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The antiproliferative activity of cannabidiol ethyl ethers against human ora epitheloid carcinoma cells

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Running title: The antiproliferative activity of cannabidiol

SUMMARY

Cannabidiol derivatives (1, 2 and 3), and 5-fluorouracil (4, 5-FU) were tested for their growth inhibitory effects against human oral epitheloid carcinoma cell lines (KB) using two different 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and sulforhodamine B protein (SRB) assay. These compounds showed a potent inhibitory activity *in vitro* in the micromolar range against KB cell lines. In general, the antitumor activity of these compounds (1, 2, 3 and 4) was in a dose-dependent over the micromolar concentration ranges from 1 μM to 100 μM. The comparison of IC_{50} values of these compounds in tumor cell lines shows that their susceptibility to these compounds decreases in the following order : CBD > 5-FU > CBDME > CBDDE by the MTT assay and SRB assay. Cannabidiol derivatives (1, 2 and 3), and 5-FU were tested for their cytotoxic effects on NIH 3T3 fibroblasts using two different MTT assay and SRB assay. These compounds exhibited potent cytotoxic activities *in vitro* in the micromolar range against NIH 3T3 fibroblasts. In general, the cytotoxic acivities of these compounds (1, 2, 3 and 4) were in a dose-dependent over the micromolar concentration range 1 μM to 100 μM. The comparison of CD_{50} values of these compounds on NIH 3T3 fibroblasts shows that their susceptibility to these compounds decreases in the following order; CBD > 5-FU > CBDDE > CBDME by MTT assay, CBD > 5-FU > CBDME > CBDDE by SRB assay. These results suggest that cannabidiol (1, CBD) retains the most growth-inhibitory activity against KB cell lines.

Key Words : Cannabidiol; 5-Fluorouracil; Growith-inhibitory activity; Cytotoxic effect; KB cell; MTT assay; SRB assay

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INTRODUCTION

Cannabidiol(1, CBD) is a non-psychoactive constituent of *Cannabis sativa L.* Both hashish and marijuana are derived from cannabis and have been used for centuries for their medicinal and psychotomimetic properties (Mechoulam *et al.*, 1970). CBD has been reported to possess potentially important pharmacological properties as an antiepileptic (Consroe *et al.*, 1981), anxiolytic (Guimaraes *et al.*, 1990), and as an antidyskinetic agent (Conti *et al.*, 1988). In a recent paper (Baek *et al.*, 1996; Baek *et al.*, 1995), we reported the significant antitumor efficacy of cannabigerol in a IC_{50} value of 31.30 μM *in vitro* MTT assay. Jung (1998) reported that cannabinoids and 5-fluorouracil (4, 5-FU) exhibit their growth inhibitory effects against human oral epitheloid carcinoma (KB) cell lines using two different 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay and sulforhodamine B protein (SRB) assay. These compounds showed potent inhibitory activity *in vitro* in the micromolar range against KB cell lines. In general, the antitumor activity of these compounds was in a dose-dependent over the micromolar concentration range 1 μM to 100 μM. Cannabinoids and 5-fluorouracil were tested for their cytotoxic effects on NIH 3T3 fibroblasts using two different MTT assay and SRB assay. Baek *et al.* (1998) reported that cannabigerol shows the least cytotoxic activity on NIH 3T3 fibroblasts. It has exhibited the highest growth-inhibitory activity against KB cell lines. In hopes of finding better therapeutic agents for antitumor activity, we report here on cannabinoids and their antitumor activities against human oral epitheloid carcinoma cell lines.

MATERIALS AND METHODS

1. Instruments

IR spectra were recorded on a Perkin- Elmer 457 grating infrared spectrophotometer. ^{1}H-NMR spectra were obtained on a Bruker WH-200 and WH-300 pulsed FT spectrometers. Chemical shifts are given in parts per million downfield from Me_4Si internal standard. Mass spectra were recorded on a Varian Mat CH-5 mass spectrometer. Analytical TLC was performed by using commercially available silica plates (polygram sil N-HR/UV_{254}), and the plates were visualized with fast blue phenol reagent. Medium pressure liquid chromatography was performed on an ALTEX glass column, 1 meter long, diameter 9 mm internal using an FMI pump and silica gel 60 (230 - 400 mesh) purchased from Merck. Fractions were collected with LKB 2070 or LKB 7000 fraction collectors at a rate of 2 - 10 ml/min.

2. Chemicals

5-FU, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide, fetal ovine serum (FBS), sulforhodamine B protein were obtained from Sigma Chemical Co., Ltd. (St. Louis, USA). Ethyl iodide were purchased from Aldrich Chemical Co., Ltd. (Milwaukee, U.S.A.). CBD was kindly provided by Prof. R. Mechoulam in the Department of Natural Products at School of Pharmacy, Hebrew University, Israel. All other chemicals were of reagent grade.

3. Tumor cell lines and culture conditions

In vitro RPMI-1640 medium was supplemented with 10% FBS, 100 μg/ml streptomycin, 100 units/ml penicillin at 37 ℃ and 5% carbon dioxide. Cells were dissociated with 0.25% trypsin just before transferring for experiment and were counted by Hemocytomer. The cytotoxic activities of cannabidiol derivatives (1, 2 and 3), and 5-FU against KB cells and NIH 3T3 fibroblasts were measured by the MTT and SRB methods.

4. Ethylation of cannabidiol

Cannabidiol (1,256 mg, 4.0 mmol) was dissolved in dry DMF (40 ml) and added ethyl iodide (2.0 mmol) and potassium carbonate (200 mg, 1.45 mmol). The reaction mixture was stirred for 4 hrs at room temperature, and ether (200 ml) was added. The reaction mixture was washed with brine, filtered, then dried over anhydrous magnesium sulfate and evaporated under reduced pressure. The residue was separated by medium pressure liquid chromatography, eluted with ethyl acetate-petroleum ether (v/v 5 : 95) to give cannabidiol monoethyl ether (2, CBDME, 124 mg, 7.8%) and cannabidiol diethyl ether (3, CBDDE, 370 mg, 23.1%). These compounds were identified by comparison of their spectral data (TLC, MS, NMR and IR) with those published or by direct comparison with an authentic sample (Baek, 1986).

5. Evaluation of antitumor activity

The antitumor activity of cannabidiol derivatives (1, 2 and 3), and 5-FU was determined by the modification of the literature methods (Mosmann, 1983 ; Carmichael *et al.*, 1987; Keepers *et al.*, 1991). All experimental data were expressed as the mean ± S.D. of triplicate experiments.

6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl -2H-tetrazoliumbromide assay

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product which can be measured spectrophotometrically (Mosmann, 1983; Carmichael *et al.*, 1987). KB cell lines were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^4) were cultured for 48 hrs at 37 ℃ in a humidified 5% CO_2 incubator in the presence or absence of cannabidiol derivatives (1, 2 and 3), and 5-FU.

7. Sulforhodamine B protein assay

The SRB assay was performed essentially according to the method of Skehan *et al.* (1990). The methods of plating and incubation of cells were identical to those cells of the MTT assay.

8. Evaluation of toxicity : cytotoxicity assay

In order to determine the cytotoxicity mediated by compounds (1, 2, 3 and 4), the colorimetric assay was used. These compounds were serially diluted in EMEM (Eagle's minimum essential medium) with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells/ml). After one hour, fresh culture medium was supplied to a total volume of 1 μM – 100 μM. On the third day of incubation in at 37 ℃ an incubator MTT terazolium dye (5 mg/ml ; 20 ㎕/well ; Polyscience, Inc. Warrigton, PA) was added to the cells. After 3 hrs, the absorbance was measured at 540 nm using ELISA reader. All experimental data were expressed as the mean ± S.D. of three experiments. The 50% cytotoxic dose (CD_{50}) was calculated using the computer program.

9. Morphology

Changes in the morphology of KB cells cultured in a medium with compounds (1, 2, 3 and 4) were documented by microphotography.

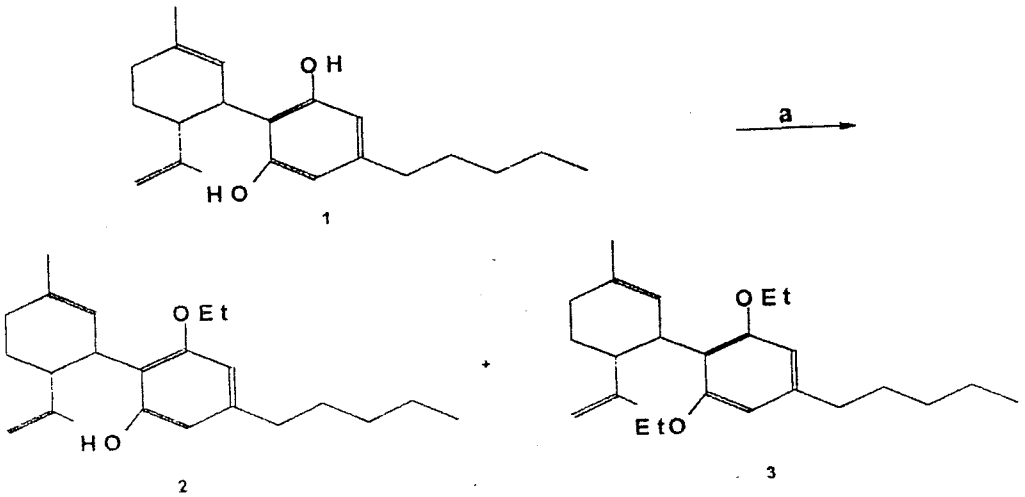
10. Statistical Analysis

All values, expressed as mean ± S. D., were statistically analyzed through analysis of Studeant's t-test. The P value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

1. Synthesis of cannabidiol ethyl ethers

CBDME and cannabidiol diethyl ether (3, CBDDE) are reported as a synthetic or natural product. The synthesis of these compounds resulted from the coupling of CBD to ethyl iodide; both prepared by literature methods (Baek, 1986). The structure of cannabidiol ethyl ethers (2, 3) was confirmed by spectral data. ^{1}H-NMR spectrum of CBDME showed the presence of nonequivalent aromatic protons (6.23 and 5.95 ppm). The phenolic OH of CBDME was observed in the IR spectrum as broad exchangeable signal at 3464 cm.$^{-1}$ However, CBDDE showed less polar than CBDME. ^{1}H-NMR spectrum of this compound (3) showed the presence of equivalent aromatic protons (6.30 ppm). The phenolic OH of CBDDE was not observed in the IR spectrum (Fig. 1).



a. CH_3CHI, K_2CO_3, DMF, RT. Overnight

Fig. 1. Ethylation of cannabidiol (1)

2. *In vitro* antitumor activity

Table 1 shows the potent antitumor activities of compounds (1, 2, 3, and 4) against KB cells. In general, the antitumor activities of these compounds were in a dose-dependent, and the susceptibility of the cancer cell lines to CBD was quite sensitive (Table 1). The value of IC_{50} of cannabidiol derivatives (1, 2 and 3) and 5-FU showed that CBD exerts the most potent antitumor activity in the MTT assay with toxicity in the SRB assay (Tables 1 and 2). A colorimetric assay was used to detect the *in vitro* antitumor activivities mediated by CBD 1 against KB cell lines. As shown in figures 2 and 3, CBD, 1-mediated antitumor activities was rapidly increased in the MTT assay when its concentration was increased from control μM to 25 μM. However, CBD, 1 was a little changeable in the MTT assay when its concentration was raised from 25 μM to 100 μM. 5-FU 4 as a reference rapidly increased in the MTT assay when its concentration was increased from 1 μM to 50 μM (Fig. 2). As shown in Fig. 3, CBDME, 2 and CBDDE, 3 did not show increasing effets in the SRB

assay when their concentrations were increased from control μM to 100 μM. CBD, 1 showed gradually increased antitumor activity when its concentration was increased from 25 μM to 100 μM. However, 5-Fu 4 showed rapidly increased antitumor activity when its concentration was increased from 1 μM to 50 μM (Fig. 3).

Table 1. The antitumor activities of cannabidiol derivatives (1, 2 and 3) and 5-FU and on KB cell lines. Comparison of IC_{50} for cannabidiol derivatives (1, 2 and 3) and 5-FU by SRB assay and MTT assay.

Compounds[a]	IC_{50} (μM)[b]	
	MTT assay	SRB assay
CBD	19.22	41.42
CBDME	85.58	743.56
CBDDE	59.13	808.77
5-FU	44.36	45.20

[a]Each compound was examined in four concentrations in triplicate experiments. [b]IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

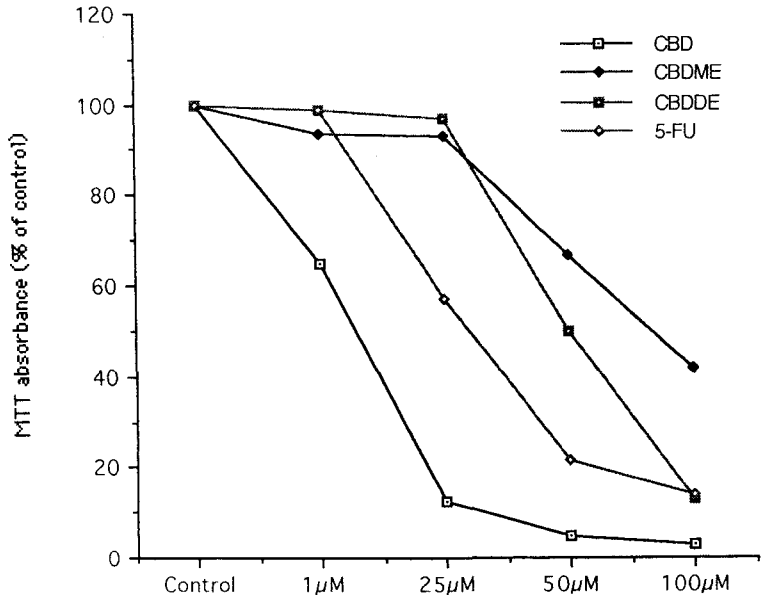


Fig. 2. *In vitro* antitumor activities of cannabidiol derivatives (1, 2 and 3) and 5-FU by the MTT assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of KB cells (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

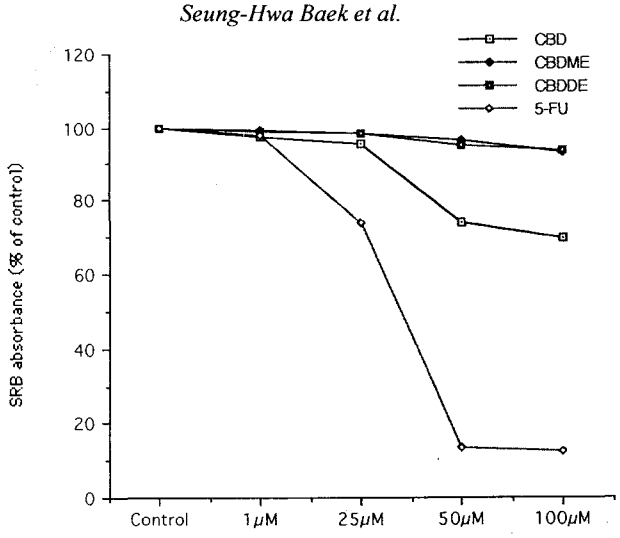


Fig. 3. *In vitro* antitumor activities of cannabidiol derivatives (1, 2 and 3) and 5-FU by the SRB assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of KB cells (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

3. Toxicity

A colorimetric assay was used to detect the *in vitro* cytotoxicity mediated by cannabidiol CBD. As shown in Fig. 4, CBD - mediated cytotoxicity was rapidly increased in the MTT assay when its concentration was raised from 1 μM to 50 μM. 5-FU as a reference rapidly increased in the MTT assay when its concentration was raised from 1 μM to 25 μM. However, 5-FU was a little changeable in the MTT assay when its concentration was increased from 25 μM to 100 μM (Fig. 4).

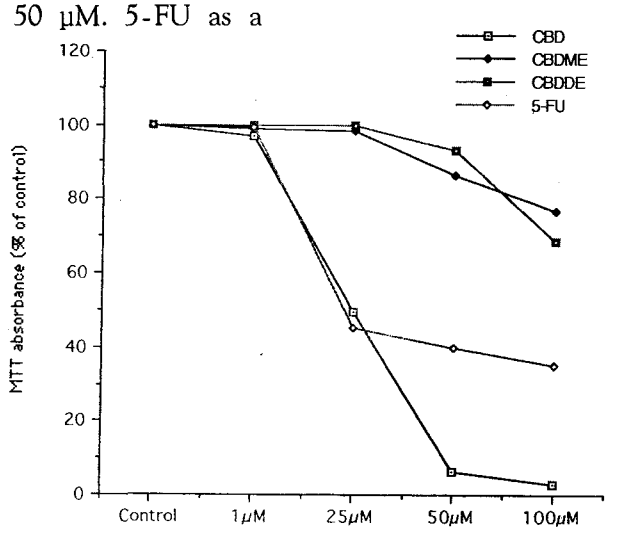


Fig. 4. *In vitro* cytotoxic effects of cannabidiol derivatives (1, 2 and 3) and 5-FU by the MTT assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments

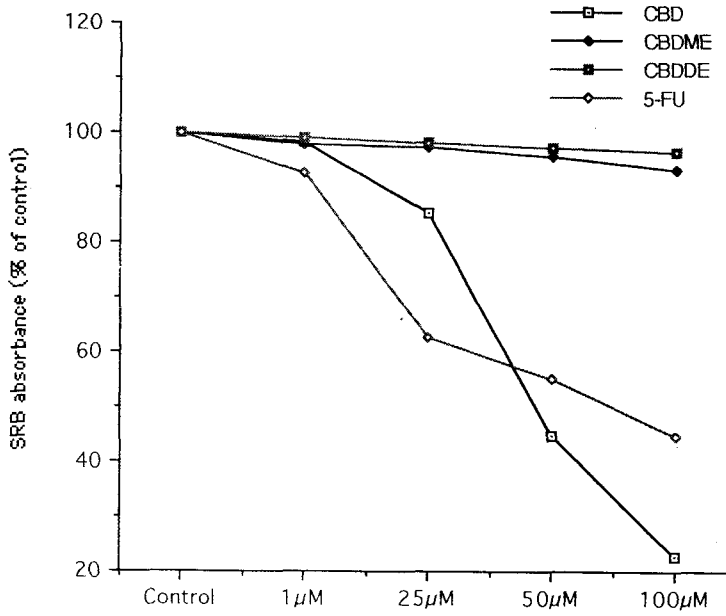


Fig. 5. *In vitro* cytotoxic effects of cannabidiol derivatives (1, 2 and 3) and 5-FU by the SRB assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

Table 2. The cytotoxic effects of cannabidiol derivatives (1, 2 and 3) and 5-FU on NIH 3T3 fibroblasts. Comparison of CD_{50} for the cytotoxic effects of cannabidiol derivatives (1, 2 and 3) and 5-FU by the SRB assay and the MTT assay.

Compounds[a]	CD_{50} (μM)[b]	
	MTT assay	SRB assay
CBD	36.27	60.25
CBDME	209.69	890.78
CBDDE	173.17	1647.4
5-FU	41.27	75.90

[a]Each compound was examined in four concentrations in triplicate experiments.
[b]CD_{50} represents the concentration of a compound required for 50% cytotoxic dose of cell growth

As shown in Fig. 4, 5, CBD, 1 - mediated cytotoxicity was rapidly increased in the SRB assay when its concentration was increased from 1 μM to 100 μM. However, 5-FU showed similar effects. CBDME, 2 and CBDDE, 3 did not increase in the SRB assay when their concentrations were increased from control μM to 100 μM. The IC_{50} values determined by MTT and SRB methods were 36.27 μM and 60.25 μM, respectively (Table 2).

4. Biological activity

The present study shows the *in vitro* growth inhibitory activities of cannabidiol derivatives (1, 2 and 3) and 5-FU as a reference against KB cell lines (Fig. 5 and Fig. 6). 5-FU is commonly used as a therapeutic agent to treat cancers of the large bowel. However, therapy with 5-FU as a single agent has only limited success. Additive agents or modulators for 5-FU effect are needed for more effective treatment of this highly resistant malignancy (Boersma *et al.*, 1993).

Table 1 shows the potent antitumor activities of cannabidiol derivatives (1, 2 and 3) and 5-FU against KB cells. In general, the antitumor activities of these compounds (1, 2, 3 and 4) were in a dose-dependent over the micromolar concentration range 1 μM to 100 μM, and the susceptibility of KB cells to these compounds was quite different. The comparison of IC_{50} values of these compounds in tumour cell lines shows that their susceptibility to these compounds decreses in the following order: CBD > 5-FU > CBDME > CBDDE by the MTT assay and the SRB assay (Table 1). CBD was the most effective growth inhibitor of KB cell lines, producing an IC_{50} of about 19 μM by the MTT assay and 41 μM by the SRB assay. The SRB assay was compared with the MTT assay for *in vitro* chemosensitivity testing of KB cells. The MTT assay appeared to be more sensitive than the SRB assay, with a better linearity with cell number and higher reproducibility. The antitumor activity of CBD exhibit more active than that of 5-FU, as a reference compound, on KB cells in the MTT and SRB assay (Table 1).

Table 2 shows the cytotoxic effects of cannabidiol derivatives (1, 2 and 3) and 5-FU against NIH 3T3 fibroblasts. In general, the cytotoxic effects of these compounds (1, 2, 3 and 4) were in a dose-dependent manner over the concentration range 1 μM to 100 μM, and the susceptibility of NIH 3T3 fibroblasts to these compounds was quite different (Table 2). The comparison of CD_{50} values of these compounds in NIH 3T3 fibroblasts shows that their susceptibility to these compounds decreases in the following order; CBD > 5-FU > CBDDE > CBDME by the MTT assay, CBD > 5-FU > CBDME > CBDDE by the SRB assay (Table 2). CBD was the most cytotoxic effect of NIH 3T3 fibroblasts, producing a CD_{50} of about 36 μM in MTT assay and 60 μM in the SRB assay. CBD was more potent than 5-FU as a reference compound. CBD exhibits more active than 5-FU on NIH 3T3 fibroblasts in MTT assay and SRB assay (Table 2). CBD is structually related to olivetol with, a known inhibitory effect (Baik *et al.*, 1993), inhibited the most effective growth-inhibitory activity against the tested cancer cell lines in the MTT assay. The compounds (1, 2, 3 and 4) used are known to inhibit the activity of several enzymes of the arachidonate cascade including cyclooxygenase and lipoxygenase (Evans *et al.*, 1987) and to both stimulate and inhibit phospholipase A2 activity (Evans *et al.*, 1987). The actions of the cannabinoids on membrane associated enzymes are complex and in a dose-dependent. Although these actions are possibly associated with the ability of the cannabinoids to act as anticancer agents (Plasse *et al.*, 1991), specific structural alterations may be critical in determining enzyme targets. CBD has been selected as

lead compounds for further examinations (Fig. 1 and 6).

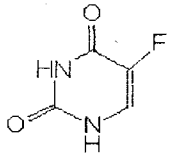


Fig. 6. The structure of 5-fluorouracil (4)

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