0022-3565/02/3021-359–368$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 302, No. 1
4852/990837
Printed in U.S.A.
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JPET 302:359–368, 2002

The Journal of PHARMACOLOGY And Experimental Therapeutics

Characterization of the Diarylether Sulfonylester (−)-(*R*)-3-(2-Hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate (BAY 38-7271) as a Potent Cannabinoid Receptor Agonist with Neuroprotective Properties

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Received December 17, 2001; accepted March 21, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

(−)-(*R*)-3-(2-Hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate (BAY 38-7271) is a new high-affinity cannabinoid receptor subtype 1 (CB1 receptor) ligand (K_i = 0.46–1.85 nM; rat brain, human cortex, or recombinant human CB1 receptor), structurally unrelated to any cannabinoid receptor ligand known so far. BAY 38-7271 was characterized as a CB1 receptor agonist in 5-[γ^{35}S]-thiophosphate triethylammonium salt binding assays using rat or human CB1 receptors. In the rat hypothermia assay, BAY 38-7271 induced a dose-dependent reduction in body temperature (minimal effective dose = 6 μg/kg, i.v.); whereas in rats trained to discriminate the CB1/CB2 receptor agonist (−)-*cis*-3-[2-hydroxy-4(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol (CP 55,940; 0.03 mg/kg, i.p.) from vehicle, BAY 38-7271 induced complete generalization (3 μg/kg, i.v.). In both in vivo models, a specific CB1 receptor-mediated mechanism was confirmed by demonstrating that the effects of CP 55,940 and BAY 38-7271 were blocked by pretreatment with the selective CB1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride. In the rat traumatic brain injury model, BAY 38-7271 demonstrated highly potent and efficient neuroprotective properties when administered as a 4-h infusion immediately after induction of subdural hematoma (70% infarct volume reduction at 100 ng/kg/h). Even when applied with a 3-h delay, a significant neuroprotective efficacy could be observed (59% infarct volume reduction at 300 ng/kg/h). The neuroprotective potential of BAY 38-7271 was confirmed in a rat model of focal cerebral ischemia induced by permanent occlusion of the middle cerebral artery. It is concluded that the CB1/CB2 receptor agonist BAY 38-7271 shows pronounced neuroprotective properties that do not result from drug-induced hypothermia and that occur in a dose range devoid of typical cannabinoid-like side effects.

Historical and anecdotal reports suggest that *Cannabis sativa* has a wide spectrum of therapeutic effects, including analgesic, appetite stimulating, anti-emetic, antiglaucomic, and antispastic effects (Joy et al., 1999). Scientific studies on the pharmacology of cannabis were advanced considerably by the identification of the cannabinoid Δ^9-THC as the major active constituent of cannabis predominantly responsible for its therapeutic and psychoactive effects (Gaoini and Mechoulam, 1964), the cloning of the centrally located CB1 receptor (Matsuda et al., 1990) and the peripherally located CB2 receptor (Munro et al., 1993), and the identification of selective CB1 and CB2 receptor antagonists (for review, see Barth, 1998). Both receptors are negatively coupled to adenylate cyclase through heterotrimeric $G_{i/o}$ proteins (Pertwee, 1997). The CB1 receptor was found to be also negatively coupled to N- and P/Q-type voltage sensitive Ca^{2+}- and D-type K^+ channels and positively coupled to A-type and inward-rectifying K^+ channels (Pertwee, 1997; Mu et al., 1999). Activation of CB1 receptors leads to cell hyperpolarization and inhibition of neurotransmitter release (Kim and Thayer, 2000; Gerdeman and Lovinger, 2001).

Arachidonylethanolamide (anandamide) was described as the first endogenous ligand for cannabinoid receptors (Devane et al., 1992). Later on, ethanolamides of other fatty acids (Hanus et al., 1993) and 2-arachidonoyl glycerol (Sugiura et al., 1995) were identified as additional putative endocannabi-

ABBREVIATIONS: BAY 38-7271, (−)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-sulfonate; B_{max}, maximal specific binding; CB1 receptor, cannabinoid receptor subtype 1; CB2 receptor, cannabinoid receptor subtype 2; CP 55,940, (−)-*cis*-3-[2-hydroxy-4(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol; CL, confidence limits; Δ^9-THC, (−)-Δ^9-tetrahydrocannabinol; [^{35}S]GTPγS, 5-[γ^{35}S]-thiophosphate triethylammonium salt; HU-210, (−)-11-OH-Δ^8-tetrahydrocannabinol-dimethylheptyl; K_d, equilibrium dissociation constant; K_i, dissociation constant for inhibitor binding; SDH, acute subdural hematoma; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride; $T_{1/2}$, half-life time; TBI, traumatic brain injury; pMCA-O, permanent focal middle cerebral artery occlusion; WIN 55,212-2, (*R*)-4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphtalenylcarbonyl)-6*H*-pyrrolo[3,2,1-*ij*]quinolin-6-one.

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noids. Although the complex physiological role of the endogenous cannabinoid system is not fully understood, it can be assumed that it is involved in the modulation of the release of neurotransmitters, such as glutamate (for review, see Piomelli et al., 2000).

One of the key events caused by brain ischemia after stroke or traumatic brain injury (TBI) is the excessive release of the excitatory neurotransmitter glutamate, which triggers a cascade of intracellular processes leading finally to neuronal death (Lipton, 1999). Based on the crucial role of glutamate-mediated events in neuronal death, a variety of therapeutic research strategies have focused on either postsynaptic inhibition of glutamate transmission by means of glutamate receptor antagonists (Boxer and Bigge, 1997) or inhibition of ischemia-evoked glutamate release by neuronal hyperpolarization (e.g., via 5-HT_{1A} receptor stimulation; Mauler et al., 2001). Because cannabinoid receptor activation may lead to an inhibition of glutamate release, it can be hypothesized that cannabinoid receptor agonists will have neuroprotective properties (for further discussion, see Piomelli et al., 2000).

Neuroprotective efficacy of cannabinoid receptor agonists has been demonstrated in several models. In vitro, the nonclassical CB1/CB2 receptor agonist WIN 55,212-2 was reported to prevent excitotoxicity of hippocampal neurons (Shen and Thayer, 1998). In addition, these authors demonstrated that WIN 55,212-2-induced neuroprotection could be blocked by the selective CB1 receptor antagonist SR 141716A, suggesting a CB1 receptor-mediated mechanism. Although the classical cannabinoid Δ^9-THC has been suggested to act in similar models via receptor-independent mechanisms (Hampson et al., 1998, 2000), recent studies indicate that the neuroprotective effects of this compound were mediated by activation of CB1 receptors (Abood et al., 2001). Also, in rat models of transient global cerebral ischemia and focal cerebral ischemia, cannabinoid receptor agonists were reported to have neuroprotective properties (Nagayama et al., 1999). Interestingly, CB1 receptor expression was found to be 4-fold increased in the penumbra after transient middle cerebral artery occlusion (Jin et al., 2000). This finding suggests that the endogenous cannabinoid system may have become more sensitive after brain ischemia and that CB1 receptor agonists may offer a unique therapeutic opportunity under these conditions.

It was the aim of the present study to characterize the activity of the diarylether sulfonylester derivative BAY 38-7271 (Fig. 1) at CB1 receptors in a number of in vitro and in vivo assays, and to evaluate its neuroprotective properties in animal models of TBI and brain ischemia.

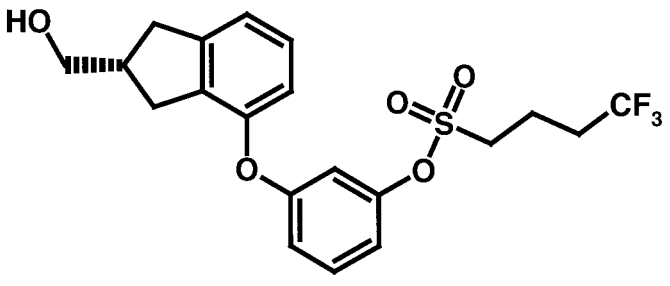

Fig. 1. Structure of BAY 38-7271.

Materials and Methods

Receptor Binding Studies

Membranes containing the human recombinant CB1 and CB2 receptor were purchased from Receptor Biology, Inc. (Beltsville, MD). For additional studies, brain membrane preparations of Wistar rats were used. The brains were dissected and the cerebellum was removed, followed by homogenization with a Potter-Elvehjem homogenizer (1200 rpm, 15 strokes) in 10 volumes of 20 mM Tris × HCl (pH 7.4, 25°C). The suspension was centrifuged for 10 min at 1,000*g*, and the supernatant was transferred to a new tube and centrifuged again at 40,000*g* for 30 min. Finally, the supernatant was discharged, and the pellet was homogenized in 20 mM Tris × HCl (pH 7.4, 25°C). Aliquots of the membrane preparations were stored at −140°C over liquid nitrogen. Human brain cortex membranes were prepared according the method described above and were obtained from Analytical Biological Services, Inc. (Wilmington, DE).

Receptor interaction screening was performed at Panlabs, Inc. (Taipei, Republic of China) as described in the respective assay protocols. BAY 38-7271 was tested in the initial recommended concentration (10 μM) in duplicate; if active (≥50% inhibition), concentration-response curves were performed.

Binding studies at cannabinoid receptors were performed as follows: radioligand ([^{3}H]BAY 38-7271, 2.2–2.4 nM), test compound, and membranes were dissolved in 50 mM Tris × HCl, pH 7.4; 2.5 mM EDTA; 5 mM $MgCl_2$; and 5 mg/ml BSA, fatty acid free (final volume = 200 μl). The reaction mixtures were incubated for 90 min at 30°C and terminated by rapid vacuum filtration over GF/C filters (presoaked for 90 min in 50 mM Tris × HCl, pH 7.4; Whatman, Clifton, NJ) using a cell harvester (Brandel, Inc., Gaithersburg, MD). To reduce unspecific binding, filters were washed 12 times with 1 ml of ice-cold wash buffer (50 mM Tris × HCl, pH 7.4, and 0.05% BSA, fatty acid free). Remaining radioactivity was counted in a scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Protein concentrations were 20, 24, 11, and 100 μg/tube for rat brain membranes, recombinant human CB1 and CB2 receptors, and human cortex membranes, respectively. Determination of K_d and B_{max} were performed in separate experiments in which the concentration of radioligand was increased stepwise.

Agonist-Stimulated [^{35}S]GTPγS Binding

Test compound, 0.05 nM [^{35}S]GTPγS, and membranes (rat cortex = 25 μg of protein; human cortex = 50 μg of protein; prepared as described above) were incubated at 30°C for 1 h in assay buffer (50 mM Tris × HCl, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 25 μM GDP, and 1 mg/ml BSA, fatty acid free, pH 7.4) in a final volume of 1 ml. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters and rinsed three times with 3 ml of ice-cold buffer (50 mM Tris × HCl, pH 7.4) using a Brandel cell harvester. The remaining radioactivity was counted in a scintillation counter (Packard BioScience, Dreieich, Germany).

Behavioral Studies

Animals. Male Wistar rats were purchased from Harlan-Winkelmann (Borchen, Germany). Body weight upon arrival at the laboratory was around 160 g. Rats were group housed (hypothermia assay, three to four animals per cage) or individually housed (drug discrimination) in macrolon type III cages under a normal 12-h light period (light on at 7:00 AM). The animals had unrestricted access to food in the hypothermia assay and restricted access to food in the drug discrimination assay (approximately 13–15 g/day, standard pellets; Ssniff Spezialdiäten GmbH, Soest, Germany). In both assays, rats were offered water ad libitum. Room temperature was maintained at 22 to 23°C. Experimental protocols and conditions conformed with the German regulations on animal welfare.

Hypothermia Assay. Different groups of rats (in general, n = 7–8 per group) were treated with vehicle or various doses of a test

compound. Their body temperature was oesophagally measured repeatedly at fixed time points. Time points measured included: 5 min before, and 7.5, 15, 30, and 60 min, or 5, 10, 20, 40, 80, and 120 min (i.p. dose-response determination) after drug administration. For the antagonism test, temperature was measured 5 min before administration of SR 141716A (1 mg/kg, i.p.) or vehicle. Sixty minutes after i.p. pretreatment, rats were administered i.v. with the agonist (i.e., 0.02 mg/kg HU-210, 0.02 mg/kg BAY 38-7271, 0.05 mg/kg CP 55,940, 0.6 mg/kg WIN 55,212-2, and 1 mg/kg Δ^9-THC) or vehicle, and temperature was measured again after 7.5, 15, 30, and 60 min. For graphical presentation, results were expressed as temperature change in degrees Centigrade relative to baseline value and were corrected for the temperature change observed in the vehicle control group.

Drug Discrimination Assay. Sessions were performed in sound- and light-attenuated standard operant chambers (Coulborn Instruments, PA). The chambers were equipped with two levers equidistant from a food tray between the levers. Food reinforcement (45 mg of precision pellets; Bio-Serv, Inc., Frenchtown, NJ) was delivered by an automated food dispenser located outside the chamber. Ventilation and masking noise were provided by a fan mounted on the wall of the chamber. A white light was switched on during the sessions, which were conducted between 9:00 and 12:00 AM.

In general, the procedure as described by De Vry and Jentzsch (1998) was followed. After initial encouragement to lever press for food reinforcement, the rats were trained to discriminate CP 55,940 (0.03 mg/kg, i.p.; *T*, 30 min, $n = 16$) from vehicle in a standard two-lever, fixed ratio 10 food-reinforced operant procedure. Daily sessions were conducted that were terminated either after 50 reinforcers or after 10 min, whichever came first. For one-half of the animals, responding on the left lever was reinforced after CP 55,940, for the other half responding on this lever was reinforced after vehicle. The rats were injected with drug or vehicle according to the following sequence: D-D-V-D-V//V-D-V-V-D//D-V-D-V-V//D-D-V-D-V (D, drug; V, vehicle; and //, no sessions during the weekends) with repetition. Discrimination criterion consisted of 10 consecutive sessions in which no more than nine responses occurred on the nonreinforced lever before the first reinforcer was obtained. Test sessions were performed when this number of incorrect responding was not more than four on three consecutive training sessions and when at least 20 reinforcers were obtained per session. During test sessions, responding on the selected lever, i.e., the lever on which 10 responses accumulated first, was reinforced for the remainder of the session. Generalization and antagonism tests were separated by at least three training sessions in which vehicle and drug were correctly discriminated, i.e., fewer than five incorrect responses before the first reinforcer. The animals were tested with different doses of the training compound (0.003–0.03 mg/kg, i.p.) before being submitted to a time-dependence study. Generalization tests were performed 30 min after application of the test compound (except for HU-210, where the injection-test interval was 120 min, and the time-dependence studies, where 0.03 mg/kg of CP 55,940 and BAY 38-7271 was tested 15–240 min after application). BAY 38-7271 was tested after i.p. and i.v. administration, to ascertain that the quality of the stimulus was not essentially different after different routes of administration and to demonstrate potency equivalence of the doses. In the antagonism study, pretreatment with SR 141716A (or vehicle; i.p.) occurred 10 min before treatment with CP 55,940 or BAY 38-7271 (0.03 mg/kg or vehicle, i.p.). In general, each dose of a test compound was tested in 6 to 8 rats, randomly allocated to each test condition. For the drug discrimination assay, test results were expressed as the percentage of rats that selected the drug lever (percent drug lever selections). In addition, the percentage of animals that selected a lever (either drug or vehicle lever) was determined as an index of behavioral disruption (i.e., percent lever selections).

Neuroprotection Studies

Animals. Male Long Evans rats (180–380 g, Møllegard & Bomholtgaard A/S, Ry, Denmark) were used for the permanent focal middle cerebral artery occlusion (pMCA-O) experiments, and Wistar rats (230–300 g, Harlan-Winkelmann, Borchen, Germany) were used for the acute subdural hematoma (SDH) experiments. The animals were allowed to adapt to housing conditions for at least 1 week before they were subjected to the study. They were housed in groups of three to five individuals in macrolon cages (type III, Ebeco, Castrop-Rauxell, Germany) bedded with saw dust. The animal housing room and the laboratory for surgery were climate controlled and continuously illuminated from 6:00 AM until 6:00 PM. Room temperature was about 21°C; relative humidity was about 50%. Food (Altromin 1324; Altromin Spezialfutterwerk GmbH, Lange, Germany; or R/M-H; V1534-00 DDb; Ssniff Spezialdiäten GmbH, Soest, Germany) and water were available ad libitum. Experimental protocols and conditions conformed with the German regulations on animal welfare.

Acute Subdural Hematoma (SDH). The animals were anesthetized with the inhalation anesthetic isofluran (Forene, Abbott GmbH, Wiesbaden, Germany or Isofluran-Baxter, Baxter Deutschland GmbH, Unterschleiβheim, Germany mixed with $\cong$ 28% O_2 in N_2O to 5–1% v/v concentration). Subdural hematoma was induced according to a standard surgical procedure (Miller et al., 1990) with the following minor modifications. The top of the head was shaved and the skin was disinfected and opened with a longitudinal midline cut. A small part of the periosteum was removed, and a burr hole was drilled into the skull with the stereotaxic coordinates: −1 mm caudal, −2.8 mm lateral to the bregma (Paxinos and Watson, 1982). The dura was carefully opened, and a specially designed plastic cannula was inserted into the subdural space between the dorsal surface of the brain and the dura. Thereafter, the cannula was fixed in position with a tissue glue (Histoacryl; B. Braun Surgical GmbH, Melsungen, Germany). Nonheparinized autologous blood was collected by puncture of the tail vein and injected directly via the prefixed cannula into the subdural space (total volume of 0.2 ml within 4 min). After that, the probe was shortened and closed with cyanacrylate glue (Histoacryl). The skin wound was closed with suture clips. During the surgery and the continuous i.v. infusion of the drug or vehicle, the body temperature was monitored and maintained within the physiological range (37.0 ± 0.5°C) using a warming pad. After recovery from anesthesia, the animals were returned to their home cage. Seven days after surgery, the rats were decapitated, and their brains were rapidly removed and frozen in 2-methyl butane cooled down to −30°C on dry ice. Serial coronal sections (20 μm thick) were cut throughout the entire infarcted area with a standard distance of 500 μm using a cryostat microtome (CM 3050, Leica Vertrieb GmbH, Bensheim, Germany; and HM 500 OM, Microm Laborgeräte GmbH, Walldorf, Germany). Slide-mounted brain sections were stained with cresyl fast violet. The volume of the cortical infarct was determined by an operator blinded to the group composition with a computer-assisted image analysis system (Optimas, BioScan, Inc., Edmonds, WA). Infarct volumes were expressed in cubic millimeters (mean ± 1 S.E.M.). For comparison of individual experiments, infarct volumes of treatment groups were expressed as the percentage from the respective controls, which were set to 100%.

Permanent Middle Cerebral Artery Occlusion (pMCA-O). Under general anesthesia (Forene, Abbott GmbH, Wiesbaden, Germany; Isofluran-Baxter, Baxter Deutschland GmbH, Unterschleiβ-heim, Germany mixed with $\cong$ 28% O_2 in N_2O to 5–1% [v/v] concentration) the MCA was occluded unilaterally based on the surgical procedure described by Bederson et al. (1986). The left temporal-parietal region of the head was shaved and the skin was disinfected and opened between the orbit and the external ear canal. After a midline incision, the temporal muscle was divided and pulled aside with surgical hooks to make the lateral aspect of the skull free. The facial nerve, major facial arteries and veins, the lateral eye muscles,

the intra- and extraorbital lacrimal glands, and the zygomatic bone were left intact. Under an operating microscope, a small burr hole was drilled directly under the zygomatic arc, 1 to 2 mm rostrally to its caudal origin from the saquamosal bone. After careful opening of the dura, the exposed MCA and its branches were permanently occluded between the olfactory tract and the inferior cerebral vein proximal to the lenticulostriate branch by microbipolar electrocoagulation (Bipolator 50, Fischer MET GmbH, or Bipol 50, Stockert GmbH, Freiburg, Germany). To avoid recanalization, the occluded vessels were removed. Muscle and skin wounds were closed with surgical suture or with tissue glue (Histoacryl). During surgery and drug/vehicle infusion, the body temperature was monitored using a rectal temperature probe and maintained between 36.5 and 37.5°C with a heating pad. After recovery from anesthesia, the animals were returned to their home cage. Further proceedings were similar to those described under the SDH procedure.

Data Analysis. If not otherwise indicated, all in vitro biochemical experiments were performed in duplicate or triplicate and were repeated at least three times. K_i values were calculated according to the Cheng-Prusoff equation from the respective IC_{50} values obtained from concentration-response curves with at least six concentrations. Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values from agonist-stimulated values. Data analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). For the hypothermia assay, data were evaluated by analysis of variance; followed, where appropriate, by Tukey's post hoc comparisons. The minimal effective dose was defined as the lowest dose that produced a statistically significant ($P < 0.05$) hypothermic effect of at least −0.7°C (mean temperature difference as compared with vehicle control), at any of the time points measured. Least-square linear regression analysis was used to estimate ED_{50}, ID_{50}, and $T_{1/2}$ values and the corresponding 95% confidence limits (CL) after log-probit conversion of the data. ED_{50} or ID_{50} values with nonoverlapping CL limits were considered to be significantly different. Generalization was considered to be complete if at least 80% drug lever selections was obtained; whereas antagonism was considered to be complete if less than 20% drug lever selections were obtained.

Drugs and Chemicals. BAY 38-7271, CP 55,940, HU-210, and SR 141716A were synthesized by the Department of Chemical Research, Business Group Pharma, Bayer AG. [^{3}H]BAY 38-7271 was obtained from the Department of Radiochemistry. WIN 55,212-2 was purchased from Research Biochemicals, Inc. (Natick, MA), and Δ^9-THC was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). If not otherwise stated, all other chemicals were of highest available purity and purchased from Merck KGaA (Darmstadt, Germany). Compounds were suspended in a solvent containing 2% (drug discrimination) or 5% (hypothermia assay) Solutol HS 15 (12-hydroxystearic-acid ethoxylate; BASF AG, Ludwigshafen, Germany) and 2% (drug discrimination) or 5% (hypothermia assay) ethanol (ethanol absolute, 99.8%; Riedel-de Haen, Sellze, Germany) for i.p. administration. For i.v. bolus administration, compounds were suspended in distilled water and Cremophor EL (1%; BASF, Ludwigshafen, Germany). Application volume was 2 ml/kg (drug discrimination) or 5 ml/kg (hypothermia assay). For neuroprotection experiments, BAY 38-7271 was dissolved in absolute ethanol corresponding 1% of final volume. Thereafter, 10% cyclodextrin solution (Pharmaceutical Technology, Bayer AG, Leverkusen, Germany) was added to reach the final volume. Ready-made solution was administered intravenously as continuous infusion, the application volume was 4 ml/kg/h.

Results

Receptor Binding

Saturation Studies. The results of saturation experiments are summarized in Table 1. [^{3}H]BAY 38-7271 binding was saturable at CB1 and CB2 receptors (Fig. 2) and Scatchard analysis fitted best with the one site model. As compared with CP 55,940 no significant difference in B_{max} and K_d could be detected on rat brain membranes, recombinant CB1 receptors, and human cortex membranes. Depending on tissue and species, both B_{max} and K_d values differed only by a factor of approximately 3 (values from human cortex membranes excluded). At human cortex membranes, preliminary experiments revealed slightly lower B_{max} values for both compounds as compared with rat brain membranes; whereas no significant differences in K_d values could be detected. At CB2 receptors, BAY 38-7271 and CP 55,940 showed comparable B_{max} and K_d values, and for both compounds there was no evidence for selectivity toward either receptor subtype.

Competition Studies. K_i values of competition studies at CB1 receptors, as obtained from rat brain membranes (Fig. 3A), human cortex membranes (Fig. 3B), recombinant human CB1 receptors (Fig. 3C), and recombinant human CB2 receptors (Fig. 3D) are summarized in Table 2. At all CB1 receptors, BAY 38-7271 was similar in potency to CP 55,940. As compared with the reference compounds tested, the ranking order of potency was: HU-210 > BAY 38-7271 ≥ CP 55,940 > WIN 55,212-2 ≫ Δ^9-THC. At CB2 receptors (Fig. 3D; Table 2), BAY 38-7271 was approximately 10-fold less potent than CP 55,940. At CB2 receptors, HU-210 was again the most potent receptor ligand, followed by WIN 55,212-2, CP 55,940, BAY 38-7271, and Δ^9-THC. At human cortex membranes (Fig. 3B), K_i values for BAY 38-7271, CP 55,940, and Δ^9-THC were similar to those found at human recombinant CB1 receptors. Similar to HU-210, CP 55,940, and Δ^9-THC, BAY 38-7271 did not display CB receptor subtype selectivity; whereas WIN 55,212-2 showed some degree of selectivity toward CB2 receptors (approximately 30 times higher versus CB1 receptors). Results from further screening investigations revealed only minor interactions with other (noncannabinoid) binding sites. Among 123 receptors tested, significant binding was detected at the adenosine A_3 receptor (IC_{50} = 7.5 μM), the peripheral $GABA_A$ benzodizepine receptor (IC_{50} = 971 nM), the melantonin ML_1 receptor (IC_{50} = 3.3 μM), and at the monoamine transporter (IC_{50} = 1.7 μM).

TABLE 1

B_{max} and K_d values of BAY 38-7271 and CP 55,940 at rat brain membranes, human cortex membranes, human recombinant CB1, and human recombinant CB2 receptors (radioligand [^{3}H]BAY 38-7271)

Compound	Rat Brain Membranes		Human Cortex Membranes		Recombinant Human CB1 Receptor		Recombinant Human CB2 Receptor	
	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d
	pM/mg	*nM*	*pM/mg*	*nM*	*pM/mg*	*nM*	*pM/mg*	*nM*
BAY 38-7271	3.01 ± 0.46	1.84 ± 1.44	0.23[a]	2.10[a]	0.87 ± 0.13	2.91 ± 0.30	1.88 ± 0.21	4.24 ± 0.18
CP 55,940	3.32 ± 0.32	2.37 ± 1.49	0.18[a]	1.29[a]	0.47 ± 0.18	1.10 ± 0.39	1.75 ± 0.17	4.20 ± 0.28

[a] Values from one preliminary experiment; all other values are the mean and standard deviation of at least three independent experiments, each performed in triplicate.

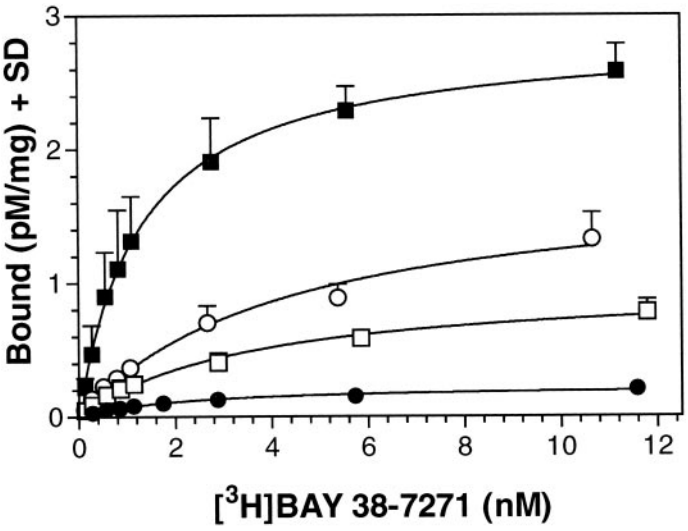

Fig. 2. Saturation curves of [^{3}H]BAY 38-7271 at rat brain membranes (■), human cortex membranes (●), recombinant human CB1 receptors (□), and recombinant human CB2 receptors (○). Values for human cortex membranes were from one preliminary experiment; all other values are the mean and standard deviation of at least three independent experiments, each performed in triplicate.

Signal Transduction. Signal transduction studies on human cortex membranes (Fig. 4A) revealed high efficacy for BAY 38-7271 (63.4 ± 2.3% over baseline level at 10 μM) and CP 55,940 (64.3 ± 0.8%); whereas Δ^9-THC displayed relatively low efficacy (15.7 ± 1.9%). Therefore, both BAY 38-7271 and CP 55,940 can be characterized as full agonists; whereas the profile of Δ^9-THC suggests that the compound behaves as a partial agonist. Also on rat cortex membranes (Fig. 4B), CP 55,940 (51.9 ± 3.8% over baseline level at 10 μM) and BAY 38-7271 (52.6 ± 5.6%) were characterized as full agonists with a similar degree of potency and efficacy; whereas Δ^9-THC (13.0 ± 2.6%) seemed to be a relatively weak partial agonist. When the individual maximal effects were used for calculation, EC_{50} values at rat brain membranes were 7.55 ± 0.76 nM for BAY 38-7271, 18.7 ± 3.9 nM for CP 55,940, and 27.6 ± 10.6 nM for Δ^9-THC, respectively, and EC_{50} values at human cortex membranes were 15.8 ± 2.76 nM for BAY 38-7271, 10.6 ± 0.9 nM for CP 55,940, and 25.7 ± 3.8 nM for Δ^9-THC, respectively.

Hypothermia Assay

BAY 38-7271, CP 55,940, HU-210, WIN 55,212-2, and Δ^9-THC induced a dose-dependent reduction in body temperature after both i.v. (Fig. 5A) and i.p. (Fig. 5B) administration. In general, the potency and time dependence of BAY 38-7271 was similar to that of CP 55,940 (Figs. 5 and 6B). The time-dependence data indicated that the hypothermia induced by BAY 38-7271 had an early onset of action, was maximal between 15 and 30 min after i.v. administration (Fig. 6A) and around 40 min after i.p. administration (data not shown). As in the case of the other reference compounds tested (as demonstrated for CP 55,940 in Fig. 7B; data not shown for the other compounds), the hypothermia induced by BAY 38-7271 was significantly attenuated by pretreatment with the CB1 receptor antagonist SR 141716A (Fig. 7A). In general, for each compound, the occurrence of hypothermia coincided with the emergence of dose-dependent behavioral side effects, such as sedation, ptosis, belly or side lying, and catalepsy; and similar to the hypothermic effects, these behavioral side effects could be antagonized by pretreatment with SR 141716A (data not shown).

Drug Discrimination Assay

All rats learned to discriminate CP 55,940 (0.03 mg/kg, i.p.) from vehicle; the median number of sessions to reach criterion was 38 (range: 29–103 sessions). The generalization obtained with CP 55,940 was dose-dependent and complete at 0.03 mg/kg (Fig. 8A) and occurred in the absence of behavioral disruption (100% lever selections at each dose). As shown in Fig. 8A, dose-dependent and complete generalization was also obtained with HU-210, WIN 55,212-2 and Δ^9-THC; complete generalization was obtained at 0.01, 1, and 3 mg/kg, i.p., respectively (ED_{50} values in Table 3). For each of these compounds, generalization occurred in the absence of behavioral disruption (100% lever selections at each dose; except for 3 mg/kg Δ^9-THC, where one of six rats tested failed to select a lever). Similar to the reference compounds, BAY 38-7271 was found to generalize dose dependently to the CP 55,940 cue, with complete generalization being obtained at 0.003 and 0.03 mg/kg, after i.v. and i.p. administration, respectively (Fig. 8A). As with the other compounds, generalization was obtained in the absence of behavioral disruption (100% lever selection at each dose). Under the present experimental conditions (i.p. administration; T, 30 min), BAY 38-7271 was found to be approximately equipotent to CP 55,940. Time-dependence studies indicated that the discriminative effects of BAY 38-7271 (0.03 mg/kg) reached maximal intensity within 30 min after i.p. administration and disappeared gradually within 4 h ($T_{1/2}$, 163 min; 95% CL, 82–325 min; 100% lever selections at each dose; data not shown). Similar studies with CP 55,940 (tested at an estimated equipotent dose of 0.03 mg/kg, i.p.) indicated that the latter compound had a similar half-life ($T_{1/2}$, 180 min; 95% CL, 120–270 min; 100% lever selections at each dose; data not shown) but a slightly slower onset of action (maximal effect obtained at 1 h). Pretreatment with SR 141716A dose dependently and completely blocked the discriminative effects of BAY 38-7271 and CP 55,940 (Fig. 8B; Table 3). SR 141716A (0.3–10 mg/kg, tested alone, or in combination with vehicle) induced mainly vehicle-appropriate discriminative responding (maximal effect obtained at 0.3 mg/kg, 17% drug lever selections; at 1–10 mg/kg, 0% drug lever selections). SR 141716A induced response rate disruptive effects at 10 mg/kg.

Neuroprotective Efficacy in the Rat pMCA-O and SDH Model

Injection of autologous blood into the subdural space resulted in a clot formation over a part of the ipsilateral frontal cortex (area 2 and area 1), forelimb and hindlimb area, and parietal cortex (area 1). As a consequence of the hematoma formation, ischemic necrosis developed in the underlying brain tissue corresponding to parts of the primary motor and primary somatosensory cortex. Sometimes shrinkage of the ipsilateral dorsal hippocampus was seen with some loss of the CA1 pyramidal cells. In a negligible number (1–2%) of animals, the hematoma and the ischemic damage extended across the interhemispheric fissure and involved a small part of the contralateral cingulate cortex. The infarct volumes of control animals obtained in this model were 113 ± 12 mm^3. When BAY 38-7271 was given as a 4-h infusion starting

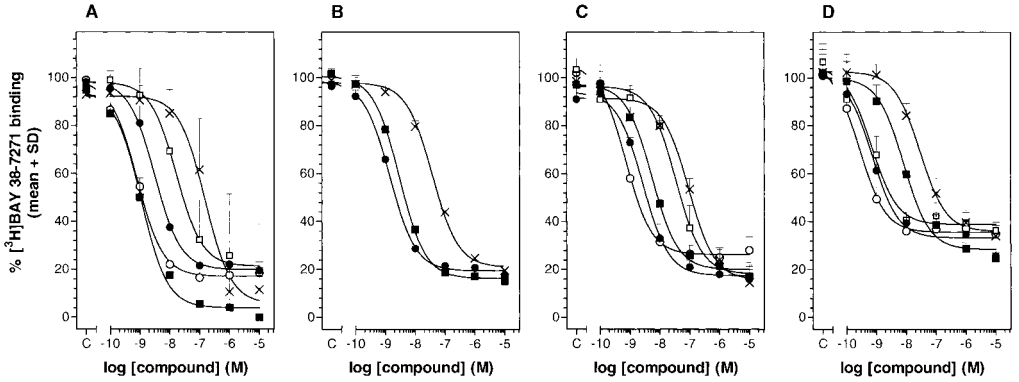

Fig. 3. Competition curves of BAY 38-7271 (■), CP 55,940 (●), Δ^9-THC (×), WIN 55,212-2 (□), and HU-210 (○) at rat brain membranes (A), human cortex membranes (B), human recombinant CB1 receptors (C), and human recombinant CB2 receptors (D), using [^{3}H]BAY 38-7271 as radioligand. Values are the mean and standard deviation of at least three independent experiments, each performed in triplicate. For K_i values, see Table 2. C, control.

TABLE 2

K_i of BAY 38-7271 and reference compounds at rat brain membranes, human cortex membranes, and recombinant human CB1 and CB2 receptors (radioligand [^{3}H]BAY 38-7271)

Values are the mean and standard deviation of at least three independent experiments, each performed in triplicate.

Compound	Rat Brain Membranes	Human Cortex Membranes	Recombinant Human CB1 Receptor	Recombinant Human CB2 Receptor
			nM	
BAY 38-7271	0.46 ± 0.19	1.09 ± 0.05	1.85 ± 0.71	5.96 ± 0.83
CP 55,940	1.30 ± 0.29	0.51 ± 0.03	1.10 ± 0.20	0.52 ± 0.13
HU-210	0.39 ± 0.13	N.T.	0.41 ± 0.05	0.14 ± 0.11
Δ^9-THC	77.3 ± 10.2	13.7 ± 1.6	15.3 ± 1.6	25.06 ± 7.14
WIN 55,212-2	6.87 ± 1.11	N.T.	11.7 ± 7.6	0.41 ± 0.31

N.T., not tested.

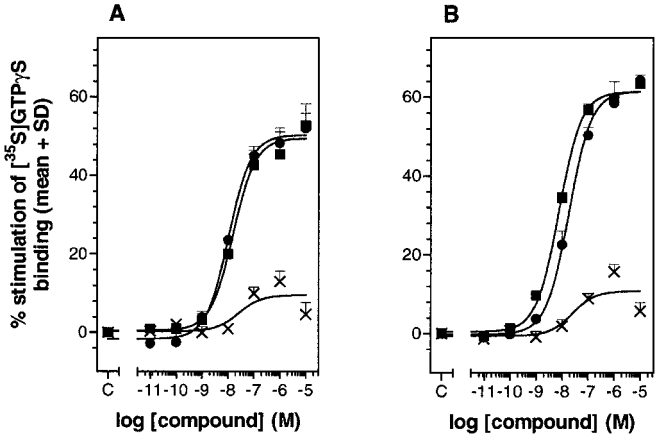

Fig. 4. Signal transduction efficacy of BAY 38-7271 (■), CP 55,940 (●), and Δ^9-THC (×) on human cortex (A) and rat cortex (B) membranes, using the [^{35}S]GTPγS binding technique. Values are the mean and standard deviation of at least three independent experiments each performed in triplicates. C, control (baseline activity was set to 0).

directly after the induction of SDH, mean infarct volume reductions of 49, 61, 70 (35.7 ± 6.7 to 43.3 ± 7.6 mm^3), and 62% were found at the doses of 1, 10, 100, and 1000 ng/kg/h, respectively (Fig. 9, A and C). A graded decrease of efficacy was observed at lower (0.1 ng/kg/h; 37% infarct volume reduction) and higher (10,000 ng/kg/h; 38% infarct volume reduction) doses.

When BAY 38-7271 was administered with a delay of 3 h after induction of SDH, mean infarct volume reductions of 36, 59, and 48% could be observed at the doses of 100, 300, and 1000 ng/kg/h, respectively (Fig. 9B). BAY 38-7271 also displayed neuroprotective efficacy in the rat pMCA-O model (infarct volume of controls were 124 ± 7.6 mm^3), reducing significantly the infarct volume by 27% at 1000 ng/kg/h (Fig. 10). At lower and higher doses, less neuroprotective efficacy was obtained, indicating that the dose-response curve was relatively flat (Fig. 10).

Discussion

For the in vitro characterization of BAY 38-7271 as a cannabinoid receptor ligand, recombinant human CB1 and CB2 receptors and rat brain and human cortical membranes expressing naturally occurring CB1 receptors were selected. As compared with a number of classical and nonclassical cannabinoid receptor agonists, saturation experiments using [^{3}H]BAY 38-7271 and Scatchard analysis revealed B_{max} and K_d values for BAY 38-7271 that were most similar to those found for CP 55,940. Although some of these data were of a preliminary nature, the binding data obtained with the reference compounds were well within the range of previously published data (Rinaldi-Carmona et al., 1994; Glass et al., 1998; Pertwee, 1999). BAY 38-7271 can be considered to be a selective CB1/CB2 receptor ligand as a broad receptor binding screening revealed no evidence for equipotent interactions with other targets than cannabinoid receptors. Thus, the range between specific cannabinoid receptor binding and interaction with other binding sites was at least 2 orders of magnitude.

Because cannabinoid receptors are coupled to G-proteins, the pharmacological effects of cannabinoid receptor ligands are not only dependent on receptor ligand interaction but also on signal transduction efficacy and various other conditions, such as cell- and/or area-dependent G-protein composition, ability of G-protein assembly and disassembly efficacy

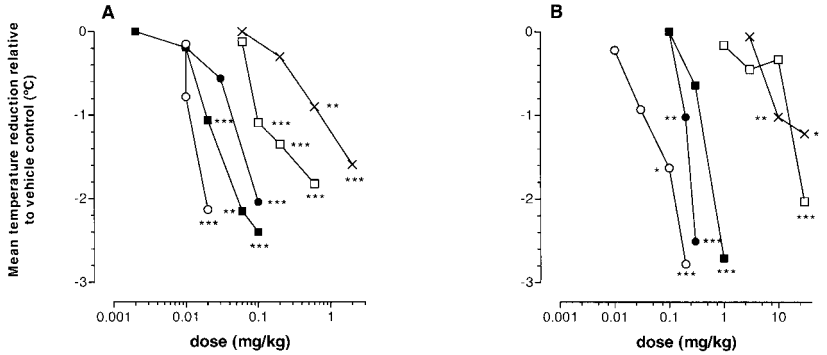

Fig. 5. Hypothermic effects of BAY 38-7271 (■), CP 55,940 (●), Δ^9-THC (×), WIN 55,212-2 (□), and HU-210 (○) after i.v. (A) or i.p. (B) administration. The 0 value on the *x*-axis represents the baseline value determined before administration of the compound. The dose-response curves shown represent the injection-time interval at which maximal effects were obtained. Maximal effects for i.v. administration were obtained at 15 min for WIN 55,212-2, at 60 min for HU-210, and for all other compounds at 30 min (n = 7–14). For i.p. administration, maximal effects were determined at 20 min for WIN 55,212-2, at 80 min for HU-210 and CP 55,940, and at 40 min for all other compounds (n = 7–8). ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ compared with vehicle control.

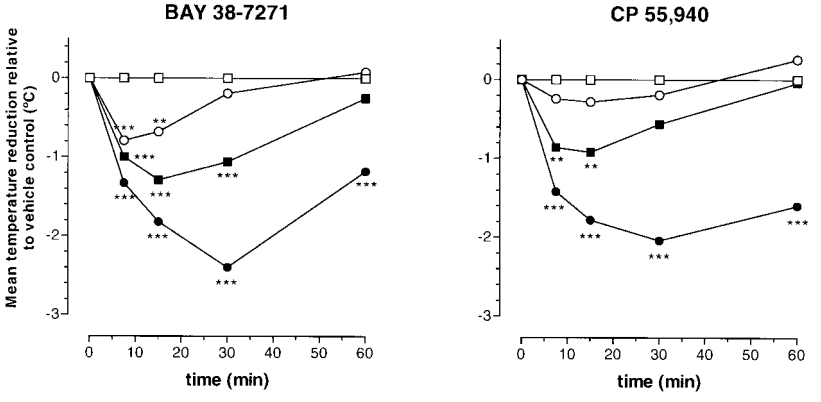

Fig. 6. Time and dose dependence of the hypothermic effects of BAY 38-7271 [0.006 (○), 0.02 (■), and 0.1 (●) mg/kg] and CP 55,940 [0.01 (○), 0.03 (■), and 0.1 (●) mg/kg] after i.v. administration. The 0 value on the *x*-axis represents the baseline value determined before administration of the compound (n = 7–14); ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ compared with vehicle control.

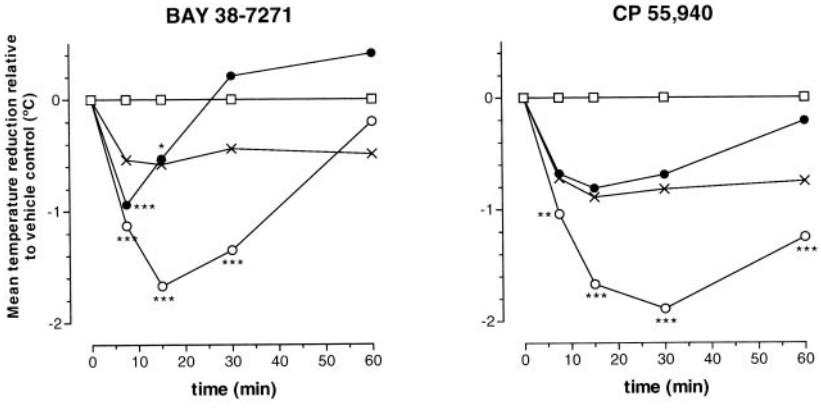

Fig. 7. Attenuation of the hypothermic effects of BAY 38-7271 [0.02 mg/kg i.v./vehicle i.p. (○), vehicle i.v./1 mg/kg i.p. SR 141716A (×), and 0.02 mg/kg i.v./1 mg/kg i.p. SR 141716A (●)], and CP 55,940 [0.05 mg/kg i.v./vehicle i.p. (○), vehicle i.v./1 mg/kg i.p. SR 141716A (×), and 0.05 mg/kg i.v./1 mg/kg i.p. SR 141716A (●)], by SR 141716A administered 60 min before i.v. administration of the CB1 receptor agonist. The 0 value on the *x*-axis represents the baseline value, as determined immediately before administration of the pretreatment. Control rats received vehicle i.v./vehicle i.p. (n = 7); ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ compared with vehicle control.

under different circumstances, number of active/passive receptors, and receptor reserve (Prather et al., 2000). Consistent with previous studies using the agonist stimulated [^{35}S]GTPγS binding technique, the present study characterized Δ^9-THC as a relatively weak CB1 receptor partial agonist; whereas BAY 38-7271 and CP 55,940 were characterized as CB1 receptor full agonists. On both rat brain and human cortex membranes, the latter compounds were nearly indistinguable in terms of potency and efficacy (Burkey et al., 1997; Petitet et al., 1998).

The in vitro characterization of BAY 38-7271 as a highly potent CB1 receptor agonist was confirmed in the rat hypothermia assay and the rat CP 55,940 drug discrimination assay, two behavioral models highly sensitive to CB1 receptor activation (Martin et al., 1991; Wiley et al., 1995a). After i.v. or i.p. administration, the hypothermic effects of BAY 38-7271 were comparable in magnitude to those induced by the reference compounds HU-210, CP 55,940, and WIN 55,212-2 and somewhat more pronounced than those induced by Δ^9-THC. This finding may indicate that these compounds differ with respect to their intrinsic activity and is compatible with the observation that the former compounds have a higher level of intrinsic activity at CB1 receptors than Δ^9-THC (see above).

The order of potency obtained with these compounds is consistent with other data seen in similar hypothermia assays (Martin et al., 1991; Rinaldi-Carmona et al., 1994) and is in agreement with the present binding data. Further evi-

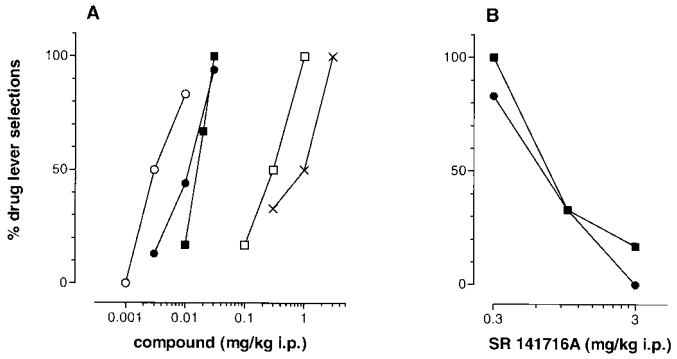

Fig. 8. A, dose-dependent generalization induced by BAY 38-7271 (■), CP 55,940 (●), Δ^9-THC (×), WIN 55,212-2 (□), and HU-210 (○) in rats trained to discriminate CP 55,940 (0.03 mg/kg i.p) from vehicle. Compounds were administered i.p. 30 min before test, except for HU-210, which was administered 120 min before test (n = 6–16). B, dose-dependent antagonism of the discriminative stimulus effects induced by BAY 38-7271 and CP 55,940 (both tested at 0.03 mg/kg, i.p.) by pretreatment with SR 141716A. The antagonist was administered 10 min before CP 55,940, BAY 38-7271, or vehicle (n = 6).

dence for an involvement of CB1 receptors in the hypothermia assay was suggested by the finding that pretreatment with the selective CB1 receptor antagonist SR 141716A attenuated the hypothermic effects of the various CB1 receptor agonists (present study; Rinaldi-Carmona et al., 1994; Compton et al., 1996; Mansbach et al., 1996). The CB1 receptor agonist profile of BAY 38-7271 as obtained in the hypothermia assay was confirmed in a drug discrimination assay in which rats were trained to discriminate a relatively low dose of CP 55,940 (0.03 mg/kg, i.p.) from vehicle. It has been previously demonstrated that the discriminative stimulus induced by a CB1 receptor agonist is highly sensitive and specific (Wiley et al., 1995a,b). Moreover, it is very likely that the cue produced by a CB1 receptor agonist is centrally mediated because it was found that intracerebral administration of Δ^9-THC induced drug-appropriate responding in rats trained to discriminate this compound from vehicle after systemic administration (Kallman et al., 1984). Each of the CB1 receptor agonists tested in the drug discrimination assay induced complete generalization; the order of potency closely resembled that obtained in the hypothermia assay and the in vitro binding assays. This finding supports the suggestion that the discriminative effects of these compounds are mediated by activation of CB1 receptors. This suggestion is also supported by the finding that the discriminative effects of BAY 38-7271 and CP 55,940 could be blocked in a dose-dependent and complete manner by the CB1 receptor antagonist SR 141716A. These findings are in accordance with other studies, which reported that the discriminative effects of Δ^9-THC, CP 55,940, or WIN 55,212-2 could be blocked by the CB1 receptor antagonist (Wiley et al., 1995a,b; Mansbach et al., 1996; Pério et al., 1996).

As mentioned previously (see the introduction), activation of CB1 receptors leads via various mechanisms to cell hyperpolarization and inhibition of neurotransmitter release (Kim and Thayer, 2000; Gerdeman and Lovinger, 2001). Based on these findings, CB1 receptor agonists are hypothesized to have neuroprotective efficacy, as assessed in TBI or brain ischemia models. To further test this hypothesis, we characterized the neuroprotective properties of BAY 38-7271 in the rat SDH model, which represents a preclinical model of TBI, and in the pMCA-O model, which represents a preclinical model of stroke. It was found that BAY 38-7271 displayed potent and pronounced neuroprotective efficacy in the rat SDH model. Interestingly, postponement of administration of BAY 38-7271 to 3 h after induction of SDH did only result in a slight reduction of neuroprotective potency, whereas the efficacy was maintained. It can be assumed that the neuroprotective properties of BAY 38-7271 result from CB1 receptor activation because the compound was found to be a highly selective CB receptor agonist, and its neuroprotective effects were obtained at doses that at least partly coincide with those to be active in the CB1 receptor-selective drug discrimination assay. Even though BAY 38-7271 was able to induce dose-dependent hypothermia, it can be argued that hypothermia itself is not responsible for the observed neuroprotective effects. Thus, special care was taken during the neuroprotection experiments to maintain the body temperature of the animals at 37 ± 0.5°C during the whole experiment. In addition, it was found that optimal neuroprotection was obtained at doses that were lower than those that induced hypothermia. Interestingly, the doses needed for maximal neuroprotection in the SDH model were extremely low, which suggests either a favorable pharmacokinetic profile (i.e., enrichment of the compound in the brain tissue under these experimental conditions) or a sensitized functional interaction with those CB1 receptors that are rapidly up-regulated after the cerebral insult (Jin et al., 2000).

Although it remains unclear why the efficacy and potency of BAY 38-7271 seemed to be considerably lower in the rat

TABLE 3

Summary of test results in rats trained to discriminate CP 55,940 (0.03 mg/kg, i.p.) from vehicle

Generalization tests were performed 30 min after application of the test compound (except for HU-210, where the injection-test interval was 120 min). In the antagonism study, pretreatment with SR 141716A (or vehicle; i.p.) occured 10 min before treatment with CP 55,940 or BAY 38-7271 (0.03 mg/kg or vehicle, i.p.; n = 6–16 rats, randomly allocated to each test condition).

Compound	Application	Type of test	% Drug Lever Selections ED_{50}	95% CL
			mg/kg	*mg/kg*
BAY 38-7271	i.p.	Generalization	0.015	0.010–0.023
BAY 38-7271	i.v.	Generalization	0.0004	0.0001–0.0011
CP 55,940	i.p.	Generalization	0.009	0.005–0.016
HU-210	i.p.	Generalization	0.004	0.002–0.010
WIN 55,212-2	i.p.	Generalization	0.24	0.10–0.55
Δ^9-THC	i.p.	Generalization	0.58	0.28–1.23
SR 141716A + vehicle	i.p.	Generalization	>10	
SR 141716A + CP 55,940 (0.03)	i.p.	Antagonism	0.65	0.35–1.20
SR 141716A + BAY 38-7271 (0.03)	i.p.	Antagonism	1.14	0.49–2.68

CL, confidence limits.

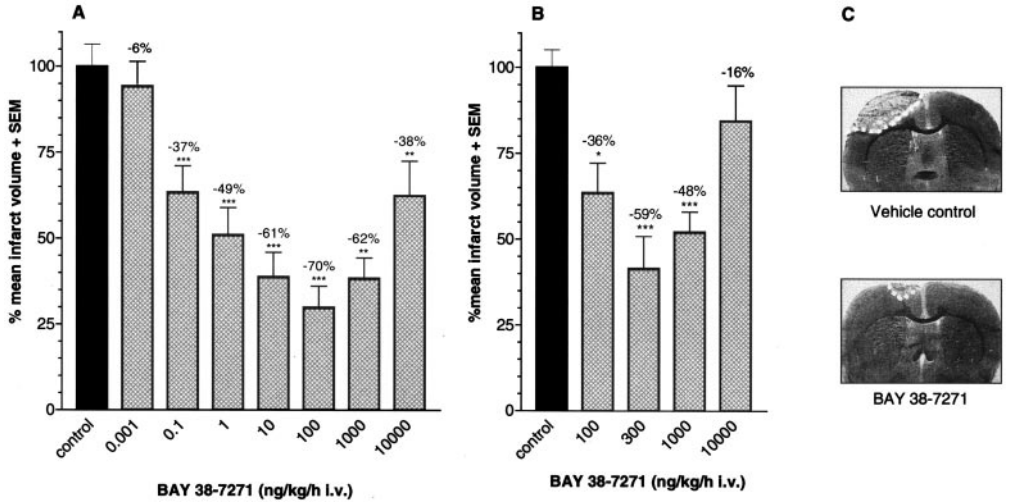

Fig. 9. Neuroprotective efficacy of BAY 38-7271 in the rat SDH model, administered directly after induction of SDH (A) or administered with a 3-h delay (B). BAY 38-7271 was administered as continuous i.v. infusion for 4 h. Infarct volumes were determined 7 days after SDH. Infarct volumes were calculated as percentage of infarct volumes of the control group, which was set to 100%. Values above bars indicate the percentage of infarct volume reduction compared with controls. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ ($n = 8$–12). C, histological sections of a vehicle control and a drug-treated animal (100 ng/kg/h) administered directly after induction of SDH. The dotted line indicates infarct area.

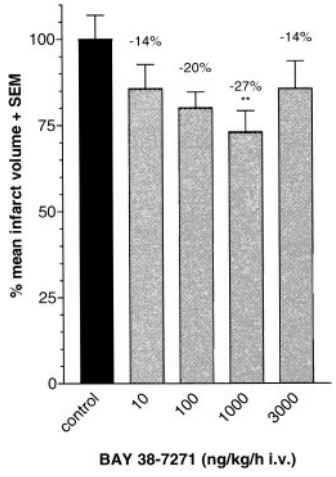

Fig. 10. Neuroprotective efficacy of BAY 38-7271 in the rat pMCA-O model. BAY 38-7271 was administered as continuous i.v. infusion for 4 h directly after occlusion of the media cerebri artery. Infarct volumes were determined 7 days after pMCA-O. Infarct volumes were calculated as the percentage of infarct volumes of the control group, which was set to 100%. Values above bars indicate the percentage of infarct volume reduction compared with controls. ⋆⋆, $P < 0.01$ ($n = 9$–38).

pMCA-O model, it is possible that the compound more sensitively attenuates those processes that are mainly involved in neuronal cell death caused by TBI than those that are involved in the generation of infarcts in stroke pathogenesis. Consistent with the present findings, the nonclassical cannabinoid receptor agonist WIN 55,212-2 was reported to show also neuroprotective effects in a rat model of focal cerebral ischemia and the transient global cerebral ischemia model (Nagayama et al., 1999). Although it was demonstrated in the latter study that the neuroprotective effects of WIN 55,212-2 are mediated by activation of CB1 receptors, it remains to be tested whether this compound is also more effective in a TBI model than in a stroke model.

In conclusion, BAY 38-7271 was characterized as a highly potent and selective CB1/CB2 receptor agonist with pronounced neuroprotective efficacy. As the doses needed for maximal neuroprotective efficacy were significantly lower than those inducing typical cannabinoid-like side effects, it is to be expected that the compound may offer a novel therapeutic approach with a favorable therapeutic window for the treatment of TBI and cerebral ischemia.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of K.-H. Augstein, M. Borsch, G. Eckel, M. Faβbender, J. Frielingsdorf, K. R. Jentzsch, A. Klein, H. Otto, and H. Struppeck. We thank Dr. U. Pleiβ (Bayer AG) for the synthesis of [^{3}H]BAY 38-7271.

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