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CB1 and CB2 receptors are novel molecular targets for Tamoxifen and 4OH-Tamoxifen

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ABSTRACT

Tamoxifen (Tam) is classified as a selective estrogen receptor modulator (SERM) and is used for treatment of patients with ER-positive breast cancer. However, it has been shown that Tam and its cytochrome P450-generated metabolite 4-hydroxy-Tam (4OH-Tam) also exhibit cytotoxic effects in ER-negative breast cancer cells. These observations suggest that Tam and 4OH-Tam can produce cytotoxicity via estrogen receptor (ER)-independent mechanism(s) of action. The molecular targets responsible for the ER-independent effects of Tam and its derivatives are poorly understood. Interestingly, similar to Tam and 4OH-Tam, cannabinoids have also been shown to exhibit anti-proliferative and apoptotic effects in ER-negative breast cancer cells, and estrogen can regulate expression levels of cannabinoid receptors (CBRs). Therefore, this study investigated whether CBRs might serve as novel molecular targets for Tam and 4OH-Tam. We report that both compounds bind to CB1 and CB2Rs with moderate affinity (0.9–3 μM). Furthermore, Tam and 4OH-Tam exhibit inverse activity at CB1 and CB2Rs in membrane preparations, reducing basal G-protein activity. Tam and 4OH-Tam also act as CB1/CB2R-inverse agonists to regulate the downstream intracellular effector adenylyl cyclase in intact cells, producing concentration-dependent increases in intracellular cAMP. These results suggest that CBRs are molecular targets for Tam and 4OH-Tam and may contribute to the ER-independent cytotoxic effects reported for these drugs. Importantly, these findings also indicate that Tam and 4OH-Tam might be used as structural scaffolds for development of novel, efficacious, non-toxic cancer drugs acting via CB1 and/or CB2Rs.

1. Introduction

Tamoxifen (Tam) is a selective estrogen receptor modulator (SERM) [1] and is one of the most commonly prescribed chemotherapeutic agents for treatment of estrogen receptor (ER)-positive breast cancer [2]. It has been established that the efficacy of Tam against ER-positive breast cancer results primarily from its ability to act as an ER antagonist [3]. However, in addition to efficacy in ER-positive forms of breast cancer, it has been shown that Tam and its P450-generated metabolite, 4-hydroxy-Tam (4OH-Tam), exhibit cytotoxic effects in types of breast cancer that do not express ERs [4,5]. Tam is also effective against several types of cancer derived from tissues not sensitive to estrogen, such as melanoma [6], glioma [7] and pancreatic cancer [8]. Furthermore, Tam appears to be efficacious for treatment of a variety of other diseases not related to cancer, including atherosclerosis [9], osteoporosis [10] and rheumatoid arthritis [11]. These observations suggest that Tam and 4OH-Tam produce therapeutic effects via both ER-dependent and -independent mechanism(s).

The molecular targets responsible for ER-independent effects of Tam and its derivatives are poorly understood; however, several candidates have been proposed [12]. Tam produces cytotoxicity in human prostate cancer cells [13] and astrocytoma [14] via inhibition of protein kinase C (PKC). ER-independent apoptotic effects of Tam in breast cancer cells appear to be mediated via activation of caspases 6, 7, and 9 [15], whereas inhibition of AKT and JNK and sustained activation of MAP kinase and ERK produces cell death in neuronally-derived glioma cells [16]. Additional molecular

Abbreviations: 4OH-Tam, 4OH-Tamoxifen; AC, adenylyl cyclase; CB1R, cannabinoid receptor type 1; CB2R, cannabinoid receptor type 2; CHO, Chinese hamster ovary; CP-55,940, 5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol; DMEM, Dulbecco's modification of Eagle's medium; ER, estrogen receptor; GPCR, G-protein coupled receptor; [^{35}S]GTPγS, guanosine 5'-O-(3-[^{35}S]thio)triphosphate; hCB2, human CB2 receptors; hMOR, human mu-opioid receptors; IBMX, isobutylmethyl-xanthine; SERM, selective estrogen receptor modulator; Tam, Tamoxifen; WIN-55,212-2, [(3*R*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone.

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mechanisms that may underlie ER-independent effects have been reported. Tam has been shown to reduce angiogenesis required for malignant growth and metastasis via inhibition of vascular endothelial growth factor (VEGF) secretion in breast cancer cells [17], and to reverse multi-drug resistance (MDR) in an *in vivo* animal model of colorectal carcinoma by interaction with mdr1 protein [18].

Cannabinoids are compounds originally isolated from the marijuana plant (*Cannabis sativa*) that have been shown to reduce tumor growth and progression in many cellular and animal models by acting at two G-protein coupled receptors (GPCRs), cannabinoid receptors type 1 and 2 (CB1R and CB2R) [19]. CB1Rs are expressed in high abundance throughout the CNS, while CB2Rs are expressed predominantly in immune cells and non-neuronal tissues [20]. It is important to note that, like Tam and 4OH-Tam, cannabinoids exhibit anti-proliferative and apoptotic effects in ER-negative breast cancer cells [21]. Cannabinoids are similarly efficacious against cancer types not derived from estrogen sensitive tissues, including melanoma [22], glioma [23] and pancreatic cancer [24]. These compounds inhibit tumor angiogenesis via modulation of VEGF activity [25], and reverse MDR in a leukemia cell line [26]. In addition, estradiol (E2) regulates expression levels of CB1Rs in human primary tumor colon cancer cell lines making it an estrogen-responsive receptor [27].

Based on the similarity between many of the ER-independent effects of Tam and the actions of cannabinoids, this study investigated whether CBRs are novel molecular targets for Tam and 4OH-Tam. We report that Tam and 4OH-Tam bind to CB1 and CB2Rs and act as inverse agonists, suggesting that many of their ER-independent effects may be mediated via CBRs.

2. Material and methods

2.1. Materials

Tam, 4OH-Tam, and GDP were purchased from Sigma Aldrich (St. Louis, MO). WIN-55,212-2, CP-55,940, O-2050, and morphine were obtained from Tocris Bioscience (Ellisville, MO). GTPγS was procured from EMD Chemical (Gibbstown, NJ). [^{3}H]CP-55,940 (174.6 Ci/mmol) was purchased from PerkinElmer (Waltham, MA) and [^{35}S]GTPγS (1250 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Mice

The University of Arkansas for Medical Sciences IACUC committee approved the animal use protocol employed in this study. All efforts were made to minimize animal suffering and reduce the number of animals used. B6SJL mice were obtained from an in-house breeding colony. All animals were maintained on a 12 h light/dark cycle with free access to food and water. Following anesthesia with isoflurane, mice were sacrificed and brains harvested by decapitation and snap frozen using liquid nitrogen for membrane preparation.

2.3. Cell culture

CHO-K1 cells were stably transfected with the human CB2 receptor (CNR2; CHO-hCB2) [28] or the human mu-opioid receptor (MOR, CHO-hMOR) [29]. CHO cells stably expressing hCB1 receptors (CNR1; CHO-hCB1) were purchased from DiscoveRx Corporation (Fremont, CA). CHO-hCB2, CHO-hMOR and CHO-Wild-Type (-WT) cells were cultured in DMEM (Mediatech Inc., Manassas, VA). CHO-hCB1 cells were cultured in F-12 K media (ATCC, Manassas, VA). Culture media contained 10% FCS (Gemini Bioproducts, Sacramento, CA), 0.05 IU/mL penicillin, and 50 mg/mL streptomycin (Invitrogen, Carlsbad, CA). For stably transfected cells, 250 μg/mL of Geneticin (G418; Sigma–Aldrich, St. Louis, MO) was added. All cells were maintained in a humidified chamber at 37 °C with 5% CO_2, harvested weekly, and only cells from passages 5–15 were used in experiments.

2.4. Radioligand binding assays

Receptor binding experiments were conducted as described previously [28]. Briefly, membranes (100 μg) from mouse brain, CHO-hCB1, or CHO-hCB2 cells were incubated with increasing concentrations of test compound (100 nM to 30 μM) and a fixed concentration of the radiolabeled non-selective CB1/CB2R agonist, [^{3}H]-CP-55,940 (0.2 nM; equivalent to its apparent K_D for each receptor subtype and thus predicted to result in ~50% receptor occupancy). After reaching equilibrium binding (90 min), samples were rapidly filtered and the bound radioactivity was assessed by liquid scintillation counting (LSC).

2.5. G-protein activation assays

[^{35}S]GTPγS binding assays were conducted as described previously [30] in buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM MgCl2 and 0.1% BSA. Each reaction contained membrane protein (mouse brain, CHO-hCB1, or CHO-hCB2; 50 μg), test compound (10 μM), [^{35}S]GTPγS (0.1 nM) and GDP (10 μM). Non-specific binding was defined by 10 μM of non-radioactive GTPγS. After 30 min at 30 °C, reactions were terminated by filtration and bound radioactivity was determined by LSC. Specificity of drug effect was determined by co-incubation of Tam or 4OH-Tam with a receptor saturating concentration of the CB1R-neutral antagonist O-2050 (1 μM) [31] or by comparison of drug modulation of [^{35}S]GTPγS activity between CBR-transfected and CHO-WT membranes.

2.6. Adenylyl cyclase (AC) assays

Activity of the intracellular effector AC in intact CHO-hCB1 or -hCB2 cells was measured as reported previously [32]. Cells were seeded into 24 well plates and cultured to confluence. Cells were treated with culture medium containing 0.9% NaCl, 500 μM 3-isobutyl-1-methlyxanthine (IBMX), and 2 μCi/well [^{3}H]adenine. After 2 h at 37 °C, the [^{3}H]adenine mixture was removed and increasing concentrations of each test compound (0.1 nM to 30 μM) were added for 15 min in a Krebs–Ringer–Hepes buffer containing 500 μM IBMX and 10 μM forskolin. Reactions were terminated with 50 μl of 2.2 N HCl and [^{3}H]cAMP, separated by alumina column chromatography, and quantified by LSC. Specificity of drug effect was determined by co-incubation of Tam or 4OH-Tam with a receptor saturating concentration of the CB1R-neutral antagonist O-2050 (1 μM) [31] or by comparison of inhibition of AC activity between CBR-expressing and CHO-WT cells.

2.7. Statistical analysis

All data are expressed as the mean ± SEM, and results were obtained from a minimum of three separate experiments, each performed in triplicate. Curve fitting and statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). Non-linear regression for one-site competition was used to determine the IC_{50} for competition receptor binding. The Cheng-Prusoff equation [33] was employed to convert the experimental IC_{50} values obtained from competition receptor binding experiments to K_i values (a quantitative measure of receptor affinity). Curve fitting of concentration-effect curves via non-linear

regression was also employed to determine the ED_{50} (measure of potency) and E_{MAX} (measure of efficacy) for the GTPγS binding and AC-experiments. A one-sample *t*-test was used to determine whether the modulation of G-protein or AC-activity produced by a 10 μM concentration of test compounds was significantly different than basal levels.

3. Results and discussion

3.1. Tam and 4OH-Tam bind to human CB1 and CB2Rs with moderately high affinity

As demonstrated by competition receptor binding studies, Tam and 4OH-Tam produce complete displacement of the non-selective CB1/CB2 agonist [^{3}H]CP-55,940 from human CB1 and CB2Rs, with affinities (K_i values) of 3.2 ± 0.60 or 1.4 ± 0.25 μM, respectively (Fig. 1A, $N = 3$ each). Both compounds bind to CB1Rs expressed in mouse brain with similar affinity to that demonstrated for human CB1Rs (data not shown). Tam and 4OH-Tam also bind to human CB2Rs with slightly higher affinities of 1.9 ± 0.31 and 0.9 ± 0.13 μM, respectively (Fig. 1B, $N = 4$ each). These results indicate that Tam and 4OH-Tam bind with relatively high affinity to human CB1 and CB2Rs, with a slight selectivity for CB2Rs. Furthermore, 4OH-Tam has slightly higher affinity for both CBRs than does Tam.

Interestingly, while this study was in preparation, Kumar and Song published data showing that raloxifene, a benzothiophene-based SERM that is structurally distinct from the triphenylethylene-based Tam [34], binds to CB2Rs [35]. Unfortunately, the ability of raloxifene to bind to CB1Rs was not examined. Although an actual measure of affinity (e.g., K_i or K_D) was not reported, a value of approximately 100 nM might be predicted based on the competition receptor binding data reported in that study. These findings suggest that CBR affinity might be a property common to both benzothiophene and triphenylethylene-based SERMs.

3.2. Tam and 4OH-Tam act as CB1 and CB2R inverse agonists to regulate G-protein activity in membrane homogenates

CB1 and CB2Rs are members of the Gi/Go-linked class of G-protein coupled receptors [20]. As such, the binding of agonists to these receptors results in activation of G-proteins. Activated G-proteins exchange GTP for GDP and thus when membranes are incubated with [^{35}S]GTPγS (a radiolabeled form of GTP), the degree of G-protein activation can be quantified by measuring increases in the binding of [^{35}S]GTPγS to G-proteins. The well characterized full CB1/CB2 agonist CP-55,940 (1 μM) activates G-proteins in CHO-hCB1 (55%) and CHO-hCB2 (27%), but not CHO-WT (4%) membranes (Fig. 1C). In marked contrast, a receptor saturating concentration (10 μM) of Tam and 4OH-Tam reduces G-protein activity below basal levels in CHO-hCB1 (−33%, −28%) and CHO-hCB2 (−31%, −23%), but not in CHO-WT (−6%, 1%) membranes. Co-incubation with the neutral CB1R antagonist O-2050 (1 μM) [31] also significantly attenuates the reduction in G-protein activity below basal levels produced by Tam and 4OH-Tam in mouse brain membranes (data not shown). These results indicate that Tam and 4OH-Tam act as inverse agonists at CB1 and CB2Rs, blocking activation of Gi-proteins by constitutively active CBRs [36].

3.3. Tam and 4OH-Tam act as CB1 and CB2R inverse agonists to regulate AC-activity in intact whole cells

CBRs activate the Gi subclass of G-proteins, which then proceed to inhibit the activity of the downstream intracellular effector AC, resulting in a reduction in intracellular cAMP levels [20]. The CB1/

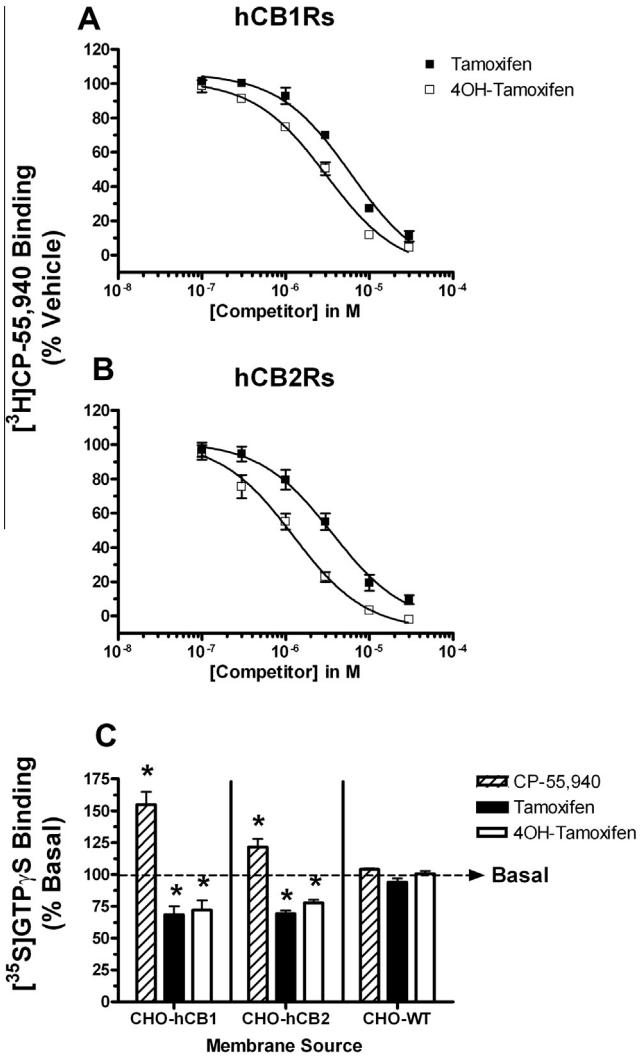

Fig. 1. Tam and 4OH-Tam bind to human CB1 and CB2 receptors with moderately high affinity and act as inverse agonists to regulate G-protein activity. Specific binding was determined by incubating 0.2 nM of [^{3}H]CP-55,950 with increasing concentrations of Tam or 4OH-Tam and 100 μg of membranes prepared from (A) CHO-hCB1 or (B) CHO-hCB2 cells (see Section 2). The Cheng-Prusoff equation [33] was used to convert the experimental IC_{50} values obtained from competition receptor binding experiments to K_i values, a quantitative measure of receptor affinity. (C) Membranes (50 μg) prepared from CHO-hCB1, CHO-hCB2 or CHO-WT cells were incubated in the presence of 0.1 nM [^{35}S]GTPγS with a receptor saturating concentration of CP-55,950 (1 μM), Tam (10 μM) or 4OH-Tam (10 μM) (see Section 2). Modulation of G-protein activity by compounds in non-transfected CHO-WT cells was evaluated to determine if the observed drug effects were mediated specifically via CB1 and CB2 receptors. The mean ± SEM of basal G-protein activity in the presence of test compounds is presented. *Significantly different from basal G-protein activity ($P < 0.05$; One sample *t*-test).

CB2 full agonist CP-55,940 (1 μM) inhibits AC activity in intact CHO-hCB1 (79%) and CHO-hCB2 (86%), but not CHO-hMOR (8%) cells (Fig. 2A). The well-characterized mu-opioid agonist morphine (1 μM) inhibits AC activity only in intact CHO-hMOR (66%) cells. A receptor saturating concentration of 4OH-Tam (10 μM) instead increases AC activity (as reflected by increased levels of cAMP) above basal levels in intact CHO-hCB1 (17%) and CHO-hCB2 (175%), but not in CHO-hMOR (1%) cells. Tam (10 μM) appears to be a less efficacious inverse agonist at CBRs than 4OH-Tam, significantly increasing intracellular cAMP levels in CHO-hCB2 (36%), but not CHO-hCB1 (2%) cells. These results indicate that Tam and 4OH-Tam also act as inverse agonists at CB1 and CB2 receptors to regulate activity of the downstream intracellular effector AC, reducing

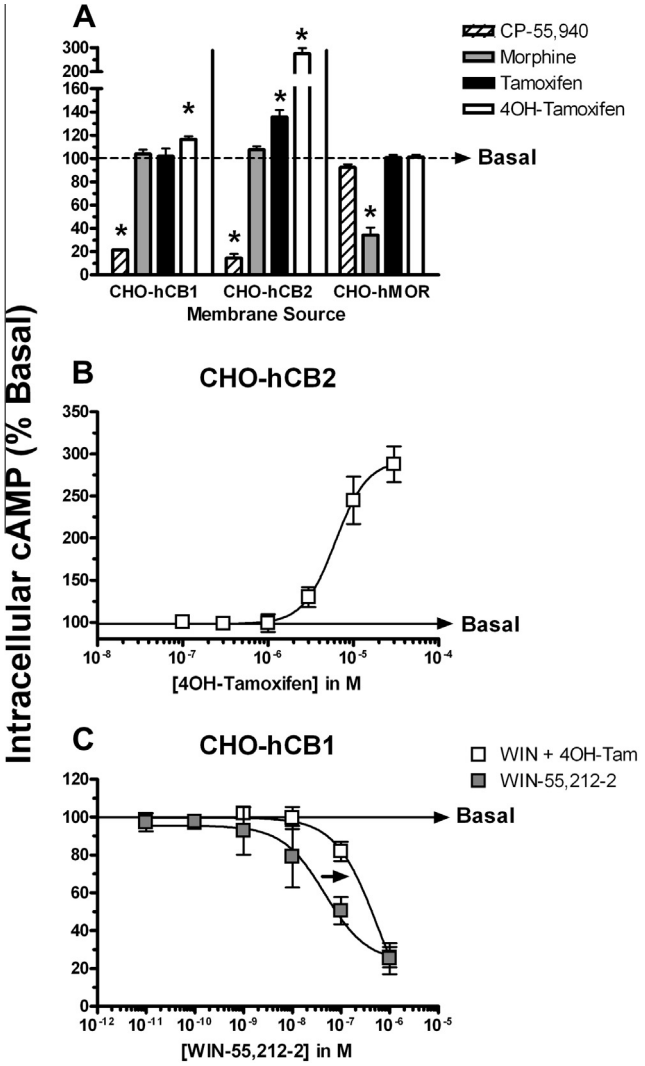

Fig. 2. Tam and 4OH-Tam act as inverse agonists at human CB1 and CB2 receptors to regulate the intracellular effector AC activity in intact cells. (A) Modulation of forskolin-stimulated AC-activity was evaluated by incubating intact CHO-hCB1, CHO-hCB2 or CHO-hMOR cells with a receptor saturating concentration of CP-55,950 (1 μM), morphine (1 μM), Tam (10 μM) or 4OH-Tam (10 μM) as described in the Section 2. AC-inhibition produced by the hMOR agonist morphine was evaluated as a positive control in CHO-hMOR cells. The mean ± SEM of basal cAMP levels in the presence of test compounds is presented. (B) Increasing concentrations of 4OH-Tam (100–10 μM) modulated AC-activity in a concentration-dependent manner in CHO-hCB2 cells. (C) Co-incubation with 4OH-Tam produced a parallel shift-to-the-right in the ability of the CB1/CB2 agonist WIN-55,212–2 to inhibit AC-activity in CHO-hCB1 cells, indicative of CB1 receptor antagonism. *Significantly different from basal cAMP levels ($P < 0.05$; One sample t-test).

AC inhibition produced by constitutively active CB receptors. Furthermore, 4OH-Tam is a very efficacious inverse agonist at CB2 receptors.

4OH-Tam also produces a concentration-dependent increase in cAMP levels in intact CHO-hCB2 cells with a potency (5.8 ± 0.3 μM) (Fig. 2B) that is similar to its affinity for CB2 receptors as determined by competition receptor binding (0.9 μM) (Fig. 1B). Demonstration of concentration-dependence and correlation between the potency for effector regulation and receptor affinity, provide additional confirmation that 4OH-Tam indeed acts as a CB2-inverse agonist.

Although 4OH-Tam produces relatively little (though significant) elevations in cAMP levels in CHO-hCB1 cells, co-incubation with this compound produces an 8-fold parallel shift-to-the-right in the potency for the well characterized full CB1/CB2 agonist WIN-55,212-2 to inhibit AC activity (Fig. 2C, $K_b = 1.6 \pm 0.4$ μM). This demonstrates that under conditions in which CB1Rs show little constitutive activity, 4OH-Tam acts as an antagonist with a K_b value very similar to its affinity for CB1Rs (Fig. 1A; 1.4 μM). Therefore, depending upon the level of CBR constitutive activity, Tam and 4OH-Tam can act as either antagonists or inverse agonists.

Raloxifene was also reported to act as an inverse agonist at CB2Rs to regulate AC-activity in transfected human embryonic kidney cells [35]. This finding, combined with the present work, suggests that these structurally distinct SERMs appear to share the ability to act as inverse agonists at CB2Rs. However, it is interesting to note that although raloxifene (a benzothiophene SERM) appears to have a higher affinity for this receptor, the triphenylethylene SERM, 4OH-Tam, appears to be a much more efficacious CB2R inverse agonist, producing almost 10-fold higher elevations in cAMP levels (193% - Fig. 2B versus 20% - [35], respectively). Dissimilar CB2R density between examined cell lines might account for some of the differences observed.

CB1 and CB2Rs are novel molecular targets for Tam and 4OH-Tam and may serve as a mechanism underlying the ER-independent effects often reported for these drugs. Tam and 4OH-Tam bind with very high affinity to ERs (K_i values in the low nM and even sub-nM range) [1] and their ability to act as ER antagonists is clearly responsible for therapeutic effects in ER-positive breast cancer [2]. However, Tam also produces a variety of ER-independent effects, which usually necessitate higher doses than those required to elicit ER-dependent effects [37]. Based on the similarity between many of the ER-independent effects of Tam and the actions of cannabinoids (see Section 1), this study investigated whether CBRs are novel molecular targets for Tam and 4OH-Tam.

As hypothesized, both Tam and 4OH-Tam were found to bind to CB1 and CB2Rs with moderately high affinity. Since the majority of current evidence indicates that the anticancer actions of cannabinoids are due to activation of CB1 and CB2Rs by agonists [38], it might have been predicted that Tam and 4OH-Tam would act as CB1 and/or CB2R agonists; however, our data indicates that they function as inverse agonists. Studies are accumulating that support the alternate view that, in some instances, enhancement of endocannabinoid signaling may serve to promote cancer progression (for review see [39]). In support of this theory, blocking CB1R signaling by inverse agonists has been shown to reduce tumor growth in thyroid cancer [40] and mantle-cell lymphoma [41]. Furthermore, CB1 inverse agonists (including Tam) have been shown slow tumor progression in ER-negative and ER-positive breast cancer [4,42] and colon cancer [43,44], and reduce tumor neoangiogenesis [17,45]. Based on these prior studies and the data presented here, it can be speculated that the novel CBR inverse agonist actions of Tam and 4OH-Tam contribute to many of the high dose and/or ER-independent anti-cancer effects observed for these compounds. In any case, results reported in the present study indicate that both benzothiophene and triphenylethylene SERMs might be used as structural scaffolds for development of a novel class of potent, efficacious and non-toxic anti-cancer drugs acting via CB1 and/or CB2Rs.

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References

[1] H. Rochefort, Mechanism of action of high-affinity antiestrogens, an overview, Am. J. Clin. Oncol. 14 (Suppl 2) (1991) S1–S4.

[2] A. Karn, A.K. Jha, S. Shrestha, B. Acharya, S. Poudel, R.B. Bhandari, Tamoxifen for breast cancer, J. Nepal Med. Assoc. 49 (2010) 62–67.
[3] R.R. Love, Tamoxifen therapy in primary breast cancer: biology, efficacy, and side effects, J. Clin. Oncol. 7 (1989) 803–815.
[4] R.R. Perry, Y. Kang, B. Greaves, Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells, Ann. Surg. Oncol. 2 (1995) 238–245.
[5] V.K. Todorova, Y. Kaufmann, S. Luo, V.S. Klimberg, Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake, Cancer Chemother. Pharmacol. 67 (2011) 285–291.
[6] J.R. Beguerie, J. Xingzhong, R.P. Valdez, Tamoxifen vs. non-tamoxifen treatment for advanced melanoma: a meta-analysis, Int. J. Dermatol. 49 (2010) 1194–1202.
[7] L. Mastronardi, F. Puzzilli, A. Ruggeri, Tamoxifen as a potential treatment of glioma, Anticancer Drugs 9 (1998) 581–586.
[8] S. Tomao, A. Romiti, B. Massidda, M.T. Ionta, A. Farris, A. Zullo, A. Brescia, L. Santuari, L. Frati, A phase II study of gemcitabine and tamoxifen in advanced pancreatic cancer, Anticancer Res. 22 (2002) 2361–2364.
[9] K.S. Stamatelopoulos, J.P. Lekakis, N.A. Poulakaki, C.M. Papamichael, K. Venetsanou, K. Aznaouridis, A.D. Protogerou, T.G. Papaioannou, S. Kumar, S.F. Stamatelopoulos, Tamoxifen improves endothelial function and reduces carotid intima-media thickness in postmenopausal women, Am. Heart J. 147 (2004) 1093–1099.
[10] L. Gennari, D. Merlotti, F. Valleggi, G. Martini, R. Nuti, Selective estrogen receptor modulators for postmenopausal osteoporosis: current state of development, Drugs Aging 24 (2007) 361–379.
[11] S. Williams, B. Michael, D. Mewar, E. Tunn, Inflammatory osteoarthritis which was precipitated by Arimidex and resolved with tamoxifen, British Medical Journal Case Reports, 2010 (2010).
[12] T.A. Bogush, E.A. Dudko, E.A. Bogush, B.E. Polotskii, S.A. Tiuliandin, M.I. Davydov, Tamoxifen non-estrogen receptor mediated molecular targets, Oncol. Rev. 6 (2012) e15.
[13] C. Rohlff, M.V. Blagosklonny, E. Kyle, A. Kesari, I.Y. Kim, D.J. Zelner, F. Hakim, J. Trepel, R.C. Bergan, Prostate cancer cell growth inhibition by tamoxifen is associated with inhibition of protein kinase C and induction of p21(waf1/cip1), Prostate 37 (1998) 51–59.
[14] T.R. Sharif, M. Sharif, A novel approach for examining the anti-proliferative effect of protein kinase C inhibitors against human astrocytoma cells, Int. J. Oncol. 13 (1998) 685–692.
[15] A. Thiantanawat, B.J. Long, A.M. Brodie, Signaling pathways of apoptosis activated by aromatase inhibitors and antiestrogens, Cancer Res. 63 (2003) 8037–8050.
[16] Y. Feng, J. Huang, Y. Ding, F. Xie, X. Shen, Tamoxifen-induced apoptosis of rat C6 glioma cells via PI3K/Akt, JNK and ERK activation, Oncol. Rep. 24 (2010) 1561–1567.
[17] S. Garvin, C. Dabrosin, Tamoxifen inhibits secretion of vascular endothelial growth factor in breast cancer *in vivo*, Cancer Res. 63 (2003) 8742–8748.
[18] L.Z. Shen, Y.B. Hua, X.M. Yu, Q. Xu, T. Chen, J.H. Wang, W.X. Wu, Tamoxifen can reverse multidrug resistance of colorectal carcinoma in vivo, World J. Gastroenterol. 11 (2005) 1060–1064.
[19] G. Velasco, C. Sanchez, M. Guzman, Towards the use of cannabinoids as antitumour agents, Nat. Rev. Cancer 12 (2012) 436–444.
[20] A.C. Howlett, Pharmacology of cannabinoid receptors, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 607–634.
[21] M.M. Caffarel, C. Andradas, E. Perez-Gomez, M. Guzman, C. Sanchez, Cannabinoids: a new hope for breast cancer therapy?, Cancer Treat Rev. 38 (2012) 911–918.
[22] C. Blazquez, A. Carracedo, L. Barrado, P.J. Real, J.L. Fernandez-Luna, G. Velasco, M. Malumbres, M. Guzman, Cannabinoid receptors as novel targets for the treatment of melanoma, FASEB J. 20 (2006) 2633–2635.
[23] I. Galve-Roperh, C. Sanchez, M.L. Cortes, T. Gomez del Pulgar, M. Izquierdo, M. Guzman, Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation, Nat. Med. 6 (2000) 313–319.
[24] A. Carracedo, M. Gironella, M. Lorente, S. Garcia, M. Guzman, G. Velasco, J.L. Iovanna, Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes, Cancer Res. 66 (2006) 6748–6755.
[25] C. Blazquez, M.L. Casanova, A. Planas, T. Gomez Del Pulgar, C. Villanueva, M.J. Fernandez-Acenero, J. Aragones, J.W. Huffman, J.L. Jorcano, M. Guzman, Inhibition of tumor angiogenesis by cannabinoids, FASEB J. 17 (2003) 529–531.
[26] M.L. Holland, J.A. Panetta, J.M. Hoskins, M. Bebawy, B.D. Roufogalis, J.D. Allen, J.C. Arnold, The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells, Biochem. Pharmacol. 71 (2006) 1146–1154.
[27] M. Notarnicola, C. Messa, A. Orlando, M. Bifulco, C. Laezza, P. Gazzerro, M.G. Caruso, Estrogenic induction of cannabinoid CB1 receptor in human colon cancer cell lines, Scand. J. Gastroenterol. 43 (2008) 66–72.
[28] J.L. Shoemaker, B.K. Joseph, M.B. Ruckle, P.R. Mayeux, P.L. Prather, The endocannabinoid noladin ether acts as a full agonist at human CB2 cannabinoid receptors, J. Pharmacol. Exp. Ther. 314 (2005) 868–875.
[29] K.A. Seely, L.K. Brents, L.N. Franks, M. Rajasekaran, S.M. Zimmerman, W.E. Fantegrossi, P.L. Prather, AM-251 and rimonabant act as direct antagonists at mu-opioid receptors: Implications for opioid/cannabinoid interaction studies, Neuropharmacology 63 (2012) 905–915.
[30] K.A. Seely, L.K. Brents, A. Radominska-Pandya, G.W. Endres, G.S. Keyes, J.H. Moran, P.L. Prather, A Major Glucuronidated Metabolite of JWH-018 Is a Neutral Antagonist at CB1 Receptors, Chem. Res. Toxicol. 25 (2012) 825–827.
[31] A. Gardner, P.E. Mallet, Suppression of feeding, drinking, and locomotion by a putative cannabinoid receptor 'silent antagonist', Eur. J. Pharmacol. 530 (2006) 103–106.
[32] M. Rajasekaran, L.K. Brents, L.N. Franks, J.H. Moran, P.L. Prather, Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors, Toxicol. Appl. Pharmacol. 269 (2013) 100–108.
[33] Y. Cheng, W. Prusoff, 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 (1973) 3099–3108.
[34] S.R. Goldstein, S. Siddhanti, A.V. Ciaccia, L. Plouffe Jr., A pharmacological review of selective oestrogen receptor modulators, Human Reprod. Update 6 (2000) 212–224.
[35] P. Kumar, Z.H. Song, Identification of raloxifene as a novel CB2 inverse agonist, Biochem. Biophys. Res. Commun. 435 (2013) 76–81.
[36] V.J. Aloyo, K.A. Berg, W.P. Clarke, U. Spampinato, J.A. Harvey, Inverse agonism at serotonin and cannabinoid receptors, Prog. Mol. Biol. Transl. Sci. 91 (2010) 1–40.
[37] S. Saji, K. Kuroi, Application of selective estrogen receptor modulators for breast cancer treatment according to their intrinsic nature, Breast Cancer 15 (2008) 262–269.
[38] D.J. Hermanson, L.J. Marnett, Cannabinoids, endocannabinoids, and cancer, Cancer Metast. Rev. 30 (2011) 599–612.
[39] S. Pisanti, P. Picardi, A. D'Alessandro, C. Laezza, M. Bifulco, The endocannabinoid signaling system in cancer, Trends Pharmacol. Sci. 34 (2013) 273–282.
[40] G. Portella, C. Laezza, P. Laccetti, L. De Petrocellis, V. Di Marzo, M. Bifulco, Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis, FASEB J. 17 (2003) 1771–1773.
[41] J. Flygare, K. Gustafsson, E. Kimby, B. Christensson, B. Sander, Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma, FEBS Lett. 579 (2005) 6885–6889.
[42] D. Sarnataro, S. Pisanti, A. Santoro, P. Gazzerro, A.M. Malfitano, C. Laezza, M. Bifulco, The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits human breast cancer cell proliferation through a lipid raft-mediated mechanism, Mol. Pharmacol. 70 (2006) 1298–1306.
[43] E. Motylewska, H. Lawnicka, G. Melen-Mucha, Oestradiol and tamoxifen inhibit murine Colon 38 cancer growth and increase the cytotoxic effect of fluorouracil, Endokrynologia Polska 58 (2007) 426–434.
[44] A. Santoro, S. Pisanti, C. Grimaldi, A.A. Izzo, F. Borrelli, M.C. Proto, A.M. Malfitano, P. Gazzerro, C. Laezza, M. Bifulco, Rimonabant inhibits human colon cancer cell growth and reduces the formation of precancerous lesions in the mouse colon, Int. J. Cancer 125 (2009) 996–1003.
[45] S. Pisanti, P. Picardi, L. Prota, M.C. Proto, C. Laezza, P.G. McGuire, L. Morbidelli, P. Gazzerro, M. Ziche, A. Das, M. Bifulco, Genetic and pharmacologic inactivation of cannabinoid CB1 receptor inhibits angiogenesis, Blood 117 (2011) 5541–5550.