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Comparative topical anti-inflammatory activity of cannabinoids and cannabivarins

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ABSTRACT

A selection of seven phytocannabinoids representative of the major structural types of classic cannabinoids and their corresponding cannabivarins was investigated for *in vivo* topical anti-inflammatory activity in the Croton oil mouse ear dermatitis assay. Differences in the terpenoid moiety were far more important for anti-inflammatory activity than those at the C-3 alkyl residue, suggesting the involvement not only of cannabinoid receptors, but also of other inflammatory end-points targeted by phytocannabinoids.

1. Introduction

Phytocannabinoids are meroterpenoids biogenetically derived from the prenylation of a 3-alkylresorcinyl derivative [1]. They are typical constituents of hemp (*Cannabis sativa* L., Cannabinaceae), and no other natural source is known, although cannabinoid-like moieties have been found in some prenylated aromatics [2]. In classic phytocannabinoids, the resorcinyl alkyl residue is a *n*-pentyl group, but these compounds are commonly accompanied by trace amounts of lower homologues with a shorter, *n*-propyl side chain, derived from the prenylation of a pentaketide (divarinic acid) rather than a hexaketide precursor (olivetolic acid) [1]. Because of a mutation in polyketide synthesis, some strains of hemp accumulate this type of C3-phytocannabinoids, known as cannabivarins, at the expense of classic C5-phytocannabinoids [1]. The alkyl residue is a critical element of the phytocannabinoid pharmacophore [3], and important differences between the biological profiles of cannabinoids and cannabivarins have, in fact, been reported. Thus, while Δ^9-tetraidrocannabinol (Δ^9-THC; **1a**) is the archetypal agonist of cannabinoid receptors CB_1 and CB_2, its lower homologue Δ^9-tetrahydrocannabivarin (Δ^9-THCV; **1b**) behaves as a "neutral" antagonist toward CB_1 and as an agonist for CB_2 [4,5]. These observations, and the growing interest for the medicinal properties of minor phytocannabinoids [3], provided a rationale for undertaking a comparative study on the biological profile of cannabinoids and cannabivarins.

Within the many facets of the phytocannabinoid preclinical potential, the topical anti-inflammatory activity is one of the best documented [6], but differences in end-points and experimental protocols make it difficult to assess the relative potency of the compounds investigated. The Croton oil mice ear assay [7] is an *in vivo* anti-inflammatory test, capable to discriminate between the profiles of various agents. It can

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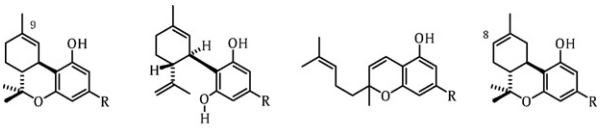
rank them in comparison with various non-steroidal anti-inflammatory drugs (NSAIDs) as well as corticosteroids, and was therefore selected for this study, comparing the anti-inflammatory activity of the three archetypal anti-inflammatory cannabinoids [Δ^9-THC (**1a**), CBD (**2a**) and CBC (**3a**)] with that of their corresponding cannabivarins.



	R
1a	n-C_5H_{11}
1b	n-C_3H_7
2a	n-C_5H_{11}
2b	n-C_3H_7
3a	n-C_5H_{11}
3b	n-C_3H_7
4a	n-C_5H_{11}
4b	n-C_3H_7

2. Experimental

2.1. General

Silica gel 60 (70–230 mesh) was used for gravity column chromatography. Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates and were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Croton oil and indomethacin were purchased from Sigma–Aldrich (Milan, Italy). Ketamine hydrochloride (Inoketam 100) was purchased from Virbac s.r.l. (Milan, Italy). The other chemicals of analytical grade were purchased from Carlo Erba (Milan, Italy). Male CD-1 mice weighing 28–32 g were supplied by Harlan Laboratories (San Pietro al Natisone, Italy).

2.2. Plant material

A single non-psychotropic cannabivarin plant was identified in a landrace from Sicily (Italy) during a large screening of germplasm. Selection and self pollination of this plant made it possible to develop inbred lines S1 with different contents of cannabivarins, estimated from approximately 5% to 53% of the content of the respective pentyl cannabinoids by GC analysis. Plants were cultivated in a greenhouse at CRA-CIN, Rovigo (Italy). The seedlings were kept under artificial light for 18 h photoperiod for the first 5 weeks, and flowering was next induced by reducing the photoperiod to 12 h of light until plants were harvested (9 weeks old). Plants were judged mature when the inflorescences showed the brown mature stigma, and were then harvested at the end of the flowering and seed formation stage. A voucher specimen is available from SC (ecobise@gmail.com).

2.3. Isolation of CBDV (**2b**) and CBCV (**3b**)

Dried plant material (inflorescences, 404 g) was powdered and then heated in a ventilated oven at 120 °C for 4 h to decarboxylate pre-cannabinoids to cannabinoids. After cooling to room temperature, the plant material was extracted with acetone (2×2 L) at room temperature, yielding 15 g of a dark-black oil, that was dissolved in methanol and filtered through a short column of RP-silica gel (ca. 50 g) to remove waxes and pigments. The resulting purified extract (70 g) was fractionated by gravity column chromatography on silica gel (200 g), using a petroleum ether-acetone gradient, from 98:2 to 90:10. Fractions 8–22 afforded 3.6 g (0.89%) CDB (**2a**) as a white powder, while fractions 24–37 gave a mixture of **2b** and **3b** (2.1 g). These compounds were further separated on neutral alumina (50 g), using a petroleum ether-EtOAc gradient (from 95:5 to 70:30). Fractions 6–14 yielded 1.74 g (0.43%) CBDV (**2b**), and fractions 35–54 160 mg (0.04%) CBCV (**3b**), identified by comparison of their spectroscopic data with those reported in the literature [8,9].

2.4. Synthesis of Δ^8-THCV (**4b**) from CBDV (**2b**)

A solution of CBDV (**2b**, 200 mg, 0.70 mmol) and *p*-toluensulfonic acid (13 mg, 0.075 mmol) was refluxed for 2 h. After cooling, the reaction was worked up by dilution with EtOAc and washing with sat. $NaHCO_3$. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated, affording a brownish oil. This was purified by gravity column chromatography on silica gel (7 g), using petroleum ether-EtOAc 9:1 as eluant, affording 192 mg (96%) Δ^8-THCV (**4b**) as an orange powder, identified by comparison with the spectroscopic data reported in the literature [4].

2.5. Anti-inflammatory activity

Topical inflammation was induced on the right ear (surface: about 1 cm^2) of anaesthetised mice (145 mg/kg ketamine hydrochloride, intraperitoneally) applying 80 µg of Croton oil dissolved in 15 µl acetone. The left ear remain untreated. Control animals received only the irritant solution, whereas other animals received both the irritant and the tested substances [7]. After 6 h, at the maximum oedema formation in control mice, animals were sacrificed and a punch (6 mm Ø) was taken from both the treated and the untreated ears to evaluate the oedematous response. Oedema was quantified by the difference in weight between the punches taken from the treated and the controlateral ears. The anti-inflammatory activity was expressed as percent inhibition of the oedematous response in animals treated with the test substances in comparison to that of control mice [7]. Ten animals were used for each group of treatment. All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC).

2.6. Statistical analysis

Pharmacological data were analyzed by one-way analysis of variance, followed by the Dunnett's test for multiple comparisons of unpaired data, and a probability level lower than 0.05 was considered as significant. The dose giving 50% inhibition of the oedematous response (ID_{50}) was calculated by linear regression analysis of the dose–response curves, using the Pharmacologic Calculation System software [10].

3. Results and discussion

Capitalizing on the availability of a variety of *Cannabis* rich in cannabivarins, CBDV (**2b**) [8] and CBCV (**3b**) [9] were

obtained by isolation. Starting from CBDV (**2b**), we next attempted to synthesize Δ^9-THCV (**1b**), but, despite considerable experimentation, only its more stable Δ^8-isomer (Δ^8-THCV, **4b**) [11] could be obtained, even under conditions that are reported to produce, in a regiospecific way, Δ^9-THC (**1a**) from CBD (**2a**) [12]. Δ^8-THC was therefore enclosed in the reference group of classic cannabinoids to act as a tetrahydrocannabinol reference. Δ^8-THC (**4a**) and Δ^9-THC (**1a**) show similar agonistic affinity for cannabinoid receptors (CBs) [3], as do, in antagonistic way, Δ^8-THCV (**4b**) and Δ^9-THCV (**1b**) for CB_1 [4], suggesting that the biological profile of the two regio-isomers is very similar.

The anti-inflammatory activity of cannabinoids has long been known, and has been shown to involve mechanism(s) that go beyond the activation of CB [5–13]. Thus, Δ^9-THC (**1a**), the major psychotropic constituent of marijuana and the archetypal CBs ligand, shows an impressive anti-inflammatory activity, outperforming aspirin and hydrocortisone in the carrageenan-induced paw oedema model in rats [14], and being also active topically in the tetradecanoyl phorbol-acetate (TPA)-induced mice ear erythema assay [15]. On the other hand, also non-psychotropic cannabinoids like cannabidiol (CBD, **2a**) [16] and cannabichromene (CBC, **3a**) [17,18] show outstanding potency in *in vivo* assays of inhibition of inflammatory responses, despite their negligible activity as activators of CBs [3]. The excellent activity of CBD against a series of molecular targets of relevance for inflammation, like vanilloid receptor TRPV1, cycloxigenase-2 and inducible nitric oxide synthase, and its increasing activity on adenosine signalling might underlie these observations [12,19,20], whose clinical potential was supported by the activity of CBD in a rodent model of osteo-arthrosis [21]. To assess the relative anti-inflammatory potency of cannabinoids and cannabivarins, their anti-oedematous activity was evaluated at the dose range of 0.1–1 μmol/cm^2, and compared to that of the non-steroidal anti-inflammatory drug (NSAID) indomethacin. The psychoactive compounds Δ^9-tetraidrocannabinol (Δ^9-THC, **1a**), Δ^8-tetraidrocannabinol (Δ^8-THC, **4a**) as well as the cannabinoid antagonist Δ^8-tetraidrocannabivarin (Δ^8-THCV, **4b**) exerted a significant dose-dependent oedema inhibition, which ranged from about 20% (0.1 μmol/cm^2) to about 70% (1 μmol/cm^2) (Fig. 1). The non-psychoactive cannabinoid cannabichromene

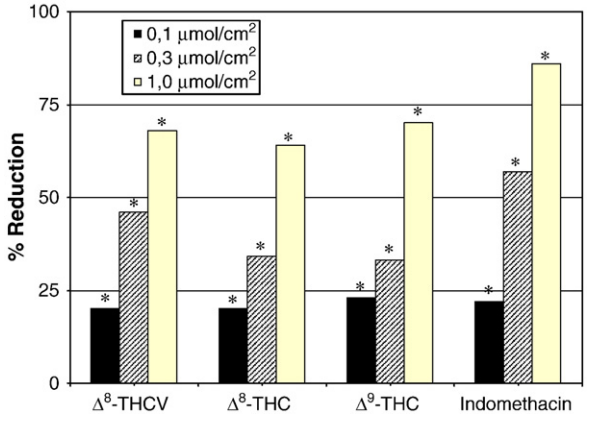


Fig. 1. Dose-dependent anti-inflammatory activity of Δ^8-THCV, Δ^8-THC, Δ^9-THC and indomethacin (*$p<0.05$ at the analysis of variance, as compared to controls).

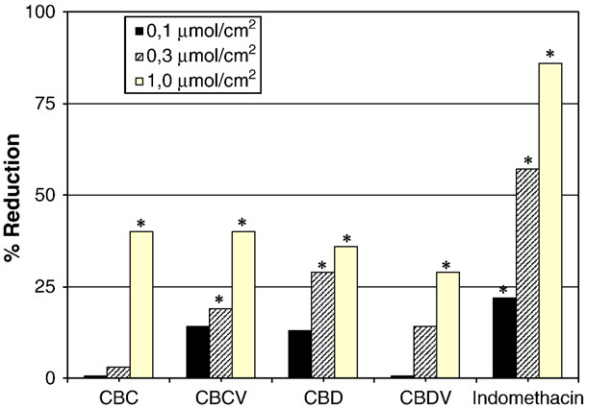


Fig. 2. Dose-dependent anti-inflammatory activity of CBC, CBCV, CBD, CBDV and indomethacin (*$p<0.05$ at the analysis of variance, as compared to controls).

(CBC, **3a**), cannabidiol (CBD, **2a**), and their lower homologues CBCV (**3b**) and CBDV (**2b**) showed a lower potency (Fig. 2), with oedema reduction at the highest tested dose (1 μmol/cm^2) being only 40% for CBC (**3a**) and CBCV (**3b**), 36% for CBD (**2a**) and 29% for CBDV (**2b**), respectively. The NSAID indomethacin could affect a significant oedema reduction, ranging from 22% to 86%, depending on the tested doses (0.1–1 μmol/cm^2) (Figs. 1, 2).

To better compare the anti-inflammatory potency of the tested compounds, the dose capable to inhibit the oedematous response by 50% (ID_{50}) was evaluated for each compound. Δ^9-THC (**1a**) and its analogues Δ^8-THC (**4a**) and Δ^8-THCV (**4b**) showed comparable ID_{50} values, in the range of 0.46–0.55 μmol/cm^2, and were only about two fold less potent than the reference drug indomethacin ($ID_{50} = 0.25$ μmol/cm^2). Conversely, the ID_{50} of the non-psychoactive cannabinoid CBD (**2a**) and CBC (**3a**) and of their lower homologues CBDV (**2b**) and CBCV (**3b**) was higher than 2 μmol/cm^2, much less active than indomethacin (Table 1).

These results suggest that the tricyclic tetrahydrocannabinol motif necessary for binding to cannabinoid receptors, either in an agonistic or an antagonistic way [4,5], is important also for the topical anti-inflammatory activity of phytocannabinoids, since the lack of this feature was detrimental for activity. The contribution of cannabinoid receptors to the outcome of the assay is, however, difficult to assess, owing not only to the complex receptor profile of tetrahydrocannabivarins, but also to the possible involvement of TRPA1, another target of

Table 1
Anti-inflammatory potency of cannabinoids (ID_{50} values).

Substance	ID_{50} (μmol/cm^2)
Δ^9-THC	0.46
Δ^8-THCV	0.41
Δ^8-THC	0.55
CBC	n.e.
CBCV	2.38
CBD	2.42
CBDV	n.e.
Indomethacin	0.25

n.e.: not evaluable by the Pharmacologic Calculation System software [9].

phytocannabinoids [19]. On the other hand, our data show conclusively that, at least for topical anti-inflammatory activity, differences in the terpenoid moiety of phytocannabinoids are far more important than those in the alkyl residue, with the highest potency being consistently associated to compounds having a tricyclic skeleton.

References

[1] ElSohly MA, Slade D. Life Sci 2005:539–48.
[2] Woelkartm K, Sao-Ahen OM, Bauer R. Curr Top Med Chem 2008;8: 173–86.
[3] Stern E, Lambert DM. Chem Biodiv 2007:1707–28.
[4] Pertwee RG, Thomas A, Stevenson LA, Ross RA, Varvel SA, Lichtman AH, Martin BR, Razdan RK. Br J Pharmacol 2007;150:586–94.
[5] Bolognini D, Costa B, Maione S, Comelli F, Marini P, Di Marzo V, Parolaro D, Ross RA, Gauson LA, Cascio MG, Pertwee R. Br J Pharmacol 2010, doi:10.1111/j.1476-5381.2010.00756.x.
[6] Burstein SH, Zurier RB. AAPS J 2009;11:109–19.
[7] Tubaro A, Dri P, Delbello G, Zilli C, Della Loggia R. Agents Actions 1985;17:347–9.
[8] Vollner L, Bieniek D, Korte F. Tetrahedron Lett 1969:145–7.
[9] Shoyama Y, Hirano H, Oda M, Somehara T, Nishioka I. Chem Pharm Bull 1975;23:1894–5.
[10] Tallarida RJ, Murray RB. Manual of pharmacologic calculation with computer program. New York: Springer Verag; 1986.
[11] Gill EW. J Chem Soc (C) 1971:579–82.
[12] Gaoni Y, Mechoulam R. Tetrahedron 1966;22:1481–8.
[13] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Trends Pharmacol Sci 2009:515–27.
[14] Sofia RD, Nalepa SD, Harakal JJ, Vassar HBJ. Pharmacol Exp Ther 1973;186:646–55.
[15] Formukong EA, Evans AT, Evans FJ. Inflammation 1988;12:361–71.
[16] Costa B, Colleoni M, Conti S, Parolaro D, Franke C, Trovato AE, Giagnoni G. Naunyn Schmiedeberg Arch Pharmacol 2004;369:294–9.
[17] Turner CE, Elsohly MA. J Clin Pharmacol 1981;21:283S–91S.
[18] Wirth PW, Watson ES, ElSohly M. Life Sci 1980;26:1991–5.
[19] De Petrocellis L, Vellani V, Schiano-Morello A, Marini P, Magherini PC, Orlando P, Di Marzo VJ. Pharmacol Exp Ther 2008;325:1007–15.
[20] Bisogno T, Hanuš L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V. Br J Pharmacol 2001;134:845–52.
[21] Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreakos E, Mechoulam R, Feldmann M. Proc Natl Acad Sci USA 2000;97:9561–6.