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SR147778, A NEW POTENT AND SELECTIVE ANTAGONIST OF THE CB1 CANNABINOID RECEPTOR. BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION

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RUNNING TITLE

SR147778 a new CB1 cannabinoid receptor antagonist

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ABBREVIATIONS: ANOVA, analysis of variance; hCB1, human brain cannabinoid receptor; hCB2, human peripheral cannabinoid receptor; CHO, Chinese hamster ovary; CL, confidence limit; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; SIP, schedule induced ethanol polydipsia; SR147778, (5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-N-(1-piperidinyl)-1H-pyrazole-3-carboxamide)

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ABSTRACT

Based on binding, functional and pharmacological data, this study introduces SR147778 as a highly potent, selective and orally active antagonist for the CB1 receptor. This compound displays nanomolar affinity (K_i = 0.56 and 3.5 nM) for both the rat brain and human CB1 recombinant receptors, respectively. It has low affinity (K_i = 400 nM) for both the rat spleen and human CB2 receptors. Furthermore it shows no affinity for any of the over 100 targets investigated (IC_{50} >1 µM). *In vitro*, SR147778 antagonizes the inhibitory effects of CP 55,940 on both the mouse vas deferens contractions (pA_2 value = 8.1) and on forskolin-stimulated adenylyl cyclase activity in the U373 MG cell lines (pA_2 value = 8.2) but not in CHO cells permanently expressing the hCB2. SR147778 is able to block the MAP kinase activity induced by CP 55,940 in CHO cell line expressing hCB1 (IC_{50} = 9.6 nM) but was inactive in cells expressing hCB2. After oral administration, SR147778 displaced the *ex vivo* [^{3}H]-CP 55,940 binding to mouse brain membranes (ED_{50} = 3.8 mg/kg) with a long duration of action whereas it did not interact with the CB2 receptor expressed in the mouse spleen. Using different routes of administration, SR147778 (0.3 to 3 mg/kg) is shown to antagonize pharmacological effects (hypothermia, analgesia and gastrointestinal transit) induced by WIN55212-2, in mice. Finally, per se, SR147778 (0.3 to 10 mg/kg) is able to reduce both ethanol or sucrose consumption in mice and rats and food intake in fasted and non-deprived rats.

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Marijuana (*Cannabis sativa L.*) has been used for many centuries as a recreational drug or as a therapeutic agent. It is now well established that tetrahydrocannabinol (Δ^9-THC), the main active component of marijuana, the endocannabinoids (Devane et al., 1992; Mechoulam et al., 1995) as well as synthetic cannabinoid receptor agonists mediate their cellular effects through specific cannabinoid receptors, members of the G protein-coupled receptor super family. To date, two cannabinoid receptors have been identified, CB1 (Matsuda et al., 1990; Gerard et al., 1991) and CB2 (Munro et al., 1993). CB1 is predominantly expressed in the brain (Westlake et al., 1994), but also in some peripheral tissues (Gérard et al., 1991; Bouaboula et al., 1993; Shire et al., 1995). The CB2 subtype is found mainly in immune cells (Munro et al., 1993; Galiègue et al., 1995) but also in rat microglial cells (Walter et al., 2003). Both receptors mediate their effects via a pertussis toxin-sensitive GTP-binding regulatory protein. They are negatively and positively coupled to the adenylyl cyclase (Howlett and Fleming, 1984) and other different signaling pathways (Frodin et al., 1994; Bouaboula et al., 1995 a, b), respectively. To date there is increasing evidence for the existence of other subtypes of cannabinoid receptors although the genes that encode them have not yet been identified. Putative receptors included central and vascular endothelial non CB1/ non CB2 receptors (Jarai et al., 1999; Breivogel et al., 2001; Begg et al., 2003).

An intensive research effort is aimed to understand the role of the endocannabinoid system in the pathophysiology of the central nervous system. Cannabinoids have been reported to stimulate hunger and increase appetite in humans and in rodents, particularly for sweet palatable food (Abel, 1975; Cota et al., 2003). These effects may be explained by the interaction of the cannabinoid system with the most important neuronal networks and metabolic pathways involved in the control of food intake. A number of studies have highlighted the role of the endogenous cannabinoid system in feeding regulation.

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In addition, cannabinoid agonists are able to stimulate the activity of mesolimbic dopaminergic neurons and enhance brain-stimulation-induced reward (Gardner et al., 1998). Reportedly, the endogenous cannabinoid system can modulate the actions of drugs of abuse, in particular nicotine. The results obtained with the CB1 receptor antagonist SR141716 (RIMONABANT) (Cohen et al., 2002) have suggested that endogenous cannabinoid system tonically increases the sensitivity of animal species to reinforcers such as nicotine or ethanol, possibly by modulating brain reward systems. These results indicated that a cannabinoid antagonist may be useful as an aid for smoking cessation and for the treatment of alcoholism.

Finally, several lines of evidence suggest that the endocannabinoid system may play an important role in controlling memory processes. It has been shown that Δ^9-THC impairs learning and memory in rodents (Mazzola et al., 2003) and primates (Evans, 1992). These effects seem to be mediated by CB1 receptors (Lichman et al., 1996; Wolff and Leander, 2003). The results obtained with SR141716 suggest that a cannabinoid CB1 receptor antagonist can improve consolidation processes and thus may be useful in treating certain memory disorders.

To date several groups have become interested in the development of new drugs for investigating the *in vivo* dysfunction of the endocannabinoid system.

This paper introduces SR147778 [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-N-(1-piperidinyl)-1H-pyrazole-3-carboxamide, Fig. 1], as a highly potent, selective and orally active antagonist for CB1 receptor. It is expected that this compound may have a potential as a therapy for the treatment of diseases where the endocannabinoid system is implicated.

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Materials and Methods

Materials. [^{3}H]-CP 55,940 (111.9 Ci/mmol) was purchased from New-England Nuclear Corporation (Paris, France). γ-[^{33}P] ATP (112.9 Ci/mmol), cAMP scintillant proximity assay and Biotrack p42/p44 MAP kinase kits were from Amersham (Les Ulis, France). CP 55,940 and WIN55212-2 were purchased from Tocris Cookson Incorporated (Ellisville, USA) and Research Biochemicals (Illkirch, France), respectively. SR147778 was synthesized at Sanofi Recherche. Stock solutions of drugs were dissolved in dimethyl sulfoxide at 10^{-2} M and stored at -20°C. Tissue culture reagents were from Gibco (Eragny, France). Culture flasks (Costar and Falcon) were purchased from Dutscher (Brumath, France) and Becton Dickinson (Le Pont de Claix, France). The human astrocytoma cell line U373 MG was obtained from ATCC. A04 standard rat laboratory diet was obtained from UAR, France.

Animals. Male Sprague Dawley rats (180-250 g) obtained from Charles River (France) were used for *in vitro* and food intake studies. Male wistar rats (300 g) obtained from Charles River (France) were used for schedule-induced polydipsia (SIP) studies. Male CD1 mice (20-25g) obtained from Charles River (France) were used for *ex vivo* binding and gastrointestinal studies. Male Swiss mice (30-35 g) obtained from CERJ, Le Genet S^{t} Isle (France) were used for isolated vas deferens preparations. Male OF1 (25-30g) and C57Bl6 mice (25-30g) were obtained from (IFFA CREDO, France) and used for WIN55212-2 interaction studies (hypothermia and analgesia) and ethanol intake, respectively.

Drug preparation and administration. For *in vivo* experiments, drugs were suspended with 0.01% Tween 80 in saline (NaCl 0.9%) for iv administration or distilled water for other routes. SR147778 was administered by intraperitoneal (ip), oral (po) or subcutaneous (sc) route in a volume of 20 ml/kg body weight. WIN55212-2 was injected iv (in a volume of 10 ml/kg body weight), sc or ip (in a volume of 20 ml/kg body weight). The Ethical Committee

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for Laboratory Animals of Sanofi-Synthelabo Recherche had approved the protocols. They were carried out in accordance with the European Directive 86/609/EEC.

Expression of human CB1 and CB2 receptor in Chinese Hamster Ovary (CHO) cells and Cell culture.

To generate stable CHO transformants, human CB1 or CB2 cDNA were cloned in a vector optimized for expression of recombinant proteins in CHO cells as previously described (Shire et al., 1995; Shire et al., 1996). Vectors were transfected into CHO dihydrofolate-reductase-negative cells by a precipitation method. CHO cells were trypsined 48 hours after transfection and selected at a density of 5 x 10^5 cells/dish onto a culture medium A [Minimum essential Medium-glutamine medium, heat-inactivated dialysed foetal-calf serum (FCS, 10%), gentamicin (20 mg/liter), L-proline (40 mg/liter), pyruvate sodium (0.5 mM), and anti-PPLO agent (1%)]. After 10 days surviving clones were recovered and cultivated in the same medium containing fungizone (0.1%). Cells were used between the third and the 22nd passage.

U373 cells were cultivated as described by Bouaboula et al., (1995 b). Briefly they were grown as monolayers in Dulbecco's modified Eagle' medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) 1 % vitamins, and 1mM sodium pyruvate.

Membrane preparations. Membranes were isolated from CHO cells expressing either hCB1 or hCB2 as described previously (Rinaldi-Carmona et al., 1996; Shire et al., 1996). Briefly, cells were washed twice with phosphate buffered saline (PBS), scraped into 50 mM Tris-HCl, pH 7.7 (buffer A), crushed in a Polytron for 1 min at 7000 rpm/min then centrifuged for 15 min at 1100 g at 4°C. The supernatant was centrifuged for 1 hour at 105,000 g. The pellet was resuspended in buffer A and protein concentration measured. Membranes were stored at -80 °C until use. Membranes were prepared from the brain or the spleen of rats killed by

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decapitation. The brain (without the cerebellum) and the spleen were removed and homogenized 30 seconds at 4°C in buffer A (Tris-HCl 50 mM, pH7.4) in a Polytron for 30 seconds at 7000 rpm/min then centrifuged for 10 min at 1100 g. The supernatant was centrifuged for 30 minutes at 45,000 g. The pellet was resuspended in buffer A and protein concentration measured. Membranes were stored at -80 °C until use.

Binding experiments. For *in vitro* binding assays, experiments were carried as described previously (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1995). Briefly, membranes (10-100 µg) were incubated at 30°C with [^{3}H]-CP 55,940 (0.2 nM) in 1 ml of buffer A for 1 hour. A rapid filtration technique using Whatman GF/C filters (pre-treated with polyethyleneimine 0.5 % (w/v)) and a 48-well filtration apparatus (Brandel) was used to harvest and rinse labeled membranes (3 x with 5 ml cold buffer A containing 0.25 % BSA). The radioactivity bound to the filters was counted with 4 ml of biofluor liquid scintillant. Non-specific binding was determined in the presence of 1 µM CP 55,940. For selectivity studies, binding assays were carried out using standard protocols.

For *ex vivo* experiments, SR147778 was administered per os to mice before they were killed by decapitation. The brain (without the cerebellum) and the spleen were removed and homogenized in a Polytron for 30 seconds at 7000 rpm/min in buffer A. Binding assays were performed with aliquots of brain or spleen homogenates as described above.

Isolated mouse vas deferens preparations. Assays were performed as described previously (Rinaldi-Carmona et al., 1994). Drugs were added once the contractile responses to electrical stimulation were reproducible. Preparations were exposed to cumulative increasing concentrations of CP 55,940 (10^{-11} to $3x10^{-7}$M) to obtain concentration-response curves either in the absence (control) or in the presence of SR147778 (10, 30, 100 nM) added at a fixed concentration 60 min before the first concentration of CP 55,940.

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Adenylyl cyclase assays. cAMP accumulation studies were carried out in U373 MG cells, as described previously (Rinaldi-Carmona et al., 1996). Briefly, U373 MG cells grown to confluence were washed with PBS and incubated for 5 min at 37°C in 1 ml of PBS containing 0.25% acid free BSA, IBMX (0.1 mM), RO20-1724 (0.1 mM), supplemented or not (basal activity) with drugs. Forskolin was added (10 µM) and cells were incubated for 20 min at 37°C. The reaction was terminated by rapid aspiration of the assay medium and addition of 1 ml of ice-cold 50 mM Tris, 4 mM ethylenediaminetetraacetic acid. Aliquots from supernatant were dried and the cAMP concentration was determined by radioimmunoassay. In PTX experiments, cells were cultured in the presence of the toxin (100 ng/ml) for 24 h before treatment with forskolin.

Mitogen-activated protein kinase (MAPK) activity. MAPK activity was measured in hCB1 or hCB2 CHO cells as described previously (Rinaldi-Carmona et al., 1996). Briefly, cells grown to 80% confluence were maintained in medium containing 0.5% foetal calf serum for 24 hour prior to the application of ligands. After treatment with drugs, cells were washed at 4°C with 0.5 ml of buffer A [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N-tetraacetic acid, 1 mM Na_3VO_4] and lysed for 15 min in buffer A supplemented with 1% Triton X100, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The solubilized cell extracts were clarified by centrifugation at 14,000 x g for 15 min at 4°C. Aliquots were removed and stored at –80°C until use. Phosphorylation assays were carried out for 30 min at 30°C (linear assay conditions) with γ^{33}

P ATP by using the Biotrack p42/p44 MAP kinase enzyme system. The radioactivity incorporated was determined by liquid scintillation counting.

Hypothermia and analgesia induced by WIN55212-2. Tests were performed as described previously (Rinaldi-Carmona et al., 1994). Briefly, mice were administered with SR147778

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(0 to 10 mg/kg) either ip (30 minutes) or po (1 hour) before the intravenous injection of WIN55212-2 (0.8 mg/kg). Rectal temperatures were measured in isolated mice with a thermocouple probe (Physitemp) just before administration of SR147778 and 30 minutes after iv injection of WIN55212-2. For analgesia studies, mice were administered sc with WIN55212-2 (3 mg/kg), 30 minutes before being exposed to a focused noxious beam of radiant heat applied to the underside of a right hind paw (paw-flick test) automatically measured. A cutoff time at 22s was used to prevent blistering. SR147778 was administered by oral route 30 minutes before WIN55212-2 administration.

Gastrointestinal transit. Transit was assessed 30 min after a standard charcoal meal given by gavage as described previously (Colombo et al., 1998). Briefly, mice were administered with vehicle or SR147778, 30 min (3 mg/kg, ip) or 1 hour (10 mg/kg, po), before the intraperitoneal administration of 0.15 mg/kg CP 55,940 or 0.3 mg/kg WIN55212-2. Test meal was given 30 min, CP 55,940, or immediately after, WIN55212-2, administration. 30 minutes after mice were killed by decapitation and intestines were removed from the pylorus to the cecum. The distance covered by head of the marker was measured and expressed as percent of the total length of the small intestine.

Voluntary ethanol consumption. The test was performed as described previously (Arnone et al., 1997). Briefly, one week before testing, mice were singly housed in cages equipped with two bottles of plain water. Mice were then subjected to five daily 6 hours test sessions during which they had a free choice between one bottle of water or one filled with a 10% (v/v) non-sweetened ethanol solution. The quantities of the different liquids consumed were measured by weighing the bottle before and after each session. The bottles were regularly checked for possible leakage. SR147778 was administered 30 minutes before each experiment session.

Spontaneous sucrose drinking. Two weeks prior to testing, rats caged individually were given access to a sucrose solution (5 %) in their home-cage during daily 4 hours sessions

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(without food and water). At the end of the training periods, rats displaying a poor sucrose drinking response were discarded from the study. On the day of the test, rats were administered orally with SR147778 or vehicle, in a volume of 2 ml/kg one hour before the presentation of the sucrose solution. The amount of the sucrose solution drunk by each rat (n = 6-8 per group) was measured by weighing each bottle before and at the end of the 4-hour drinking.

Food intake in 18-hour fasted rats. Over a period of 10 days before the experiment, rats were fasted for 18 hours, and allowed access to food for only 6 hours between 10 a.m. and 4 p.m. each day. Water was available ad libitum. At the end of this adaptation phase, SR147778 or vehicle were administered orally, in a volume of 5 ml/kg, at 9 a.m., one hour after, a preweighed amount of food (A04 standard rat laboratory diet) was introduced into the cage, and then food intake was measured (taking into account spillage) at different times.

Spontaneous feeding in non-deprived rats. Rats were maintained in a reversed light-dark cycle (light off 9 a.m.-9 p.m.) with food and water ad libitum. On the day of the experiment, food was removed from the cage at 8 a.m., and animals were administered orally with SR147778 or vehicle in a volume of 5 ml/kg, at 9 a.m. (at the beginning of the dark phase). One hour after, a preweighed amount of food (A04 standard rat laboratory diet) was introduced into the cage and then food intake was measured (taking into account spillage) at different times.

Schedule induced ethanol polydispsia. Rats were randomly allocated to drinking either water or ethanol in a protocol of schedule-induced polydipsia (SIP, Falk et al., 1972). They were food deprived (food available for one hour per day) but not water deprived. They were trained in operant chambers (40.5cm x 31 cm x 31 cm, Imetronic, Pessac, France) fitted with a food tray where food pellets (45 mg, Bioserv, Phymep, Paris) were delivered every 60 s (FT-60s). Visits to the food tray were automatically recorded. A metal drinking tube

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protruded into the chamber near the food magazine and was connected to a drinking bottle. Licks of the drinking tube were numbered by the closure of an electric circuit. Bottles were weighed to the nearest 0.1g at the end of each SIP session. Both groups were trained for two weeks, five days per week, under the SIP procedure until they drank about 10 ml of plain water which was then substituted for slightly sweetened solutions (0.05% weight/volume saccharose) of either water or ethanol. Over the next two weeks of training, the concentration of ethanol was progressively increased by steps of 2% to reach the final 10% concentration. Fluid consumption was allowed to stabilise over five daily training sessions. Then, each group (ethanol or water) was divided into two subgroups: one treated with one dose of SR147778 administered ip one hour before testing, and the other subgroup administered with the corresponding vehicle. As a control SR141716 was tested at 10 mg/kg in the same conditions. The four subgroups of rats were tested once a week (one hour test session) with a different dose of SR147778 and trained daily (one hour training session) the four remaining days. In the same rats and in the same experimental conditions the activity of the reference CB1 antagonist, SR141716, had previously been investigated at 10 mg/kg ip. Data are presented as the mean quantities of water or ethanol consumed during each test session ; they were analysed with Student'*t* test to compare the quantities of water or ethanol drank after administration of SR147778 or vehicle during a given test session.

Data analysis. Data from competition experiments (IC_{50}, EC_{50}, DE_{50}) were analyzed using a non-linear least-squares method. K_d values of 0.09 ± 0.01 nM (brain), 0.309 ± 0.031 nM (hCB1), K_d values of 0.17 ± 0.03 nM (spleen) and K_d values of 0.49 ± 0.11 nM (hCB2) were used to determine K_i values. A Schild plot was constructed to estimate the pA_2 value in mouse vas deferens and adenylyl cyclase studies. Statistical significance was determined by Student's *t*-test and $p^* < 0.05$ was considered significant. For *in vivo* pharmacological tests,

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statistical analysis was performed using ANOVA followed by Dunnett's *t*-test. ID_{50} values were calculated using Pharmacofit method.

Results

Interaction of SR147778 with CB1 receptors *in vitro*

As shown in Fig. 2, SR147778 displaced in a concentration-dependent manner [^{3}H]-CP 55,940 specifically bound to its high affinity receptor in rat brain membranes whereas it displayed low affinity for the cannabinoid receptor expressed in rat spleen. The concentration-response curves gave K_i values of 0.56 ± 0.09 and 349 ± 24 nM (three experiments) for brain and spleen, respectively. Furthermore, in membranes isolated from CHO cells expressing hCB1, SR147778 was a potent inhibitor of [^{3}H]-CP 55,940 binding sites with a K_i value of 3.5 ± 0.29 nM (three experiments), whereas it displayed only low affinity for membranes from CHO cells expressing hCB2, K_i = 442 ± 30 nM (three experiments). These results show that SR147778 is selective for CB1 versus CB2 receptors.

Receptor binding profile of SR147778

At 1 µM, SR147778 had no affinity (% inhibition<50%) for any of the other types of receptors, enzymes or ion channels investigated including: angiotensin II (AT_1, AT_2), CGRP, endothelin (ET_A, ET_B), galanin, muscarinic (M_1, M_2, M_3, M_4, M_5), bonbesin (B_1, B_2), histamine (H_1, H_2, H_3), dopamine (D_1, D_2, D_3, $D_{4.4}$, D_5), adrenergic (α_1, α_{2A}, α_{2B}, α_{2C}, β_1, β_2), adenosine (A_1, A_{2a}, A_3), opiate (κ, µ), neurotensin, cholecystokinin (A, B), benzodiazepine (central, peripheral), sigma 1, imidazoline (I_2), tachykinin (NK_1, NK_2, NK_3), neuropeptide Y (Y_1, Y_2), melatonine (ML1), phencyclidine, vasopressin (V_{1A}, V_2), VIP, somatostatin, glycine (strychnine sensitive and insensitive), AMPA, kainate, NMDA, GABA, 5-hydroxytryptamine ($5\text{-}HT_{1A,B,D}$, $5\text{-}HT_{2A,C}$, $5\text{-}HT_3$, $5\text{-}HT_4$, $5\text{-}HT_{5A}$, $5\text{-}HT_6$, $5\text{-}HT_7$), PAF, TNFα, IP_3, LTB_4, LTD_4,

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TAX_2/PGH_2, P_{2X}, P_{2Y}, glucocorticoid, VIP, Cl^-, Na^+ ($site_1$, $site_2$), Ca^{++} (N, L, DHP) and K^+ (ATP sensitive, voltage-dependent, Ca^{++} dependent). These findings show that SR147778 is a very specific ligand for CB1 receptors.

SR147778 is an antagonist of the CB1 receptor *in vitro*

In the mouse vas deferens, SR147778 at concentration up to 1 µM did not produce clear concentration-dependent effects on twitch contractions. Only a moderate and almost straight decrease in amplitude was observed as seen in the vehicle-treated control preparations during the same time period (20 % inhibition). This effect probably resulted from spontaneous time-dependent changes during long tension recordings. As shown in Fig. 3, SR147778 produced a significant concentration-dependent rightward and almost parallel shift of the concentration response-curve for CP 55,940 (10 nM SR147778, pD_2 value of CP 55,940 = 8.47 ± 0.04, 30 nM SR147778, pD_2 value of CP 55,940 = 8.03 ± 0.06, 100 nM SR147778, pD_2 value of CP 55,940 = 7.38 ± 0.04 versus control pD_2 value of CP 55,940 = 8.70 ± 0.07, seven experiments). A pA_2 value of 8.06 ± 0.13 was found for SR147778.

As previously described (Bouaboula et al., 1995 a), U373MG cells highly expressed the hCB1 receptor. In these cells, the cannabinoid agonist CP 55,940 inhibited in a concentration-dependent manner, the forskolin-induced accumulation of cAMP with an IC_{50} value of 1.8 ± 0.3 nM (six experiments), whereas the selective CB2 agonist, JWH133, was ineffective (23% inhibition at 1 µM). At 300 nM of CP 55,940 a maximum inhibition of 75 % was observed. This effect was PTX dependent (not shown). SR147778, which produced no effect on basal adenylyl cyclase activity up to 1 µM, completely reversed the inhibition exerted by CP 55,940 (10 nM) in U373MG cells with an EC_{50} value of 9.4 [CL95%: 4-21] nM (two experiments). As shown in Fig. 4, in these cells, SR147778 produced a significant concentration-dependent rightward and almost parallel shift of the concentration response-

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curve for CP 55,940 (10 nM SR147778, IC_{50} value of CP 55,940 = 12 ± 0.2; 100 nM SR147778, IC_{50} value of CP 55,940 = 23 ± 2 nM; 300 nM SR147778, IC_{50} value of CP 55,940 = 60 ± 5 nM versus control IC_{50} value of CP 55,940 = 2.0 ± 0.3 nM). A pA_2 value of 8.2 was calculated for SR147778. Interestingly, SR147778 alone was able to stimulate in a concentration-dependent manner (EC_{50} = 1.7[CL95%: 1.3-2.4] nM; two experiments) the forskolin-sensitive adenylyl cyclase activity with a maximum effect at 3 µM (2-3 fold stimulation). These effects were completely prevented by PTX pretreatment of the cells (not shown). In these cells, the CB2 antagonist, SR144528, did not block the inhibitory action of CP 55,940 up to 1 µM. In addition, at 3 µM, SR147778 had no significant effect on CP 55,940 inhibited forskolin-sensitive adenylyl cyclase activity in hCB2 CHO cells (not shown).

In hCB1 or -CB2 CHO cells, CP 55,940 activated the 42-and 44 KDa MAPKs in a concentration-dependent manner (Bouaboula et al., 1995 a; Bouaboula et al., 1996) with an EC_{50} value of about 6 nM and a maximum of activation of 3.5 fold that occurred at 300 nM. As shown in Fig. 5, SR147778 was able to produce a concentration-dependent inhibition of MAPK activity stimulated by 10 nM CP 55,940 in hCB1 CHO cells with an IC_{50} value of 9.6 [CL95%: 6.6-14] nM (two experiments) whereas in hCB2 CHO cells it had no effect up to 1 µM. Per se, SR147778 was able to inhibit the basal MAPK activity (IC_{50} = 60 [CL95%: 23-120] nM) with a maximum effect at 300 nM (40% of inhibition). Taken together, these *in vitro* results indicate that SR147778 is a potent and selective antagonist of the CB1 versus the CB2 receptor with inverse agonist properties.

***In vivo* interaction of SR147778 with brain cannabinoid receptors.**

As shown in Fig. 6A, after oral administration, SR147778 totally displaced the specific binding of [^{3}H]-CP 55,940, measured *ex vivo*, to mouse brain homogenates in a dose-

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dependent manner with median effective dose value (ED_{50}) of 3.8 (95% CL: 3.2-4.6) mg/kg (two experiments). In contrast, SR147778 did not interact with the cannabinoid receptor expressed in the mouse spleen (CB2) up to 30 mg/kg. The occupancy by SR147778 of the brain cannabinoid receptor was time-dependent and significant for at least 24 hours after oral administration at 10 mg/kg (Fig. 6B).

***In vivo* antagonism by SR147778 for CB1 receptors in mice.**

The *in vivo* antagonism of SR147778 for the brain cannabinoid receptors was investigated in several animal models. As shown in Fig. 7, SR147778 (ip and po) significantly and dose-dependently reversed hypothermia (Fig. 7A) induced by WIN55212-2 with ID_{50} values of 0.3 and 0.4 mg/kg, respectively (po/ip ratio near unity). In addition, SR147778 (1 mg/kg, po) had a significant long lasting effect, superior to 24h (data not shown). SR147778 (po) was also able to significantly and dose-dependently reverse the increase of withdrawal latencies induced by WIN55212-2 on the paw-flick test (Fig. 7B) with a linear regression [$F(1,72) = 16.7$, $p<0.001$] and an ID_{50} value of 0.4 mg/kg. Finally, SR147778 at 10 mg/kg (po) or 3mg/kg (ip) was able to totally block the gastrointestinal transit delay induced by CP 55,940 (0.15 mg/kg) or WIN55212-2 (0.3 mg/kg) (fig. 7C). Taken together, these results indicate that SR147778 is a potent and orally active antagonist of the CB1 receptor in mice.

Intrinsic activity of SR147778 in rodent.

As shown in Fig. 8, SR147778 significantly decreased ethanol consumption in the 0.3 to 3 mg/kg dose range with a linear regression [$F(1,45) = 48.85$, $p<0.001$]. This effect was maintained upon repeated administration from sessions 1 to 5. The water intake was increased at 1 and 3 mg/kg probably due to a compensatory effects (data not shown). As already reported (Falk et al., 1972**)** control rats trained in the SIP procedure drank more water than 10% ethanol solution (22.2 to 26.55 ml versus 15.8 to 16.8 ml (Table 1). SR147778 did not modify water consumption from 0.3 to 10 mg/kg ip, although it elicited a non-significant

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trend to decrease water consumed at the highest dose (Student t test, t (df:14) = 1.85, p = 0.085). In contrast, SR147778 dose-dependently reduced ethanol intake, with significant and consistent effects observed from 1 mg/kg to 10 mg/kg (Student t tests (df:14 df): t = 3.36, p = 0.004; t = 4.71, p = 0.001 and t = 3.52, p = 0.003, for 1, 3 and 10 mg/kg, respectively). In the same test conditions, the CB1 antagonist SR141716 yielded similar results. It selectively reduced ethanol drinking without modifying water intake: ethanol consumption, 15.1 +/- 0.9 g and 8.7 +/- 1.5 g after SR141716 0 and 10 mg/kg ip respectively (Student t test, t (df: 14) = 3.63, p = 0.003), and water consumption, 24.0 +/- 2.5 g and 21.9 +/- 1.8 g after SR141716 0 and 10 mg/kg ip respectively (Student t test, t (df: 14) = 0.639, p = 0.50). During the experiments, all the pellets were eaten. As shown in Fig. 9, vehicle-treated rats displayed a very intense cumulative daily sucrose drinking (21.5 ± 3.2 g after two weeks of habituation). SR147778 markedly and dose-dependently decreased sucrose solution consumption. This effect was significant (*p<0.05) at the dose of 3 mg/kg. As shown in Fig. 10 and 11, SR147778 was able to dose-dependently decrease the food intake either in fasted or in non-deprived (in reversed light dark cycle) rats. These effects were maintained over the entire period studied and significant from the dose of 3 mg/kg (*p<0.05, **p<0.01) and long lasting up to 24 hours.

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Discussion

Several lines of evidence suggest that the cannabinoid system is involved in various physiological functions such as memory and learning, pain, feeding and appetite, reinforcement and motor coordination. Its implication in pathophysiological conditions is beginning to be elucidated and consequently this endogenous system appears as an important therapeutic target for the treatment of disorders such as obesity, drug addiction, ethanol abuse and cognitive disorders. New drugs from research in this area will provide useful tools to confirm this hypothesis. In the present study we describe a new potent, selective and orally effective CB1 cannabinoid receptor antagonist.

In vitro SR147778 has a high affinity in the nanomolar range, for the rat brain and the human CB1 receptors with low affinity for the CB2 receptor. In addition, the interaction of SR147778 with the CB1 receptors is highly specific since no significant affinity was found for over 100 receptors, ion channels or enzymes investigated. Functional studies performed *in vitro,* in tissue or cells highly expressing hCB1 indicated that SR147778 behaved as a competitive and selective antagonist against the responses induced by the cannabinoid agonist CP 55,940. As previously seen with SR141716 (Bouaboula et al., 1997), the effect observed with SR147778 on its own forskolin-sensitive adenylyl cyclase activity and on basal MAP kinase activity is consistent with an inverse agonist property. Based upon the *ex vivo* [^{3}H]-CP 55,940 binding studies, SR147778 appears to occupy the brain CB1 but not CB2 receptors and to cross the blood–brain barrier with a long duration of action in mice.

Δ^{9}-THC, endocannabinoids (AEA, 2AG) and synthetic cannabinoid agonists (CP 55,940 and WIN55,212-2) produce a number of effects in mice (hypothermia, antinociception, hypoactivity and catalepsy) that are known as the tetrad of cannabinoid-induced behaviors. These behaviors are of central origin and are thought to be mediated by the cannabinoid CB1

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receptor (Rinaldi-Carmona et al., 1994). Our experimental results showed that SR147778 was able to block both the hypothermia and the analgesic effects of WIN55,212-2 in mice suggesting that SR147778 is an effective antagonist of the CB1 receptor *in vivo*. Along the same lines, it is well established that the effects of cannabinoid agonists (CP 55,940 and WIN55212-2) on gut motility *in vitro* and *in vivo* (Pinto et al., 2002) are mediated by both central and enteric nervous CB1 receptors in rodents and humans. Our data showed that SR147778 counteracted the inhibitory action of either CP 55,940 or WIN55212-2 on mouse intestinal motility confirming its CB1 receptor antagonist properties in this animal model.

Recent advances in the understanding of the neurobiological basis of alcoholism suggest that the pharmacological and behavioral effects of alcohol are mediated through its action on neuronal signal transduction pathways and receptors that are coupled to G-proteins. The implication of the endogenous cannabinoid signaling system in the development of tolerance to drugs of abuse including alcohol was recently demonstrated. Chronic alcohol treatment increases the synthesis of endocannabinoids (AEA and 2AG) and down-regulates brain-CB1-receptors and their function (Basavarajappa and Hungund, 2002). In addition, the CB1 receptor antagonist SR141716 blocks voluntary alcohol consumption in rodents (Arnone et al., 1997; Sera et al., 2002). Similarly, activation of the CB1 receptor system promotes alcohol craving, suggesting a role for the CB1 receptor gene in excessive alcohol drinking behavior and development of alcoholism (Schmidt et al., 2002). The activity of SR147778 on ethanol intake is specific as the excessive amount of water compulsorily consumed in the SIP procedure is not modified by the drug. Although SIP is highly sensitive to food motivation this differential action clearly shows that the effect of SR147778 is unrelated to its potential action on appetitive motivation, but that it is excess alcoholic consumption which is regulated. The excessive drinking elicited by SIP makes it an attractive paradigm for investigating ethanol consumption ; but SIP also activates the pituitary adrenal axis and a

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wealth of clinical data links alcoholism with stress. However there is no experimental evidence for a role of CB1 receptors in regulating stress processes. Alternatively, schedule induced ethanol drinking has been used to establish ethanol as a positive reinforcer ; the reduction in ethanol intake when water intake is unchanged after treatment by SR147778 could suggest that blockade of CB1 receptors dampens the rewarding efficacy of ethanol. This hypothesis is further supported by the similar activity of SR141716, the reference CB1 antagonist in the same protocol and its role in reducing the incentive value of palatable foods or drinks. As reported for SR141716, the reduction of ethanol intake by SR147778 observed in both rats and mice indicates that the endogenous cannabinoid system may play a critical role in the appetitive value of alcohol in these experimental models of human alcoholism.

Numerous articles suggest that smoking cannabis stimulates hunger, and selectively increases the appetite for sweet and palatable food. Starting in the 70's, a series of well controlled scientific studies were conducted to further characterize this effect (Cota et al., 2003). An increased desire for sweet food (marshmallows) was noted in subjects smoking marijuana while others studies noted that the appetite stimulation was dependent on the route of administration, the dose used, the social environment and the satiety status. In addition, studies using a variety of behavioral paradigms indicated that endocannabinoids may play a very specific role in the control of appetite. In this regard, experiments conducted with Δ^9-THC and endocannabinoids (AEA and 2-AG) showed that endocannabinoids, injected in specific areas (nucleus accumbens shell, ventromedial hypothalamus) linked to eating motivation, induced overeating in pre-satiated animals and produced a strong increase in food intake in rodents. Finally, with the recent development of potent and specific compounds able to antagonize CB1 actions, it has been shown that hyperphagic effects induced by cannabinoid receptor agonists were mediated by CB1 activation. Indeed several authors have reported that SR141716 selectively attenuates the consumption of palatable

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food or drink (Simiand et al., 1998) in marmosets and reduced craving in rats (Arnone et al., 1997). Our present work shows that SR147778 was also able to reduce sucrose drinking and food intake in rats, a species which is known to display a strong preference for polysaccharide and sucrose solution. Furthermore SR147778 decreased in a dose-dependent manner eating in both non-deprived rats and fasted rats. This latter effect is of particular interest considering the increase motivation for food consumption in fasted animals. Thus, this study clearly confirmed the key role of the CB1 receptor in modulating appetite although the mechanisms by which the CB1 cannabinoid system modulate excessive food intake require further investigation.

Taken together these results strongly suggest that SR147778 may be considered as a useful tool to elucidate the respective physiological or pathophysiological roles of central (CB1) cannabinoid receptors. Further studies will help to confirm the potential therapeutic role of this new CB1 receptor antagonist in the clinical setting.

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Legends for figures

Fig. 1. Chemical Structure of SR147778.

Fig. 2. Displacement of [^{3}H]-CP 55,940 binding to rat and CHO cell membranes by SR147778. Binding assays were carried out at 30°C as described under "Materials and Methods" using hCB1 (●), hCB2 (▲), rat brain (■) or rat spleen (◆) membranes, 0.2 nM [^{3}H]-CP 55,940 and increasing concentrations of SR147778. Data are from one experiment out of 3 performed in duplicate and are expressed as the percentage of specific binding in the absence of SR147778 (100 %).

Fig. 3. Effect of SR147778 on CP 55,940-induced inhibition of twitch contractions in the mouse vas deferens. Cumulative concentration-response curves for CP 55,940 (10^{-6} to 10^{-11}M) on the amplitude of twitch contractions elicited by electrical field stimulation of the mouse vas deferens obtained in the absence (●)(control) and in the presence of SR147778 at 10 nM (■), 30 nM (▲) and 100 nM (◆). Assays were performed as described in "Materials and Methods". Data are expressed as a percentage of control values after incubation with SR147778. Each point is the mean value ± S.E.M of 7 determinations.

Fig. 4. Effect of SR147778 on CP 55,940-induced inhibition of forskolin-sensitive adenylyl cyclase activity in U373 MG cells. Cumulative concentration-response curves for CP 55,940 (10^{-7} to 10^{-11}M) on forskolin stimulated cAMP levels was obtained in the absence(●)(control) and in the presence of SR147778 at 10 nM (■), 100 nM (▲) and 300 nM (◆). cAMP levels were measured as described under "Materials and Methods". Data are from 1 representative experiment performed in triplicate. Data are expressed as a percentage of control values after incubation with SR147778. Each point is the mean value ± S.E.M of 3 determinations.

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Fig. 5. Effect of SR147778 on CP 55,940-induced MAPK stimulation in CHO cells expressing hCB1 (●) or hCB2 (■) receptors. Growth-arrested hCB1 or hCB2 CHO cells were treated with various concentrations of SR147778 in the presence of 10 nM CP 55,940 for 15 min. MAPK activity (42- and 44 KDa MAPK) was measured in cell lysates as described under "Materials and Methods". Data are from one representative experiment out of two performed in triplicate and are expressed as the percentage of CP 55,940 effect at 10 nM in the absence of SR147778 (100 %).

Fig. 6. Displacement of the specific [^{3}H]-CP 55,940 binding to its sites by SR147778 after oral administration. A) Mice were administered with increasing doses of SR147778. They were then sacrificed one hour after these administrations. Brains (●) were then removed and *ex vivo* binding assays were performed using [^{3}H]-CP 55,940 as described under "Materials and Methods". Data are from one representative experiment out of two performed in six replicates obtained from three animals. B) Time course of the brain cannabinoid receptor occupancy by SR147778 after oral administration. Mice were administered with 1 (●), 3 (■) or 10 (▲) mg/kg of SR147778. They were sacrificed at different times after drug administration. The brain was removed and binding studies were performed as described under "Materials and Methods". Data are means ± S.E.M. of six values obtained from three animals. Data are expressed as percentage of displacement of the specific binding in tissues of untreated mice (100 %).*=$p<0.05$, **=$p<0.01$ versus control group (Dunnett's *t*-test).

Fig. 7. Effects of SR147778 on hypothermia (A), analgesia (B) and gastrointestinal transit (C) induced by a cannabinoid agonist. A) Mice were administered with increasing doses of SR147778 either ip (●), 30 minutes, or po (○), 1 hour, before the intravenous injection of WIN55212-2 (0.8 mg/kg), respectively. Then the test was performed as described under

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"Materials and Methods". Data are expressed as variation of temperatures between the measures before SR147778 administration and 30 minutes after WIN55212-2 injection. *=p<0.05, **=p<0.01 versus WIN55212-2 control group (Dunnett's *t*-test). B) Mice were administered with vehicle (▭), or increasing doses of SR147778 by oral route (▬) 30 min before the subcutaneous administration of 3 mg/kg of WIN55212-2. Then the paw-flick test was performed as described under "Materials and Methods". Data are expressed as the paw withdrawal latencies in seconds. *=p<0.05, **=p<0.01 versus WIN55212-2 control group (Dunnett's *t*-test). In the absence of WIN55212-2 (control) a value of latency of 4.3 ± 3.4 seconds was found. C) Mice were administered with vehicle or SR147778, 30 min (3 mg/kg, ip) or 1 hour (10 mg/kg, po), before the intraperitoneal administration of 0.15 mg/kg CP 55940 or 0.3 mg/kg WIN55212-2. Test meal was given either 30 min after CP 55,940 or immediately after WIN55212-2 administration. Animals were sacrificed and the transit was assessed as described under "Materials and Methods". Data are means ± S.E.M. of values obtained from four animals. Data are expressed as percentage of gastrointestinal transit in intestine of untreated mice. Dunnett's *t* test was used for comparisons between the CP55,940 or WIN55212-2 groups versus the control group, **=p<0.01; between the SR147778 group versus the CP55,940 group, $$=p<0.01; between the SR147778 group versus the WINN55212-2 group ££=p<0.01.

Fig. 8. Effect of SR147778 on ethanol intake. Vehicle (▭) or increasing doses of SR147778 (▬) were administered subcutaneously 30 minutes before each experiment session. Then the test was performed as described under "Materials and Methods". Data are expressed as the daily mean intake in g/kg. *=p<0.05, **=p<0.01 versus control group (Dunnett's *t*-test).

Fig. 9. Effect of SR147778 on sucrose solution (5%) drinking. Following a training period (two weeks) rats were administered orally, 1 hour before the drinking session with vehicle

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(▭) or increasing doses of SR147778 (▬). Then the test was performed as described under “Materials and Methods”. Results are expressed as the mean ± S.E.M. for each treatment group. A one-way ANOVA followed by Dunnett's *t* test was used for comparisons versus the control group, *=p<0.05, **=p<0.01.

Fig. 10. Effect of SR147778 on food intake in 18-hour fasted rats. Following an adaptation phase (10 days) rats were administered orally with vehicle or increasing doses of SR147778. 1 hour after the test was performed as described under “Materials and Methods”. Results are expressed as the mean ± S.E.M. for each treatment group. A one-way ANOVA followed by Dunnett's *t* test was used for comparisons versus the control group, *=p<0.05, **=p<0.01.

Fig. 11. Effect of SR147778 on spontaneous feeding in non-deprived rats. Rats, maintained in a reversed light-dark cycle, were administered orally with vehicle or increasing doses of SR147778. 1 hour after the test was performed as described under “Materials and Methods”. Results are expressed as the mean ± S.E.M. for each treatment group. A one-way ANOVA followed by Dunnett's *t* test was used for comparisons versus the control group, *=p<0.05, **=p<0.01.

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TABLE 1 Dose-response relationship of SR147778 versus the corresponding vehicle on either water or 10% ethanol intakes (g) during one schedule-induced polydispsia in rats. (** p < 0.01 Student's *t* test; ns = non statically significant).

SR147778 **mg/kg**	**10% ethanol (g)** **Mean ± S.E.M.**	**water (g)** **Mean ± S.E.M.**
0	16.8 ± 1.1	26.5 ± 1.7
0.3	14.8 ± 1.2 (ns)	25.0 ± 2.1 (ns)
0	16.9 ± 0.8	25.0 ± 1.7
1	13.5** ± 0.5	25.6 ± 1.5(ns)
0	16.4 ± 0.6	22.2 ± 2.1
3	10.5** ± 1.1	25.2 ± 1.3 (ns)
0	15.8 ± 0.9	22.3 ± 1.8
10	10.8** ± 1.1	18.6 ± 0.9 (ns)

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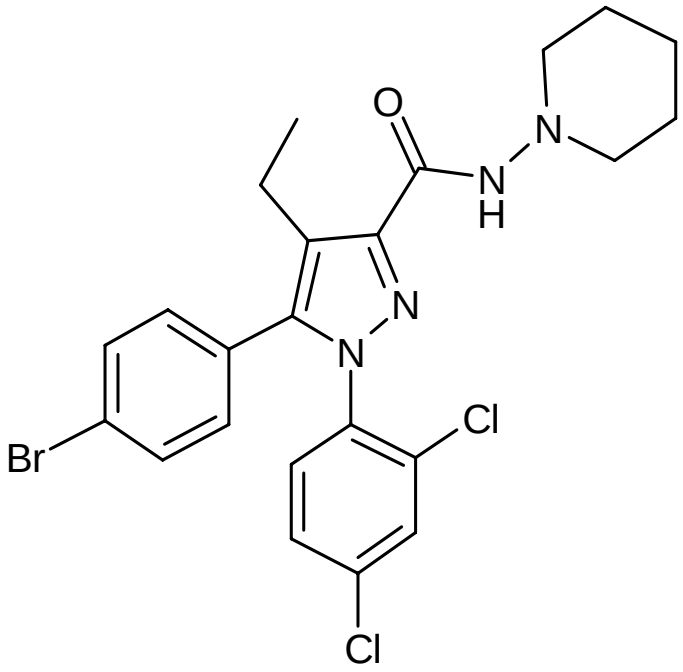

Fig. 1.

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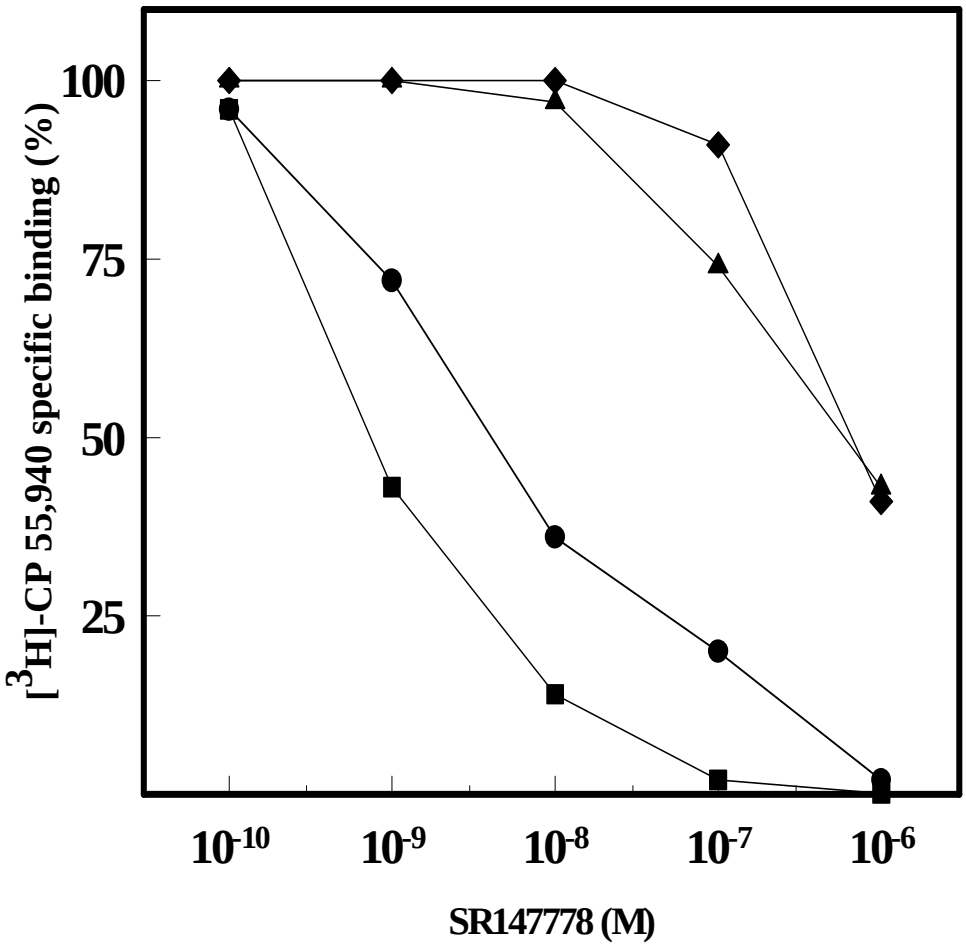

Fig. 2.

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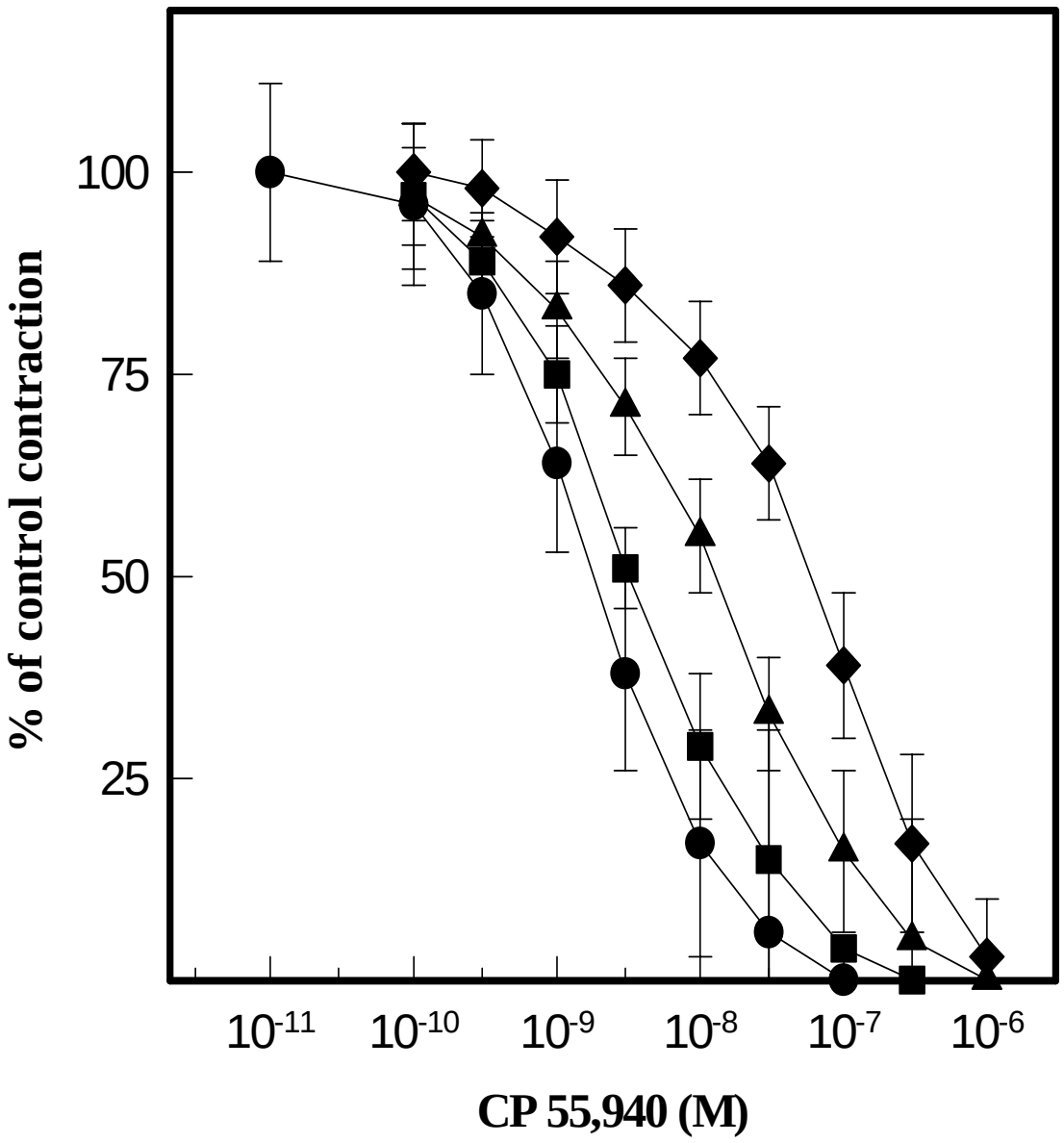

Fig. 3.

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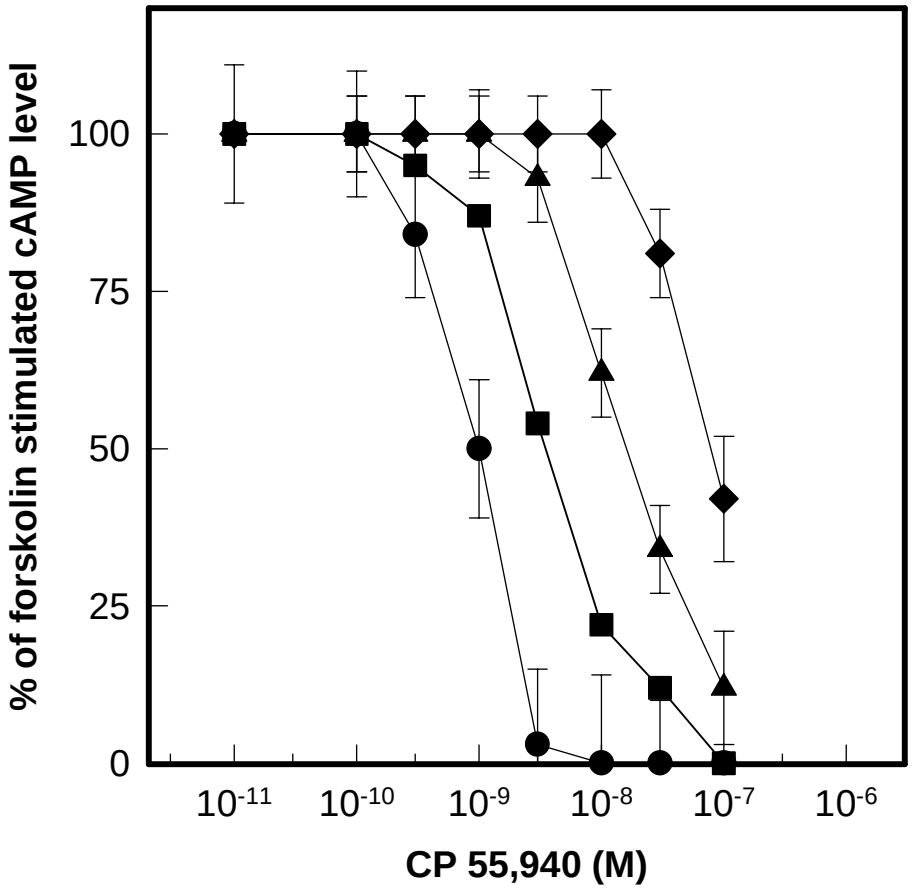

Fig. 4.

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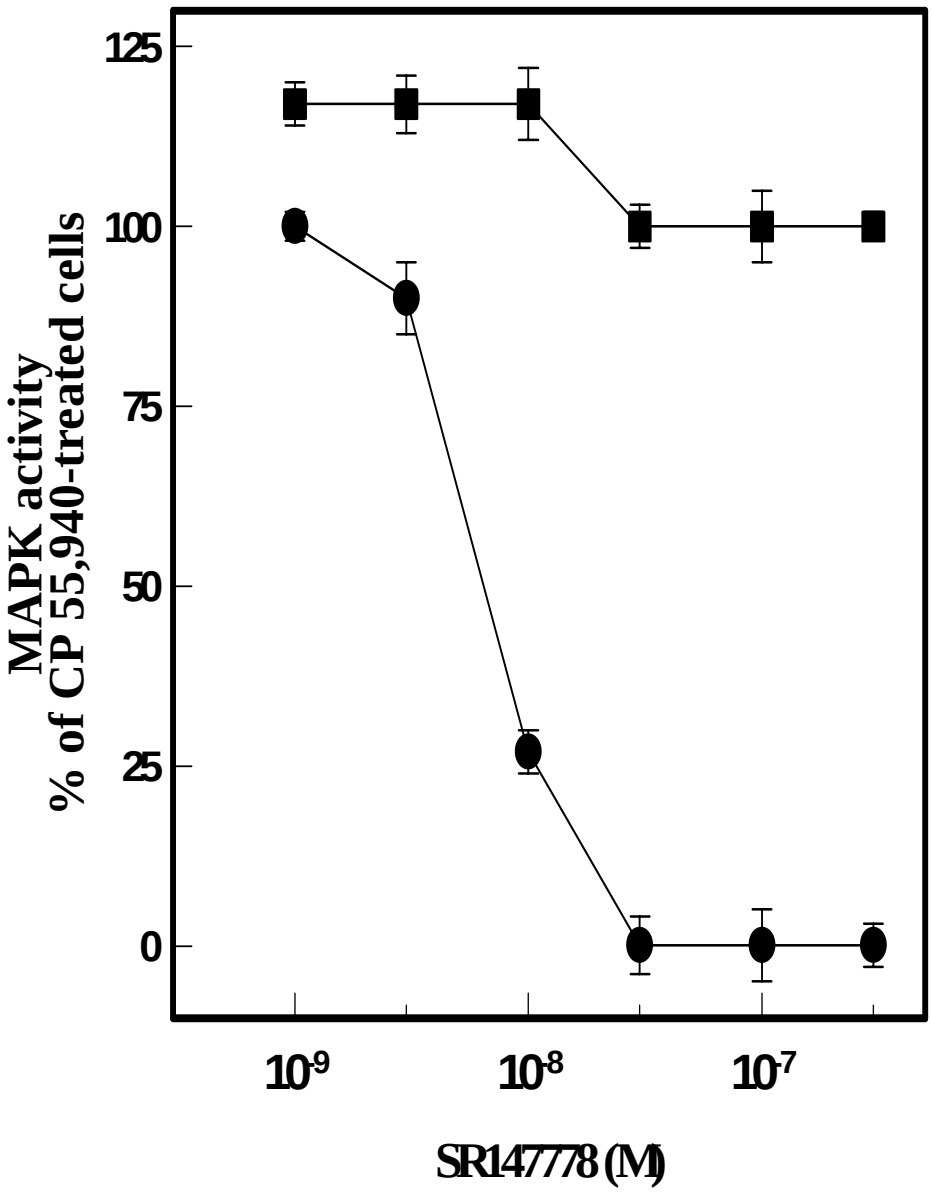

Fig. 5.

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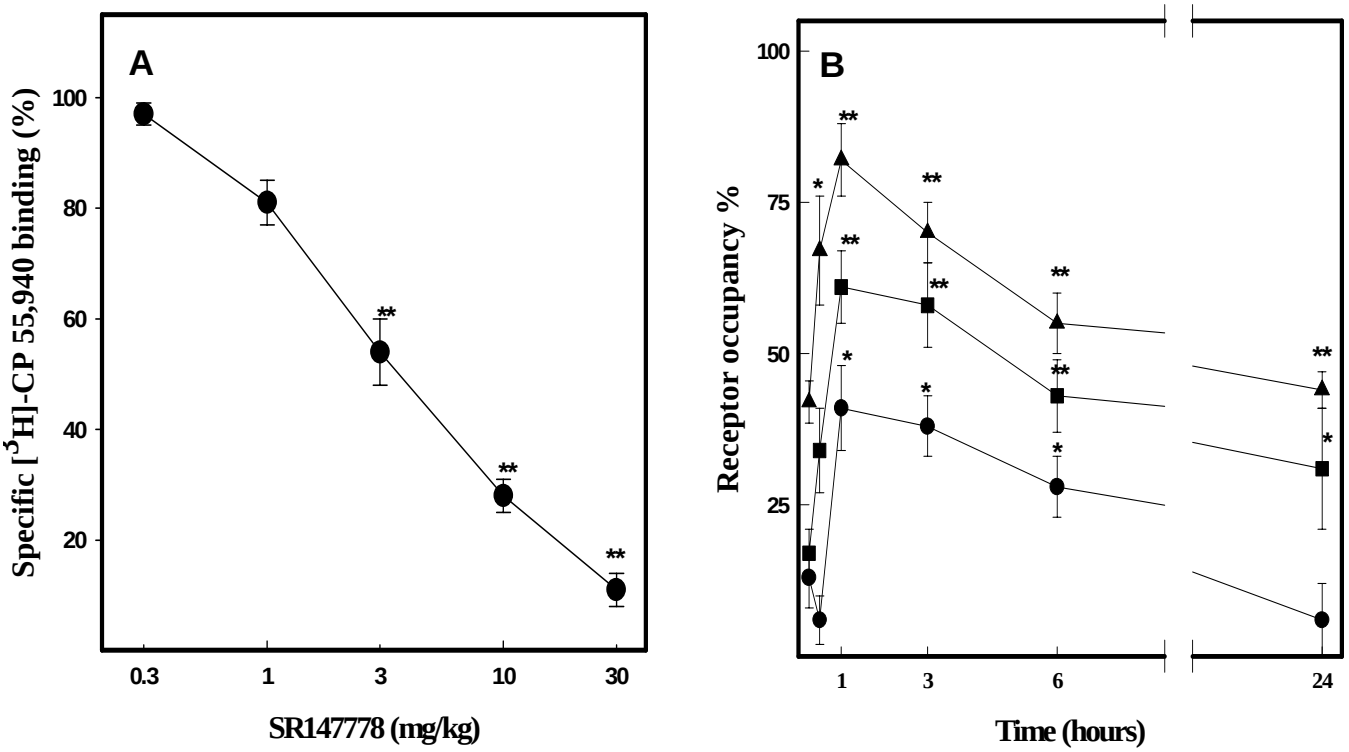

Fig. 6.

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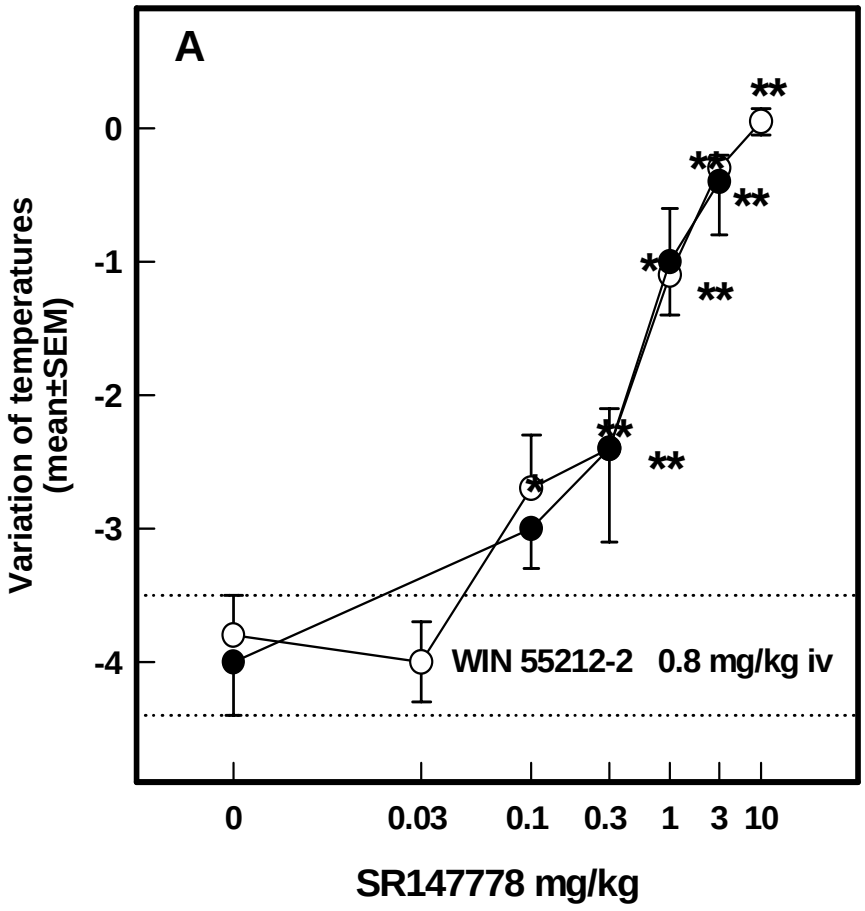

Fig. 7A.

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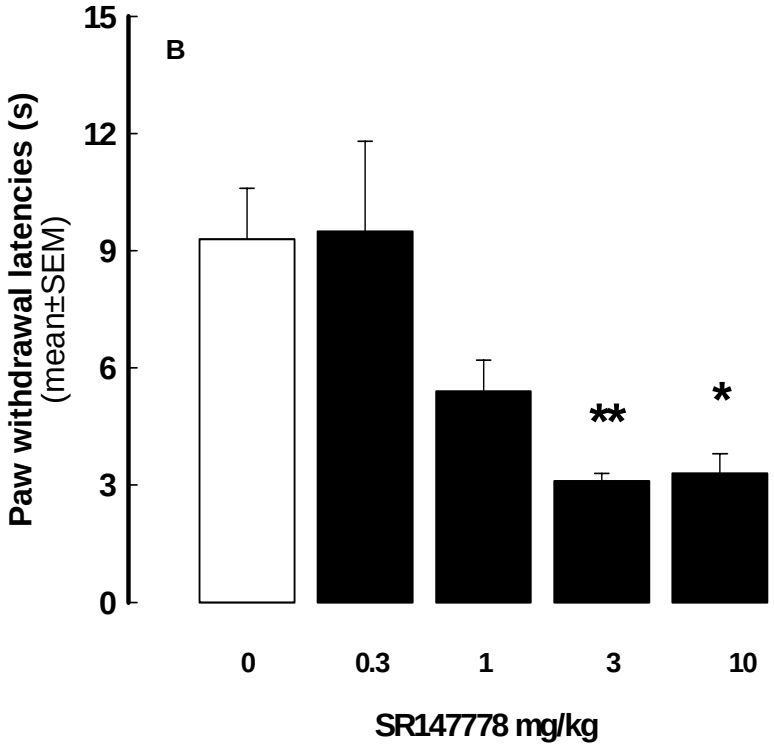

Fig. 7B.

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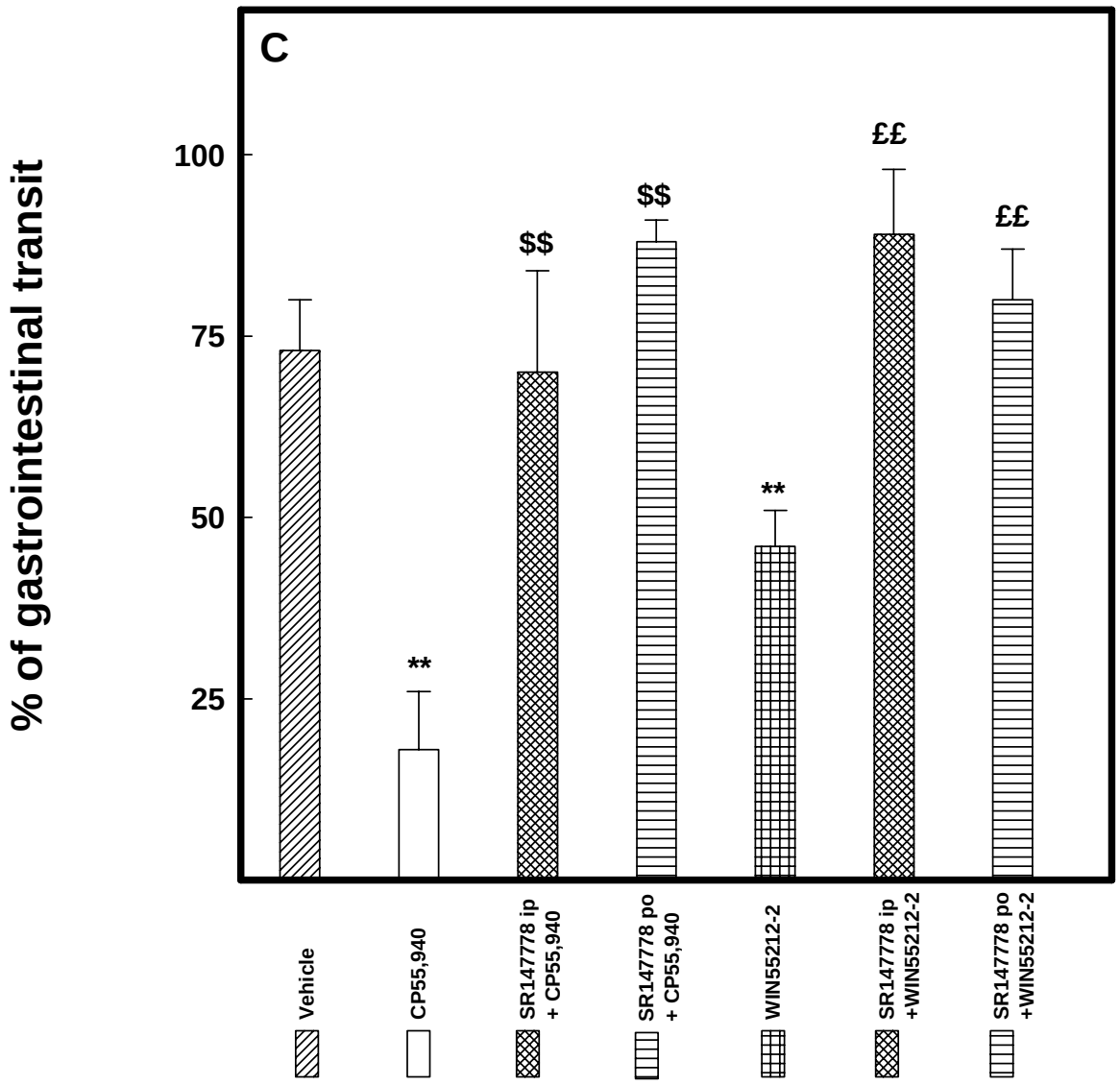

Fig. 7C.

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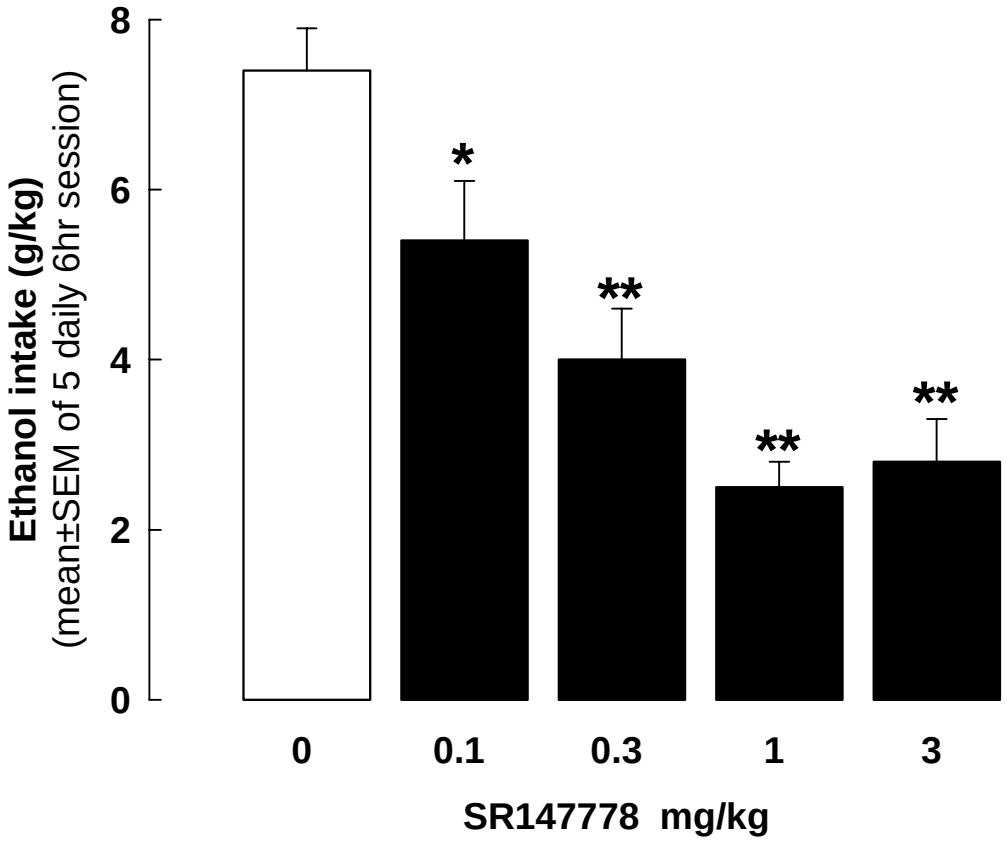

Fig. 8.

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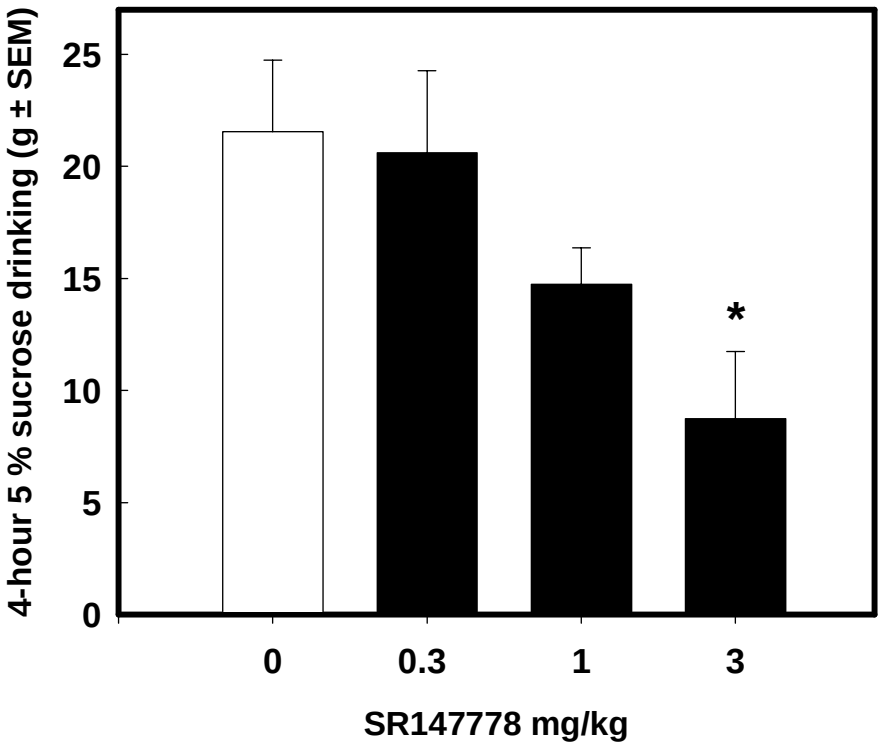

Fig. 9.

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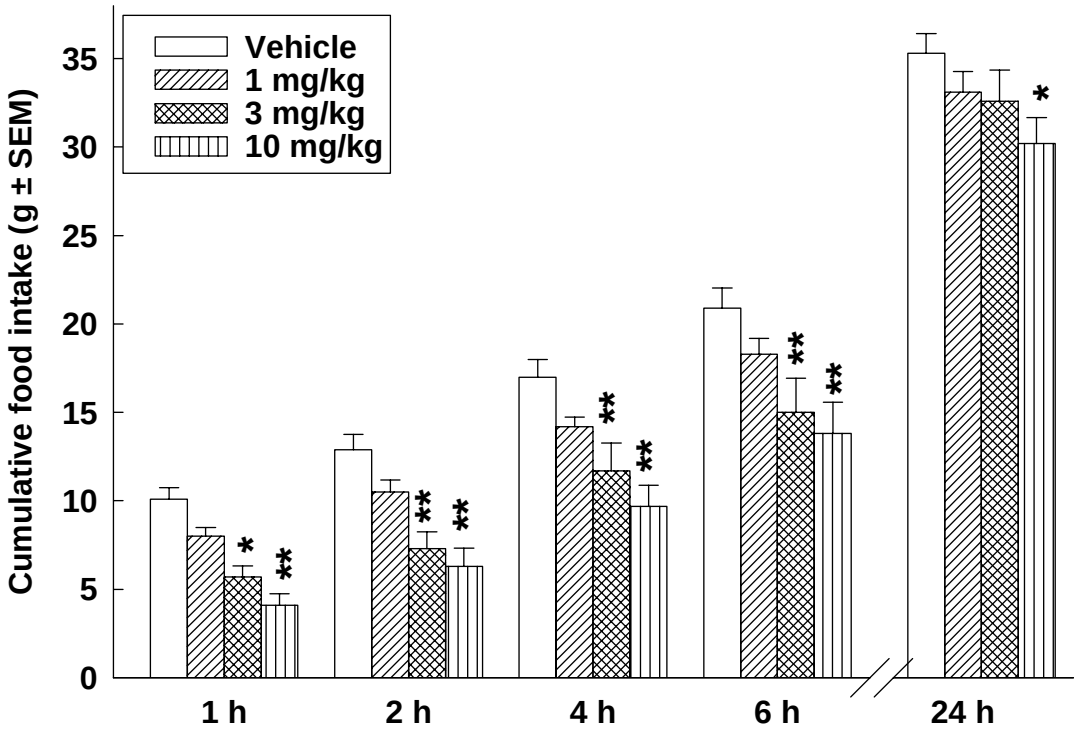

Fig. 10.

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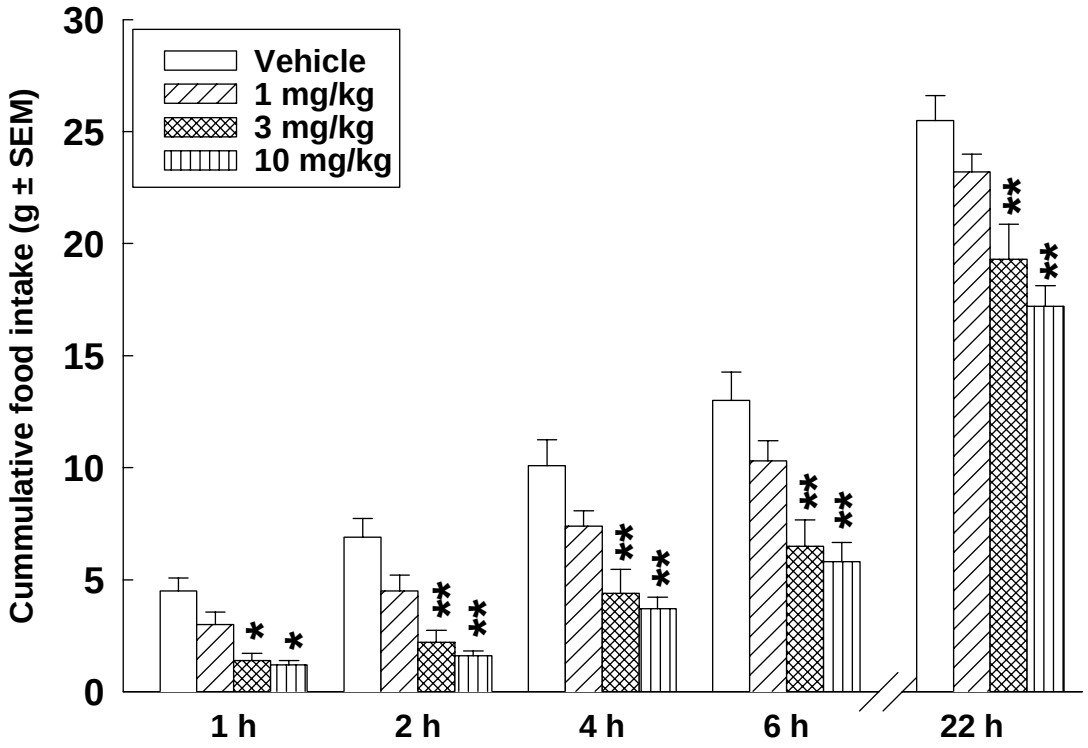

Fig. 11.