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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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ABSTRACT

The dietary polyphenols *trans*-resveratrol [5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol; found in red wine] and curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1*E*,6*E*-heptadiene-3,5-dione] (found in curry powders) exert anti-inflammatory and antioxidant effects via poorly defined mechanisms. It is inte[illegible] esting that cannabinoids, derived from the marijuan[illegible] (*Cannabis sativa*), produce similar protective eff[illegible] and CB2 receptors. We examined whether [illegible] curcumin, and ASC-J9 [1,7-bis(3,4-[illegible] droxy-1*E*,4*E*,6*E*-heptatriene-3-one[illegible] ligands at cannabinoid rece[illegible] CB1 and mouse CB1 [illegible] playing only micr[illegible] teristic of in[illegible] protein activit[illegible] ovary (CHO)-h[illegible] neutral CB1 anta[illegible] ag-onize G-protein a[illegible] in intact CHO-hCB1 cells [illegible] neutral antagonists, producing [illegible] reas competitively antag[illegible] n of adenylyl cy-[illegible] st profile in cells, [illegible] of adenylyl cy-[illegible] t. In mice, the [illegible] pothermia pro-[illegible] administration, the [illegible] in mice similar to that [illegible] inverse agonist. Finally, *trans*-[illegible] hare common structural motifs with [illegible] inoid receptor ligands. Collectively, we [illegible] s-resveratrol and curcumin act as antagonists/[illegible] gonists at CB1 receptors at dietary relevant concen-[illegible] ns. Therefore, these polyphenols and their derivatives might be developed as novel, nontoxic CB1 therapeutics for obesity and/or drug dependence.

Dietary polyphenols, such as resveratrol [5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol] (found in red wine) and curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1*E*,6*E*-heptadiene-3,5-dione] (found in curry powders), have been used safely for centuries as traditional medicines. As a consequence, increasing scientific investigation suggests that they may 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 seem to be related to their antioxidant (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 in-

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ABBREVIATIONS: ASC-J9, 1,7-bis(3,4-dimethoxyphenyl)-5-hydroxy-1*E*,4*E*,6*E*-heptatriene-3-one; [^{3}H]CP-55,950, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; CHO, Chinese hamster ovary; h, human; 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; CP-55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; ANOVA, analysis of variance; HU-210, (−)-11-hydroxy-δ(8)-tetrahydrocannabinol-dimethylheptyl; AM-251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; m, mouse; O-2050, (6*aR*,10*aR*)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6*a*,7,10,10*a*-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran; rimonabant, 5-(*p*-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-piperidinopyrazole-3-carboxamide hydrochloride; AM1241, (*R*,*S*)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1*H*-indole.

volve multiple receptor- and nonreceptor-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 seem to share many characteristics with this newly discovered, uncharacterized resveratrol receptor. Originally isolated from the marijuana plant (*Cannabis sativa*), both synthetic and naturally occurring cannabinoids, such as Δ^9-tetrahydrocannabinol, produce their effects by acting at two G-protein-coupled receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993). CB1 receptors are expressed in high abundance throughout the central nervous system, whereas CB2 receptors are expressed predominantly in immune cells and non-neuronal tissues. Cannabinoids acting at both receptors produce antioxidant (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. It is important that 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 seem to act as CB
antagonists/inverse agonists and share common st
motifs with other known cannabinoid recepto
important that these results indicate th
one of the highest affinity targets
veratrol and curcumin and m
for future development

Materials

All drugs used
(Ellisville, MO). [^{3}H
Ci/mmol) were purc
Sciences (Waltham,
from Vitrax (Placenia,
Thermo Fisher Scientifi

ence
γS (1250
and Analytical
/mmol) was obtained
agents were purchased from
n, MA).

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 and 95% air at 37°C in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml Geneticin (G418).

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 nM (CHO-hCB1) [^{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. Nonspecific binding was defined as binding observed in the presence of 1 μM nonradioactive CP-55,940. Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ) followed by two washes with ice-cold binding buffer. Analysis of the binding data were performed using the nonlinear regression (Curve Fit) function of GraphPad Prism version 4.0b (GraphPad Software Inc., San Diego, CA) to determine the concentration of the drug that displaced 50% of [^{3}H]CP-55,940 (IC_{50}). A measure of affinity (K_i) was derived from the IC_{50} values utilizing the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

[^{35}S]GTPγS Binding

[^{35}S]GTPγS binding
with minor modifications as describe
al., 2005) in a buffer containing
, 10 mM $MgCl_2$, and 0.1%
ion contained 100
B1) of membrane
nd 10 μM GDP.
active GTPγS.
terminated by
ined by liquid scintil-

evels in Intact Cells

[^{3}H]adenine-labeled ATP pools to cAMP was
ional measure of cannabinoid activity (Shoemaker et
). CHO-hCB1 cells were seeded into 24-well plates and
ured to confluence. Dulbecco's modified Eagle's medium containing 0.9% NaCl, 500 μM 3-isobutyl-1-methylxanthine, and 2 μCi/well [^{3}H]adenine was added to the cells for 2 h 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 Institutional Animal Care and Use Committee and conducted in accordance with the United States Public Health Service policy on humane care and use of laboratory animals. Male and female B6SJL mice were obtained from an in house breeding colony.

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

Body Weight Reduction Experiments. Age- and weight-matched mice were injected intraperitoneally with the indicated

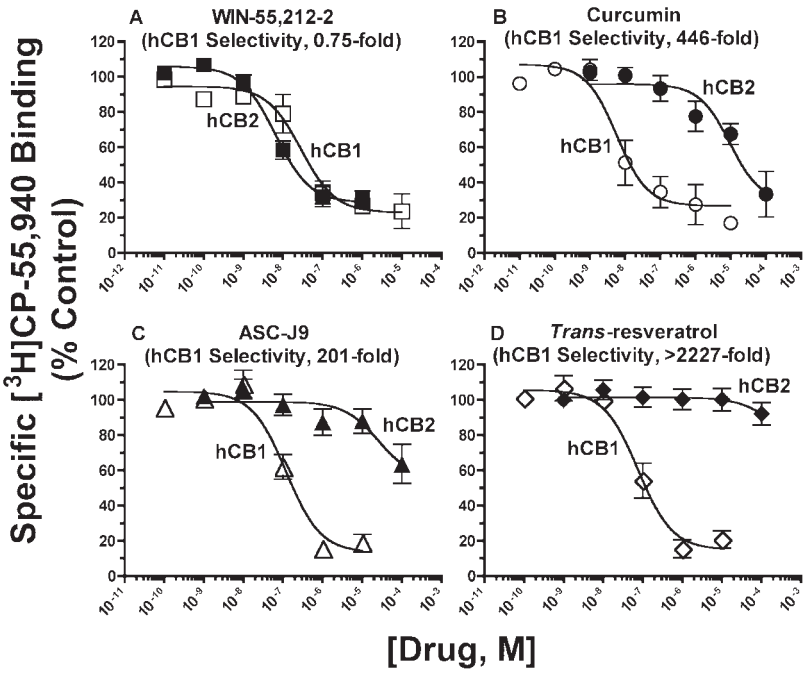

Fig. 1. *trans*-Resveratrol, curcumin, and the curcumin analog ASC-J9 selectively bind with nanomolar affinities to human CB1 receptors stably expressed in CHO cells. Membranes prepared from CHO-hCB1 and CHO-hCB2 cells were incubated with 0.5 or 0.1 nM CB1/CB2 ligand [^{3}H]CP-55,940 and increasing concentrations of WIN-55,212-2 (A), curcumin (B), ASC-J9 (C), and *trans*-resveratrol (D). Results are expressed as the percentage 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-Prusoff equation and are presented in Table 1.

doses of test drugs twice daily for 3 days. Body weight (in grams) was recorded each morning before drug injection and finally at 9:00 AM on day 4 of the study, 12 h after the last drug dose. Animals were fed ad libitum during the 3-day experiment. The injection vehicle for these experiments contained 50% polyethylene glycol and 50% saline.

Statistical Analysis

Curve-fitting and statistical analysis were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Differences between experimental groups were analyzed by one-way ANOVA followed by a Dunnett's post hoc test. Differences between two groups were analyzed by paired Student's *t* test. Differences were considered significant when $P < 0.05$. IC_{50} values were obtained from two

***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 pmol/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 pmol/mg protein (Shoemaker et al., 2005). [^{3}H]CP-55,940 binds nonselectively to hCB1 and hCB2 receptors expressed in CHO cells, with a K_d of 1.0 ± 0.3 or 0.38

mpetition binding
ve cannabinoid
alent nanomo-
5) and hCB2
1). In contrast,
ively bind to hCB1,
1, B–D). Curcumin is 446-
B1 with an affinity of 5.9 ± 2.1
g a K_i for hCB2 of over 2 μM (Fig. 1B).
log of curcumin, although demonstrating a
wer affinity (64 ± 17 nM; $n = 3$), also binds to hCB1
h a 201-fold selectively over hCB2 (13 ± 1.3 μM; $n = 4$)
(Fig. 1C). *trans*-Resveratrol 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). It is important that *cis*-resveratrol 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).

It is curious that approximately 10 to 15% of 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 nonradioactive drugs were employed for competition. It is possible that the residual biding was due, in part, to the use of nonradioactive CP-55,940 to define nonspecific binding. Un-

TABLE 1
Selectivity of polyphenols for human CB1 and CB2 receptors

Drug	K_i				Selectivity
	mCB1	hCB1	hCB2	hCB2/hCB1	
			nM		
WIN-55,212-2	3.4 ± 1.6 (5)	7.7 ± 1.3 (5)	5.8 ± 1.2 (4)	0.75	Nonselective
Curcumin	73.1 ± 23.5 (7)	5.9 ± 2.1 (6)	2600 ± 900 (4)	446	CB1
ASC-J9	190 ± 110 (8)	64 ± 17 (3)	13,000 ± 1300 (4)	201	CB1
trans-Resveratrol	270 ± 160 (6)	45 ± 17 (3)	>100,000 (4)	>2227	CB1

der certain conditions, employing the same nonradioactive compound to define nonspecific binding of the radioactive compound may identify binding erroneously as specific, when in reality it is nonspecific 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 nonselective cannabinoid agonist HU-210 (1 μM) (data not shown). Results from these experiments revealed that nonradioactive 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 nonradioactive CP-55,940 to define nonspecific binding. Although 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 nonselective 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 [illegible] 2A; $n = 3$). In marked contrast, when tested [illegible] phenols produce a concentration-dep[illegible]

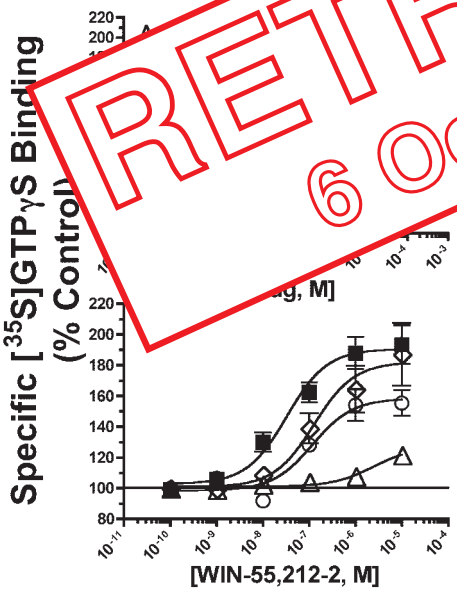

Fig. 2. *trans*-Resveratrol, curcumin, and ASC-J9 act as antagonists/inverse agonists at human CB1 receptors in membrane preparations of CHO-hCB1 cells. A, CHO-hCB1 membranes were incubated with 0.1 nM [^{35}S]GTPγS in the presence of increasing concentrations of the CB1 agonist WIN-55,212-2 (filled squares), curcumin (open circles), ASC-J9 (open triangles), or *trans*-resveratrol (open diamonds) alone. Results are expressed as the percentage of the specific [^{35}S]GTPγS binding. The ED_{50} and IC_{50} values are presented under *Results*. 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 under *Results*.

[^{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, $n = 3$) compared with their high nanomolar affinity for hCB1 receptors (Fig. 1). Consistent with an antagonist/inverse agonist profile, coincubation with a fixed concentration of each polyphenol that produced minimal reduction of [^{35}S]GTPγS binding alone resulted in a significant 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). It is interesting that coincubation [illegible] ASC-J9 resulted in a much greater, 63-fold redu[illegible] the potency of WIN-55,212-2 to activate G[illegible] O-hCB1 membranes (2500 ± 640 nM

***trans*-Re[illegible] ASC-J9 Act as Neutr[illegible] eceptors in In-**

[illegible]ors activate the [illegible]ivity of the ef-[illegible]nabinoid ago-[illegible]er, and inverse [illegible]ular cAMP (Fig. 3). [illegible]ration-dependent reduction [illegible]cAMP levels in intact CHO-hCB1 [illegible]12.0 ± 4.6 nM ($n = 8$; Fig. 3, A and B). [illegible]rast, exposure of CHO-CB1 cells to all three [illegible]s with concentrations as high as 10 μM did not [illegible] 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, coincubation 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). It is interesting that 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 cyclase activity (El-Mowafy and Alkhalaf, 2003).

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. Before conducting in vivo studies in mice, in vitro studies were conducted to determine the affinity and

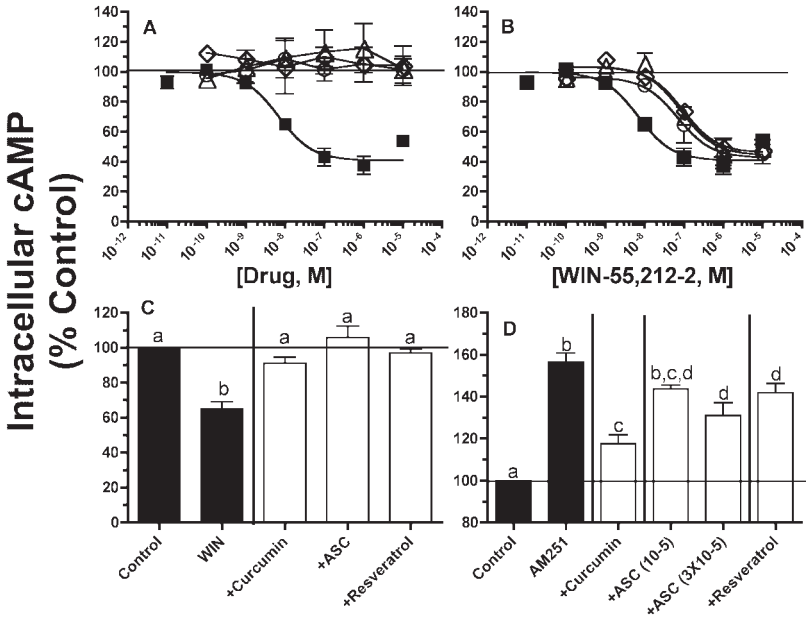

Fig. 3. *trans*-Resveratrol, curcumin, and ASC-J9 act as neutral antagonists at human CB1 receptors in intact CHO-hCB1 cells. A, forskolin (10 μM)-stimulated adenylyl cyclase assays were conducted in whole CHO-hCB1 cells. Intracellular cAMP levels were measured in response to increasing concentrations 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 percentage of cAMP levels measured in the presence of the indicated drug concentrations, compared with that observed in the absence of drugs (i.e., percentage of control). B, WIN-55,212-2 concentration-effect curves for inhibition of forskolin-stimulated adenylyl cyclase activity were determined in the absence (filled squares) or presence of a single, fixed 10 μM concentration of curcumin (open circles), ASC-J9 (open triangles), or *trans*-resveratrol (open diamonds). The IC_{50} values determined in A and B are presented under *Results*. C, curcumin (10 μM), ASC-J9 (10 μM), and *trans*-resveratrol (10 μM) significantly block inhibition of adenylyl cyclase activity produced by 10 nM WIN-55,212-2. D, curcumin (10 μM), ASC-J9 (30 μM), and *trans*-resveratrol (10 μM) significantly attenuate stimulation of adenylyl cyclase activity produced by 10 nM inverse agonist AM-251. Values designated with different letters above the error bars are significantly different (one-way ANOVA followed by Dunnett's post hoc comparison, $P < 0.05$).

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 pmol/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 ± ... Fig. 4A; Table 1) is similar to that obs... membranes (Fig. 1). Furthermore, ... to mCB1 receptors with high... same rank order of pot... Curcumin binds ... (n = 7), follo... resveratrol (2... nM, n = 8). ... brain membran... Fig. 4B). Similar to th... nols produce concentration... S]GTPγS binding to mouse brain ...). However, curcumin and ASC-J9 act as in... sts at lower concentrations (IC_{50} = 660 ± 370 nM ... 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 the brain, *trans*-resveratrol acts a pure neutral antagonist, whereas 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), whereas the decrease in [^{35}S]GTPγS binding produced by the putative inverse agonists curcumin and ASC-J9 was reversed by coincubation with the neutral CB1 antagonist O-2050 (Fig. 4C, center and right).

In Mice, Acute Administration of *trans*-Resveratrol, Curcumin, and ASC-J9 Antagonizes 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 determine whether 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 can... 940 was examined (Fig. 5, A ... aneous injections, CP-55... in body temper... 25 mg/kg (0.23–... per dose) (Fig. ... dose of CP-... a half-maximal ... ce polyphenols tested ... ent reversal of CP-55,940-... a rank order of potency (IC_{50}) for ... eratrol (14 mg/kg) > curcumin (52 mg/... 8 mg/kg) (Fig. 5A). Curcumin is most efficacious, resulting in a complete antagonism of hypothermia. ... cative of competitive antagonism, coadministration 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% confidence interval; 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). In addition, 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). Because 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 (10 mg/kg) administered twice daily for 3 days results in a significant ($p < 0.01$) weight loss of 2.8 ± 0.47 g (Fig. 5C, left; n = 6). Likewise, repeated administration of curcumin produces a dose-related weight loss, equivalent to that produced by AM-251 (Fig. 5C, center; n = 5). Although slightly

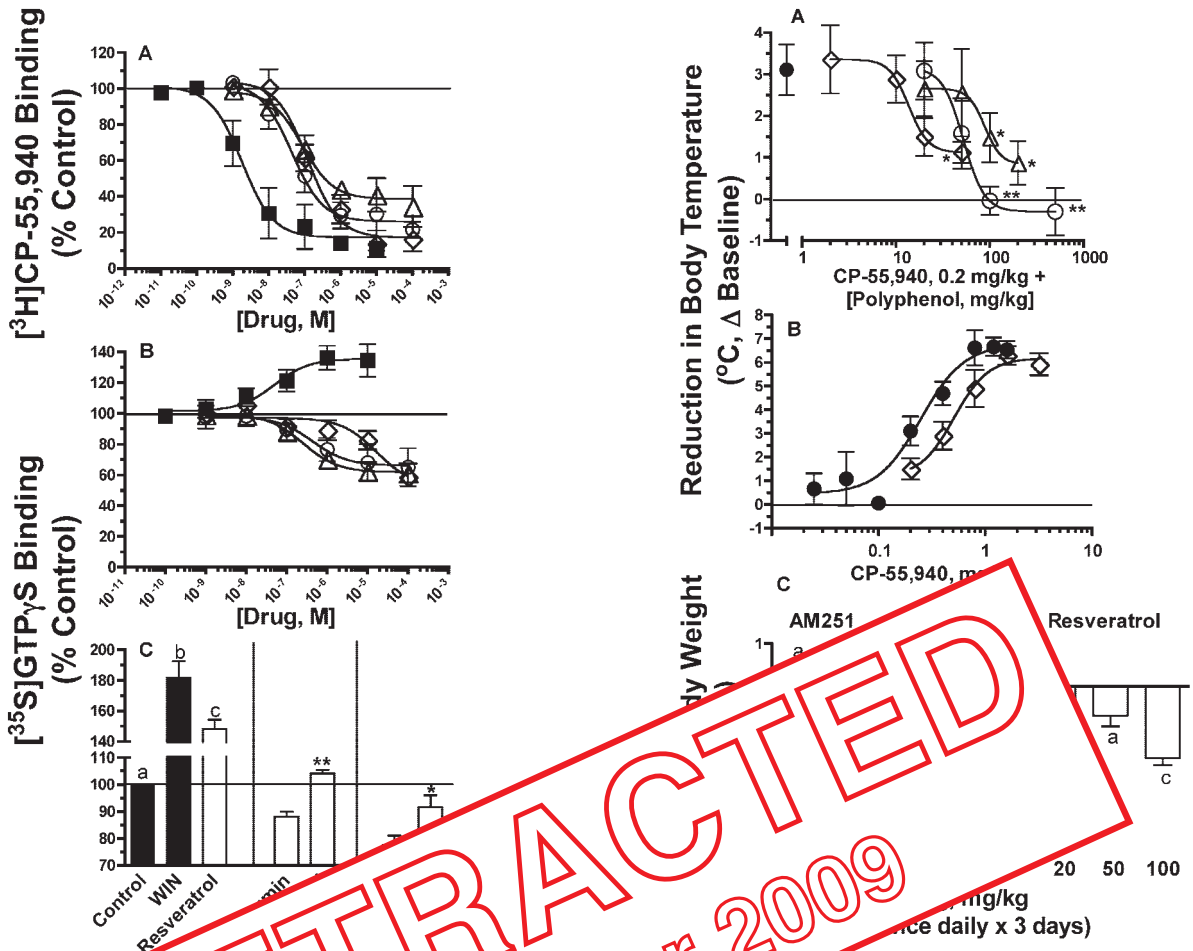

Fig. 4. *trans*-Resv affinity to, and tors in membra membranes were and increasing con (open circles), ASC monds). Results are [3H]CP-55,940 binding. B, mo ted with 0.1 nM [35S]GTPγS in the pres ations of WIN-55,212-2 (filled squares), curcu SC-J9 (open triangles), or *trans*-resveratrol (open c c. Results are expressed as the percentage of the specific γS binding. C, *trans*-resveratrol (10 μM) attenuates stimulatio of [35S]GTPγS binding produced by 1 μM CB1 agonist WIN-55,212-2. The neutral CB1 antagonist O-2040 (10 μM) blocks the inhibition of basal [35S]GTPγS binding produced by curcumin (1 μM) and ASC-J9 (300 nM). Values designated with different letters above the error bars are significantly different (one-way ANOVA followed by a Dunnett's post hoc comparison, $P < 0.05$). ∗ and ∗∗, significantly different from the percentage of [35S]GTPγS binding produced by curcumin or ASC-J9 alone (unpaired Student's *t* test, $P < 0.05$, 0.01).

resveratrol, curcumin, and ASC-J9 antagonize duced by a CB1 agonist and repeated treatment reduces similar to that produced by a CB1 inverse agonist. A, hypo- produced by 0.2 mg/kg CB1/CB2 agonist CP-55–940 was dose- pendently reduced by pretreatment with curcumin (open circles), ASC-J9 (open triangles), or *trans*-resveratrol (open diamonds). 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. C, twice daily intraperitoneal injections of curcumin or *trans*-resveratrol resulted in a dose-dependent reduction in body weight of mice similar to that produced by the CB1 antagonist/inverse agonist AM-251. ∗ and ∗∗, 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). Values designated with different letters above the error bars are significantly different (one-way ANOVA followed by a Dunnett's post hoc comparison, $P < 0.05$).

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

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 America, Inc., Sunnyvale, CA) with structure minimizations performed with a PM5 wave function 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. Although the cyclohexyl group of O-1422 is not present in *trans*-resveratrol, the dimethylheptyl side chain (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 (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 three-dimensional pharmacophore model of CB1-selective ligands recently proposed by Wang et al. (2008). For example, an aromatic region (A) and a hydrophobic region (B), which are located in aromatic rings containing electron-withdrawing groups, are present in all three molecules. Furthermore, the amide car-

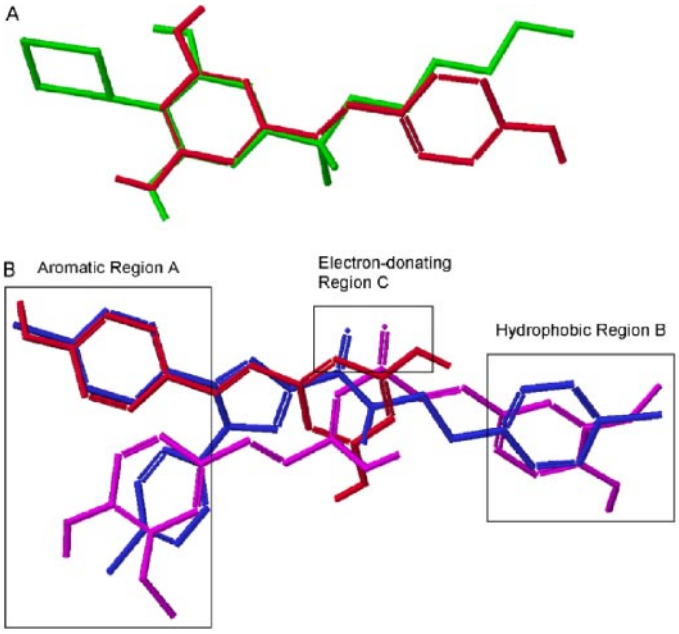

Fig. 6. In silico comparison of the structures of *trans*-resveratrol and curcumin with known cannabinoid receptor ligands reveals common structural motifs. A, CAChe molecular modeling software reveals that the favored conformation of *trans*-resveratrol (in red) is similar to a novel synthetic resorcinol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

bonyl (of rimonabant), the carbonyl [illegible] phenol (of *trans*-resveratrol) [illegible] oxygens (hydrogen bond [illegible] middle region; hence [illegible] ing region C[illegible].

Based on in[illegible] et al. (1999), i[illegible] tained in regio[illegible] some combinati[illegible] W5.43(279), F5.42[illegible] probable that the h[illegible] might interact with [illegible] case, it is clear that [illegible] several common struct[illegible] ligands, and these motifs probably contribute to their ability to bind with high affinity to CB1 receptors.

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 seem 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, nontoxic 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 antioxidant (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 nonreceptor-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, although *trans*-resveratrol inhibits the activity of quinone reductase 2, with a dissociation constant of 35 to 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 Iκβ kinase (1 μM) (Kundu et al., 2006) and lipoxygenase (3.7 μM) (Jang[illegible] al., 1997). Likewise, curcumin inhibits the activity [illegible]en synthase kinase-2β with an IC_{50} of 63 nM [illegible] 2008); however, significantly greater [illegible] uired to reduce the aggregation [illegible] et al., 2005) or inhibit[illegible] I) (Hayeshi et al., [illegible] for most other [illegible] rs clearly play [illegible] of action for [illegible] relatively low, [illegible] to produce near-full [illegible] additionally important because [illegible] ific, high-affinity, receptor-mediated [illegible] probably contributes to many of the reported [illegible] effects of these and other structurally 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 seem 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. It is most interesting that [^{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 noncannabinoid G-protein-coupled receptors, such as dopamine receptors, to which cannabinoid receptor ligands also bind.

It is interesting that all three polyphenols were shown to possess both neutral antagonist and inverse agonist proper-

ties, 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).

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 central nervous system, 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 to 2 μM parent drug after a single, acute intraperitoneal 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 c
centrations as high as 1 to 2 μM in the brains of m
relatively low dose of 2 mg/kg/day over a p
months (Begum et al., 2008). Very low d
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Based on their high nanomolar affinities for CB1 receptors reported here, if such micromolar (or even high nanomolar) 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 bind with high (or superior) affinity to CB1 receptors to mediate the in vivo effects reported here. In any case, because of 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 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), thus virtually assuring a lack of future Food and Drug Administration 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). 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. It is interesting that, 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
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ent of obesity (Pavon et al., 2008). Antioxidants
reduce many adverse consequences associated with obesity (Vincent et al., 2007). As such, novel polyphenol-derived CB1 antagonists, because of combined CB1 antagonism and anticipated antioxidant properties (Fraga, 2007), might provide additive or even synergistic improvement of obesity symptoms.

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References

Anand P, Kunnumakkara AB, Newman RA, and Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. *Mol Pharm* **4:**807–818.

Asensi M, Medina I, Ortega A, Carretero J, Baño MC, Obrador E, and Estrela JM (2002) Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic Biol Med* **33:**387–398.

Baur JA and Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* **5:**493–506.

Begum AN, Jones MR, Lim GP, Morihara T, Kim P, Heath DD, Rock CL, Pruitt MA, Yang F, Hudspeth B, et al. (2008) Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J Pharmacol Exp Ther* **326:**196–208.

Buryanovskyy L, Fu Y, Boyd M, Ma Y, Hsieh TC, Wu JM, and Zhang Z (2004) Crystal structure of quinone reductase 2 in complex with resveratrol. *Biochemistry* **43:** 11417–11426.

Bustanji Y, Taha MO, Almasri IM, Al-Ghussein MA, Mohammad MK and Alkhatib HS (2008) Inhibition of glycogen synthase kinase by curcumin: Investigation by simulated molecular docking and subsequent in vitro/in vivo evaluation. *J Enzyme Inhib Med Chem* **1:** [Epub ahead of print].

Chainani-Wu N (2003) Safety and anti-inflammatory activity of curcumin: a component of tumeric (*Curcuma longa*). *J Altern Complement Med* **9:**161–168.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1)

and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.
El-Mowafy AM and Alkhalaf M (2003) Resveratrol activates adenylyl-cyclase in human breast cancer cells: a novel, estrogen receptor-independent cytostatic mechanism. *Carcinogenesis* **24:**869–873.
Espín JC, García-Conesa MT, and Tomás-Barberán FA (2007) Nutraceuticals: facts and fiction. *Phytochemistry* **68:**2986–3008.
Fraga CG (2007) Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* **59:**308–315.
Gutierrez T, Farthing JN, Zvonok AM, Makriyannis A, and Hohmann AG (2007) Activation of peripheral cannabinoid CB1 and CB2 receptors suppresses the maintenance of inflammatory nociception: a comparative analysis. *Br J Pharmacol* **150:**153–163.
Hadi SM, Bhat SH, Azmi AS, Hanif S, Shamim U, and Ullah MF (2007) Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. *Semin Cancer Biol* **17:**370–376.
Hampson AJ, Grimaldi M, Axelrod J, and Wink D (1998) Cannabidiol and (−)delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci U S A* **95:**8268–8273.
Han YS, Bastianetto S, Dumont Y, and Quirion R (2006) Specific plasma membrane binding sites for polyphenols, including resveratrol, in the rat brain. *J Pharmacol Exp Ther* **318:**238–245.
Hayeshi R, Mutingwende I, Mavengere W, Masiyanise V, and Mukanganyama S (2007) The inhibition of human glutathione *S*-transferases activity by plant polyphenolic compounds ellagic acid and curcumin. *Food Chem Toxicol* **45:**286–295.
Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, et al. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275:**218–220.
Jones D (2008) End of the line for cannabinoid receptor 1 as an anti-obesity target? *Nat Rev Drug Discov* **7:**961–962.
Klein TW (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol* **5:**400–411.
Kundu JK, Shin YK, and Surh YJ (2006) Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin in vivo: NF-kappaB and AP-1 as prime targets. *Biochem Pharmacol* **72:**1506–1515.
Lekli I, Szabo G, Juhasz B, Das S, Das M, Varga E, Szendrei L, Gesztelyi R, Varadi J, Bak I, et al. (2008) Protective mechanisms of resveratrol against ischemi
reperfusion-induced damage in hearts obtained from Zucker obese rats: th
GLUT-4 and endothelin. *Am J Physiol Heart Circ Physiol* **294:**H85
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, and Bonner T
of a cannabinoid receptor and functional expression of th
346:561–564.
Muccioli GG (2007) Blocking the cannabinoid
apeutic promises. *Chem Biodivers* **4:**180
Munro S, Thomas KL, and Abu-Sh
peripheral receptor for canna
Pan MH, Huang TM, an
reduction and glu
Pavon FJ, Serran
dez-Silva FJ, Ma
eral antagonism
ripherally acting neutral cannabinoid receptor antagonist, in Zucker rats. *J Neuroendocrin* **20 (Suppl 1):**116–123.
Rahman I, Biswas SK, and Kirkham PA (2006) Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* **72:**1439–1452.
Scalbert A, Manach C, Morand C, Rémésy C, and Jiménez L (2005) Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* **45:**287–306.
Shoemaker JL, Joseph BK, Ruckle MB, Mayeux PR, and Prather PL (2005) The endocannabinoid noladin ether acts as a full agonist at human CB2 cannabinoid receptors. *J Pharmacol Exp Ther* **314:**868–875.
Singh M, Arseneault M, Sanderson T, Murthy V, and Ramassamy C (2008) Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. *J Agric Food Chem* **56:**4855–4873.
Smith PB, Compton DR, Welch SP, Razdan RK, Mechoulam R, and Martin BR (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther* **270:**219–227.
Song ZH, Slowey CA, Hurst DP, and Reggio PH (1999) The difference between the CB(1) and CB(2) cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN55212-2 for CB(2). *Mol Pharmacol* **56:**834–840.
Stevenson DE and Hurst RD (2007) Polyphenolic phytochemicals–just antioxidants or much more? *Cell Mol Life Sci* **64:**2900–2916.
Surh YJ, Kundu JK, Na HK, and Lee JS (2005) Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J Nutr* **135**:2993S–3001S.
Tilak JC, Banerjee M, Mohan H, and Devasagayam TP (2004) Antioxidant availability of turmeric in relation to its medicinal and culinary uses. *Phytother Res* **18:**798–804.
Vincent HK, Innes KE, and Vincent KR (2007) Oxidative stress and potential interventions to reduce oxidative stress in ov ight and obesity. *Diabetes Obes Metab* **9:**813–839.
Wang H, Duffy RA, Boykow GC, Ch nd Madison VS (2008) Identification of novel cannabinoi ts by using virtual screening with a pharmacophore 446.
Wang YJ, He F, and f resveratrol in the experimental cere 534–536.
Wiley JL van A, Razdan RK, and M for the development of Pharmacol Exp Ther
okar SS, Chen PP, ibits formation of ces amyloid in vivo.
, Grayson GK, Hooker BA, Dart tro pharmacological characterization nabinoid CB(2) receptor? *Br J Pharmacol*

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