

3-[2-Cyano-3-(trifluoromethyl)phenoxy]phenyl-4,4,4-trifluoro-1-butanesulfonate (BAY 59-3074): A Novel Cannabinoid CB₁/CB₂ Receptor Partial Agonist with Antihyperalgesic and Antiallodynic Effects

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ABSTRACT

3-[2-Cyano-3-(trifluoromethyl)phenoxy]phenyl-4,4,4-trifluoro-1-butanesulfonate (BAY 59-3074) is a novel, selective cannabinoid CB₁/CB₂ receptor ligand ($K_i = 55.4, 48.3, \text{ and } 45.5 \text{ nM}$ at rat and human cannabinoid CB₁ and human CB₂ receptors, respectively), with partial agonist properties at these receptors in guanosine 5'-[γ -³⁵S]-thiophosphate triethyl-ammonium salt ([³⁵S]GTP γ S) binding assays. In rats, generalization of BAY 59-3074 to the cue induced by the cannabinoid CB₁ receptor agonist (-)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-butanesulfonate (BAY 38-7271) in a drug discrimination procedure, as well as its hypothermic and analgesic effects in a hot plate assay, were blocked by the cannabinoid CB₁ receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR 141716A). BAY 59-3074 (0.3–3 mg/kg, p.o.) induced antihyperalgesic and antiallodynic effects against thermal or mechanical stimuli in rat

models of chronic neuropathic (chronic constriction injury, spared nerve injury, tibial nerve injury, and spinal nerve ligation models) and inflammatory pain (carrageenan and complete Freund's adjuvant models). Antiallodynic efficacy of BAY 59-3074 (1 mg/kg, p.o.) in the spared nerve injury model was maintained after 2 weeks of daily administration. However, tolerance developed rapidly (within 5 days) for cannabinoid-related side effects, which occur at doses above 1 mg/kg (e.g., hypothermia). Uptitration from 1 to 32 mg/kg p.o. (doubling of daily dose every 4th day) prevented the occurrence of such side effects, whereas antihyperalgesic and antiallodynic efficacy was maintained/increased. No withdrawal symptoms were seen after abrupt withdrawal following 14 daily applications of 1 to 10 mg/kg p.o. It is concluded that BAY 59-3074 may offer a valuable therapeutic approach to treat diverse chronic pain conditions.

Understanding of the pharmacology of *Cannabis sativa* L. was advanced considerably by the identification of (-)- Δ^9 -tetrahydrocannabinol (Δ^9 -THC) as the major active constituent of cannabis (Gaoini and Mechoulam, 1964), the cloning of the (predominantly) centrally located cannabinoid CB₁ receptor and the peripherally located cannabinoid CB₂ receptor, as well as the discovery of selective synthetic agonists and antagonists and endogenous ligands such as anandamide (for review, see Pertwee, 1999, 2001). Although the com-

plex physiological role of the endocannabinoid system is not fully understood, accumulating evidence indicates that it is intimately involved in the perception and modulation of pain (Piomelli et al., 2000; Richardson, 2000; Pertwee, 2001). Thus, it has been reported that various cannabinoid CB₁/CB₂ receptor agonists, including Δ^9 -THC, WIN 55,212-2, CP 55,940, HU-210, as well as anandamide (or its more stable analog methanandamide) show analgesic effects in animal models of acute (nonpathological) pain, such as the hot plate, tail-flick, or formalin assays (for review, see Pertwee, 2001). More importantly, however, cannabinoids and endocannabinoids were reported to have antihyperalgesic and/or antial-

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ABBREVIATIONS: Δ^9 -THC, (-)- Δ^9 -tetrahydrocannabinol; WIN 55,212-2, (*R*)-4,5-dihydro-2-methyl-4-(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6*H*-pyrrolo[3,2,1-*ij*]quinolin-6-one; CP 55,940, (-)-*cis*-3-[2-hydroxy-4(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)-cyclo-hexanol; HU-210, (-)-11-OH- Δ^8 -tetrahydrocannabinol-dimethylheptyl; BAY 59-3074, 3-[2-cyano-3-(trifluoromethyl)phenoxy]phenyl-4,4,4-trifluoro-1-butanesulfonate; [³⁵S]GTP γ S, guanosine 5'-[γ -³⁵S]-thiophosphate triethyl-ammonium salt; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydro-chloride; BAY 38-7271, (-)-(*R*)-3-(2-hydroxymethylindanyl-4-oxy)phenyl-4,4,4-trifluoro-1-butanesulfonate; %MPE, percent maximal possible effect; CL, confidence limit.

lodynic effects in various animal models of chronic neuropathic pain, such as the chronic constriction injury model (Herzberg et al., 1997; Mao et al., 2000; De Vry et al., 2004b), the L5 spinal nerve ligation model (Bridges et al., 2001), and the partial sciatic nerve ligation model (Fox et al., 2001; Monhemius et al., 2001). This is particularly relevant because neuropathic pain is generally considered to be relatively unresponsive to opiates (Mao et al., 2000) and difficult to treat effectively (Sindrup and Jensen, 1999; Martin and Eisenach, 2001). In addition, some of these compounds also show antihyperalgesic and/or antiallodynic effects in rodent models of acute inflammatory pain, such as the intraplantar carrageenan model (Richardson et al., 1998; Hedley et al., 1999; Clayton et al., 2002; Conti et al., 2002) and chronic inflammatory pain model, such as the intraplantar complete Freund's adjuvant model (Smith et al., 1998; Martin et al., 1999). Interestingly, the antihyperalgesic and antiallodynic effects of cannabinoids were typically obtained at doses that are generally lower than the doses needed to induce analgesic effects against acute, nonpathological pain (De Vry et al., 2004a). Although the mechanism underlying this increased potency of cannabinoids against chronic, pathological pain is not entirely clear, it was found that neuropathic pain coincided with an up-regulation of cannabinoid CB₁ receptor mRNA in the thalamus (Siegling et al., 2001). Because this up-regulation was observed in the contralateral but not ipsilateral thalamus compared with the unilateral nerve damage, it was suggested that the increased sensitivity of the endocannabinoid system is specifically related to the modulation of pain processing. Under such conditions, cannabinoid CB₁ receptor partial agonists may offer an attractive approach to treat chronic pain at doses that produce few (if any) side effects and with a minimal risk of producing tolerance (Piomelli et al., 2000).

The present study characterized the activity of the novel butanesulfonate derivative BAY 59-3074 (Fig. 1) at cannabinoid receptors in a number of *in vitro* and *in vivo* assays and evaluated its analgesic, antihyperalgesic, and antiallodynic properties in rat models of acute and chronic pain. In addition, the liability of the compound to induce tolerance to its antihyperalgesic/antiallodynic effects and physical dependence upon abrupt withdrawal was assessed.

Materials and Methods

Receptor Binding. Human brain cortex membranes were obtained from Analytical Biological Services Inc. (Wilmington, DE). Membranes containing the human recombinant cannabinoid CB₂ receptor for binding assays were purchased from Receptor Biology, Inc. (Beltsville, MD). For additional studies, brain membrane preparations from male Wistar rats were used. Brains were dissected, and the cerebellum was removed; this was followed by homogeniza-

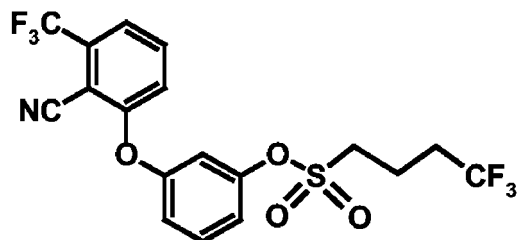


Fig. 1. Structure of BAY 59-3074.

tion 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 1,000g, and the supernatant was transferred to a new tube and centrifuged again at 40,000g 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 to the same method.

Binding studies at cannabinoid receptors were performed as follows. Radioligand ([³H]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 MgCl₂, and 5 mg/ml of fatty acid-free BSA (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% fatty acid-free BSA). Remaining radioactivity was counted in a scintillation counter (Beckman Coulter, Fullerton, CA). Protein concentrations were 20, 11, and 100 μg/tube for rat brain membranes, recombinant human cannabinoid CB₂ receptors, and human cortex membranes, respectively.

Further noncannabinoid target interaction screening was performed at Panlabs, Inc. (Taipei, Taiwan) as described in the respective assay protocols. BAY 59-3074 was tested at the recommended initial concentration (10 μM) in duplicate; if active (≥50% inhibition), concentration-response curves were determined.

Agonist-Stimulated [³⁵S]GTPγS Binding. Rat brain and human cortex membranes were prepared as described above, and human recombinant cannabinoid CB₂ receptors were prepared from cannabinoid CB₂ receptor-expressing Chinese hamster ovary cells. Cells were abraded from plates and washed three times in phosphate-buffered saline (pH 7.4). The pellet was suspended in 50 mM Tris × HCl, pH 7.4, and followed by two homogenization and centrifugation steps (40,000g for 30 min). Finally, the pellet was resuspended in 50 mM Tris × HCl, pH 7.4, and aliquots of the membrane preparations were stored at -140°C over liquid nitrogen. Test compound, 0.05 nM [³⁵S]GTPγS, and membranes (rat cortex = 25 μg of protein, human cortex = 50 μg of protein, and human recombinant cannabinoid CB₂ receptor = 25 μg of protein) were incubated at 30°C for 1 h in assay buffer (50 mM Tris × HCl, 100 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 25 μM GDP, and 1 mg/ml of fatty acid-free BSA, 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 (HsdCpb: WU; Borchon, Germany) or Charles River (CrI: Wi/Wu; Sulzfeld, Germany; carrageenan and complete Freund's adjuvant model). Body weight upon arrival at the laboratory ranged from about 160 to 250 g (130–150 g for the carrageenan and complete Freund's adjuvant model). Rats were housed by group (hypothermia/hot plate assay, 2 to 4 animals per cage) or individually (drug discrimination, physical dependence study) 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, except for the drug discrimination assay, where food access was restricted (approximately 13–15 g/day, standard pellets) (Ssniff Spezialdiäten GmbH, Soest, Germany). In all assays, rats were offered water *ad libitum*. Room temperature and relative humidity were maintained at 22 ± 1°C and 55 ± 5%, respectively. Experimental protocols and conditions conformed with the German regulations on animal welfare.

Hypothermia Assay. Rats ($n = 6-8$ per group) were orally treated by gavage with CP 55,940 (0, 0.3–3 mg/kg), Δ^9 -THC (0, 15–60 mg/kg), and BAY 59-3074 (0, 5–20 mg/kg). Body temperature was esophageally measured repeatedly at fixed time points after p.o. administration. Measurement of core body temperature was performed with a digital thermocouple laboratory thermometer (Model BAT-12; Technical and Scientific Equipment GmbH, Bad Homburg, Germany) equipped with a temperature measurement probe for esophageal use (Model RET-2; Technical and Scientific Equipment GmbH). Time points measured included 5 min before and 0.5, 1, 2, 4, and 6 h after administration (2 and 4 h for CP 55,940). For the antagonism test, temperature was measured 5 min before administration of SR 141716A (0, 0.6–6 mg/kg, i.p.) or vehicle. One hour after i.p. pretreatment, rats ($n = 8$ per group) received BAY 59-3074 (10 mg/kg, p.o.) or vehicle, and temperature was measured again after 1, 2, and 4 h.

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

After initial encouragement to lever press for food reinforcement, rats were trained to discriminate the highly selective and potent cannabinoid CB₁ receptor full agonist BAY 38-7271 (Mauler et al., 2002) (0.05 mg/kg, i.p.; $t = 30$ min and $n = 24$) from vehicle in a standard two-lever, fixed ratio 10 food-reinforced operant procedure, as described by De Vry and Jentzsch (2002). Daily sessions were conducted that were terminated either after 50 reinforcers or after 10 min, whichever came first. For half the animals, responding on the left lever was reinforced after BAY 38-7271; 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., less than five incorrect responses before the first reinforcer. Animals were first tested with BAY 38-7271 (0 and 0.006–0.1 mg/kg, i.p.; $t = 30$ min). Further generalization tests were performed with CP 55,940 (0.01–0.1 mg/kg, p.o.; $t = 1$ h), Δ^9 -THC (0.1–3 mg/kg, p.o.; $t = 1$ h) and BAY 59-3074 (0.1–1 mg/kg, p.o.; $t = 1$ h; 0.1–1 mg/kg, i.p.; $t = 30$ min). In a time-dependence study, BAY 59-3074 was tested 0.5, 1, 2, and 4 h following p.o. administration of 0.5 mg/kg during different test sessions. In the antagonism study, pretreatment with SR 141716A (0 and 0.3–5 mg/kg, i.p.) occurred 1 h before treatment with BAY 59-3074 (0.5 mg/kg, p.o.; $t = 1$ h). In the latter study, SR 141716A (5 mg/kg, i.p.) was also tested in combination with vehicle (p.o.) according to the same application schedule. Each dose of a test compound or test compound combination was tested in 5 to 6 rats randomly allocated to each test condition (except for the generalization tests with both training conditions after i.p. administration, which were performed in all rats).

Hot Plate Acute Analgesia Model. Rats ($n = 6-8$ per group) were orally treated with CP 55,940 (0, 0.3–3 mg/kg), Δ^9 -THC (0, 10–60 mg/kg), and BAY 59-3074 (0, 1–10 mg/kg), and latency to react in a hot plate assay was tested. The hot plate apparatus (25 ×

25 cm; Ugo Basile, Comerio, Italy), equipped with a clear Plexiglas cylinder, was gradually heated to 52°C about 30 min before the start of the experiment. The device guaranteed a temperature stability with a variation of 0.2°C. Reaction latency time (s) was scored with a foot-operated stopwatch. After being placed on the hot plate, animals were observed for the occurrence of vocalization, licking or lifting of the hindpaw, or jumping. As soon as one of these behavioral symptoms occurred, the reaction latency was recorded, and the animals were taken from the hot plate. If no reaction occurred within 40 s, animals were taken from the hot plate and given the latency score of 40 s (cutoff). The hot plate was wiped clean before testing the next animal. Time points measured included 5 min before and 2 and 4 h (CP 55,940), 1 and 2 h (Δ^9 -THC), or 2, 4, and 6 h (BAY 59-3074) after administration. For the antagonism test, reaction latency was measured 5 min before administration of SR 141716A (0, 0.6–6 mg/kg, i.p.) or vehicle. One hour after i.p. pretreatment, rats ($n = 8$ per group) received BAY 59-3074 (10 mg/kg, p.o.) or vehicle, and reaction latency was measured again after 1, 2, and 4 h.

Chronic Constriction Injury Model of Neuropathic Pain. In general, the method described by Bennett and Xie (1988) was followed. Rats were anesthetized with pentobarbital (Nembutal; Sanofi, Libourne, France; 50 mg/kg, i.p.). The right common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic trifurcation, about 12 mm of nerve was freed of adhering tissue, and four ligatures (100% mercerized cotton thread; strength, 40) were tied loosely around it with about 1-mm spacings. The length of nerve thus affected was 6 to 8 mm long. Great care was taken to tie the ligatures so that the diameter of the nerve was seen to be just barely constricted when viewed with 40× magnification. The desired degree of constriction retarded, but did not arrest, circulation through the superficial epineural vasculature and sometimes produced a small, brief twitch in the muscle surrounding the exposure. The incision was closed in layers. In sham-operated controls, an identical dissection was performed on the same side, except that the sciatic nerve was not ligated.

For assessment of thermal hyperalgesia, rats were placed upon an elevated floor of window glass (model 400 Complete Mesh Stand; IITC Inc., Woodlands Hill, CA) at least 5 min before the start of the experiment. They stood or sat quietly in this situation after an initial few minutes of exploration. Thermal hyperalgesia was assessed by means of a radiant heat source with an infrared strength of 35 and an automatic cutoff of 32 s, which was aimed beneath the glass floor at the plantar surface of the operated hindpaw (model 7370; Ugo Basile). The radiant heat source's aperture measured 5 × 10 mm; it was placed at right angles to the hindpaw and positioned so that the proximal half of the plantar surface was irradiated. Stimulus onset was operated manually and activated a timer that was controlled by a photocell positioned to receive light reflected from the hindpaw. The hindpaw withdrawal reflex, which consisted of the abrupt withdrawal and/or licking of the paw, interrupted the photocell's light and automatically stopped the timer. Latencies were measured with a 0.1-s accuracy level. For assessment of mechanical allodynia, animals were placed in mesh-wired cages at least 5 min before the start of the experiment. Mechanical allodynia was assessed by means of the von Frey test (Digital Transducer Indicator equipped with von Frey fibers; IITC Inc.). The von Frey fibers device was gently lifted to the middle plantar surface of the operated hindpaw until the paw was abruptly withdrawn and/or licked. As soon as this reaction occurred, the required pressure was indicated in grams, and this value was considered to be the individual threshold value.

For the dose-response experiment, rats ($n = 10-12$ per group) were tested twice per week during the first 3 weeks following operation. During the 3rd week following operation, the first test (baseline control test) was performed to check whether the animals showed thermal hyperalgesia and mechanical allodynia (tested in this particular order). Baseline values were considered to be valid if the mean reaction latency values were around 20 and 10 s in the

sham and ligated groups, respectively, and the mean von Frey test values were around 70 to 90 and 20 to 30 g, respectively. The experimental design included a vehicle-treated sham and ligated control group and three ligated groups that received 0.3, 1, and 3 mg/kg BAY 59-3074 p.o. ($t = 1$ h). In this model, as well as in the other neuropathic and inflammatory pain models, testing occurred "double-blind"; i.e., drug administrator and behavioral observers were unaware of the identity of the injection. In an additional experiment, sham and ligated groups of rats ($n = 7$ –12 per group) were prepared and tested as described above, and the effect of BAY 59-3074 (1 mg/kg, p.o.; $t = 1$ h) on mechanical allodynia was tested in separate groups of rats either 1 or 7 weeks after operation.

Tibial Nerve Injury Model of Neuropathic Pain. In general, the method described by Hofmann et al. (2003) was followed. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.). Distal to the trifurcation of the left sciatic nerve, the tibial branch of the sciatic nerve was axotomized (transected), whereas the sural and common peroneal nerves remained uninjured. In sham-operated controls, an identical dissection, but without axotomization, was performed. Assessment of thermal hyperalgesia and mechanical allodynia and baseline requirements for pharmacological testing were similar to those described above (chronic constriction injury model).

The experimental design for the dose-dependence study (performed 9 weeks postoperation) included a vehicle-treated sham and axotomized control group, as well as three sham control and axotomized groups that received 0.3, 1, and 3 mg/kg of BAY 59-3074 p.o., respectively ($t = 1$ h and $n = 6$ –8 per group). A similar group design was used for a time-dependence study (performed 6 weeks postoperation) in which separate groups ($n = 7$ –8 per group) were tested 1, 2, and 4 h after oral treatment with 1 mg/kg BAY 59-3074.

Spared Nerve Injury Model of Neuropathic Pain. In general, the method described by Decosterd and Woolf (2000) was followed. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.). Distal to the trifurcation of the left sciatic nerve, the tibial and common peroneal branches of the sciatic nerve were axotomized, whereas the sural nerve remained uninjured. In sham-operated controls, an identical dissection, but without axotomization, was performed. Assessment of thermal hyperalgesia and mechanical allodynia and baseline requirements for pharmacological testing were similar to those described above.

The experimental design for the dose-dependence study (performed 16 weeks postoperation) included a vehicle-treated sham and axotomized control group, as well as three sham control and axotomized groups that received 0.3, 1, and 3 mg/kg of BAY 59-3074 p.o., respectively ($t = 1$ h and $n = 6$ –8 per group). A similar group design was used for a time-dependence study (performed 6 weeks postoperation) in which separate groups ($n = 6$ –8 per group) were tested 1, 2, and 4 h after oral treatment with 1 mg/kg BAY 59-3074. To test whether the antihyperalgesic and antiallodynic effects of BAY 59-3074 were maintained after repeated administration ("tolerance experiment"), two groups of axotomized rats received the compound (1 mg/kg, p.o.; $t = 1$ h) either once daily for 14 days or acutely on day 14 after 13 daily applications of vehicle, whereas a sham and axotomized control group was treated for 14 days with vehicle according to the same application schedule ($n = 10$ –12 per group). The tolerance experiment was performed 4 weeks postoperation, and testing included the assessment of thermal hyperalgesia and mechanical allodynia. The rapid up-titration of the daily dose of BAY 59-3074 was tested in a final experiment to determine whether it would be a successful strategy to avoid the occurrence of behavioral side effects without compromising antihyperalgesic/antiallodynic efficacy ("dose-escalation experiment"). In this study, which started 3 weeks and ended 5 weeks postoperation, the daily dose given to the axotomized group ($n = 12$) was doubled every 4th day, starting from 1 mg/kg (day 19 postoperation) and culminating to 32 mg/kg p.o. (day 35 postoperation). Control groups included a sham and axotomized group that received daily vehicle applications ($n = 12$ per group). Assessment of thermal hyperalgesia and mechanical allodynia was performed 1 h

after the first application of each dosing step. Because cannabinoid-induced hypothermia coincided with other cannabinoid-related behavioral side effects, it can be considered to be a representative surrogate parameter for such side effects; therefore, the effect of BAY 59-3074 on body temperature was assessed 5 min before and 55 min after the 1st and 3rd application of each dosing step.

Spinal Nerve Ligation Model of Neuropathic Pain. In general, the method described by Kim and Chung (1992) was followed. Anesthesia was initiated via a face mask with a nitrous oxide/oxygen (70/30%) mixture and 3% isoflurane (Forene; Abbott GmbH, Wiesbaden, Germany) and maintained throughout the surgical procedure with the nitrous oxide/oxygen mixture and 3 to 1.5% isoflurane. The rat was placed in a prone position, and the left paraspinal muscles were separated from the spinous processes at the L4-S2 level. Under microscopic control, part of the L6 transverse process was removed, and the L5 spinal nerve was identified and carefully dissected free from the adjacent L4 spinal nerve. Distal to the dorsal root ganglion and proximal to the formation of the sciatic nerve, the L5 nerve was tightly ligated using 6–0 silk suture. Finally, the muscle layer and skin were sutured. The procedure for sham surgery was similar to that of the experimental group, except that the spinal nerve was exposed and not ligated. Assessment of mechanical allodynia was similar to that described above, with the exception that each test included three trials that were averaged to yield individual withdrawal values. After surgery, the exclusion criteria for the L5-ligated rats were disturbed motor behavior, such as paw dragging or dropping, and/or failure to exhibit subsequent mechanical allodynia (mean averaged baseline values around 40–50 and 20–30 g in the sham and ligated groups, respectively). The experimental design included a vehicle-treated sham and ligated control group and two ligated groups that received 1 and 3 mg/kg of BAY 59-3074 p.o. ($t = 1$ h, 5 weeks postoperation, $n = 10$ per group).

Carrageenan Model of Inflammatory Pain. The carrageenan inflammatory pain model was adapted from Hargreaves et al. (1988). Rats received a single s.c. injection of 100 μ l of carrageenan (1.5%; Sigma-Aldrich, St. Louis, MO; sham-control group received a 100- μ l saline injection) into the plantar surface of the right hindpaw. This injection resulted in an acute local inflammation and an accompanying thermal hyperalgesia that lasted from about 2 to at least 6 h after carrageenan injection. Assessment of thermal hyperalgesia was performed by measuring the latency time to retract the hindpaw from a radiant heat source (stimulus current, 4.3–4.4 A; automatic cutoff of 21 s) that was aimed at the plantar surface of the hindpaw from beneath a glass floor (University Anesthesia Research & Development Group, University of California San Diego, La Jolla, CA). The study contained a vehicle-treated sham group, a vehicle-treated carrageenan group, and three carrageenan groups that received either 1, 2, or 3 mg/kg BAY 59-3074 ($n = 14$ –15 per group) given orally 205 min after intraplantar injection. The reaction latency to thermal stimulation was measured 140, 160, 180, and 200 min after intraplantar injection and 20, 40, and 60 min after drug administration. Baseline values were considered to be valid if the test values were around 4 to 5 and 12 to 14 s in the carrageenan and control groups, respectively.

Complete Freund's Adjuvant Model of Inflammatory Pain. In general, the method described by Bertorelli et al. (1999) was followed. Rats received a single s.c. injection of complete Freund's adjuvant into the plantar surface of the right hindpaw (100 μ l of liquid paraffin containing 50 μ g of heat-killed and dried Mycobacterium tuberculosis) (Difco, Detroit, MI). Sham controls received a 100- μ l saline injection. For assessment of mechanical allodynia, rats were placed in mesh-wired cages at least 40 min before testing. Mechanical allodynia was assessed by using the von Frey test (electronic von Frey system; Somedic, Hörby, Sweden). Mechanical stimulation was applied by a stainless steel monofilament (tip diameter, 0.7 mm) that was attached to an electronic force transducer with an amplifier and computer system. The electronic force transducer recorded changes in pressure from the moment the monofilament was

applied to the inflamed paw until the animal withdrew its paw. The filament was applied to the middle plantar surface of the paw. Pressure changes were automatically recorded and subsequently analyzed by the electronic von Frey system (DASYLab software; Somedic). The threshold for mechanical allodynia was defined as the force (in grams) at which the rat withdrew its paw. Baseline values were considered to be valid if the test values were around 35 to 40 and 10 to 15 g in the control and inflamed groups, respectively. Mechanical allodynia developed within 3 days and lasted for at least 2 weeks following injection of complete Freund's adjuvant. The study contained a vehicle-treated sham group, a vehicle-treated inflamed group, and two inflamed groups that received either 1 or 3 mg/kg BAY 59-3074 p.o. ($n = 9-10$ per group). The threshold for mechanical allodynia was measured 5 times at 40, 60, and 80 min after administration, and the mean value was calculated for each time point.

Physical Dependence Liability Assessment. In general, the method described by Aceto et al. (1996) was followed. For 14 consecutive days, four groups of rats ($n = 8$ per group) were treated p.o. once a day between 8:00 and 10:00 AM. with 1, 3, or 10 mg/kg BAY 59-3074 or vehicle. From day 1 to day 15, body temperature was measured esophageally 5 min before and 1 h after the daily drug administration (on day 15 no applications took place). Body weight was recorded every day immediately after the first body temperature measurement. On day 15, 24 h after the last application, withdrawal symptoms were scored during a 1-h period by means of a 10-min time sampling behavioral checklist. Thus, rats were observed every 10th min for the occurrence (value 0 if absent, 1 if weakly present, and 2 if strongly present) of each of the following parameters: turning left (360°), turning right (360°), walking backward, digging, wet dog shake, tremor, forepaw treading, boxing, raised hindpaw, scratching, grooming or rubbing face, grooming body, licking, biting, tongue rolling, ptosis, and arched back.

Data Analysis. Biochemical experiments were performed in triplicate and repeated 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]GTP γ S 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 Inc., San Diego, CA). For the behavioral assays, data were generally analyzed by analysis of variance and followed, where appropriate, by Tukey's or Bonferroni's post hoc comparisons. If data were not normally distributed, analysis was performed with the Kruskal-Wallis test and followed by Dunnett's test. For graphical presentation of the body temperature data, results were expressed as mean absolute body temperature or temperature change in degrees Celsius relative to baseline value and corrected for the temperature change observed in the vehicle control group. For ED_{50} value calculation, a temperature reduction of 2.5°C (compared with vehicle control) was defined as 100% effect. For calculation of the ID_{50} value of SR 141716A in the antagonism study, the hypothermic effect induced by BAY 59-3074 (10 mg/kg) was defined as 100% effect. For ED_{50} value calculation and graphical presentation of the hot plate assay results, efficacy was expressed as %MPE by the following equation: %MPE = $100 \times (\text{test latency} - \text{control latency}) / (\text{cutoff time} - \text{control latency})$. For calculation of the ID_{50} value of SR 141716A in the antagonism study, the reaction latency response induced by BAY 59-3074 (10 mg/kg) was defined as 100% effect. The other pain models were considered to be valid if a statistically significant ($P < 0.05$) difference was obtained between the mean withdrawal latencies/thresholds of the sham group and each experimental pain group (during baseline assessment) and between the vehicle-treated sham group and vehicle-treated experimental pain group (during pharmacological testing). BAY 59-3074 was considered to be effective if the dependent variable as obtained in the drug-treated experimental pain group was significantly increased compared with the vehicle-treated experimental pain group. Efficacy was calculated as the difference of the mean values obtained

in the drug-treated and vehicle-treated experimental pain groups and was expressed as a percentage of the difference of the mean values obtained in the vehicle-treated sham group and vehicle-treated experimental pain group. Least-square linear regression analysis was used to estimate ED_{50} , ID_{50} , and $t_{1/2}$ values and the corresponding 95% CL after log-probit conversion of the data. ED_{50} or ID_{50} values with nonoverlapping CL limits were considered to be significantly different. In the drug discrimination experiment, generalization and antagonism were considered to be complete if at least 80% and less than 20% drug lever selections were obtained, respectively. For the physical dependence study, a separate analysis of variance was performed on the individual scores for each behavioral symptom, as well as body weight, whereas for the body temperature assay, the individual differences between post- and preapplication measurements were calculated for data presentation and analysis. Individual behavioral scores were obtained by adding all values (0, 1, or 2) over the 1-h sampling period (six time samples resulting in individual scores between 0 and 12).

Drugs and Chemicals. Δ^9 -THC was purchased from Sigma-Aldrich. Other compounds were synthesized by the Medical Chemistry and Radiochemistry Department (Bayer HealthCare, Wuppertal, Germany). Other chemicals were of the highest available purity and were purchased from Merck (Darmstadt, Germany). Compounds were suspended in a solvent containing 2.5 to 5% Solutol HS 15 (12-hydroxystearic-acid ethoxylate) (BASF AG, Ludwigshafen, Germany), 2.5 to 5% ethanol (ethanol absolute, 99.8%) (Riedel-de Haën, Seelze, Germany), and distilled water, except for BAY 59-3074, which was suspended in 10% Cremophor EL (BASF AG) and distilled water for p.o. administration. Application volume was 5 ml/kg (except for 2 ml/kg in the drug discrimination study).

Results

Receptor Binding. K_i values of competition studies at cannabinoid receptors as obtained from rat brain (Fig. 2A), human cortex (Fig. 2B), and recombinant human cannabinoid CB_2 receptors (Fig. 2C) are summarized in Table 1. Compared with the reference cannabinoids, the ranking order of potency was similar at each cannabinoid receptor subtype: CP 55,940 \gg BAY 59-3074 = Δ^9 -THC. Similar to CP 55,940 and Δ^9 -THC, BAY 59-3074 did not display selectivity toward either cannabinoid receptor subtype. Results from further screening investigations revealed only minor interactions with other (noncannabinoid) binding sites. Among 214 receptors or enzymes tested, significant binding or activity was only detected at the L-type calcium channel (dihydropyridine site, $K_i = 5.6 \mu\text{M}$; phenylalkylamine site, $K_i = 10.6 \mu\text{M}$), GABA $_A$ (peripheral, $K_i = 0.38 \mu\text{M}$), monoamine transporter ($K_i = 6.1 \mu\text{M}$), sigma site ($K_i = 6.0 \mu\text{M}$), β -lactamase ($IC_{50} = 30.2 \mu\text{M}$), monoamine oxidase-A ($IC_{50} = 2.0 \mu\text{M}$), and extracellular signal-regulated serine/threonine kinase-1 ($IC_{50} = 56.5 \mu\text{M}$).

Agonist-Stimulated [35 S]GTP γ S Binding. Signal transduction studies on rat brain membranes (Fig. 3A) revealed high signal transduction efficacy for CP 55,940 ($64.3 \pm 0.8\%$ over baseline level at $10 \mu\text{M}$), moderate efficacy for BAY 59-3074 ($29.1 \pm 1.1\%$ at $1 \mu\text{M}$), and relative low efficacy for Δ^9 -THC ($15.7 \pm 1.9\%$ at $1 \mu\text{M}$). CP 55,940 can therefore be characterized as a full agonist, whereas BAY 59-3074 and Δ^9 -THC behave as partial agonists. Also, on human cortex membranes (Fig. 3B), CP 55,940 ($51.9 \pm 3.8\%$ over baseline level at $10 \mu\text{M}$) seemed to be a full agonist with a similar degree of potency and efficacy as obtained on rat brain membranes, whereas BAY 59-3074 ($21.1 \pm 1.5\%$ at $1 \mu\text{M}$) and Δ^9 -THC ($13.0 \pm 2.6\%$ at $1 \mu\text{M}$) were again characterized as partial agonists. On recombi-

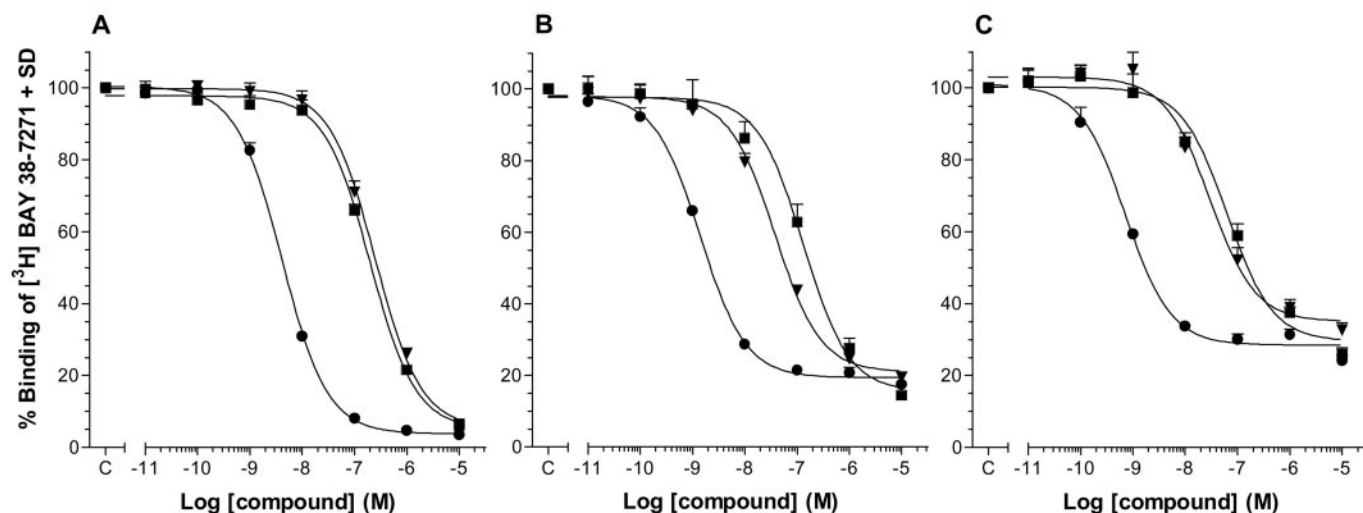


Fig. 2. Competition curves of BAY 59-3074 (■), CP 55,940 (●), and Δ^9 -THC (▼) at rat brain membranes (A), human cortex membranes (B), and human recombinant cannabinoid CB₂ receptors (C) using [³H]BAY 38-7271 as a radioligand. Values are the mean and standard deviation of at least three independent experiments, each performed in triplicate. For K_i values, see Table 1. C, control.

TABLE 1

K_i values of BAY 59-3074, CP 55,940, and Δ^9 -THC at rat brain membranes, human cortex membranes, and recombinant cannabinoid CB₂ receptors (radioligand [³H]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 Cannabinoid CB ₂ Receptor
	<i>nM</i>		
BAY 59-3074	55.4 ± 5.3	48.3 ± 9.2	45.5 ± 6.3
CP 55,940	1.15 ± 0.06	0.51 ± 0.03	0.54 ± 0.10
Δ^9 -THC	69.7 ± 11.6	13.7 ± 1.6	22.9 ± 7.8

nant human cannabinoid CB₂ receptors, the ranking order of potency and efficacy was similar to that observed on cannabinoid CB₁ receptors. Thus, CP 55,940 was characterized as a cannabinoid CB₂ receptor full agonist (250 ± 18% at 10 μ M), whereas BAY 59-3074 (113 ± 18% at 10 μ M) and Δ^9 -THC (67.3 ± 9.9% at 10 μ M) behaved as partial agonists. When the individual maximal effects were used for calculation, EC₅₀ values at rat brain membranes were 18.7 ± 3.9 nM, 122.7 ± 20.9 nM, and 27.6 ± 10.6 nM for CP 55,940, BAY 59-3074, and Δ^9 -THC, respectively. EC₅₀ values at human cortex membranes were 10.6 ± 0.9 nM, 142.7 ± 11.6 nM, and 25.7 ± 3.8 nM for CP 55,940, BAY 59-3074, and Δ^9 -THC, respectively. On recombinant cannabinoid CB₂ receptors, EC₅₀ values were 0.97 ± 0.06 nM, 15.8 ± 8.0 nM, and 15.4 ± 3.0 nM for CP 55,940, BAY 59-3074, and Δ^9 -THC, respectively.

Hypothermia Assay. BAY 59-3074 (5–20 mg/kg), CP 55,940 (0.3–3 mg/kg), and Δ^9 -THC (15–60 mg/kg) induced a dose-dependent reduction in body temperature after p.o. administration (ED₅₀ value with 95% CL: 7.2, 3.6–14.3 mg/kg; 1.4, 0.7–2.8 mg/kg; and >60 mg/kg, respectively) (Fig. 4A). BAY 59-3074-induced hypothermia, obtained at 0.5, 2, and 4 h after administration of 5, 10, and 20 mg/kg, respectively, reached a maximal effect of -0.9°C ($P > 0.05$), -2.0°C ($P < 0.001$), and -2.0°C ($P < 0.001$) compared with vehicle treatment (Fig. 4B) and was dose-dependently blocked by pretreatment with SR 141716A (ID₅₀ value with 95% CL: 1.71, 1.00–2.95 mg/kg and 1.13, 0.68–1.91 mg/kg, as assessed 1

and 2 h after administration of 10 mg/kg BAY 59-3074, respectively) (Fig. 4C). In general, for each compound, the occurrence of hypothermia coincided with the emergence of dose-dependent behavioral side effects, such as sedation and ptosis; 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 the cannabinoid CB₁ receptor agonist BAY 38-7271 (0.05 mg/kg, i.p.) from vehicle, with the median number of sessions to reach criterion being 52 (range, 26–78 sessions; De Vry and Jentsch, 2002). The generalization obtained with the training drug was dose-dependent and reached 100% drug lever selections at 0.05 mg/kg i.p. (ED₅₀ value with 95% CL: 0.018, 0.012–0.026 mg/kg; data not shown) and occurred in the absence of behavioral disruption (100% lever selections at each dose, except for the 0.1-mg/kg dose, at which 1 of 11 rats tested did not select a lever). Dose-dependent and complete generalization was also obtained with CP 55,940 and Δ^9 -THC (Fig. 5A), with 100% generalization being obtained at 0.1 and 3 mg/kg p.o., respectively (ED₅₀ values with 95% CL: 0.023, 0.010–0.056 mg/kg and 0.43, 0.20–0.93 mg/kg, respectively). For each of these compounds, generalization occurred in the absence of behavioral disruption (100% lever selections at each dose). Similar to the reference compounds, BAY 59-3074 induced complete generalization both after p.o. (100% generalization at 0.5 mg/kg, ED₅₀ value with 95% CL: 0.17, 0.09–0.31 mg/kg) (Fig. 5A) and i.p. administration (100% generalization at 1 mg/kg, ED₅₀ value with 95% CL: 0.24, 0.10–0.55 mg/kg) (data not shown) in the absence of behavioral disruption. The discriminative effects of BAY 59-3074 (0.5 mg/kg, p.o.) reached maximal intensity between 0.5 and 1 h after administration and disappeared gradually within 4 h ($t_{1/2}$ and 95% CL: 175; 116–263 min; 100% lever selections at each dose) (Fig. 5B). Pretreatment with SR 141716A dose-dependently and completely blocked the discriminative effects of BAY 59-3074 (0% generalization at 5 mg/kg, ID₅₀ value with 95% CL: 1.15, 0.42–3.16 mg/kg; 1 of 6 rats tested failed to select a lever at both the 3- and 5-mg/kg dose) (Fig. 5C). SR 141716A (5 mg/kg, tested in

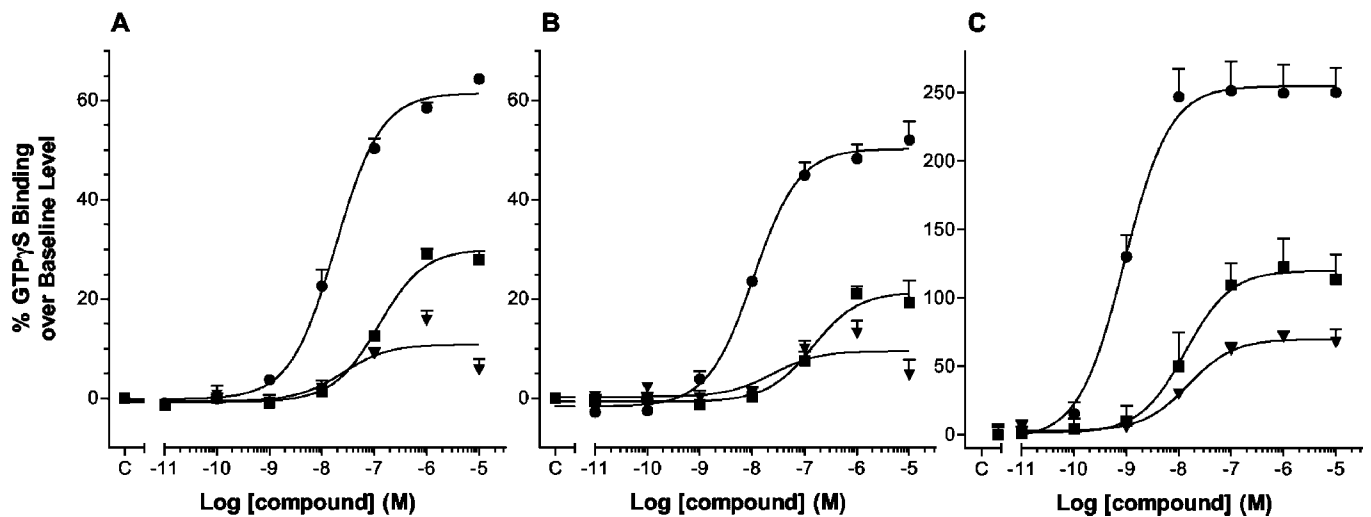


Fig. 3. Signal transduction efficacy of BAY 59-3074 (■), CP 55,940 (●), and Δ⁹-THC (▼) on rat brain membranes (A), human cortex membranes (B), and recombinant human cannabinoid CB₂ receptors (C). Values are the mean and standard deviation of at least three independent experiments, each performed in triplicate. C, control (baseline activity was set to 0).

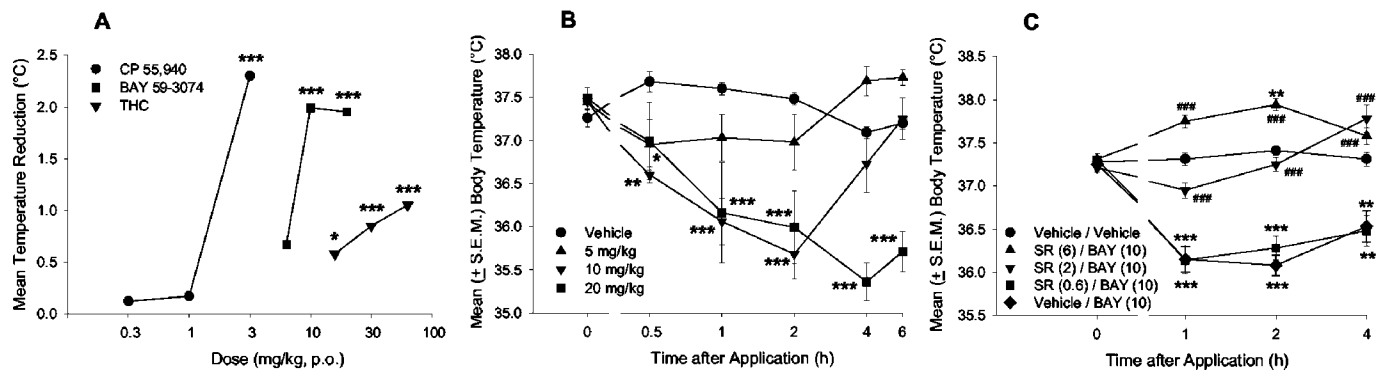


Fig. 4. A, hypothermia induced by BAY 59-3074, CP 55,940, and Δ⁹-THC in rats. Curves show activity at time point of maximal activity (CP 55,940, 4 h; Δ⁹-THC, 1 h; and BAY 59-3074, 2 h after p.o. administration). B, effect of BAY 59-3074 on body temperature. The 0 value on the x-axis represents the baseline value as determined 5 min before administration of the compound. C, antagonism of the hypothermia induced by BAY 59-3074 (BAY; 10 mg/kg, p.o.) by pretreatment with SR 141716A (SR; dose in milligrams per kilogram, i.p.). The antagonist was administered 1 h before BAY 59-3074 ($n = 6-8$ per group). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with vehicle/(vehicle) control; ###, $P < 0.001$ compared with vehicle/BAY 59-3074.

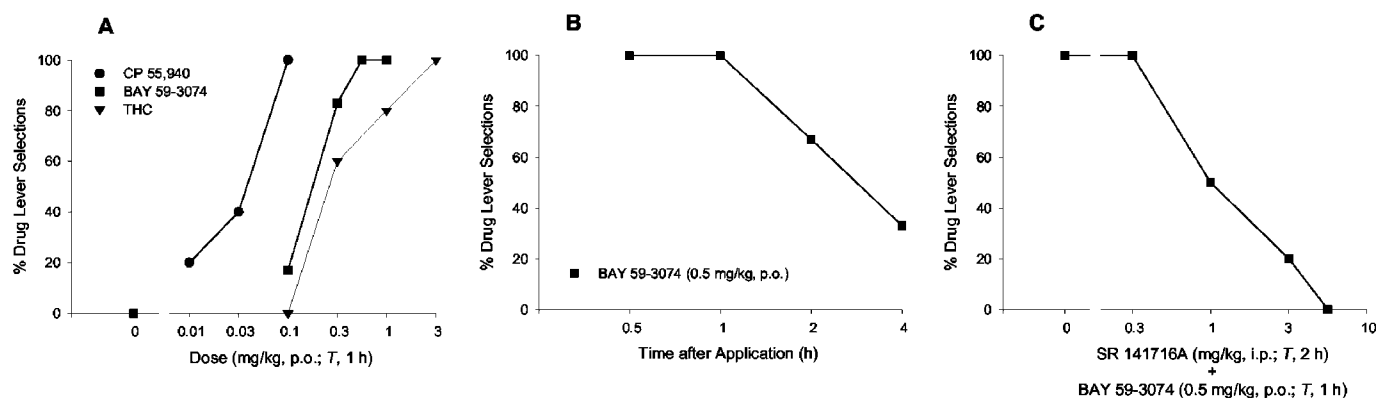


Fig. 5. A, generalization induced by BAY 59-3074, CP 55,940, and Δ⁹-THC in rats trained to discriminate the cannabinoid CB₁ receptor agonist BAY 38-7271 (0.05 mg/kg, i.p.) from vehicle in a two-lever procedure. The extent of generalization induced by the training conditions was 6% (vehicle) and 88% (BAY 38-7271) drug lever selections. B, time dependence of the generalization induced by BAY 59-3074. C, antagonism of the generalization induced by BAY 59-3074 by pretreatment with SR 141716A. The antagonist was administered 1 h before BAY 59-3074. The 0 time point reflects the baseline value obtained 5 min before the first application; the 1, 2, and 4 h time points reflect time points after the second application ($n = 5-8$ per dose).

combination with vehicle) induced vehicle-appropriate discriminative responding (0% drug lever selections; 1 of 6 rats tested did not select a lever).

Hot Plate Acute Analgesia Model. BAY 59-3074, CP 55,940, and Δ⁹-THC dose-dependently increased reaction latency in the hot plate assay after p.o. administration (ED₅₀

value with 95% CL: 6.7, 3.7–12.1 mg/kg; 2.1, 1.4–3.2 mg/kg, and 68.0, 34.1–135.6 mg/kg, respectively) (Fig. 6A). BAY 59-3074-induced increase in reaction latency reached a maximal effect of 73% at 2 h, lasted for at least 6 h after administration of 10 mg/kg (Fig. 6B), and was dose-dependently blocked by pretreatment with SR 141716A (ID₅₀ value with 95% CL: 1.28, 0.75–2.19 mg/kg, as assessed 1 h after administration of 10 mg/kg BAY 59-3074) (Fig. 6C). CP 55,940 and Δ^9 -THC reached a maximal effect of 83 and 54% and was obtained at 4 and 1 h after administration of 3 and 60 mg/kg, respectively.

Chronic Constriction Injury Model of Neuropathic Pain. At the time of testing, thermal hyperalgesia and mechanical allodynia were considered to be present if the baseline and test values were within the defined range (see *Materials and Methods*), and a statistically significant difference between the sham and each ligated group was present on the day preceding pharmacological testing (data not shown). BAY 59-3074 dose-dependently and completely reversed thermal hyperalgesia, with a maximal effect of 171% at 3 mg/kg p.o. (ED₅₀ value with 95% CL: 0.25, 0.09–0.70 mg/kg) (Fig. 7A), and partially reversed mechanical allodynia, with a maximal effect of 58% at 3 mg/kg (ED₅₀ value with 95% CL: 2.23, 0.87–5.71 mg/kg) (Fig. 7B). In the additional experiment, mechanical allodynia was present in the ligated groups tested 1 and 7 weeks following operation (mean withdrawal threshold in the vehicle-treated sham and experimental pain groups: 76.3 and 22.5 g and 89.6 and 26.9 g, respectively; both $P < 0.001$). BAY 59-3074 (1 mg/kg, p.o.) induced a relatively similar, partial reversal of mechanical allodynia at both time points (compound effect: 47%, $P < 0.001$ and 61%, $P < 0.01$ at 1 and 7 weeks postoperation, respectively).

Tibial Nerve Injury Model of Neuropathic Pain. In the dose-response experiment, only mechanical allodynia was significantly present at the time of testing. BAY 59-3074 partially reversed mechanical allodynia, with a maximal effect of 56% at 1 mg/kg p.o. (ED₅₀ value with 95% CL: 1.63, 0.35–7.65 mg/kg) (Fig. 8A). Although thermal hyperalgesia was only weakly present in this experiment, the symptom was completely absent after 1 and 3 mg/kg BAY 59-3074 (data not shown). BAY 59-3074 did not affect the reaction latency to thermal stimulation or the withdrawal threshold

to mechanical stimulation in the corresponding sham groups (data not shown). In the time-dependence study, thermal hyperalgesia and mechanical allodynia were both present at the time of testing (mean reaction latency and withdrawal threshold values in the vehicle-treated sham and axotomized group: 30.2 and 15.0 s and 90.1 and 33.4 g, respectively; both $P < 0.001$). The antihyperalgesic effect of BAY 59-3074 (1 mg/kg, p.o.) was maximal at 1 h postapplication (99% effect) and lasted for at least 4 h (68% effect at this time point), whereas the antiallodynic effect was somewhat less pronounced but showed the same time dependence (38, 26, and 18% effect at 1, 2, and 4 h postapplication, respectively). Again, reaction latency and withdrawal threshold values were not affected by BAY 59-3074 in the corresponding drug-treated sham groups tested at 1, 2, and 4 h postapplication (data not shown).

Spinal Nerve Ligation Model of Neuropathic Pain. The symptom of mechanical allodynia was significantly present at the time of testing (5 weeks postoperation). BAY 59-3074 partially reversed mechanical allodynia, with a maximal effect of 68% at 1 mg/kg p.o. (Fig. 8B).

Spared Nerve Injury Model of Neuropathic Pain. In the dose-response experiment, the symptoms of thermal hyperalgesia and mechanical allodynia were both significantly present at the time of testing. BAY 59-3074 almost completely reversed thermal hyperalgesia, with a maximal effect of 71% at 1 mg/kg p.o. (ED₅₀ value with 95% CL: 1.04, 0.33–3.30 mg/kg) (Fig. 9A), and completely reversed mechanical allodynia, with a maximal effect of 93% at 3 mg/kg (ED₅₀ value with 95% CL: 1.22, 0.52–2.90 mg/kg) (Fig. 9B). BAY 59-3074 did not affect the reaction latency to thermal stimulation or withdrawal threshold to mechanical stimulation in the corresponding sham groups (data not shown). In the time-dependence study, thermal hyperalgesia and mechanical allodynia were both present at the time of testing (mean reaction latency and withdrawal threshold values in the vehicle-treated sham and axotomized group: 27.8 and 18.1 s and 82.0 and 39.4 g, respectively; both $P < 0.05$). The antihyperalgesic effect induced by BAY 59-3074 (1 mg/kg, p.o.) was maximal at 1 h postapplication (94% effect) and lasted for at least 4 h (65% effect at this time point), whereas the anti-allodynic effect was less pronounced (32, 9, and 17%

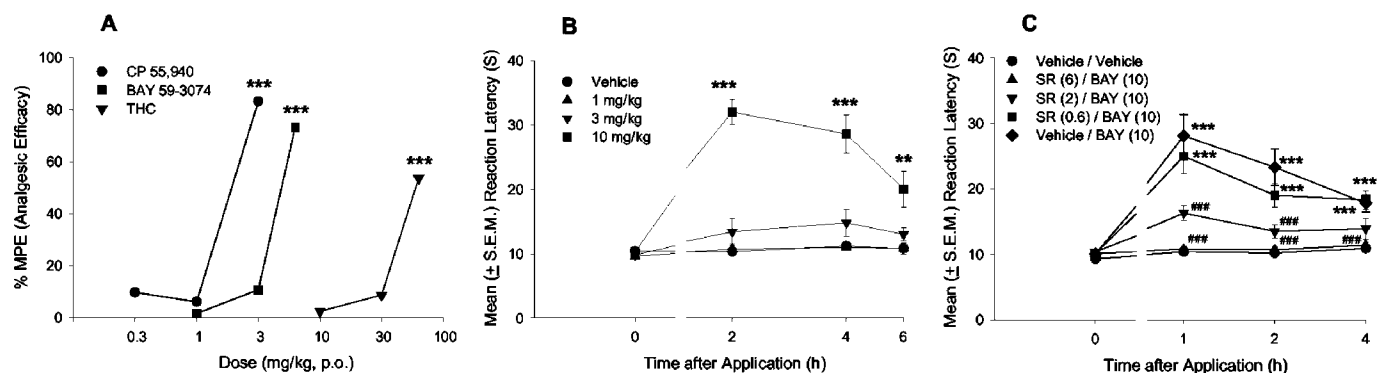


Fig. 6. A, analgesia induced by BAY 59-3074, CP 55,940, and Δ^9 -THC in rats, as assessed in the hot plate assay (52°C). Curves show activity at the time point of maximal activity (CP 55,940: t , 4 h; Δ^9 -THC: t , 1 h; BAY 59-3074: t , 2 h after p.o. administration). B, dose and time dependence of analgesia induced by BAY 59-3074. The 0 value on the x-axis represents the baseline value, as determined 5 min before administration of the compound. C, antagonism of analgesia induced by BAY 59-3074 (BAY; 10 mg/kg, p.o.) by pretreatment with SR 141716A (SR; dose in milligrams per kilogram, i.p.). The 0 time point reflects the baseline value obtained 5 min before the first application; the 1, 2, and 4 h time points reflect time points after the second application ($n = 8$ per group). **, $P < 0.01$ and ***, $P < 0.001$ compared with vehicle/(vehicle) control; ###, $P < 0.001$ compared with vehicle/BAY 59-3074.

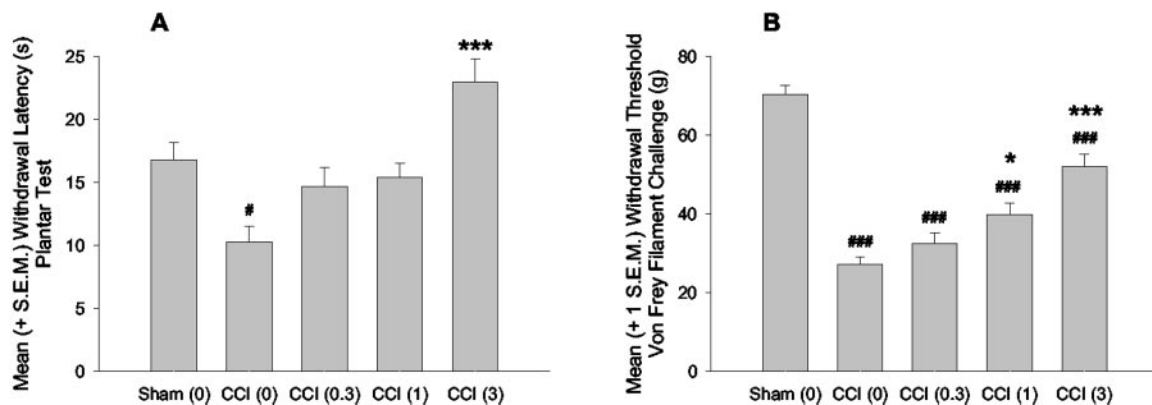


Fig. 7. A and B, reversal of thermal hyperalgesia (A) and mechanical allodynia (B) by BAY 59-3074 (dose in milligrams per kilogram, p.o.; *t*, 1 h) in the chronic constriction injury (CCI) model of neuropathic pain (*n* = 10–12 per group). *, *P* < 0.05 and ***, *P* < 0.001 compared with vehicle-treated ligated control; #, *P* < 0.05 and ###, *P* < 0.001 compared with vehicle sham control.

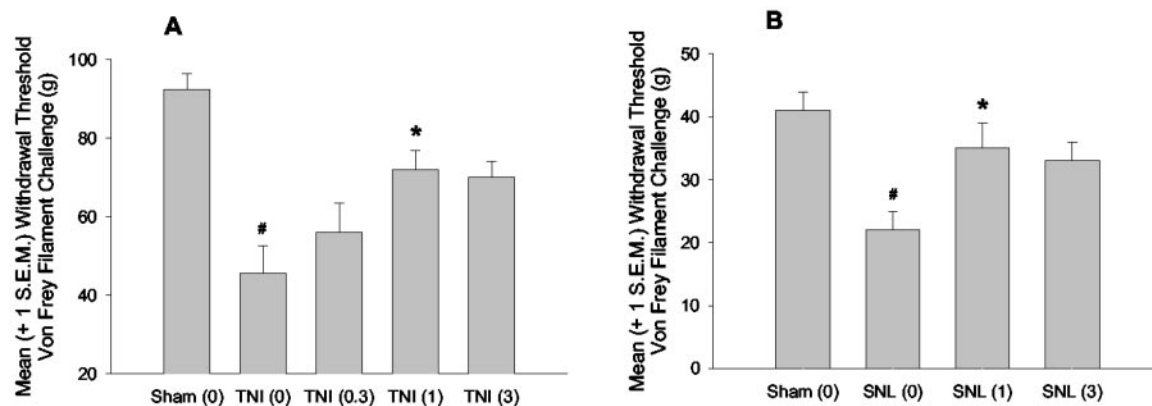


Fig. 8. Reversal of mechanical allodynia by BAY 59-3074 (dose in milligrams per kilogram, p.o.; *t*, 1 h) in the (A) tibial nerve injury (TNI; *n* = 7–8 per group) and (B) spinal nerve ligation (SNL) model of neuropathic pain (*n* = 10 per group). *, *P* < 0.05 compared with vehicle-treated TNI/SNL control; #, *P* < 0.05 compared with vehicle sham control.

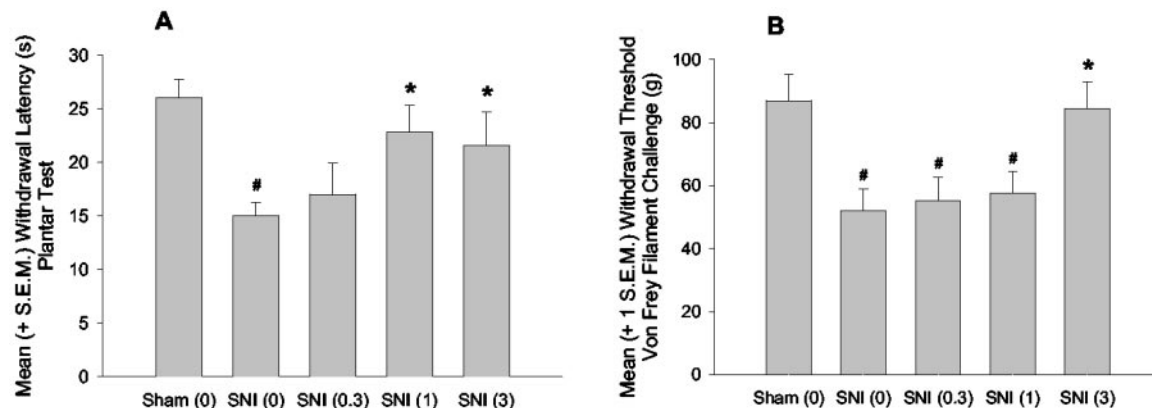


Fig. 9. A and B, reversal of thermal hyperalgesia (A) and mechanical allodynia (B) by BAY 59-3074 (dose in milligrams per kilogram, p.o.; *t*, 1 h) in the spared nerve injury (SNI) model of neuropathic pain (*n* = 6–8 per group). *, *P* < 0.05 compared with vehicle-treated axotomized control (SNI); #, *P* < 0.05 compared with vehicle sham control.

effect at 1, 2, and 4 h postapplication, respectively). Again, reaction latency and withdrawal threshold values were not affected by BAY 59-3074 in the corresponding drug-treated sham groups tested at 1, 2, and 4 h postapplication (data not shown). In the repeated administration study, only mechanical allodynia was clearly present at the time of testing (mean withdrawal threshold values in the vehicle-treated sham and axotomized group: 83.6 and 31.6 g, respectively; *P* < 0.001). BAY 59-3074 (1 mg/kg, p.o.) induced a similar antiallodynic

effect after acute (38% effect, *P* < 0.05) and subacute administration (45% effect, *P* < 0.05).

In the dose-escalation experiment, mechanical allodynia and thermal hyperalgesia were present when assessed in the vehicle-treated control groups at the first day of each dose step. Thus, the mean reaction latency values in the vehicle-treated sham and axotomized group were 26.8 and 18.7 s (day 19), 26.0 and 16.8 s (day 23), 27.8 and 19.4 s (day 26), 29.0 and 17.0 s (day 29), 28.9 and 16.8 s (day 32), and 30.1

and 19.9 s, respectively ($P < 0.05$ at each time point), whereas the mean withdrawal threshold values were 47.8 and 25.1 g (day 19), 56.9 and 17.3 g (day 23), 78.5 and 29.8 g (day 26), 78.9 and 20.7 g (day 29), 67.4 and 20.9 g (day 32), and 72.4 and 22.2 g, respectively ($P < 0.05$ at each time point). The antihyperalgesic effect of BAY 59-3074 after the increasing dose steps was 128% (1 mg/kg), 99% (2 mg/kg), 124% (4 mg/kg), 98% (8 mg/kg), 116% (16 mg/kg), and 94% (32 mg/kg, $P < 0.05$ at each dose), whereas the antiallodynic effect was 17% (1 mg/kg), 18% (2 mg/kg), 46% (4 mg/kg, $P < 0.05$), 45% (8 mg/kg, $P < 0.05$), 48% (16 mg/kg, $P < 0.05$), and 44% (32 mg/kg, $P < 0.05$). Interestingly, however, BAY 59-3074 did not affect body temperature at any of the doses tested. Thus, mean body temperature ranged from 36.2 to 36.6°C during the baseline measurements, 36.4 to 37.0°C after treatment in the vehicle-treated sham group, 36.1 to 36.7°C during the baseline measurements, 36.5 to 37.2°C after treatment in the vehicle-treated axotomized group, 36.3 to 36.7°C during the baseline measurements, and 36.6 to 37.3°C after treatment in the BAY 59-3074-treated axotomized group, respectively. Although not systematically scored, there was no indication for the occurrence of other cannabinoid-related behavioral side effects at any of the doses of BAY 59-3074 tested, supporting the selection of hypothermia as a representative surrogate parameter for such side effects.

Carrageenan Model of Inflammatory Pain. Thermal hyperalgesia was present when assessed 5 min before and 20 to 60 min after drug administration (200–265 min after carrageenan injection). BAY 59-3074 induced a dose-dependent reversal of thermal hyperalgesia, with a maximal effect of 97% at 3 mg/kg p.o. 40 min after administration (ED_{50} value with 95% CL: 1.40, 1.12–1.75 mg/kg) (Fig. 10A). At 20 and 60 min, the compound was less effective (maximal effect: 34%, $P < 0.05$ and 52%, $P < 0.001$, respectively).

Complete Freund's Adjuvant Model of Inflammatory Pain. Mechanical allodynia was present at the time of testing (6 days after injection of complete Freund's adjuvant). BAY 59-3074 induced a dose-dependent reversal of mechanical allodynia, with a maximal effect of 93% at 3 mg/kg p.o. 40 min after administration (ED_{50} value with 95% CL: 0.84, 0.40–1.78 mg/kg) (Fig. 10B). At 60 and 80 min, the compound was less effective (maximal effect: 51%, $P < 0.001$ and 25%, $P < 0.01$, respectively).

Physical Dependence Liability Assessment. Overall, body weight was reduced after 3 ($P < 0.05$) and 10 mg/kg BAY 59-3074 ($P < 0.001$) but not after 1 mg/kg (Fig. 11A). From day 3 on, the daily increase of body weight was similar in each group. Also, at day 15, one day after the termination of drug application, the groups did not differ in their daily body weight increase, indicating that withdrawal did not affect body weight. BAY 59-3074 dose-dependently affected body temperature (Fig. 11B). Drug-induced hypothermia was, however, only obtained on the first 3 days of application, indicating that tolerance developed rapidly for this effect. Although not systematically scored in the present experiment, it was observed that BAY 59-3074 also induced dose-dependent behavioral side effects (ptosis, sedation, and body and side lying), with a minimal effective dose of 3 mg/kg. Again, complete tolerance to these behavioral side effects occurred within 4 days (i.e., four applications). Withdrawal also did not affect body temperature (day 15). As assessed during a 1-h observation period performed 24 h after the last injection of the subacute drug treatment regimen, there was no evidence for the emergence of behavioral withdrawal symptoms in any of the drug-treated groups (across all behavioral symptoms, mean scores ranged from 0.0 to 1.2; none of the scores was significantly different from control) (Table 2). Further occasional observations (not explicitly scored during the present experiment) failed to reveal any behavioral withdrawal symptoms during the 2 days following the last application.

Discussion

For the *in vitro* characterization of BAY 59-3074 as a cannabinoid receptor ligand, rat brain and human cortex membranes expressing native cannabinoid CB_1 receptors, as well as recombinant human cannabinoid CB_2 receptors, were used. The binding data obtained with the reference cannabinoids CP 55,940 and Δ^9 -THC were well within the range of previously published data (Rinaldi-Carmona et al., 1994; Pertwee, 1999; Mauler et al., 2002). As assessed in agonist-stimulated [35 S]GTP γ S binding assays using rat brain and human cortex membranes or recombinant human cannabinoid CB_2 receptors, BAY 59-3074 was characterized as a cannabinoid CB_1/CB_2 receptor partial agonist. Consistent with previous studies using this technique, CP 55,940 and

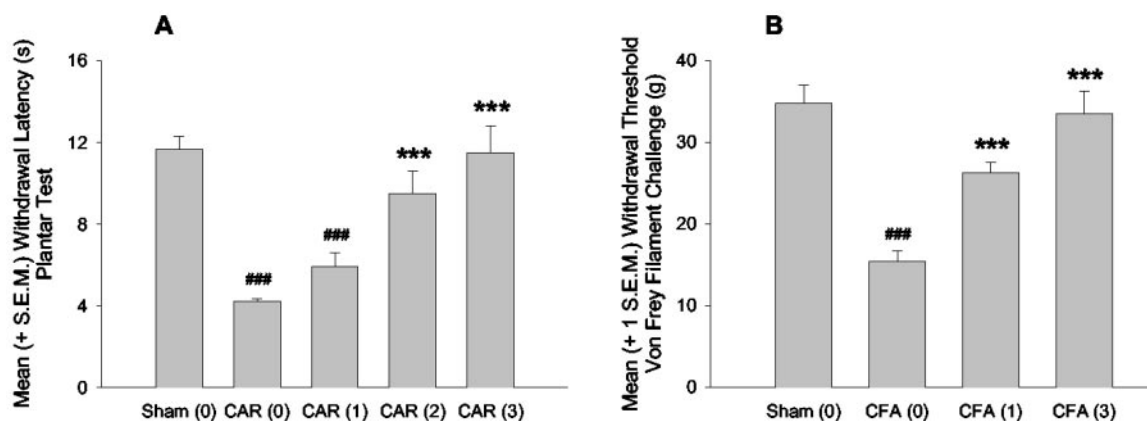


Fig. 10. A and B, reversal of thermal hyperalgesia (A) in the carrageenan (CAR; $n = 14$ –15 per group) and mechanical allodynia (B) in the complete Freund's adjuvant (CFA) model of inflammatory pain ($n = 10$ per group) by BAY 59-3074 (dose in milligrams per kilogram, p.o.; t , 40 min). ***, $P < 0.001$ compared with vehicle-treated CAR/CFA control; ###, $P < 0.001$ compared with vehicle sham control.

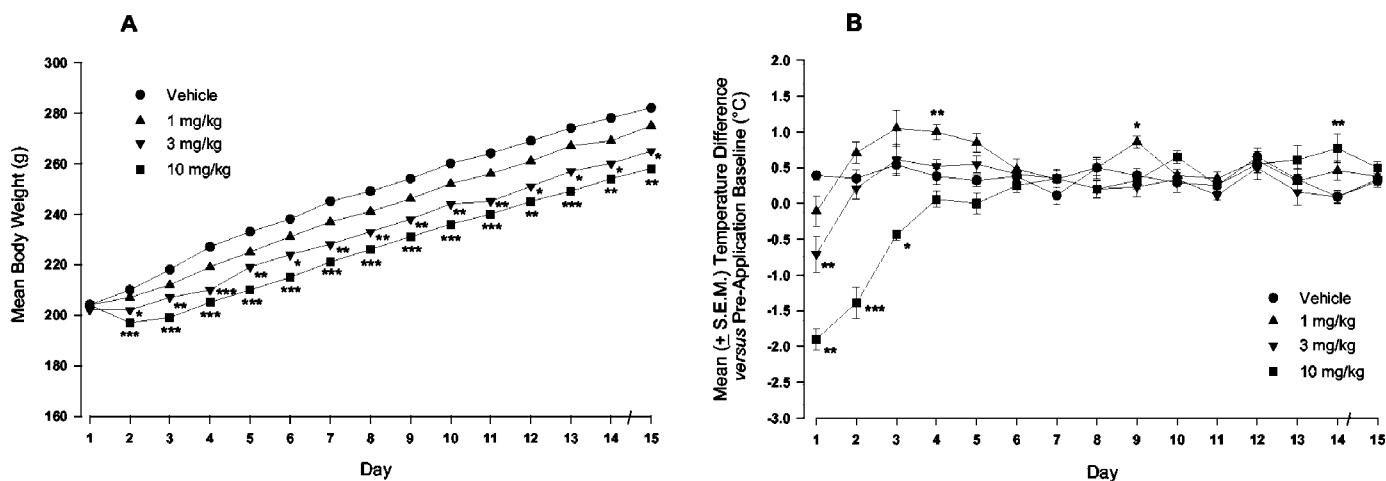


Fig. 11. Effect of repeated treatment with BAY 59-3074 (p.o.) on body weight (A) and temperature (B). Rats were treated once a day for 14 days. Body weight was recorded immediately before the daily injection. Temperature was measured immediately before and 1 h after each daily injection, and the difference between both measurements is shown. On day 15, no application was given ($n = 8$ per group). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with vehicle control.

TABLE 2

Behavioral symptoms induced by abrupt withdrawal after repeated treatment with vehicle and 1, 3, or 10 mg/kg BAY 59-3074 p.o. once a day for 14 consecutive days

Symptoms were scored on day 15 24 h after the last application and are represented as mean \pm S.E.M. values obtained during a 1-h observation period. None of the scores obtained after drug treatment was statistically different from the scores obtained after vehicle treatment. $n = 8$ per group.

Behavioral Symptom	Vehicle	1 mg/kg	3 mg/kg	10 mg/kg
Turning left (360°)	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Turning right (360°)	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Walking backwards	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Digging	0.0 \pm 0.0	0.2 \pm 0.1	0.0 \pm 0.0	0.0 \pm 0.0
Wet dog shake	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Tremor	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Forepaw treading	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Boxing	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Raised hindpaw	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Scratching	0.0 \pm 0.0	0.0 \pm 0.0	0.7 \pm 0.2	0.6 \pm 0.3
Grooming face	0.0 \pm 0.0	0.0 \pm 0.0	0.2 \pm 0.1	0.5 \pm 0.3
Grooming body	0.0 \pm 0.0	0.2 \pm 0.1	1.2 \pm 0.3	0.6 \pm 0.3
Licking	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Biting	0.0 \pm 0.0	0.0 \pm 0.0	0.3 \pm 0.2	0.1 \pm 0.1
Tongue rolling	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Ptosis	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Arched back	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0

Δ^9 -THC behaved as cannabinoid CB₁/CB₂ receptor full and partial agonists, respectively (Burkey et al., 1997; Petit et al., 1998; Mauler et al., 2002). On both cannabinoid receptor subtypes, the level of intrinsic activity of BAY 59-3074 seemed to be between that of CP 55,940 and Δ^9 -THC. BAY 59-3074 is considered to be a selective cannabinoid CB₁/CB₂ receptor ligand as a broad receptor binding, and enzyme activity screen revealed no evidence for interactions of comparable potency with targets other than cannabinoid receptors. Thus, the margin between specific cannabinoid receptor binding and interaction with other binding sites was at least one order of magnitude.

The in vitro characterization of BAY 59-3074 as a cannabinoid CB₁ receptor agonist was confirmed in the rat hypothermia assay and BAY 38-7271 drug discrimination assay, two behavioral models highly sensitive to cannabinoid CB₁ receptor activation (Martin et al., 1991; De Vry and Jentsch, 2002). After oral administration, both the potency and efficacy of BAY 59-3074 were intermediate between that of CP 55,940 and Δ^9 -THC in producing hypothermia. The order of potency and extent of hypothermia obtained with these com-

pounds is consistent with other data seen in similar hypothermia assays (Martin et al., 1991; Rinaldi-Carmona et al., 1994; Mauler et al., 2002; De Vry et al., 2004a) and compatible with their affinity and intrinsic activity estimation at these receptors (see above). Further evidence for an involvement of cannabinoid CB₁ receptors in the hypothermia assay is indicated by the finding that pretreatment with the selective cannabinoid CB₁ receptor antagonist SR 141716A (Rinaldi-Carmona et al., 1994) blocks the hypothermic effects of various cannabinoid CB₁ receptor agonists, including BAY 59-3074 (present study; Rinaldi-Carmona et al., 1994; Mauler et al., 2002; De Vry et al., 2004a;).

The cannabinoid CB₁ receptor agonist profile of BAY 59-3074 was also confirmed in a drug discrimination assay in which rats were trained to discriminate the highly selective and potent cannabinoid CB₁ receptor full agonist BAY 38-7271 (De Vry and Jentsch, 2002; Mauler et al., 2002) from vehicle. It was previously demonstrated that the discriminative stimulus induced by cannabinoid CB₁ receptor full agonists is highly sensitive and specific (Wiley et al., 1995; De Vry and Jentsch, 2002; Mauler et al., 2002). All three can-

nabinoid CB₁ receptor agonists tested in the drug discrimination assay induced complete generalization, with the order of potency closely resembling that obtained in the hypothermia assay and the in vitro binding assays. This finding supports the suggestion that the discriminative effect of these compounds is mediated by activation of cannabinoid CB₁ receptors. This suggestion is also supported by the finding that their discriminative effect, including that of BAY 38-7271, is blocked by SR 141716A (Wiley et al., 1995; De Vry and Jentsch, 2002, 2003; Mauler et al., 2002). The finding that the partial agonists Δ^9 -THC and BAY 59-3074 induce the same level of generalization as the full agonist CP 55,940 is most likely due to a high degree of receptor reserve of the cannabinoid receptor population mediating the discriminative effect of these compounds (for discussion, see Gifford et al., 1999; De Vry and Jentsch, 2003).

It has been suggested that the endogenous cannabinoid system is intimately involved in the perception and modulation of pain (Pertwee, 2001; Piomelli et al., 2000; Richardson, 2000), and cannabinoids, including Δ^9 -THC, WIN 55,212-2, CP 55,940, and HU-210, as well as anandamide and methanandamide, were found to have analgesic effects in animal models of acute and chronic pain (for review, see Pertwee, 2001). Of significant interest is the consistent observation that various cannabinoids and endocannabinoids have antihyperalgesic and/or antiallodynic effects in animal models of chronic neuropathic and inflammatory pain (for references, see Introduction). In addition, it was reported that cannabinoids are able to attenuate capsaicin-induced hyperalgesia, a model that has been suggested to reflect sensitization processes underlying chronic pain (Li et al., 1999; Johanek et al., 2001). In the present study, BAY 59-3074 showed antihyperalgesic and/or antiallodynic effects in various models of neuropathic pain, including the chronic constriction injury model (Bennett and Xie, 1988), the L5 spinal nerve ligation model (Kim and Chung, 1992), the spared nerve injury model (Decosterd and Woolf, 2000), and the recently described tibial nerve injury model (Hofmann et al., 2003), as well as in models of inflammatory pain, such as the carrageenan model (Hargreaves et al., 1988) and complete Freund's adjuvant model (Bertorelli et al., 1999). Interestingly, the antihyperalgesic and antiallodynic effects of cannabinoids, including BAY 59-3074, could be obtained at doses that were generally lower than the doses that induced analgesic effects against acute, nonpathological pain (see also De Vry et al., 2004a). This finding may indicate that the chronic pain models are more sensitive to the pharmacological effects of BAY 59-3074 than the acute pain models. Alternatively, it could imply that the endocannabinoid system becomes more sensitive to the analgesic effects of cannabinoids in chronic pain conditions. As discussed by Richardson et al. (1998), Drew et al. (2000), Piomelli et al. (2000), and Siegling et al. (2001), possible mechanisms for such increased sensitivity include 1) increased expression of cannabinoid CB₁ and/or CB₂ (-like) receptors, 2) changes in cannabinoid CB₁ and/or CB₂ (-like) receptor function and/or G-protein coupling, 3) altered formation/release of endocannabinoids such as anandamide and 2-arachidonolglycerol, or 4) synergism with other endogenous ligands that are active only during chronic pain conditions.

It is generally accepted that the antihyperalgesic/antiallodynic activity of cannabinoids is predominantly mediated

through activation of cannabinoid CB₁ receptors located in central and peripheral nervous structures involved in the processing of pain (such as the thalamus, brain stem, spinal cord, and peripheral sensory neurons). Nevertheless, the relative contribution of these anatomical substrates, as well as the possible contribution of peripheral cannabinoid CB₂ or CB₂-like receptors, remains unclear (Piomelli et al., 2000; Pertwee, 2001). Although it was found that pretreatment with the selective cannabinoid CB₁ receptor antagonist blocked the analgesic effect of BAY 59-3074 in the hot plate model of acute pain, it remains to be determined to what extent analgesia can also be affected by a selective cannabinoid CB₂ receptor antagonist and to what extent these antagonists are able to block antihyperalgesic/antiallodynic effects of the compound.

Of possible clinical interest is the observation that the antihyperalgesic and antiallodynic effects of BAY 59-3074 could be obtained at doses that did not induce typical cannabinoid-related behavioral side effects, such as ptosis, sedation, catalepsy, or hypothermia. This observation may indicate that the compound could have a relatively favorable therapeutic window (at least with respect to tolerability). It should be recognized, however, that the antihyperalgesic and antiallodynic effects occurred at doses that also produced discriminative stimulus effects. This could imply that therapeutic efficacy coincides with cannabimimetic subjective effects.

Interestingly, as assessed after repeated administration of a fixed daily dose or rapid up-titration of the daily dose, it seemed that the antihyperalgesic and antiallodynic effects of BAY 59-3074 were relatively resistant to tolerance development, whereas tolerance developed rapidly to the typical cannabinoid-related behavioral side effects (occurring at higher doses). Although more extensive dose-response analysis is required to substantiate this impression, it may indicate that tolerance develops differentially to the antihyperalgesic/antiallodynic effects and the behavioral side effects of the compound (for discussion, see De Vry et al., 2004a). The possible occurrence of differential tolerance development may also be clinically important, because it suggests that the therapeutic window increases after repeated administration of the compound. Finally, it should be mentioned that abrupt withdrawal following daily administration of BAY 59-3074 (up to 10-fold the therapeutically effective dose) for 2 weeks did not induce clear behavioral withdrawal symptoms. Although further studies (such as precipitated withdrawal studies) are needed to fully assess the physical dependence liability of BAY 59-3074, this initial study suggests that it may be low.

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