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Selective blockade of the hydrolysis of the endocannabinoid 2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents

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Monoacylglycerol lipase (MAGL) represents a primary degradation enzyme of the endogenous cannabinoid (eCB), 2-arachidonoylglycerol (2-AG). This study reports a potent covalent MAGL inhibitor, SAR127303. The compound behaves as a selective and competitive inhibitor of mouse and human MAGL, which potently elevates hippocampal levels of 2-AG in mice. *In vivo*, SAR127303 produces antinociceptive effects in assays of inflammatory and visceral pain. In addition, the drug alters learning performance in several assays related to episodic, working and spatial memory. Moreover, long term potentiation (LTP) of CA1 synaptic transmission and acetylcholine release in the hippocampus, two hallmarks of memory function, are both decreased by SAR127303. Although inactive in acute seizure tests, repeated administration of SAR127303 delays the acquisition and decreases kindled seizures in mice, indicating that the drug slows down epileptogenesis, a finding deserving further investigation to evaluate the potential of MAGL inhibitors as antiepileptics. However, the observation that 2-AG hydrolysis blockade alters learning and memory performance, suggests that such drugs may have limited value as therapeutic agents.

The endocannabinoid (eCB) system is formed by the G protein-coupled receptors, CB1 and CB2, and their main transmitters, 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide; AEA)¹. eCBs play an important neuromodulatory role in the peripheral and central nervous system (CNS), modulating a wide range of physiological and pathological processes, including cognition, emotion, mood, appetite and pain². Alterations in the eCB system have been observed in a variety of diseases across therapeutic areas. For example, changes in tissue concentrations of AEA and 2-AG have been observed in pain and inflammation^{3,4}, immunological disorders⁵, neurological and psychiatric conditions⁶, obesity and metabolic syndromes^{7,8} and cancer⁹. These observations have fueled significant interest in the development of eCB-manipulating drugs to treat these conditions^{10–12}. For example, CB1 antagonism has been demonstrated to limit the risks of cardiovascular disease and type 2 diabetes^{13–17}, but also to produce a number of undesirable CNS side effects¹⁸. As of today the efforts to uncouple these beneficial and unwanted effects have not been successful, thus limiting the therapeutic value of direct CB1 receptors ligands.



Inhibition of 2-AG and AEA catabolism has been suggested to represent an alternative avenue to modulate the eCB system^{19–22}. The signaling functions of these two transmitters are terminated by enzyme hydrolysis in processes principally mediated by the serine hydrolases monoacylglycerol lipase (MAGL)²³ and fatty acid amide hydrolase (FAAH)²⁴, respectively. While FAAH inhibitors have been discovered, characterized in several animal models of disease and are in pharmaceutical development (for a recent review, see²⁵), MAGL inhibitors have lagged behind. However, they may offer certain advantages over FAAH inhibitors because of their greater eCB specificity^{19,26,27}.

Several compounds with selectivity for MAGL have been described (for reviews, see^{19,22,28}). For example, lead MAGL inhibitors from the O-aryl carbamate class, such as JZL184 and its analog KML29 show good selectivity (>100-fold) for MAGL over FAAH and most other serine hydrolases^{29,30}. However, JZL184 inhibits several carboxylesterase enzymes in peripheral tissues, has cross-activity with FAAH after repeated administration and appears to be less potent in inhibiting MAGL in rats when compared to mice^{31,32}. Moreover, all potent inhibitors of MAGL activity reported thus far are claimed irreversible because they produce their action by forming covalent bonds with reactive cysteine or serine residues. The only exception is the naturally occurring terpenoid, pristimerin, which inhibits MAGL activity with high potency through a reversible mechanism²⁸. The most extensively studied selective MAGL inhibitors are JZL184 and KML29. When administered in mice, both drugs dramatically decreased brain MAGL hydrolysis activity and concomitantly elevated 2-AG levels in the brain. These metabolic and biochemical modifications were accompanied by several CB1- or CB2-dependent behavioral effects, such as analgesia and anxiolysis^{30,33–38}. Importantly, while JZL184 elicited cannabimimetic untoward effects, including hypothermia, and hypomotility²⁹, KML29 was devoid of such effects³⁸.

Here, we report the detailed characterization of a structurally distinct carbamate compound that acts as a highly potent and selective MAGL inhibitor, here named SAR127303 (Figure 1). While our data confirm that MAGL inhibition translates into antinociceptive effects *in vivo*, we also provide new evidence that blockade of this enzyme affects cognitive processes and epileptogenesis.

Methods

Ethics statement. All experimental procedures described herein were carried out in accordance with the “Guide and Care and Use of Laboratory Animals” (National Institutes of Health) and approved by the Sanofi Institutional Animal Care and Use Committee (studies conducted in the USA) or the Animal Ethics Committee of Sanofi (studies conducted in France).

Animals. Animals had access to food and water ad libitum with a 12-h light/dark cycle (lights on at 7:00 a.m.). The following species and strains were used: (1) mice: C57BL/6J, CB17 SCID, CD1, NMRI, OF1 and Swiss; (2) Rats: Sprague-Dawley, Wistar and Wistar Han (see below for further details). All testing was performed during the light (day) cycle.

Drugs. SAR127303, diazepam, rimonabant, SR144528, WIN55,512-2 (Sanofi Medicinal Chemistry, France), levetiracetam (Advanced Technology & Industrial Co. Ltd, Hong Kong), kainic acid, pentylentetrazol and MK-801 (Sigma RBI, St Quentin Fallavier, France) were dissolved or suspended in distilled water with 0.6%

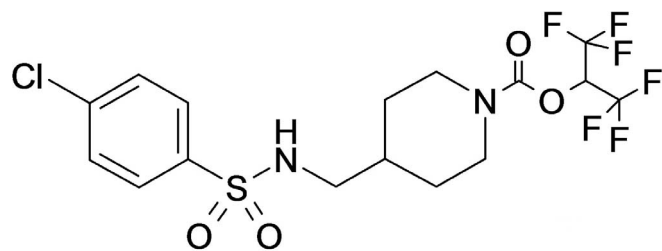


Figure 1 | Chemical structure of SAR127303.

methylcellulose and the addition of 0.1% Tween 80 (Sigma RBI) (unless otherwise indicated) in *in vivo* studies and suspended in DMSO at 10 mM in *in vitro* experiments. Formalin (Sigma RBI) was dissolved in 0.9% saline and phenylbenzoquinone (Sigma RBI) was solubilized in a 0.9% saline solution containing 1% alcohol. SSR411298 and JZL184 were synthesized by Sanofi Medicinal Chemistry. Doses refer to the weight of the free base. All drug solutions were prepared fresh daily.

In vitro selectivity profile. The effect of SAR127303 on approximately 240 different receptors, ion channels, enzymes, transporters, kinases and serine hydrolases (see Supplementary Material and Supplementary Table S1 for details) was evaluated at contract research organizations (CEREP, ActivX) using established protocols or through internal studies. IC₅₀ were determined in case significant activity was observed at 10 μM (≥50% inhibition).

Activity of SAR127303 in MAGL biochemical assay. Biochemical activity of SAR127303, reference MAGL inhibitor JZL 184 and FAAH inhibitor SSR411298 were assessed using human recombinant MAGL (rhMAGL) and 4-nitrophenylacetate as a substrate using MAGL Inhibitor Screening kit (Cayman Chemical, Cat. 705192) according to manufacturer’s instructions. IC₅₀ was calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc.).

LC-MS evidence of covalent modification of MAGL Ser122 by SAR127303. Recombinant human MAGL (2.5 μM) was pre-incubated with different concentrations of SAR127303 for 2 hours. Resulting samples were digested with chymotrypsin overnight. The mixtures were then injected into AB Sciex Qtrap 5500 LC/MS/MS System for quantitative monitoring of two unmodified peptides containing Ser 122 (LHSMGGAIAIL is the peptide and LLHSMGGAIAIL is with a mis-cleavage). Peptide derived from MAGL C-terminus (HKELPEVTNSVF) was used as control. Separation was performed on 1.0 × 150 mm Waters HSS T3 LC column with the flow rate of 0.2 mL/min.

Protein crystallization and data collection. Crystals of the apo-protein were obtained in MES 50 mM pH 6.0, MPD 40% (v/v) at 4°C. They were used for soaking experiments in presence of 1 mM of SAR127303 overnight at 4°C. The cryo-protecting solution contained 10% MPD (v/v), 30% PEG2000 MME and 1 mM of SAR127303. A 2.36 Å resolution dataset was collected at ESRF (beamline ID14-2). The structure of MAGL-SAR127303 was solved by molecular replacement using the apo-structure (PDB code 3JW8) as the search model. Protein cloning, expression and purification were described elsewhere³⁹. Coordinates and structure factors for MAGL in complex with SAR127303 have been deposited in the Protein Data Bank under the accession code 4UUQ.

Plasma exposure and brain penetration. SAR127303 was administered p.o. to CB17 SCID male mice (Charles River) in 20% Glycofurol, 10% Vitamin E TPGS in water. Plasma and brain samples were harvested at the indicated time points and stabilized in the presence of 0.4 mg/ml PMSF. SAR127303 was detected in plasma and in tissue using established LC/MS protocols.

Functional activity. MAGL activity of brain homogenates prepared from mice treated with SAR127303. Mice were euthanized 1 hour after oral administration of SAR127303 or vehicle. Brains were rapidly removed and immediately frozen at –80°C for conservation. The day of MAGL activity measurement, brains were homogenized in buffer containing Tris-HCl 10 mM (pH=8.0); 150 mM NaCl; 1 mM EDTA. The hydrolysis reaction was performed at room temperature in phosphate buffer 50 mM, pH8, fatty acid free BSA 1 mg/ml, in 96 wells filtration plates. Briefly, mouse brain homogenates (treated by SAR127303 or vehicle) were incubated 20 minutes with 50 μM of a mix of cold and tritiated 2-oleoylglycerol radiolabeled on its glycerol moiety (0.027 μCi/well). The reaction was stopped by the addition of activated charcoal (activated charcoal 5 M diluted in NaCl 1.5 M and HCl 0.5 M). Plate was then mixed for 10 minutes. The [³H]glycerol produced was recovered by vacuum filtration prior to scintillation counting (plate reader). MAGL activity for each mouse is expressed in % of MAGL activity measured in vehicle treated mice. A non specific MAGL activity, obtained by the measure of 2-AG hydrolysis in the presence of an excess of SAR127303 in wells containing vehicles treated mice brain homogenate, is subtracted from each value. This non specific hydrolysis represents about 20% of total 2-AG hydrolysis. Data were analyzed with a one-way ANOVA followed, when appropriate, by a Dunnett’s test.

Brain lipid profiling. The levels of several monoacylglycerols (MAGs) and free fatty acids (FFAs) were measured in the whole brain of mice that received vehicle or SAR127303 (MAGs: 10 or 30 mg/kg, p.o.; FFAs: 10, 30 or 60 mg/kg p.o.) and that were sacrificed 4, 8 and 24 h later. Brain tissues were rapidly homogenized in Tris-EDTA buffer (pH=7.4) using Precellys 24 tissue homogenizer and an aliquot was used for protein measurements. Lipids were extracted using chloroform/methanol solvent (1:2) containing 0.025 N HCl. After centrifugation and supernatant withdrawal, the samples were dried by gentle nitrogen flow. The residue was reconstituted in methanol solvent for LC/MS analysis. Quantitation of monoacylglycerols (MAGs) was performed on the ABSciex QTrap 5500 MS system coupled with Agilent 1290 Infinity UHPLC system using Waters Xbridge C18 column (2.1 × 100 mm) for separation. MAG abundance was normalized to protein concentration in the sample. The following MAGs were measured: MAG 16:0,



1-hexadecanoyl-rac-glycerol; MAG 18:0, 1-monoacylglyceride; MAG 18:1, 1.1-(11Z-Octadecenoyl)-rac-glycerol; MAG 18:2, 1-(9Z,12Z-octadecadienoyl)-rac-glycerol; MAG 20:4, 2-arachidonylglycerol. The following FFAs were measured: Palmitic acid, palmitoleic acid, stearic acid, vaccenic acid, linoleic acid, arachidonic acid and docosahexaenoic acid. Two-way ANOVA (treatment x time) was used to assess overall effect of treatment, and Dunnett's post test was used to compare differences between treatment groups.

Levels of 2-AG, AEA, PEA and OEA in the hippocampus of mice treated with SAR127303. The levels of 2-AG, AEA, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) were measured in the hippocampus of CD1 mice that received either vehicle (0.1 mL/10 g, p.o.) or SAR127303 (8 mg/kg, p.o.) and that were sacrificed 1, 2, 6 and 24 h later. Hippocampus (right and left) were frozen and weighed before storage until analysis. For 2-AG analysis, each sample was homogenized in 1 mL of heptane/isopropanol 3/2 containing 1 μ M of 2-AG-d8 (Internal standard for 2-AG), centrifuged at 12000 g for 7 minutes at 4°C.

Supernatant was evaporated and dissolved in 150 μ L of acetonitrile (ACN) and 150 μ L of H₂O for injection (100 μ L) in an on-line SPE-LC-MS/MS. The on-line SPE (Solid Phase Extraction) coupled to the LC (Liquid Chromatography) was performed with a "Symbiosis" apparatus from SparkHolland using a C18 HD, 7 μ M cartridge (2 \times 10 mm as internal diameter \times length) and a Hypersil gold C18 (1.9 μ m, 50 \times 2.1 mm) column respectively for the on-line extraction and separation. The elution was performed at 0.25 mL/min with a ACN/H₂O + ACNH₄ 2 mM gradient, both solvent containing 0.1% of HCOOH. The end eCBs were identified and quantified by a triple quadrupole mass spectrometry detector (Quantum Ultra from Thermo Electron Corporation) equipped with an ESI probe and working in the SRM mode (Selective Reaction Monitoring). The concentrations of each compound were calculated according to external calibration method. Data were analyzed with a one-way ANOVA followed by a Dunnett's test.

Mouse tetrad experiments and elevated plus-maze. The potential cannabimimetic activity of SAR127303 in 8-week-old OF1 mice (Charles Rivers Laboratories, L'Arbresle, France) was evaluated using the following indices: locomotor activity, nociception in the tail flick test, catalepsy in the bar test, and hypothermia. The effects were compared to those obtained with the CB1 receptor agonist, WIN55,212-2 at 15 mg/kg. In addition, SAR127303 effects on anxiety-related behaviors were assessed in the elevated plus-maze test. For determining hypomotility each mouse was placed in an actimeter device consisting of a cylinder (20 cm diameter, 9.5 cm high, Apex, France) equipped with two perpendicular light beams located 1.5 cm above the floor and locomotor activity (number of interrupted light beams) was recorded for a period of 30 min. The tail flick analgesia meter consists of a stimulation unit (containing the halogen lamp for the heat stimulus) and an electronic control unit (Columbus Instruments, Columbus, Ohio, USA). The mouse is placed with its tail protruding within a restraining tube on the platform of the Stimulus Unit. The animal's tail is positioned on a slot of adjustable width equipped with a groove that guarantees a correct placement of the rodent's tail. A remote foot-switch controls the test start/stop allowing rapid hands-free experiments. A photo beam detects the tail flick and the latency is automatically presented on a digital display on the Control Unit. Measurements of reaction time are given with a 0.01 s precision. A cut-off time of 20 s is set to avoid tissue damage. Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75 cm diameter) that was elevated 4.5 cm above the surface. Mice that remained motionless with their paws on the bar for 10 s (with the exception of respiratory movements) were scored as cataleptic. Hypothermia by inserting a thermocouple probe 2.0 cm into the rectum. The elevated plus-maze test is based on that described by Lister et al (1990). The testing apparatus was made of grey polyvinylchloride (PVC) and elevated to a height of 48 cm with two open/unprotected (30 \times 5 cm) and two enclosed arms (30 \times 5 \times 20 cm) arranged so that the arms of the same type were opposite to each other. To prevent mice falling off, a rim of Plexiglas (0.25 cm high) surrounded the perimeter of the open arms. The light intensities measured in the open arms, closed arms and on the central platform were 70, 20 and 50 lux, respectively. For testing, mice were placed in the center of the maze facing a closed arm, for a free exploration period of 5 min. A camera was mounted above the maze and a video tracking software (EthoVision XT 3.0, Noldus, Wageningen, Netherlands) was used to record the behavior of mice. Results are expressed as mean ratio of time spent in open arms to total time spent in both open and closed arms. SAR127303 and the positive control, diazepam, were administered p.o. 60 min before testing. Data were analyzed with a one-way ANOVA followed, when appropriate, by a Dunnett's test.

Effects of SAR127303 in models of pain. The phenylbenzoquinone (PBQ)-induced writhing model of acute visceral pain in mice. PBQ (2 mg/kg in 10 mL/kg) or vehicle was injected i.p. in male OF1 mice (24–29 g, Charles Rivers Laboratories, L'Arbresle, France). Each mouse was placed in an individual box containing sawdust and the intensity of nociceptive behavior was quantified by counting the total number of writhes (contraction of the abdominal muscle together with a stretching of hind limbs or rotation of the trunk) occurring between 5 and 15 min after the PBQ injection. The intensity of the writhing response was expressed as the total writhing over the 10 min period. Experiment 1 (dose-response): SAR127303 was administered orally 60 min prior to testing; Experiment 2 (antagonism by the CB1 receptor antagonist, rimonabant): SAR127303 and rimonabant were administered orally or intraperitoneally 60 and 30 min prior to testing, respectively. Experiment 3 (antagonism by the CB2 receptor antagonist, SR144528): SAR127303 and SR144528

were administered orally or intraperitoneally 60 or 90 min prior to testing, respectively. Data were analyzed with a Kruskal-Wallis test followed, when appropriate, by a Wilcoxon two-tailed comparisons test. These analyses, and all subsequent ones were performed using the SAS software (SAS Institute Inc., Cary, NC, USA).

The formalin test of inflammatory pain in mice. Twenty microliters of diluted formalin (2.5%) were injected subcutaneously (s.c.) into the dorsal surface of the right hind paw of male OF1 mice (26–30 g, Charles Rivers Laboratories, L'Arbresle, France) using a microsyringe. The incidence of spontaneous behavior was scored every 3 minutes for a period of 45 minutes after formalin injection. Briefly, the scores were: 0 = normal behavior of the hind limb to support the body; 1 = slight touching of the injected paw on the glass surface to lightly support or not support the body; 2 = total withdrawal of the injected paw; and 3 = licking, biting or shaking of the injected paw.

The data collected between 0 and 15 minutes post-formalin injection represent the acute phase (nociceptive phase), and the data collected between 15 and 45 minutes represent the late phase (inflammatory phase). Experiment 1 (acute dosing): Sixty minutes before the formalin insult, the animals were orally administered with the vehicle or SAR127303; Experiment 2 (repeated dosing): SAR127303 was administered once-a-day for five consecutive days. The last dosing occurred on day five 60 min before formalin injection. The areas under the curves (AUCs [0–15 min] and [15–45 min]) of the vehicle-treated and SAR127303-treated group at 10 mg/kg (acute and repeated dosing) were compared with a Wilcoxon test. We have tested the unique dose of 10 mg/kg in this test because it produces a nearly complete inhibition of both MAGL activity and writhes in the PBQ test. This latter being a good predictor of dose-efficacy in the formalin test.

Effects of SAR127303 in models of cognitive processes. In vitro long term potentiation (LTP). Following halothane anaesthesia, the brain of 3- to 8-week-old rats (Janvier Labs, Le Genest Saint Isle, France) was dissected and hippocampal slices were prepared using a cooled vibroslicer (Leica Biosystems, Nanterre, France). Following recovery for at least 1 hour at room temperature in oxygenated artificial cerebrospinal fluid (aCSF containing in mM: NaCl 126; KCl 3.5; NaH₂PO₄ 1.1; CaCl₂ 2.2; MgCl₂ 1.3; NaHCO₃ 25; D-glucose 10; pH 7.2–7.4), slices were transferred on microelectrode arrays (MEAs, Multi Channel Systems, Reutlingen, Germany) under visual control using a CCD camera (Microvision Instruments, Evry, France). The hippocampal slices were placed in close contact with the largest number of recording electrodes within the CA1 area. The field population spikes (fPS) were elicited in the hippocampal CA1 area every 30 s by injection of biphasic currents (–120 μ A/ +120 μ A, 60 μ s, MC Stimulus, Multi Channel Systems). fPS amplitudes were recorded 10 minutes before and 30 minutes after LTP induction (High Frequency Stimulation, 100 Hz for 1 second, twice with a 30 seconds interval) in the absence (control) or in the presence of SAR127303. SAR127303 was superfused at 1 or 10 μ M in a CSF, 30 minutes before and 30 minutes after LTP induction. All recordings were performed at a controlled temperature of 32°C (\pm 1°C).

Effects of 2-AG and SAR127303 on [³H]ACh release evoked by electrical depolarisation in rat hippocampal slices. Tissues were obtained from male Sprague-Dawley rats (200–250 g). After death, brains were removed and hippocampus was quickly dissected out. Hippocampal slices (500 μ m) were suspended in 20 mL Krebs buffer saturated with 5% CO₂ in O₂. Composition of Krebs medium (in mM) was: NaCl 125, KCl 5, CaCl₂ 1.2, MgCl₂ 1.1, NaHCO₃ 20, NaH₂PO₄ 1.2, glucose 10, pH 7.4. [³H]-Acetylcholine ([³H]-ACh), 100 nM was added and the incubation was carried out at 37°C for 30 min. After incubation, slices were rinsed three times with Krebs medium. 30 μ L of Krebs medium containing hippocampal slices were transferred into each superfusion chamber between two platinum electrodes, 1 cm apart (Brandel system, USA). The slices were superfused at a rate of 0.5 ml/min. Collection of 3 min serial fractions (1.5 mL) began after a 42 min wash period. Electrical stimulation consisted of 2 ms monophasic rectangular pulses (2 Hz, 160 mA, 1 min) (Brandel stimulator, USA) and was done during the 6th fraction. CTP was perfused from 9 min before the electrical stimulation to the end. At the end of the experiments, tissue slices were collected and solubilized in Soluene-350 (Perkin-Elmer). Total tritium activity in the 3 min fractions and samples with solubilized tissue slices was measured by liquid scintillation spectrometry. The fractional efflux of [³H]-ACh was estimated as a percentage of the amount of radioactivity in the superfusate fraction relative to the total amount of radioactivity in the slices at the beginning of the experiment. The amount of radioactivity at the beginning of the experiment was estimated as the sum of radioactivity measured in the solubilized slices at the end of the super-fusion and the total radioactivity collected in the medium samples from the beginning to the end of the experiment. To determine stimulated release, the basal efflux of radioactivity in the fraction prior to stimulation was subtracted from each of the subsequent five electrical depolarization fractions, the values then being summed. The statistical analyses of the biochemical results were performed using Student's t-test.

Effect of SAR127303 on in vivo hippocampal ACh release in rats.

Surgery and microdialysis. Sprague-Dawley rats (Charles River, France) weighing 250–350 g, were anaesthetized with Zoletil® 50 (60 mg/kg, i.p.) in NaCl (0.9%, 10 mL/kg of body weight) and placed in a stereotaxic apparatus. A guide cannulae was implanted at the following coordinates according to the atlas of Paxinos and Watson³⁰: 5.3 mm posterior to bregma, 4.8 mm lateral to bregma and 8 mm down from the dorsal surface). The microdialysis probe (4 mm outer diameter 0.5 mm; CMA 12, CMA/Microdialysis, Stockholm, Sweden) was perfused at a constant flow



rate of 2 $\mu\text{L}/\text{min}$ using a microinjection pump (CMA 100, CMA/Microdialysis, Stockholm, Sweden) with a gassed Ringer's solution containing (in mM): NaCl, 147; KCl, 4; CaCl_2 , 1.2; MgCl_2 , 1; pH 7.4. To reduce ACh degradation in the dialysate, 0.3 μM of neostigmine (Sigma-Aldrich, Saint Quentin Fallavier, France) was added to the Ringer's solution perfused in the probe. Microdialysis sampling started 90 min after the probe was placed into the hippocampus. Serial samples were collected at 20 minutes and analyzed using high performance chromatography (HPLC) with electrochemical detection. The position of the probe was verified histologically at the end of extracellular ACh experiment⁴¹.

Treatment. SAR127303 was suspended with Tween 80 (Polyoxyethylene sorbitan mono, 1 drop, Sigma-Aldrich) and methylcellulose 0.6% (Sigma-Aldrich, Saint Quentin Fallavier, France) in distilled water and was administered intraperitoneally (i.p.) (5 mL/kg body weight) after a control period of 60 minutes.

Determination of extracellular ACh. ACh levels were measured in 20-min dialysate samples (40 μL) by an high pressure liquid chromatography system (HPLC) and electrochemical detection using a similar technique previously described by Damsma et al.⁴². Briefly, 40 μL of samples were injected through a refrigerated Microsampler (CMA-200; Carnegie Medicine AB, Stockholm, Sweden) onto a reverse-phase C18 column (Kromasil 5 μm , 50 \times 2 mm) preloaded with sodium laurylsulphate (Sigma-Aldrich, Saint Quentin Fallavier, France). The mobile phase consists of a 0.15 M potassium phosphate buffer (pH = 8.0) containing 600 mg/L of tetramethylammonium chloride (Merck, Nogent sur Marne, France) and was delivered by a dual-piston HPLC pump Waters 515 (Waters Associates, CA, USA) at a flow rate of 0.5 mL/min. ACh was converted into hydrogen peroxide and betaine in a post-column enzyme-reactor (BAS.MF-6151). Hydrogen peroxide resulting from the enzymatic reaction was then detected using a platinum electrode (Antec, Leiden, The Netherlands) set at + 500 mV versus an Ag/AgCl reference electrode. The chromatographic column, the enzyme reactor and the electrochemical cell were maintained at 30°C. The detection limit of the assay was approximately 3 pg/40 μL . Extracellular ACh levels (pg/40 μL) were expressed by comparing the area of the ACh peak measured in the dialysate with that detected in a standard solution. The area corresponds to the oxidation current of ACh and is proportional to the concentration of the neurotransmitter in the dialysate sample.

Statistical analysis and data presentation. ACh levels in samples were converted to a percentage of the mean value of the 3 fractions before treatment (fractions -40, -20 and 0 minutes). The effect of SAR127303 in brain regions was presented by comparing to baseline variation during the post-treatment period (fractions 20 to 140 minutes). The stability of the 3 fractions used to define the 100% basal period was checked by performing a two-way ANOVA with repeated measures on basal ACh levels according to "Treatment" and "Time" (repeated factor) at -40, -20 and 0 minutes. For the effect of SAR127303, a two-way ANOVA with repeated measures was carried out on transformed percent ACh levels from 20 to 140 minutes according to "Treatment" and "Time" (repeated factor). In case of significance of the "Treatment" and "Time" interaction, a Winer analysis is performed to study the global effect of factor "Treatment" for each level of "Time" factor. Then, if the "Treatment" global effect is significant at a given level of "Time", a Dunnett's test is performed to compare the treatment versus vehicle treated group at this (these) time(s).

The novel object recognition task in mice. The test apparatus was based on that described by Ennaceur and Delacour⁴³ in rats and adapted for use in mice⁴⁴. The apparatus consisted of a uniformly lit (20 lx) PVC enclosure (52 L \times 52 W \times 40 H cm) with a video camera positioned 160 cm above the bench. The observer was located in an adjacent room fitted with a video monitoring system. The experiment consisted of 3 sessions. During a first session, male Swiss mice (25–30 g; Janvier Labs, Le Genest Saint Isle, France) were allowed to become familiar with the experimental environment for 5 min (S1). Time spent active (animal moving around with or without sniffing and exploration) was measured. 24 hours later, the animals were again placed in the enclosure in the presence of two identical objects for the amount of time necessary to spend 15 s exploring these two objects to a limit of 5 min (exploration was defined as the animal having its head within 2 cm of the object while looking at, sniffing, or touching it) (S2). After a forgetting interval of 60 min, mice were placed again in the enclosure with a previously presented familiar object and a novel object for a period of 4 min (S3). Time spent exploring the familiar and novel objects was recorded. For a short-term forgetting delay, during the recall session, normal mice spent more time exploring the novel object compared to the familiar one. That reflects a remembering of the familiar object. Short-term memory was impaired by MK-801 injection, mice spending the same amount of time exploring both object, reflecting a forgetting of the familiar object and a short-term visual memory deficit. Experiment 1 (dose-response): SAR127303 was administered orally immediately following S2. Experiment 2 (repeated): SAR127303 was administered orally twice-a-day for 4 days. The last administration was performed on day 5, immediately after S2. Experiment 2 (antagonism by rimonabant): SAR127303 and rimonabant were administered orally or i.p., respectively, immediately following S2. Data were expressed as ratio of novelty index: [(time exploring novel object)/(time exploring novel object + time exploring familiar object) \times 100]. Data (time exploring each of the 2 objects, in seconds) were analyzed for habituation and acquisition sessions, using a one-way ANOVA assessed on the variables "locomotion" and

"time" necessary to reach 20-second active exploration for 2 identical objects, respectively. For the recall test sessions, the novelty index ratio (expressed in percentage) was analyzed with a one-way ANOVA followed, when appropriate, by a Dunnett's (Experiments 1) or Newman-Keuls (Experiments 2 and 3) analysis for individual comparisons. These analyzes, and all subsequent ones were performed using the SAS software (SAS Institute Inc., Cary, NC, USA).

The Y-maze test in mice. The Y-maze consisted of 3 arms in gray PVC in the shape of a Y. Arms were 28 cm long, 6 cm wide with walls 15 cm high. Movement was tracked manually using homemade software by an experimenter located in an adjacent room via a camera mounted directly above the maze. The animal was placed in an arm facing the center (Arm A) for 5 min. A correct alternation occurred when the animal moved to the other 2 arms without retracing its steps (i.e. Arm A to B to C). Movements such as ABA were incorrect. Based on the movement over the entire session, the percentage of correct alternations was calculated (i.e. Total number of alternation \times 100)/(Total number of arm entries-2). SAR127303 was administered orally 60 min and PCP was given i.p. 30 min prior to testing. Thirty minutes later, NMRI mice (16–18 g, Janvier Labs, Le Genest Saint Isle, France) were placed in the apparatus and recording was started. Statistics performed on total arm entries and percentage alternation consisted of using a one-way ANOVA, followed by a post-hoc Dunnett's test for individual comparisons.

The Morris water maze in rats. The Morris water maze apparatus consisted of a PVC pool (1.20 m diameter \times 0.60 m high), filled with thermostated water (23 \pm 2°C) to a depth of 35 cm, with the addition of milk to render the water opalescent. A Plexiglas escape platform (12 cm diameter) was placed into the pool, 1 cm below the water surface and 10 cm from the wall. The test room contained several permanent extra-maze cues such as posters, flag, etc. on walls. A video-tracking camera (placed 200 cm above the center of the pool surface) monitored the trajectory of the rat [male Wistar Han strain, 225–250 g (Janvier Labs, Le Genest Saint Isle, France) at the start of the study] and the video signal was transmitted to a computer in an adjacent room and analyzed using the VIDEOTRACK® system (View Point Ltd, Champagne au Mont d'Or, France). The platform was placed at one of four possible cardinal locations NW, SE, NE, and SW, and NW for learning session 1, 2, 3, 4, and 5, respectively. Each learning session consisted of three trials, with a maximal duration of 120 s and an intertrial interval of 30 s. Latency times (in seconds) to find the hidden platform were recorded during each trial of each learning session. If the rat located the platform within the maximum time allowed (120 s), it was left on the platform for 30 s. If the rat did not locate the platform within the time limit, it was gently placed on it for a 30-s period. At the start of each trial of each day, the rat was gently placed at the periphery of the maze, opposite of the platform (ie for the first trial E, for the NW quadrant). For each subsequent learning session, a similar cardinal rule was applied (i.e. N, W, N, and E for the SE, NE and SW, and NW quadrant, respectively). All rats received administrations of SAR127303 (p.o.) or vehicle, 60 min before the first trial of each day. Data (latency times to reach the platform for each individual trial during the 3-day learning sessions were analyzed with a two-way ANOVA, with the treatment as the between factor, and the trials for each learning session as the within factor. Post-hoc analysis was performed with Newman-Keuls post-hoc tests. The data on the first trial were compared using an ANOVA to ensure that the groups do not display different performance levels at start.

Effects of SAR127303 in models of seizures. *The 6-Hz electroshock-induced seizures in mice.* SAR127303 (30 mg/kg) and the reference anticonvulsant agent levetiracetam (20 mg/kg) were administered orally or i.p. to OF1 mice (12–14 g) 60 or 30 min prior to test, respectively. An electrical stimulus (6 Hz, 0.2 ms rectangular pulse, 3 s duration, 32 mA) was applied to both corneas. Animals were closely observed and rated for seizure behavior according to a modified Racine scale⁴⁵. Kruskal-Wallis multiple comparisons test was used to assess potential differences between treatment groups.

The pentylenetetrazol (PTZ) seizure threshold test in mice. The seizure threshold was determined by the infusion of 8 mg/mL of PTZ at a rate of 0.7 mL/min via a flexible plastic catheter into the tail vein of freely moving OF1 mice (22–24 g) using an infusion pump. The convulsant dose for each endpoint was calculated in mg/kg PTZ. SAR127303 (30 mg/kg) and the reference benzodiazepine diazepam (3 mg/kg) were administered orally or i.p., 60 or 30 min prior to the beginning of PTZ infusion, respectively. One-way ANOVA was used to assess overall effect of treatment, and Dunnett's post test was used to compare differences between treatment groups.

The corneal kindling model in mice. C57BL/6J mice (22–24 g) received twice-daily subconvulsive corneal stimulations of 8 mA/0.8 ms at 60 Hz for 2 s for 11 days. Following the acquisition of kindling at day 5, mice were allowed a two-day stimulation free period. Stimulations were at least 90 min apart. Mice were considered kindled when displaying five consecutive stage five seizures according to a modified Racine scale. Mice were divided into the following groups: (1) Control: they were treated with saline from day 1 to day 5, and from day 8 to day 11; (2) Acute SAR127303: mice were treated with saline from day 1 to day 5, and from day 8 to day 10, and on day 11 they received a single oral administration of 30 mg/kg SAR127303; (3) Repeated SAR127303: these mice were administered 10 or 30 mg/kg SAR127303 orally once-a-day from day 1 to day 5, and from day 8 to day 10. On treatment days, animals were administered saline or SAR127303 after the second stimulation, except on day 11, where the administrations were performed between the two stimulations. Two-way ANOVA (treatment \times time) with repeated measures on



factor time was used to assess overall effect of treatment, and Winer post test was used to compare differences between treatment groups.

The kainic acid-induced seizures test in mice. SAR127303 (30 mg/kg) was administered orally to C57BL/6 mice (22–24 g) 30 min prior to the i.p. injection of 30 mg/kg kainic acid. Thirty min later, animals were closely observed and rated for seizure behavior according to a modified Racine scale⁴⁵. Kruskal-Wallis multiple comparisons test was used to assess potential differences between treatment groups.

Results

In vitro selectivity profile. SAR127303 behaves as a highly selective inhibitor of mouse and human MAGL ($IC_{50} = 3.8$ and 29 nM, respectively). With the exception of ABHD6, the drug does not modify the activities of other human serine hydrolases (e.g. FAAH, DPP7, APEH) (Figure S1) nor interact with a variety of potential targets, including 170 kinases, ion channels, neurotransmitter transporters, and receptors, including CB1 and CB2.

Activity of SAR127303 in MAGL biochemical assay. SAR127303 potently inhibited rhMAGL ($IC_{50} = 48$ nM). Published MAGL tool compound JZL184 has lower potency ($IC_{50} = 0.48$ μ M), while FAAH inhibitor SSR411298 did not show any activity in this assay ($IC_{50} > 10$ μ M) (Figure 2).

LC-MS evidence of covalent modification of MAGL Ser122 by SAR127303. Mass spectrometry based mapping identified Ser 122 as the covalent binding site for SAR127303 (data not shown). Subsequent treatment of recombinant human MAGL with different concentrations of SAR127303 resulted in dose-dependent modification of MAGL protein, as indicated by the decrease in unmodified MAGL peptides containing Ser 122. The abundance of the control peptide derived from MAGL C-terminus was not affected (Figure 3).

Protein crystal structure. MAGL inhibitor JZL184 has a chemical structure which is close to that of SAR629 described elsewhere³⁹. They both adopt a Y-shaped structure and their binding modes to human MAGL are similar and were described either by X-ray crystallography³⁹ or molecular modelling³⁰. The chemical structure of SAR127303, described here, does not possess a Y-shape and its binding mode, revealed by X-ray crystallography, shows that it can adopt two slightly different conformations. Indeed, as for the MAGL-SAR629 structure, the complex MAGL-SAR127303 crystallizes with two molecules in the asymmetric unit. Both molecules, although globally similar, adopt some structural differences as depicted in figure 4A. These differences mainly concern the position of the α -helix A4 of the LID and its surrounding loops.

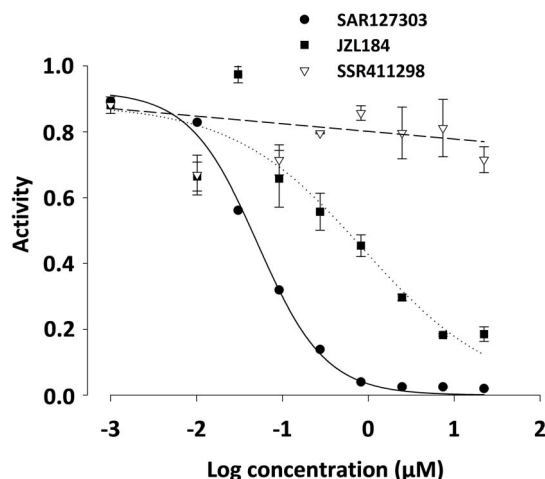


Figure 2 | Activity of SAR127303 in MAGL biochemical assay. Data are presented as mean \pm S.D.; N=3. SAR127303 and JZL184 are MAGL inhibitors; SSR411298 is a FAAH inhibitor.

A.

MPEESSRRTTQPSIPYQDLPHLVNADGQYLFCRYWKPTGTPKALIFV
SHGAGEHSGRYEELARMLMGLDLLVFAHDHVGHGQSEGERMVVS
DFHVFVRDVLQHVDSMQKDYPLPVFLLGHSMMGGAIILTAERP
GHFAGMVLISPLVLANPESATTFKVLAAKVLNLVLPNLSLGPIDSSVLS
RNKTEVDIYNSDPLICRAGLKVCFGIQLLNAVSRVERALPKLTVPFLLL
QGSADRLLCDSKGAYLLMELAKSQDKTLKIYEGAYHVLHKELPEVTNS
VFHEINMWVSQRTATAGTASPP

B.

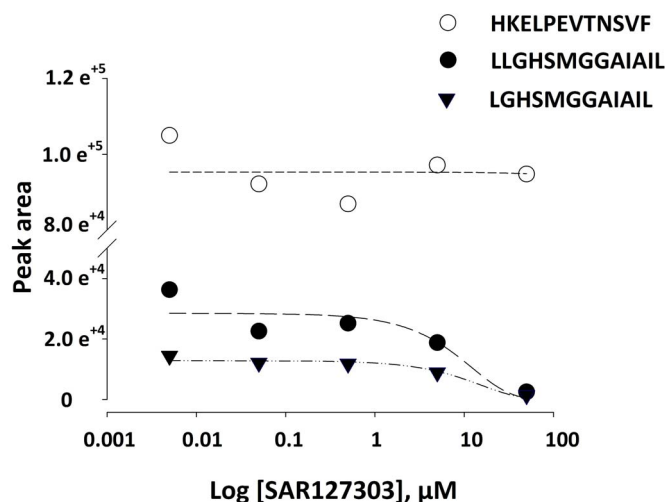


Figure 3 | LC-MS evidence of covalent modification of MAGL Ser122 by SAR127303. (A) MAGL protein sequence. Peptide containing Ser122 and control peptide sequences are underlined. (B) Dose-dependent decrease in unmodified Ser122-containing peptides in the presence of SAR127303.

One of these loops leads to different interactions with SAR127303 in molecule A and B in the crystal.

SAR127303, in return also depicts a slightly different orientation of its sulfonyl moiety. This leads to some differences in the protein-ligand interactions between molecule A and B (Figure 4B).

Aside from the covalent bond with Ser132, SAR127303 interacts with MAGL in the following way: the carbonyl oxygen of the carbamate function is H-bonded to main-chain nitrogen atoms of Ala61 and Met133, the hexafluoro-propanol moiety being removed during catalysis. The protein interaction with the piperidine moiety occurred through van der Waals interactions on one side with Leu251 but makes no contact on the other side. One oxygen atom of the sulfonyl function interacts in both molecules of the asymmetric unit with Asn162 main-chain nitrogen. In molecule A, this sulfonyl oxygen further makes water-mediated interactions with Asn162 side-chain nitrogen and Leu251 main-chain carbonyl oxygen. In molecule B, a water molecule is found H-bonded to the nitrogen atom of the main scaffold, but seems too distant to any other atom of the protein. The chloro-phenyl moiety of SAR127303 fits in a pocket lined by Leu215, Gly220, Leu223 and Leu224 residues in one side and Ser165, Ala166 in the other side. Gly220 main-chain and, only in molecule A, Ser165 side-chain oxygen atoms are the only polar atoms at a short distance of the chloro-phenyl moiety. The two different binding modes observed here can be explained by the fact that the loop to which belongs Asn162 is very flexible and the residues interacting with the chloro-phenyl moiety of SAR127303 (Ser165, Ala166) are not localized equivalently in both molecules of the asymmetric unit, resulting in different binding modes for the inhibitor.

It is important to note that several constructs have been tested to maximize success in expression rate and subsequent purification and crystallization. The best construct in terms of crystallizability and

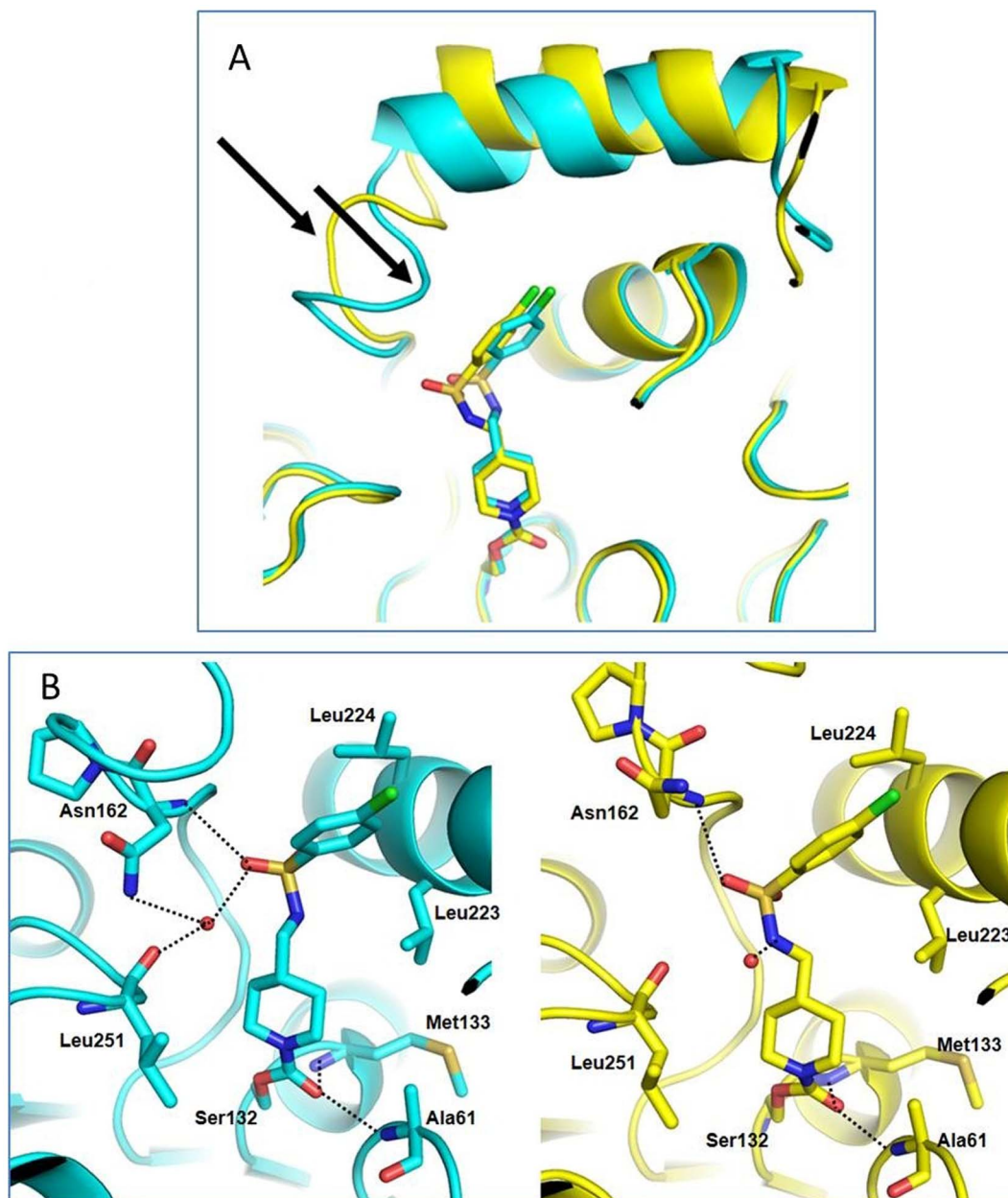


Figure 4 | A. Superimposition of SAR127303 active site in both molecules of the asymmetric unit (molecule A in cyan, molecule B in yellow). The flexible loops, interacting with the chlorophenyl and sulfonyl moieties of SAR127303 are pointed by arrows. B. MAGL-SAR127303 interactions in molecule A (cyan) and B (yellow). Hydrogen bonds are depicted as black dashes.

expression was His-1-1313. The purified isoform of human MAGL (Q6IBG9_HUMAN) has 313 aminoacids in comparison to 303 for the isoform described previously (Q99685_HUMAN), which explains the numbering difference (Ser122 in the LC-MS experiment vs Ser132 here).

Brain penetration and exposure. Single oral administration of SAR127303 at 10 and 30 mg/kg resulted in near dose-proportional increase in compound concentration in mouse plasma and brain. Plasma and brain C_{max} were observed at 4 hours after SAR127303 administration (Figure S2 and Table S2).

Functional activity. MAGL activity of brain homogenates prepared from mice treated with SAR127303. SAR127303 decreased MAGL activity in mouse brain in a dose-dependent fashion, an effect which reached statistical significance at 3 mg/kg p.o. [$F(5,36) = 42.09$, $P < 0.001$] (Figure 5A). Time-course analysis indicated that

enzyme activity was inhibited markedly for up to 6 hours after administration at the doses tested. Although at 24 hours post-administration, enzyme activity tended to return to baseline levels, it remained significantly inhibited [$F(4,30) = 188.26$, $P < 0.001$] (Figure 5B).

Brain lipid profiling. Treatment with SAR127303 (10 and 30 mg/kg p.o.) resulted in significant increase in major MAGL substrate in the brain, 2-arachidonoylglycerol [2-AG, MAG20:4: $F(4,27) = 2.70$, $P < 0.05$]. Basal concentrations of 2-AG at 4, 8 and 24 hours were 248, 218 and 209 $\mu\text{g/ml}$, respectively. Levels of other monoacylglycerols were not significantly altered, highlighting exquisite specificity of SAR127303 [MAG16:0: $F(4,27) = 0.19$, $P = 0.94$; MAG18:0: $F(4,27) = 1.95$, $P = 0.13$; MAG18:1: $F(4,27) = 0.43$, $P = 0.78$; MAG18:2: $F(4,27) = 0.68$, $P = 0.61$]. In the 10 mg/kg group, 2-AG levels declined to basal levels by 24 hours. In contrast, it remained elevated in 30 mg/kg-treated mice 24 hours after compound admin-

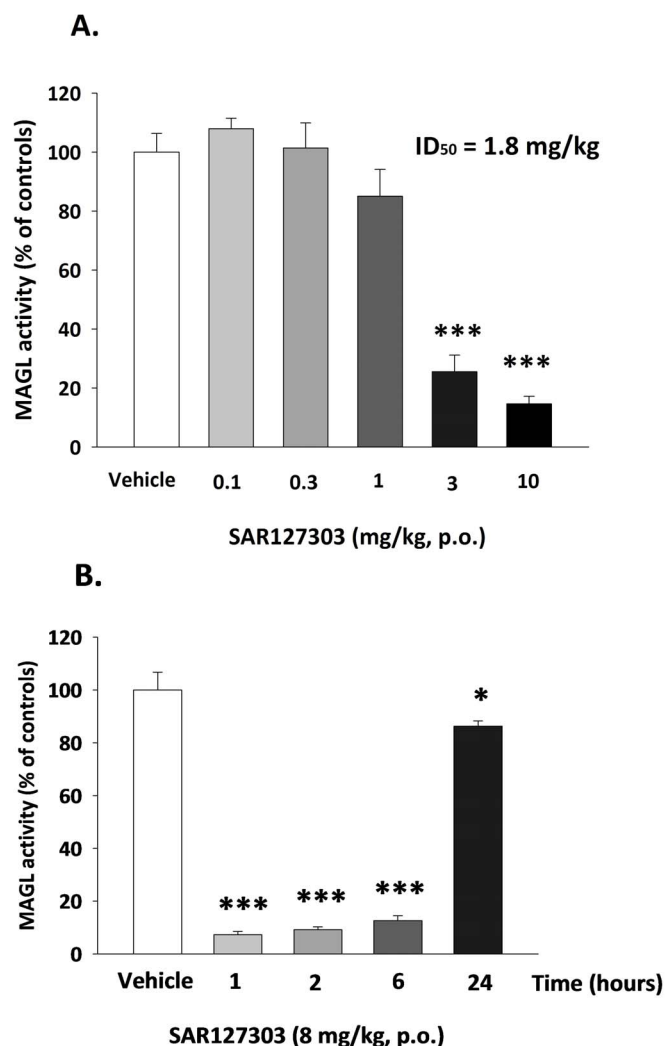


Figure 5 | MAGL activity of brain homogenates from mice treated with SAR127303. Data are presented as mean + S.E.M.; * $P < 0.05$, *** $P < 0.001$; $N = 7$ per group.

istration (data not shown). Figure 6 shows that at 10 mg/kg, SAR127303, produced a 4-fold increase in 2-AG as compared to control levels. This effects lasted at least for 8 hours. The highest dose of SAR127303 (i.e. 30 mg/kg) produced a 4- (at 4 and 24 hours) or 5- (at 8 hours) fold increase in 2-AG. None of the FFAs measured were modified significantly by SAR127303 up to 60 mg/kg p.o. (data not shown).

Levels of 2-AG, AEA, OEA and PEA in the hippocampus of mice treated with SAR127303. SAR127303 significantly increased hippocampal 2-AG levels at 8 mg/kg p.o. [$F(4,43) = 229.22$, $P < 0.001$], but failed to affect AEA, PEA and OEA levels in this structure when administered at 8 mg/kg p.o. (Figure 7). The effect on 2-AG reached statistical significance at 1, 2 and 6 h post-administration (Dunnett's test for all time-points: $P < 0.001$). Twenty-four hours after the administration of the drug, 2-AG levels returned to baseline ($P = 0.94$) (Figure 7).

Mouse tetrad experiments and elevated plus-maze. SAR127303 did not elicit antinociception in the tail-flick test, catalepsy, hypothermia, nor decrease locomotor activity at doses up to 30 mg/kg p.o. over the entire 24-h testing period (Figure S3). Moreover, free observation revealed no hyperreflexia as observed with previous MAGL inhibitors. This was in contrast to the CB1 receptor agonist WIN55,512-2 at 15 mg/kg i.p., which produced significant antino-

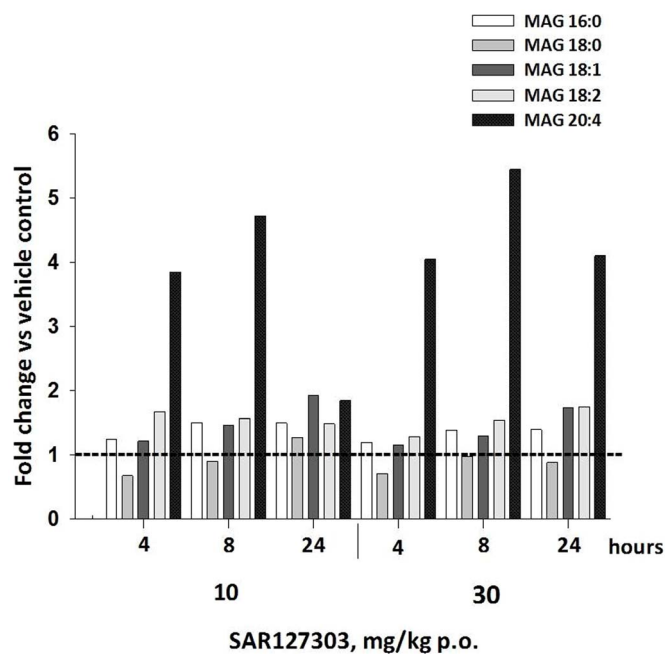


Figure 6 | Brain lipid profiling. Monoacylglycerol (MAG) abundance in SAR127303-treated samples was normalized to vehicle-treated samples harvested at the same time and expressed as fold induction. $N = 4$ mice per group. MAG 16:0, 1-hexadecanoyl-rac-glycerol; MAG 18:0, 1-monoacylglyceride; MAG 18:1, 1,1-(11Z-Octadecenoyl)-rac-glycerol; MAG 18:2, 1-(9Z,12Z-octadecadienoyl)-rac-glycerol; MAG 20:4, 2-arachidonylglycerol.

ciceptive [$F(25,205) = 8.50$, $P < 0.001$], hypothermic [$F(20,168) = 13.16$, $P < 0.001$] and cataleptic [$F(30,252) = 10.96$, $P < 0.001$] effects for at least 4 hours. Moreover, the drug decreased significantly locomotor activity [$F(5,60) = 12.28$, $P < 0.001$]. In the elevated plus-maze, SAR127303 at 10 mg/kg p.o. significantly decreased percentage of time in open arms, while the prototypical anxiolytic drug diazepam produced the opposite effects [$F(4,55) = 9.93$, $P < 0.001$] (Figure S4).

Effects of SAR127303 in models of pain. *The phenylbenzoquinone (PBQ)-induced writhing model of acute visceral pain-like behavior in mice.* In the dose-response experiment, Kruskal-Wallis analysis indicated a global main effect of treatment ($\chi^2 = 25.418$, $P < 0.001$). Further analysis showed that SAR127303 produced a significant reduction in writhing response at 1 and 3 mg/kg (Figure 8A). In the antagonism experiments, the CB1 receptor blocker rimonabant ($\chi^2 = 18.570$, $P < 0.001$), but not the CB2 antagonist, SR144528 ($\chi^2 = 29.829$, $P < 0.001$), blocked the anti-writhing effects of SAR127303 at 3 mg/kg p.o. (Figures 8B and 8C).

The formalin test of inflammatory pain-like behavior in mice. Results of the effects of SAR127303 on pain induced by formalin in mice are shown in Figure 9A and B. In the first experiment, acute treatment with the MAGL inhibitor caused a significant diminution of the late phase [inflammatory phase: AUC (15–45 min): Two-sided Wilcoxon test for factor treatment: $S = 100$, $P < 0.001$], but not the early [nociceptive phase: AUC (0–15 min): $S = 71.5$, $P = 0.75$] phase induced by formalin. In the second experiment, where SAR127303 was administered orally for five consecutive days, a similar suppressive effect was also observed in the late phase [AUC (15–45 min): $S = 97.5$, $P < 0.001$], and again not in the acute phase [AUC (0–15 min): $S = 85$, $P = 0.08$].

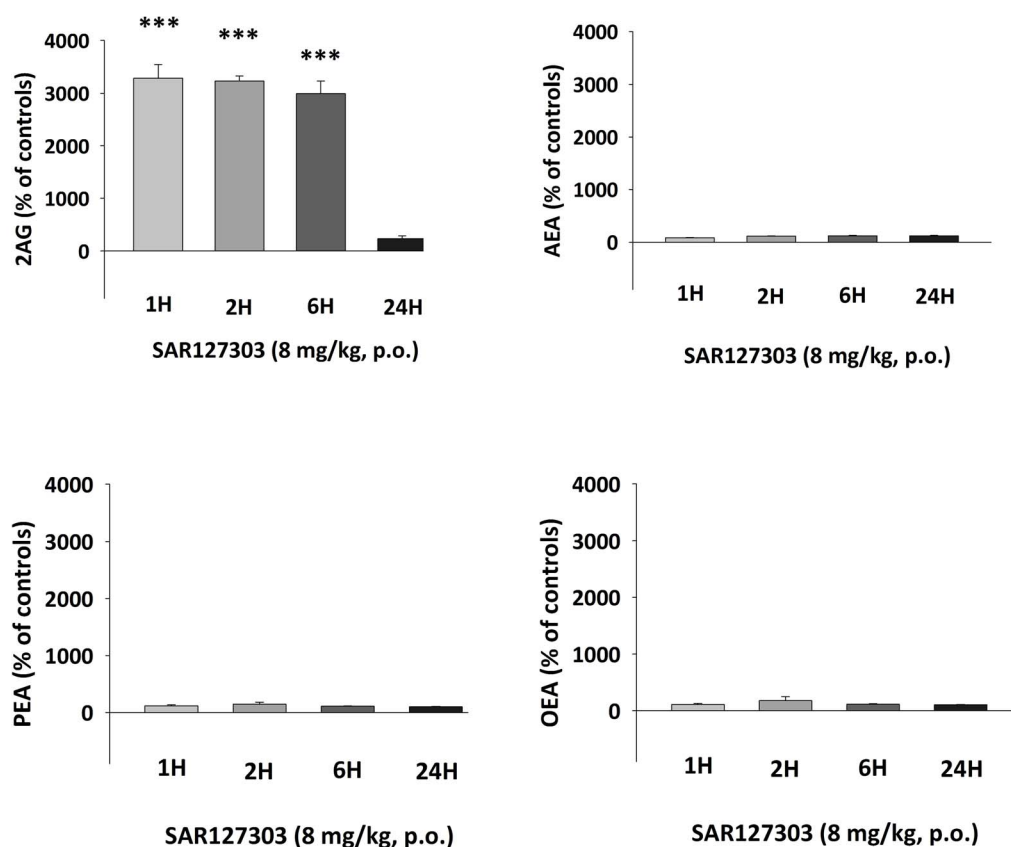


Figure 7 | Levels of 2-AG, AEA, PEA and OEA in the hippocampus of mice treated with 8 mg/kg SAR127303. Data are presented as mean \pm S.E.M.; *** $P < 0.001$ vs baseline level; $N = 3$ per group.

Effects of SAR127303 in models of cognitive processes

In vitro long term potentiation (LTP). In hippocampal slices from vehicle-treated rats, HFS induced a stable LTP, lasting more than 30 minutes. At 1 μM , SAR127303 was found to be completely inactive, while at 10 μM , a slight, albeit non-significant, decrease in LTP amplitude was observed. SAR127303 did not modify baseline fPS amplitude suggesting no effect on basal synaptic transmission (Figure S5).

Effect of 2-AG and SAR127303 on [^3H]ACh release evoked by electrical depolarisation in rat hippocampal slices. 2-AG (10^{-9} , 10^{-8} and 10^{-7} mol/L) (Figure 10A) and SAR127303 (10^{-7} and 10^{-6} mol/L) (Figure 10B) induced a dose-dependent inhibition on [^3H]-ACh release evoked by electrical depolarization in hippocampal slices (-7, -39% and -53% for 2-AG, and -12% and -36% for SAR127303). The effect of 2-AG at 10^{-8} mol/L was completely reversed by the CB1 receptor antagonist, rimonabant (10^{-7} mol/L) (Figure 10C).

Effect of SAR127303 on in vivo hippocampal ACh release in rats. The average level of basal extracellular ACh in the hippocampus (25.25 ± 3.763 pg/40 μL) was defined as the mean of 30 values corresponding to the 10 animals of the experiment and that each represents the mean of the 3 fractions (-40, -20 and 0 minute) before SAR127303 or its vehicle administration. In freely-moving rats, the i.p. administration of SAR127303 induced a global significant decrease of extracellular levels of ACh [$F(1,8) = 10.35$, $P = 0.0123$]. The onset of the decrease was observed immediately after the injection and is significant at 40, 60, 100 and 120 min compared to vehicle treated group (Figure 10D).

The novel object recognition task in mice

Dose-response. Under a protocol in which the two objects were presented one hour following exposure to the familiar object, vehicle-treated animals spent more time exploring the novel one

(17.0 ± 2.2 versus 8.4 ± 1.3 s). The relative time spent exploring the novel object for these animals was significantly above the chance level of fifty (i.e. 65.9 ± 5.4) (Student's t-test: $P = 0.014$) (Fig. 11A). This preferential investigation of the novel object was abolished by administration of 0.125 mg/kg of MK-801 and by SAR127303 at 1 and 10 mg/kg p.o. immediately after presentation of the familiar object. One-way ANOVA indicated a significant treatment effect on novelty index ratio [$F(4,55) = 3.64$, $P < 0.01$]. Further analysis with Dunnett's test showed that MK-801 and SAR127303 at 1 and 10 mg/kg significantly reduced the novelty index ratio as compared to control vehicle. Mice treated with MK-801 or SAR127303 spent the same amount of time investigating both objects.

Antagonism by rimonabant. The same protocol was used as in the dose-response experiment. Similar to this latter, control mice spent a greater amount of time investigating the novel object (Student's t-test: $P = 0.017$), while those treated with 1 mg/kg SAR127303 spent the same amount of time investigating both objects (Fig. 11B). However, when the drug was coadministered with the CB1 receptor antagonist rimonabant at 3 mg/kg, novelty index ratio was significantly different from chance level ($P = 0.026$). It must be noted that ANOVA did not show a significant global effect on novelty index ratio [$F(3,35) = 0.76$, $P = 0.52$].

Repeated treatment. Mice treated with vehicle for 5 days and submitted on day 5 to the same protocol as used in the dose-response experiment, spent again a greater amount of time investigating the novel object (Student's t-test: $P = 0.0003$) (Fig. 11C). Moreover, animals treated with vehicle for 4 days and which received a single administration of SAR127303 at 1 mg/kg on day 5 were unable to discriminate between both objects. Similarly, mice treated repeatedly with SAR127303 at 1 mg/kg p.o. for 5 days failed to discriminate

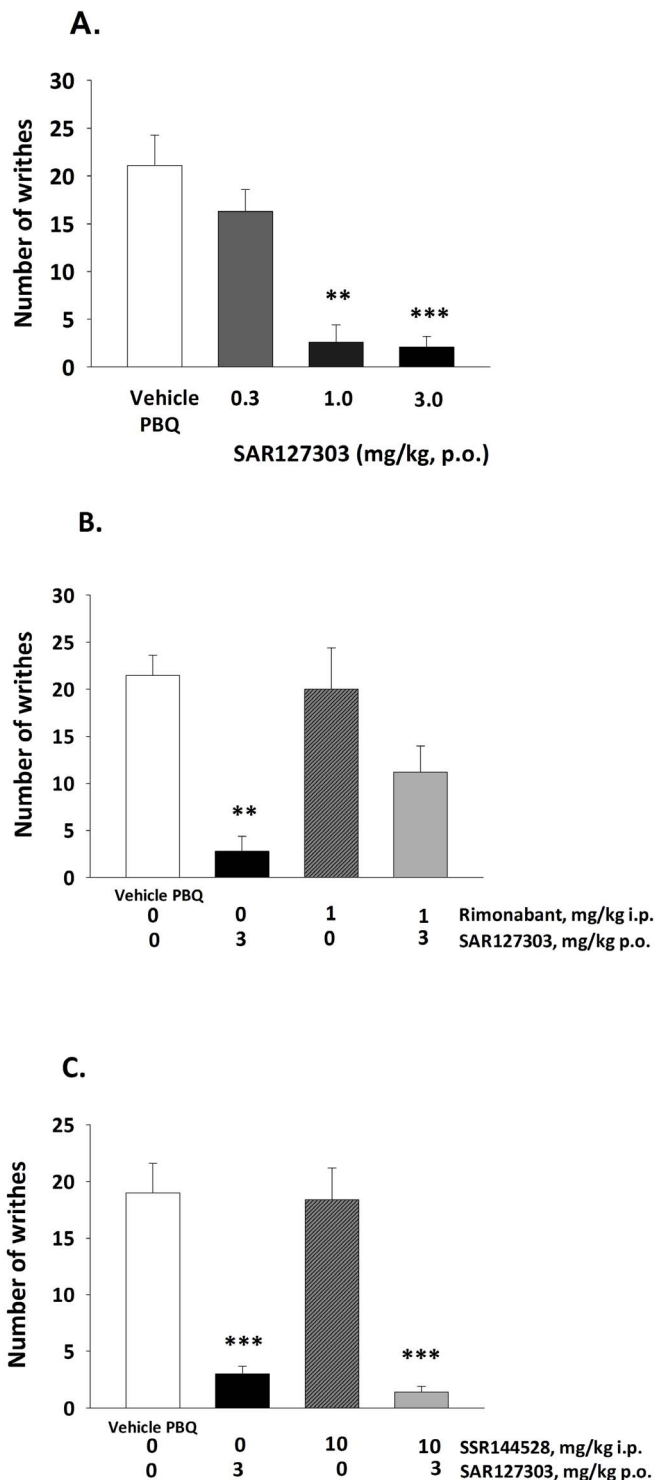


Figure 8 | Effects of SAR127303 alone (A) and in combination with the CB1 receptor antagonist, rimonabant (B), or the CB2 receptor antagonist, SSR144528 (C), against writhing induced by phenylbenzoquinone (PBQ) in mice. Each bar represents the average (+ S.E.M) writhes. Post-hoc analyses following Kruskal-Wallis test: ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated controls. $N = 10$ mice per group.

between the two objects. One-way ANOVA revealed a significant treatment effect on novelty index ratio [$F(2,24) = 3.80$, $P < 0.05$]. Post-hoc analysis using Newman-Keuls test indicated that SAR127303 when given acutely, but not repeatedly, at 1 mg/kg significantly reduced the novelty index ratio as compared to control

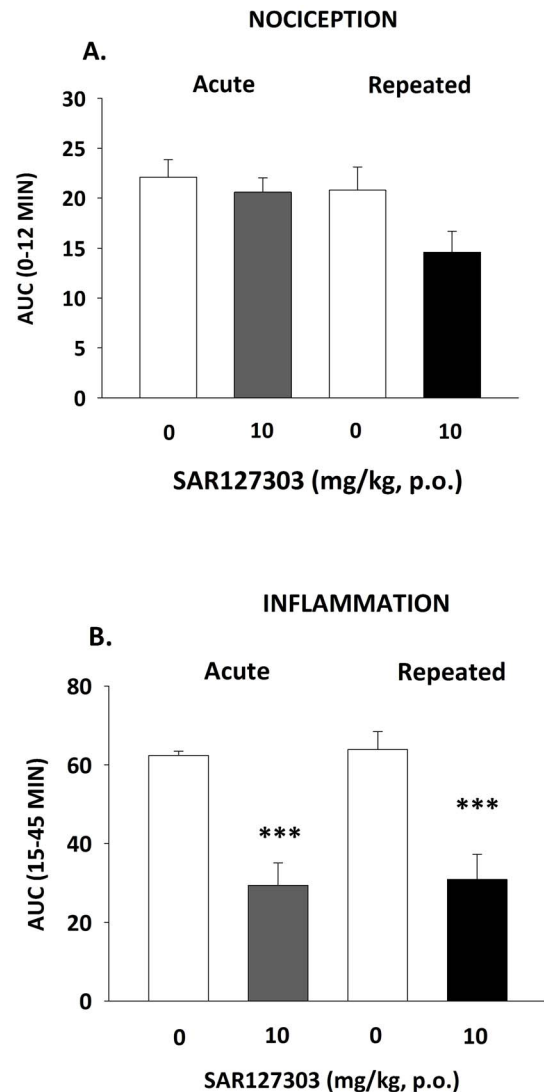


Figure 9 | Effects of acute and repeated (once-a-day for 5 consecutive days) dosing of SAR127303 on formalin-induced pain-like behavior in mice. Each bar represents the average (+ S.E.M.) area under the curve (AUC) of flinching behavior. Two-sided Wilcoxon test for factor treatment: *** $P < 0.001$ versus vehicle-treated controls. $N = 8$ mice per group.

vehicle ($P = 0.037$). However, it must be emphasized that in the repeated treatment group, statistical analysis just failed to reach significance ($P = 0.057$).

The Y-maze test in mice. ANOVA indicated a global significant effect of treatment [$F(4,49) = 6.22$, $P < 0.001$] in the dose-response experiment. Post-hoc Dunnett's *t*-test analysis showed that mice treated with the psychotomimetic PCP (1.5 mg/kg, i.p.) or with SAR127303 at 0.1, 1 and 10 mg/kg, p.o. displayed a significant reduction of spontaneous alternation compared to vehicle-treated mice (Fig. 12). No statistical difference was observed in total number of spontaneous alternation between groups in either experiment (data not shown).

The Morris water maze in rats. Comparison of the data on the first trial at day 1 did not reveal any statistical significance between groups, which would have compromised the interpretation of the learning curves. Two-way ANOVAs indicated a significant treatment effect for all three learning sessions [Day 1: $F(3,36) = 6.35$, $P < 0.01$; Day 2: $F(3,36) = 4.51$, $P < 0.01$; Day 3: $F(3,36) = 5.17$, $P <$

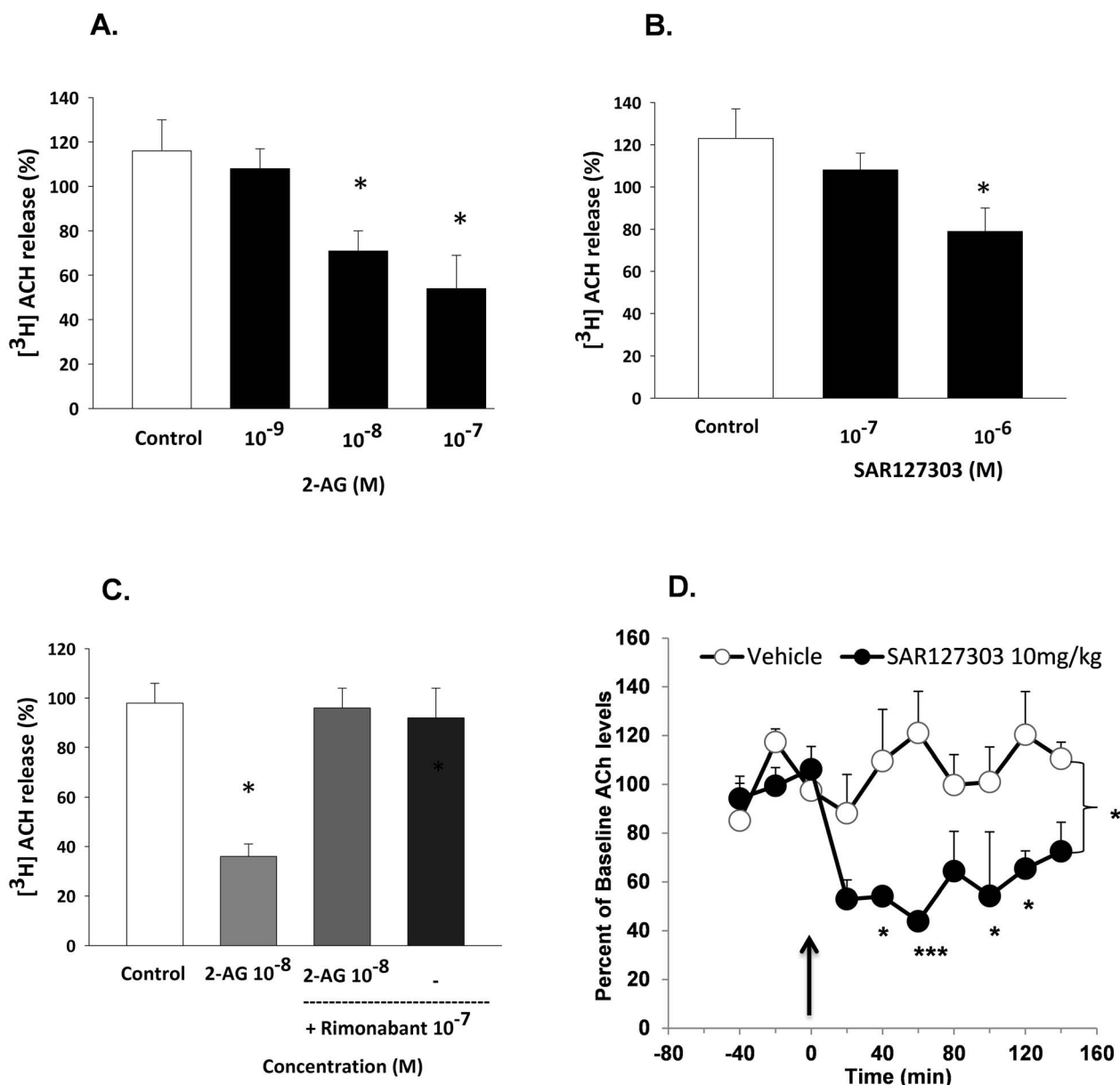


Figure 10 | Effects of 2-AG (A) and SAR127303 (B) on [³H]ACh release evoked by electrical depolarisation in rat hippocampal slices. (C) Blockade by the CB1 receptor antagonist rimobantant of the effects of 2-AG on [³H]ACh release evoked by electrical depolarisation in rat hippocampal slices. Data are expressed as a percentage of basal [³H]-ACh release. * $P < 0.05$ vs control. $N = 3$ slices/group. (D) Effect of SAR127303 on hippocampal ACh release measured by microdialysis in the ventral hippocampus of freely-moving rats. Data are expressed as percent of basal period and are Mean \pm S.E.M. of data. * $P < 0.05$, *** $P < 0.001$. Dunnett's test versus "Vehicle" treated group from 20 to 140 minutes for each level of "Time" with significant global effect of factor "Treatment". $N = 5$ rats/group.

0.01] and a significant inter-trial effects for sessions one [$F(2,72) = 10,65$, $P < 0.0001$] and two [$F(2,72) = 10,20$, $P < 0.001$]. Post-hoc analysis showed that vehicle-treated animals and those that received SAR127303 at 0.1 mg/kg p.o. displayed improved performance at sessions one and two as their latency to reach the platform was decreased significantly over trials (Figure 13). This was in contrast to rats treated with SAR127303 at 10 mg/kg, which failed to improve significantly their performance during the three learning sessions. Similarly, animals from the 1 mg/kg group did not improve significantly their performance at day 1 and 3. However, a significant decrease in latency was observed the second day for trial three. In addition inter-group comparisons showed a significant difference between vehicle-treated animals and rats treated with SAR127303 at 10 mg/kg on day 1 (trials 2 and 3), day 2 (trial 3) and on day 3 (trial 3), and at 1 mg/kg at day 1 (trial 2) (Figure 13).

Effects of SAR127303 in models of seizures. *The 6-Hz electroshock-induced seizures, pentylenetetrazol (PTZ) seizure threshold and kainic acid-induced seizures tests in mice.* SAR127303 was devoid of any protective activity against seizures induced both by 6-Hz stimulation and by the convulsants PTZ and kainic acid (Table S4, supplementary section). This was in contrast to the reference anticonvulsant, levetiracetam ($\chi^2 = 9.7$, $P < 0.01$), and the benzodiazepine, diazepam [$F(2,39) = 59,05$, $P < 0.001$], which afforded full protection in the 6-Hz and PTZ tests, respectively.

The corneal kindling model in mice. Daily treatment with SAR127303 30 mg/kg p.o. delayed the acquisition while decreasing the expression of kindled seizures [$F(16,920) = 5.56$, $P < 0.001$] (Fig. 14A). As compared to the vehicle control group mean seizure severity proved to be significantly lower at all stimulation sessions from day 3. The

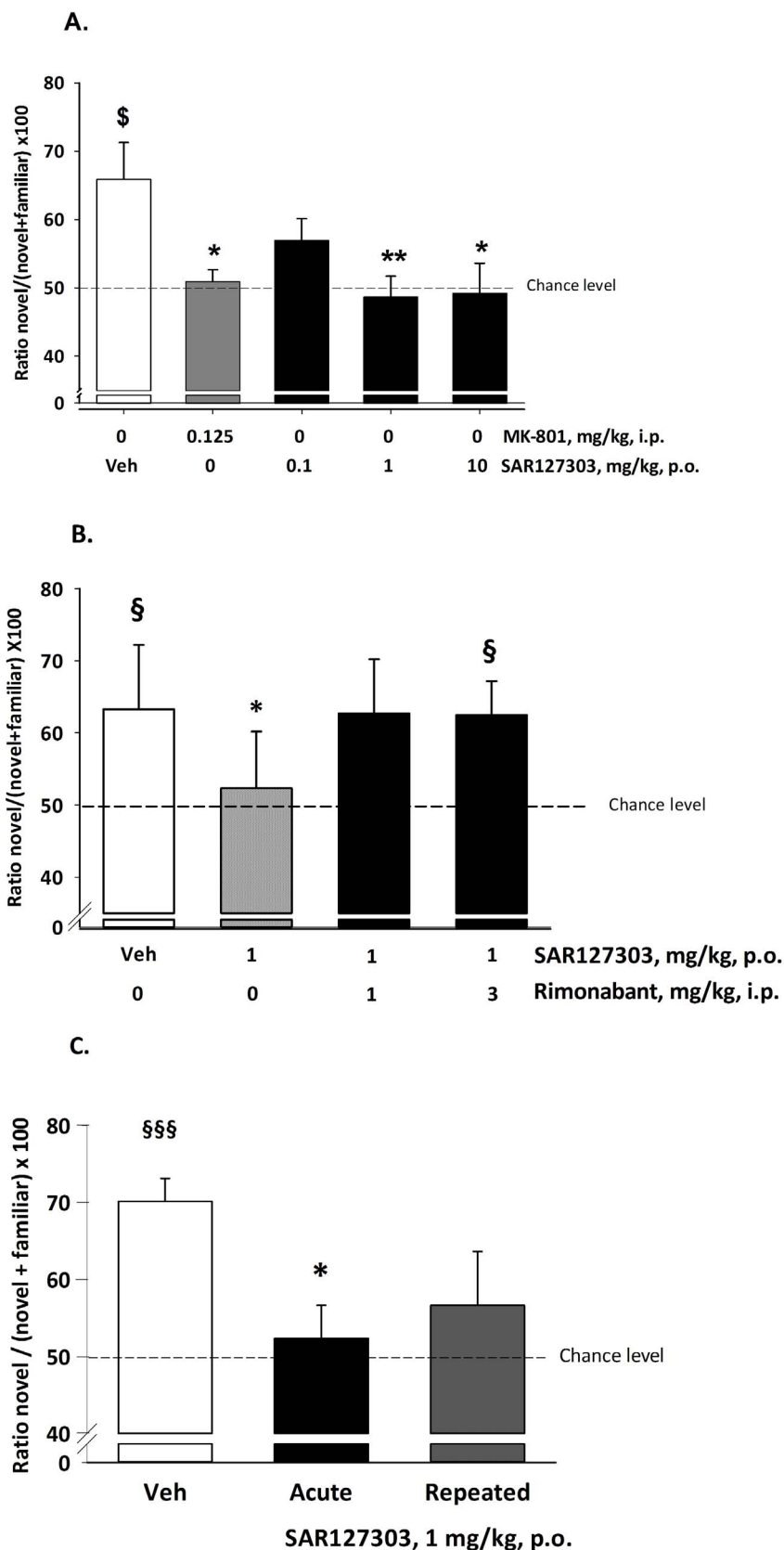


Figure 11 | Effects of oral administration of SAR127303 on short-term visual episodic memory in mice using the novel object recognition test. (A) Dose-response versus the NMDA receptor antagonist MK-801; (B) Antagonism of the effects of SAR127303 by the CB1 receptor antagonist, rimonabant; (C) Comparison of the effects of acute and repeated (5 days, twice-a-day) treatment of SAR127303. Each bar represents the average (+ S.E.M) novelty index ratio. Student's t-test: § $P < 0.05$ versus chance level of 50; Post-hoc analyses following one-way ANOVA: * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated controls. $N = 9$ to 13 mice per group.

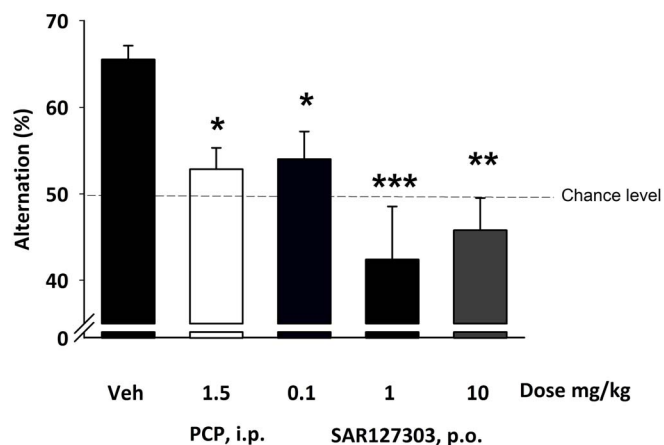


Figure 12 | Effects of oral administration of SAR127303 on spatial working memory in mice using the Y-maze test. (A) Dose-response versus the non-competitive NMDA receptor antagonist, PCP; (B) Antagonism of the effects of SAR127303 by the CB1 receptor antagonist, rimonabant. Bars represent number of arm entries (mean + S.E.M.). Post-hoc analyses following one-way ANOVA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated controls. $N = 10$ to 12 mice per group.

lowest dosage of SAR127303 used in the present study (10 mg/kg p.o.) produced no significant effect on kindling acquisition. When fully-kindled vehicle-treated mice were treated with 30 mg/kg p.o. SAR127303 on day 11 prior to stimulation, seizure severity proved to be significantly lower as compared to control animals. This effect lasted at least up to 24 h post-dosing [$F(2,36) = 6.49$; $P < 0.01$] (Figure 14B).

Discussion

Enhancing eCB signaling has been considered as a potential strategy to treat several diseases, including affective and neurodegenerative disorders, cancer, gastrointestinal inflammation and pain¹⁰. Preparations based on Δ^9 -tetrahydrocannabinol and its synthetic analogues, which activate directly CB receptors, are used by cancer patients undergoing chemotherapy to increase appetite and decrease nausea, but these compounds produce major central side effects, which seriously limits their use on a routinely basis. However, new generations of endocannabinoid-enhancing compounds that indirectly enhance the functionality of endocannabinoid receptors by inhibiting their catabolism have been discovered. These novel drugs are thought to be more selective and safer than direct agonists based on the assumption that they are active only at sites of on-going production of eCBs¹⁰. Here we describe SAR127303, a highly selective, brain penetrant and orally-active inhibitor of the 2-AG-degrading enzyme, MAGL.

Characterization of the mechanism of action of SAR127303. Our data show that SAR127303 has outstanding selectivity for MAGL in the brain, inhibiting one additional serine hydrolase, ABHD6⁴⁶. Animals treated with SAR127303 display marked and sustained inhibition of MAGL in the absence of effect on FAAH that correlated with ~30-fold elevations in endogenous 2-AG, but not AEA levels in the hippocampus. Comparable inhibitory effects have been observed with recombinant human MAGL. In this latter assay, SAR127303 was 10 times more potent than the reference MAGL inhibitor, JZL184. The increased levels of 2-AG following SAR127303 were maintained for a substantial period of time (≥ 6 hours), but tended to return to baseline by 24 h post-treatment, as did MAGL activity. These observations are consistent with the brain exposure of the inhibitor, which was no longer detected at this time point. To assess whether elevation in 2-AG produced behavioral effects which

are CB1-dependent, we tested SAR127303 in the "tetrad test" for cannabinoid behavior. Our findings show that SAR127303 was completely devoid of such effects up to 30 mg/kg, results which somewhat contrast with those of previous studies showing that MAGL inhibitors produce some cannabimimetic effects in these assays^{29,38,47}. Our protein crystallization data may provide some tentative explanation for these differences. The flexibility of SAR127303 might prevent a full structuration of MAGL, especially the amphipathic helix A4 described as the "anchoring" helix to the cell membrane. This could lead to different localisation of MAGL in brain tissues thus leading to different pharmacological effects.

Characterization of SAR127303 in models of inflammatory pain.

In the rodent skin, spinal cord or peripheral nerves eCB levels can increase following the administration of irritants or inflammatory stimuli (for reviews, see refs. 2,11,48). These modifications have been suggested to be a beneficial response to decrease proinflammatory mediators and re-establishing homeostasis. This idea is substantiated by numerous findings showing that pharmacological inhibition or genetic inactivation of endocannabinoid hydrolysis (targeting MAGL or FAAH) produces antinociceptive effects in a variety of preclinical assays related to inflammatory pain (for recent reviews, refs. 19,49–51). Moreover, both CB1 and CB2 agonists exert antinociceptive and anti-inflammatory actions in these rodent models. The current results are in line with these findings as they show that systemic administration of SAR127303 reduces pain-like behavior in an acute model of inflammatory pain (i.e. formalin) and one model of visceral pain sensation (i.e. phenyl-p-benzoquinone). In the formalin test in mice, these effects were maintained following repeated administration of a dose slightly higher than the lowest dose (i.e. 10 vs 8 mg/kg) producing near-complete inhibition (~90%) of MAGL in this species. This effect is somewhat unexpected considering previous reports describing the effects of MAGL inhibitors (i.e. JZL184 and KML29) in pain assays, which showed that prolonged and almost complete (~80%) inhibition of the enzyme is associated with tolerance to their antinociceptive effects as a probable results of impaired eCB-dependent synaptic plasticity and desensitized brain CB1 receptors^{32,37,38}. It is tempting to suggest that the lack of tolerance to the anti-pain effects of repeated administration of SAR127303 can be explained by its differential impact on synaptic plasticity and CB1 receptors as compared to existing compounds. SAR127303 inhibits both MAGL and ABHD6, a feature not shared by JZL184 and KML29^{29–31}. The α/β hydrolase ABHD6 has been demonstrated to be strategically positioned to regulate neuronal production of 2-AG that reaches pre-synaptic CB1 receptors, while 2-AG at post-synaptic CB1 receptors is thought to be under the control of MAGL^{46,52}. This redundancy has been suggested to provide the eCB system with a greater capacity to control the duration and magnitude of 2-AG signaling. It can be speculated that when both enzymes are simultaneously inhibited, this action may increase and/or prolong the efficacy of 2-AG at CB1 receptors, preventing for example the occurrence of tolerance to the effects of protracted 2-AG hydrolysis blockade. To check this hypothesis it would be interesting to assess the function of the CB1 receptor, using GTP γ S binding for example, to verify if no desensitization occurred under the current experimental conditions. Another possibility would be to use a selective MAGL inhibitor in combination with an ABHD6 inhibitor to determine if there is no functional tolerance following repeated administration. Finally, it would be worth testing the effects of repeated treatment of SAR127303 in other models of inflammatory pain, such as the carrageenan-induced mouse paw oedema test for example, to determine if the lack of tolerance to the anti-inflammatory action of the drug in the formalin test can be generalized to other experimental situations involving inflammatory response.

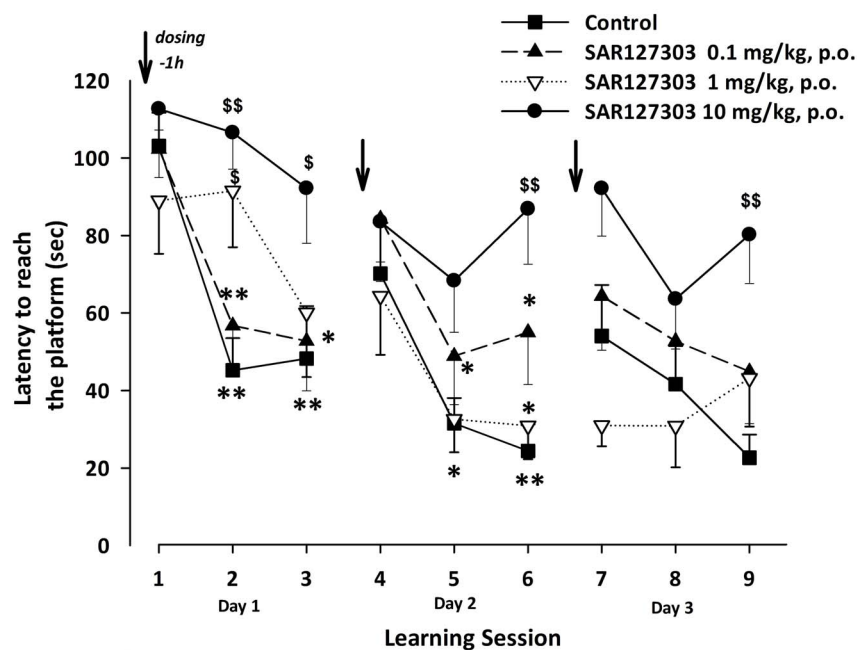


Figure 13 | Effects of oral administration of SAR127303 on spatial reference memory in rats using the Morris water maze. Each line represents the average (\pm S.E.M) latency to reach the platform across trial and learning sessions. Post-hoc analyses following a two-way ANOVA: * $P < 0.05$ and ** $P < 0.01$, vs Trial 1 on each day; \$ $P < 0.05$ and \$\$ $P < 0.01$ vs control-treated rats on each Trial. $N = 10$ rats per group.

Our data further demonstrate that the antinociceptive effects of SAR127303 in the visceral pain model are CB1-, but not CB2-dependent, as demonstrated by the finding that the CB1 antagonist rimonabant, but not the CB2 antagonist SSR144528, blocks this action. These findings agree with previous studies showing that the antinociceptive activity of MAGL inhibitors is mediated by a CB1 receptor mechanism¹⁹. However, the anti-inflammatory pain effects of SAR127303 certainly involve physiological systems and receptor types beyond the CB1 receptor as hypothesized recently by Alhouayek et al.⁵³ who proposed that inflammation involving 2-AG can be mediated by its metabolite arachidonic acid or oxidation of 2-AG by inflammation-induced enzymes such as cyclooxygenase-2 or lipoxygenases, depending the tissue or cellular subtype.

Characterization of SAR127303 in models of learning and memory.

Extensive evidence indicates that eCBs modulate cognitive processes throughout species (for a recent review, see ref. 54). There is general agreement that activation of the eCB system impairs memory acquisition and consolidation. For example, numerous studies have demonstrated that systemic administration of direct (i.e. the CB1 agonists Δ^9 -THC or WIN55,212-2) and indirect (i.e. the FAAH inhibitor, URB597) cannabinoid receptor agonists before or after learning impairs subsequent cognitive performance in a variety of memory assays, such as the MWM, contextual fear conditioning and object recognition in rodents. However, little is known about the cognitive effects of enhanced eCB signalling following selective manipulation of 2-AG. Although in a previous study JZL184 was found to impair MWM performance in mice, it did so only at a dose that also inhibits FAAH (i.e. 40 mg/kg)^{30,33,55}. Here we show that oral administration of SAR127303 over a wide dose-range dramatically impaired acquisition and consolidation in tasks addressing various forms of hippocampal-dependent memory, including spatial, episodic or working memory. Importantly, the memory disruptive effects of MAGL inhibition were blocked by rimonabant, indicating a CB1 receptor mechanism of action. Moreover, repeated administration of SAR127303 did not alter its amnesic action. Pharmacological blockade of MAGL, has been reported to result in dramatic

elevation in brain 2-AG levels⁵⁶, suggesting that selective increase in brain 2-AG levels is associated with memory disruption.

It is likely that at least parts of the cognitive action of SAR127303 is mediated through the activation of hippocampal cannabinoid receptors as a result of the elevation of 2-AG levels produced by the drug. CB1 receptors are highly expressed in hippocampal regions, including the dentate gyrus, CA1, and CA3^{57,58}. Moreover, LTP of CA1 synaptic transmission and hippocampal acetylcholine release, two hallmarks of memory function, were both decreased by SAR127303 in the current study. Interestingly, direct application of 2-AG on rat hippocampal slices inhibits the release of acetylcholine evoked by electrical stimulation in a CB1-dependent manner (current results) and LTP of hippocampal field EPSPs⁵⁹. While the role of other transmitters, such as glutamate or GABA, in the cognitive effects of SAR127303 cannot be excluded, our current data agree with the widely accepted notion that the learning and memory impairments produced by increased eCB signaling is the consequence of a decrease in the release of neurotransmitters below the levels necessary to trigger long-term synaptic modifications which are involved in memory formation⁶⁰.

Characterization of SAR127303 in models of epilepsy.

eCBs have been shown to be increased after the induction of seizures, and synthesized and released on demand to protect against excitotoxic damage through CB1 receptor activation⁶¹. For example, proconvulsant agents such as pilocarpine, kainate or picrotoxin elevate brain levels of AEA or 2-AG when administered to rodents⁶²⁻⁶⁴. These findings prompted speculation that pharmacological modulation of eCB tone might represent a strategy for treating epilepsy. In line with this idea, several reports have demonstrated that eCBs and drugs that increase eCB levels can indeed decrease excitatory vulnerability and seizure susceptibility⁶⁵⁻⁷⁰. These studies used natural and synthetic CB1 receptor agonists or FAAH inhibitors, but none has investigated the effects of selective inhibition of MAGL in animal models for seizures and epilepsy. Here we show that SAR127303 is inactive in the 6-Hz procedure and following the administration of the convulsant PTZ and kainic acid, procedures

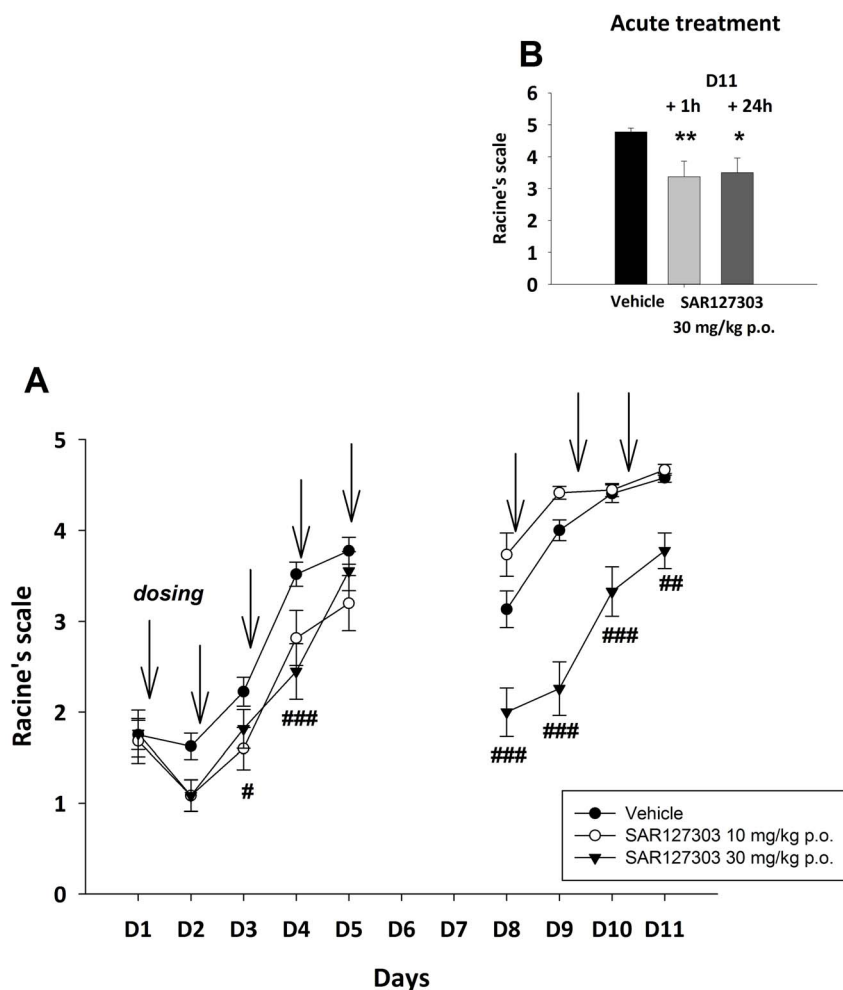


Figure 14 | (A). Effect of different dosages of once daily treatment of SAR127303 on acquisition of kindling, i.e. development of seizure severity upon repeated transcorneal stimulation. Treatment and kindling were interrupted over the weekend on days 6 and 7. (B). Effect of SAR127303 at 30 mg/kg in fully-kindled vehicle-treated mice 11 days after the beginning of kindling. Animals were tested either 60 min or 24 h after drug administration. Data are mean values \pm S.E.M shown for each stimulation session. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, * $P < 0.05$, ** $P < 0.01$ compared to vehicle group. $N = 26$ to 58 mice per group.

claimed to model certain aspects of pharmacoresistant and generalized tonic-clonic seizures in mice⁷¹. This is in contrast to the corneal kindling model of partial epilepsy in mice⁷², where two-week administration of SAR127303 not only delayed the acquisition of kindled seizures, but also decreased their expression. Moreover, in fully kindled animals, a single administration of the drug reduced seizure severity in a long lasting manner as the effect persisted when kindling was continued following wash-out. It is noteworthy that the minimal active dose in the kindling model (30 mg/kg) was higher than those in the pain and cognition models (~1-3 mg/kg), indicating that greater 2-AG hydrolysis inhibition is required to achieve efficacy against seizures. These data indicate that administration of the compound inhibits seizure initiation and protects against focal seizure activity. It can be hypothesized that SAR127303 causes interference with seizure progression by inhibiting propagation of activity from the focus, suggesting that it has the potential to retard the development of epileptogenesis. The mechanisms underlying the neuroprotective action of MAGL blockade remain to be determined. As indicated above, convulsant agents increase brain levels of 2-AG⁷³. It can be hypothesized as was suggested for AEA, that this effect occurs to protect neurons from the effects of altered discharge activity through modulation of calcium and potassium channels, and additional entails a wide range of prosurvival signal transducers via

CB1-dependent events^{61,68,74}. However, to determine if CB1 receptors were involved in the current effects of SAR127303 in the kindling model, additional experiments using CB1 and perhaps CB2 receptor antagonists are warranted. Moreover, the observation that the drug produced these effects at a relatively high dose compared to its effects in the pain and cognition models may indicate the involvement of additional mechanisms, which need to be investigated in future studies.

Conclusion. In conclusion, our study demonstrates that the novel selective and orally-active inhibitor of 2-AG hydrolysis, SAR127303 has anti-inflammatory effects, thus confirming therapeutic potential of MAGL inhibitors against inflammatory pain. Moreover, it provides the first evidence that blockade of 2-AG hydrolysis interferes with the epileptogenesis process and may represent a potential new avenue for the treatment of partial epilepsy. However, our data also reveal that SAR127303 impairs memory performance at a dose-level overlapping with that producing antinociceptive and antiepileptic effects, indicating that MAGL inhibitors are of limited utility as therapeutic agents. Nevertheless, it cannot be ruled out that these compounds may be potentially used to treat diseases associated with the inability to extinguish maladaptive behaviors, such as those for example seen in post-traumatic stress disorder. To verify this idea it would be interesting



in future experiments to assess potential effects of SAR127303 on aversive memory extinction, using for example contextual fear extinction. Moreover, MAGL inhibitors may prove useful in chronic conditions where collateral effects on memory performance may be acceptable (e.g., cancer pain). Finally, the inhibitory properties of SAR127303 of both MAGL and ABHD6, which may explain some of the differences between the current effects and those observed with previous selective MAGL inhibitors deserve further attention.

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Author contributions

G.G. wrote the main manuscript text; P.P., S.B. and T.B. prepared figures; A.J., A.R., B.Z., F.S., F.V., H.A., J.E., J.H., L.B., M.L.G., N.M., N.R., Q.Y., S.B., S.Br., T.B., T.H., T.L., T.T., V.M. carried out experiments, D.F., D.W., F.A., J.E., L.E., M.D., O.E.B., P.A., P.G., P.P., T.B., V.M., V.Mi. reviewed the manuscript.

Additional information

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