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The dietary polyphenols *trans*-resveratrol and curcumin selectively bind human CB1 cannabinoid receptors with nanomolar affinities and function as antagonists/inverse agonists

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Running Title: Polyphenols bind CB1 receptors and function as antagonists

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Non-Standard Abbreviations:

CHO, Chinese hamster ovary; hCB1, human CB1 receptors; hCB2, human CB2 receptors; [^{35}S]GTPγS, guanosine 5'-O-(3-[^{35}S]thio)triphosphate; mCB1, mouse CB1 receptors

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Abstract:

The dietary polyphenols *trans*-resveratrol (found in red wine) and curcumin (found in curry powders) exert anti-inflammatory and anti-oxidant effects via poorly defined mechanisms. Interestingly, cannabinoids, derived from the marijuana plant (*Cannabis sativa*), produce similar protective effects via CB1 and CB2 receptors. We examined whether *trans*-resveratrol, curcumin and ASC-J9 (a curcumin analog) act as ligands at cannabinoid receptors. All three bind to hCB1 and mCB1 receptors with nanomolar affinities, displaying only micromolar affinities for hCB2 receptors. Characteristic of inverse agonists, the polyphenols inhibit basal G-protein activity in membranes prepared from CHO-hCB1 cells or mouse brain, that is reversed by a neutral CB1 antagonist. Furthermore, they competitively antagonize G-protein activation produced by a CB1 agonist. In intact CHO-hCB1 cells, the polyphenols act as neutral antagonists, producing no effect when tested alone, while competitively antagonizing CB1 agonist mediated inhibition of adenylyl cyclase activity. Confirming their neutral antagonist profile in cells, the polyphenols similarly attenuate stimulation of adenylyl cyclase activity produced by a CB1 inverse agonist. In mice, the polyphenols dose-dependently reverse acute hypothermia produced by a CB1 agonist. Upon repeated administration, the polyphenols also reduce body weight in mice similar to that produced by a CB1 antagonist/inverse agonist. Finally, *trans*-resveratrol and curcumin share common structural motifs with other known cannabinoid receptor ligands. Collectively, we suggest that *trans*-resveratrol and curcumin act as antagonists/inverse agonists at CB1 receptors at dietary relevant concentrations. Therefore, these polyphenols and their derivatives might be developed as novel, non-toxic CB1 therapeutics for obesity and/or drug dependence.

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Introduction:

Dietary polyphenols, such as resveratrol (found in red wine) and curcumin (found in curry powders), have been used safely for centuries as traditional medicines. Consequently, increasing scientific investigation suggests that they may indeed prove useful as therapeutics for a broad range of conditions (Scalbert et al., 2005), from inflammatory diseases (Rahman et al., 2006), to cancer (Hadi et al., 2007). The protective effects of resveratrol and curcumin appear to be related to their anti-oxidant (Fraga, 2007) and anti-inflammatory (Surh et al., 2005) properties. Although the specific mechanisms responsible for these beneficial effects remain unclear, the beneficial effects *in vitro* generally require relatively high concentrations (>1 μM) and are thought to involve multiple receptor and non-receptor mediated processes (Stevenson and Hurst, 2007).

Recently, it has been reported that resveratrol and other polyphenols bind with high affinity to a distinct, yet unidentified, plasma membrane bound receptor that occurs in high density throughout the brain (Han et al., 2006). Cannabinoid receptors appear to share many characteristics with this newly discovered, uncharacterized resveratrol receptor. Originally isolated from the marijuana plant (*Cannabis satvia*), both synthetic and naturally occurring cannabinoids such as Δ^9-THC produce their effects by acting at two G-protein coupled receptors (GPCRs); CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993). CB1 receptors are expressed in high abundance throughout the central nervous system, while CB2 receptors are expressed predominantly in immune cells and non-neuronal tissues. Cannabinoids acting at both receptors produce anti-oxidant (Hampson et al., 1998) and anti-inflammatory (Klein, 2005) effects, similar to that reported for resveratrol and curcumin. Therefore, the current studies were conducted to determine whether two important dietary polyphenols, resveratrol and curcumin, and an analog of curcumin (ASC-J9) act as ligands at cannabinoid receptors. Importantly, our study identifies the human CB1 cannabinoid receptor as a high affinity target for all three polyphenols; resveratrol (K_i = 45 nM), curcumin (K_i = 6 nM) and ASC-J9 (K_i = 64 nM, an analog of curcumin). Furthermore, all polyphenols examined appear to act as CB1 antagonists/inverse agonists and share common structural motifs with other known cannabinoid receptor ligands. Importantly, these results indicate that CB1 receptors are

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one of the highest affinity targets identified to date for resveratrol and curcumin and may have significant implications for future development of novel, non-toxic CB1 ligands.

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Methods:

Materials. All drugs used in this study were obtained from Tocris Bioscience (Ellisville, MO). [^{3}H]CP-55,950 [(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol] (168 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). [^{3}H]Adenine (26 Ci/mmol) was obtained from (Vitrax; Placenia, CA). All other reagents were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

Cell Culture. CHO-K1 cells stably expressing hCB1 receptors (CHO-hCB1) were a generous gift from Dr. Debra A. Kendall (University of Connecticut, Storrs, CT). Stably transfected CHO-hCB2 cells were generated in our laboratory (Shoemaker et al., 2005). Cells were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37^oC in DMEM with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml geneticin.

Membrane Preparation. Brain tissue was collected from decapitated male and female B6SJL mice obtained from an in house breeding colony. Whole brains were pooled before beginning homogenization. Pellets of frozen/thawed cells or freshly harvested brain tissue were resuspended in a homogenization buffer containing 50 mM Hepes pH 7.4, 3 mM $MgCl_2$, and 1 mM EGTA. Using a 40 mL Dounce glass homogenizer (Wheaton, Philadelphia PA), samples were subjected to 10 complete strokes and centrifuged at 18,000 rpm for 10 min at 4°C. After repeating the homogenization procedure twice more, the samples were resuspended in Hepes buffer (50 mM, pH 7.4) and subjected to 10 strokes utilizing a 7 mL glass homogenizer. Membranes were stored in aliquots of approximately 1 mg/mL at –80°C.

Competition Receptor Binding. Increasing concentrations of WIN-55,212-2 or different polyphenols were incubated with 0.1 nM (mouse brain or CHO-hCB2) or 0.5 (CHO-hCB1) nM of [^{3}H]CP-55,940 in a final volume of 1 mL of binding buffer as described previously (Shoemaker et al., 2005). Each binding assay contained 100 (mouse brain or CHO-hCB2) or 150 (CHO-hCB1) µg of membrane protein and reactions were incubated for 90 min at room temperature with mild agitation. Non-specific binding

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was defined as binding observed in the presence of 1 μM of non-radioactive [^{3}H]CP-55,940. Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters followed by two washes with ice-cold binding buffer. Analysis of the binding data was performed using the non-linear regression (Curve Fit) function of GraphPad Prism® v4.0b to determine the concentration of the drug that displaced 50% of [^{3}H]CP (IC_{50}). A measure of affinity (K_i) was derived from the IC_{50} values utilizing the Cheng-Prushoff equation [Cheng, 1973 #45].

[^{35}S]GTPγS binding. [^{35}S]GTPγS binding assays were performed with minor modifications as described previously (Shoemaker et al., 2005) in a buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$ and 0.1% bovine serum albumin. Each binding reaction contained 100 (mouse brain or CHO-hCB2) or 150 (CHO-hCB1) μg of membrane protein, cannabinoid ligands, 0.1 nM [^{35}S]GTPγS and 10 μM of GDP. Non-specific binding was defined by 10 μM of non-radioactive GTPγS. Following incubation at 30°C for 2 hr, the reaction was terminated by filtration and bound radioactivity determined by liquid scintillation counting.

Measurement of cAMP Levels in Intact Cells. The conversion of [^{3}H]adenine labeled ATP pools to cyclic AMP was used as a functional measure of cannabinoid activity (Shoemaker et al., 2005). CHO-hCB1 cells were seeded into 24 well plates and cultured to confluence. DMEM containing 0.9% NaCl, 500 μM 3-isobutyl-1-methlyxanthine, and 2 μCi/well [^{3}H]adenine was added to the cells for 2 hrs at 37°C. The [^{3}H]adenine mixture was removed and the cannabinoids were added for 15 min in a Krebs-Ringer–Hepes buffer containing 500 μM 3-isobutyl-1-methlyxanthine and 10 μM forskolin. The reaction was terminated with 50 μL of 2.2 N HCL and [^{3}H]cAMP separated by alumina column chromatography.

Animal Studies.

Mice. Animal use protocols employed in this study were approved by the University of Arkansas for Medical Sciences IACUC committee and conducted in accordance with the USPHS policy on humane care and use of laboratory animals. Male and female B6SJL mice were obtained from an in house breeding colony.

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Hypothermia Experiments. Body temperature of age- and weight-matched mice was measured by a digital thermometer (Fisher Scientific, Model 17025) inserted ~1 cm into the rectum. Body temperature was measured 1 hr after a s.c. injection of CP-55,940, a time interval resulting in maximal hypothermia (data not shown). When testing CB1 antagonism, drugs were given 30 min prior to CP-55,940 injections by the i.p. route. For all experiments, body temperature was measured prior to any injection, 30 min after antagonist or vehicle injection and 1 hr after injection of CP-55,940. The injection vehicle used for these experiments contained 50% polyethyleneglycol and 50% saline.

Body Weight Reduction Experiments. Age- and weight-matched mice were injected i.p. with the indicated doses of test drugs twice daily for 3 days. Body weight (in gms) was recorded each morning prior to drug injection and finally at 9 AM on day 4 of the study, 12 hr after the last drug dose. Animals were fed *ad libitum* during the 3 day experiment. The injection vehicle for these experiments contained 50% polyethyleneglycol and 50% saline.

Statistical analysis. Curve-fitting and statistical analyses were conducted utilizing GraphPad Prism® v4.0b (GraphPad Software, Inc.; San Diego, CA). Data obtained from three or more experimental groups were analyzed by a one-way ANOVA, followed by a Dunnett's *post-hoc* comparison of individual groups. A non-paired Student's *t*-test was employed to statistically compare data obtained from two experimental groups.

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Results:

***Trans*-resveratrol, curcumin and the curcumin analog ASC-J9 selectively bind with nanomolar affinities to human CB1 receptors stably expressed in CHO cells.** Homologous competition receptor binding with the CB1/CB2 agonist [^{3}H]CP-55,940 showed that stably transfected CHO-hCB1 cells express a density of CB1 cannabinoid receptors of 0.26 ± 0.14 pmole/mg protein (N=3; data not shown). Saturation binding studies with [^{3}H]CP-55,940 demonstrated that CHO-hCB2 cells express a density of hCB2 receptors of 1.4 ± 0.24 pmole/mg protein (Shoemaker et al., 2005). [^{3}H]CP-55,940 binds non-selectively to hCB1 and hCB2 receptors expressed in CHO cells with a K_D of 1.0 ± 0.3 or 0.38 ± 0.06 nM for each, respectively. Competition binding (Fig. 1A) demonstrates that the non-selective cannabinoid agonist WIN-55,212-2 [(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] also has relatively equivalent nanomolar affinity (K_i) for hCB1 (7.7 ± 1.3 nM; N=5) and hCB2 (5.8 ± 1.2 nM; N=4) receptors (Fig 1A, Table 1). In contrast, all three polyphenols examined selectively bind to hCB1, relative to hCB2 receptors (Fig. 1B-1D). Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1E,6E-heptadiene-3,5-dione] is 446-fold selective, binding to hCB1 with an affinity of 5.9 ± 2.1 nM (N=6), while having a K_i for hCB2 of over 2 μM (Fig. 1B). ASC-J9 [1,7-bis(3,4-dimethoxyphenyl)-5-hydroxy-1E,4E,6E-heptatriene-3-one], an analog of curcumin, while demonstrating a slightly lower affinity (64 ± 17 nM; N=3), also binds to hCB1 with a 201-fold selectively over hCB2 (13 ± 1.3 μM; N=4) (Fig. 1C). *Trans*-resveratrol [5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol] is highly selective, binding to hCB1 with a K_i of 45 ± 17 nM (N=3), while failing to significantly displace [^{3}H]CP-55,940 from hCB2 at concentrations up to 100 μM (Fig. 1D). Importantly, *cis*-resveratrol [5-[(1Z)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol] failed to displace [^{3}H]CP-55,940 from hCB1 at concentrations up to 100 μM (data not shown). All polyphenols (100 μM) fail to reduce [^{3}H]CP-55,940 binding in wild-type CHO cells (data not shown).

Curiously, approximately 10-15% residual [^{3}H]CP-55,940 binding was observed in both CHO-hCB1 and CHO-hCB2 homogenates for all ligands examined (including WIN-55,212-2), even when high concentrations of the non-radioactive drugs were employed for competition. It is possible that the residual biding was due, in part, to the use of non-radioactive CP-55,940 to define non-specific binding. Employing the same

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non-radioactive compound to define non-specific binding of the radioactive compound may under certain conditions erroneously identify as specific, binding which is in reality non-specific, but inhibitable. Therefore, experiments were performed to compare the maximal displacement of [^{3}H]CP-55,940 produced by CP-55,940 (1 μM) and a second high affinity non-selective cannabinoid agonist HU-210 [(–)-11-Hydroxy-delta(8)-tetrahydrocannabinol-dimethylheptyl] (1 μM) (data not shown). Results from these experiments revealed that non-radioactive CP-55,940 and HU-210 produce near identical maximal displacement of [^{3}H]CP-55,940 in membrane homogenates prepared from mouse brain, CHO-hCB1 and CHO-hCB2 cells. This suggests that the residual [^{3}H]CP-55,940 binding observed for all cannabinoid ligands tested was not due to the use of non-radioactive CP-55,940 to define non-specific binding. While the exact reason for the observed residual binding is unknown, it is possible that the highly hydrophobic properties of the ligands tested, relative to CP-55,940, might contribute these results.

***Trans*-resveratrol, curcumin and ASC-J9 act as antagonists/inverse agonists at human CB1 receptors in membrane preparations of CHO-hCB1 cells.** To determine the intrinsic activity concerning G-protein function, the ability of the three polyphenols to modulate [^{35}S]GTPγS binding in CHO-hCB1 membranes was examined (Fig. 2). Characteristic of agonists, the non-selective full CB1/CB2 agonist WIN-55,212-2 produces a concentration-dependent increase of approximately 90% in the binding of [^{35}S]GTPγS to CHO-hCB1 membranes with an ED_{50} of 31 ± 6.4 nM (Fig 2A; N=3). In marked contrast, when tested alone, all polyphenols produce a concentration-dependent decrease of [^{35}S]GTPγS binding to CHO-hCB1 membranes. All polyphenols (100 μM) fail to produce any change in [^{35}S]GTPγS binding to membranes prepared from wild-type CHO cells (data not shown). This suggests that the polyphenols act as inverse agonists, suppressing G-protein activation produced by constitutively active hCB1 receptors. However, the potency (*e.g.,* IC_{50}) of the polyphenols required to observe inverse agonism is relatively low (curcumin, 1.3 ± 0.3 μM, N=3; ASC-J9, 56 ± 22 μM, N=3; *trans*-resveratrol, 47 ± 17 μM, N=3) when compared to their high nanomolar affinity for hCB1 receptors (Fig. 1). Consistent with an antagonist/inverse agonist profile, co-incubation with a fixed concentration of each polyphenol that produced minimal reduction of [^{35}S]GTPγS binding alone, resulted in a significant

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reduction in the potency of the agonist WIN-55,212-2 to activate G-proteins (Fig. 2B). Both curcumin and *trans*-resveratrol produced a significant ($p<0.05$), 3-fold shift-to-the right in the concentration-effect curve of WIN-55,212-2 (+curcumin, 120 ± 3.5 nM, N=3; +*trans*-resveratrol, 130 ± 25 nM, N=3) (Fig. 2B). Interestingly, co-incubation with ASC-J9 resulted in a much greater, 63-fold reduction in the potency of WIN-55,212-2 to activate G-proteins in CHO-hCB1 membranes (2500 ± 640 nM, N=3).

***Trans*-resveratrol, curcumin and ASC-J9 act as neutral antagonists at human CB1 receptors in intact CHO-hCB1 cells.** Cannabinoid receptors activate the Gi/Go-class of G-proteins, modulating the activity of the effector adenylyl cyclase. Consequently, cannabinoid agonists reduce, neutral antagonists do not alter, and inverse agonists increase the levels of intracellular cAMP (Fig. 3). WIN-55,212-2 produced a concentration-dependent reduction of over 60% in intracellular cAMP levels in intact CHO-hCB1 cells, with an ED_{50} of 12.0 ± 4.6 nM (N=8; Fig 3A-B). In marked contrast, exposure of CHO-CB1 cells to all three polyphenols with concentrations as high as 10 μM did not alter intracellular levels of cAMP (Fig. 3A). Therefore, all polyphenols tested act as neutral antagonists, rather than inverse agonists, in intact CHO-hCB1 cells. Indicative of competitive antagonism, co-incubation with a fixed concentration of each of the polyphenols with the agonist WIN-55,212-2 resulted in a significant ($p<0.05$), 7 to 10-fold, parallel shift-to-the-right in the concentration-effect curve (WIN+curcumin, 130 ± 65 nM, N=5; WIN+ASC-J9, 90 ± 25 nM, N=4; WIN+*trans*-resveratrol, 100 ± 25 nM, N=4) (Fig. 3B). Characteristic of neutral antagonists, all polyphenols examined attenuated not only the inhibitory effects of the agonist WIN-55,212-2 (Fig. 3C), but also the stimulatory action of the inverse agonist AM-251 (Fig. 3D). Lastly, neither WIN-55,212-2 nor any of the polyphenols tested altered intracellular cAMP levels in wild-type CHO cells not transfected with hCB1 (data shown). Interestingly, the inability of *trans*-resveratrol to alter cAMP levels in CHO cells suggests that these cells respond differently than MCF-7 breast cancer cells, in which resveratrol has been shown to directly stimulate adenylyl cylcase activity (El-Mowafy and Alkhalaf, 2003).

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Similar to human CB1 receptors, *trans*-resveratrol, curcumin and ASC-J9 bind with nanomolar affinity to, and act as antagonists/inverse agonists at, mouse CB1 receptors in membrane preparations of whole brain tissue. Prior to conducting *in vivo* studies in mice, *in vitro* studies were conducted to determine the affinity and intrinsic activity of the polyphenols at mouse CB1 receptors (Fig. 4). Homologous competition receptor binding with [^{3}H]CP-55,940 showed that mouse brain membranes contain a density of mCB1 cannabinoid receptors of 0.59 ± 0.14 pmole/mg protein, to which CP-55,940 binds with an affinity (K_D) of 2.6 ± 0.55 nM (N=3, data not shown). The affinity of WIN-55,212-2 for brain mCB1 receptors (3.4 ± 1.5 nM, N=5; Fig. 4A, Table 1) is similar to that observed in CHO-hCB1 membranes (Fig. 1). Furthermore, all three polyphenols bind to mCB1 receptors with high nM affinity and with the same rank order of potency as observed for hCB1 (Table 1). Curcumin binds mCB1 with the highest affinity (73 ± 24 nM, N=7), followed by similar, but lower, K_i values for *trans*-resveratrol (270 ± 160 nM, N=6) and ASC-J9 (190 ± 110 nM, N=8). WIN-55,212-2 activates G-proteins in mouse brain membranes with an ED_{50} of 76 ± 38 nM (N=3; Fig. 4B). Similar to that observed for hCB1, all polyphenols produce concentration-dependent inhibition of [^{35}S]GTPγS binding to mouse brain membranes (Fig. 4B). However, curcumin and ASC-J9 act as inverse agonists at lower concentrations (IC_{50} = 660 ± 370 nM, N=3; 360 ± 86 nM, N=3, respectively) than *trans*-resveratrol (IC_{50} = 6.7 ± 2.5 μM, N=4). This suggests that at mCB1 receptors in brain, *trans*-resveratrol acts a pure neutral antagonist, while curcumin and ASC-J9 are inverse agonists. Consistent with these predictions, the neutral antagonist *trans*-resveratrol significantly attenuated G-protein activation by WIN-55,212-2 (Fig. 4C, left panel), while the decrease in [^{35}S]GTPγS binding produced by the putative inverse agonists curcumin and ASC-J9 was reversed by co-incubation with the neutral CB1 antagonist O-2050 [(6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran] (Fig. 4C, center and right panels).

In mice, acute administration of *trans*-resveratrol, curcumin and ASC-J9 antagonize hypothermia produced by a CB1 agonist. Cannabinoid agonists produce a classic tetrad of effects in mice (hypothermia, analgesia, catalepsy and reduced locomotor activity), mediated by activation of CB1 receptors (Smith et al., 1994). To

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determine if the polyphenols act as antagonists/inverse agonists at mCB1 receptors *in vivo* (as predicted by *in vitro* assays), the ability of each compound to antagonize hypothermia produced by the cannabinoid agonist CP-55,940 was examined (Fig. 5A-B). One hour after s.c. injections, CP-55,940 produces a dose-related decrease in body temperature in mice of over 6°C with an ED_{50} of 0.25 mg/kg (0.23-0.28, 95% C.I.; N=5-22 mice per dose) (Fig. 5B). When co-administered with a 0.2 mg/kg dose of CP-55,940, employed to produce approximately a half-maximal effect (-3.1 ± 0.82^{o}C, N=22), all three polyphenols tested produce a significant dose-dependent reversal of CP-55,940-induced hypothermia with a rank order of potency (IC_{50}) for reversal of *trans*-resveratrol (14 mg/kg) > curcumin (52 mg/kg) > ASC-J9 (88 mg/kg) (Fig. 5A). Curcumin is most efficacious, resulting a complete antagonism of hypothermia. Indicative of competitive antagonism, co-administration with a fixed dose of *trans*-resveratrol (5 mg/kg) results in a significant ($p<0.05$), parallel rightward shift in the hypothermia dose-effect curve for CP-55,940 to 0.51 mg/kg (0.48-0.55, 95% C.I.; N=5 mice per dose). CP-55,940-induced hypothermia is completely blocked by the CB1 antagonist/inverse agonist AM-251 (10 mg/kg, data not shown). Additionally, when administered alone, polyphenol concentrations that produce maximal antagonism of hypothermia induced by CP-55,940 (*trans*-resveratrol, 50 mg/kg; curcumin, 500 mg/kg; ASC-J9, 200 mg/kg), produce no hypothermia (data not shown).

In mice, repeated administration of *trans*-resveratrol and curcumin produces dose-dependent reduction in body weight, similar to that produced by the CB1 antagonist/inverse agonist AM-251. CB1 antagonists/inverse agonists produce reductions in food intake and body weight in mice (Pavon et al., 2008). Since *in vitro* assays suggest that all polyphenols tested act as antagonists/inverse agonists at mCB1, the ability of *trans*-resveratrol and curcumin to reduce body weight in mice was examined (Fig. 5C). As anticipated, the CB1 antagonist/inverse agonist AM-251 [N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (10 mg/kg) administered twice daily for 3 days results in a significant ($p<0.01$) weight loss of 2.8 ± 0.47 gms (Fig. 5C, left panel; N=6). Similarly, repeated administration of curcumin produces a dose-related weight loss, equivalent to that produced by AM-251 (Fig. 5C, center panel; N=5). Although slightly higher doses are

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required, *trans*-resveratrol also results in significant ($p<0.05$), dose-dependent weight loss (Fig. 5C, right panel, N=5).

***In Silico* comparison of the structures of *trans*-resveratrol and curcumin with known cannabinoids reveals common structural motifs.** Molecular modeling studies employing CAChe® molecular modeling software (Fujitsu) with structure minimizations performed with a PM5 wavefunction in water reveals that the favored conformation of trans-resveratrol (Fig. 6A, in red) is similar to that of a series of novel synthetic resorcinol-derived cannabinoids (Wiley et al., 2002), as graphically illustrated by comparison with the resorcinol O-1422 (Fig. 6A, in green). When the resorcinol rings of both molecules are overlaid, the similarities are striking. While the cyclohexyl group of O-1422 is not present in trans-resveratrol, the dimethylheptyl sidechain (also present in many other cannabinoids) of O-1422 is similar in length to the trans-double bond and phenol ring of resveratrol.

In addition, a subsequent overlay of the CB1-selective ligand rimonabant [5-(p-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-piperidinopyrazole-3-carboxamide hydrochloride] (Fig. 6B, in blue), trans-resveratrol (Fig. 6B, in red) and curcumin (Fig. 6B, in purple) reveals several areas of similarity that closely match a 3D pharmacophore model of CB1-selective ligands recently proposed by Wang *et al.* (Wang et al., 2008). For example, an aromatic region (A) and a hydrophobic region (B) in which are located aromatic rings containing electron-withdrawing groups, are present in all three molecules. Furthermore, the amide carbonyl (of rimonabant), the carbonyl (of curcumin) and the phenol (of trans-resveratrol) all contain electron-donating oxygens (hydrogen bond acceptors) and are all located in the middle region, hence designated as electron-donating region C.

Based on inferences drawn from a model proposed by Song *et al.* (Song et al., 1999), it might therefore be predicted that aromatic rings contained in region A of these ligands likely interact with some combination of CB1 receptor residues F3.25(189), W5.43(279), F5.42(278) Y5.39(275). Moreover, it is also probable that the hydrophobic region B of these compounds might interact with CB1 receptor residue F3.36(200). In any case, it is clear that trans-resveratrol and curcumin share several common structural motifs with known cannabinoid ligands, and these motifs likely contribute to

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their ability to bind with high affinity to CB1 receptors.

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Discussion:

The most significant finding of this study is the identification of human CB1 cannabinoid receptors as a high affinity target for three distinct polyphenols; *trans*-resveratrol (K_i = 45 nM), curcumin (K_i = 6 nM) and ASC-J9 (K_i = 64 nM, an analog of curcumin). All polyphenols examined appear to act as CB1 antagonists/inverse agonists, at dietary relevant concentrations, in both *in vitro* and *in vivo* assays. Furthermore, *in silico* comparison of the structures of *trans*-resveratrol and curcumin with known cannabinoids reveals common structural motifs. Coupled with their proven safety, these studies indicate that *trans*-resveratrol, curcumin and/or their derivatives might be developed as novel, non-toxic CB1 therapeutics for use in obesity, diabetes, drug dependence and additional disease states in which CB1 antagonists have shown efficacy.

Polyphenols, including *trans*-resveratrol and curcumin, are known to produce many biological effects by acting on multiple targets (Stevenson and Hurst, 2007). *Trans*-resveratrol and curcumin are very efficacious anti-oxidant (Fraga, 2007) and anti-inflammatory (Surh et al., 2005) agents, however, their *in vitro* effects require relatively high concentrations (>1 μM) and are thought to involve multiple receptor and non-receptor mediated processes. Therefore, the specific molecular mechanisms responsible for these effects remain unclear. This study identifies CB1 receptors as one of the highest affinity targets for *trans*-resveratrol and curcumin reported to date. For example, while *trans*-resveratrol inhibits the activity of quinone reductase 2 (QR2) with a dissociation constant of 35-50 nM (Buryanovskyy et al., 2004), much higher concentrations are required to stimulate adenylyl cyclase (800 nM) (El-Mowafy and Alkhalaf, 2003) or inhibit the activity of Ikappaβ kinase (1 μM) (Kundu et al., 2006) and lipooxygenase (3.7 μM) (Jang et al., 1997). Similarly, curcumin inhibits the activity of glycogen synthase kinase-2β with an IC_{50} of 63 nM (Bustanji et al., 2008), however, significantly greater concentrations are required to reduce the aggregation of β-amyloid (800 nM) (Yang et al., 2005) or inhibit glutathione S-transferases (0.04-5 μM) (Hayeshi et al., 2007). Therefore, when compared with the affinity for most other identified targets, it is likely that CB1 receptors clearly play an important role in the molecular mechanism of action for *trans*-resveratrol and curcumin, requiring relatively low, physiologically attainable concentrations to produce near full CB1 receptor occupancy.

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The present findings are additionally important because they identify a specific, high affinity, receptor-mediated mechanism that likely contributes to many of the reported beneficial effects of these and other structural related polyphenols in a variety of disease states. For example, both CB1 antagonists/inverse agonists and polyphenols (including *trans*-resveratrol and curcumin) are efficacious anti-inflammatory agents (Rahman et al., 2006; Muccioli, 2007) and appear to be promising therapeutics for use in cardiovascular disease, cancer, stroke and diabetes (Scalbert et al., 2005). In addition, curcumin has been used for centuries in the traditional Indian Ayurveda system of medicine to reduce the hallucinatory effects of many psychotropic drugs including hashish, a potent form of cannabis (Tilak et al., 2004). However, the most direct evidence supporting our observations that certain polyphenols may produce actions through CB1 receptors is provided by the recent report that *trans*-resveratrol and several other polyphenols bind to a specific, yet unidentified, binding site in rat brain (Han et al., 2006). Similar to CB1 receptors, these binding sites are localized to plasma membranes, expressed in high density and widely distributed throughout the brain. Most interestingly, [^{3}H]*trans*-resveratrol binds to these unidentified sites with an affinity (K_D) of 220 nM, very similar to its affinity (K_i) for mCB1 receptors of 270 nM reported in this study. It is certainly possible that [^{3}H]*trans*-resveratrol might also bind to the orphan receptor GPR55, or to other non-cannabinoid GPCRs such as dopamine receptors, to which cannabinoid receptor ligands also bind.

Interestingly, all three polyphenols were shown to possess both neutral antagonist and inverse agonist properties, depending on the assay or tissue/cell homogenate examined. These data suggest that the polyphenols tested might act as protean agonists at CB1 receptors, similar to that recently described for the CB2 ligand AM-1241 (Yao et al., 2006). A protean agonist is a compound that changes its apparent intrinsic activity to exhibit agonist, antagonist or inverse agonist activity at the same receptor, depending on the specific assay systems employed for detection. Alternatively, a more simple explanation for the current observations might be due to differences between assay conditions used for the GTPγS binding assay (employing membrane homogenates and relatively high concentrations of guanine nucleotides), relative to that employed for the cAMP assay (employing whole cells).

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Trans-resveratrol and curcumin, like most polyphenols, are extensively and rapidly metabolized by glucuronidation and sulfation in the liver and other tissues (Singh et al., 2008). This predicts that relatively poor bioavailability, particularly in the CNS, might preclude observation of significant antagonism of effects mediated by central CB1 receptors in mice as reported here. However, even with such unfavorable pharmacokinetic properties, peak serum concentrations in mice of approximately 1-2 μM of parent drug following a single, acute intra-peritoneal injection of moderate doses (~20-100 mg/kg) of either *trans*-resveratrol (Asensi et al., 2002) or curcumin (Pan et al., 1999) have been reported. In addition, curcumin can accumulate to concentrations as high as 1-2 μM in the brains of mice chronically fed a relatively low dose of 2 mg/kg/day (Begum et al., 2008). Very low doses of *trans*-resveratrol protect against neuronal damage following cerebral ischemia, providing evidence that this polyphenol is also able to cross the blood-brain-barrier in sufficient concentrations to provide neuroprotection (Wang et al., 2003). Lastly, in humans, consumption of a single oral 7.5 μg/kg dose of dietary *trans*-resveratrol contained in red wine results in a serum concentration of ~26 nM and a 25 mg per 70 kg of body weight oral dose of pure *trans*-resveratrol results in a serum concentration of ~37 nM ([reviewed in [Baur, 2006 #1682]). Although no chronic consumption studies in humans have been conducted, it might be predicted that serum levels of *trans*-resveratrol occurring in daily red wine drinkers might be even higher than those observed following a single exposure. Based on their high nM affinities for mCB1 receptors reported here, if such μM (or even high nM) concentrations of *trans*-resveratrol, curcumin or ASC-J9 are attained in the brain, near full receptor occupancy would be predicted. Alternatively, it is also certainly possible that a metabolite of *trans*-resveratrol and/or curcumin might also bind with high (or superior) affinity to CB1 receptors to mediate the *in vivo* effects reported here. In any case, due to the potential therapeutic promise of these drugs in a number of disease states, several methods to improve their systemic bioavailability, including the development of liposomal and nanoparticle preparations, are actively being pursued (Anand et al., 2007). Based on the present findings, future development of polyphenol-based CB1 ligands should include similar studies to improve systemic bioavailability.

Activation of peripheral CB1 receptors is effective at suppressing inflammation that leads to chronic pain states (Gutierrez et al., 2007). However, the potential use of

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current CB1 agonists for this application is severely limited by concurrent stimulation of central CB1 receptors resulting in unacceptable psychotropic side effects. Furthermore, the CB1 antagonist/inverse agonist rimonabant is very effective for management of obesity (Pavon et al., 2008). However, several adverse effects, presumed to be mediated via blockade of central CB1 receptors, resulted in the recent discontinuance of all ongoing clinical trials of rimonabant in Europe (Jones, 2008) and thus virtually assuring a lack of future FDA approval for use in the United States. Several studies indicate that the metabolic benefits of CB1 antagonists/inverse agonists in obese animals is due to action at peripheral, but not central, CB1 receptors (Pavon et al., 2008). Indeed, results from the present study demonstrating that repeated administration of curcumin or *trans*-resveratrol produces a dose-dependent reduction in body weight provide additional evidence for this observation. Interestingly, although not attributed to action at CB1 receptors, others also report that trans-resveratrol reduces body weight in Zucker obese rats (Lekli et al., 2008). Therefore, polyphenol-derived, peripherally restricted CB1 agonists or antagonists might be developed as a novel class of non-toxic cannabinoids. The observation that high doses of either *trans*-resveratrol (Espin et al., 2007) or curcumin (Chainani-Wu, 2003) appear to be well tolerated and produce a very limited number of adverse side effects in humans provides further support for this hypothesis.

Lastly, as an additional advantage, it is likely that polyphenol-derived CB1 ligands could be developed that posses multiple therapeutic actions due to their pleiotropic action at several distinct targets simultaneously, in addition to their action at CB1 receptors. Such novel CB1 antagonists/inverse agonists might be particularly useful for the treatment of several disease states. For example, current CB1 antagonists/inverse agonists appear to be very efficacious for the management of obesity (Pavon et al., 2008). Anti-oxidants also reduce many adverse consequences associated with obseity (Vincent et al., 2007). As such, novel polyphenol-derived CB1 antagonists, due to combined CB1 antagonism and anticipated antioxidant properties (Fraga, 2007), might provide additive or even synergistic improvement of obesity symptoms.

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Legends for figures:

Fig. 1. *Trans*-resveratrol, curcumin and the curcumin analog ASC-J9 selectively bind with nanomolar affinities to human CB1 receptors stably expressed in CHO cells. Membranes prepared from CHO-hCB1 and CHO-hCB2 cells were incubated with 0.5 or 0.1 nM of the CB1/CB2 ligand [^{3}H]CP-55,940 and increasing concentrations of WIN-55,212-2 (**panel A**), cucumin (**panel B**), ASC-J9 (**panel C**) and *trans*-resveratrol (**panel D**). Results are expressed as the percent of specific [^{3}H]CP-55,940 binding. The IC_{50} values obtained were converted to a measure of receptor affinity (K_i) by employing the Cheng-Prushoff equation and are presented in Table 1.

Fig. 2. *Trans*-resveratrol, curcumin and ASC-J9 act as antagonists/inverse agonists at human CB1 receptors in membrane preparations of CHO-hCB1 cells. ***Panel A***; CHO-hCB1 membranes were incubated with 0.1 nM [^{35}S]GTPγS in the presence of increasing concentrations of the CB1 agonist WIN-55,212-2 (filled squares), curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds) alone. Results are expressed as the percent of the specific [^{35}S]GTPγS binding. The ED_{50} and IC_{50} values are presented in the Results section. ***Panel B***; WIN-55,212-2 concentration-effect curves for [^{35}S]GTPγS binding were determined in the absence (filled squares) or presence of a single, fixed concentration of curcumin (3 μM, open circles), ASC-J9 (3 μM, open triangles) or *trans*-resveratrol (10 μM, open diamonds). The ED_{50} values are presented in the Results section.

Fig. 3. *Trans*-resveratrol, curcumin and ASC-J9 act as neutral antagonists at human CB1 receptors in intact CHO-hCB1 cells. ***Panel A***; Forskolin (10 μM) stimulated adenylyl cyclase assays were conducted in whole CHO-hCB1 cells. Intracellular cAMP levels were measured in response to increasing concentrations of the CB1 agonist WIN-55,212-2 (filled squares), curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds) alone. Data are presented as the % of cAMP levels measured in the presence of the indicated drug concentrations, compared to that observed in the absence of drugs (*i.e.,* % of Control). ***Panel B***; WIN-55,212-2 concentration-effect curves for inhibition of forskolin-stimulated adenylyl cylclase activity were determined in the absence (filled squares) or presence of a single, fixed 10 μM

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concentration of curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds). The IC_{50} values determined in panels A and B are presented in the Results section. ***Panel C***; Curcumin (10 μM), ASC-J9 (10 μM) and *trans*-resveratrol (10 μM) significantly block inhibition of adenylyl cyclase activity produced by 10 nM of WIN-55,212-2. ***Panel D***; Curcumin (10 μM), ASC-J9 (30 μM) and *trans*-resveratrol (10 μM) significantly attenuate stimulation of adenylyl cyclase activity produced by 10 nM of the inverse agonist AM-251.

[a-b]Values designated with different letters above the error bars are significantly different (One-way ANOVA followed by a Dunnett's *post-hoc* comparison, $P<0.05$).

Fig. 4. *Trans*-resveratrol, curcumin and ASC-J9 bind with nanomolar affinity to, and act as antagonists/inverse agonists at, mouse CB1 receptors in membrane preparations of whole brain tissue. ***Panel A***; Mouse brain membranes were incubated with 0.1 nM of the CB1/CB2 ligand $[^3H]$CP-55,940 and increasing concentrations of WIN-55,212-2 (filled squares), curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds). Results are expressed as the percent of specific $[^3H]$CP-55,940 binding. ***Panel B***; Mouse brain membranes were incubated with 0.1 nM $[^{35}S]GTP\gamma S$ in the presence of increasing concentrations of WIN-55,212-2 (filled squares), curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds) alone. Results are expressed as the percent of the specific $[^{35}S]GTP\gamma S$ binding. ***Panel C***; *Trans*-resveratrol (10 μM) attenuates stimulation of $[^{35}S]GTP\gamma S$ binding produced by 1 μM of the CB1 agonist WIN-55,212-2. The neutral CB1 antagonist O-2040 (10 μM) blocks the inhibition of basal $[^{35}S]GTP\gamma S$ binding produced by curcumin (1 μM) and ASC-J9 (300 nM).

[a-b]Values designated with different letters above the error bars are significantly different (One-way ANOVA followed by a Dunnett's *post-hoc* comparison, $P<0.05$). [*,**]Significantly different from the % of $[^{35}S]GTP\gamma S$ binding produced by curcumin or ASC-J9 alone (Unpaired Student's *t*-test, $P<0.05$, 0.01).

Fig. 5. In mice, *trans*-resveratrol, curcumin and ASC-J9 antagonize hypothermia produced by a CB1 agonist and repeated treatment reduces body weight similar to that produced by a CB1 inverse agonist. ***Panel A***; Hypothermia produced by 0.2

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mg/kg of the CB1/CB2 agonist CP-55-940 was dose-dependently reduced by pretreatment with curcumin (open circles), ASC-J9 (open triangles) or *trans*-resveratrol (open diamonds). ***Panel B***; Pretreatment of mice with a single, fixed 5 mg/kg dose of *trans*-resveratrol resulted in a 2-fold parallel shift-to-the-right in the dose-response curve for hypothermia produced by CP-55-940. ***Panel C***; Twice daily i.p. injections of curcumin or *trans*-resveratol resulted in a dose-dependent reduction in body weight of mice similar to that produced by the CB1 antagonist/inverse agonist AM-251.

[*,**]Significantly different from the hypothermia produced by CP-55,940 alone (One-way ANOVA followed by a Dunnett's *post-hoc* comparison, P<0.05, 0.01).

[a-b]Values designated with different letters above the error bars are significantly different (One-way ANOVA followed by a Dunnett's *post-hoc* comparison, P<0.05).

Fig. 6. *In Silico* comparison of the structures of *trans*-resveratrol and curcumin with known cannabinoid receptor ligands reveals common structural motifs. ***Panel A***; CAChe® molecular modeling software reveals that the favored conformation of *trans*-resveratrol (in red) is similar to a novel synthetic resorcinol cannabinoid O-1422 (in green). ***Panel B***; Curcumin (in purple) and *trans*-resveratrol also share aromatic, hydrophobic and electron-donating regions similar to that occurring in the CB1 selective ligand rimonabant (in blue).

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Table 1: Selectivity of Polyphenols for Human CB1 and CB2 Receptors.

	Ki (nM)				
Drug	***mCB1***	***hCB1***	***hCB2***	***hCB2/ hCB1***	***Selectivity***
WIN-55,212-2	3.4 ± 1.6 (5)	7.7 ± 1.3 (5)	5.8 ± 1.2 (4)	0.75	Non-Selective
Curcumin	73.1 ± 23.5 (7)	5.9 ± 2.1 (6)	2600 ± 900 (4)	446	CB1
ASC-J9	190 ± 110 (8)	64 ± 17 (3)	13000 ± 1300 (4)	201	CB1
Trans-Resveratrol	270 ± 160 (6)	45 ± 17 (3)	>100,000 (4)	>2227	CB1

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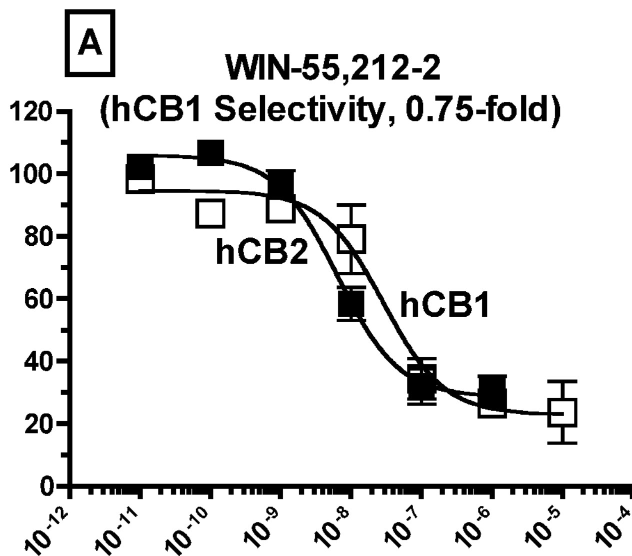
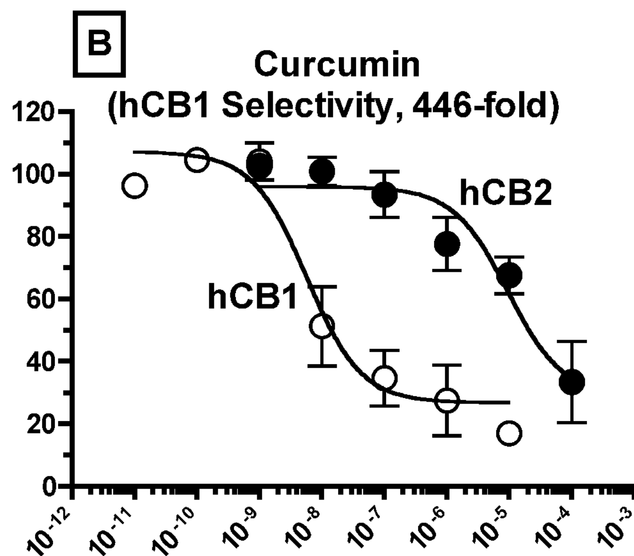
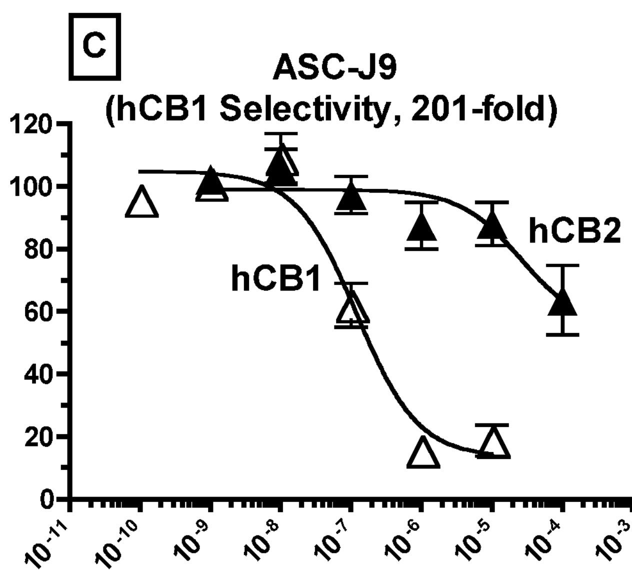
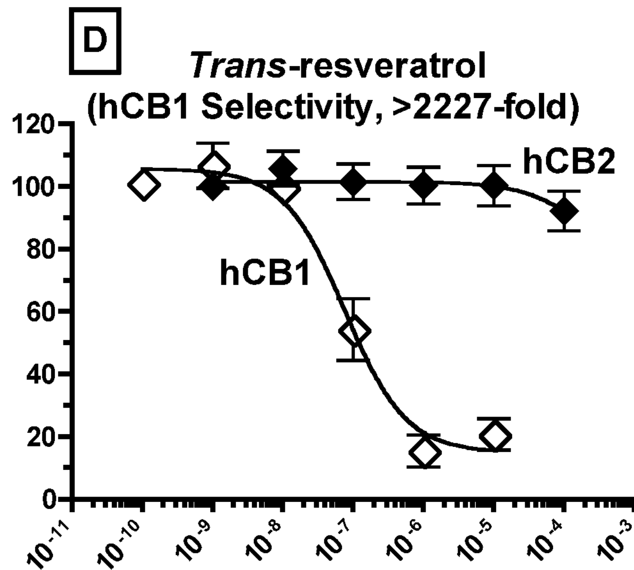
Figure 1









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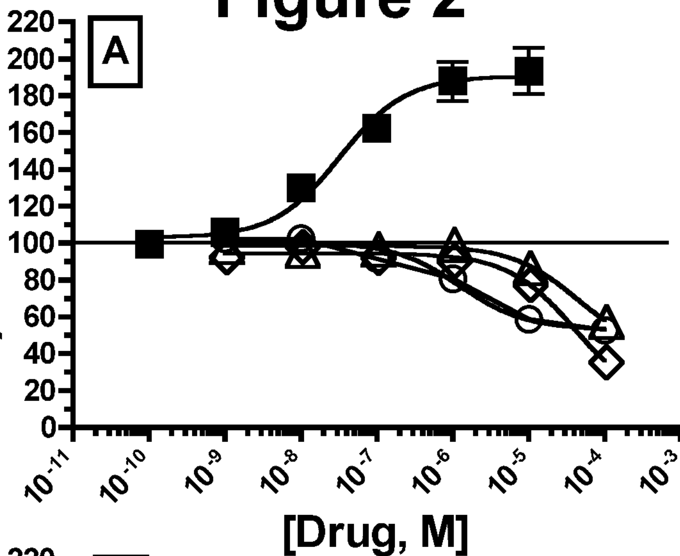


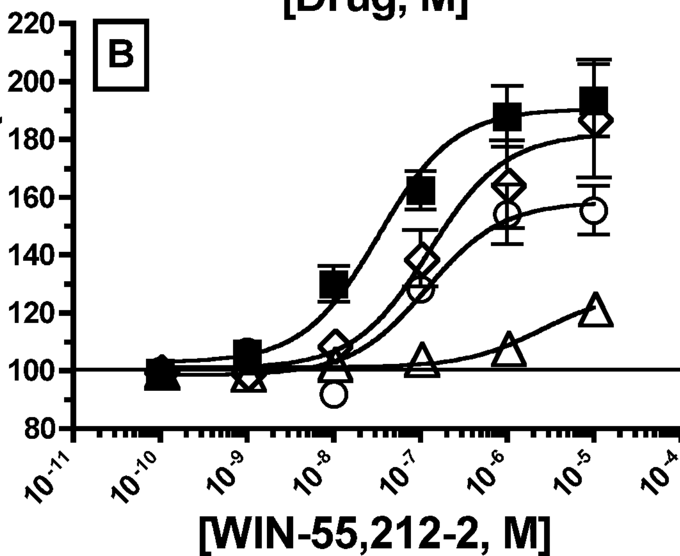
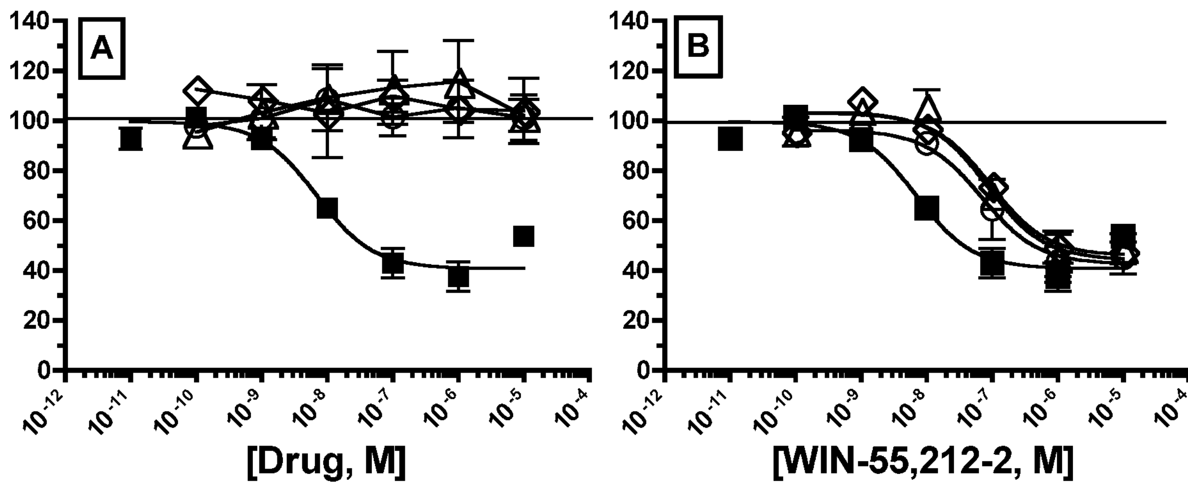


Figure 3



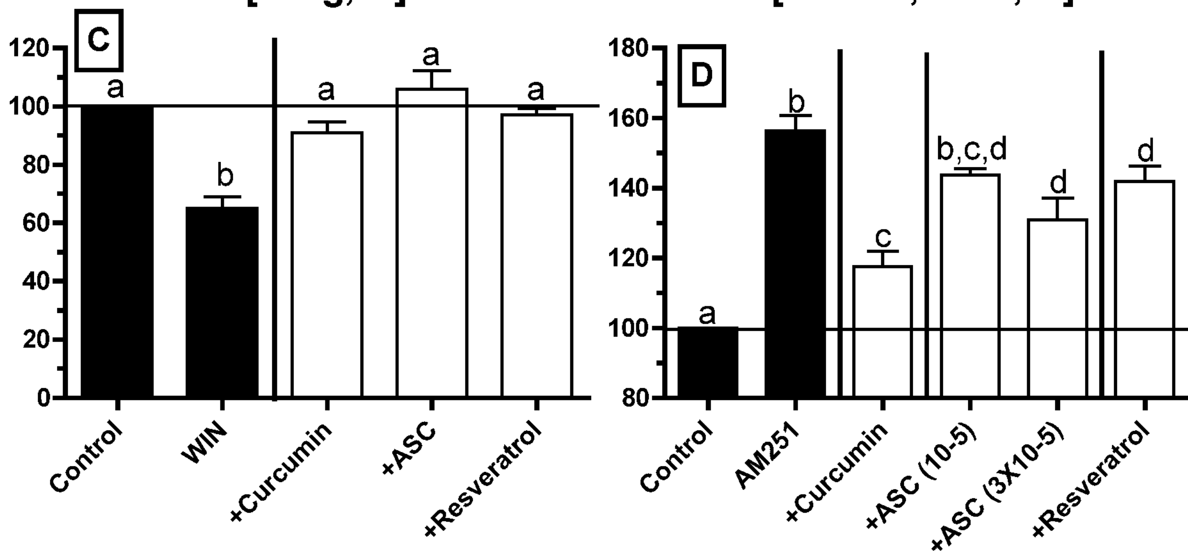


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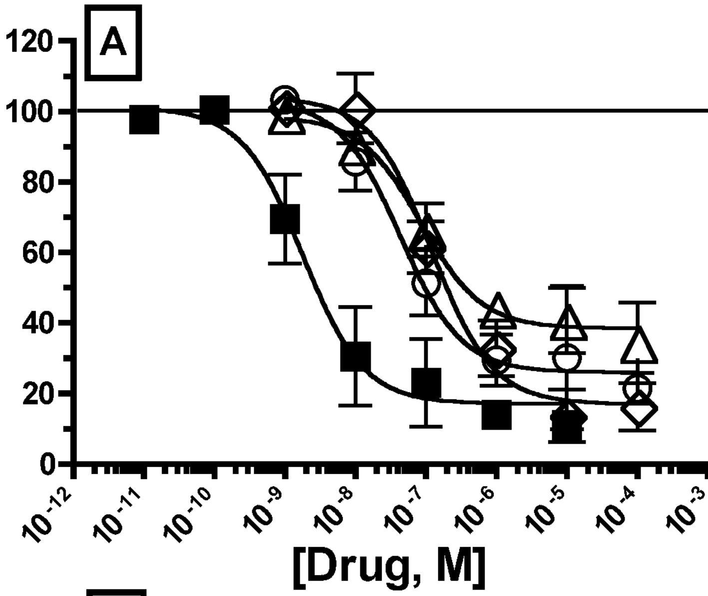
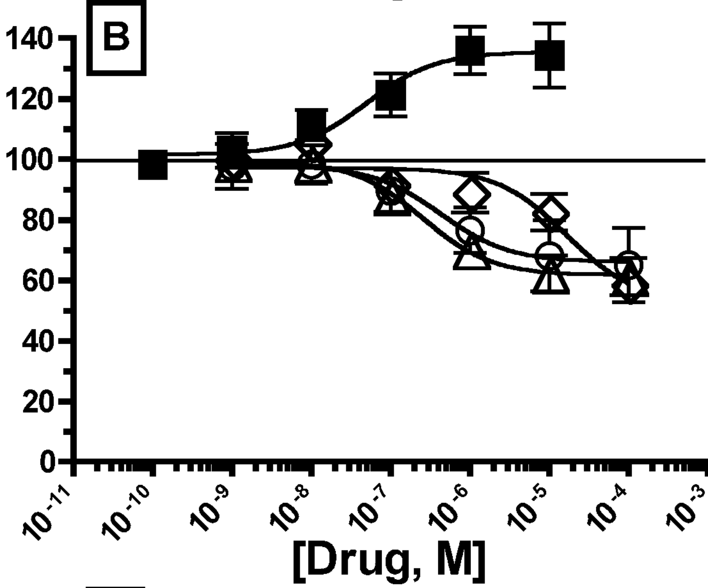
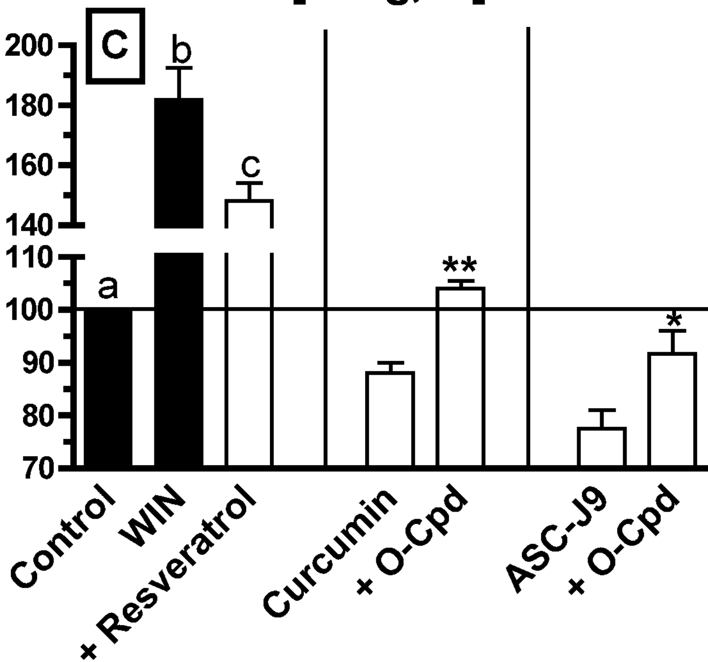
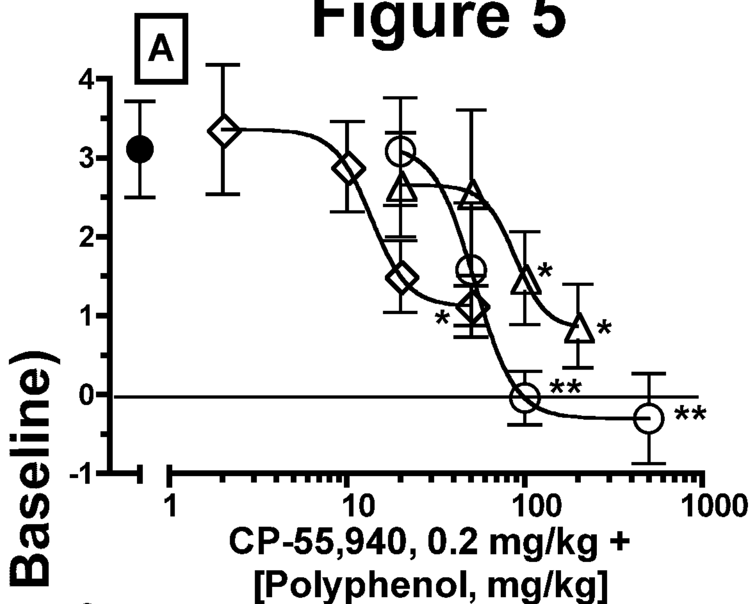
Figure 4









B

CP-55,940, mg/kg

C

AM251

Curcumin

Resveratrol

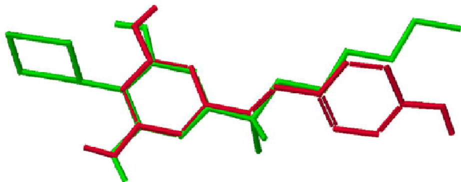
Change in Body Weight (Grams)

Drug, mg/kg

(i.p. twice daily x 3 days)

Figure 6

A



B

Aromatic Region A

Electron-donating
Region C

Hydrophobic Region B