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Cannabinoids inhibit human keratinocyte proliferation through a non-CB1/CB2 mechanism and have a potential therapeutic value in the treatment of psoriasis

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Summary

Background: Cannabinoids from cannabis (*Cannabis sativa*) are anti-inflammatory and have inhibitory effects on the proliferation of a number of tumorigenic cell lines, some of which are mediated via cannabinoid receptors. Cannabinoid (CB) receptors are present in human skin and anandamide, an endogenous CB receptor ligand, inhibits epidermal keratinocyte differentiation. Psoriasis is an inflammatory disease also characterised in part by epidermal keratinocyte hyper-proliferation.
Objective: We investigated the plant cannabinoids Δ-9 tetrahydrocannabinol, cannabidiol, cannabinol and cannabigerol for their ability to inhibit the proliferation of a hyper-proliferating human keratinocyte cell line and for any involvement of cannabinoid receptors.
Methods: A keratinocyte proliferation assay was used to assess the effect of treatment with cannabinoids. Cell integrity and metabolic competence confirmed using lactate-dehydrogenase and adenosine tri-phosphate assays. To determine the involvement of the receptors, specific agonist and antagonist were used in conjunction with some phytocannabinoids. Western blot and RT-PCR analysis confirmed presence of CB1 and CB2 receptors.
Results: The cannabinoids tested all inhibited keratinocyte proliferation in a concentration-dependent manner. The selective CB2 receptor agonists JWH015 and BML190 elicited only partial inhibition, the non-selective CB agonist HU210 produced a concentration-dependent response, the activity of theses agonists were not blocked by either CB1/CB2 antagonists.

Abbreviations: CBG, cannabigerol; CBD, cannabidiol; THC, tetrahydrocannabinol; CB, cannabinoid

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Conclusion: The results indicate that while CB receptors may have a circumstantial role in keratinocyte proliferation, they do not contribute significantly to this process. Our results show that cannabinoids inhibit keratinocyte proliferation, and therefore support a potential role for cannabinoids in the treatment of psoriasis.

1. Introduction

Psoriasis, a common inflammatory skin condition notable for the manifestation of unsightly lesions ('scale') that develop within the epidermis, affects between 2 and 4% of the population. Morbidity is significant, and causes distress in many patients. The pathogenesis and aetiology of psoriasis are complex and our understanding incomplete, but in brief, psoriasis can be characterised by epidermal keratinocyte hyper-proliferation accompanied by the infiltration and increased expression of pro-inflammatory mediators into the skin. The most significant mediators involved are those that are associated with a dominant Th1 cytokine profile. For further information, see reviews, e.g. Bowcock and Krueger [1], Krueger and Bowcock [2]. There are several treatments currently available for psoriasis, however most have unacceptable side effects and are considered inadequate, and so research continues into new therapeutic strategies.

Cannabinoids, the active constituents of the plant *Cannabis sativa* (sometimes known as 'phytocannabinoids', to distinguish them from endocannabinoids), and their derivatives are known to have anti-inflammatory properties [3] and are reported to have an inhibitory effect on rapidly proliferating tumorigenic cell lines [4]. It is widely accepted that these compounds elicit their activity via the G-protein coupled receptors CB1 and CB2, both of which are widely distributed throughout the body. Cannabis and cannabinoids have known anti-inflammatory activity in other autoimmune diseases that have similar characteristics to psoriasis, such as rheumatoid arthritis and Crohn's disease, and have been shown to alter immune function by influencing cytokine expression [5]. Importantly, they have been shown to transform the predominantly pro-inflammatory Th1 type expression to the more anti-inflammatory Th2 type profile [6]. Other anti-inflammatory and immunological effects of cannabinoids have been observed in a number of *in vivo* and *in vitro* models, and provide evidence that could support a potential application for psoriasis. Recent studies have shown CB receptors to be present in human skin [7] and that anandamide, an endogenous CB receptor ligand, inhibits epidermal keratinocyte differentiation [8]. Despite all this, little research, if any, has been undertaken concerning the therapeutic potential of cannabinoid-based preparations in the treatment of psoriasis. This is probably due, at least in part, to the psychoactive properties of cannabis, which could be considered undesirable side effects in the treatment of such a disease. However, not all cannabinoids elicit psychoactive effects but can still exhibit strong anti-inflammatory activity [9]. In addition, cannabinoids are lipophilic and are therefore readily absorbed through the skin. This is traditionally the preferred route of delivery for treating psoriasis, because of its localised expression: it also avoids most of the adverse side effects associated with systemic therapy. These considerations have led to the hypothesis that cannabinoids may have a potential therapeutic application for the treatment of psoriasis. Here we initially report the differential effects of a number of phytocannabinoids on a hyper-proliferating human keratinocyte cell line.

2. Materials and methods

The phytocannabinoids THC, CBD, CBN and CBG were isolated from a hexane extract of *C. sativa*, grown domestically under controlled hydroponics conditions. Structures were verified by analysis of NMR spectroscopy. Synthetic CB receptor ligands, HU210, AM251, AM630, JWH015 and BML190 were all purchased from Tocris (UK).

Keratinocyte proliferation was assessed using a sulforhodamine B (SRB) assay developed and described by Skehan et al. [10]. A limited number of normal keratinocytes were initially tested in parallel to transformed HPV-16 human keratinocytes. Observations for both cell lines were comparable in the presence of the phytocannabinoids but were only viable for up to passage three; for this reason the principal cells selected for use in these experiments were HPV-16 E6/E7 transformed human skin keratinocytes (ATCC; CRL-2309 KERTr, Promochem, UK). Cells were cultured in EpiLife™ medium and keratinocyte growth supplements (Cascade Biologicals). Dulbecco's phosphate buffered saline (PBS), Versene (EDTA Solution) and trypan blue were purchased from Gibco (UK) Ltd. Trypsin and trypsin blocker were supplied by TCS Cell Works, UK.

Normal aseptic cell culture techniques, with incubation at 37 °C and 5% CO_2, were utilized and upon reaching 60—80% confluence in T75 culture flasks, keratinocytes were detached using trypsin and counted using trypan blue exclusion. Cell density was adjusted to 5×10^{-1} ml^{-1} in fresh media and 10,000 cells (200 μl) were seeded into 96 well plates (Costar®, Corning) and incubated for a further 24 h to allow the cells to adhere to the bottom of the wells. After 24 h, the medium was removed and substituted with test compounds that had previously been dissolved in dimethyl sulfoxide (DMSO) and diluted 100-fold into EpiLife™ growth medium. In all cases, both an appropriate DMSO control and a media control were used. After a further 72 h incubation, cells were fixed and stained using SRB, and cell density determined colormetrically. Cell integrity and metabolic competence, to ensure that the observed effect was not due to simple cytotoxicity, were confirmed by using lactate-dehydrogenase [11] and adenosine tri-phosphate assays [12]. Analysis was performed using Graph Pad Prism, and by using classical sigmoidal concentration response curves, the inhibitory concentration (IC50) values were calculated. Each experiment was conducted in duplicate with six in-plate replicates.

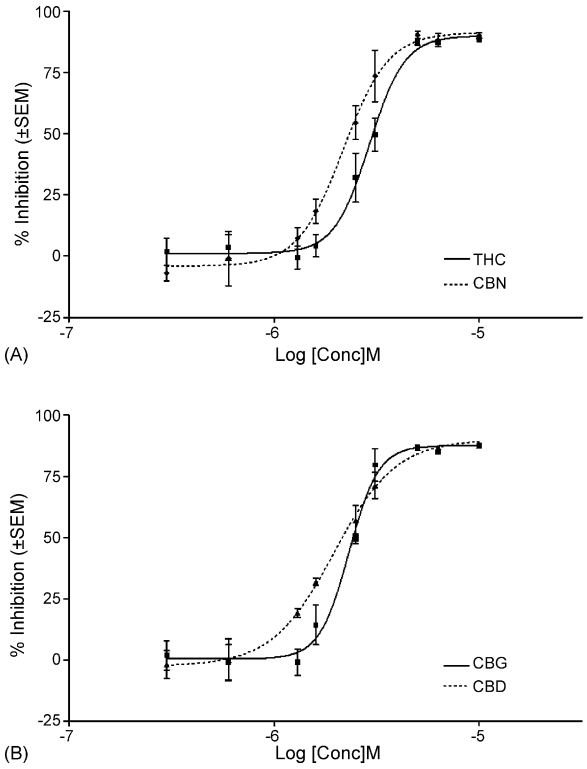

Fig. 1 Keratinocyte proliferation results after 72 h exposure to the isolated phytocannabinoids ($n = 4$). (A) Δ9-Tetrahydrocannabinol (THC) (IC50 = 2.9 μM) and cannabinol (CBN) (IC50 = 2.1 μM). (B) Cannabidiol (CBD) (IC50 = 2 μM) and cannabigerol (CBG) (IC50 = 2.3 μM).

3. Results and discussion

The isolated phytocannabinoids tested, Δ-9 THC, CBN, CBD and CBG, all inhibited keratinocyte proliferation in a concentration-dependant manner (Fig. 1), with average IC50 values of 2.3 μM; the highest IC50 being for Δ-9 THC at 2.7 μM and the lowest CBD at 2 μM. Maximum inhibition of proliferation by all cannabinoids was achieved at between 3 and 5 μM except in the case of CBG, which occurred within the 2.5—3 μM range. These compounds showed anti-proliferative potencies of the same order in this cell line, with CBG and CBD eliciting the greatest overall activity; CBD having the lowest maximum inhibitory concentration, and CBD having the lowest IC50 value. Metabolic competence and cell integrity of all compounds tested, at concentrations of 100 and 200 μM, was confirmed using the ATP and LDH assays, and showed that these observations were not the result of simple cytotoxicity (data not shown). Our results suggest that superficially all the cannabinoids tested have a similar effect on cell proliferation. However it is known that neither CBD nor CBG are significantly active at CB receptors, possessing much weaker binding affinities than THC or CBN. This indicates more than one mechanism of action, none of which involve CB receptors to any great extent: a premise also supported by the steep gradient of the dose—response curves which is a characteristic of a non-specific effect. There is a precedent for this phenomenon in that both THC and CBD have been shown to exhibit significantly similar pharmacological affects in the same model, but while THC did so by a CB1 dependant mechanism and was blocked by the addition of the CB antagonist SR141716, it was shown that CBD activity was independent of CB1 blockade [13]. There is also more recent evidence that CDB exhibits activity in a tumor cell migration model mediated through a CB receptor independent mechanism [14].

Although our results suggest a CB receptor-independent mechanism, in order to confirm this, and ascertain directly whether CB receptors are involved in keratinocyte proliferation, it was first established by Western blot and RT-PCR analysis that the receptors were indeed expressed by the cell line. This HPV-16 keratinocyte cell line is not as well characterised as normal keratinocytes (NEKs), so the presence of CB receptors were also confirmed in normal keratinocytes for comparison (Fig. 2(A)). Furthermore, the compounds JWH015 and BML190,

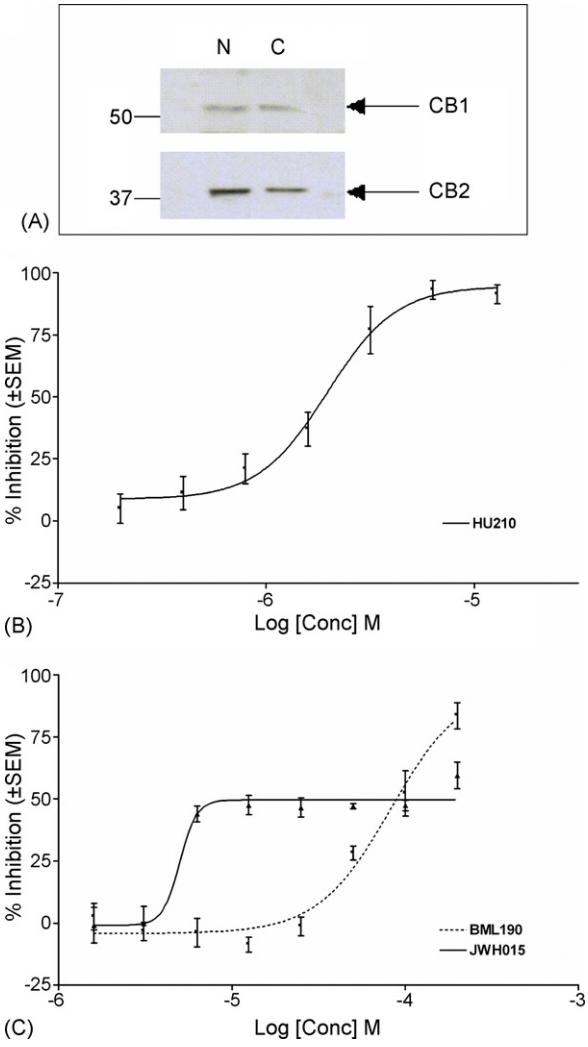

Fig. 2 (A) Western blot showing both CB1 and CB2 at 52 and 40 kDa, respectively, in both normal human epithelial keratinocytes (N) and immortalised HPV-16 keratinocytes (C). CB1 and CB2 antibodies supplied by Cayman Chemicals. (B) Keratinocyte proliferation results after 72 h exposure to CB agonist HU210 (IC50 = 1.7 μM) (n = 3). (C) Keratinocyte proliferation results after 72 h exposure to CB2 agonists BML-190 and JWH015 (n = 4).

which are selective CB2 receptor agonists, and the potent CB agonist HU210, were tested in the proliferation assay (Fig. 2). JWH015 demonstrated only partial agonist activity, not achieving more than 50% inhibition even at the highest concentrations tested (200 μM), a similar result to that produced at only 6.3 μM. BML190 demonstrated an inhibitory affect only at concentrations greater than 50 μM. The weak activity of JWH015 and BML190 compared to the more significant activity of the phytocannabinoids (which are much less potent and less selective agonists) indicates that while CB2 receptors may well have a circumstantial or mediatory role in keratinocyte proliferation, they do not contribute significantly to this process. The CB agonist HU210 demonstrated a concentration dependant response with an IC50 value of 1.7 μM (Fig. 2(B)) indicating a possible role of CB1 receptor or a combined effect of both CB1 and CB2. Further confirmation was investigated using the CB1 and CB2 antagonists, AM251 and AM630, respectively. In the unlikely event of any involvement of the vanilloid receptor (TRPV1), iodoresiniferatoxin (I-RTX, a TRPV1 antagonist) was also included in these experiments. However, I-RTX also had no significant effect on the inhibition of proliferation induced by the phytocannabinoids, thus eliminating any contribution from TRPV1 activation. Both CB1 and CB2 receptor antagonists tested also failed to inhibit the effects of either the phytocannabinoids (effect on THC and CBD shown in Fig. 3(A)) or the synthetic agonists BML190, HU210 or JWH015 (data not shown). Finally, it was observed that although not significantly so, the addition of a CB receptor antagonist seemed to potentiate the inhibitory effect of the agonists, shifting the response curves to the left. This observation was more notable at higher concentrations. Further investigations showed that the antagonist themselves demonstrated an ability to inhibit proliferation in a concentration dependant manner (Fig. 3(B)).

These results indicate that the activity of cannabinoids on keratinocyte proliferation cannot be explained by the activation of CB1 or CB2 receptors alone, and that the predominant mechanism is not via the classical CB receptor pathway. Since this work was undertaken it has been reported that the G-protein coupled receptor identified as GPR55, can also act as a receptor for some cannabinoids [15]. Data on this novel receptor is limited and controversial, and its involvement and expression in the epidermis has not yet been described.

In speculating further about a putative mechanism of action, it is possible that peroxisome proliferator-activated receptor-gamma (PPAR-γ), a receptor for thiazolidinedione ligands, may be involved. The PPAR-γ receptor has recently been shown to be yet another CB receptor [16], and agonists and antagonists have been identified as potential therapeutic targets for various epidermal disorders including psoriasis [17]. Moreover PPAR-γ agonists (for example the thiazolidinediones) have been shown by several authors to inhibit the proliferation of epithelial cells (for example see Bhagavathula et al. [18]), as well as normalising the histological appearance of human psoriatic skin in organ culture [19]. This remains to be investigated, as the wider roles of PPAR-γ are revealed, although clearly, the data presented here may be a consequence of cannabinoid/PPAR-γ interactions result-

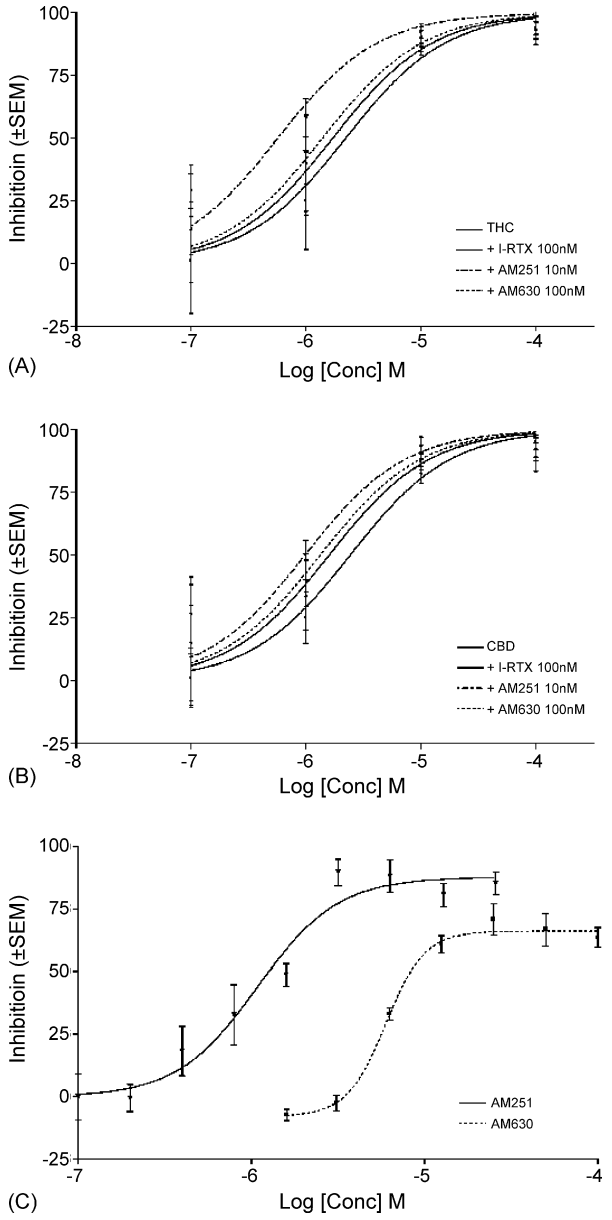

Fig. 3 (A) Result showing the effect of receptor antagonists on the anti-proliferative activity of both THC and CBD. I-RTX was included to rule out the TRVP1 receptor as a possible mediator of this activity. Analysis by two-way ANOVA and Bonferroni post-tests show that there is no significant difference between the control and the inclusion of the antagonists. (B) Cannabinoid receptor antagonists, AM251 (CB1) and AM630 (CB2), independently inhibit keratinocyte proliferation in a concentration dependant manner ($n = 6$).

ing in the inhibition of cell proliferation. Many inhibitors of keratinocyte proliferation, such as interferon-gamma, tetradecanoyl phorbol acetate and Vitamin D3, are inducers of keratinocyte differentiation and it would be useful to investigate further the mechanism of action of cannabinoids by looking at the expression of differentiation markers such as transglutaminase, in the treated HPV transformed keratinocyte cell line. Our results show clearly that cannabinoids inhibit the proliferation of keratinocytes, thus demonstrating a therapeutic potential for the treatment of psoriasis, but further investigation is urgently needed to identify the mechanism by which they act in order to realise this potential.

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