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**TITLE PAGE**

# Long-lasting *in vivo* effects of the cannabinoid CB1 antagonist AM6538

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Running title: CB1 antagonist effects of AM6538 in vivo

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**Non-standard abbreviations**

AM6538: 4-(4-(1-(2,4-Dichlorophenyl)-4-methyl-3-(piperidin-1-ylcarbamoyl)-1*H*-pyrazol-5-yl)phenyl)but-3-yn-1-yl nitrate

AM4054: 9β-(Hydroxymethyl)-3-(1-adamantyl)-hexahydrocannabinol

THC: $\Delta^9$-Tetrahydrocannabinol

WIN 55,212: ((*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

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**ABSTRACT**

AM6538 is a cannabinoid antagonist that binds CB1 receptors expressed in HEK-293 cells in a wash-resistant manner. The effects of AM6538 in live animals has not previously been established. We characterized the antagonist effects of AM6538 in male mice, using a warm-water tail-withdrawal assay, and in male squirrel monkeys trained to discriminate the CB1 agonist AM4054 from vehicle. The cannabinoid agonists WIN 55,212, $\Delta^9$-tetrahydrocannabinol (THC), and AM4054 all produced 100% maximum possible antinociceptive effects in mice following vehicle pretreatment. One hour pretreatment with increasing doses of AM6538 (0.1-10 mg/kg) produced first rightward, then downward shifts of the agonist dose-effect functions. Rimonabant, 1-10 mg/kg, produced parallel rightward shifts of the AM4054 dose-effect functions, and baseline effects of AM4054 were nearly recovered within 24 h following 10 mg/kg rimonabant. In contrast, in mice treated with 10 mg/kg AM6538, antagonism of THC or AM4054 lasted up to 7 days. AM6538 also antagonized the discriminative stimulus effects of AM4054 in a squirrel monkeys in a dose-related manner, and the effects of 3.2 mg/kg AM6538 endured for more than 7 days. The effective reduction in CB1 receptor reserve was used to calculate the relative efficacy (*tau* values) of WIN 55,212, THC, and AM4054 in mice and of AM4054 monkeys, with results indicating that THC has a lower efficacy than WIN 55,212 or AM4054 in mice. These results demonstrate that AM6538 is a long-acting CB antagonist *in vivo*, and further suggest that differences in CB efficacy can be revealed in behavioral assays following AM6538 treatment.

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## Introduction

Cannabinoids such as marijuana and related plant-based cannabis products (phytocannabinoids) are the most commonly used illicit drugs worldwide (UNODC, 2014). This widespread usage may be attributable, in part, to the acceptance that $\Delta^9$-tetrahydrocannabinol (THC), the principal psychoactive constituent of marijuana, is considered to be relatively safe in comparison to other abused drugs (Nutt et al., 2007). In contrast, newer synthetic cannabinoids have been associated with greater toxicity than phytocannabinoids. The reasons for the different risk profiles of THC and synthetic cannabinoids are not clear. One possible explanation is that the adverse effects of synthetic cannabinoids result from having higher agonist efficacy at cannabinoid (CB) receptors than THC. As in other drug classes, relatively low efficacy may confer a degree of safety (Liguori et al., 1996); whereas greater toxicity might accompany cannabinoids with higher efficacy.

Using *in vitro* methods, cannabinoid agonist efficacy has been characterized by equating maximal response with maximal efficacy. For example, using assays of forskolin-stimulated cAMP accumulation, β-arrestin recruitment, GTPγS-binding, or $K^+$ channel activation, studies have consistently revealed that older and newer synthetic cannabinoids including WIN 55,212, CP 55,940, JWH-073 and AB-PINACA, have greater efficacy than THC (Banister et al., 2015; Brents et al., 2012; Govaerts et al., 2004). Such differences in maximal agonist effects are less apparent in whole animals, perhaps because most behavioral assays of cannabinoids were developed to identify THC-like effects of novel compounds (Little et al., 1988). Nonetheless, there are some conditions under which THC appears to be a partial agonist *in vivo*. For example, the dose-effect function for hypothermic effects of THC in Swiss-Webster mice reveals a plateau at approximately 6$^o$C below baseline for doses greater than 30 mg/kg THC, whereas other



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cannabinoid agonists are able to decrease body temperature by ≥8°C (Paronis et al., 2012; Tai et al., 2015). These relatively limited hypothermic effects have allowed THC to be used as an antagonist of the synthetic cannabinoids AM2389 or JWH-018. In other studies, an indirect approach involving cannabinoid tolerance was used, based on similar studies of opioid tolerance demonstrating that the magnitude of tolerance or cross-tolerance varies inversely with agonist efficacy (Paronis and Holtzman, 1992; Walker et al., 1997). Thus, in THC-treated monkeys, greater tolerance is seen to the discriminative stimulus effects of THC than cross-tolerance to the THC-like effects of JWH-073, JWH-018, or CP 55,940, suggesting that THC has lower efficacy than the other three cannabinoid agonists (Hruba et al., 2012). Unfortunately, *in vivo* studies involving chronic administration to produce tolerance or drug interactions to establish relative agonist/antagonist profiles are time consuming and, hence are not amenable to assaying a range of compounds simultaneously.

An alternative method of determining agonist efficacy is to quantify the responses to drugs following treatment with irreversible antagonists to inactivate a proportion of the available receptors. According to receptor theory, the shape and position of agonist dose-effect functions following receptor inactivation is dependent on their efficacy: low efficacy agonists do not produce maximal effects following receptor depletion and so their dose-effect functions are shifted downward; in contrast, dose-effect functions of full agonists, which have receptor reserves, are shifted initially to the right and are shifted downward only following higher doses of the antagonist (Furchgott and Bursztyn, 1967). These changes in the agonist dose-effect functions are reflected in differences in the value of the transducer ratio (*tau*, $\tau$), a tissue-dependent measure of the operational efficacy of agonists (Black et al., 1985). One distinct advantage of this approach is that it can be applied in cell-based functional assays, tissue

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preparations, or whole animals (Leff and Dougall, 1989; Nickolls et al., 2011; Walker et al., 1998). Calculations of *tau* thus have been used to distinguish full and partial agonists at several different types of receptors (Koek et al., 2000; Madia et al., 2012), and a recent paper described calculation of *tau* values for two cannabinoid allosteric agonists, in combination with the orthosteric agonist CP 55,940, in HEK293 cells (Laprairie et al., 2017). To date, however, the method of receptor inactivation has not been used to quantify differences in cannabinoid agonist efficacy *in vivo*.

A novel structural analog of rimonabant, AM6538, has been described as forming a tight attachment to cannabinoid CB1 receptors (Hua et al., 2016). AM6538 exhibits high affinity for CB1 receptors and competes with CP 55,940 and THC in assays of cAMP inhibition, β-arrestin recruitment, and GTPγS-binding. However, preincubation of membrane preparations with AM6538 reveal that AM6538 binding is wash-resistant, suggesting it forms a pseudo-irreversible bond at CB1 receptors. This tight binding ability allows AM6538 to stabilize the CB1 receptor, permitting identification of the crystal structure of modified human CB1 receptors expressed in HEK293F cells (Hua et al., 2016). In view of this property of AM6538 *in vitro*, we sought to use AM6538 as a pseudo-irreversible antagonist *in vivo*. The effects of AM6538 pretreatment were examined in an assay of antinociception in mice, and a CB1-agonist drug discrimination assay in monkeys. Here, we present findings that AM6538 has long-lasting cannabinoid antagonist effects in mice and nonhuman primates. Further, our data suggest that AM6538 can be used as a tool to delineate the relative efficacies of cannabinoid agonists *in vivo*.

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**Methods.**

**Antinociception.** *Subjects*. Male CD-1 mice (Charles River Laboratories, Wilmington MA) weighing 25-30 g at the start of the study, were housed 4/cage in a climate-controlled vivarium with food and water available *ad libitum*. Prior to testing, mice were habituated by being exposed twice to handling and the experimental methods described below. All experiments were performed during the light portion of the light/dark cycle. All studies were approved by the Northeastern University Animal Care and Use Committee, in accordance with guidelines established by the National Research Council.

*Apparatus and Behavioral Procedure*. Antinociception was determined using a warm water tail-withdrawal assay. A water bath maintained water temperature at 52.0 ± 0.5°C. Each mouse was gently hand-held while the distal 2-3 cm of the tail was immersed in the water; latency to tail-withdrawal was measured using a stopwatch, with a cut-off time of 8s to avoid tissue damage. Baseline latencies were determined twice on each test day with a 10 min interval between determinations.

*Injection Procedures***.** Complete dose-effect functions for agonists were generated in each mouse in single sessions using cumulative dosing procedures similar to those described previously (Chopda et al., 2016). Briefly, 60 min after an injection, tail-withdrawal latencies were determined and, then, mice were injected with the next dose; each injection increased the total cumulative dose by 0.5 log units. This procedure was repeated until the tail-withdrawal latency reached the cut-off or no longer increased with subsequent increases in drug dose. Pilot studies with 3 mg/kg AM6538 indicated that antagonism of AM4054 occurred in less than 30 min, but reached maximum effects at 1-2 h after injection. Thus, agonist dose-effect functions were initially determined 60 min after injection with vehicle or with graded doses of AM6538 or

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rimonabant. To assess the time course of antagonism, effects of AM4054 or THC were re-determined in sessions that began at various times, ranging from 1 to 7 days, after injection of 10 mg/kg of AM6538 or rimonabant. Each mouse was tested once or twice, with at least one week between test sessions.

**AM4054 Discriminative Stimulus Effects**. *Subjects*. Six adult male squirrel monkeys (*Saimiri sciureus*) were used in the procedures described here. Experimental sessions were conducted 5 days a week (Monday–Friday). The experimental protocol for the present studies was approved by the Institutional Animal Care and Use Committee at McLean Hospital. Subjects were maintained in a facility licensed by the U.S. Department of Agriculture and in accordance with the guidelines established by the National Research Council.

*Apparatus*. During experimental sessions, subjects sat in a Plexiglas chair within a ventilated sound- and light-attenuating chamber as described previously (Delatte and Paronis, 2008). The front panel of the chair was outfitted with two response levers that were positioned 6 cm left and right of center. Each lever-press with a force of at least 0.25 N closed a microswitch, produced an audible click, and was recorded as a response. Red stimulus lights were mounted behind the transparent front panel of the chair, approximately 10 cm above each response lever. Before each session, a shaved portion of each subject's tail was coated with electrode paste and placed under brass electrodes for the delivery of brief, low-intensity current (see below). Experimental events and data collection were controlled by Med Associates (St. Albans, VT) interfacing equipment and operating software.

*Behavioral Procedure*. The subjects were previously trained to discriminate the pre-session administration of 0.01 mg/kg of the cannabinoid agonist AM4054 or its vehicle by responding on one of two levers. Briefly, subjects initially were trained to terminate visual stimuli associated

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with the delivery of brief, low-intensity current (200 ms; 3 mA) across the electrodes by depressing one of the two response levers. The active lever varied until subjects reliably terminated visual stimuli by responding on either lever. Subsequently, both levers were present in all sessions and the active lever was signaled only by a pre-session injection: one lever was active only following the i.m. injection of the CB1 agonist AM4054 (0.01 mg/kg, i.m.), and the other lever was active only following i.m. injection of vehicle; right and left lever assignments were counterbalanced among subjects. Following initial training, each subsequent session began with a 30-minute timeout period during which all lights were extinguished and responding had no programmed consequences. After the timeout period, two red stimulus lights above each lever were illuminated and completion of 10 consecutive responses [fixed ratio (FR) 10] on the active lever extinguished all stimulus lights and initiated a 50-second timeout. Responses on the inactive lever reset the FR requirement. Current delivery was scheduled for delivery every 10 seconds until either the FR 10 was completed on the correct lever or 30 seconds elapsed, whichever came first.

*Drug Testing*. Tests for generalization of AM4054 to the training stimulus were conducted when a subject's discrimination performance was at least 90% accurate for four of the last five training sessions and on the immediately preceding session. Procedurally, test sessions differed from training sessions in two ways. First, 10 consecutive responses on either lever extinguished the stimulus lights and associated current delivery, and initiated the 50-second timeout. Second, no current deliveries were scheduled during test sessions so as to preclude possible stimulus-induced enhancement of responding. Other schedule contingencies were unchanged. Using cumulative-dosing procedures, graded doses of AM4054 were administered at the beginning of sequential components of the test session (i.e., at the onset of the 30-minute timeout periods that

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initiated session components). Test sessions began at various times, 1 hour to 7 days, after injection of AM6538.

**Drugs.** $\Delta^9$-THC and rimonabant were obtained from the National Institute on Drug Abuse [(NIDA), Rockville, MD]; WIN-55-212 [((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] was purchased from Sigma-Aldrich (St. Louis, MO). AM4054 [9β-(hydroxymethyl)-3-(1-adamantyl)-hexahydrocannabinol] and AM6538 4-(4-(1-(2,4-dichlorophenyl)-4-methyl-3-(piperidin-1-ylcarbamoyl)-1*H*-pyrazol-5-yl)phenyl)but-3-yn-1-yl nitrate were synthesized at the Center for Drug Discovery, Northeastern University. All compounds were prepared in 5% ethanol, 5% emulphor-620 (Rhodia, Cranbury, NJ) and 90% saline, and further diluted with saline. Drug doses are expressed in terms of the weight of free base.

**Measurement of Effects and Statistical Analysis.** Tail withdrawal latencies are expressed as a percentage of maximum possible effect (%MPE ± SEM), calculated using the formula: %MPE = [(test latency - baseline latency)/(8 - baseline latency)] × 100. The two primary dependent measures in discrimination studies are response distribution across the two levers and overall response rate. Response distribution (% AM4054 lever responses) was calculated by dividing the number of responses on the lever associated with the injection of AM4054 by the total number of responses (excluding any responses during timeout periods). Response rate was calculated by dividing the total number of responses on both levers by the total session time (excluding all timeout periods). Doses of drugs were considered to substitute fully when response distribution was 90% AM4054 lever and response rates were sufficiently high ( >0.2 responses/s) to calculate a meaningful response distribution values. Data were plotted and analyzed with doses expressed as log transformed values using GraphPad Prism v. 5.02 (GraphPad Software, San Diego



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California) to obtain $ED_{50}$ and *tau* values along with 95% CI; values were considered to be different if there was no overlap of the 95% CI. Nonlinear regression analysis of grouped data, applying constant values for maximum (100%) and minimum (0%) effects, were used to calculate $ED_{50}$ values of the agonists. Mean dose-effect functions for each agonist were simultaneously fit to the operational model for agonism to obtain *tau*-values for each curve, applying common values for slope and $K_A$ and constant values for maximum (100%) and minimum (0%) effects. The fraction of available receptors remaining was calculated as the ratio of *tau* values before and after AM6538 treatment. Response rates obtained for each dose-response function were analyzed using 1-way ANOVA, followed by Dunnett's multiple comparison test, comparing data from each dose to response rates obtained after saline and 0.1 mg/kg AM4054 during training sessions; significance for all tests was set at $P < 0.05$.

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## Results

### Antinociception.

**Control values**. AM6538 did not alter the latency to tail-withdrawal responses whereas AM4054, THC, and WIN 55,212 produced near maximal antinociceptive effects. Baseline tail-flick latencies in vehicle treated mice averaged 2.1 sec, and ranged from 1.4 to 2.7 sec. One hour after pretreatment with 0.1, 0.3, 3.0, or 10 mg/kg AM6538, average tail-flick latencies were unchanged (2.0, 2.1, 2.0, and 2.2 sec, respectively). Cumulative injections of AM4054 dose-dependently increased tail-flick latencies in vehicle pretreated animals, with an $ED_{50}$ value of 0.38 mg/kg (see Table 1). THC and WIN 55,212 had effects that were qualitatively similar to AM4054, but with lesser potency. The control $ED_{50}$ values were 18.2 mg/kg for THC and 2.8 mg/kg for WIN 55, 212.

**Antagonist effects of AM6538**. One hour pretreatment with AM6538 dose-dependently antagonized the antinociceptive effects of AM4054. As shown in Figure 1A, the dose of 0.3 mg/kg AM6538 produced a rightward shift in the AM4054 dose-effect function, increasing the $ED_{50}$ to 0.56 mg/kg and 3 mg/kg AM6538 further increased the ED50 value to 2.47 mg/kg, significantly different from the control $ED_{50}$ value (see Table 1). The dose of 10 mg/kg AM6538 decreased the maximum obtained effect, resulting in a flattening of the AM4054 dose-effect function. Also in Figure 1, AM6538 similarly antagonized the antinociceptive effects of WIN 55,212. The dose of 0.3 mg/kg AM6538 increased the $ED_{50}$ of WIN 55,212 to 4.0 mg/kg and after pretreatment with 3.0 mg/kg AM6538, a dose of 100 mg/kg WIN 55,212 resulted in only 71% of the maximum possible antinociceptive effect and significantly increasing the $ED_{50}$ value (Table 1). AM6538 was more potent in antagonizing the effects of THC (Fig. 1B). The doses of 0.3, 3.0 and 10 mg/kg all flattened the THC dose-effect function, and 300 mg/kg THC produced

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less than 50% of the maximum possible effect precluding the determination of $ED_{50}$ values. A lower dose of 0.1 mg/kg AM6538 marginally (less than 2-fold) increased the $ED_{50}$ of THC to 31.4 mg/kg.

**AM6538 Time-course**. Figure 2 depicts the recovery of the antinociceptive effects of THC and AM4054 at different times following treatment with 10 mg/kg AM6538. Antagonism of AM4054 by AM6538 was greatest 1 h after injection; the dose of 10 mg/kg AM4054 yielded only 37±11% of the maximum possible effect. At 24-48 h after injection of AM6538 there was some recovery in the effects of AM4054: 10 mg/kg AM4054 produced nearly full antinociceptive effects, though this represented a significant rightward shift of the AM4054 dose-effect function (Fig. 2A). The $ED_{50}$ of AM4054 at 24 h after AM6538 was 2.46 mg/kg, slightly more than ¾ log-units to the right of the control function, and at 48h after AM6538 the AM4054 $ED_{50}$ was still significantly greater than the control value (see Table 2). Baseline effects of AM4054 were fully recovered by 7 days after the AM6538 injection, with an $ED_{50}$ value of 0.25 mg/kg.

As shown in Figure 2B, a similar pattern of recovery was obtained with THC: at 48 h after AM6538, 300 mg/kg THC produced approximately 60% of the maximum possible antinociceptive effects and the $ED_{50}$ value of THC was 197 mg/kg, reflecting an approximately 1 log-unit rightward shift of the dose-effect function. Seven days after treatment with AM6538, 100 mg/kg THC produced 73±8% of the maximum possible antinociceptive effect, and the THC $ED_{50}$ was 32.3 mg/kg (Table 2).

**Rimonabant Antagonism**. The effects of AM4054 were also determined following treatment with 1 -10 mg/kg rimonabant, with results shown in Figure 3A. Rimonabant produced a surmountable rightward shift in the AM4054 dose-effect function. In these studies, the $ED_{50}$ of

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AM4054 under control conditions (i.e., 30 minutes after vehicle pretreatment) was 0.30 mg/kg (0.23, 0.38). Following treatment with 1 and 3 mg/kg rimonabant, the AM4054 $ED_{50}$ was increased to, respectively, 0.42 mg/kg (0.32, 0.55) and 0.63 mg/kg (0.44, 0.90). The dose of 10 mg/kg rimonabant produced a 10-fold shift in the AM4054 dose-effect function, with an $ED_{50}$ of 3.04 mg/kg (2.23, 4.13). As depicted in Figure 3B, there was nearly full recovery of AM4054's effects from this dose of rimonabant within 24 h, with an $ED_{50}$ value of 0.44 mg/kg (0.32, 0.60).

**Drug Discrimination.**

**Control values**. During the training days preceding test days, squirrel monkeys responded nearly exclusively on the vehicle lever during vehicle training cycles and nearly exclusively on the drug lever during AM4054 training cycles. Mean response rates (±SEM) did not differ ($t_{(5)}$=0.16) during vehicle and AM4054 training cycles and were 3.26 (± 0.40) and 3.19 (± 0.41) responses/sec, respectively. As shown in Figure 4, cumulative injections of AM4054, 0.001–0.01 mg/kg, produced dose-related increases in responding on the AM4054-associated lever without altering response rates ($F_{(4,25)}$=0.50). Full substitution (≥90% responding on the drug lever) occurred following cumulative injection of the training dose of 0.01 mg/kg AM4054, and the group average $ED_{50}$ was 0.004 mg/kg (0.002, 0.008).

**Antagonist effects of AM6538**. In initial experiments, monkeys received 1 or 3 mg/kg AM6538 1 h prior to test sessions in which the dose-effect function of AM4054 was re-determined. As seen in Figure 4, both doses of AM6538 antagonized the effects of AM4054, resulting in significant rightward shifts of the dose effect function with no decrease in the maximum effects. Following 1 mg/kg AM6538, the $ED_{50}$ of AM4054 was increased more than 6-fold to 0.025 mg/kg (0.018, 0.036) and after 3 mg/kg AM6538 the $ED_{50}$ value was 0.24 mg/kg (0.13, 0.44) or

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60-fold higher than control the control value. A 1-way ANOVA revealed significant differences in response rates ($F_{(12,48)}$=2.128; P=0.0324) however post-hoc analysis identified no differences from control values obtained with saline or 0.01 mg/kg AM4054.

**AM6538 Time-course**. Figure 5 shows the duration of antagonism following 3 mg/kg AM6538. As was seen with antinociceptive effects in mice, the antagonism of the discriminative stimulus effects of AM4054 effects was greatest 1 h after injection of AM6538 followed by a gradual recovery towards control over the seven days of testing. Thus, significant antagonism was apparent at 2 and 4 days after AM6538, and there was still a slight rightward shift in the AM 4054 dose-effect function (more than 2-fold) at 7 days. The $ED_{50}$ values of AM4054 at the different times are as follows: 0.033 mg/kg (0.016, 0.067) at 2 days, 0.015 mg/kg (0.008, 0.026) at 4 days and 0.009 mg/kg (0.006, 0.014) at 7 days after 3 mg/kg AM6538. A 1-way ANOVA revealed no differences in response rates ($F_{(18,70)}$=1.202; P=0.28).

**Agonist efficacy**.

Tables 3 and 4 present *tau* values calculated for THC, WIN 55,212, and AM4054; these values were used to estimate the fraction of available receptors remaining after AM6538 treatment. The top row in Table 3 lists initial *tau* values for THC, WIN 55,212, and AM4054; in antinociception studies, these values were not significantly different. AM6538 dose-dependently decreased the *tau* values for all cannabinoid agonists, reflecting reductions in the available receptors. Following 0.3 mg/kg AM6538, the *tau* value for THC was decreased to 1.3, indicating the fraction of receptors that remained available was 27% whereas for WIN 55,212 and AM4054 the fraction of available receptors were reduced to 78% and 72% of control levels, respectively. The higher dose of 3.0 mg/kg AM6538 resulted in 12% of receptors remaining for THC, and 31% and 27% for WIN 55,212 and AM4054, respectively. The initial *tau* value for AM4054 in the



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squirrel monkey discrimination studies was higher than those obtained for any of the cannabinoid agonists in mice, yet also was highly susceptible to reduction by AM6538: the fraction of remaining receptors was 18% after treatment with 1 mg/kg AM6538 and was further reduced to 4% after 3 mg/kg.

Table 4 presents *tau* values for THC and AM4054 at different times after 10 mg/kg AM6538 in antinociceptive studies in mice, and for AM4054 after 3 mg/kg AM6538 in drug discrimination studies in monkeys. The dose of 10 mg/kg AM6538 initially reduced the *tau* value of THC to 0.4, indicating only 9% of available receptors remained; these values increased to 29% at 48 h and 69% at 7 days following the administration of 10 mg/kg AM6538. The *tau* values for AM4054 also increased progressively over time. In mice, the dose of 10 mg/kg AM6538 yielded a *tau* value for AM4054 of 1.0, reflecting 10% of receptors remained available and this increased to 25% and 41% at 24 h and 48 h after AM6538, respectively. Full recovery occurred 7 days after AM6538 and, possibly, even a slight overshoot occurred, as the *tau* value of 14.6 reflected 145% of the baseline receptor availability. The AM4054 *tau* values obtained in squirrel monkeys also increased over time and the fraction of available receptors at 48 h and 7 days were 14% and 46%, respectively.

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**Discussion**

The three cannabinoid agonists tested – THC, WIN 55,212, and AM4054 - all produced maximum antinociception in mice when warm water was used as the nociceptive stimulus. Previous studies have shown that the antinociceptive effects of THC and WIN 55,212 are antagonized by rimonabant, consistent with CB1-receptor mediation (Marshell et al., 2014; Rinaldi-Carmona et al., 1994). The antagonism of AM4054-induced antinociception by rimonabant in the present studies confirm that these effects also are also likely to be CB1-receptor mediated. As expected for a competitive antagonist, rimonabant pretreatment resulted in surmountable rightward shifts of the AM4054 dose-effect function. There were some lingering antagonist effects of the highest dose of rimonabant tested, 10 mg/kg, at 24 hours after injection, however these effects were small. These results are in keeping with previous characterizations of the pharmacokinetic profile of rimonabant in mice (Petitet et al., 1999; Rinaldi-Carmona et al., 1994).

Like rimonabant, AM6538 also dose-dependently antagonized the antinociceptive effects of the three cannabinoid agonists in mice. Lower pretreatment doses of AM6538 produced relatively modest rightward shifts of the agonist does-effect functions, whereas higher AM6538 doses produced larger rightward shifts and decreased the maximum effect of each agonist. Moreover, AM6538 produced enduring antagonism, with robust effects on the THC dose-effect function evident up to 7 days after treatment.

The effects of AM4054 were further studied in a drug discrimination assay in squirrel monkeys. Previous studies from this laboratory have demonstrated that these effects of AM4054 are CB1-receptor mediated in squirrel monkeys (Kangas et al., 2013). As was seen in the antinociception studies, AM6538 also dose-dependently shifted the dose-effect function for the

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discriminative stimulus effects of AM4054 rightward, and these effects persisted for at least four to seven days. Taken together, our results in male subjects demonstrate that AM6538 can be an effective long-acting cannabinoid antagonist *in vivo*, across different types of behavioral assays and in different species.

AM6538 is a structural analog of rimonabant that was developed as a ligand capable of stabilizing the CB1 receptor. Functional *in vitro* studies recently revealed that the interaction between CP 55,940 and AM6538 is competitive when the ligands are co-administered, yet pre-incubation of CB1 receptors with AM6538 results in wash-resistant binding, suggesting that it forms noncovalent, but tight, attachments with cannabinoid CB1 receptors (Hua et al., 2016). Although the chemical nature of this interaction remains to be determined, the long duration of action and decreases in maximal agonist effects reported here in AM6538-treated animals is consistent with the conclusion that AM6538 acts as a pseudo-irreversible CB1 receptor antagonist *in vivo*.

Receptor elimination has been used in the past to identify receptor mechanisms of particular drug responses, to isolate receptors of interest, and to characterize drug efficacy. Advances in genetic disruption have led to increased use of transgenic mice to address these issues, no doubt due to the selectivity afforded by gene targeting, as well as the possibility of complete elimination of the receptor in question (Rohrer and Kobilka, 1998). Nonetheless, pharmacological manipulation of receptor number has distinct advantages, namely the ability to use graded doses to produce graded effects and, of translational value, cross-species verification of effect. We took advantage of these properties to determine the relative efficacy of different cannabinoid agonists in mice, and for a single agonist in two species.

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Apparent agonist efficacy was quantified by obtaining *tau* values using the operational model of receptor function, as derived by Black and Leff (Black et al., 1985). Most studies that have employed this analysis used alkylating ligands to decrease receptor number; however, similar analyses have been applied using either pseudo-irreversible antagonists or antisense (Chen et al., 1996; Walker et al., 1998). In the present studies, the pseudo-irreversible antagonist AM6538 reduced the fraction of available receptors for THC more than for either WIN 55,212 or AM4054. In conjunction with prior studies using more indirect methods to study THC efficacy, these data support the view that THC is a partial agonist at cannabinoid CB1 receptors (Hruba et al., 2012; Paronis et al., 2012; Shen and Thayer, 1999).

In addition to their translational value, another benefit of using pharmacological methods to decrease available receptors is the ability to track recovery of function. Accordingly, the *tau* values for AM4054 and THC increased progressively over time following pre-treatment with AM6538 in the present studies, reflecting time-dependent recovery of the receptor population. Although absolute *tau* values were different in the mouse antinociceptive studies and squirrel monkey drug discrimination studies, we found qualitatively similar effects of AM6538 as an antagonist of AM4054 in both mice and squirrel monkeys. Thus, the *tau* values obtained in both species decrease systematically as a function of AM6538 dose and showed recovery is a function of time after administration.

In summary, the data presented here are consistent with the view that AM6538 acts as a pseudo-irreversible cannabinoid antagonist in different behavioral assays and species and that AM6538 can be effectively used to evaluate the apparent efficacy of cannabinoid full and partial agonists. A similar type of analysis may be applicable to other cannabinoid drugs and other

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behavioral assays. Additionally, AM6538 may be useful in future studies which require temporary reductions in cannabinoid receptor availability.

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## AUTHORSHIP CONTRIBUTION

Participated in research design: Paronis, Chopda, Bergman

Conducted experiments: Chopda, Zakarian

Contributed new reagents: Vemuri, Makriyannis

Performed data analysis: Paronis, Chopda

Wrote or contributed to the writing of the manuscript: Paronis, Chopda, Bergman

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**FOOTNOTES**

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## FIGURE LEGENDS

**Figure 1**. AM6538 antagonizes the antinociceptive effects of cannabinoid agonists in mice. Panel A: Effects of AM4054 after 1 hour pretreatment with vehicle or 0.3-10.0 mg/kg AM6538; Panel B: Effects of THC after 1 hour pretreatment with vehicle or 0.1-3.0 mg/kg AM6538; Panel C: Effects of WIN 55,212 after 1 hour pretreatment with vehicle or 0.3 or 3.0 mg/kg AM6538. Each point represents the mean of 6-8 mice; vertical bars indicate ±SEM. Ordinates: percent of maximal antinociceptive effect; Abscissae: cumulative drug dose in mg/kg.

**Figure 2**. Antagonism the antinociceptive effects of AM4054 (panel A) or THC (panel B) 1 hour after administration of vehicle (open symbols) or at different times, 1 hour – 7 days, after administration of 10 mg/kg AM6538 in mice; other details as in figure 1. Data in vehicle treated animals and 1 h after AM6538 are re-plotted from Figure 1 to facilitate comparison.

**Figure 3**. Antagonism of the antinociceptive effects of AM4054 by rimonabant in mice. Panel A: effects of AM4054 after 30 min pretreatment with 1-10 mg/kg rimonabant; Panel B: effects of AM4054 after 30 min pretreatment with vehicle or 30 min or 24 hour pretreatment with 10 mg/kg rimonabant; other details as in figure 1.

**Figure 4**. Discriminative stimulus effects of AM4054 in squirrel monkeys alone or 1 hour after pretreatment with 1 or 3 mg/kg AM6538. Each point represents the mean of 3-6 monkeys; vertical bars indicate ±SEM. Ordinates: (top) percent responding on the AM4054-associated lever, (bottom): response rates expressed as responses per second. Abscissae: cumulative drug



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dose in mg/kg body weight; points above "S" and "AM" show control data from saline and 0.01 mg/kg AM4054 training cycles, respectively**.**

**Figure 5.** Discriminative stimulus effects of AM4054 at different times, 1 hour – 7 days, after administration of 3 mg/kg AM6538 in monkeys; other details as in Figure 4. Data in vehicle treated animals and 1 h after AM6538 are the same as those presented in Figure 4.

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Table 1. $ED_{50}$ values in mg/kg (with 95%CI) obtained in the mouse antinociception assay following 1 hour pretreatment with AM6538.

| AM6538 pretreatment | Agonist | | |
|---|---|---|---|
| | THC | WIN 55,212 | AM4054 |
| Control | 18.2 (11.9, 27.8) | 2.8 (1.9, 4.4) | 0.38 (0.29, 0.50) |
| 0.1 mg/kg | 31.4 (16.1, 61.2) | ---[a] | ---[a] |
| 0.3 mg/kg | >300[b] | 4.0 (2.4, 6.7) | 0.56 (0.36, 0.87) |
| 3 mg/kg | >300[b] | 18.0* (8.1, 40.4) | 2.47* (1.18, 5.21) |

[a] not determined

[b] Maximum obtained effect <50%, $ED_{50}$ value not calculated

* Different from control value ($P < 0.05$)

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Table 2. THC and AM4054 $ED_{50}$ values in mg/kg (with 95%CI) obtained in the mouse antinociception assay at various times after pretreatment with 10 mg/kg AM6538.

| Time after 10 mg/kg AM6538 | Agonist | |
|---|---|---|
| | THC | AM4054 |
| 1 h | >300[b] | >10[b] |
| 24 h | ---[a] | 2.46*<br>(1.69, 3.58) |
| 48 h | 196.8*<br>(85.8, 451.6) | 1.22*<br>(0.98, 1.51) |
| 7 days | 32.3<br>(19.6, 53.2) | 0.25<br>(0.20, 0.33) |

[a] not determined

[b] Maximum obtained effect <50%, $ED_{50}$ value not calculated

* Different from control value (presented in Table 1, P <0.05)

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Table 3 – *Tau* values (with 95%CI) obtained 1 hour after AM6538 in mice (antinociception) or monkeys (drug discrimination).

| | Agonist | | | |
|---|---|---|---|---|
| AM6538 pretreatment | THC (mouse) | WIN 55,212 (mouse) | AM4054 (mouse) | AM4054 (monkey) |
| control | 4.6 (1.6, 13.6) | 6.8 (1.3, 35.0) | 10.1 (2.4, 42.8) | 60.1 (10.8, 333.6) |
| 0.1 mg/kg | 3.2 (1.3, 7.9) | ---[a] | ---[a] | ---[a] |
| 0.3 mg/kg | 1.3 (0.8, 2.0) | 5.4 (1.1, 26.0) | 7.3 (1.8, 28.9) | ---[a] |
| 1 mg/kg | ---[a] | ---[a] | ---[a] | 10.7 (2.1, 54.6) |
| 3 mg/kg | 0.6* (0.4, 0.9) | 2.1* (0.9, 5.4) | 2.7 (1.1, 7.0) | 2.2* (0.9, 5.4) |
| 10 mg/kg | 0.4* (0.2, 0.7) | ---[a] | 1.0* (0.6, 1.7) | ---[a] |

[a] not determined

* Different from control value ($P < 0.05$)

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Table 4– *Tau* values (with 95%CI) obtained at different times after 10 mg/kg AM6538 in mice (antinociception) or 3 mg/kg AM6538 in monkeys (drug discrimination).

| | Agonist | | |
|---|---|---|---|
| Time after AM6538 | THC (mouse) | AM4054 (mouse) | AM4054 (monkey) |
| | 10 mg/kg AM6538 | | 3 mg/kg AM6538 |
| control | 4.6 (1.6, 13.6) | 10.1 (2.4, 42.8) | 60.1 (10.8, 333.6) |
| 1 h | 0.4* (0.2, 0.7) | 1.0* (0.6, 1.7) | 2.2* (0.9, 5.4) |
| 24 h | ---[a] | 2.6 (1.0, 6.5) | ---[a] |
| 48 h | 1.3 (0.8, 2.1) | 4.1 (1.3, 13.2) | 8.5 (1.8, 40.4) |
| 4 day | ---[a] | ---[a] | 16.9 (3.2, 88.2) |
| 7 day | 3.2 (1.3, 7.7) | 14.6 (3.2, 65.8) | 27.5 (4.9, 154.5) |

[a] not determined

* Different from control value ($P < 0.05$)



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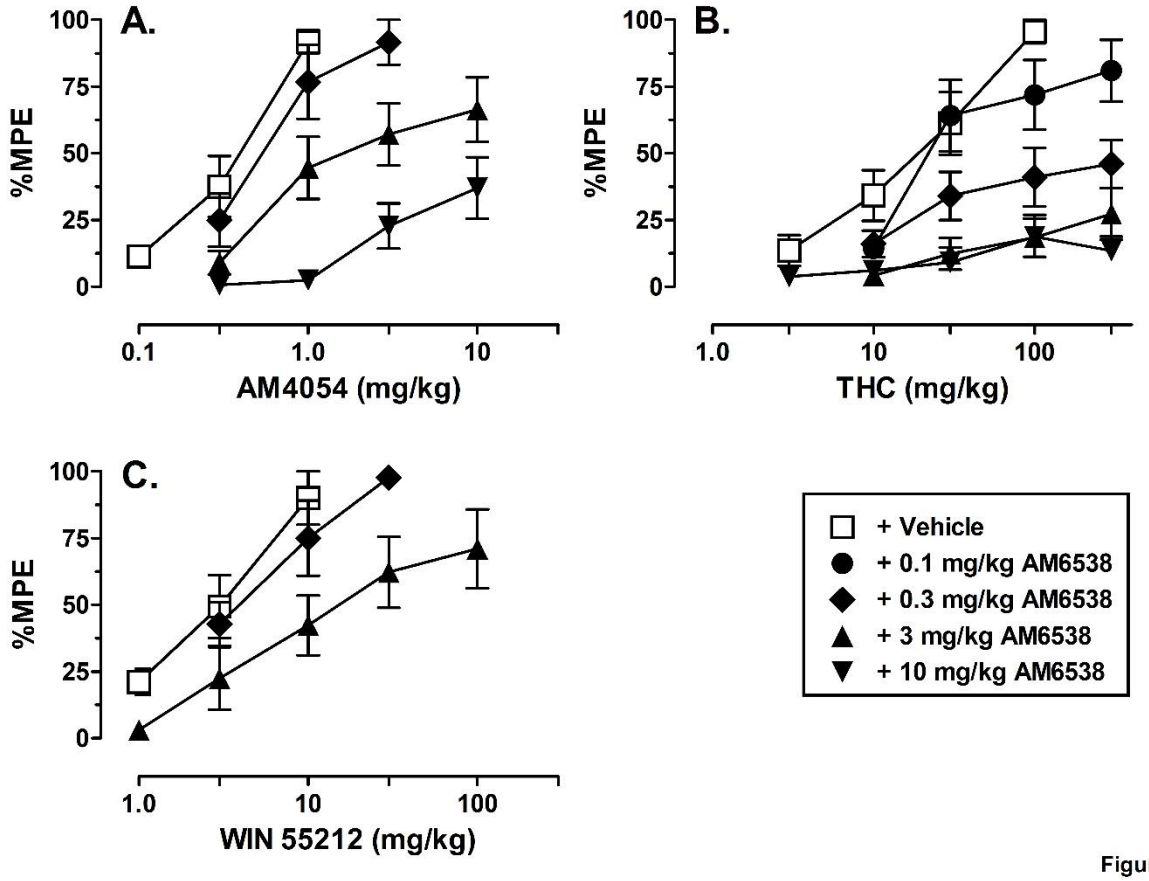


Figure 1

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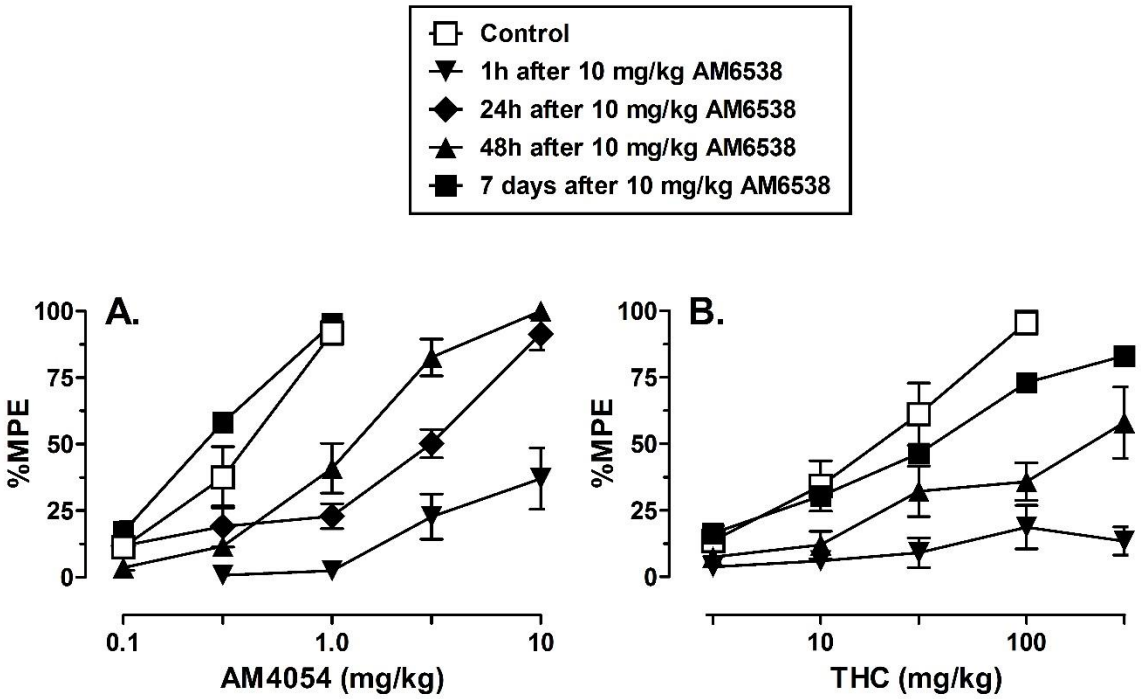


**Figure 2**

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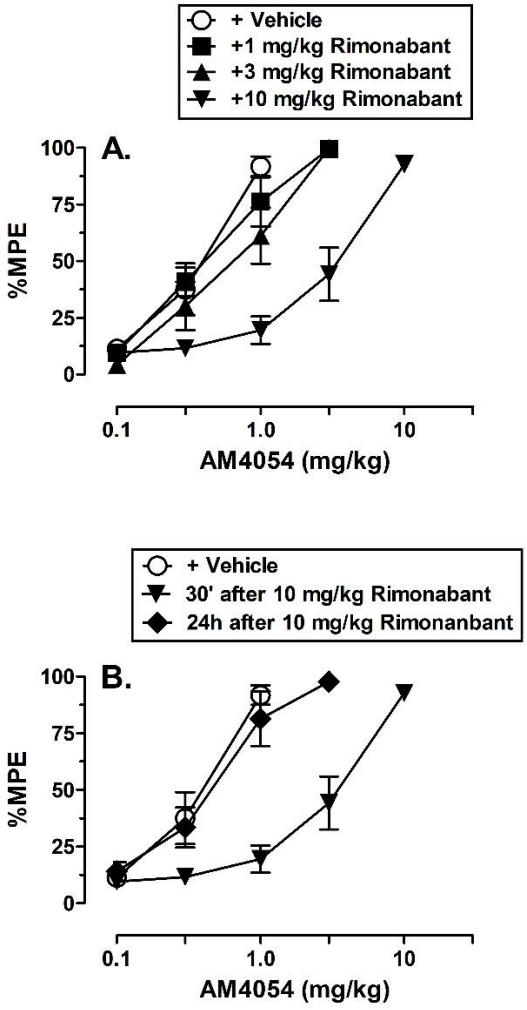


**Figure 3**

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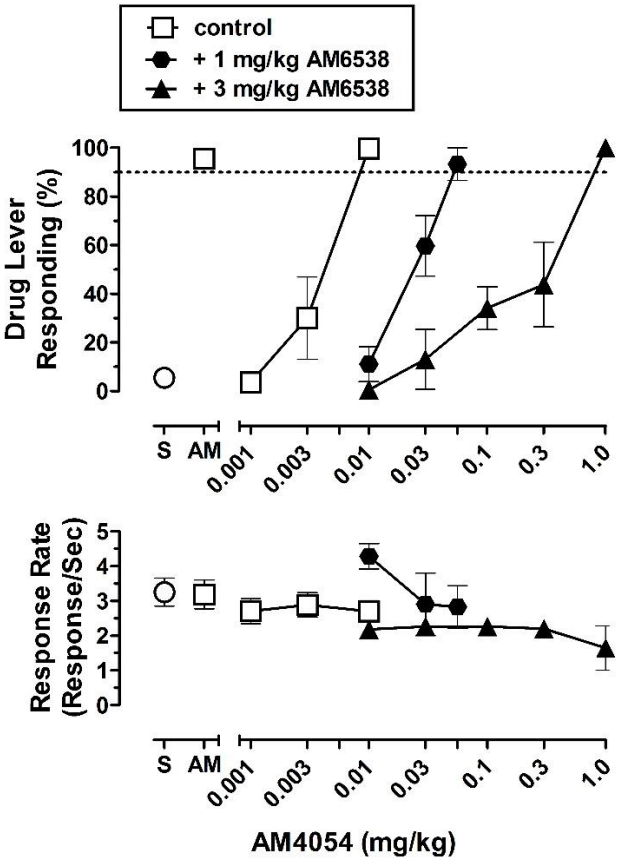


Figure 4

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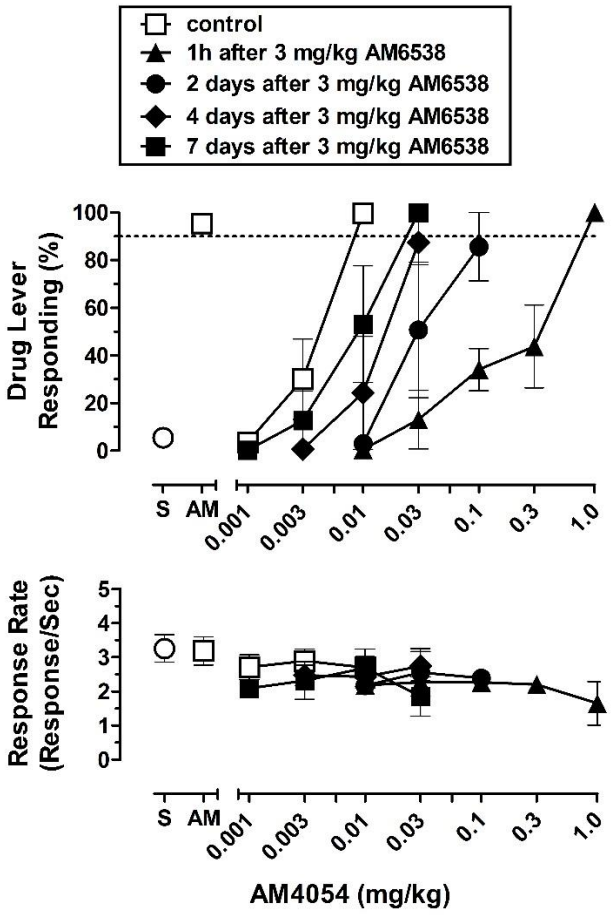


Figure 5

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