Neuropharmacology and Analgesia

CP47,497-C8 and JWH073, commonly found in 'Spice' herbal blends, are potent and efficacious CB$_1$ cannabinoid receptor agonists

Brady K. Atwood [a,c], Donghoon Lee [b], Alex Straiker [a], Theodore S. Widlanski [b], Ken Mackie [a,*]

[a] The Gill Center and the Department of Psychological & Brain Sciences, Indiana University, 1101 E. 10th St, Bloomington, IN, 47405, USA
[b] Department of Chemistry, Indiana University, 800 E. Kirkwood Ave., Bloomington, IN, 47405, USA
[c] Program of Neurobiology and Behavior, University of Washington, T-471 Health Sciences Center, 1959 NE Pacific St, Seattle, WA, 98195, USA

ARTICLE INFO

ABSTRACT

'Spice' is an herbal blend that has been reported to produce cannabis-like effects when smoked and is marketed as an alternative to marijuana. Synthetic additives have been identified in numerous 'Spice' preparations from different sources. Common among many of the preparations were the compounds JWH018 and a dimethyloctyl variant of CP47,497 (CP47,497-C8) and, more recently JWH073. The synaptic effects of each of these compounds were uncharacterized. We previously reported that JWH018 is a potent and efficacious CB$_1$ cannabinoid receptor agonist. In this study we have examined the abilities of CP47,497-C8 and JWH073 to inhibit neurotransmission in cultured autaptic hippocampal neurons. Each inhibited EPSCs with an efficacy and potency similar to JWH018. We also analyzed these compounds' effects on promoting internalization of CB$_1$ receptors in HEK293 cells stably expressing CB$_1$ receptors. Similar to our neurotransmission data, CP47,497-C8 internalized CB$_1$ in a fashion indistinguishable from JWH018. However, JWH073 was less potent and produced slower internalization than JWH018 and CP47,497-C8. It appears that 'Spice' contains a number of cannabinoid receptor agonists that activate CB$_1$ receptors to inhibit synaptic transmission with similar potencies and efficacies. It is highly probable that the cannabis-like effects of 'Spice' are due to the presence of these and analogous synthetic additives acting on CB$_1$ receptors.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Cannabis sativa (cannabis, marijuana or hashish) is a widely used plant preparation with well-known psychoactive effects (Ameri et al., 1999; Costa, 2007; Howlett, 2002; Howlett et al., 2002; Pertwee, 2008). 'Spice' is an herbal blend that is used recreationally for its cannabis-like effects and promoted as an alternative to marijuana (Auwarter et al., 2009; Hudson et al., 2010; Lindigkeit et al., 2009; Uchiyama et al., 2010; Vardakou et al., 2010; Zimmermann et al., 2009). Its use as a recreational drug has resulted in numerous analyses of its chemical constituents (Auwarter et al., 2009; Lindigkeit et al., 2009; Uchiyama et al., 2010). These have led to changes in its legal status (Griffiths et al., 2010; Lindigkeit et al., 2009; McLachlan, 2009; Vardakou et al., 2010), although this has not been without debate (Hammersley, 2010). Recent reports confirm similar physiological responses from 'Spice' use and cannabis use (Muller et al., 2010; Zimmermann et al., 2009). Mass spectrometry analyses of different 'Spice' preparations reveal that these products contain diverse synthetic cannabinoid additives (Auwarter et al., 2009; Hudson et al., 2010; Lindigkeit et al., 2009; Uchiyama et al., 2010; Vardakou

et al., 2010). The cannabinoid JWH018 (Fig. 1A) was common among many of the different products first analyzed (Auwarter et al., 2009). These products also contain CP47,497-C8 (Fig. 1B), a variant of CP47,497 (extending the dimethylheptyl sidechain to a dimethyloctyl one) (Melvin et al., 1993). A third compound, JWH073 (Fig. 1C), the butyl homolog of JWH018, has appeared in more recently tested samples, replacing JWH018 in some cases (Lindigkeit et al., 2009). Interestingly Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of *Cannabis sativa*, was not reported present in the samples of 'Spice' that were analyzed, suggesting that the psychoactive effects were likely due to these synthetic cannabinoid additives (Auwarter et al., 2009).

The CB$_1$ cannabinoid receptor is the receptor primarily responsible for mediating the psychoactive effects of THC (Huestis et al., 2001; Monory et al., 2007). CB$_1$ is abundantly expressed in the central nervous system, particularly in areas linked to behaviors affected by THC (Mackie, 2005). In neurons, CB$_1$ is primarily found on axon terminals where it modulates neurotransmission (Chevaleyre et al., 2006; Katona et al., 2006; Mackie, 2005). CB$_1$ activation reduces cellular excitability and the probability of neurotransmitter release (Shen et al., 1996). This ability to inhibit neurotransmission allows both exogenous (such as THC) and endogenous cannabinoid agonists to modulate neuronal communication, and may underlie the psychoactivity of THC. Prolonged activation of CB$_1$ receptors results

---

* Corresponding author. Tel.: +1 812 855 2042; fax: +1 812 856 7187.
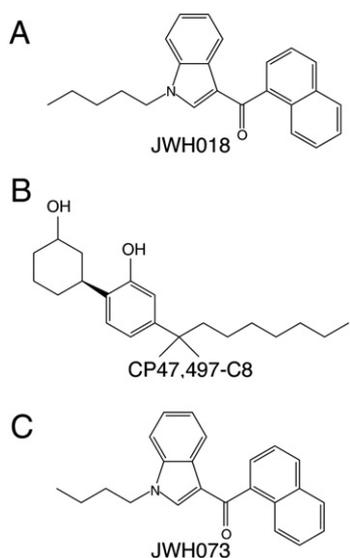*E-mail address:* kmackie@indiana.edu (K. Mackie).

Fig. 1. The chemical structures of (A) JWH018, (B) CP47,497-C8, and (C) JWH073.

characterization (Aung et al., 2000; Compton et al., 1992; Griffin et al., 1998; Melvin et al., 1984, 1993; Wiley et al., 1998). We sought to further characterize these two 'Spice' additives using assays that are relevant to the physiological effects of drugs of abuse: modulation of neurotransmission and promotion of receptor internalization.

2. Materials and Methods

2.1. Materials

Drugs and reagents were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Sigma-Aldrich (St Louis, MO, USA). JWH018 was generously provided by John Huffman and was synthesized as described in Huffman et al. (1994). Rimonabant (SR141716) was obtained from the National Institute of Drug Abuse drug supply. Chrm1-EGFP mice were obtained from the Mutant Mouse Regional Resource Center (University of California, Davis, CA, USA). DSMO was used as a solvent for JWH018 and rimonabant. Ethanol was used for JWH073 and the CP47,497-C8 compounds.

2.2. Synthesis of CP47,497-C8 Stereoisomers

cis- and trans-CP47,497-C8 were synthesized using a modified method of previously reported procedures (Melvin et al., 1984) (Fig. 2).

2.3. Cell Culture and Transfection

All procedures used in this study were approved by the Animal Care Committees of Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Hippocampal neurons isolated from the CA1–CA3 region of chrm1-EGFP mice were cultured on micro-islands as previously described

in desensitization of the receptor and its internalization (Hsieh et al., 1999; Jin et al., 1999; Roche et al., 1999). These processes are thought to be involved in tolerance to drugs of abuse such as THC (Wu et al., 2008).

We previously characterized JWH018 as a potent and efficacious CB_1 receptor agonist based on its ability to inhibit neurotransmission and promote CB_1 receptor internalization (Atwood et al., 2010). These data indicate that JWH018 may promote the cannabis-like effects of 'Spice.' However CP47,497-C8 and JWH073 are also present in many samples of 'Spice.' Like JWH018 these compounds only had limited
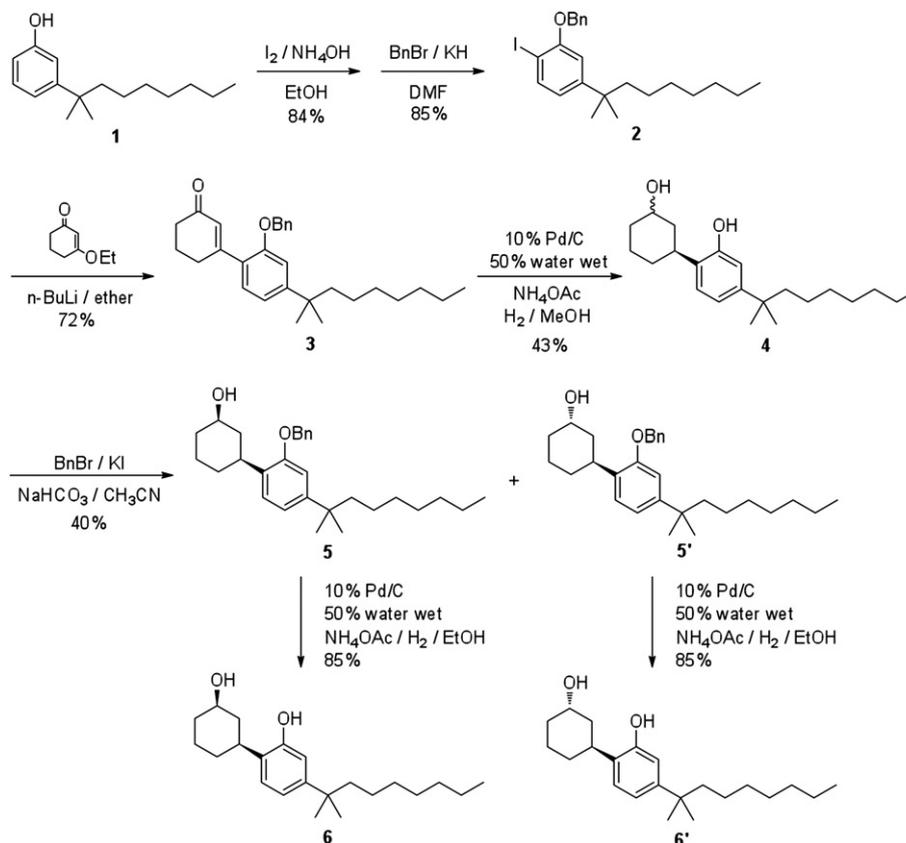

Fig. 2. Scheme for the synthesis of cis- and trans-CP47,497-C8.

(Atwood et al., 2010; Bekkers and Stevens, 1991; Furshpan et al., 1976; Straiker and Mackie, 2005). Cultures were grown in high-glucose (20 mM) minimum essential media (MEM) containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than 3 h after removal from culture medium (Straiker and Mackie, 2005).

CB_1 stable cell lines were made as previously described (Brown et al., 2002; Daigle et al., 2008). Stable clones uniformly expressing CB_1 were expanded and used for the internalization assays. Cells were grown in Dulbecco's modified Eagle's media with 10% fetal bovine serum and penicillin/streptomycin (GIBCO, Carlsbad, CA, USA) at 37 °C in 5% CO_2.

2.4. Electrophysiology

All electrophysiological experiments were performed exclusively on excitatory autaptic neurons. Autaptic neurons are neurons grown on islands of permissive substrate that form synapses (autapses) with themselves. Stereoisomeric mixtures of CP47,497-C8 and JWH073 were tested on cells from at least two different preparations. Individual stereoisomers of CP47,497-C8 were tested on cells from one preparation. Whole-cell, voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA USA). The extra-cellular solution contained (mM): NaCl 119, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 30 and HEPES 20. Continuous flow of solution through the bath chamber ($\sim$2 ml min^{-1}) ensured rapid drug application and clearance. Drugs were prepared as a stock, then diluted into extracellular solution at their final concentration and used within one hour (final solvent concentration was 0.01%). Solvents had no effect on EPSC magnitude. Recording pipettes of 2–4 MΩ were filled with solution containing (mM): potassium gluconate 121.5, KCl 17.5, NaCl 9, $MgCl_2$ 1, HEPES 10, EGTA 0.2, MgATP 2 and LiGTP 0.5. Access resistance was monitored and only cells with a stable access resistance were included for data analysis.

Electrophysiological experiments were carried out as previously described (Atwood et al., 2010). Briefly, the stimulus protocol used was to elicit an unclamped action current with a 1.0 ms depolarizing step every 20 s from a holding potential of -70 mV. The magnitude of excitatory postsynaptic currents (EPSCs) was calculated by integrating the evoked current to yield charge (in pC). Data were acquired at a sampling rate of 5 kHz.

2.5. Internalization Assays

The extent of CB_1 internalization was analyzed as previously described (Daigle et al., 2008). The extent of internalization was calculated as the average integrated intensities of the drug treated wells divided by the average integrated intensities of the untreated wells and is expressed as percentages of initial intensity (reflecting the original amount of cell surface receptors). Solvents had no effect on internalization (final solvent concentration was 0.1%).

2.6. Statistical Analysis

Data are reported as mean with 95% confidence interval (CI) (IC50, EC50 and t1/2) or as mean $\pm$ S.E.M. (other values). Nonlinear regression was used to fit the concentration response curves and the time course of internalization. Student's t-tests and one-way ANOVA were used to compare drug treatments and were used where indicated. Bonferroni's Multiple Comparisons Test was used to compare JWH018, CP47,497-C8 (and its individual stereoisomers) and JWH073 treatments at specific time points and drug concentrations. Statistical significance is indicated as follows: ***P<0.0001, **P<0.01, and *P<0.05. All graphs and statistical analyses were

generated using GraphPad Prism 4.0 software (Hearne Scientific Software, Chicago, IL, USA).

3. Results

3.1. CP47,497-C8 and JWH073 Inhibit Neurotransmission by Activating CB_1 Receptors

As seen in Fig. 3A, bath application of CP47,497-C8 reduced EPSC size. This suppression of EPSCs was concentration dependent with an IC_{50} of 15.4 nM (2.0 to 118.7 nM) and a maximal suppression at 1 μM to $51.6 \pm 8.0\%$ of baseline (Fig. 3C). Furthermore, as seen in Fig. 3A and D, the effect of CP47,497-C8 was reversed by rimonabant, a CB_1 receptor antagonist. JWH073 also decreased the magnitude of EPSCs (Fig. 3B) in a concentration-dependent manner (Fig. 3C). The effect of JWH073 tended to be less potent than that of CP47,497-C8 with an IC_{50} of 49.4 nM (19.5 to 125.0 nM), although it was somewhat more efficacious (maximal suppression to $31.5 \pm 4.0\%$ of baseline at 1 μM, P = 0.0504 vs CP47,497-C8). Its effects were also due to CB_1 receptor activation as seen by the reversal produced by rimonabant treatment in Fig. 3B and D. Data from previous experiments demonstrated that rimonabant has no effect on EPSC size on its own ($96 \pm 4.0\%$ of baseline with 100 nM treatment) (Straiker and Mackie, 2005).

3.2. CP47,497-C8 and JWH073 also Stimulate CB_1 Receptor Internalization

We next examined the ability of these two compounds to promote CB_1 receptor internalization. To ensure a better comparison with our previous report on the effects of JWH018 and to account for differences in passage number and other cell culture variables, we tested JWH018 alongside our analyses of CP47,497-C8 and JWH073. Fig. 4A shows the time course of internalization due to prolonged treatment with 100 nM CP47,497-C8, JWH073 and JWH018. In these experiments, JWH018 promoted CB_1 receptor internalization with a half-life of 22.9 min (15.4 to 44.9 min) and reached a plateau of $50.7 \pm 2.6\%$ of basal surface levels. For CP47,497-C8 internalization occurred with a nearly identical half-life of 23.1 min (16.1 to 40.6 min) and reached a maximal internalization of $51.4 \pm 2.2\%$ of basal surface levels at 6 h. Previous work suggested that a maximal amount of CB_1 internalization was usually obtained by 2 to 3 h (Atwood et al., 2010; Daigle et al., 2008). However, JWH073 produced a much slower rate of receptor internalization than either JWH018 or CP47,497-C8 with a 74.2 min (53.7 to 119.7 min) half-life (non-overlapping confidence interval with JWH018 or CP47,497-C8). The maximal internalization plateau of JWH073 treatment was $57.5 \pm 3.1\%$ of basal surface levels. There was no significant difference between any of the drugs' maximal internalization levels at 6 h (One-way ANOVA, P = 0.18). However at all other time points a one-way ANOVA analysis revealed a significant difference between the levels of internalization produced by the different drugs. We also performed an analysis of the concentration-dependence of internalization to compare the potencies of these three compounds (Fig. 4B). CP47,497-C8 (2 h treatment) produced profound CB_1 internalization in a concentration-dependent manner with an EC_{50} of 4.4 nM (1.6 to 12.5 nM) and a maximal internalization at 1 μM of $52.7 \pm 4.3\%$ of basal surface levels. We also revisited the ability of JWH018 to promote CB_1 receptor internalization in order to compare its potency to that of CP47,497-C8's. JWH018 promoted CB_1 internalization with an EC_{50} of 10.1 nM (4.1 to 24.6 nM) with a maximal internalization at 1 μM of $45.2 \pm 2.0\%$ of basal surface levels. JWH073 was less potent and efficacious than both CP47,497-C8 and JWH073 with an EC_{50} of 45.6 nM (21.8 to 95.4 nM; non-overlapping CI with CP47,497-C8) and an E_{max} of $61.2 \pm 2.5\%$ of basal surface levels. One-way ANOVA analysis showed that there was a significant difference between the
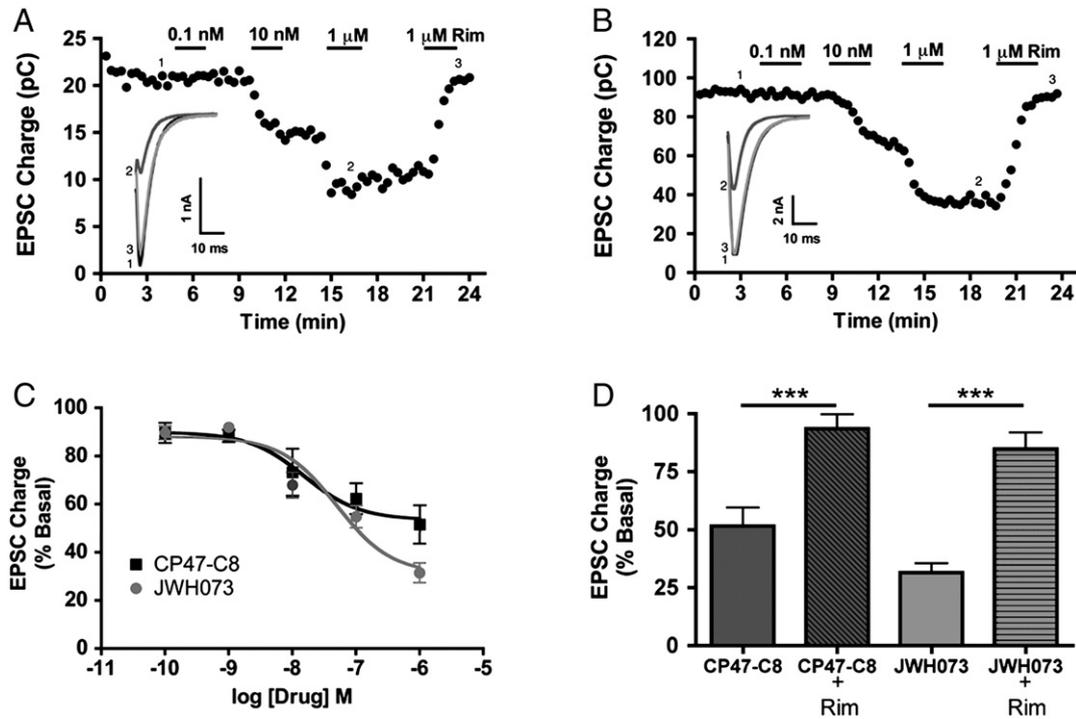
Fig. 3. CP47,497-C8 and JWH073 decreases neurotransmitter release by activating CB1 receptors. (A) A representative experiment showing a time course of EPSC inhibition by CP47,497-C8 with the indicated concentrations. 1 μM rimonabant reverses CP47,497-C8 inhibition. Inset shows representative traces for three indicated time points. (B) A representative experiment showing a time course of EPSC inhibition by JWH073 with the indicated concentrations. 1 μM rimonabant, a CB1 receptor antagonist, reverses JWH073 inhibition. Inset shows representative traces for three indicated time points. (C) Concentration dependence of CP47,497-C8- and JWH073-induced suppression of neurotransmission ($n = 5$ to 8 for each concentration tested). (D) Quantification of 1 μM rimonabant inhibitory effect on 1 μM drug treatment ($n = 7$ to 8 for each concentration) values are presented as mean $\pm$ S.E.M. where applicable. Data analyzed using unpaired Student's t-tests. ***$P < 0.0001$.

amounts of internalization produced by these drugs at 10 nM ($P < 0.018$), 100 nM ($P < 0.0001$), and at 1 μM ($P < 0.0092$). Fig. 4C shows that in all cases 1 μM rimonabant prevented internalization due to treatment with 1 μM of each of these drugs, demonstrating that internalization by these drugs is due to CB_1 receptor activation. Treatment with 1 μM rimonabant alone had no effect on CB_1 surface levels ($99.4 \pm 2.7\%$ of basal surface levels, data not shown).

3.3. The Individual cis- and trans-Stereoisomers of CP47,497-C8 Produce Similar Effects as the Stereoisomeric Mixture

Fig. 5A provides the structures of the cis- and trans- stereo-isomers of CP47,497-C8. As seen in Fig. 5B no significant differences were observed between the EPSC suppression achieved by treatment with the stereoisomeric mixture and either individual stereoisomer at each of the concentrations tested. Each stereo-isomer's suppressive effects were reversed by 1 μM rimonabant treatment. Fig. 5C and D shows example time courses of recordings from autaptic neurons treated with increasing concentrations of cis-CP47,497-C8 and trans-CP47,497-C8, respectively. In our inter-nalization assays we found similar time courses (Figs. 4A and 5E) of the cis- and trans-stereoisomeric mixture and the individual stereoisomers (cis-CP47,497-C8: $t_{1/2} = 26.8$ min (19.7 to 41.9 min), plateau = $48.4 \pm 2.4\%$ of basal surface levels; and trans-CP47,497-C8: $t_{1/2} = 34.2$ min (24.2 to 58.5 min), plateau = $47.7 \pm 3.1\%$ basal surface levels). In addition, as demonstrated in Fig. 5F (see also Fig. 4B), we found the potency and maximal efficacies of the cis- and trans-stereoisomers to be nearly identical (cis-CP47,497-C8: $EC_{50} = 1.9$ nM (0.9 to 3.9 nM), $E_{max} = 53.8 \pm 2.4\%$ of basal surface levels and trans-CP47,497-C8: $EC_{50} = 2.7$ nM (0.9 to 7.8 nM), $E_{max} = 54.9 \pm 3.7\%$ of basal surface levels). 1 μM rimonabant also prevented the internalization produced by 1 μM of each compound (cis-CP47,497-C8: $P = 0.0021$ and trans-CP47,497-C8: $P = 0.0035$).

Statistical analyses found no significant differences between each of the cis- and trans-stereoisomers and the stereoisomeric mixture at any time point or concentration.

4. Discussion

Following the original identification of synthetic cannabinoid additives in different preparations of 'Spice' herbal blends (Auwarter et al., 2009) we extended the characterization of JWH018 to demonstrate that it is indeed a potent and efficacious CB_1 receptor agonist (Atwood et al., 2010). The original report along with several more recent ones confirmed that in addition to JWH018, 'Spice' also can contain CP47,497-C8 and, more recently, JWH073 (Auwarter et al., 2009; Hudson et al., 2010; Lindigkeit et al., 2009; Uchiyama et al., 2010). Both of these latter compounds have been shown to have high affinity for CB_1 and to produce some of the same effects as other cannabinoids (Aung et al., 2000; Compton et al., 1992; Melvin et al., 1984, 1993; Wiley et al., 1998). As with JWH018, we confirmed our hypothesis that these additional additives inhibit neurotransmission and promote CB_1 receptor internalization as CB_1 receptor agonists.

Activation of presynaptic CB_1 receptors suppresses neurotrans-mitter release (Shen et al., 1996). In autaptic hippocampal neuronal cultures, this is observed as a reduction in the magnitude of EPSCs following treatment with a CB_1 receptor agonist (Straiker and Mackie, 2005). Both CP47,497-C8 and JWH073 inhibit neurotransmission. Interestingly the concentration–response curve of CP47,497-C8 is nearly identical to that from our previous characterization of JWH018. We determined that in our autaptic hippocampal neuron cultures, JWH018 decreases EPSC magnitudes to a maximum at of 48.0% at 1 μM and with an IC_{50} of 14.9 nM (Atwood et al., 2010), quite similar to the data we report here for CP47,497-C8 ($IC_{50} = 15.4$ nM, $E_{max} = 51.6\%$). The potencies of JWH018 and CP47,497-C8 in this preparation are similar to WIN55,212 ($IC_{50} = 18.1$ nM), a well
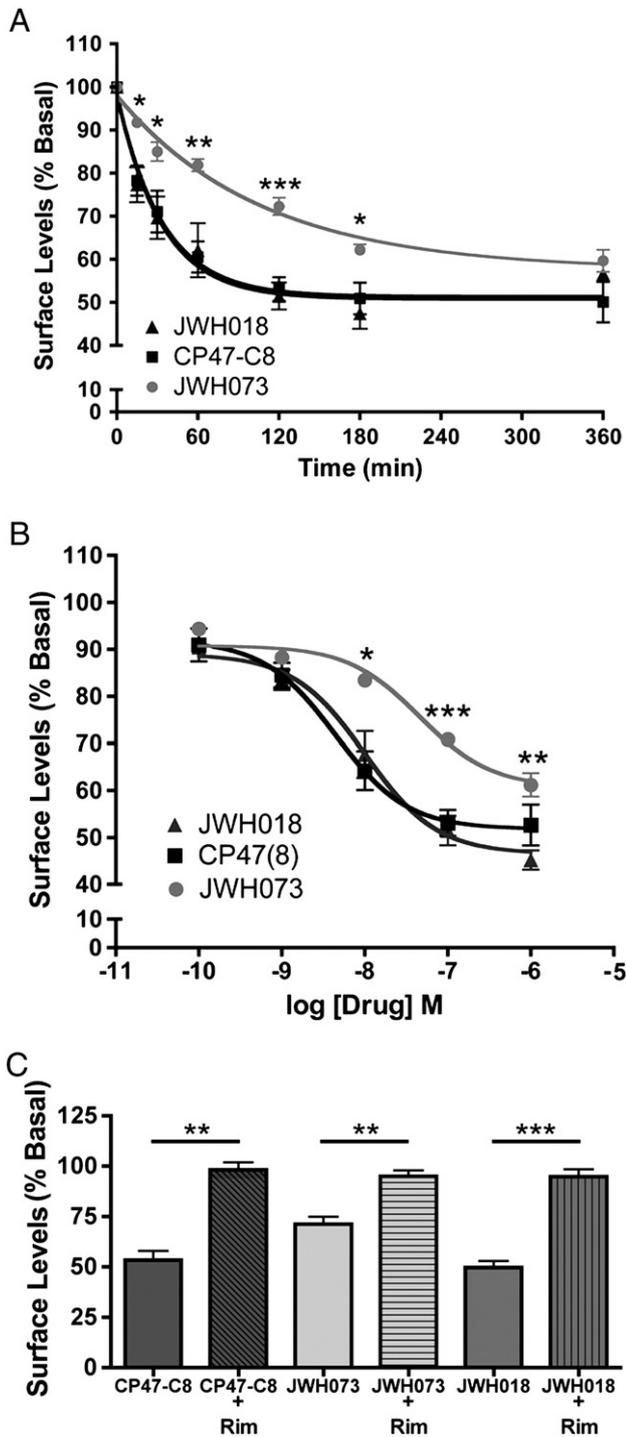
Fig. 4. Prolonged treatment with CP47,497-C8 or JWH073 promotes CB1 receptor internalization. (A) In CB1 expressing HEK293 cells 100 nM CP47,497-C8, JWH073 or JWH018 treatment results in robust receptor internalization (3 replicate samples from 5 to 14 independent experiments, except for 360 min time point: 3–4 experiments). (B) Following 2 h of exposure to increasing concentrations of each drug, CB1 internalized in a concentration-dependent manner (3 replicate samples from 4 to 14 independent experiments). (C) 1 µM rimonabant prevents receptor internalization by each drug treatment. Values are presented as mean ± S.E.M. Data in (A) and (B) analyzed using one-way ANOVA with Bonferroni's multiple comparison test. Data in (C) analyzed using unpaired Student's t-test. *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

described cannabinoid receptor agonist (Straiker and Mackie, 2005). Based on their similar structures and nearly identical affinities for CB$_1$ (Aung et al., 2000) we hypothesized that we would not find any significant difference between JWH018 and JWH073 in inhibiting

neurotransmission. In comparing the suppression produced by JWH073 with that produced by JWH018 and CP47,497-C8, we also found no significant differences at any concentration, however at 1 µM the effects of JWH073 trended towards being more efficacious (one-way ANOVA, $P = 0.0651$). JWH073 also appeared to be slightly less potent than the other two compounds with an IC$_{50}$ of 49.4 nM. As we previously noted for JWH018 (Atwood et al., 2010), the effects of these compounds significantly differ from that of THC, which produces no inhibition of EPSCs, but does produce cannabinoid receptor desensitization (Atwood et al., 2010; Straiker and Mackie, 2005). In our previous report on the effects of JWH018 on neurotransmission we determined that the most likely site of action of JWH018 was the presynaptic CB$_1$ receptors (Atwood et al., 2010). We accomplished this by measuring paired-pulse ratios, spontaneous EPSCs, and the coefficient of variation before and after JWH018 treatment. Since JWH073 and CP47,497-C8, like JWH018, are CB$_1$ receptor agonists it is highly likely that these compounds also act at presynaptic CB$_1$ receptors. The results presented here confirm that in addition to JWH018, other synthetic agonists found in 'Spice' products also inhibit neurotransmission via CB$_1$ receptors.

As noted in a recent paper that reported a case study of a patient undergoing cannabis-like withdrawal symptoms after cessation of 'Spice' use (Zimmermann et al., 2009), tolerance and withdrawal are important factors to consider when characterizing the actions of drugs of abuse. Receptor desensitization and internalization are two processes thought to play a role in promoting physiological tolerance to drugs (Koch and Höllt, 2008; Wu et al., 2008). Analysis of µ-opioid receptor internalization has suggested an inverse relationship between internalization and receptor desensitization (Koch et al., 2005). Work done with CB$_1$ cannabinoid receptors demonstrated that agonists that produce less internalization, such as THC, produce more rapid receptor desensitization (Wu et al., 2008). In light of these data, we also tested the ability of each of these compounds found in 'Spice' to induce CB$_1$ receptor internalization. JWH073 appeared to produce much slower internalization (105.4 min) than JWH018 (38.4 min) and CP47,497-C8 (23.0 min).

In the study that first identified these synthetic cannabinoids in samples of 'Spice,' two racemic mixtures of different diastereomers of CP47,497-C8 were identified: one with an axial hydroxyl group, trans-CP47,497-C8, and the other with an equatorial one, cis-CP47,497-C8 (Fig. 5A) (Auwarter et al., 2009). This difference significantly influences the in vivo potency of CP47,497-C8. cis-CP47,497-C8 is more potent in vivo than trans-CP47,497-C8, although metabolic inter-conversion of these compounds may occur (Melvin et al., 1984). Curiously, we found no significant differences between the two stereoisomers of CP47,497-C8 and with the stereoisomeric mixture. We had hypothesized based on its decreased potency in vivo, that the axial alcohol stereoisomer, trans-CP47,497-C8, would be less potent in reducing the size of EPSCs in our neuronal cultures than either the mixture or the equatorial alcohol stereoisomer, cis-CP47,497-C8. The decreased potency in vivo may be therefore due to pharmacokinetic mechanisms rather than a reduced efficacy at CB$_1$. We were also surprised to see no significant differences in our measurements of internalization between the stereoisomers of CP47,497-C8 and the mixture, although trans-CP47,497-C8 may have a slightly slower time course of internalization (34.2 min) that that of the mixture (23.1 min) or cis-CP47,497-C8 (26.8 min). The EC$_{50}$ of this stereoisomer fell between its counterpart and that of the mixture. Once again, the decreased potency of this stereoisomer observed in vivo may be a result of pharmacokinetic differences rather than fundamentally different pharmacodynamics.

In our previous report, JWH018 and WIN55,212 internalized CB$_1$ to a similar extent (Atwood et al., 2010), placing both within the category of high endocytotic agonists (Wu et al., 2008). Since CP47,497-C8 produced a nearly identical extent and rate of internalization as JWH018, it is likely that each of these compounds will also
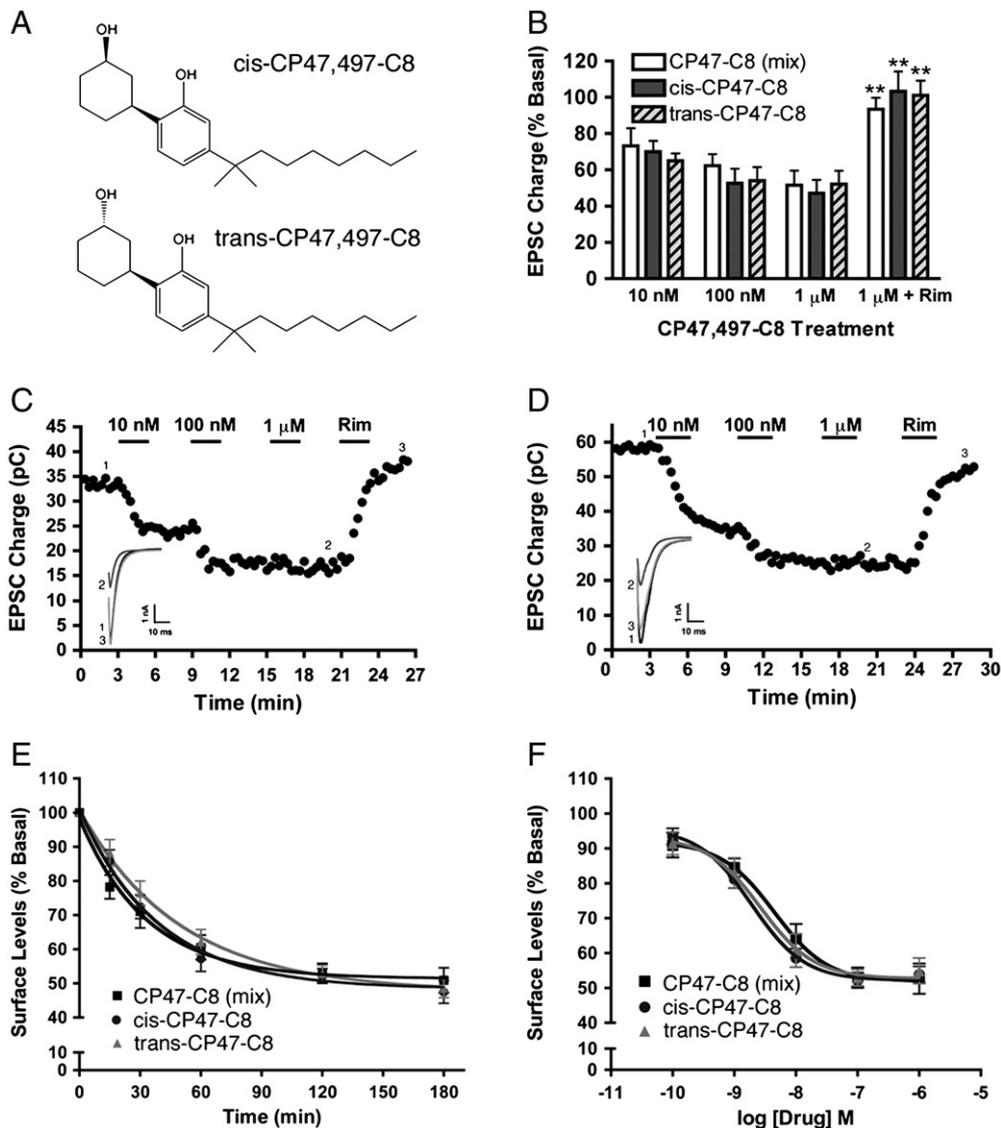
Fig. 5. Individual stereoisomers of CP47,497-C8 do not differ from the stereoisomeric mixture in their effects on neurotransmission and CB1 receptor internalization. (A) Chemical structures of cis-CP47,497-C8 and trans-CP-47,497-C8. (B) Summary of the amount of EPSC suppression achieved by indicated concentrations of each stereostereoisomer. Data from Fig. 3C included for ease of comparison. 1 μM rimonabant treatment reverses the effects of treatments with 1 μM of each drug. **: $P<0.01$ vs. 1 μM drug treatments (unpaired Student's t-tests). (C) A representative experiment showing a time course of EPSC inhibition by cis-CP47,497-C8 with the indicated concentrations. 1 μM rimonabant reverses inhibition. Inset shows representative traces for three indicated time points. (D) A representative experiment showing a time course of EPSC inhibition by trans-CP47,497-C8 with the indicated concentrations. 1 μM rimonabant reverses inhibition. Inset shows representative traces for three indicated time points. (E) In CB1 expressing HEK293 cells 100 nM cis-CP47,497-C8 or trans-CP47,497-C8 treatment results in robust receptor internalization (3 replicate samples from 5 to 10 independent experiments). Data from Fig. 4A have been included for ease of comparison. (F) Following 2 h of exposure to increasing concentrations of each drug, CB1 was internalized in a concentration-dependent manner (3 replicate samples from 5 to 10 independent experiments). Values are presented as mean ± S.E.M where appropriate. Data analyzed using one-way ANOVA with Bonferroni's multiple comparison tests (except where indicated in (B)).

produce less receptor desensitization than THC. On the other hand JWH073 produced much slower internalization than the other compounds. In comparing potencies, CP47,497-C8 and JWH018 are the most potent. From our earlier experiments with WIN55,212 we found that the potency of WIN55,212 (19 nM) (Atwood et al., 2010) is higher than JWH073 but less than JWH018 and CP47,497-C8. Based on the data from Wu et al. (2008), JWH073 may produce much more rapid receptor desensitization than JWH018 and CP47,497-C8. It remains to be determined to what extent each of these compounds produces tolerance *in vivo*. Based on the case study mentioned above, it is likely that these compounds will produce tolerance *in vivo*.

Following the original paper highlighting the presence of JWH018 in 'Spice' (Auwarter et al., 2009) and our original characterization of JWH018 (Atwood et al., 2010), this compound has gained considerable media attention. A more recent analysis has reported a reduction

in the levels of JWH018 in samples of 'Spice' accompanied by an appearance of JWH073 (Lindigkeit et al., 2009), although different samples vary. Other labs have also found JWH073 in similar products (Hudson et al., 2010; Uchiyama et al., 2010). The CP47,497-C8 compound remains a consistent presence in many different products (Auwarter et al., 2009; Hudson et al., 2010; Lindigkeit et al., 2009; Uchiyama et al., 2010). Other synthetic additives have also been noted (Hudson et al., 2010). Based on these reports and our current data, it appears that different preparations of 'Spice' contain one or more synthetic cannabinoid agonists that are each independently capable of inhibiting neurotransmission potently and with high efficacy. Since THC is most likely not present in 'Spice,' it is likely that the psychoactive effects produced by 'Spice' are due to these synthetic cannabinoids and their effects on neurotransmission. In addition these compounds can promote CB_1 receptor internalization, although to different degrees, and

this may play a role in the development of tolerance to 'Spice.' We predict that in the future, that in addition to the compounds tested here, other cannabinoid agonists will also be identified in samples of 'Spice.' Indeed, JWH398 and JWH250 have recently been identified in samples of 'Spice' in the United Kingdom and in Germany (Vardakou et al., 2010). It is also highly probable any new compounds identified in 'Spice' will also be potent and efficacious cannabinoid agonists and produce similar effects on neurotransmission and receptor internalization.

Acknowledgements

References

Ameri, A., Wilhelm, A., Simmet, T., 1999. Effects of the endogeneous cannabinoid, anandamide, on neuronal activity in rat hippocampal slices. Br. J. Pharmacol. 126, 1831–1839.

Atwood, B.K., Huffman, J., Straiker, A., Mackie, K., 2010. JWH018, a common constituent of 'Spice' herbal blends, is a potent and efficacious cannabinoid CB(1) receptor agonist. Br. J. Pharmacol. 160, 585–593.

Aung, M.M., Griffin, G., Huffman, J.W., Wu, M., Keel, C., Yang, B., Showalter, V.M., Abood, M. E., Martin, B.R., 2000. Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB(1) and CB(2) receptor binding. Drug Alcohol Depend. 60, 133–140.

Auwarter, V., Dresen, S., Weinmann, W., Muller, M., Putz, M., Ferreiros, N., 2009. 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? J. Mass Spectrom. 44, 832–837.

Bekkers, J.M., Stevens, C.F., 1991. Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. Proc. Natl Acad. Sci. USA 88, 7834–7838.

Brown, S.M., Wager-Miller, J., Mackie, K., 2002. Cloning and molecular characterization of the rat CB2 cannabinoid receptor. Biochim. Biophys. Acta 1576, 255–264.

Chevaleyre, V., Takahashi, K.A., Castillo, P.E., 2006. Endocannabinoid-mediated synaptic plasticity in the CNS. Annu. Rev. Neurosci. 29, 37–76.

Compton, D.R., Johnson, M.R., Melvin, L.S., Martin, B.R., 1992. Pharmacological profile of a series of bicyclic cannabinoid analogs: classification as cannabimimetic agents. J. Pharmacol. Exp. Ther. 260, 201–209.

Costa, B., 2007. On the pharmacological properties of delta9-tetrahydrocannabinol (THC). Chem. Biodivers. 4, 1664–1677.

Daigle, T.L., Kearn, C.S., Mackie, K., 2008. Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. Neuropharmacology 54, 36–44.

Furshpan, E.J., MacLeish, P.R., O'Lague, P.H., Potter, D.D., 1976. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc. Natl Acad. Sci. USA 73, 4225–4229.

Griffin, G., Atkinson, P.J., Showalter, V.M., Martin, B.R., Abood, M.E., 1998. Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5′-O-(3-[35S] thio)-triphosphate binding assay in rat cerebellar membranes. J. Pharmacol. Exp. Ther. 285, 553–560.

Griffiths, P., Sedefov, R., Gallegos, A., Lopez, D., 2010. How globalization and market innovation challenge how we think about and respond to drug use: 'Spice' a case study. Addiction 105, 951–953.

Hammersley, R., 2010. Dangers of banning spice and the synthetic cannabinoid agonists. Addiction 105, 373.

Howlett, A.C., 2002. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 68–69, 619–631.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002.

International union of pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.

Hsieh, C., Brown, S., Derleth, C., Mackie, K., 1999. Internalization and recycling of the CB1 cannabinoid receptor. J. Neurochem. 73, 493–501.

Hudson, S., Ramsey, J., King, L., Timbers, S., Maynard, S., Dargan, P.I., Wood, D.M., 2010. Use of high-resolution accurate mass spectrometry to detect reported and previously unreported cannabinomimetics in "herbal high" products. J. Anal. Toxicol. 34, 252–260.

Huestis, M.A., Gorelick, D.A., Heishman, S.J., Preston, K.L., Nelson, R.A., Moolchan, E.T., Frank, R.A., 2001. Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. Arch. Gen. Psychiatry 58, 322–328.

Huffman, J.W., Dong, D., Martin, B.R., Compton, D.R., 1994. Design, synthesis and pharmacology of cannabimimetic indoles. Bioorg. Med. Chem. Lett. 4, 563–566.

Jin, W., Brown, S., Roche, J.P., Hsieh, C., Celver, J.P., Kovoor, A., Chavkin, C., Mackie, K., 1999. Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. J. Neurosci. 19, 3773–3780.

Katona, I., Urban, G.M., Wallace, M., Ledent, C., Jung, K.M., Piomelli, D., Mackie, K., Freund, T.F., 2006. Molecular composition of the endocannabinoid system at glutamatergic synapses. J. Neurosci. 26, 5628–5637.

Koch, T., Höllt, V., 2008. Role of receptor internalization in opioid tolerance and dependence. Pharmacol. Ther. 117, 199–206.

Koch, T., Widera, A., Bartzsch, K., Schulz, S., Brandenburg, L.O., Wundrack, N., Beyer, A., Grecksch, G., Höllt, V., 2005. Receptor endocytosis counteracts the development of opioid tolerance. Mol. Pharmacol. 67, 280–287.

Lindigkeit, R., Boehme, A., Eiserloh, I., Luebbecke, M., Wiggermann, M., Ernst, L., Beuerle, T., 2009. Spice: a never ending story? Forensic Sci. Int. 191, 58–63.

Mackie, K., 2005. Distribution of cannabinoid receptors in the central and peripheral nervous system. Handb. Exp. Pharmacol. 299–325.

McLachlan, G., 2009. Taking the spice out of legal smoking mixtures. Lancet 374, 600.

Melvin, L.S., Johnson, M.R., Harbert, C.A., Milne, G.M., Weissman, A., 1984. A cannabinoid derived prototypical analgesic. J. Med. Chem. 27, 67–71.

Melvin, L.S., Milne, G.M., Johnson, M.R., Subramaniam, B., Wilken, G.H., Howlett, A.C., 1993. Structure-activity relationships for cannabinoid receptor-binding and analgesic activity: studies of bicyclic cannabinoid analogs. Mol. Pharmacol. 44, 1008–1015.

Monory, K., Blaudzun, H., Massa, F., Kaiser, N., Lemberger, T., Schutz, G., Wotjak, C.T., Lutz, B., Marsicano, G., 2007. Genetic dissection of behavioural and autonomic effects of delta(9)-tetrahydrocannabinol in mice. PLoS Biol. 5, e269.

Muller, H., Sperling, W., Kohrmann, M., Huttner, H.B., Kornhuber, J., Maler, J.M., 2010. The synthetic cannabinoid Spice as a trigger for an acute exacerbation of cannabis induced recurrent psychotic episodes. Schizophr. Res. 118, 309–310.

Pertwee, R.G., 2008. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydro-cannabivarin. Br. J. Pharmacol. 153, 199–215.

Roche, J.P., Bounds, S., Brown, S., Mackie, K., 1999. A mutation in the second transmembrane region of the CB1 receptor selectively disrupts G protein signaling and prevents receptor internalization. Mol. Pharmacol. 56, 611–618.

Shen, M., Piser, T.M., Seybold, V.S., Thayer, S.A., 1996. Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. J. Neurosci. 16, 4322–4334.

Straiker, A., Mackie, K., 2005. Depolarization-induced suppression of excitation in murine autaptic hippocampal neurones. J. Physiol. 569, 501–517.

Uchiyama, N., Kikura-Hanajiri, R., Ogata, J., Goda, Y., 2010. Chemical analysis of synthetic cannabinoids as designer drugs in herbal products. Forensic Sci. Int. 198, 31–38.

Vardakou, I., Pistos, C., Spiliopoulou, C., 2010. Spice drugs as a new trend: mode of action, identification and legislation. Toxicol. Lett. 197, 157–162.

Wiley, J.L., Compton, D.R., Dai, D., Lainton, J.A., Phillips, M., Huffman, J.W., Martin, B.R., 1998. Structure-activity relationships of indole- and pyrrole-derived cannabinoids. J. Pharmacol. Exp. Ther. 285, 995–1004.

Wu, D.F., Yang, L.Q., Goschke, A., Stumm, R., Brandenburg, L.O., Liang, Y.J., Höllt, V., Koch, T., 2008. Role of receptor internalization in the agonist-induced desensitization of cannabinoid type 1 receptors. J. Neurochem. 104, 1132–1143.

Zimmermann, U.S., Winkelmann, P.R., Pilhatsch, M., Nees, J.A., Spanagel, R., Schulz, K., 2009. Withdrawal phenomena and dependence syndrome after the consumption of "spice gold". Dtsch. Arztebl. Int. 106, 464–467.