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SHORT COMMUNICATIONS

Actions of cannabis constituents on enzymes of arachidonate metabolism: anti-inflammatory potential

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The anti-inflammatory and analgesic properties of cannabis herb [1] (*Cannabis sativa* L) have been associated with non-cannabinoid constituents which reduce prostaglandin (PG) levels, both *in vitro* and *in vivo* [2, 3]. In contrast, cannabinoids have been shown to inhibit PGE_2 formation only at high concentrations, and at lower levels, actually stimulate PG release [2].

Nevertheless, cannabinoids have been reported to have beneficial actions in certain inflammatory conditions, notably asthma [4]; they may also inhibit cyclo-oxygenase at high concentrations [5, 6]. In view of the likely complexity of the actions of cannabis herb on levels of PG's *in vivo*, we have continued our evaluation of the anti-inflammatory potential of cannabis constituents by examining their effects in several cell-free systems, and attempting to correlate this information with activity in cell culture and animal models.

In this report we describe the effects of cannabinoids and other constituents on enzymes involved in arachidonate metabolism.

Materials and methods

Preparation of plant extracts. Cultivated cannabis was extracted as previously described [2, 3]. Both the petroleum ether and the ethanolic extracts were concentrated by evaporation on a rotary evaporator and aliquots were then dried to constant weight in a vacuum oven. For biological testing 1 mg ml stock solutions were diluted with 50% aqueous ethanol.

Other test compounds. Cannabinoids and olivetol were obtained from Makor Chemicals (Tel Aviv) and used without further purification. The flavone (**2**), was isolated from the ethanolic extract as previously described [2]. The structures of these compounds are given in Fig. 1.

Preparation of seminal vesicle microsomes. Ovine seminal vesicles were obtained from Northeast Biomedical Laboratories, Uxbridge; 100 g of frozen glands were homogenised in 1.5 vol. 0.1 M phosphate buffer (pH 8.0) containing 5 mM EDTA, 5 mM diethyldithiocarbamate and 1 mM phenol at 4° using a blender. The homogenate was centrifuged at 12,000 *g* for 15 min and the pellet discarded. The supernatant was centrifuged at 100,000 *g* for 45 min and the microsome pellet stored at −70° in 50% glycerol.

Assay of cyclo-oxygenase activity. Enzyme activity in the microsome preparation was measured using a Clark oxygen electrode (Yellow Springs, Ohio) [7]. The microsomes were suspended in 0.1 M phosphate buffer containing 0.1 mM phenol, at a concentration of between 1–10 mg/ml depending on activity, and maintained during the assay at 4° before introduction into the assay mixture. The assay was carried out at 30°. The assay mixture contained 0.5 ml of the microsome preparation and 10 μl of the inhibitor solution in a total volume of 1.9 ml. The reaction was initiated by adding arachidonic acid (Sigma, 200 μM) in 100 μl buffer. Activity was determined by measuring the initial linear rate of oxygen uptake, and assay conditions were selected so that control suspensions utilised 5–10% of oxygen present in the buffer at saturation with air. Indomethacin (Sigma) was used as a control both before and after each assay.

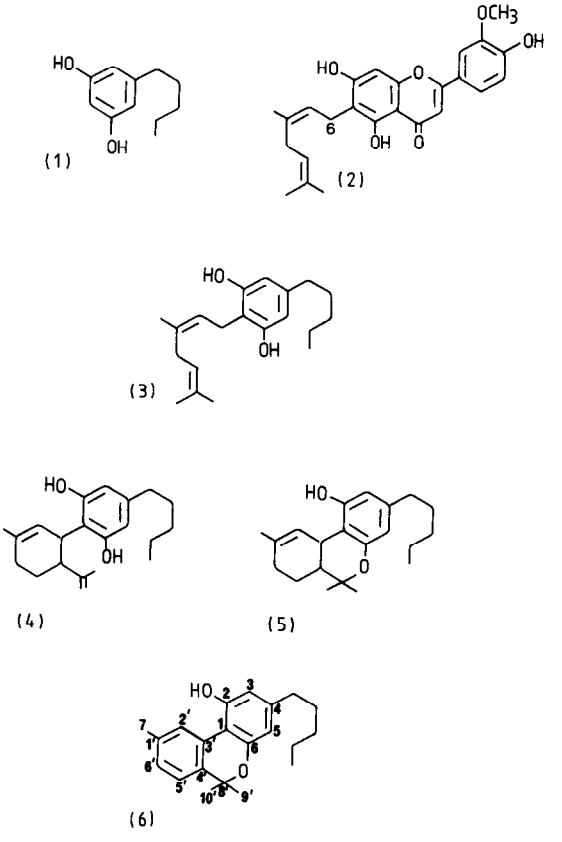

Fig. 1. Structures of cannabis constituents; 1. Olivetol. 2. Cannflavone-2. 3. Cannabigerol. 4. Cannabidiol. 5. Tetrahydrocannabinol. 6. Cannabinol.

Assay of soybean lipoxygenase activity. The activity of soybean lipoxygenase (Sigma) was measured in a direct spectrophotometric assay by measuring the increase in absorbance at 236 nm due to the formation of lipid peroxide [8]. The assay was carried out at 30°, using a reaction mixture containing 500 units of enzyme and 10 μl of inhibitor solution in borate buffer (pH 9.0, 2.9 ml of 0.1 M). Arachidonic acid (200 μM) in 100 μl of buffer were added to initiate the reaction, and the initial linear rate of increase in absorbance was measured to determine enzyme activity.

Results and discussion

Cannabis sativa has traditionally been used in the treatment of a number of inflammatory and allergenic conditions, including arthritis and asthma. The active constituents of this herb are believed to be the cannabinoids (Fig. 1) and although the cannabinoid receptor remains to be identified, previous work has concentrated upon their ability to affect dependent hormonal activity including secondary amines [9] and prostaglandins [2, 3].

Our results confirm that several constituents of cannabis can inhibit cyclo-oxygenase activity *in vitro*. The activities of the cannabinoids (IC_{50} in the range 30–50 μM) were similar. In addition the non-cannabinoid compounds (**1**) and (**2**) were also shown to be inhibitors of cyclo-oxygenase. Of significance was the observation that the oil and the ethanolic extracts induced inhibition of this enzyme (Table 1). These extracts were more effective on a weight basis than the constituents themselves, suggesting that either still more effective cyclo-oxygenase inhibitors remain to be isolated from the plant, or that potentiation occurs with individual compounds. Potentiation of *in vivo* effects has previously been observed in the cannabinoids [10].

The mechanism of action of these compounds was also studied on soybean lipoxygenase in a lipid peroxidation system which is well characterised [8]. Once again the activities of the cannabinoids were in a similar range with IC_{50}; 2.2–9.2 μM. The non-cannabinoid constituents (**1**) and (**2**) were ineffective at concentrations of up to 50 μM. (Table 2). Preliminary kinetic evaluation of the inhibitory effects of the cannabinoids on the enzyme indicate a complex mechanism of action. There was evidence of competitive inhibition at low inhibitor/high substrate concentrations, and non-competitive inhibition predominating at high inhibitor/low substrate concentrations, giving rise to curved reciprocal plots (Fig. 2). This is characteristic of compounds which interfere with peroxide activation of both cyclo-oxygenase and lipoxygenase [11], suggesting that the cannabinoids might share a common mode of action with other phenolic anti-oxidants. Cannabis constituents are probably dual inhibitors of both cyclo-oxygenase and lipoxygenase. Burstein and co-workers [12] have recently reported biphasic activation of cyclo-oxygenase by analogues of tetrahydrocannabinol. We did not observe a stimulatory action of cannabinoids, including tetrahydrocannabinol (**5**) at lower concentrations, but this might have been due to the inclusion of phenol as an activator. Clearly, this characteristic requires further evaluation, despite the observations [13, 14] that some phenols are wholly inhibitory in action.

The significance of functional groups on the actions of cannabis constituents as inhibitors of these enzymes can be facilitated by reference to Fig. 1. All compounds which exhibit a C-2 phenolic moiety were effective cyclo-oxygenase inhibitors. The introduction of an alkyl side chain at C-1 of the phenolic ring conferred inhibitor activity against lipoxygenase, which was marginally maximal when the alkyl side chain was cyclised to the terpene form as in compound (**4**). The formation of an epoxide and the aromatisation of the side chain as in compound (**6**) reduced the effectiveness of that compound as an inhibitor of lipoxygenase. This

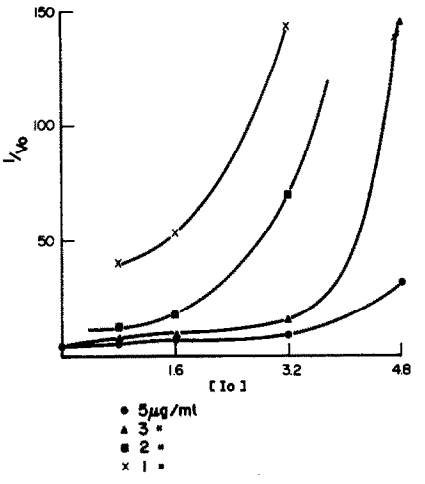

Fig. 2. Dixon plot of inhibitory action of cannabinol on soybean lipoxygenase at varying substrate concentrations.

observation may also hold for compound (**2**), in that the C-6 hydroxyl and the keto function are known to interact by hydrogen bonding [15].

Comparison of these results (Tables 1 and 2) with those previously reported by us [10] on models of inflammation suggest that that both olivetol (**1**) and the flavone (**2**) are orally active anti-inflammatory agents acting primarily upon cyclo-oxygenase. The anti-inflammatory potential of the cannabinoids presents a more complex problem. These compounds have been shown to suppress tetradecanoylphorbolacetate (TPA) induced release of prostaglandin from cell culture [2] and to inhibit TPA-induced erythema of mouse skin [10]. Protein kinase C is believed to be the phorbol ester receptor site [16], but we have demonstrated that the cannabinoids (data not shown) only prevent TPA activation of that kinase in a cell-free system, at concentrations in excess of 100 μM. Possibly the inflammation induced by TPA is of mixed aetiology [17], or the cannabinoids are metabolised during absorption through mouse skin. The stimulation of phospholipase A_2 has been suggested as a mechanism for the activity of tetrahydrocannabinol [18] and it has been demonstrated that a number of related natural cannabinoids will produce a biphasic activation and inhibition of that enzyme [19]. Further work is required to clarify the complex actions of

Table 1. Inhibition of cyclo-oxygenase activity by constituents of cannabis. Data was the mean of eight experiments

Compound	Maximum conc. tested mg/l	Maximum inhibition (%)	I.C.$_{50}$ mg/l (±SE)	μM
Indomethacin	10	88	5.1 (1.13)	14.2
Cannabis oil	10	100	7.5	—
Ethanolic extract	10	55	6.7	—
1.	50	85	27.5 (6.34)	152.8
2.	25	60	7.0 (1.19)	16.0
3.	20	25	—	—
4.	20	80	12.5 (2.57)	39.8
5.	20	65	10.0 (2.22)	31.8
6.	20	95	11.7 (4.36)	37.7

Table 2. Inhibition of soybean lipoxygenase activity by constituents of cannabis

Compound	Maximum conc. tested mg/ml	Maximum inhibition (%)	I.C.$_{50}$ (±SE) (μM)
1.	50	inactive	—
2.	50	40	—
3.	30	98	2.2 (0.6)
4.	30	96	2.9 (0.2)
5.	30	83	3.2 (0.5)
6.	30	79	9.2 (0.3)

Data was the mean of five experiments

these compounds on the enzymes of the prostaglandin pathway.

In this communication the actions of cannabis constituents on enzymes of arachidonate metabolism have been determined. The results confirm that the non-cannabinoid constituents are primarily cyclo-oxygenase inhibitors whilst the cannabinoids are dual inhibitors of both cyclo-oxygenase and lipoxygenase. Preliminary kinetic studies suggest that the cannabinoids share a common mode of action with other phenolic anti-oxidants.

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REFERENCES

1. E. W. Gill, W. D. M. Paton and R. G. Pertwee, *Nature, Lond.* **228**, 134 (1970).
2. M. L. Barrett, D. Gordon and F. J. Evans, *Biochem. Pharmac.* **34**, 2019 (1985).
3. J. W. Fairbairn and J. T. Pickens, *Br. J. Pharmac.* **72**, 401 (1981).
4. L. Vachon, M. X. Fitzgerald, N. H. Solliday, I. A. Gould and E. A. Gaenster, *New Engl. J. Med.* **288**, 9851 (1973).
5. H. Spronk, J. Luteijn and C. Salemink, *Biochem. Pharmac.* **27**, 607 (1978).
6. S. Burstein, E. Levin and C. Varanelli, *Biochem. Pharmac.* **22**, 2905 (1973).
7. W. L. Smith and W. E. M. Lands, *Biochemistry* **11**, 3276 (1972).
8. J. Magee in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer) p. 411, Academic Press, New York (1965).
9. S. Ferri, G. Costa, G. Muran, A. M. Panics, E. Speroni and R. Amigo Reina, *Psychopharmacology* **75**, 144 (1981).
10. E. A. Formukong, A. T. Evans, E. M.Williamson and F. J. Evans, *J. Pharm. Pharmac.* **38**, 18P (1986).
11. W. E. M. Lands and A. M. Handel, *Prostaglandins and Related Substances* (Eds. C. Pace Asciak and F. Granstrom) p. 203. Elsevier, Amsterdam (1983).
12. S. Burstein, Personal Communication (1986).
13. J. Bauman, F. V. Bruchhausen and G. Wurm, *Prostaglandins* **20**, 627 (1980).
14. F. E. Dewhirst, *Prostaglandins* **20**, 209 (1980).
15. L. Crombie, W. M. L. Crombie and S. V. Jamieson, *Tetrahedron Lett.* **21**, 3607 (1980).
16. U. Kikkawa, Y. Takai, Y. M. Tanaka, R. Miyake and Y. Nishizuka, *J. biol. Chem.* **258**, 11442 (1983).
17. R. J. Schmidt and F. J. Evans, *Archs. Toxicol.* **44**, 279 (1980).
18. H. E. White and R. L. Tansik, *Prostaglandins and Medicine* **4**. 409 (1980).
19. A. T. Evans, E. A. Formukong and F. J. Evans, *6th Int. Conf. Prostaglandins and Related Compounds,* Florence, 386p. Raven Press, New York (1986).

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Monoclonal antibodies specific for 1–4 benzodiazepines

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Benzodiazepines (BZD)* are an important class of pharmacologically active molecules [1] widely used as anxiolytics, hypnotics, anticonvulsants or muscle relaxants. They exert their pharmacological effects through interaction with γ aminobutyric acid receptors [2, 3] localised on neurons, probably acting at the synaptic level [4]. "Peripheral" binding sites for BZDs have also been demonstrated in kidney, heart, lung and on thrombocytes [5, 6] but their function is not known.

Minor modifications to the BZD structure can lead to dramatic changes in pharmacological properties: thus the anticonvulsant drug clonazepam (for BZD structural formula see Fig. 1) is specific for receptors of the neuronal type whereas RO5-4864, which is structurally closely related, is specific for the peripheral binding sites [7] and does not show BZD-like pharmacological activity. Flunitrazepam, a centrally active BZD, binds to both types of receptor [8].

One approach to the analysis of the structure/activity relationships of these drugs is to use immunological methods to develop antibodies that can be considered as models for receptors. In this respect monoclonal antibodies specific for clonazepam have already been described [9, 10].

The present report concerns the production and purification of murine monoclonal antibodies directed towards flunitrazepam. The binding specificity and the immunochemical characteristics of three of these are described.

* Abbreviations used: BSA, bovine serum albumine; BGG, bovine γ-globulins; PBS, phosphate buffer saline; FNZ, flunitrazepam; BZD, benzodiazepine; ABTS, 2-2' azino-di-(3 ethylbenzthiazoline sulfonic acid); mAb, monoclonal antibody; tBu, tertiobutyl.

Materials and methods

Chemicals. Bovine serum albumin V, bovine γ globulin Cohn fraction II and adipic acid dihydrazide agarose were obtained from the Sigma Chemical Co., 1-(3-dimethylaminopropyl-)-3-ethyl-carbodiimide hydrochloride from