Delta(9)-tetrahydrocannabinol Inhibits 17β-Estradiol-induced Proliferation and Fails to Activate Androgen and Estrogen Receptors in MCF7 Human Breast Cancer Cells

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Abstract. *Background: Delta(9)-tetrahydrocannabinol (THC) exerts palliative effects in cancer patients, but produces adverse effects on the endocrine and reproductive systems. Experimental evidence concerning such effects is controversial. Whether THC exhibits estrogenic or androgenic activity in vitro was investigated. Materials and Methods: Estrogenic effects of THC were analyzed in vitro by measuring the proliferation of estrogen-sensitive MCF7 cells. Androgenic activity was investigated by the A-Screen assay that measures androgen-dependent inhibition of proliferation of the androgen receptor (AR)-positive human mammary carcinoma cell line, MCF7-AR1. Results: In contrast to 17β-estradiol, included as positive control with an EC_{50} value (concentration required for 50% of maximal 17β-estradiol-induced proliferation) of 1.00×10^{-12} M, THC failed to induce cell proliferation in the MCF7 cell line at concentrations between 10^{-13} and 10^{-4} M. THC inhibited 17β-estradiol-induced proliferation in wild-type MCF7 and MCF7-AR1 cells, with an IC_{50} value of 2.6×10^{-5} M and 9×10^{-6} M, respectively. Conclusion: THC failed to act as an estrogen, but antagonized 17β-estradiol-induced proliferation. This effect was independent of the AR expression level.*

Prolonged nausea and emesis that frequently accompany the administration of cancer therapeutics can be so severe that some patients stop treatment. Cannabinoids, including delta(9)-tetrahydrocannabinol (THC), exert palliative effects in cancer patients by preventing vomiting and nausea, stimulating appetite, and by inhibiting pain (1). Several clinical trials have compared the effectiveness of THC with placebo or with another antiemetic drugs (2-4). Cannabinoids showed some antiemetic efficacy, but did not

prevent nausea and vomiting in two-thirds of patients. Therefore, cannabinoids would not be indicated as a first-line treatment for nausea and vomiting, but they may be used as adjuvant treatment to enhance the effects of existing antiemetic medications, since mechanisms of action differ from other agents. Many patients with advanced cancer suffer also from lack of appetite, weight loss and anorexia. Cachexia is estimated to account for 20% of cancer deaths (5). Many studies have demonstrated that THC and other cannabinoids exert a stimulatory effect on appetite, also in advanced cancer patients (1, 6).

Cannabis compounds have also been previously reported to exert antiproliferative actions through different cellular mechanisms, by inhibiting tumor angiogenesis and metastasis, induction of apoptosis, and cell cycle arrest (1). One aspect to be considered in the use of THC for cancer patients, in particular those with hormone-dependent tumors, are possible interactions of the drug with endocrine mechanisms. The evidence for such interactions is controversial. Interactions of cannabinoid compounds with estrogen receptors have been studied, with conflicting results (7-9).

We investigated possible estrogenic or androgenic activities of THC in two tests on human breast cancer cell lines that make use of the fact that androgens and estrogens exert opposing effects on the growth of malignant human breast tissues (10, 11). Androgens have been found to inhibit proliferation (12, 13), whereas estrogens to have mitogenic effects (14). Estrogenic activity was analyzed by measuring proliferation of the estrogen-sensitive human breast cancer MCF7 cell line. A possible androgenic action of THC was investigated by the A-Screen assay that measures androgen-dependent inhibition of proliferation of the androgen receptor (AR)-positive human mammary carcinoma cell line, MCF7-AR1. This cell line has been stably transfected with a full human AR (11); it expresses approximately five times more AR than do wild-type MCF7 cells. MCF7-AR1 cells retain the capacity to proliferate in response to estrogen treatment. Androgens inhibit estrogen-induced proliferation and cells arrest in G0/G1 phase (11, 15).

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Materials and Methods

Cell lines and culture conditions. MCF7 (MCF7-BUS) human breast cancer cells and MCF7-AR1 cells were kindly given by Ana Soto and Carlos Sonnenschein, Tufts University, Boston. MCF7 cells were at passage 10, MCF7-AR1 cells at passage 18. Samples from frozen stocks were used for a maximum of 6-13 passages. The MCF7-AR1 cell line has been stably transfected with a full human AR (11). MCF7 and MCF7-AR1 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) with phenol red (GIBCO, Grand Island, NY, USA) supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, GIBCO) and 2 nM L-glutamine (GIBCO). This medium was used as the growth medium to maintain the cells in culture. Phenol red is commonly used in media as a pH-indicator, but is also a weak estrogen (16). Cells become proliferatively quiescent when transferred into phenol red-free DMEM supplemented with 10% charcoal-dextran-treated FBS (CD-FBS, steroid free), 2 nM L-glutamine, 10 mM Hepes buffer 1 M (GIBCO, Basel, Switzerland). This medium was used as the experimental medium.

Drugs. 17β-Estradiol was purchased from Calbiochem (Richmond, USA), methyltrienolone (R1881) from NEN Life Science Products (Boston, MA, USA), 5α-dihydrotestosterone (DHT) from Fluka (Buchs, Switzerland), and delta(9)-tetrahydrocannabinol (THC) from Lipomed (Arlesheim, Switzerland). THC was initially dissolved in DMSO (Sigma-Aldrich, Schnelldorf, Germany) to a concentration of 20 mM and stored at –20°C. THC was further diluted with medium for the *in vitro* experiments.

Estrogenic and androgenic activity. To assess potential estrogenic activity of THC, the E-Screen assay was conducted, as described in recent studies (17-20). Briefly, on the first day, MCF7 cells were trypsinized and plated into Costar 96-well plates at an initial density of 3'000 cells per well in 100 μl experimental medium, and allowed to attach. On the second day, 100 μl of experimental medium containing different dilutions of THC were added into each well to give final concentrations between 10^{-13} and 10^{-4} M. Cells were incubated for six days. The assay compares the cell number of MCF7 cells in the absence of any estrogens (untreated control), in the presence of 10^{-10} M 17β-estradiol (positive control) and in the presence of a range of test chemical concentrations. Potential anti-estrogenic activity was investigated by comparing the cell number of wild-type MCF7 cells treated with 10^{-10} M 17β-estradiol in the absence and presence of THC.

Potential actions on androgen receptors (AR) were analyzed in a comparable setting by the A-Screen assay. In this test, proliferation of MCF7-AR1 cells is induced by 10^{-10} M 17β-estradiol. The A-screen assay measures the inhibition of this proliferative response by known androgens. The effect of methyltrienolone (R1881) and 5α-dihydrotestosterone (DHT) (included as positive controls) was compared with that of the test chemical, THC (11, 15, 21).

Cell density was assessed as changes in total protein using a sulforhodamine B assay (22). Briefly, the experimental medium was gently discarded, then fixed for 30 min with 100 μl cold (4°C) 10% trichloro-acetic acid (TCA) (Merck, NJ, USA). Plates were left for 30 min at 4°C and subsequently washed 5 times with deionized water. Plates were then left to dry at room temperature for at least

24 h. Cells were stained by adding 100 μl 0.4% sulforhodamine B (SRB, Sigma Chemical Co, St. Louis, MO, USA) in 1% acetic acid to each well for 15-20 min at room temperature, protected from light, in order to avoid fading of the color. SRB was then removed and the plates were washed 5 times with 1% acetic acid before air-drying. Bound SRB was solubilized with 100 μl 10 mM Tris buffer, pH 10.6 (unbuffered Tris-base solution), and plates were left on a plate shaker for at least 10 min. The absorbance was read in a 96-well plate reader (Anthos Labtec 2001 plate reader, Anthos Labtec, Salzburg, Austria) at 492 nm subtracting the background measurement at 620 nm. IC_{50} values were calculated from the regression curve as described elsewhere (21, 23).

Viability assay. To assess the effect of THC on cell viability of MCF7 breast cancer cells, cells were trypsinized and plated into 24-well plates at an initial density of 2.5×10^4 cells per well in 1 ml growth medium and allowed to attach. After 24 h, the growth medium was aspirated and replaced with 0.5 ml experimental medium alone, with experimental medium in the presence of 10^{-10} M 17β-estradiol and with 0.5 ml experimental medium in presence of 10^{-10} M 17β-estradiol with a range of THC concentrations. After incubation for six days, samples were harvested and viable and non-viable cells were determined by trypan blue dye exclusion assay (24).

Statistical analysis. The results were expressed as the mean and standard error (SEM). Dose-response curves and IC_{50} values with their 95% confidence intervals were calculated from the regression curve with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) software.

Results

Effect of THC on MCF7 cell proliferation. To test whether THC is able to act as an estrogenic compound, we examined its effect on MCF7 cell proliferation. As shown in Figure 1A, THC was not able to stimulate the proliferative response above the level of the untreated control at a wide concentration range (10^{-13}-10^{-4} M). In contrast, 17β-estradiol, used as positive control, showed a dose-dependent increase of optical density as an index of cell number (Figure 1A). Stimulation of proliferation was maximal at 10 to 100×10^{-12} M 17β-estradiol and the EC_{50} value (concentration required for 50% of maximal 17β-estradiol-induced proliferation) was 1.00×10^{-12} M, in the range of earlier studies.

THC inhibits 17β-estradiol-induced cell proliferation in MCF7-AR1 and MCF7 cells. We next assessed possible agonistic activity of THC on androgen receptors (AR). To this end, we stimulated proliferation of MCF7-AR1 cells by 17β-estradiol (10^{-10} M) and exposed them to known AR agonists and to different concentrations of THC. Estrogen-induced proliferation was reduced by the AR agonists R1881 and DHT (Figure 2A), with IC_{50} values of 4.41×10^{-11} and 4.44×10^{-11} (data for R1881 and DHT from (21)). THC likewise inhibited 17β-estradiol-induced proliferation of
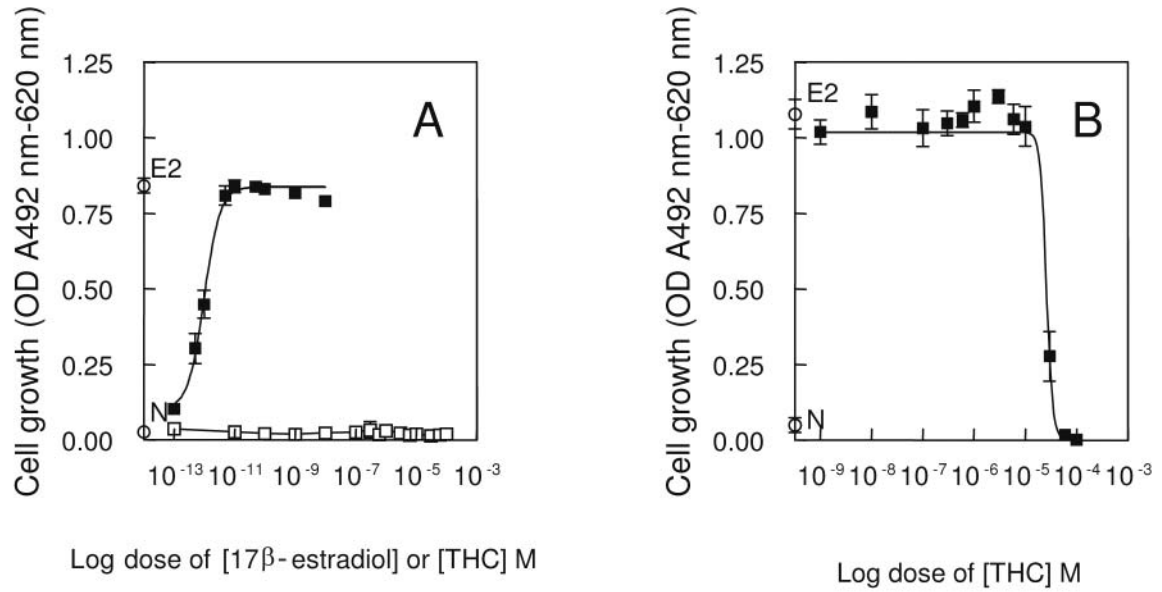
Figure 1. *Effect of THC (open square) and 17β-estradiol (filled square) on MCF7 cell proliferation in vitro (A). Inhibition of 17β-estradiol-induced MCF7 cell proliferation by THC (B). Mean±SEM. N, experimental medium only; E2, 10^{-10} M 17β-estradiol alone. Data from two independent experiments.*
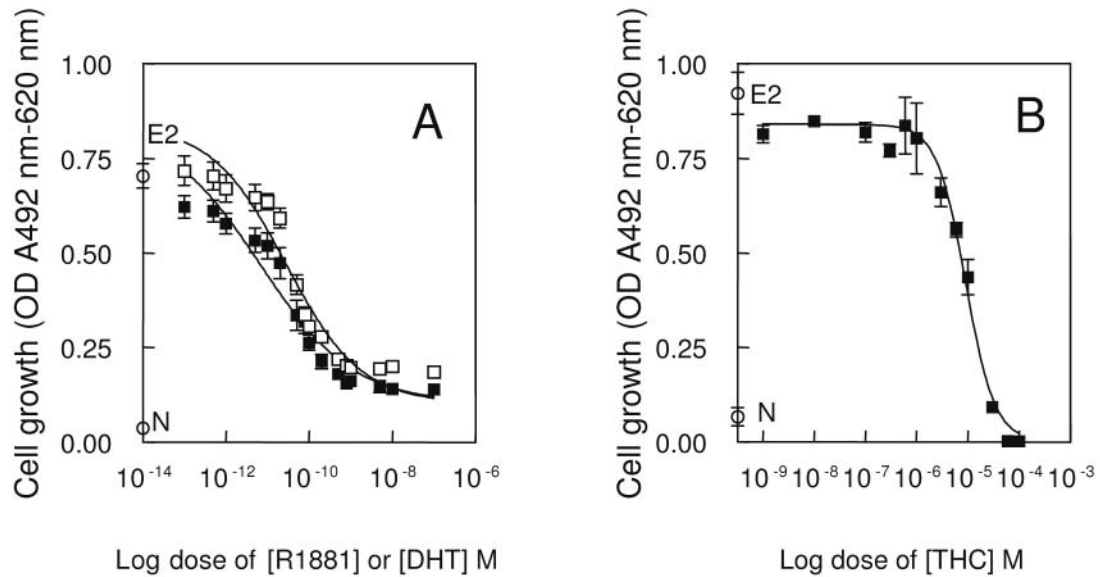

Figure 2. *Inhibition of 17β-estradiol-induced cell proliferation of MCF7-AR1 cells (A) by methyltrienolone (R1881; filled square) and 5α-dihydrotestosterone (DHT; open square), and (B) by THC. N, experimental medium only; E2, 10^{-10} M 17β-estradiol alone. Results are presented as the mean±SEM of at least two independent experiments. Data for R1881 and DHT from (21).*

MCF7-AR1 cells in a concentration-dependent manner (Figure 2B), with an IC_{50} value of 9×10^{-6} M (7.7×10^{-6} – 1.1×10^{-5}, 95% confidence interval).

In order to answer the question whether the inhibitory effect of THC might be due to an AR agonistic action or to antagonism of 17β-estradiol, we added THC to MCF7 cells in the presence of 10^{-10} M 17β-estradiol. Again, THC reduced 17β-estradiol-induced proliferation of MCF7 cells to a similar extent (Figure 1B), with an IC_{50} value of 2.6×10^{-5} M (8.5×10^{-6} – 8.0×10^{-5}, 95% confidence interval).

Possible cytotoxic effect of THC on MCF7 cells. Next, the trypan blue dye exclusion assay was used to examine THC-mediated cytotoxicity and to assess cell viability upon exposure to THC. The drug reduced 17β-estradiol-induced total cell number and viability of MCF7 cells in a dose-dependent manner (Figure 3). The results showed that THC inhibits MCF7 cell viability by reducing the number of viable cells, but not by inducing a significant cytotoxic cell response. MCF7 cells appear to be resistant to THC-induced cytotoxic cell death (Figure 3).

Discussion

In the present study, we used biological assays to assess potential estrogenic and androgenic activities of THC. The effect of the positive control, 17β-estradiol, on MCF7 cells with an EC_{50} value corresponding to the range reported in earlier studies (18-20, 25), indicates that the cell system was able to detect ER agonists. Using this sensitive and well-established assay, THC failed to show any estrogenic activity *in vitro*.

Interaction studies of THC with estrogen receptors so far have yielded conflicting results. THC has been demonstrated to bind to rat uterine estrogen receptors (26), whereas another study failed to observe any competition at the estrogen receptor in rodent uterus (8). By addressing the question of whether THC behaves as an estrogen receptor agonist in studies of uterine growth, results have again been conflicting (7-9). Our investigation confirms earlier data that THC (≤10 μM) is unable to stimulate MCF7 cell proliferation (25, 27), and extends these data to a broader range of THC concentrations.

To our knowledge, the current study analyzed for the first time the potential androgenic activity of THC. For demonstration of androgenic activity, the MCF7-AR1 cell assay was employed (11, 15), which makes use of the inhibitory effect of androgens on estrogen-induced proliferation of MCF7-AR1 cells. Validation experiments with known androgens, R1881 and DHT (21), showed a good sensitivity of the cells, with IC_{50} values of 4.41×10^{-11} and 4.44×10^{-11} comparable with earlier studies (11). THC reduced 17β-estradiol-induced proliferation of MCF7-AR1 cells in a concentration-dependent manner, with an IC_{50} value of 9×10^{-6} M.

The effect of THC on MCF7-AR1 cells might be interpreted as an indication of the androgenic activity of the test chemical. However, it could also result from an antiestrogenic effect. THC added to (wild-type) MCF7 cells in the presence of 10^{-10} M 17β-estradiol inhibited cell proliferation in a very similar and concentration-dependent manner, with an IC_{50} value of 2.6×10^{-5} M. Since MCF7-AR1 cells express approximately five times more AR than wild-type MCF7 cells (11), it seems improbable that the inhibitory effect of THC on 17β-estradiol-induced

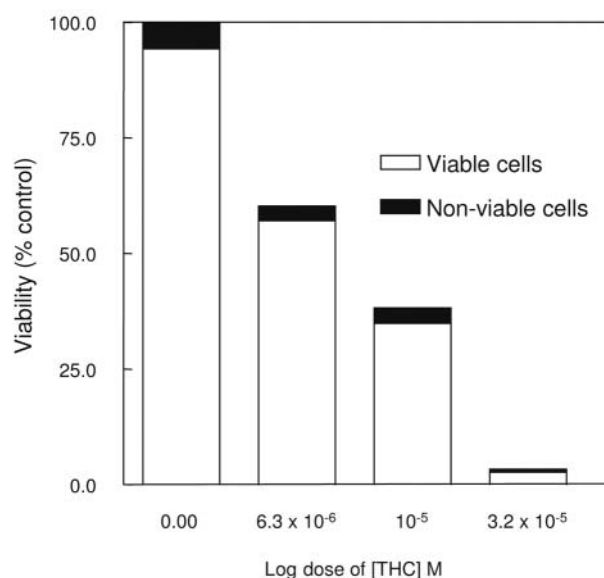Figure 3. *Effect of THC on cell viability of MCF7 cells. THC reduced the viability of MCF7 cells in the presence of 10^{-10} M 17β-estradiol as determined by trypan blue dye exclusion assay without increasing the number of dead cells. Results are presented as fractions of viable and non viable cells and are expressed as a percentage of the control (exposed to 10^{-10} M 17β-estradiol alone) total (viable and non viable) cell number. Data from two independent experiments.*

cell proliferation was mediated by AR. The data would rather be compatible with an antiestrogenic activity of THC. However, antiestrogenic effects of THC have been excluded in a recent study using the human breast cancer cell proliferation assay, a recombinant human estrogen receptor assay, and a reporter gene assay (28). Cytotoxic effects as a basis of the reduction of cell proliferation by THC can also be excluded because THC reduced the viability of MCF7 cells by reducing the cell number of viable cells rather than by inducing cytotoxic cell death. Thus, the effect on the two cell lines may be due to a THC-specific mode of action. There is growing evidence that THC, through activation of cannabinoid receptors, reduces human breast cancer cell proliferation at micromolar concentrations (29), mainly by blocking cell cycle progression (1, 29).

In conclusion, THC fails to act as an estrogen or androgen and appears to reduce 17β-estradiol-induced proliferation of breast cancer cell lines by a mechanism which is independent of AR and probably does not involve ER either. These results support the notion that THC controls cell proliferation through activation of cannabinoid receptors, independently of AR and ER, and thus might also be used in patients with hormone-sensitive tumors.

Acknowledgements

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