could have subtle effects on the neurones, without immediate effects on the neuronal firing rate. If, on the other hand, RS-45041-190 is an antagonist, then in order to see an effect in functional studies *in vivo* there would have to be an endogenous ligand bound to its receptors, derived either from neural release or from the circulation. If no effect was seen, it is possible that such an endogenous ligand was not present at its binding sites, at least under the conditions in which the test was conducted. Thirdly, we investigated only a selection of some of the known functions of the majority of these highlighted brain regions. For example, with regard to the hypothalamic nuclei for which [^{3}H]-RS-45041-190 showed high density binding (MacKinnon *et al.*, 1995c), we did not pursue any studies into endocrine effects of RS-45041-190. Similarly, the autoradiography study with [^{3}H]-RS-45041-190 highlighted the arcuate nucleus and other nuclei (medial habenular nucleus, dorsal raphe nucleus, locus coeruleus and nucleus tractus solitarius) which are some of the targets of its efferent fibres (Sim & Joseph, 1991); these authors proposed this as a descending system modulating nociception, but we did not examine the effects of RS-45041-190 on nociception.

The presence of high density binding sites for [^{3}H]-RS-45041-190 in areas implicated in central cardiovascular control, such as the nucleus tractus solitarius, area postrema and dorsomedial hypothalamic nucleus (MacKinnon *et al.*, 1995c), led us to expect cardiovascular responses to this agent. However, no obvious effects occurred, either after intravenous administration (both in normotensive and hypertensive rats), or after i.c.v. administration. Imidazoline binding sites of the I_1 subtype have been found in the ventromedullary areas involved in the control of blood pressure and are thought to be involved in the hypotensive action of clonidine (Ernsberger *et al.*, 1990). The lack of effect of RS-45041-190 on blood pressure may reflect its low affinity for I_1 receptors (MacKinnon *et al.*, 1995c).

RS-45041-190 had no effect on tail skin temperature at a dose of 1 mg kg^{-1}, s.c., indicating that it is not interacting with α_2-adrenoceptors at this dose, as blockade of vascular α_2-adrenoceptors in the rat tail under these experimental conditions raises tail skin temperature by vasodilatation (Redfern *et al.*, 1995). Many centrally-acting agents have effects on core temperature, but this was not observed with RS-45041-190 in conscious rats. An effect of RS-45041-190 on arousal might be expected because of the high density of binding sites for this agent in the locus coeruleus (MacKinnon *et al.*, 1995c), a nucleus which has long been associated with vigilance, arousal and sleep-wakefulness (see Carlson, 1991). However, RS-45041-190 was devoid of effects on arousal, assessed by barbiturate sleeping time in mice and activity in rats, and on motor co-ordination, assessed by rotarod performance in rats.

Against the background of negative effects on the CNS, any effects of RS-45041-190 on food intake would tend to suggest a specific effect on appetite. Studies with idazoxan have suggested that its stimulatory effect on food intake in rats may be due to an action at I_2 receptors (Jackson *et al.*, 1991), although the lack of selectivity of idazoxan precludes the definitive implication of I_2 receptors in the regulation of appetite. The functional role of I_2 receptors on food intake was explored directly in the present study by use of RS-45041-190. RS-45041-190 significantly increased food consumption at 10 and 25 mg kg^{-1}, i.p. Although it is possible that this hyperphagic effect of RS-45041-190 was due to an indirect effect, hypoglycaemia can probably be ruled out, as work from our laboratory has indicated that RS-45041-190 (0.1 mg kg^{-1}, i.v.) does not affect plasma levels of glucose, insulin or free fatty acids in conscious dogs (unpublished observations). If it is indeed a central effect, one can only speculate on its site of action; in our autoradiography study (MacKinnon *et al.*, 1995c), [^{3}H]-RS-45041-190 shows high density binding to various nuclei in the hypothalamus, a brain region known to be involved in appetite (see Luiten *et al.* 1987), and to the nucleus tractus solitarius, which contains neurones involved in relaying gustatory information (Doetsch & Erickson, 1970). Apart from the obvious requirement to determine whether RS-45041-190 is an agonist or antagonist at I_2 imidazoline receptors, more sophisticated studies, such as analysis of feeding patterns and nutrient selection (Blundell & Latham, 1982; Leibowitz, 1992; Rowland, 1993) are required with this agent before the role of its receptors in feeding behaviour can be established.

In the present study, idazoxan (10 mg kg^{-1}, i.p.) not only increased food consumption but also significantly increased water consumption, which was unaffected by RS-45041-190. Given the high density of binding of [^{3}H]-RS-45041-190 in the subfornical organ (MacKinnon *et al.*, 1995c), an area thought to mediate the dipsogenic effect of angiotensin II (see Phillips, 1987), we were surprised to find that RS-45041-190 had no acute effect on water intake. This suggests that I_2 receptors in the subfornical organ are not involved in the regulation of water consumption. In view of the fact that both the subfornical organ and area postrema are highly vascularized (Gross, 1992) it is possible that, in our autoradiography study (MacKinnon *et al.*, 1995c), [^{3}H]-RS-45041-190 was labelling binding sites on microvessels in these two brain structures, rather than on neurones. As for the dipsogenic effect of idazoxan, Jackson *et al.* (1991) concluded that this is most probably due to renal α_2-adrenoceptor blockade, as they observed similar effects with the peripherally selective α_2-antagonist, L-659,066 (however, as these authors pointed out, because the subfornical organ is readily accessible to blood-borne agents [i.e., no blood-brain barrier; Gross, 1992], L-659,066 could still be acting at this site).

In conclusion, RS-45041-190 is a selective, high affinity ligand at I_2 imidazoline receptors, and is an ideal agent for the study of I_2 receptor function. The high selectivity of RS-45041-190 for I_2 imidazoline receptors suggests that the hyperphagic effect of RS-45041-190 is mediated directly via I_2 receptors and that I_2 receptors may have a physiological role in the mod-

ulation of appetite. Our *in vivo* CNS studies have focused on a selection of the known functions of the principal brain regions highlighted in the autoradiography study with this agent (MacKinnon *et al.*, 1995c); further studies with RS-45041-190 may well reveal roles for I_2 imidazoline receptors in other physiological systems.

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