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Assessment of rimonabant-like adverse effects of purported CB1R neutral antagonist / CB2R agonist aminoalkylindole derivatives in mice

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Abstract

Background: Cannabinoids may be useful in the treatment of CNS disorders including drug abuse and addiction, where both CB1R antagonists / inverse agonists and CB2R agonists have shown preclinical efficacy. TV-5–249 and TV-6–41, two novel aminoalkylindoles with dual action as neutral CB1R antagonists and CB2R agonists, previously attenuated abuse-related effects of ethanol in mice. **Purpose:** To further characterize these drugs, TV-5–249 and TV-6–41 were compared with the CB1R antagonist / inverse agonist rimonabant in assays relevant to adverse effects and cannabinoid withdrawal.

Procedures and Findings: The cannabinoid tetrad confirmed that TV-5–249 and TV-6–41 were devoid of CB1R agonist effects at behaviorally-relevant doses, and neither of the novel drugs induced rimonabant-like scratching. Generalized aversive effects were assessed, and rimonabant and TV-5–249 induced taste aversion, but TV-6–41 did not. Schedule-controlled responding and observation of somatic signs were used to assess withdrawal-like effects precipitated by rimonabant or TV-6–41 in mice previously treated with the high-efficacy CB1R agonist JWH-018, or vehicle. Rimonabant and TV-6–41 dose-dependently suppressed response rates in all subjects, but TV-6–41 did so more potently in JWH-018-treated mice than in vehicle-treated mice, while rimonabant equally suppressed responding in both groups. Importantly, rimonabant elicited dramatic withdrawal signs, but TV-6–41 did not.

Conclusions: These findings suggest differences in both direct adverse effects and withdrawal-related effects elicited by rimonabant, TV-5–249 and TV-6–41, which could relate to neutral CB1R antagonism, CB2R agonism, or a combination of both. Both mechanisms should be

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6.1 Conflicts of interest

There are no conflicts of interest.

explored and exploited in future drug design efforts to develop pharmacotherapies for drug dependence.

Keywords

cannabinoid receptors; inverse agonist; neutral antagonist; scratching; taste aversion; precipitated withdrawal

1.1 Introduction

The endocannabinoid system is involved in various factors relevant to alcohol and drug abuse (Parsons and Hurd, 2015; Volkow *et al.*, 2017; Zlebnik and Cheer, 2016). In particular, the selective CB1R antagonist/inverse agonist rimonabant (formerly SR141716A) decreases alcohol consumption in animals, possibly by indirect modulation of dopaminergic neurotransmission (Arnone *et al.*, 1997; Vasiljevik *et al.*, 2013; Vinod *et al.*, 2008). In addition, activating CB2 receptors modulates the reinforcing effects of ethanol (Ishiguro *et al.*, 2007) and protects against alcohol-induced liver disease (Louvét *et al.*, 2011) in mice. Unfortunately, human trials of rimonabant relevant to drug abuse were discontinued due to adverse psychiatric effects, including suicidal ideation, nausea, seizure, anxiety and depression (Alger, 2013; Moreira and Crippa, 2009), and similar adverse events resulted in its withdrawal from the European market, where it had previously been used as a weight loss drug. These adverse events have been ascribed to the inverse agonist properties of rimonabant at CB1Rs in the CNS (Bergman *et al.*, 2008; Tai *et al.*, 2015); thus, the development of a drug combining CB1R neutral antagonist and CB2 agonist properties may offer a new strategy for treating alcohol and drug abuse with a reduced potential for psychiatric side effects.

We previously demonstrated that a metabolite (M4) of the indole-derived synthetic cannabinoid naphthalen-1-yl-(1-butyldol-3-yl)methanone (JWH-073) acts as a neutral antagonist of CB1R-mediated G-protein activation and attenuates hypothermia induced by the CB1R agonist naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) in mice, although M4 unexpectedly exhibited low efficacy CB1R agonist properties in terms of inhibiting activity of the downstream intracellular effector adenylyl cyclase (Brents *et al.*, 2012). In our efforts to develop improved compounds with pure neutral antagonist properties at CB1R, we have also reported that two different analogues of M4, 1-butyl-7-methoxy-3-(naphthalen-1-ylmethyl)-1H-indole (TV-5–249) and 1-butyl-7-methoxy-3-(naphthalen-2-yl)-1H-indole (TV-6–41), demonstrated markedly reduced CB1R efficacy relative to M4 and acted as full CB2 agonists in the *in vitro* adenylyl cyclase assay (Vasiljevik *et al.*, 2013). Both analogues antagonized CB1R-mediated hypothermia *in vivo*, blocked establishment of an ethanol conditioned place preference, and decreased voluntary consumption of a 10% ethanol solution (Vasiljevik *et al.*, 2013). Collectively, these data suggested that indole-derived cannabinoids may serve as a probe for the development of treatments for alcohol dependence.

With specific regard to cannabis dependence, it is not currently known whether abuse of high-efficacy synthetic cannabinoids (SCBs) would result in a more extreme abstinence

syndrome than typically observed following discontinuation of marijuana use, but reports of SCB withdrawal have accumulated in the literature (Zimmermann *et al.*, 2009, Nacca *et al.*, 2013, Macfarlane and Christie, 2015, Sampson *et al.*, 2015). Given the adverse effects associated with acute and long-term abuse of SCBs (Fantegrossi *et al.*, 2014, Hohmann *et al.*, 2014, Luciano and Perazella, 2014; Cooper 2016) it is increasingly apparent that research into potential therapeutics is warranted. Currently, there is no accepted medical treatment for cannabinoid dependence. Rimonabant remains the most well-researched pharmacotherapeutic for the treatment of cannabinoid dependence, but the inverse agonist properties of this drug result in not only attenuation of the pharmacological effects elicited by cannabinoid agonists, but also in disruption of constitutive CB1R activity. This suggests that cannabinoid withdrawal precipitated by rimonabant, and the direct adverse effects of rimonabant in cannabinoid agonist-naïve subjects, may not be solely attributed to antagonism of CB1Rs, but may also be exacerbated by the negative efficacy of rimonabant at those binding sites. The role of CB2Rs in cannabinoid withdrawal is not well understood, but administration of CB2R agonists does not result in tolerance to CB1R-mediated effects or exacerbate CB1R-mediated cannabinoid withdrawal (Deng *et al.*, 2015). There is growing interest in the therapeutic potential of CB2R agonists to treat numerous clinical symptoms which overlap the adverse effects induced by rimonabant, including nausea (Nevalainen, 2014, Malik *et al.*, 2015), seizure and convulsion (Pertwee, 2012, Agar, 2015), and anxiety and depression (Bahi *et al.*, 2014, Kim and Li, 2015). Taken together, these findings suggest that recruitment of CB2R signaling may both lessen the intensity of cannabinoid withdrawal and protect against the adverse effects observed following rimonabant administration.

Here we test the hypothesis that drugs that combine neutral CB1R antagonism with CB2R agonism may have rimonabant-like utility as pharmacotherapeutics for cannabinoid dependence without the treatment-limiting adverse effects profile. TV-5-249 (Figure 1, left) and TV-6-41 (Figure 1, right) were tested in several *in vivo* assays to assess their behavioral effects in comparison to the classical CB1R agonist ⁹-tetrahydrocannabinol (THC), the high-efficacy CB1R agonist naphthalen-1-yl-(1-pentyl-1H-indol-3-yl)methanone (JWH-018), or rimonabant. Initially, TV-5-249 and TV-6-41 were screened for antinociceptive, cataleptic, hypothermic and locomotor suppressant effects in the cannabinoid tetrad assay (Little *et al.*, 1988, Compton *et al.*, 1992) to ensure that no effects consistent with CB1R agonism were observed *in vivo* at doses previously shown to attenuate alcohol-related behaviors and to inhibit hypothermic effects of the SCB JWH-073 in mice (Vasiljevik *et al.*, 2013). Next, an assay of drug-induced scratching behavior was used to further compare these novel aminoalkylindoles with rimonabant in terms of aversive pruritic effects (Schlosburg *et al.*, 2011). We also attempted to compare the compounds with rimonabant in behavioral assays to model aspects of adverse psychiatric effects in the mouse. Conditioned taste aversion was used to model generalized undesirable effects, as numerous CNS-active drugs which induce aversive effects decrease behavior directed towards a novel taste stimulus paired with their administration (Davis and Riley, 2010). Additionally, precipitated withdrawal was measured by scoring observable signs (e.g., Lichtman and Martin, 2002; Ford *et al.*, 2017) and by using an assay of schedule-controlled responding (Emmett-Oglesby *et al.*, 1990) in JWH-018 pretreated mice subsequently administered rimonabant or TV-6-41.

2.1 Material and Methods

2.1.1 Animals

All studies were carried out in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (National Institute of Health, 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Experiments were conducted in male NIH Swiss mice (Harlan Sprague Dawley) weighing 20–25 g on delivery and group housed, 3 to a cage, in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. Mice were maintained on a 12-h light/dark cycle (lights on at 0700h, off at 1900h) in room conditions that were maintained at $22 \pm 2^\circ\text{C}$ and 45–50% humidity. Animals were fed Lab Diet rodent chow (Laboratory Rodent Diet no. 5001, PMI Feed, St Louis, MO) and, with the exception of mice used in operant assays of food maintained responding, were fed *ad libitum* until immediately before testing. Mice used in operant assays of food-maintained responding were food restricted for the duration of all studies to maintain their weights at approximately 30 g with supplemental feeding after daily behavioral sessions. All mice were drug-naïve prior to their respective experimental testing, and separate groups of mice were used for each experiment described below.

2.1.2 Cannabinoid Tetrad

Mice ($n=6$ per dose) were sequentially tested for hypothermia, antinociception, horizontal bar immobility (i.e., catalepsy) and locomotor suppression 60 min after injection, as previously described (Marshall *et al.*, 2014). After a 60 min pretreatment, hypothermia was measured using a rectal digital thermometer (model BAT-12, PhysiTemp, Clifton, NJ) equipped with a Ret-3 mouse probe (model 50314, Stoelting Co., Dale, IL). The lubricated probe was inserted approximately 2 cm into the rectum for approximately 6 sec prior to each recording. Immediately after removal of the rectal thermometer, antinociception was measured as tail flick latency using an EMDIE-TF6 radiant heat apparatus (Emdie Instrument Co., Montpelier, VA). Each mouse was placed on the stage of the apparatus, while the tail was positioned between a radiant heat source directed onto the dorsal surface, approximately 2 cm from the base of the tail, and an automated motion sensor, which terminated the heat source and recorded the latency to tail flick. Tail flick latencies were measured from the time of initial heat stimulus contact with the tail up to the time of the tail flick. Sensitivity and light intensity were set at 150 and 369, respectively (calibrated to produce a tail flick latency between 2 and 4 sec for untreated mice). The maximum time allowed for heat exposure was 10 sec to minimize tissue damage. As soon as mice exhibited the nociceptive tail-flick response, catalepsy was assessed by the horizontal bar test, utilizing a cylindrical steel bar (0.5 cm in diameter) that was supported 4.0 cm above and horizontal to a Plexiglas platform, which was covered with a paper towel to provide better traction. To begin the test trial, a mouse was placed into a species-atypical position with its forelimbs on the horizontal bar and its hindlimbs on the platform, in such a way that the mouse assumed a rearing posture. Catalepsy was measured as the length of time each mouse maintained both forelimbs in an elevated position on the bar. Mice that remained immobile (with the exception of respiratory movements) were considered cataleptic. The maximum time

allowed on the bar was 30 sec. Immediately following assessment of cataleptic effects, locomotor activity was measured using plastic activity boxes (26.67 cm x 20.96 cm x 15.24 cm). The base of each box was marked in order to divide it into four equally-sized quadrants. Each mouse was placed into a separate activity box and quadrant crossings (defined as all four paws crossing from one quadrant into another) were scored for 5 min using an overhead video camera. In this manner, all four measures were sequentially obtained, in this order, from each mouse used in these studies. Each mouse completed the entire tetrad assessment within 6 min after a 60 min pretreatment. Activity boxes were thoroughly cleaned and dried prior to each use.

2.1.3 Drug-elicited Scratching

Observation chambers were used to score scratching for each mouse (n=6 per group). Chambers consisted of a cylindrical clear glass container (radius 4.0 cm, height 16.5 cm) sealed with a ventilated cover, providing 360° of visibility of movement and ample air circulation. Observation chambers were used to score the duration of scratching within a 60 min period immediately following a single injection of either rimonabant, TV-5-249, TV-6-41 or vehicle. Scratching was defined as using the hind paw to scrape the skin. This behavior was chosen based on previous findings that reported a change in frequency of scratching after a single administration of a CB1R antagonist or when used to induce CB1R antagonist precipitated withdrawal (Cook *et al.*, 1998, Lichtman *et al.*, 2001). Data were collected in 5 min bins at the 5–10, 15–20, 25–30, 35–40, 45–50 and 55–60 min time intervals, then summed as total scratching scored per treatment.

2.1.4 Operant Responding

Mice (n=6 per group) were trained and tested in operant conditioning chambers (Med Associates, St. Albans, VT) enclosed within light- and sound-attenuating boxes. Fans mounted above the operant conditioning chambers ran during sessions to mask extraneous sounds that may have occurred during the session. Ambient light was provided by a bulb in the top center of the front panel (house light). Two lighted nose-poke apertures were located on the front panel of the chamber and were 12.1 cm apart, with the reinforcement aperture centered between them. A photobeam crossed the threshold of each nose-poke opening, and breaking a photobeam in either nose-poke was registered as a response and produced an audible click. Both nose-poke apertures were “active” in all sessions. Reinforced responses operated a liquid dipper, allowing a 5-sec access period to 0.01 ml of evaporated milk (Kroger Brand, Cincinnati, OH) that was 50% diluted with water. This access period was immediately followed by a 10-sec timeout in which the house light was turned off and no responses were registered. Control and data collection for training and testing sessions was accomplished with Med Associates interface and operating software.

Training sessions ended after either 60 min or after 60 reinforcements, whichever occurred first. Mice were incrementally trained to respond under a terminal fixed ratio (FR) 5 schedule reinforced by presentation of evaporated milk in daily sessions (i.e., 5 responses on the nose-poke aperture resulted in a milk presentation) using procedures similar to those previously described (Fantegrossi *et al.*, 2009, Murnane *et al.*, 2009). Initial training sessions used a FR1 schedule of reinforcement, meaning that a single response on either nose-poke

aperture resulted in a milk presentation. Every 20th reinforcer earned incremented the FR by 1, and mice were thus shaped to a terminal FR5 across sessions. Testing began when response rates varied no more than 20% for 3 consecutive training sessions.

2.1.5 Conditioned Taste Aversion (CTA)

Mice (n=6 per group) were trained for CTA as described above (see *2.1.4 Operant Responding*), and tested using procedures similar to those previously published (Hyatt and Fantegrossi, 2014). During CTA trials, an identical schedule was used to reinforce responding, but the milk reinforcer for these sessions was flavored with strawberry syrup (Kroger, Cincinnati, OH), at a ratio of 1 ml syrup to 8 ml milk. Immediately after each session where flavored milk was available, mice were removed from the chamber, injected with 10 mg/kg rimonabant, TV-5-249, TV-6-41 or saline, and then returned to the home cage in the colony room. Thus, the flavored milk reinforcer was contiguously paired with a drug or saline after each strawberry milk trial. Three “recovery” sessions, where unflavored milk reinforced responding, were interposed between each taste aversion trial.

2.1.6 Induction of Cannabinoid Dependence

Three days prior to JWH-018 administration, mice (n=6 per group) were handled and injected with the cannabinoid vehicle to habituate them to experimental conditions. Subsequently, the same animals were treated daily with 3.0 mg/kg JWH-018 for 5 consecutive days. Immediately prior to each injection, rectal temperatures were obtained as described above (see *2.1.2 Cannabinoid Tetrad*), and then re-determined 60 min later in order to gauge development of tolerance to hypothermic effects of the agonist. We have previously used this same regimen of JWH-018 administration in mice to induce dramatic and persistent tolerance to hypothermic effects, as well as downregulation and desensitization of CB1Rs in the hypothalamus (Tai *et al.*, 2016), and have observed robust rimonabant-precipitated observable withdrawal signs following this same dosing regimen (Ford *et al.*, 2017).

2.1.7 Precipitated Cannabinoid Withdrawal - observable withdrawal signs

After obtaining the final rectal temperature (see *2.1.6 Induction of Cannabinoid Dependence*), mice (n=6 per group) were administered 10 mg/kg rimonabant (to precipitate withdrawal), equivolume vehicle or 10 mg/kg TV-6-41. Immediately after these injections, mice were individually placed into observation chambers (described above in *Drug-elicited Scratching*) beneath a video camera, and behaviors were recorded for 60 minutes. Videos were subsequently scored for validated observable signs of cannabinoid withdrawal, including front paw tremor, face rubbing and rearing (e.g., Lichtman and Martin, 2002; Ford *et al.*, 2017).

2.1.8 Precipitated Cannabinoid Withdrawal - schedule-controlled responding

Mice (n=6 per group) were trained for this procedure as described above (see *2.1.4 Operant Responding*). Prior to precipitating withdrawal, mice were pretreated with 3 mg/kg

JWH-018 or vehicle once daily for 5 consecutive days (the same injection schedule as described in *2.1.6 Induction of Cannabinoid Dependence*). On Day 6, mice were

administered cumulative doses (0.1, 0.3, 1.0, 3.0 and 10.0 mg/kg) of rimonabant, TV-6–41 or saline and placed into operant chambers. Intermediate doses were assessed by administering supplemental injections to accumulate with previous injections (e.g., the first dose of 0.1 mg/kg and an intermediate dose of 0.2 mg/kg accumulated to the 0.3 mg/kg dose). After a 10 min pretreatment period following each injection, mice could respond as described above. Each dose-component of the behavioral session lasted for 10 min, or until 10 reinforcers were earned.

2.2 Drugs

All drugs were suspended in saline containing 1% ethanol and 7.8% Tween-80, and stored at 4°C until used. Injections were administered intraperitoneally (IP) in a volume of 0.01 cc / g. The doses of JWH-018 and THC were chosen based on earlier studies from our lab showing significant cannabimimetic effects of these doses in the mouse tetrad test (Marshall *et al.*, 2014; Ford *et al.*, 2017). These cannabinoid agonists acutely induce hypothermia, antinociception, catalepsy and suppress locomotor activity, and therefore served as positive controls of cannabimimetic activity. The doses of TV-5–249 and TV-6–41 were chosen based on our previous study with these compounds showing similar potency to rimonabant in attenuating hypothermic effects of JWH-073 and blunting alcohol-related behaviors in the mouse (Vasiljevic *et al.*, 2013). TV-5–249 was not measured for precipitated cannabinoid withdrawal due to the results of the CTA experiment (see Figure 4). Overall, previous findings from our laboratory provided justification for use of the single doses chosen for study in the tetrad, scratching, conditioned taste aversion, and observable withdrawal signs studies. Full dose-response assessments were performed in the studies of schedule-controlled responding, as we have not previously used that procedure in the context of cannabinoid dependence and withdrawal.

2.3 Data Analysis

Cannabinoid tetrad, scratching, and observable withdrawal signs studies used 6 mice per observation, and all animals were experimentally-naïve prior to study. Data from these were therefore compared via one-way ANOVA followed by Tukey's post-hoc test for all pairwise comparisons, except for rectal temperatures collected during the induction of cannabinoid dependence, which were statistically compared using a repeated measures one-way ANOVA, followed by Holm-Sidak's multiple comparison post-hoc test to compare each JWH-018 administration to the vehicle injection. Operant studies (conditioned taste aversion and schedule-controlled responding) also used 6 mice per group, but all subjects within a given group received multiple injections ("pairings" for the conditioned taste aversion experiments, and cumulative doses for the schedule-controlled responding experiments.) Response rate data from these studies utilizing operant responding were statistically compared using a repeated measures two-way ANOVA with one-factor repetition, followed by Holm-Sidak's multiple comparison post-hoc test for all pairwise comparisons. For these precipitated cannabinoid withdrawal studies, group ED₅₀ values were also calculated by averaging the individual interpolated ED₅₀ values for each animal in a treatment group, then each antagonist was compared to its respective vehicle group via *t*-test. All data are expressed as group means ± SEM, and any points without error bars indicate that the

variability was contained within the point. In all cases, statistical significance was judged at $p < 0.05$.

3.1 Results

3.1.1 Cannabinoid Tetrad

The cannabinoid tetrad assay was used to measure cannabimimetic effects of vehicle, 3 mg/kg JWH-018, 10 mg/kg THC, 10 mg/kg TV-5-249 or 10 mg/kg TV-6-41 at 60 min after IP injection. As compared to vehicle-treated mice, administration of 3 mg/kg JWH-018 or 10 mg/kg THC induced significant hypothermic responses ($q=14.829$ and 6.021 , respectively; $p<0.05$ for both tests), however, neither TV-5-249 nor TV-6-41 altered rectal temperature ($p>0.05$ for both comparisons) (Figure 2, upper left). Similarly, mice treated with 3 mg/kg JWH-018 and 10 mg/kg THC exhibited significant catalepsy on the horizontal bar test ($q=5.625$ and 4.618 , respectively; $p<0.05$ for both tests), but administration of TV-5-249 or TV-6-41 resulted in vehicle-like catalepsy scores ($p>0.05$ for both comparisons) (Figure 2, upper right). Administration of JWH-018, THC, TV-5-249 or TV-6-41 all resulted in vehicle-like latencies in the antinociception assay ($p>0.05$ for the overall ANOVA) (Figure 2, lower left). In the motor activity test, similar locomotor suppression was evident in mice administered JWH-018 or THC, although only motor activity elicited by JWH-018 administration was significantly different from vehicle ($q=4.622$; $p<0.05$). Neither TV-5-249 nor TV-6-41 altered locomotor activity as compared to vehicle-treated mice ($p>0.05$ for both comparisons) (Figure 2, lower right). Thus, for all tetrad measures, 10 mg/kg TV-5-249 or TV-6-41 did not induce any effects different from those observed following vehicle injection, suggesting that these ligands do not display *in vivo* effects typical of CB1R agonists at the doses tested.

3.1.2 Drug-elicited Scratching

Scratching elicited by IP injection of vehicle or 10 mg/kg rimonabant, TV-5-249 or TV-6-41 was scored in mice as previously described. As compared to vehicle (Figure 3, white bar), administration of rimonabant (Figure 3, black bar) robustly increased scratching behavior ($q=4.876$; $p<0.05$). Interestingly, injection of the novel aminoalkylindoles (Figure 3, grey bars) did not induce significantly more scratching behavior than vehicle ($p<0.05$ for both comparisons). The decreased capacity of TV-5-249 and TV-6-41 to elicit scratching compared to rimonabant is not likely a result of only potency differences among these compounds, as our previous studies with these three agents showed similar potencies against alcohol-related behaviors and to inhibit hypothermic effects of the SCB JWH-073 in mice (Vasiljevik *et al.*, 2013).

3.1.3 Conditioned Taste Aversion

Prior to pairing with post-session injections, the first session in which responding was reinforced by presentations of the novel flavored milk solution was characterized by high response rates which did not differ from those observed in sessions where unflavored milk was the reinforcer. The overall ANOVA revealed significant main effects of drug ($F=3.550$; $p<0.05$) and pairing ($F=8.424$; $p<0.05$), as well as a significant drug x pairing interaction ($F=1.896$; $p<0.05$). Repeated pairings of the flavored milk reinforcer with post-session saline

administration (Figure 4, open squares) did not significantly alter response rates as compared to those observed in the pre-injection trial (Figure 4, “0 pairing” condition) ($p>0.05$ for all comparisons). A similar finding was obtained in mice where the flavored milk reinforcer was repeatedly paired with 10 mg/kg TV-6-41 (Figure 4, grey inverted triangles), where response rates were again not significantly different from those observed in the pre-injection trial at any pairing ($p>0.05$ for all comparisons). However, repeated pairing of the flavored milk reinforcer with 10 mg/kg rimonabant (Figure 4, filled circles) significantly suppressed subsequent response rates on pairings 3 ($t=3.091$; $p<0.05$) and 4 ($t=3.764$; $p<0.05$), indicative of a conditioned taste aversion. Interestingly, a similar finding was obtained in mice repeatedly administered 10 mg/kg TV-5-241 after sessions where the flavored milk reinforcer was available (Figure 4, grey triangles), as significant decreases from the pre-injection trial rates were observed on pairings 3 ($t=4.120$; $p<0.05$) and 4 ($t=4.423$; $p<0.05$), again characteristic of a conditioned taste aversion. Across treatment conditions, response rates in the saline group did not differ from those observed in animals administered rimonabant, TV-5-249 or TV-6-41 during pairings 0, 1, or 2 ($p<0.05$ for all comparisons.) However, on pairings 3 and 4, significant differences in response rates were observed between saline and rimonabant groups ($t=2.978$ and 3.161 , respectively; $p<0.05$ for both comparisons), and between saline and TV-5-249 groups ($t=3.132$ and 3.028 , respectively; $p<0.05$ for both comparisons.) No significant differences in response rates between saline and TV-6-41 groups were obtained at any pairing ($p<0.05$ for all comparisons). Because TV-5-249 exhibited a rimonabant-like profile in this assay of generalized adverse effects, it was not studied further. However, since TV-6-41 did not induce a conditioned taste aversion, it was further compared with rimonabant in assays of cannabinoid withdrawal.

3.1.4 Induction of Cannabinoid Dependence

As expected, administration of 3 mg/kg JWH-018 elicited a significant hypothermic response 60 min after injection (Figure 5, top left) ($F=87.078$; $p<0.05$), and this effect diminished with daily administration. When compared to temperatures measured 60 min after vehicle injection, the first four administrations of 3 mg/kg JWH-018 elicited significant hypothermic responses ($t=17.890$, 11.620 , 8.513 and 6.181 ; $p<0.05$ for all comparisons). The fifth and final injection of JWH-018 did not elicit significant hypothermia ($t=1.679$; $p>0.05$), indicating the development of complete tolerance to this CB1R-mediated effect with repeated drug administration, perhaps implying the induction of dependence.

3.1.5 Precipitated Cannabinoid Withdrawal - observable withdrawal signs

Mice treated daily with 3 mg/kg JWH-018 for five consecutive days exhibited robust and statistically significant cannabinoid withdrawal signs of face rubbing ($t=7.283$; $p<0.05$) (Figure 5, top right), front paw tremor ($t=4.396$; $p<0.05$) (Figure 5, bottom left), and rearing ($t=8.653$; $p<0.05$) (Figure 5, bottom right) when administered 10 mg/kg rimonabant 60 min after the final JWH-018 injection, as compared to mice administered an equivolume injection of the cannabinoid vehicle. In contrast, mice receiving 10 mg/kg TV-6-41 exhibited dramatically reduced withdrawal signs as compared to rimonabant-treated animals. Very low levels of face rubbing were observed following treatment with TV-6-41, such that mice in this treatment group differed significantly from both vehicle-treated ($t=5.297$;

$p < 0.05$) and rimonabant-treated animals ($t = 12.580$; $p < 0.05$). Paw tremor occurred at vehicle-like levels in mice administered TV-6–41 ($t = 0.513$; $p > 0.05$), and this was significantly different than mice treated with rimonabant ($t = 4.909$; $p < 0.05$). Similarly, rearing was also observed at vehicle-like levels in mice administered TV-6–41 ($t = 0.487$; $p > 0.05$), and this was significantly different than mice treated with rimonabant ($t = 9.366$; $p < 0.05$).

3.1.6 Precipitated Cannabinoid Withdrawal - schedule-controlled responding

Mice treated daily with 3 mg/kg JWH-018 or vehicle for five consecutive days maintained high and stable response rates when repeatedly administered saline injections in a four-component test session. No systematic rate-decreasing or rate-increasing effects were observed in any subjects following repeated saline administration (Figure 6). Cumulative doses of rimonabant (Figure 6, left panel) dose-dependently suppressed response rates with a similar potency in mice previously treated with vehicle or JWH-018, with the overall ANOVA revealing significant effects of dose ($F = 22.036$; $p < 0.05$), but not of pretreatment ($F = 1.038$; $p > 0.05$) and no significant interaction ($F = 1.140$; $p > 0.05$). The ED_{50} for rimonabant-induced rate suppression was 0.67 ± 0.11 mg/kg for the vehicle-treated subjects and 0.51 ± 0.20 mg/kg for the JWH-018-treated animals, and a t-test on these values did not reach statistical significance ($t = 0.887$; $p > 0.05$). Similar to rimonabant, TV-6–41 also dose-dependently suppressed response rates in both treatment groups, however, here the overall ANOVA revealed significant main effects of dose ($F = 17.225$; $p < 0.05$) and pretreatment ($F = 6.818$; $p < 0.05$), as well as a significant dose $\times$ pretreatment interaction ($F = 3.029$; $p < 0.05$). The effects of TV-6–41 on response rates were significantly more potent (approximately 5-fold) in JWH-018-treated animals than in vehicle-treated animals, with an ED_{50} of 4.98 ± 1.30 mg/kg in vehicle-treated animals and ED_{50} of 1.04 ± 0.34 mg/kg in mice exposed to JWH-018 ($t = 57.00$; $p < 0.05$). In addition, within-dose comparisons showed significant differences between vehicle and JWH-018 pretreatments at the 1.0 and 3.0 mg/kg TV-6–41 dose conditions ($t = 4.263$ and 3.087 , respectively; $p < 0.05$ for both comparisons.)

4.1 Discussion

In these studies, we compared the effects of novel CB1R neutral antagonist / CB2R agonist aminoalkylindoles TV-5–249 and TV-6–41 with THC and JWH-018 to assess cannabimimetic effects, and also to the CB1R antagonist / inverse agonist rimonabant in several assays modeling adverse effects and cannabinoid withdrawal. The hypothesis tested by these experiments was that drugs with a mechanism of action combining neutral CB1R antagonism with CB2R agonism may have rimonabant-like utility as a pharmacotherapeutic for cannabinoid dependence without the treatment-limiting adverse effects profile. Overall, the results of these studies supported that hypothesis. It was determined that neither TV-5–249 nor TV-6–41 possessed effects consistent with *in vivo* CB1R agonism, as measured in the cannabinoid tetrad assay, at doses previously shown to blunt JWH-073-elicited hypothermia and alcohol-related behaviors in mice (Vasiljevik *et al.*, 2013). This is consistent with all of our previous *in vitro* studies with these compounds (Vasiljevik *et al.*, 2013), and suggests that the lack of significant CB1R agonism observed in binding studies is recapitulated *in vivo*. In comparison to the same dose of rimonabant, we observed dramatically attenuated scratching behavior in mice treated with either TV-5–249 or TV-6–

41. However, despite very similar pharmacological profiles in our initial studies, TV-5–249 induced a rimonabant-like conditioned taste aversion, whereas TV-6–41 had no apparent aversive effects at this dose in this assay. For this reason, only TV-6–41 was further studied in the precipitated cannabinoid withdrawal assays, where we found intriguing differences between the effects of TV-6–41 and rimonabant.

Antagonist-induced pruritus is purportedly induced by CB1R inverse agonism as opposed to neutral antagonism (Bergman *et al.*, 2008; Tai *et al.*, 2015), but until now this theory has not been directly tested *in vivo* due to the lack of a suitable neutral antagonist (Aceto *et al.*, 1996; Janoyan *et al.*, 2002). The minimal scratching observed following treatment with active doses of TV-5–249 and TV-6–41 is novel and could be a result of their lack of negative efficacy at CB1Rs. Alternatively, these compounds may simply be less potent than rimonabant in this regard, although our previous work showed TV-5–249 and TV-6–41 to be active within the same dose range as rimonabant (Vasiljevik *et al.*, 2013). Nevertheless, since these novel aminoalkylindoles also have efficacy at CB2Rs, it is also possible that CB2R agonism plays a part in their reduced pruritic effects. A more refined mechanistic explanation will be developed by further examining the effects of TV-5–249 and TV-6–41 on scratching behavior in the presence of a selective CB2R antagonist, such as SR144528 or AM630.

One of the most common adverse events associated with rimonabant use in human trials was patients experiencing nausea (FDA Briefing Document, 2007). The taste reactivity test in rats is a standard model for measuring anticipatory nausea by pairing a nausea-inducing substance with an intra-oral stimulus, resulting in the display of conditioned gaping behavior (often interpreted as subjective “disgust”) (Grill and Norgren, 1978, Parker, 2003). In this assay, the CB1R antagonist / inverse agonist AM251 induced conditioned gaping in rats, but neither the centrally active CB1R neutral antagonist AM4113 nor the peripherally constrained CB1R neutral antagonist AM6545 elicited signs consistent with nausea (McLaughlin *et al.*, 2005, Sink *et al.*, 2008, Cluny *et al.*, 2010). This suggests that nausea is not triggered by CB1R antagonism, but rather by inverse agonist efficacy at central CB1Rs. CTA can similarly be used to examine generalized undesirable effects such as nausea in rodents. In the present study, rimonabant produced a strong CTA over several pairings, perhaps consistent with its previously-demonstrated capacity to induce a nausea-like state mediated through CB1Rs. The lack of CTA produced by TV-6–41 may indicate that it does not induce a nausea-like state, which may be consistent with its lack of CB1R inverse agonist efficacy. Interestingly, TV-5–249 induced rimonabant-like aversive effects in this assay, which was surprising given that TV-5–249 produced much less scratching than rimonabant in the previous measure and exhibited a neutral CB1R antagonist profile *in vitro* (Vasiljevik *et al.*, 2013). The mechanism of the CTA induced by TV-5–249 remains unknown, but the drug clearly has a rimonabant-like aversive profile in this test, whereas its structural analogue TV-6–41 does not at the dose here tested. Since these assays were designed to identify compounds that did not induced adverse effects previously reported with rimonabant, only TV-6–41 was selected for further characterization in the precipitated cannabinoid withdrawal assays.

At the time of this report, few purported neutral CB1R antagonists exist because available CB1R antagonists also exhibit inverse agonist properties at CB1Rs. However, previous reports have identified important differences *in vitro* when directly comparing CB1R neutral antagonists to inverse agonists. For example, the CB1R antagonists / inverse agonists rimonabant and AM251 increased forskolin-stimulated cAMP levels (Sink *et al.*, 2008), but this effect was not observed in cells treated with the purported CB1R neutral antagonists AM4113 or O-2050 (Sink *et al.*, 2008; Wiley *et al.*, 2011). Furthermore, as previously mentioned, CB1R inverse agonists induced an apparent nausea-like state which was not induced by neutral antagonists (Chambers *et al.*, 2007; Sink *et al.*, 2008). Interestingly, food consumption and body weights in rats were equally reduced by both CB1R neutral antagonists and inverse agonists (Gardner and Mallet, 2006; Chambers *et al.*, 2007), although weight reduction was observed in mice during chronic treatment with rimonabant, but not with TV-5-249 or TV-6-41 (Vasiljevik *et al.*, 2013). These findings may indicate that CB1R neutral antagonists could provide the therapeutic effects of CB1R antagonism while minimizing the adverse effects profile characteristic of inverse agonists.

The present studies characterized cannabinoid withdrawal elicited by TV-6-41 and rimonabant in mice with a history of high efficacy cannabinoid agonist exposure. In JWH-018-treated mice, both TV-6-41 and rimonabant dose-dependently reduced response rates, with rimonabant being slightly more potent than the novel aminoalkylindole in this regard. Most importantly, rimonabant-induced rate suppression generally occurred with similar potency regardless of an animal's cannabinoid history, while TV-6-41 was about 5-fold more potent in suppressing rates in JWH-018-treated subjects than in cannabinoid-naïve subjects. This finding was unexpected, as we initially hypothesized that the severity of cannabinoid withdrawal induced by an inverse agonist would be greater than that of a neutral antagonist, and *in vitro* data demonstrate that neutral antagonists do not disrupt the constitutive activity of the CB1R, as indicated by a lack of effect on intracellular cAMP levels (Sink *et al.*, 2008; Cluny *et al.*, 2010; Vasiljevik *et al.*, 2013). Furthermore, CB1R inverse agonists elevate cAMP, disrupting the constitutive activity of the receptor, perhaps explaining why inverse agonists can produce dramatic *in vivo* effects even in the absence of prior agonist exposure. The failure to observe a difference in rimonabant-precipitated effects in mice pretreated with vehicle or JWH-018 might be explained by the proposition that direct rimonabant-induced suppression of response rate is a more salient effect than cannabinoid withdrawal. In other words, food-maintained behavior using these schedule parameters may be more sensitive to disruption via CB1R inverse agonist effects than by cannabinoid withdrawal. In contrast, the pattern of results obtained with TV-6-41 appears consistent with a withdrawal-mediated effect because prior treatment with the high-efficacy CB1R agonist JWH-018 dramatically increased the potency of TV-6-41 to disrupt ongoing food-maintained behavior. However, when examining observable withdrawal signs in mice challenged with rimonabant or TV-6-41 following the same daily JWH-018 regimen to elicit dependence, clear differences emerged. Rimonabant-treated animals exhibited robust and dramatic face rubbing, front paw tremor, and rearing, but none of these withdrawal-associated behaviors were different from vehicle controls in mice challenged with TV-6-41. Injection of TV-6-41 actually suppressed paw tremor to a level even lower than that observed in vehicle-treated animals, suggesting not only a reduced capacity to elicit

antagonist-precipitated withdrawal, but perhaps also a protective effect against at least some of the observable signs associated with spontaneous withdrawal.

5.1 Conclusions

These findings provide further evidence that adverse effects elicited by CB1R inverse agonists could perhaps be minimized by utilizing a neutral antagonist, while suggesting the recruitment of CB2R agonism may also attenuate some of the adverse effects associated with CB1R inverse agonists. Thus, “dual mechanism” drugs which function as neutral antagonists at CB1Rs and agonists at CB2Rs, like TV-6–41, may provide a clinical advantage over existing compounds with inverse agonist efficacy at CB1Rs. In recent years, a role for CB2Rs in drug addiction has been identified (Vlachou and Panagis, 2014), however the involvement of CB2Rs in drug dependence and withdrawal has yet to be fully elucidated.

These studies recognize the translational significance of discovering rimonabant-like cannabinoid drugs with reduced treatment-limiting adverse effects. We therefore attempted to characterize two such novel therapeutics through a rigorous battery of tests assessing several components of rimonabant-like adverse effects, using a screening regimen designed to exclude drugs from further study if they did not exhibit an improved profile as compared to rimonabant. It remains to be seen, however, if the findings here obtained with TV-6–41 would be maintained in a clinical trial. Despite the unknown translational relevance of the present drugs studied, our findings support the position that the clinical utility of CB1R inverse agonists is likely to be limited given the mounting evidence of their adverse effects, but rational drug design efforts may result in clinically useful novel therapeutic cannabinoid ligands if CB1R inverse agonist properties can be eliminated in favor of neutral antagonists and CB2R agonist properties can be added.

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Figure 1.
Structures of TV-5-249 (left) and TV-6-41 (right).

Figure 2.

Effects of vehicle; VEH (white bars), 10 mg/kg TV-6-41 and TV-5-249 (grey bars), 10 mg/kg THC or 3 mg/kg JWH-018 (black bars) in the cannabinoid tetrad. Measures of hypothermia (top left), catalepsy (top right), analgesia (bottom left) and motor suppression (bottom right) were assessed 60 min after injection. Asterisks indicate significant differences from vehicle ($p < 0.05$).

Figure 3. Effects of 10 mg/kg rimonabant (black bar), 10 mg/kg TV-5-249 or TV-6-41 (grey bars) or vehicle (white bar) on scratching behavior. Abscissa: injection condition. Ordinate: scratching score, expressed in seconds. Asterisk indicates significant difference from vehicle ($p < 0.05$).

Figure 4.

Effects of saline (white squares), 10 mg/kg rimonabant (black circles), 10 mg/kg TV-5-249 (grey triangles) or 10 mg/kg TV-6-41 (grey inverted triangles) on responding reinforced by presentation of a novel taste stimulus paired with their administration. *Abscissa*: number of pairings. Three “recovery” sessions were interposed between aversion trials, and injections were administered immediately after each trial. *Ordinate*: response rate, expressed as % of control. Asterisks indicate significant within-group differences from baseline response rate across pairings, while hash marks indicate significant between-group differences from saline within pairings ($p < 0.05$).

Figure 5.

Top left panel – Effects of daily administration of 3.0 mg/kg JWH-018 on rectal temperature in mice. *Abcissa*: vehicle test, or treatment day. *Ordinate*: change in rectal temperature, calculated as the difference in 60 min post-injection sample from pre-injection baseline. Asterisks indicate significant differences from vehicle test. **Top right panel** – Effects of vehicle, rimonabant or TV-6-41 on face rubbing in mice previously treated with JWH-018 for five days. *Abcissa*: injection of equivolume vehicle, or 10 mg/kg rimonabant or TV-6-41, administered 60 minutes after the final administration of 3.0 mg/kg JWH-018. *Ordinate*: duration of face rubbing scored during the epochs viewed over 60 min after injection. Asterisks indicate significant differences from vehicle ($p < 0.05$), and hash marks indicate significant differences from rimonabant ($p < 0.05$). **Bottom left panel** – Effects of vehicle, rimonabant or TV-6-41 on front paw tremor in mice previously treated with JWH-018 for five days. *Abcissa*: as described for top right panel. *Ordinate*: counts of paw tremor scored during the epochs viewed over 60 min after injection. Symbols for statistical comparisons as described for top right panel. **Bottom right panel** – Effects of vehicle, rimonabant or TV-6-41 on rearing in mice previously treated with JWH-018 for five days. *Abcissa*: as described for top right panel. *Ordinate*: rearing counts scored during the epochs viewed over 60 min after injection. Symbols for statistical comparisons as described for top right panel.

Figure 6.

Effects of saline and rimonabant (left panel) or saline and TV-6-41 (right panel) on milk-maintained responding in mice previously exposed to daily vehicle (white squares) or JWH-018 (black circles). *Abscissae*: number of consecutive saline injections, and cumulative dose of rimonabant (left) or TV-6-41 (right) in mg/kg. *Ordinates*: response rate, expressed as % of saline control. Asterisks indicate significant differences from response rates observed following saline administration ($p < 0.05$), and hash marks indicate significant differences between vehicle-treated and JWH-018-treated groups, within dose ($p < 0.05$).