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The Inhibition of DNA Synthesis by Cannabinoids[1]

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SUMMARY

Several of the cannabinoids found in marihuana have been shown to inhibit tumor growth and increase the life-span of mice bearing the Lewis lung adenocarcinoma. When trypsin-dispersed isolated Lewis lung cells are incubated *in vitro,* they maintain their capacity to carry out macromolecular synthesis (RNA, DNA, protein). This process can be inhibited by cytosine arabinoside, actinomycin D, or methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, whereas cyclophosphamide, an agent that must be bioactivated, was inactive. Inhibition of DNA synthesis as measured by [^{3}H]thymidine uptake into acid-insoluble material was used as an index of cannabinoid activity against isolated Lewis lung cells, L1210 leukemia cells, and bone marrow cells incubated *in vitro*. Δ^9-, Δ^8-, 1-hydroxy-3-*n* pentyl-, and 1-Δ^8-*trans*-tetrahydrocannabinol, and cannabinol demonstrated a dose-dependent inhibition of DNA synthesis whereas cannabidiol and 1-hydroxy-3-*n*-pentylcannabidiol were markedly less inhibitory in our *in vitro* cell systems. Furthermore, our *in vitro* observations with these cannabinoids are supported by *in vivo* tumor inhibition studies. Ring modifications as in cannabichromene or cannabicyclol abolish *in vitro* activity as does dihydroxylation at the 8β and 11 positions of 1-Δ^9-*trans*-tetrahydrocannabinol. Δ^9-*trans*-tetrahydrocannabinol demonstrated the least toxicity of all inhibitory cannabinoids *in vivo;* this is supported by its lesser effect on bone marrow DNA synthesis *in vitro*.

INTRODUCTION

Recently, we reported (5, 6, 12) that certain cannabinoids exhibit inhibitory activity against the Lewis lung adenocarcinoma *in vitro* and *in vivo* and that the Friend leukemia virus induced splenomegaly *in vivo*. This report expands the *in vitro* observations to include additional cannabinoids and other compounds using L1210 leukemia cells, isolated mouse bone marrow cells, and trypsin-dispersed isolated Lewis lung cells. The spectrum of effects attributed to Δ^9-THC,[2] the major psychoactive ingredient in marihuana, is varied and complex (17). To date, an understanding of any of its behavioral or biochemical effects is still in the realm of speculation. Recent investigations aimed at elucidating the cellular responses to marihuana or Δ^9-THC have shown that cultures of human lung cells show changes in DNA synthesis following exposure to marihuana smoke (9). Zimmerman and McClean (22) demonstrated in *Tetrahymena* that exposure to Δ^9-THC produced cytolysis that was preceded by the inhibition of RNA, DNA, and protein synthesis. Aberrant chromosomal changes have been reported in chronic marihuana users (21) as well as impairment of spermatogenesis and testosterone secretion (8). Mitogen-stimulated lymphocytes from marihuana users have been reported to incorporate [^{3}H]thymidine at a lower rate than did lymphocytes from control subjects (13). These observations suggest a complex interaction between the drug and the various systems studied. The investigations in our laboratories are currently directed toward the identification of active cannabinoids (structure activity relationships); proposal of structural modification of the THC ring system that alter activity; and elucidating their mechanism(s) of action. This report deals primarily with *in vitro* structure activity observations.

MATERIALS AND METHODS

Preparation of Isolated Lewis Lung Cells. Lewis lung tumors were grown in the gluteus muscle of C57BL/6 mice. Tumors were removed 14 to 18 days posttransplant, cleared of tissue debris, and cut into 1- to 2-mm sections. They were resuspended in MEM (with Earle's salts) containing 20% heat-inactivated fetal calf serum. Cells were enumerated using a Model ZB1 Coulter counter diluted in Isoton, and cell viability was monitored using trypan blue dye exclusion (0.5% trypan blue). Cells were centrifuged (600 × *g* for 10 min) and resuspended in MEM (with Earle's salts) supplemented with (for every 500 ml MEM) 5 ml 100× vitamins, 10 ml 50× amino acids, 5 ml 200 mM glutamine, 5 ml penicillin (5000 units/ml)-streptomycin (5000 μg/ml). Tissue culture reagents were obtained from Grand Island Biological Co., Grand Island, N. Y., or Flow Laboratories, Rockville, Md. Cell number was adjusted to 10^7 cells/ml and dispensed in 25-ml Erlenmeyer flasks (3 to 5 ml/flask) containing 10 μl of drug or drug vehicle (ethanol). Flasks were then allowed to equilibrate for 15 min at 37° under an atmosphere of 5% CO_2-95% O_2 with shaking in a Dubnoff metabolic bath. Radiolabel was then added and 1-ml aliquots for liquid scintillation were removed at various times. The quality of cells that one can isolate depends upon the care with which one chooses the tumor and its trypsinization. The use of old

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[2] The abbreviations used are: Δ^9-THC, 1-Δ^9-*trans*-tetrahydrocannabinol; THC, *trans*-tetrahydrocannabinol; MEM, minimal essential medium; ara-C, 1-β-D-arabinofuranosylcytosine; ED_{50}, 50% inhibition of cell proliferation; Δ^8-THC, 1-Δ^8-*trans*-tetrahydrocannabinol; ABN Δ^8-THC, 1-hydroxy-3-*n*-pentyl-Δ^8-tetrahydrocannabinol; CBD, cannabidiol; ABN-CBD, 1-hydroxy-3-*n*-pentylcannabidiol; CBN, cannabinol.

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necrotic tissue is undesirable as are extended periods of trypsinization (>2 hr). It became apparent following repeated use of the Erlenmeyer flasks that acid wash followed by alcohol-acetone rinses was insufficient in removal of cannabinoid material from the glassware. We therefore have been using 20-ml glass scintillation vials which are then discarded.

Isolation and Incubation of Mouse Bone Marrow Cells. C57BL/6 × DBA/2 F_1 (hereafter called B6D2F_1) mice were sacrificed (cervical dislocation) and the tibias and fibulas were freed of surrounding muscle and connective tissue. The distal portions of bones were removed and 1 ml heparinized MEM (1.0 unit/ml) was forced through the bone using a 1-ml syringe with a 26-gauge needle. Cells were then centrifuged (600 × *g* for 10 min), resuspended 3 times in MEM, and the nucleated cells were enumerated using a Coulter counter. Cell viability was monitored by trypan blue dye exclusion. Cell number was adjusted to 10^7 cells/ml and incubated with the drug or drug vehicle as described above.

Isolated L1210 Cells. DBA/2 mice were inoculated with 1 × 10^5 L1210 cells 6 days prior to sacrifice (cervical dislocation). L1210 cells were removed by flushing the peritoneal cavity with 10 ml unsupplemented serum-free Dulbecco's MEM containing penicillin and streptomycin. Cells were centrifuged (600 × *g* for 10 min) and resuspended in the above serum-free medium at a final concentration of 10^7 cells/ml. L1210 cells were then incubated as described for Lewis lung cells.

Radiolabel Uptake *in Vitro*. Radiolabeled [*methyl*-^{3}H] thymidine (6.7 Ci/mmole; New England Nuclear, Boston, Mass.) and/or [2-^{14}C]uridine (57 mCi/mmole; New England Nuclear) were added (10 μCi/flask) following the 15-min equilibration period of cells with drug or drug vehicle. At various times after the addition of radiolabel, 1-ml aliquots were removed and placed in 12- x 75-mm test tubes containing 2 ml 10% trichloroacetic acid (4°). The samples were mixed and then kept at 4° for at least 20 min prior to filtration on a Millipore filtering apparatus. The filters were washed 3 times with 3 volumes of 10% trichloroacetic acid (4°). Filters were then transferred to glass scintillation vials and 10 ml of toluene-Liquiflor cocktail were added. Samples were counted in a Beckman liquid scintillation spectrometer. The incorporation of radioactive precursors into acid-insoluble material (DNA) was linear over the incubation period (45 min) for Lewis lung, L1210, and bone marrow cells.

Drugs. ara-C (NSC 63878), actinomycin D (NSC 3053), methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 9544), and cyclophosphamide (NSC 26261) were kindly supplied by Dr. Ruther Geran, National Cancer Institute. Cannabinoids were supplied by Dr. Monique Braude of the National Institutes of Drug Abuse or Dr. Raj Razdan of The Sheehan Institute for Research, Cambridge, Mass.

RESULTS

Effect of Drugs on [^{3}H]Thymidine Uptake. In an attempt to evaluate our *in vitro* systems, we tested compounds of known activities. ara-C, an inhibitor of DNA synthesis (3), has been shown to be highly active against the L1210 *in vivo* (13) and is extremely active against the L1210 *in vitro* (Table 2A). Lewis lung or bone marrow cells incubated *in vitro* are apparently not as sensitive to this agent (Table 2A); this is supported by its marginal activity against the Lewis lung tumor *in vivo* (Table 1). At nontoxic doses of ara-C (10, 40 mg/kg) significant inhibition of primary tumor size was seen only at 14 days; at later times *i.e.*, 21 and 28 days, no significant inhibition of the primary tumor was observed. Significant increases in the life-span of these animals was seen only at the highest nontoxic dose used (40 mg/kg). Actinomycin D, an inhibitor of RNA synthesis (14–16), when incubated with Lewis lung cells *in vitro* in the presence of radiolabeled thymidine and uridine, inhibits only the uptake of [^{14}C]uridine into acid-precipitable material while not affecting [^{3}H]thymidine uptake. Inhibition of DNA synthesis by actinomycin D was only seen when uridine incorporation was less than 30% of control (data not shown). Methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea is a purported alkylating agent (11) that showed only marginal activity in our *in vitro* system against the Lewis lung, and cyclophosphamide which is an alkylating agent requiring bioactivation (2) showed no activity when tested against the L1210 *in vitro* (data not shown).

Effect of Cannabinoids and ara-C on [^{3}H]Thymidine Uptake *in Vitro* and *in Vivo* Tumor Inhibition. We tested several cannabinoids in our *in vitro* systems as indicated in Tables 2A and 2B. Similar data (not shown) were also obtained using radiolabeled uridine. Those cannabinoids that inhibited [^{3}H]thymidine uptake by 50% (ED_{50}) in the Lewis lung *in vitro* at μM concentrations include Δ^9-, Δ^8-, ABN Δ^8-THC, and CBN (Table 2A). Concentrations of cannabinoids in excess of 10^{-5} M were required, for CBD, ABN-CBD, cannabichromene, 8β, 11-dihydroxy, Δ^9-THC, and cannabicyclol (Table 2B). Comparisons of *in vitro* activity for several of these compounds with *in vivo* tumor inhibition can be made by comparing Tables 2 and 3. Dose-dependent inhibition of primary tumor growth is seen with those compounds that inhibited *in vitro* DNA synthesis at μM concentrations (Table 2A). Those compounds (CBD, ABN-CBD) that required larger concentrations *in vitro* either were inactive or as is seen with CBD stimulated tumor growth at either 25 or 200 mg/kg (Table 3). Significant (>25%) increases in the life-span of Lewis lung tumor-bearing mice were seen only with Δ^9-, Δ^8-, ABN Δ^8-THC, and CBN (data not shown). The inhibition of DNA synthesis in the L1210 *in vitro* is depicted in Table 2. A comparison of cannabinoid activity in this system is of interest since basically the activities fall into the same 2 groups seen with the Lewis lung. Although Δ^9-THC has an ED_{50} greater than Δ^8-THC, ABN Δ^8-THC, or CBN, it is at least 10 times more effective than the other cannabinoids (Table 2). The cannabinoids have not been shown to have any activity against the L1210 *in vivo* (11) and, if one compares the best *in vitro* activity against any of the cannabinoids (CBN) with ara-C, a drug that can cure the L1210 *in vivo*, there is approximately a 100-fold difference in their ED_{50}'s. Comparison of cannabinoid activity against normal bone marrow cells *in vitro* is seen in Table 2. Δ^9-THC was the only cannabinoid that inhibited both Lewis lung DNA synthesis *in vitro* and tumor growth *in vivo* while

Table 1

The effect of ara-C in Lewis lung adenocarcinoma in vivo

Groups of B6D2F$_1$ male mice were inoculated i.m. with 5×10^5 Lewis lung tumor cells. ara-C was administered i.p. daily for 10 consecutive days beginning 24 hr after tumor implantation.Tumor weights were calculated from caliper measurements by:

$$\frac{w = a \times b^2}{2}$$

where a is the long axis (mm), b is the short axis (mm), and w is weight (mg).

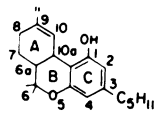
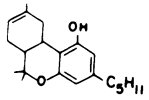
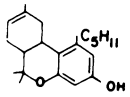
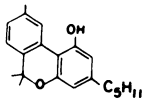
Dose (mg/kg)	Tumor wt (mg) (posttransplant) Day 14	Day 21	Day 28	% increase in life-span
0	2700 ± 200[a] (8)	7000 ± 300 (8)	9600 ± 400 (7)	
10	1600 ± 100 (7)	5500 ± 300 (7)	10000 (1)	0
40	1400 ± 60 (8)	6500 ± 500 (8)	9700 ± 600 (5)	25.4
80		Toxic		

[a] Mean ± S.E. of the tumor weights was derived from the number of mice indicated in parentheses.

Table 2A

The effect of cannabinoids and ara-C on in vitro DNA synthesis

Cells were prepared and incubated with drug or drug vehicle as described in "Materials and Methods." The drug dose that produces ED_{50} of DNA synthesis was calculated using the method of Litchfield and Wilcoxin (10). Values for which an ED_{50} was calculated had slopes that were not significantly different except for ara-C and represent the 15-min incubation time. Drugs were made up fresh daily in ethanol (10 μl/flask) and preincubated with cells for 15 min prior to the addition of radiolabel [^{3}H]thymidine, (10 μCi/flask). Following a 15-min incubation of cells with [^{3}H]thymidine the amount of trichloroacetic acid-insoluble (DNA) radioactivity present was: 25,000 cpm/10^7 cells, Lewis lung; 20,000 cpm/10^7 cells, L1210; 6,000 cpm/10^7 cells, bone marrow.

Drug	Structure	ED_{50}(M) Lewis lung	L1210	Bone marrow
Δ^9-THC		4.18×10^{-6}	3.26×10^{-5}	2.06×10^{-5}
Δ^8-THC		2.99×10^{-6}	8.70×10^{-6}	1.26×10^{-6}
ABN Δ^8-THC		1.48×10^{-6}	5×10^{-6}	3.56×10^{-6}
CBN		2.3×10^{-6}	2.2×10^{-6}	3.08×10^{-7}
ara-C		1.36×10^{-7}	2.53×10^{-8}	1.57×10^{-7}

exhibiting a differential effect on bone marrow DNA synthesis. This apparent selective toxicity was also seen with ara-C [bone marrow *versus* L1210, although not Lewis lung *versus* bone marrow (Table 2A)].

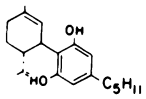
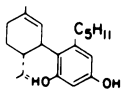
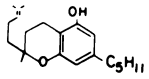
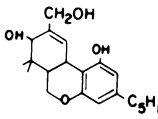
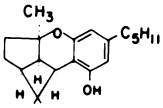
DISCUSSION

The use of isolated cell systems *in vitro* as models to study and evaluate both basic biochemical and drug mechanisms

Table 2B

The effect of cannabinoids and ara-C on in vitro DNA synthesis

Cell incubations were prepared as described in Table 2A and "Materials and Methods."

Drug	Structure	ED_{50} (M)		
		Lewis lung	L1210	Bone marrow
CBD		3.37×10^{-5}	[a]	4.89×10^{-4}
ABN-CBD		9.28×10^{-5}	1.29×10^{-2}	5.51×10^{-6}
Cannabichromene		$>10^{-4}$	$>10^{-4}$	
8β, 11-Dihydroxy Δ^9-THC		$>10^{-4}$	$>10^{-4}$	
Cannabicyclol		$>10^{-4}$		

[a] Doses of CBD (10^{-4} to 10^{-7} M) produced marked stimulation of [^{3}H]thymidine uptake into acid-precipitable material.

Table 3

The effect of cannabinoids on Lewis lung adenocarcinoma in vivo

Groups of B6D2F$_1$ male mice were inoculated with 5×10^5 Lewis lung carcinoma cells in the right hind gluteus muscle. Cannabinoids were administered by gavage daily for 10 consecutive days beginning 24 hr after tumor implantation. Tumor inhibition was determined 14 days after tumor transplant by converting caliper measurements to mg of tumor.

Drug	Tumor wt at the following doses (mg/kg)		
	0	25	200
Δ^9-THC	2000 ± 240[a]	850 ± 300	700 ± 200
Δ^8-THC	2200 ± 200	1200 ± 140	1100 ± 150
ABN Δ^8-THC	2400 ± 130	1400 ± 100	1200 ± 140
CBN	1300 ± 150	1000 ± 150	300 ± 100
CBD	2800 ± 200	4200 ± 500	3900 ± 300
ABN-CBD	2200 ± 200	1900 ± 250	2000 ± 150

[a] Mean ± S.E. derived from 8 mice/group.

represents an important investigational tool. The antitumor activity of Δ^9-THC reported by Munson *et al.* (12) is consistent with our *in vitro* observations. In addition, all those cannabinoids that we have studied *in vitro* have demonstrated a high degree of correlation when tested *in vivo* (Table 2 *versus* Table 3). We have also evaluated several drugs currently used in treating human leukemia (14). ara-C has been shown significantly to increase the survival time of mice bearing the L1210 leukemia, and indeed its activity in isolated L1210 cells incubated *in vitro* clearly supports its *in vivo* inhibition. When Δ^9-THC was evaluated *in vitro* against the L1210, inhibition of DNA synthesis was observed, although it was less than what was seen with Lewis lung cells incubated *in vitro*. When Δ^9-THC was tested *in vivo* against the L1210 leukemia, it showed no activity (12). This apparent discrepancy may be accounted for by the fact that agents that significantly prolong the survival time of L1210 mice produce significant daily cell kills (>99.9%) (18–20). The data in Table 2A would indicate that Δ^9-THC is at least 2 to 3 orders of magnitude less potent in inhibition of [^{3}H]thymidine uptake than is ara-C in the L1210. The rapid doubling time of L1210 cells *in vivo* (0.55 day), which have a comparatively short G_1 phase relative to their S phase, suggests that Δ^9-THC may not work by inhibiting the S phase of the cell cycle. The kinetics of cell growth (4, 11) in Lewis lung tumor cells *in vivo* (doubling time, 1.7 days for 100 mg of tumor) appear to make this solid tumor more amenable to control by Δ^9-THC [Munson *et al.* (12)] than is the L1210 leukemia and indicates that Δ^9-THC may work by inhibiting some other phase of the cell cycle. This possibility is currently under investigation. ara-C is approximately 10 times less potent in blocking DNA synthesis in the Lewis lung *in vitro* as it is against the L1210. This finding is supported by the observation that this agent is only marginally effective against the Lewis lung *in vivo*. These observations support the validity of our *in vitro* models in evaluating the cannabinoids. It would also appear from the data on other chemo-

therapeutic drugs that these *in vitro* sytstems may provide an appropriate milieu in which drug and biochemical studies can be carried out with a reasonable prediction of their *in vivo* activities.

Following the recent reports of Δ^9-THC activity on lymphocytes (7, 13), testosterone levels (8), chromosome damage (21), and the inhibition of the Lewis lung tumor and Friend leukemia virus-induced splenomegaly *in vivo*, we have attempted to identify the active sites on the THC molecule, to elucidate its sites of action, and to develop more potent analogs. The data from our *in vitro* systems and *in vivo* studies clearly indicate that there is no relationship between central nervous system activity and antitumor properties, since CBN and ABN Δ^8-THC are active *in vitro* and *in vivo* (12) against the Lewis lung while they do not produce any significant behavioral responses (1, 17). The location of the double bond in the A-ring (Δ^9-THC, Δ^8-THC, CBN) does not change its antitumor potency, although we have not evaluated 1-Δ^3-*trans*-tetrahydrocannabinol or other A-ring-saturated derivatives. Substitution or ring alterations on the A-ring are not compatible with activity as seen following hydroxylation (8β, 11-dihydroxy, Δ^9-THC) or alteration of the A-ring as seen with cannabichromene or cannabicyclol. The opening of the B-ring is also incompatible with activity (CBD, ABN-CBD) and in fact appears to increase [^{3}H]thymidine uptake *in vitro*. This structural change also increases the rate of tumor growth *in vivo* and decreases the life-span of the animals with tumor (12). Exchanging the alkyl and phenolic-hydroxyl groups in the C-ring appears not to alter the potency of THC (Δ^8-THC, ABN Δ^8-THC, Table 2A). General conclusions can therefore be made from our *in vitro* observations: (*a*) A-ring constituents are important for activity; this may be related to the planarity of the molecule although other physicochemical changes in the A-ring may provide a further understanding of A-ring requirements; (*b*) the integrity of the B-ring appears essential since CBD and its abnormal derivative show no inhibitory properties; (*c*) modification of the C-ring (ABN Δ^8-THC) does not alter activity. Additionally, these agents have a wide therapeutic index and toxic effects appear to be less serious with cannabinoids than they are with standard chemotherapeutic agents.

The preliminary finding that isolated bone marrow cells are not as drastically affected by Δ^9-THC as the isolated Lewis lung tumor cells represents a significant observation that we are currently pursuing. The lack of such specificity in the other active cannabinoids is supported by the weight loss noted in animals bearing the Lewis lung tumor treated with these drugs (12). In addition, it has been found (J. Levy, personal communication) that peripheral leukocyte counts from Δ^9-THC-treated animals (200 mg/kg), although initially depressed, recover and return to control levels within 4 days. We have also found (unpublished observation) that the total number of peripheral leukocytes from tumor-bearing mice treated with Δ^9-THC (25 to 200 mg/kg) for 10 days are identical to those of untreated animals. We are currently evaluating differential WBC counts from Δ^9-THC animals in order to rule out a change in a specific cell type.

An attempt to understand how the active cannabinoids produce their effects is currently under investigation. Macromolecular (DNA, RNA, protein) synthesis appears to be equally depressed by Δ^9-THC (unpublished data) although this is not related to cell death as indicated by cell viability (>95%). We have studied the action of Δ^9-THC on Lewis lung tumor cells grown in tissue culture; [^{3}H]thymidine uptake studies indicate that drug (Δ^9-THC) concentrations that inhibit thymidine uptake into acid-insoluble material by 50% do not affect the radioactivity in the acid-soluble pool. This observation indicates that Δ^9-THC is not inhibiting DNA synthesis by depressing precursor uptake (A. White and R. Carchman, unpublished observations) in Lewis lung tumor cells.

Our *in vitro* systems for evaluating and understanding the potential chemotherapeutic efficacy of cannabinoids may provide an additional mechanism not only for understanding the activity of these agents but also for accelerating the numbers and kinds of compounds that can be tested. In addition, the isolated bone marrow cells afford an opportunity to extend *in vitro* observations and offer some potential for predicting drug toxicity *in vivo*.

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