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ORIGINAL INVESTIGATION

AM2389, a high-affinity, in vivo potent CB_1-receptor-selective cannabinergic ligand as evidenced by drug discrimination in rats and hypothermia testing in mice

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Abstract
Rationale The endocannabinoid signaling system (ECS) has been targeted for developing novel therapeutics since ECS dysfunction has been implicated in various pathologies. Current focus is on chemical modifications of the hexahydrocannabinol (HHC) nabilone (Cesamet®).
Objective To characterize the novel, high-affinity cannabinoid receptor 1 (CB_1R) HHC-ligand AM2389 [9β-hydroxy-3-(1-hexyl-cyclobut-1-yl)-hexahydrocannabinol in two rodent pre-clinical assays.
Materials and methods CB_1R mediation of AM2389-induced hypothermia in mice was evaluated with AM251, a CB_1R-selective antagonist/inverse agonist. Additionally, two groups of rats discriminated the full cannabinergic aminoalkylindole AM5983 (0.18 and 0.56 mg/kg) from vehicle 20 min post-injection in a two-choice operant conditioning task motivated by 0.1% saccharin/water. Generalization/substitution tests were conducted with AM2389, AM5983, and Δ^9-tetrahydrocannabinol (Δ^9-THC).
Results Δ^9-THC (30 mg/kg)-induced hypothermia exhibited a faster onset and shorter duration of action compared with AM2389 (0.1 and 0.3 mg/kg). AM251 (3 and 10 mg/kg) attenuated/blocked hypothermia induced by 0.3 mg/kg AM2389. In drug discrimination, the order of potency was AM2389>AM5983>Δ^9-THC with ED_{50} values of 0.0025, 0.0571, and 0.2635 mg/kg, respectively, in the low-dose condition. The corresponding ED_{50} values in the high-dose condition were 0.0069, 0.1246, and 0.8438 mg/kg, respectively. Onset of the effects of AM2389 was slow with a protracted time-course; the functional, perceptual in vivo half-life was approximately 17 h.
Conclusions This potent cannabinergic HHC exhibited a slow onset of action with a protracted time-course. The AM2389 chemotype appears well suited for further drug development, and AM2389 currently is used to probe behavioral consequences of sustained ECS activation.

Keywords Cannabinoid · AM2389 · AM5983 · Δ^9-THC · AM251 · Hypothermia · Drug discrimination · Mice · Rats

Introduction

Drug discrimination is a behavioral technique similarly applicable for investigating drug effects in man and animals. For example, humans discriminating between orally ingested (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC; 25 mg) and vehicle capsules evinced orderly dose generalization gradients with approximate ED_{50} values of 8 mg (Lile et al. 2009) and 10 mg (Lile et al. 2010). Other psychotropic drugs (triazolam, hydromorphone, and methylphenidate) did not substitute, i.e., the study participants did not perceive the non-cannabinergics as producing an effect spectrum matching that of the reference compound Δ^9-THC. Such pharmacological specificity is a hallmark feature of drug discrimination (Järbe 1989). The hexahydrocannabinol (HHC) nabilone (Cesamet®), on the other hand, engendered a Δ^9-THC-like response (Lile et al. 2010).

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Nabilone was more potent (ED_{50} value≈2 mg) than Δ^9-THC, as would have been predicted from drug discrimination studies in rats where intraperitoneally (i.p.) injected nabilone was about three times more potent than Δ^9-THC (Browne and Weissman 1981; Weissman 1978). When combined in humans, the two cannabinergics resulted in additive effects (Lile et al. 2011), as would also have been predicted from previous drug discrimination studies in infrahuman subjects (Järbe 2011).

Early studies with the HHC scaffold suggested the possibility that cannabinergic-induced analgesia might be dissociable from other cannabinoid-receptor-induced "side" effects such as the "high" (Wilson et al. 1976). Although the dissociation between cannabinoid receptor 1 (CB_1R)-induced "high" and analgesia did not materialize, the planarity of the substituent at the C9 position of the terpenoid C ring turned out to be of considerable importance for CB_1R/ligand recognition/activation as demonstrated in a number of studies, including drug discrimination (Carney et al. 1979; Edery et al. 1984; Järbe et al. 1986; Reggio et al. 1991). Thus, pharmacological activity is favored where the protrusion of the C9 substituent is planar/equatorial (β) rather than axial (α). A similar stereochemistry pattern is evident also for the pharmacology of some minor Δ^9-THC metabolites (e.g., 8β,11 vs. 8α,11 di-OH-Δ^9-THC), although neither of the examined metabolites reached the potency of the parent compound Δ^9-THC or its major pharmacologically active metabolite (−)-11-hydroxy-Δ^9-THC, 11-OH-Δ^9-THC (Ford et al. 1984; Järbe and McMillan 1980). The discriminative stimulus effect of the naturally occurring minor isomer (−)-Δ^8-THC is approximately 1/2 to 1/3 as potent compared with Δ^9-THC (Balster and Prescott 1992).

Branching of the side chain of the THC and HHC templates can lead to ligands having a slow onset of action and a very protracted time-course, in addition to increased potency. The best-known example is HU210 [(−)-11-OH-Δ^8-THC-DMH], i.e., the C1'-dimethylheptyl homolog of 11-OH-Δ^8-THC which was 70 to 80 times more potent as a discriminative stimulus than the reference drug Δ^9-THC in rats and pigeons (Järbe et al. 1989). Also, the parent compound of HU210, i.e., (−)-Δ^8-THC-DMH, exhibited a slow onset and a long duration of action, especially in pigeons (Järbe et al. 1989, 1981). A HHC ligand designed with the above considerations in mind (i.e., C9 equatorial substitution and branched side chain) resulted in the CB_1R high-affinity ligand HU243 [(6a*R*,10a*R*)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[*c*]chromen-1-ol)], exhibiting a potency and time-course profile similar to that of HU210 in pigeons discriminating Δ^9-THC from vehicle (Devane et al. 1992).

Earlier work aimed at defining the pharmacophoric elements of the cannabinoid side chain led to novel Δ^8-THC analogs that bear cyclic moieties at the C1' position of the side chain (Papahatjis et al. 1998, 2002, 2003, 2007). Aforementioned studies resulted in Δ^8-THC analogs with high affinities for the two cloned cannabinoid receptors, CB_1R and CB_2R, and provided a starting point for developing more subtype-selective second-generation ligands. We investigated the effect of modifying the C3 side chain in HHCs with regard to affinity and selectivity for CB_1R and CB_2R (Nikas et al. 2010). A number of HHC analogs bearing cyclic moieties at the C1' position of the side chain pharmacophore were designed and synthesized. One of the most promising compound was the C1'–cyclobutane analog, AM2389, with remarkably high affinity in addition to displaying some selectivity (26-fold) for the CB_1R (K_i=0.16 nM) compared with the CB_2R (K_i=4.21 nM). In vivo, this ligand produced hypothermia and also displayed rimonabant (a CB_1R-selective antagonist/inverse agonist) sensitive tail-flick analgesia in rats, suggesting predominant CB_1R mediation (Nikas et al. 2010). The aim of the present studies was to extend some of these observations to mice, a species commonly used in cannabinoid research, and to drug discrimination in rats, an assay highly predictive of the human response. Given that hypothermia is not specific to cannabinergics, evaluation of CB_1R mediation for the AM2389-induced hypothermia in mice employed the selective CB_1R antagonist/inverse agonist AM251. Hypothermia represents one of the endpoints in the mouse tetrad screening assay for cannabinergics. For the drug discrimination assay, two training doses were used of the novel aminoalkylindole cannabinergic AM5983 in two groups of rats [systematic replication (Sidman 1960)]. AM5983 was previously found to be about eight times more potent than Δ^9-THC in rats, discriminating between vehicle and 3 mg/kg Δ^9-THC. Co-administration of AM5983 with rimonabant (1 mg/kg) produced an 11-fold right-ward shift of the generalization curve 30 min post-injection, suggestive of surmountable antagonism; the Δ^9-THC-like effects of AM5983 were waning 90 min post-administration (Järbe et al. 2011a). Like the phytocannabinoid Δ^9-THC, AM5983 significantly attenuated the stimulus effects of rimonabant in a discriminated drinking aversion task for rats, whereas the anandamide derived CB_1R-selective analog AM1346 was only marginally effective in this respect (Järbe et al. 2011b; see also Wiley et al. 2011). Given above considerations, it was predicted that the novel cannabinergic AM2389 would be potent and have a slow onset of action and a protracted time-course compared with similar in vivo actions of Δ^9-THC.

Materials and methods

Animals

Mice. C57BL/6 J mice (Charles River Breeding Laboratories, Wilmington, MA, USA) weighing 30 to 35 g were group housed five to a cage, in a temperature-controlled (20°C) animal facility. Mice were habituated to the animal facility for at least 1 week prior to experiments with a 12-h light/dark cycle (lights on at 7:00 AM). Mice were given free access to food and water. Experimentally naïve mice were used for each dose condition, and the mice were tested during the light phase.

Rats. Upon arrival, male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were individually housed in a colony room with an average temperature of 20°C and a 12-h light/dark cycle (lights on at 7:00 AM) with free access to food and water during a 1-week acclimation period before implementing below-described protocols (rats were trained and tested during the light phase). Animals were approximately 90 days old at the time of purchase and experimentally naïve at the time of shaping the lever pressing response (see below). After the acclimation period, the animals were accustomed during the subsequent week to being handled and injected, and access to water was gradually limited to 0.5 h/day. Animals had free access to tap water from Friday afternoon until Sunday. All rodent procedures were approved by the Animal Care and Use Committee of Northeastern University, Boston, MA, USA. The guidelines in "Principles of Animal Laboratory Care" (National Institutes of Health 1996) were followed.

Apparatus

Rectal temperature was measured in mice using a rectal probe of a digital laboratory thermometer, RET-3-ISO, type T thermocouple (Physitemp Instruments Inc, Clifton, NJ, USA).

Drug discrimination training and testing utilized eight operant conditioning chambers (Camden Instruments, Ltd., London, UK) enclosed in sound attenuating cubicles and connected to an IBM-compatible PC via an LVB interface. Behavioral sessions and data collection were conducted using a Med-PC software program (v. 1.16, Med Associates, St. Albans, VT). The chambers were capable of delivering liquid reinforcement and equipped with two retractable response levers. The levers were separated by a receptacle in which fluid could be presented by a retractable drinking cup. The reinforcer was a 5-s access to sweetened (saccharin 0.1%) water. The cup delivered 0.2 ml fluid per presentation.

Procedure

Rectal temperature in mice The lubricated probe was inserted approximately 2.0 cm into the rectum for approximately 30 s prior to each recording. The first recording occurred at time-point zero at which time injections were given followed by recordings at 20, 60, 180, and 360 min post-injection. The final recording took place 24 h after injection. The ambient temperatures ranged from 21.9 to 23.3 with a mean (±SEM) of 22.44 (±0.32)°C.

Drug discrimination in rats Rats were trained to drink from a cup accessible through a receptacle located midway between the two response levers. The animals were shaped by successive approximation to lever press for fluid until they responded ten times for each reinforcer (fixed-ratio 10 schedule of reinforcement (FR-10)). The position of drug-appropriate levers was randomly assigned among subjects so that it was to the right of the receptacle for half the subjects and left for the other half. Throughout the session, the aforementioned FR-10 schedule of reinforcement was in effect. When the house light was off, and the stimulus lights above the response levers were lit, completion of ten presses on the active lever resulted in the delivery of one reinforcer. The house light went off simultaneously with a 5-s cup presentation and illumination of the cup by a receptacle light. At the end of the 5-s reinforcement period, the stimulus lights above the levers were lit, the house light was turned off, and the FR-10 schedule of reinforcement contingency reinstated. Sessions ended by all lights in the box being turned off. Post-session supplemental drinking for 0.5 h took place in the afternoon. Pellet food (Harlan Rat Chow®, #2018) was freely available except during the operant conditioning sessions.

Discrimination training and testing We used two training doses of the full CB_1R agonist AM5983 (0.18 and 0.56 mg/kg) in two separate groups of rats (N=24; n=12), in the spirit of systematic replication (Sidman 1960). The training drug was administered i.p. (2 ml/kg) 20 min before session onset. Rats were trained for 20-min sessions, 5 days a week (Monday through Friday). The schedule of drug- or vehicle-training sessions was non-systematic, with no more than two sessions under the same training condition occurring consecutively. Which lever was correct depended upon whether AM5983 or its vehicle had been administered before the session. Responses on the inappropriate manipulandum were recorded but had no programmed consequences. To avoid potential inter-animal cues, the daily order of drug or vehicle sessions for animals trained in the same chamber was varied (Extance and Goudie 1981). The

criterion for the acquisition of the discrimination was the completion of the first FR on the correct, injection-appropriate lever on at least eight out of ten consecutive training sessions. Correct selection was defined as the number of responses for receiving the daily first reward being equal to, or less than, 14 (FRF≤14), i.e., not having pressed the state inappropriate lever more than four times before accumulating ten responses on the state-appropriate lever.

Upon reaching above criterion, testing began with different doses and drugs, and in the case of AM2389 testing, also occurred at different injection-to-test intervals spanning from 20 min to 48 h. Test (T) sessions were conducted on average three times every 2 weeks; on interim days, regular drug (D) or vehicle (V) training sessions of 20 min duration took place. Such scheduling assured that each test was preceded by at least one D and one V maintenance session. Typically, the order of sessions was: D, V, T, V, D (week 1); V, T, V, D, T (week 2); V, D, T, D, V (week 3); and D, T, D, V, T (week 4). Tests were conducted only if responding during the preceding training sessions had been correct (FRF≤14) during the initial six FR-10 cycles of the session. If incorrect, animals were retrained for at least three sessions where FRF≤14 before additional testing took place. In test sessions, fluid was delivered for ten presses on either lever for six reinforcement cycles or until 20 min had elapsed, whichever occurred first. There was one session per test day. Doses and drugs were examined in a mixed order. However, because of the long duration of action of AM2389, tests with that ligand occurred only once weekly (Thursday or Friday) to allow for a 3- to 2-day "washout" period before resuming training. For each dose and interval tested, the percentage of responding on the drug-appropriate lever was calculated from the ratio of the number of presses on the AM5983 associated lever to the total number of lever presses in a test session (excluding responding during the time-out periods). Only data for animals receiving at least one reinforcer during the test session were considered for this measure, i.e., animals must have made a minimum of ten presses on one of the two levers. Additionally, response rate (responses per second) across all subjects was calculated. This measure was based on the performance of all animals, including non-responders.

Statistics

Non-linear regression analyses of mean drug discrimination dose-generalization and time-course data after log-X transformation were performed using Prism software (v. 5, GraphPad Software, San Diego, CA; www.graphpad.com) to provide estimates of the independent variable when the co-ordinates of X intersected with Y=50 and their 95% confidence limits (ED_{50}±95% CL; regression model: log dose or log time vs. response−variable slope with the top and bottom of the curves constrained to 100 and 0). Using the F-test, the Prism program estimates if slopes are parallel or not and, if parallel, evaluates whether the intercepts are equal or not (a measure of potency). Potency ratios, i.e., the quotient between two ED_{50} values, were also computed.

Results are presented as the mean (±SEM). Significant differences regarding temperature and response rate were calculated by means of one-way analyses of variance (ANOVA), followed by Holm–Sidak multiple comparison post hoc statistical test procedure. Differences were considered significant at the $p \leq 0.05$ level.

Drugs

The levo isomer of Δ^9-THC (6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6*H*-benzo[c]chromen-1-ol), dissolved in ethanol (200 mg/ml), was kindly provided by the National Institute on Drug Abuse (NIDA; Bethesda, Maryland, USA) and stored at −20°C until used. For preparing suspensions, appropriate amounts of Δ^9-THC were withdrawn; the ethanol evaporated under a stream of nitrogen; the residue dissolved in a solution of propylene glycol and Tween-80, and stored at −20°C. Shortly before being used, the solute was slowly diluted with normal (0.9%) saline in a step-wise fashion after the solute had been sonicated for 20 to 30 min. Racemic AM5983 [(1-((1-methylpiperidin-2-yl) methyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone; K_i (CB_1)=2.1 nM; K_i (CB_2)=2.3 nM] was handled the same way as Δ^9-THC as were also AM2389 [9β-hydroxy-3-(1-hexyl-cyclobut-1-yl)-hexahydrocannabinol; K_i (CB_1)= 0.16 nM; K_i(CB_2)=4.21 nM] and AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; K_i (CB_1)=7.5 nM; K_i (CB_2)= 2,290 nM]. All compounds, except Δ^9-THC, were synthesized at the Center for Drug Discovery, Northeastern University, Boston, MA, USA. Structures of the three cannabinergics are shown in Fig. 1.

Results

Temperature

Vehicle-treated mice exhibited stable body temperature recordings over the whole time period (i.e., 20 min to 24 h post-injection; Fig. 2). Significant initial changes in body temperature were exhibited at the 20-min time-point in mice treated with 0.3 mg/kg AM2389 and 30 mg/kg Δ^9-THC compared with vehicle at the same time-point; this change persisted at the 60, 180, 360, and 1,440 min time-

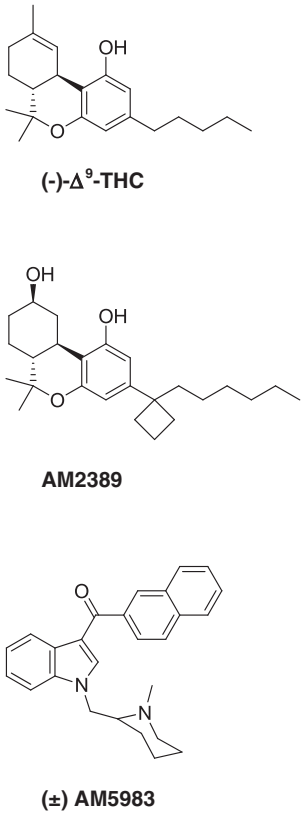

Fig. 1 Chemical structures of cannabinergics used in the study

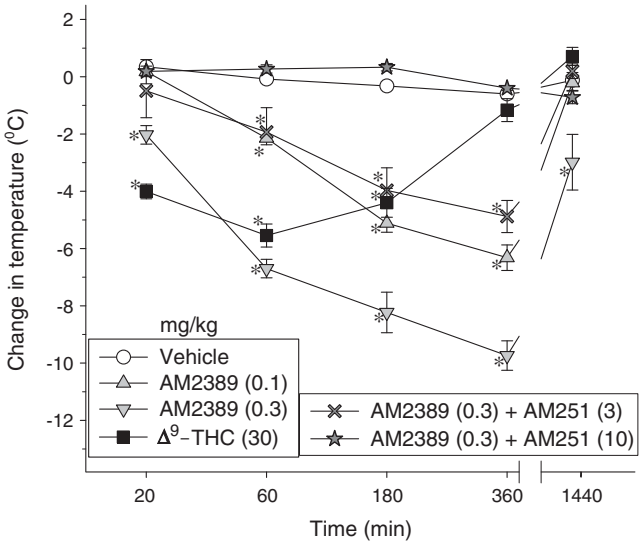

Fig. 2 Changes in temperature compared between vehicle (N=11; n= 2 to 3), AM2389 (n=5 to six per dose), Δ^9-THC alone (n=6) and AM2389 (0.3 mg/kg) plus AM251 (3 and 10 mg/kg; n=6 to 7 per each dose condition) in C57BL/6 J mice: N refers to the total number of mice used in the statistical analysis, while n refers to the number of mice run in parallel with each drug dose. Changes in temperature were recorded over time at the 20-, 60-, 180-, 360-, and 1,440-min time-points. Rectal temperatures prior to dosing averaged 37.40°C±0.18, 37.33°C±0.28, 37.30°C±0.18, 37.07°C±0.18, 37.61°C±0.18, and 37.20°C±0.30 for vehicle, AM2389 (0.1 mg/kg), AM2389 (0.3 mg/kg), AM2389/AM251 (3 mg/kg), AM2389/AM251 (10 mg/kg) and Δ^9-THC groups, respectively. Time-points were analyzed separately by means of one-way ANOVA. *Asterisk* indicates significant difference from the vehicle group at the same time-point at $p \leq 0.05$ (Holm–Sidak post hoc multiple comparison procedure involving a control mean)

points for 0.3 mg/kg AM2389 and at the 60- and 180-min time-points for Δ^9-THC. These temperature changes were significantly different from vehicle at the same time-points. Significant decreases in body temperature for 0.1 mg/kg AM2389 alone and 0.3 mg/kg AM2389 plus 3 mg/kg AM251 were initially exhibited at the 60-min time-point in comparison to vehicle at the same time-point; this decrease was even greater at the 180 and 360 min time-points. Maximum recorded effect on hypothermia was observed for all groups treated with AM2389 alone or in combination with 3 mg/kg AM251 at the 360 min time-point, while Δ^9-THC peaked at the 60-min time-point. AM251 (10 mg/kg) completely blocked the hypothermic reaction expected from administration of 0.3 mg/kg AM2983. Thus, at no time-point was there a significant difference between the rectal temperatures following the drug combination and the controls throughout the whole recording period. In addition, all groups (except 0.3 mg/kg AM2389 alone) reached temperatures similar to the vehicle group at 1,440 min post-injection. Δ^9-THC (30 mg/kg)-treated mice had similar body temperatures to the controls at the 360- and 1,440-min time-points.

Drug discrimination

Figure 3 shows the substitution pattern in tests with AM2389 at two post-injection intervals (60 and 180 min), and one interval for AM5983 (20 min) and Δ^9-THC (20 min), respectively, for rats trained to discriminate between the effects of vehicle and 0.18 mg/kg AM5983 (top panel), and the corresponding rate data (bottom panel). The ED_{50} (±95% CL) estimates and goodness of fit (r^2) for the discriminative stimulus effects of the compounds as substitutes for AM5983 (0.56 mg/kg) are listed in Table 1. The order of potency was: AM2389>AM5983>Δ^9-THC. The potency of AM2389 was significantly less at the 60 min test compared with testing at the 360 min post-injection interval [F (1, 3)=39.21; p=0.0082; Hill slopes for these two curves were not significantly different; F (1, 3)= 9.23; p>0.05]. Rate of responding was elevated for some of these tests (bottom panel) compared with the corresponding vehicle rate (mean±SEM, 0.64±0.05 responses/s).

Figure 4 shows the substitution pattern in tests with a fixed dose of 0.1 mg/kg AM2389 at different post-injection time intervals (range, 20 to 2,880 min), in rats trained to discriminate between the effects of vehicle and 0.18 mg/kg AM5983 (top panel), and the corresponding rate data (bottom panel). The estimated in vivo functional half-life of 0.1 mg/kg AM2389 as a substitute for

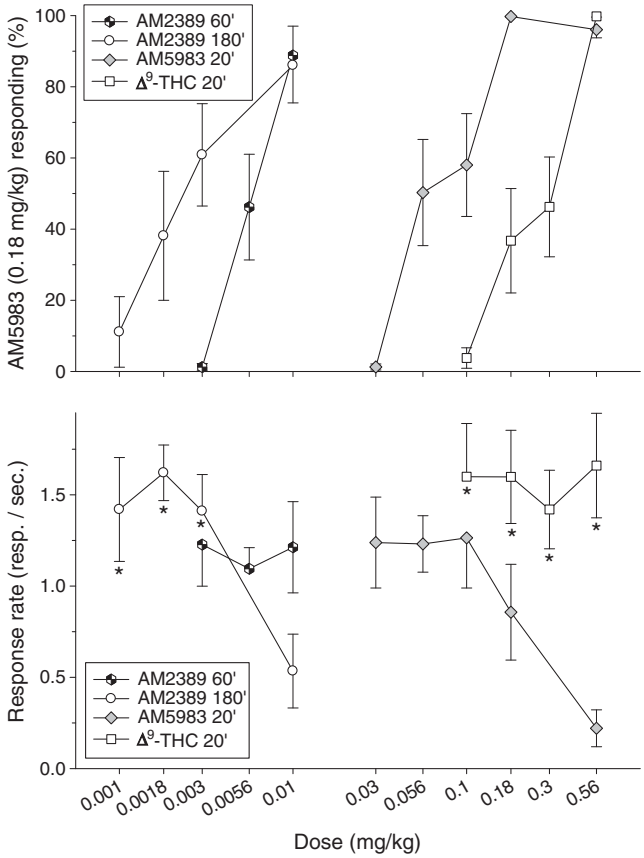

Fig. 3 Generalization test data for AM2389, AM5983, and Δ^9-THC (*top*) and the corresponding response rate data (*bottom*) for rats trained to discriminate between vehicle and 0.18 mg/kg AM5983. Tests were conducted 20 min (20') after i.p. administration of AM5983 and Δ^9-THC; AM2389 was examined separately at both 60 (*60'*) and 180 (*180'*) min post-injection. The generalization results represent the mean (±SEM) percentage of lever presses on the drug (0.18 mg/kg AM5983) appropriate lever out of the total number of lever presses emitted during a test session (*Y* axis); doses examined in milligrams per kilogram (*X* axis). Rate refers to the mean (±SEM) number of lever presses per second emitted during a test session (*Y* axis); doses in milligrams per kilogram (*X*-axis). Data points are based on one observation for each rat (n=10 to 12, AM2389_60'; n=8 to 10, AM2389_180'; n=11 to 12, AM5983_20'; and n=10 to 12, Δ^9-THC_20') and were obtained on separate test days. Test results are based on sessions of a maximum of six reinforcements or 20 min, whichever occurred first. Vehicle rate (mean±SEM) was 0.64±0.05 responses per second, based on the initial six reinforcement cycles of the non-drug maintenance sessions immediately preceding the above tests. *Asterisk* indicates significant difference from the vehicle rate at $p \leq 0.05$ (Holm–Sidak post hoc multiple comparison procedure involving a control mean)

AM5983 (0.18 mg/kg) was 1,043 (489 to 2,227) min post-injection as indicated in Fig. 3 (top panel; curve-fitting based on the data points spanning 1 to 48 h). Except for the 180 min post-injection results, rate of responding was elevated for all data points of these tests (bottom panel) compared with the corresponding vehicle rate (mean±SEM, 0.52±0.02 responses/s).

Figure 5 shows the substitution pattern in tests with AM2389 (180 min), AM5983 (20 min), and Δ^9-THC (20 min) in rats trained to discriminate between the effects of vehicle and 0.56 mg/kg AM5983 (top panel), and the corresponding rate data (bottom panel). The ED_{50} (±95% CL) estimates and goodness of fit (r^2) for the discriminative stimulus effects of the compounds as substitutes for AM5983 (0.56 mg/kg) are listed in Table 1. The order of potency was: AM2389>AM5983>Δ^9-THC. Rate of responding was elevated for some of these tests (bottom panel) compared with the corresponding vehicle rate (mean±SEM, 0.65±0.07 responses/s).

The right-ward shifts of the generalization curves as a function of the two AM5983 training doses (0.18 and 0.56 mg/kg) for the three CB_1R ligands were significant for AM2389 [F (1, 5)=23.93; p=0.0028], and Δ^9-THC [F (1, 4)=31.31; p=0.005] but not for AM5983 [F (1, 6)=5.11; p>0.05], although there was a trend in that direction for AM5983 also. The ratios for the ED_{50} values across the two training doses of AM5983 were 2.80 (AM2389), 3.20 (Δ^9-THC), and 2.18 (AM5983), respectively. All Hill slopes for the generalization gradients were parallel.

Discussion

Our findings with AM2389 in the temperature assay for mice compare nicely with previous data using rats (Nikas et al. 2010), although a comparison with Δ^9-THC was not given for rats. Thus, in mice, the onset of hypothermia after Δ^9-THC administration was faster than that of AM2389, peaked at 1 h post-administration, and returned to control levels at 6 h post-administration, and remained at control levels also at the 24 h post-administration recordings. In contrast, AM2389-induced hypothermia had a slower onset of action and produced its strongest measured response at 6 h post-administration, and regarding the higher dose of AM2389 (0.3 mg/kg), temperature had not fully recovered at the 24 h post-administration recordings. In concordance with previous tail-flick analgesia data (Nikas et al. 2010), AM2389-induced hypothermia was attenuated by a CB_1R antagonist. Thus, AM251 (3 mg/kg) diminished the 0.3 mg/kg AM2389-induced hypothermia to approximately the temperature levels produced by 0.1 mg/kg AM2389 alone. At the dose of 10 mg/kg, AM251 completely blocked the hypothermic response expected from 0.3 mg/kg AM2389 alone at all time-points, including 24 h post-administration. Thus, CB_1R mediation is strongly implicated from this outcome. Δ^9-THC-induced hypothermia is blocked by both rimonabant and AM251 in C57BL/6 J mice (McMahon and Koek 2007).

In the drug discrimination assay, the order of potency was: AM2389>AM5983>Δ^9-THC irrespective of the drug

Table 1 Summary of substitution test results

Training condition: AM5983 0.18 mg/kg vs. vehicle, 20'			
Test drug	ED_{50} (± 95% C.L.) mg/kg	r^2	Potency ratio
AM2389 60'	0.0060 (0.0038-0.0094);	0.9955	Δ^9-THC 20' / AM2389 60': 43.92
AM2389 180'	0.0025 (0.0016-0.0038);	0.9733	Δ^9-THC 20' / AM2389 180': 105.40
AM5983 20'	0.0571 (0.0494-0.1161);	0.9449	Δ^9-THC 20' / AM5983 20': 4.61
Δ^9-THC 20'	0.2635 (0.1328-0.5259);	0.9159	AM5983 20' / AM2389 60': 9.51 AM5983 20' / AM2389 180': 22.84

Training condition: AM5983 0.56 mg/kg vs. vehicle, 20'			
Test drug	ED_{50} (± 95% C.L.) mg/kg	r^2	Potency ratio
AM2389 180'	0.0069 (0.0053-0.0090);	0.9751	Δ^9-THC 20' / AM2389 20': 122.29
AM5983 20'	0.1246 (0.0797-0.1947);	0.9622	Δ^9-THC 20' / AM5983 20': 6.77
Δ^9-THC 20'	0.8438 (0.8438-0.8755);	0.9998	AM5983 20' / AM2389 20': 18.06

Note: AM2389 was examined separately at both 60 min (60') and 180 min (180') post-administration in the animals discriminating between 0.18 mg/kg AM5983 and vehicle 20 min (20') after injection (top).

training condition (0.18 or 0.56 mg/kg AM5983). Based on the 3 h post-injection interval data for AM2389, this HHC was estimated to be 105 and 122 times more potent as a discriminative stimulus than Δ^9-THC for the two (0.18 and 0.56 mg/kg AM5983) separate drug discrimination conditions, respectively. That makes AM2389 one of the most potent cannabinergics examined to date in vivo. The potency ratios for Δ^9-THC and AM5983 were 4.6 for the low-dose and 6.8 for the high-dose training conditions, respectively; the potency ratios for AM5983 and AM2389 (at 180 min post-injection) were 22.84 and 18.06, respectively. Given the relative nature of drug discrimination, ED_{50} values generally are proportional to the training dose employed (Stolerman et al. 2011). However, it has also been proposed that intrinsic activity (efficacy) can be a determinant such that the higher the training dose of a full agonist, a disproportionate amount of a low-efficacy/partial agonist is required for substitution (Bergman et al. 2000). For instance, rats discriminating between vehicle and either of two doses (0.014 and 0.03 mg/kg) of the full CB_1R agonist CP55,940 required relatively higher doses of the partial/low-efficacy agonist Δ^9-THC for generalization in the high-dose relative to the low-dose CP55,940 training condition (De Vry and Jentzsch 2003). Similarly, mice and monkeys rendered tolerant to the rate-suppressant effects of Δ^9-THC in an operant conditioning task displayed a rightward shift of the Δ^9-THC dose–response curve compared with acute administration; the dose–response curves for

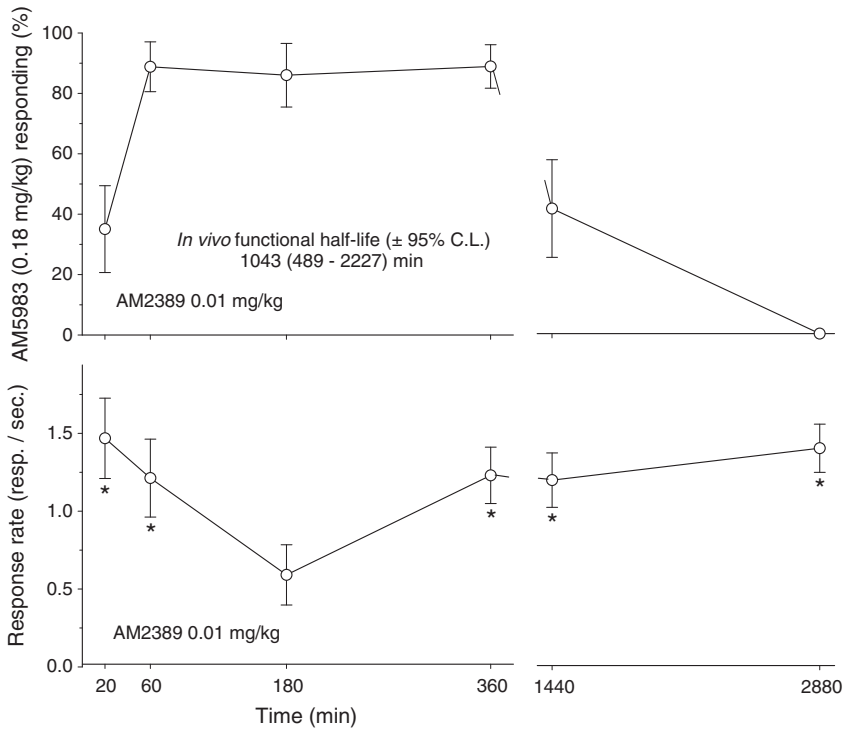

Fig. 4 Generalization test data for 0.01 mg/kg AM2389 (*top*) and the corresponding response rate data (*bottom*) for rats trained to discriminate between vehicle and 0.18 mg/kg AM5983. Tests were conducted at different post-injection times (range, 20 min to 48 h), and represent the mean (±SEM) percentage of lever presses on the drug (0.18 mg/kg AM5983) appropriate lever out of the total number of lever presses emitted during a test session (*Y* axis); elapsed time since injection of 0.01 mg/kg AM2389 (*X* axis). Rate refers to the mean (±SEM) number of lever presses per second emitted during a test session (*Y* axis); elapsed time since injection of 0.01 mg/kg AM2389 (*X* axis). Data points are based on one observation for each rat (n=8 to 12) and were obtained on separate test days. Test results are based on sessions of a maximum of six reinforcements or 20 min, whichever occurred first. Vehicle rate (mean±SEM) was 0.52±0.02 responses per second, based on the initial six reinforcement cycles of the non-drug maintenance sessions immediately preceding the above tests. *Asterisk* indicates significant difference from the vehicle rate at $p \leq 0.05$ (Holm–Sidak post hoc multiple comparison procedure involving a control mean)

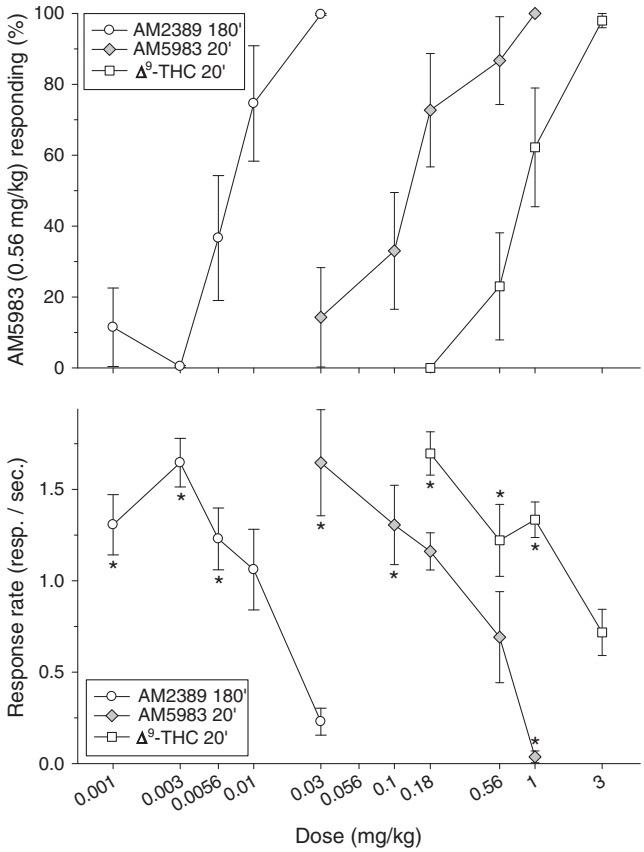

Fig. 5 Generalization test data for AM2389, AM5983, and Δ^9-THC (*top*) and the corresponding response rate data (*bottom*) for rats trained to discriminate between vehicle and 0.56 mg/kg AM5983. Tests were conducted 20 min (20') after i.p. administration of AM5983 and Δ^9-THC; AM2389 was examined at 180 (180') min post-injection. The generalization results represent the mean (±SEM) percentage of lever presses on the drug (0.56 mg/kg AM5983) appropriate lever out of the total number of lever presses emitted during a test session (*Y* axis); doses examined in milligrams per kilogram (*X* axis). Rate refers to the mean (±SEM) number of lever presses per second emitted during a test session (*Y* axis); doses in milligrams per kilogram (*X* axis). Data points are based on one observation for each rat (n=9 to 10, AM2389_180'; n=8 to 12, AM5983_20'; and n=8 to 9, Δ^9-THC_20') and were obtained on separate test days. Test results are based on sessions of a maximum of six reinforcements or 20 min, whichever occurred first. The data points for 1 mg/kg AM5983 are based on two responding rats out of 12 tested. Vehicle rate (mean± SEM) was 0.65±0.07 responses per second, based on the initial six reinforcement cycles of the non-drug maintenance sessions immediately preceding the above tests. *Asterisk* indicates significant difference from the vehicle rate at $p \leq 0.05$ (Holm–Sidak post hoc multiple comparison procedure involving a control mean)

CP55,940 and the aminoalkylindole full CB_1R agonist WIN55,212-2 were not changed as a consequence of the Δ^9-THC-induced tolerance (McMahon 2011; Singh et al. 2011). Thus, differences in efficacy may be part of the reason why the potency ratios for the partial agonist Δ^9-THC appeared to be shifted more upwards as a function of the training dose of AM5983 compared with the two full CB_1R agonists in the current study. This intriguing possibility requires further attention in order to draw a firm conclusion.

Like dimethylheptyl homologues of Δ^8-THC (e.g., HU210) and a related HHC CB_1R ligand (HU243), the onset of action was slow with AM2389 and the duration of action was long in both the hypothermia and drug discrimination assays. Thus, using a fixed dose of 0.01 mg/kg AM2389 examined at different post-administration intervals in the drug discrimination assay, we estimated that the discriminative stimulus in vivo functional half-life of AM2389 was approximately 17 h. To generate a more precise time-course estimate would require establishing full dose–response functions for each time-point in the manner done for the 1 and 3 h time-points for the AM5983 low-dose (0.18 mg/kg) training condition. That comparison clearly indicated that the effect of AM2389 was still on the rise at the 1 h time-point post-administration. Of course, we cannot state for certain that the 3 h post-administration time-point represents the peak as a complete generalization curve was not generated for the 6-h interval. However, to the extent that our previous work with long-acting cannabinergics (Δ^8-THC-DMH, HU210, and HU243) can be extrapolated to the current situation, the 3-h interval should at least be close to the peak for the discriminative stimulus effects of AM2389 (Devane et al. 1992; Järbe et al. 1989, 1981).

The temperature data also highlight that different end-points can yield different time-course data. For example, we (Järbe 1978) showed that hypothermia in rats peaked at 1 to 2 h after Δ^9-THC administration and thereafter exhibited a gradual recovery during the next 5 to 6 h of continuous recordings. The discriminative stimulus effects of Δ^9-THC were substantially diminished by 4 to 4.5 h post-administration (Järbe et al. 1981, 1986), whereas the aforementioned hypothermic effect still was significantly lower compared with the vehicle controls at those time-points (Järbe 1978).

Given that the ligand AM5983 is a racemic mixture, it is relevant to know if both isomers produce qualitatively similar effects or not. In unpublished studies, we found that, when examined separately, both isomers indeed produced qualitatively similar effects in rats, i.e., substituted for the discriminative stimulus effects produced by the racemic mixture (AM5983) used for the drug discrimination training.

AM2389 displays some selectivity for CB_1R over CB_2R (Nikas et al. 2010) whereas Δ^9-THC, HU210, and HU243 are non-selective, i.e., display essentially equal affinity for the two cannabinoid receptor subtypes (Bayewitch et al. 1995). Direct CB_2R activation or blockade did not result in substitution in animals trained to recognize the discriminative stimulus effects produced by a CB_1R agonist or a CB_1R antagonist (Järbe et al. 2004, 2006, 2008; McMahon 2006; Vann et al. 2007), ruling out direct involvement of

CB_2R in CB_1R-mediated drug discrimination. However, recent data suggest that there might be a limited amount of neuronal CB_2R present in brain even under normal, non-pathological conditions which opens up the possibility of "cross-talk" between the two cannabinoid receptors. The functional significance of this discovery remains to be more fully explored, but ligands exhibiting cannabinoid receptor subtype selectivity likely will be useful tools in such a quest (Atwood and Mackie 2010; Roche and Finn 2010). Indeed, it was recently found that systemic and centrally administered CB_2R agonists attenuated cocaine self-administration, cocaine-enhanced locomotor activity, and cocaine-induced dopamine release in the nucleus accumbens of wild-type as well as of CB_1R, but not CB_2R knockout mice. The diminished reactions to cocaine following CB_2R agonism were reversed by the CB_2R antagonist AM630 but not by the CB_1R antagonist AM251 (Xi et al. 2011). Another recent mouse study implicated CB_2R in emotional states such as anxiety (Garcia-Gutierrez et al. 2011).

As predicted at the outset of these studies, the high-affinity cannabinoid receptor ligand AM2389 was very potent and functionally acted as a cannabinergic (CB_1R), exhibiting a slow onset of action with a protracted time-course. It is further expected that, should the probe be evaluated in man, a discriminative pharmacology profile similar to that described in this report would emerge. Collectively, current and previous data (Nikas et al. 2010) lay a foundation to continue exploring the structural requirements for enhancing CB_1R over CB_2R subtype selectivity of ligands not derived from lipophilic endogenous signaling molecules. Thus, we envision that the next generation of potent drugs derived from the AM2389 chemotype will exhibit an improved "drugability" profile gearing towards enhanced water solubility and increased oral bioavailability. As a pharmacological tool, the long duration of action can be exploited for examining the cellular/physiological consequences of sustained activation of endocannabinoid receptor signaling. We are currently using AM2389 to induce tolerance in mice and subsequently examining the consequences of CB_1R antagonist precipitated displacement of the agonist from its CB_1R binding sites (Tai et al. 2011).

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