Biochemical Pharmacology 79 (2010) 1815–1826

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Falcarinol is a covalent cannabinoid CB_1 receptor antagonist and induces pro-allergic effects in skin

Marco Leonti [a], Laura Casu [a], Stefan Raduner [b], Filippo Cottiglia [a], Costantino Floris [c], Karl-Heinz Altmann [b], Jürg Gertsch [b,*]

[a] Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari, Facoltà di Farmacia, Via Ospedale 72, 09124 Cagliari, Italy
[b] Institute of Pharmaceutical Sciences, ETH Zürich, Wolfgang-Pauli-Strasse 10, CH-8057 Zurich, Switzerland
[c] Dipartimento di Scienze Chimiche, Università di Cagliari, Complesso di Monserrato, 09042 Monserrato, Italy

ARTICLE INFO

Article history:
Received 7 December 2009
Accepted 24 February 2010

Keywords:
Falcarinol
Skin irritation
CB1 cannabinoid receptor
Anandamide
Endocannabinoid system

ABSTRACT

The skin irritant polyyne falcarinol (panaxynol, carotatoxin) is found in carrots, parsley, celery, and in the medicinal plant *Panax ginseng*. In our ongoing search for new cannabinoid (CB) receptor ligands we have isolated falcarinol from the endemic Sardinian plant *Seseli praecox*.

We show that falcarinol exhibits binding affinity to both human CB receptors but selectively alkylates the anandamide binding site in the CB_1 receptor (K_i = 594 nM), acting as covalent inverse agonist in CB_1 receptor-transfected CHO cells. Given the inherent instability of purified falcarinol we repeatedly isolated this compound for biological characterization and one new polyyne was characterized. In human HaCaT keratinocytes falcarinol increased the expression of the pro-allergic chemokines IL-8 and CCL2/MCP-1 in a CB_1 receptor-dependent manner. Moreover, falcarinol inhibited the effects of anandamide on TNF-alpha stimulated keratinocytes. *In vivo*, falcarinol strongly aggravated histamine-induced oedema reactions in skin prick tests. Both effects were also obtained with the CB_1 receptor inverse agonist rimonabant, thus indicating the potential role of the CB_1 receptor in skin immunopharmacology. Our data suggest anti-allergic effects of anandamide and that falcarinol-associated dermatitis is due to antagonism of the CB_1 receptor in keratinocytes, leading to increased chemokine expression and aggravation of histamine action.

1. Introduction

The endocannabinoid system (ECS) comprises the G-protein coupled cannabinoid receptors (CB_1 and CB_2) and potentially also the orphan receptor GPR55, several endogenous arachidonic acid-derived ligands, their regulatory transport systems, and their hydrolytic enzymes (for reviews see [1–3]). Although the CB_1 receptor is the predominant cannabinoid receptor expressed in the brain it is also present in numerous peripheral tissues, often in concert with the peripheral CB_2 receptor. The major endocannabinoids arachidonoyl ethanolamide (anandamide) (Fig. 1) and 2-arachidonoyl glycerol (2-AG) (Fig. 1) non-selectively and to different degrees activate both CB receptors at nM concentrations. In skin, the activation of the ECS mediates a rather complex physiology, which remains to be investigated (for a recent review see [4]). While the CB_2 receptor has been shown to potentially transduce pro-inflammatory effects via 2-AG [5], an overall increased endocannabinoid tone appears to be beneficial [6]. Studying CB knockout mice, Karsak et al. have observed that the ECS and the CB_1 receptor can inhibit the pathogenesis of allergic contact dermatitis [6]. Interestingly, in their study it was shown that selective activation of the CB_2 receptor in skin worsened the allergic reaction, thus confirming previous data obtained by Oka et al. [7], while blockage of the CB_2 receptor by the inverse agonist SR144528 paradoxically led to a similar result [6]. By contrast, CB_1 agonists such as Δ^9-tetrahydrocannabinol (THC) and inhibition of anandamide degradation are considered to be promising therapeutic strategies to treat different forms of dermatitis [4,6]. Overall, anandamide acting via both CB_1 and CB_2 receptors appears to mediate skin protective effects. While an immunological role of the ECS in skin has already been shown, and both CB_1 and CB_2 receptors and endocannabinoids have been detected in keratinocytes and fibroblasts in the epidermis [8,9], the pleiotropic endocannabinoid signals involved in skin inflammation are complex and remain poorly understood. In this study we have identified the fatty acid-derived natural product falcarinol which possesses a reactive polyyne structure, as new functional CB receptor ligand. Interestingly, falcarinol has previously been reported to exert pharmacological effects,

* Corresponding author at: Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland.
E-mail address: gertsch@mci.unibe.ch (J. Gertsch).

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doi:10.1016/j.bcp.2010.02.015

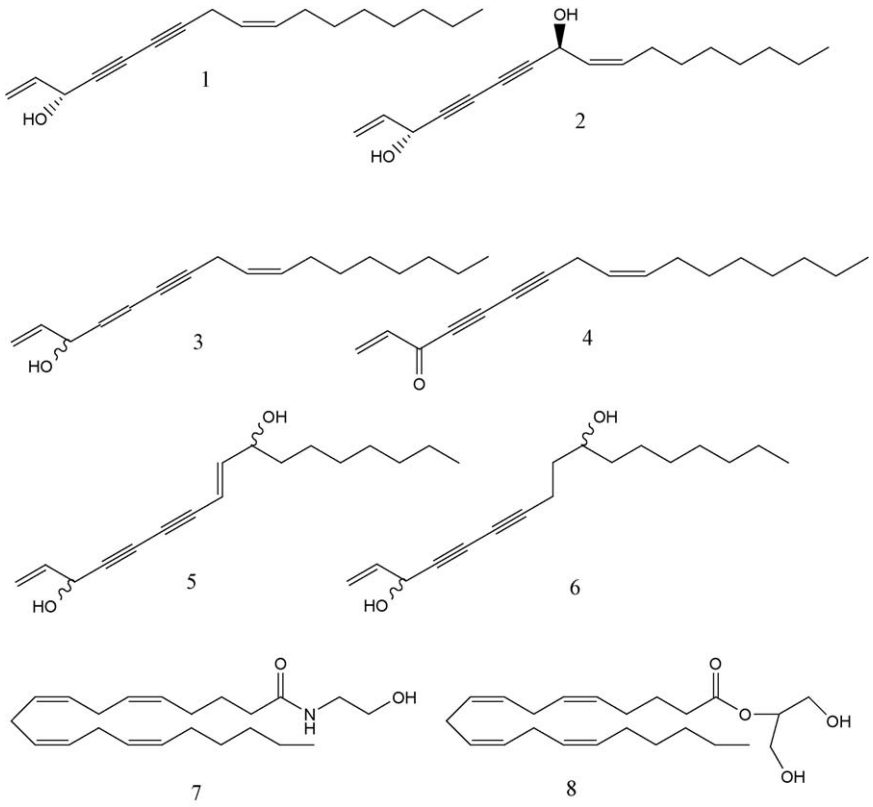

Fig. 1. Structures of natural polyynes and decomposition products (**1**–**6**), and endocannabinoids (**7**;**8**). (*R*)-Falcarinol ((*Z*)-heptadeca-1,9-diene-4,6-diyn-3-ol) (**1**), 3(*R*),8(*S*)-falcarindiol ((*Z*)-heptadeca-1,9-diene-4,6-diyn-3,8-diol) (**2**); (4*E*,9*Z*)-1,4,9-heptadecatriene-6-yn-3-ol or 4,5-dihydrofalcarinol (4,5-dihydropanaxynol) or ginsenoyne J (**3**); falcarinone ((*Z*)-heptadeca-1,9-diene-4,6-diyn-3-one) (**4**); *E*-heptadeca-1,8-diene-4,6-diyne-3,10-diol (**5**); dihydroseselidiol (heptadeca-1-ene-4,6-diyne-3,10-diol) (**6**); *N*-arachidonoyl ethanolamine (anandamide) (**7**); 2-arachidonoyl glycerol (2-AG) (**8**).

including skin irritation (dermatitis) and potential anti-carcinogenic effects in colon [10–13]. However, to date no protein targets for falcarinol have been identified. Here we show that falcarinol non-selectively binds to both CB receptors, but selectively alkylates the CB_1 receptor and induces functional signals at the CB_1 receptor. In keratinocytes these signals may lead to up-regulation of pro-inflammatory chemokines. Our data further suggest that this interaction is similar to the molecular interaction of the CB_1 receptor-selective inverse agonist rimonabant (SR141716) which is known to potentially mediate pro-inflammatory effects in skin [6]. To our knowledge, this is the first report on skin inflammation induced by externally applied CB_1 receptor antagonists (inverse agonists).

2. Materials and methods

2.1. Plant material

Stems of the endemic *Seseli praecox* (Gamisans) Gamisans (Apiaceae), a chamaephyte species growing on calcareous substrate were collected near Baunei (Ogliastra, Sardinia) in October 2006.

2.2. Chemicals

[^{3}H]-8-OH-DPAT (1 mCi/ml, 106–170 Ci/mmol), [^{3}H]-GR65630 (1 mCi/ml, 77.2 Ci/mmol), [^{3}H]-CP55,940 (1 mCi/ml, 126 Ci/mmol), [^{3}H]-RTX (1 mCi/ml, 43 Ci/mmol), [^{3}H]FMLP, and their respective ^{3}H unlabelled analogs, as well as the Ultima Gold scintillation cocktail were obtained from PerkinElmer, Switzerland. [^{3}H]Anandamide (223 Ci/mmol) was purchased from NEN Life Science Products. Rimonabant (SR141716) and SR144528 were obtained as a kind gift from Sanofi Synthelabo, France. Anandamide and WIN55,212-2 were purchased from Tocris, UK. Histamine hydrochloride was obtained from Sigma Aldrich, Switzerland. Silica gel (40–63 μm) and TLC plates with silica gel 60 and fluorescence indicator 254 nm was obtained from Merck, Solvents were obtained from VWR International, Germany. All other chemicals were from Fluka Chemie AG, Switzerland.

2.3. Receptor screen

Human CB_1, CB_2, 5-HT_{1A}, 5-HT_3, FPRL-1 overexpressing HEK-293 membranes were obtained from PerkinElmer, Switzerland. CHO-K1 cells stably expressing human TRPV1 were a gift from Dr. Zoltan Sandor, University of Debrecen, Hungary. Membrane preparations (between 7 and 20 μg) were resuspended in 0.2 ml (final volume) of binding buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mg/ml fatty acid free BSA, pH 7.4). Receptor concentrations (B_{max}) were 1.7–3.2 pmol/mg of protein. The nonspecific binding of the radioligand was determined in the presence of excess ligands. Radioligand saturated membrane preparations were incubated with 10 μM of falcarinol. After 90 min of incubation, the suspension was rapidly filtered through 0.05% polyethyleneimine presoaked GF/C glass fiber filters on a 96-well cell harvester and washed nine times with 0.5 ml of ice-cold washing buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM MgCl2, 2% BSA, pH 7.4). Radioactivity on filters was measured with a Beckman LS 6500. A displacement of more than 50% of the radioligands by 10 μM of competitor was considered to be significant. Using this setup, the nonlabeled analogs of the respective radioligands displaced the radioligands by more than 70%.

2.4. Extraction protocol, isolation process, spectroscopy and spectrometry

1 kg of dried and milled *S. praecox* stems was percolated extensively with dichloromethane (DCM) to afford 40 g of crude extract. An aliquot of 10 g of crude DCM extract was fractionated by vacuum liquid chromatography (VLC) using 115 g silica gel (0.040–0.063 mm) stationary phase and approximately 50 mL toluene/ethyl-acetate (EtOAc) for each fraction (9.3:0.7 for fractions 1–19 and 8:2 for fractions 20–28). After comparison of the 28 fractions by thin layer chromatography (TLC) they were, according to visual similarities, united resulting in a set of 12 distinct fractions. VLC fraction 2 (1.4 g of a yellow viscous liquid) was chromatographed on an open column (CC) loaded with 106 g of silica gel and a mixture of *n*-hexane/EtOAc (8.5:1.5) resulting in 11 subfractions. Subfaction 9, which corresponded to pure compound **1** (Fig. 1) was dispached for screening. However, during cargo compound **1** suffered decomposition and therefore upon arrival was repurified by high-pressure liquid chromatography (HPLC) (Waters Symmetry® C18 column (19 × 100 mm)) with a UV diode array detector (206 nm). Water and acetonitrile (ACN) was used as mobile phase with the following gradient elution program: starting with 50% ACN a linear gradient was used up to 98% ACN over 10 min, which was held for another 10 min resulting in compound **1** (t_R = 9.7 min). Altogether, compound **1** accounted for 12.5–15% of total crude extract (5–6 g).

VLC fraction 8 (320 mg) was chromatographed on an open column with 40 g silica gel and eluted with *n*-hexane/EtOAc (7:3) resulting in 12 subfractions. Compound **2** (Fig. 1) was obtained from subfraction 8, which was purified on HPLC using *n*-hexane/EtOAc (8:2, flow of 2.5 mL/min, t_R = 29 min, 23 mg).

The re-examination of purified and freezer stored fraction 2.9 (compound **1**) by TLC revealed the formation of a decomposition product. We chromatographed an aliquot of 40 mg of this mixture over 5 g silica gel on CC with 100% DCM as mobile phase. The more polar fraction contained the newly formed product together with traces of falcarinol. Normal phase HPLC with *n*-hexane/EtOAc (8.5:1.5, flow 2.5 mL/min) led to the isolation (t_R = 16.3 min, 2.5 mg) of the newly formed compound **3** (Fig. 1).

Photooxidation of freezer-stored falcarinol was induced by the exposure to sunlight for 24 h in DCM. Subsequently the DCM insoluble products were separated by normal phase SPE cartridge. The resulting DCM soluble fraction was purified by normal phase HPLC and *n*-hexane/EtOAc (8.5:1.5, flow 2.5 ml/min, 3 ml/min from min 26 onwards) resulting in compound **4** (Fig. 1, t_R = 14.4 min) and compound **5** (Fig. 1, t_R = 35 min).

VLC fraction 9 (200 mg) was chromatographed with 20 g silica gel on an open column with a mixture of DCM/EtOAc (9.3:0.7) as mobile phase. The most polar fraction (14) was further fractionated on normal phase HPLC with a mixture of DCM/EtOAc (9.5:0.5, flow 3 ml/min) affording compound **6** (Fig. 1, t_R = 34 min, 4 mg).

NMR spectra were recorded on a Bruker Avance 300 MHz and on a Varian Unity INOVA 400 MHz spectrometer. Low-resolution mass spectrometric experiments were carried out on a Saturn 2000 ion trap coupled with a Varian 3800 gas chromatograph (Varian, Walnut Creek CA). Optical rotation was measured on a Perkin-Elmer 241 polarimeter. HPLC was conducted by means of a Hewlett-Packard 1050 instrument. The column was 250 × 10 mm Spherisorb silica, particle size 5 μm (Waters), and the UV detection wavelength was 254 nm.

2.5. FACS analysis of CB receptor expression

HaCaT and primary keratinocytes (kindly obtained from Prof. Dr. Michael Detmar, ETH Zurich, Switzerland) or positive control CB_1/CB_2-transfected CHO-K1 cells (10^6) were washed in PBS (Invitrogen) supplemented with 0.1% NaN_3 and 2% FBS and incubated (1:100) with the rabbit polyclonal CB_2-specific antibody (Ab3561) or (1:200) with rabbit polyclonal CB_1-specific antibody (Ab3558) for 45 min on ice in the dark. After two washing steps, the cells were incubated (1:32) with a monoclonal anti-rabbit fluorescein isothiocyanate labeled antibody for 45 min on ice in the dark. The cells were washed twice and resuspended in 500 μl of PBS with 0.1% NaN_3 and 1% *p*-formaldehyde before analysis on a FACScan cytometer (BD Biosciences). Measurements were carried out with the CellQuest software, and relative expressions were compared with secondary antibody controls.

2.6. Radioligand displacement assays on CB_1 and CB_2 receptors

For the CB_1 receptor, binding experiments were performed in the presence of 0.39 nM radioligand [^{3}H]CP-55,940 or 0.24 nM [^{3}H]anandamide at 30 °C in siliconized glass vials together with 7.16 μg of membrane recombinantly overexpressing CB_1 (RBHCB1 M; PerkinElmer Life Sciences), which was resuspended in 0.2 ml (final volume) of binding buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mg/ml fatty acid free BSA, pH 7.4). CB_1 receptor concentration (B_{max}) was 2.5 pmol/mg of protein. Test compounds were present at varying concentrations, and the nonspecific binding of the radioligand was determined in the presence of 10 μM CP-55,940. After 90 min of incubation, the suspension was rapidly filtered through 0.05% polyethyleneimine presoaked GF/C glass fiber filters on a 96-well cell harvester and washed nine times with 0.5 ml of ice-cold washing buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 2% BSA, pH 7.4). Radioactivity on filters was measured with a Beckman LS 6500 scintillation counter in 3 ml of Ultima Gold scintillation liquid. Data collected from three independent experiments performed in triplicate were normalized between 100 and 0% specific binding for [^{3}H]CP-55,940 and [^{3}H]anandamide, respectively. These data were fitted in a sigmoidal curve and graphically linearized by projecting Hill plots, which for both cases allowed the calculation of IC_{50} values. Derived from the dissociation constant (K_D) of [^{3}H]CP-55,940 (0.18 nM for CB_1 and 0.39 nM for CB_2) and [^{3}H]anandamide (89 nM for CB_1 and 192 nM for CB_2) (*vide infra*) and the concentration dependent displacement (IC_{50} value), inhibition constants (K_i) of competitor compounds were calculated by using the Cheng-Prusoff equation as reported previously [14]. For CB_2 receptor binding studies, 3.8 μg of membrane recombinantly overexpressing CB_2 (RBXCB2 M; PerkinElmer Life Sciences) was resuspended in 0.6 ml of binding buffer (*vide supra*) together with 0.11 nM the radioligand [^{3}H]CP-55,940. The CB_2 receptor radioligand binding assay was conducted in the same manner as for CB_1. CB_2 receptor concentration (B_{max}) was 4.7 pmol/mg protein. One mg/ml of fatty acid free bovine serum albumin (BSA) was used to reduce nonspecific binding. B_{max} and K_D values of [^{3}H]CP-55,940 and [^{3}H]anandamide and were determined by PerkinElmer, Life and Analytical Sciences, Boston, USA and were in the range of values reported previously [14].

2.7. cAMP measurements

Human CB_1 and CB_2 receptor expressing CHO-K1 cells were stably-transfected cell lines obtained as a gift from Euroscreen S.A., Belgium. The cells were checked for receptor expression by immunostaining using adequate antibodies (ab3558 and ab3561, both from Abcam) in both Western blots and FACS. Cells were cultured in the presence of 500 μg/ml G418 (Gibco) in RPMI medium 1640 supplemented with 10% FCS and plated in 96-well plates at a density of 3×10^5 cells per ml and incubated over night. The antibodies were highly selective towards *h*CB_1 and *h*CB_2, respectively, but showed some non-specific binding to cells

(10–35%). After aspirating the media, the cells were chilled for 10 min at room temperature in RPMI medium 1640 (w/o supplements) containing 500 μM 3-isobutyl-1-methylxanthine. Cells were then treated with different concentrations of test-compounds and incubated for 30 min at 37 °C in a total volume of 100 μl. After another 30 min of incubation at 37 °C with 20 μM forskolin, intracellular cAMP levels were detected by HitHunter for adherent cells EFC chemiluminescent detection assay (Amersham; catalog no. 90000302) according to the manufacturer's instructions and measured on a Microlumat *Plus* Microplate Luminometer LB 96 V (EG&G Berthold). The high-affinity CB receptor ligand WIN55,212–2 was used as positive control.

2.8. Measurement of $[Ca^{2+}]_i$

HL60 CB_2-positive cells (from ATCC; CCL-240) were grown as published previously (14) washed once, and cells (10^7 cells/ml) were incubated at 37 °C for 20 min in Hanks' balanced salt solution containing fluo3/AM in a final concentration of 4 μM and 0.15 mg/ml Pluronic F-127. The cells were then diluted 1:5 in Hanks' balanced salt solution containing 1% fetal bovine serum and incubated for 40 min at 37 °C. Afterwards, the cells were washed three times and resuspended in 500 μl of Ca^{2+}-free HEPES-buffered saline, containing 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM glucose, 0.5 mM $MgCl_2$, 0.1 mM EGTA, 1 g/liter bovine serum albumin, 10 mM HEPES, pH 7.4. Prior to each measurement, the cells were incubated for 7 min in a 37 °C water bath. In some experiments the cells were pretreated for 4 min with SR144528 (1 μM). The cells were subsequently stimulated with drugs (falcarinol, falcarindiol, SR144528) and vehicle controls and analyzed with the FL1 channel on a FACScan flow cytometer equipped with a 488 nm argon laser (BD Biosciences). Because the solvent (ethanol) showed an effect on $[Ca^{2+}]_i$ in vehicle controls, this solvent effect was subtracted from each value.

2.9. HaCaT keratinocyte cell culture

HaCaT cells were cultured in calcium-free DMEM (Sigma, Switzerland), with 10% chelexed FBS (Sigma, Switzerland), 4 mM L-glutamine (Sigma, Switzerland), and supplemented with calcium chloride at 0.03 mM or 2.8 mM and 50 IU/ml penicillin and 50 μg/ml streptomycin final concentration. HaCaT cells maintained in this low calcium medium for 24 h prior to experiments showed an improved response to TNF-alpha stimulation and did not require addition of trypsin inhibitor for the cells to re-attach after EDTA–trypsin treatment. FBS was calcium-depleted by incubation with Chelex 100 resin (BioRad # 142–2832) for 1 h at 4 °C according to the BioRad protocol. The Chelex was subsequently removed using a 50 mL Millipore 0.22 μm filter unit system.

2.10. Quantifications of chemokines IL-8 and CCL2/MCP-1

For quantification of IL-8 in cell culture medium, 96-well enzyme immunoassay plates (Nunc, Rochester, NY) were coated with 0.5 μg/ml of capture antibody (mAb 208, R&D Systems) for 24 h at room temperature. The plates were washed with PBS containing 0.05% Tween 20 (wash buffer) to remove unbound antibodies. Plates were then blocked with PBS containing 1% bovine serum albumin, 5% sucrose, and 0.05% sodium azide for 2 h at room temperature. This was followed by the addition of 10–100 μl of culture medium or tissue extract per well in a total final volume of 100 μl and incubation at room temperature for 2.5 h. Recombinant IL-8 (R&D Systems) was used as standard. After multiple washes with wash buffer, the plates were incubated with biotinylated anti-human IL-8 antibody (BAF 208, R&D Systems) at 100 ng/ml for 2 h at room temperature. Plates were washed with wash buffer and 100 μl of diluted peroxidase-conjugated streptavidin (1:4000; Zymed Laboratories, San Francisco, CA) was aliquoted per well and incubated for 30 min at room temperature. The plates were then washed with wash buffer and incubated with 100 μl per well of substrate consisting of a 1:1 mixture of peroxidase solution and tetramethyl benzidine substrate (KPL, Gaithersburg, MD) for 15 min at room temperature. The enzymatic activity was stopped with 5 N sulfuric acid and optical density determined at 450 nm in a microplate reader. The IL-8 ELISA was linear from 60 to 2000 pg/ml. Human monocyte chemokine attractant protein 1 (CCL2/MCP-1) was quantified with a commercial ELISA kit (Bender MedSystems), according to manufacturers instructions. Quantification range was linear between 20 and 500 pg/ml.

2.11. Skin prick tests

The procedure was explained to the healthy volunteers participating in the prick test. Prior informed consent from volunteers was obtained according to ethical guidelines. The volunteer was symptom free and was not taking medications. The skin of the ventral forearm was clean and free of active eczema. A grid was marked with a pen at 2 cm intervals and a drop of the relevant test compound or histamine was placed on the arm at the end of each line. In our experiments, 0.5 mg of compound dissolved in ethanol (50 μl volume) were applied to the skin and left to dry for 5 min. Depending on the experiment, 50 μg of histamine was added. A lancet with 1 mm point was used to prick the skin through the drop. The lancet was wiped with a gauze between each prick, in order to prevent carry-over of substances. Formation of weals (oedema) typically occurred within 10–15 min after which the results were assessed by measuring the diameters (if not round most distant points were measured). A skin reaction of 3 mm was interpreted as positive response (erythema and wheal with pseudopodia). As a negative control solution ethanol (puriss, analytical grade) was used. The positive control solution was a histamine hydrochloride solution (Sigma Aldrich, Switzerland). In the combination tests, compounds were applied 5–10 min prior to application of histamine. The test was carried out by medical staff based on the approved study protocol.

2.12. Statistical analysis

Results are expressed as mean values ± SD or SEM (depending on experiment) for each examined group. Statistical significance of differences between groups was determined by the Student's *t*-test (paired *t*-test) with GraphPad Prism software. Outliners in a series of identical experiments were determined by Grubb's test (ESD method) with α set to 0.05. Statistical differences between treated and vehicle control groups were determined by Student's *t*-test for dependent samples. Differences between the analyzed samples were considered as significant if $p \leq 0.05$.

3. Results

3.1. Radioligand screening assays

In our ongoing search for new CB receptor ligands from plant extract libraries [14] the DCM extract of *S. praecox* stem (dissolved in DMSO) was identified to concentration-dependently displace the high-affinity non-selective cannabinoid receptor ligand [^{3}H]CP55,950 [14] from CB receptors. The positive "hit rate" (displacement > 50% at 10 μg/ml) under the screening conditions used was approximately 1 in 80. The *S. praecox* extract yielded reproducible K_i values in the low μg/ml concentration range for both CB_1 (K_i = 1.2 μg/ml) and CB_2 (K_i = 985 ng/ml) receptors,

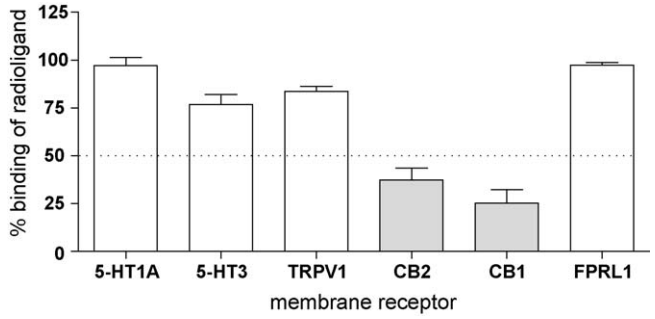

Fig. 2. Displacement of selective radioligands from different membrane proteins (serotonin 5-HT1A receptor (3%); 5-HT3 (24%) ion chanel, transient receptor potential vanilloid-1; TRPV1 channel (16%), cannabinoid type-2; CB_2 (63%); cannabinoid type-1; CB_1 (75%), formyl-peptide like-1 (FPRL-1) receptor (3%) by falcarinol (10 μM), Data are mean values of three independent experiments ± SD.

respectively. Subsequent bioactivity-guided chromatographic fractionations of the lipophilic raw extract (Supplementary Fig. 1) yielded a CB receptor binding major fraction (fraction 2.9). This fraction was purified by HPLC to yield falcarinol (compound **1**, Fig. 1), which was finally identified as the major CB receptor binding principle in *S. praecox* with apparent K_i values for CB_1 and CB_2 receptors of 590 nM to 4 μM, depending on the radioligand and assay conditions used (*vide infra*). Importantly, falcarinol was not able to significantly displace the radioligands (≥50% at 10 μM) from transient receptor potential vanilloid 1 (TRPV1) channels, formyl peptide like-1 (FPRL1) receptors, and serotonin 1A (5HT1A) receptors and 5HT3A channels (Fig. 2), thus indicating that the binding interaction detected with CB receptors was not due to nonspecific membrane effects. Moreover, falcarindiol (compound **2**, Fig. 1), a close natural analogue of falcarinol did not show detectable affinity towards CB receptors. The percentage of falcarinol in *S. praecox* stem was estimated to be approximately 0.5% of dried weight, thus identifying *S. praecox* as a major source for falcarinol.

3.2. Instability of falcarinol

In our hands, falcarinol started to decompose after purification (Supplementary Fig. 2). Freezing of the compound at −80 °C, storage in argon, and protection from light in different solvents (dimethylsulfoxide (DMSO), *n*-hexane, ethanol, and DCM did delay but not prevent its decomposition (not shown)). Isolation of this compound by both normal phase and reversed phase HPLC showed that decomposition was independent of chromatography-associated residues. In order to carry out a reliable biological characterization we first determined the apparent half-life (time to 50% decomposition) of falcarinol in DMSO, which was the vehicle used in the cellular experiments, as well as in RPMI1640 cell culture medium supplemented with 10% fetal calf serum (FCS). At room temperature, falcarinol decomposed rapidly in DMSO with an apparent half-life of less than 24 h and in cell culture medium of 5–6 h. Therefore, for the biochemical pharmacological studies falcarinol was isolated repetitively from major fraction 2.9 (Supplementary Fig. 1) and its stability was monitored by HPLC throughout the experiments. Intriguingly, after an initial decomposition, fraction 2.9 (compound **1**) stored in DCM, remained relatively stable, probably because decomposition products in the fraction quenched further decomposition.

3.3. Characterization of decomposition products and dihydroseselidiol, a new polyyne

Despite the fact that previous studies carried out with falcarinol did not stress the instability of falcarinol in biological experiments [12,13,15,16] we aimed to determine the major decomposition products spontaneously formed at room temperature. The stable endproducts were isolated by HPLC from previously purified falcarinol. The reduction product 4,5-dihydrofalcarinol (ginsenoyne J, compound **3**, Fig. 1) was found in falcarinol stored at −20 °C. The TLC control of light exposed falcarinol previously stored at −20 °C revealed traces of falcarinol and its oxidation products falcarinone (compound **4**, Fig. 1), as well as *E*-heptadeca-1,8-diene-4,6-diyne-3,10-diol (compound **5**, Fig. 1), while 4,5-dihydrofalcarinol was not detectable anymore. None of these spontaneously occurring products did displace the radioligand [^{3}H]CP55,940 from CB receptors (data not shown). Moreover, falcarinol decomposed under different temperature conditions completely lost its ability to displace [^{3}H]CP55,940 from CB receptors, thus providing evidence that the natural product falcarinol is the primary CB receptor interacting compound in the extract. Compound **6** (Fig. 1) was obtained by HPLC purification (Supplementary Fig. 1) as a transparent oily liquid and identified as dihydroseselidiol (heptadeca-1-ene-4,6-diyne-3,10-diol), a new polyacteylene. The molecular formula of $C_{17}O_2H_{26}$ was in agreement with a $[M-H]^+$ at (*m/z*) 261 in the EI-MS. ^{13}C NMR ($CDCl_3$ 300 MHz) δ: 14.1 (C-17), 15.8 (C-8), 22.8 (C-16), 25.6 (C-12) 29.2, 29.6 (C-13, C-14), 31.7 (C-15), 35.4 (C-9), 37.5 (C-11), 63.6 (C-3), 64.5 (C-6), 70.6 (C-10), 71.9 (C-5), 74.1 (C-4), 81.9 (C-7), 117.1 (C-1), 136.1 (C-2). ^{1}H NMR ($CDCl_3$ 300 MHz) δ: 0.87 (3H, t, *J* = 6.9, H-17), 1.27 (10H, m, H-12, H-13, H-14, H-15, H-16) 1.43 (2H, m, H-11), 1.65 (2H, m, H-9), 2.43 (2H, t, *J* = 6.9, H-8), 3.68 (1H, m, H-10) 4.90 (1H, *J* = 5.4, H-3), 5.24 (1H, *J* = 10.2, H-1a), 5.46 (1H, *J* = 16.8 H-1b), 5.94 (1H, m, H-2).

In contrast to seselidiol [17], dihydroseselidiol differs by the missing double bond between C-8 and C-9. The vinylic protons at δ 2.43 (H_2-8) coupled with H_2-9 at δ 1.65 while H_2-9 showed coupling with H-10 at δ 3.68. Bi-dimensional spectra (HMBC) revealed long-range heteronuclear couplings of H_2-8 with the four quartenary carbons of the diyne moiety as well as with C-9 and C-10. Protons of C-9 show HMBC connectivities with the quartenary C-7, vinylic C-8 and the hydroxy bearing C-10.

3.4. Binding interactions of falcarinol with CB_1 and CB_2 receptors

Based on the previous findings, detailed receptor binding studies were only possible with freshly isolated falcarinol and subsequent monitoring of the stability of this compound by ^{1}H NMR and HPLC. The displacement curves of [^{3}H]CP55,940 from CB receptors stably expressed in HEK293 cells obtained with falcarinol were almost identical for CB_1 and CB_2 receptors (Fig. 3) with comparable K_i values calculated from the Hill plots (hCB_1 K_i = 3.78 ± 0.23 μM; hCB_2 K_i = 2.36 ± 0.04 μM). Quite intriguingly, when using [^{3}H]anandamide as the radioligand the K_i values obtained were significantly and selectively lower for the CB_1 receptor (hCB_1 K_i = 594 ± 37 nM; hCB_2 K_i = 2.1 ± 0.16 μM). Based on this result we hypothesized that falcarinol may form a covalent (i.e. potentially irreversible) bond in the CB_1 receptor, but not in the CB_2 receptor, involving the alkylation of mercapto and amino groups, and that the putative adduct in the CB_1 receptor more closely overlaps with [^{3}H]anandamide binding than [^{3}H]CP55940 binding. To experimentally address this question we incubated 20 μM of falcarinol and rimonabant with CB_1 receptor membrane preparations for 3 h at 37 °C, then thoroughly washed the membranes with washing buffer and finally saturated the membranes with high concentrations of [^{3}H]anandamide (50 nM) prior to a final washing. As shown in Fig. 4, using this protocol the falcarinol treated membranes could not be fully saturated with excess [^{3}H]anandamide (34% of control) while the rimonabant treated membranes were saturated (96% of control). When the same experiment was performed with CB_2 receptor-transfected membranes this effect

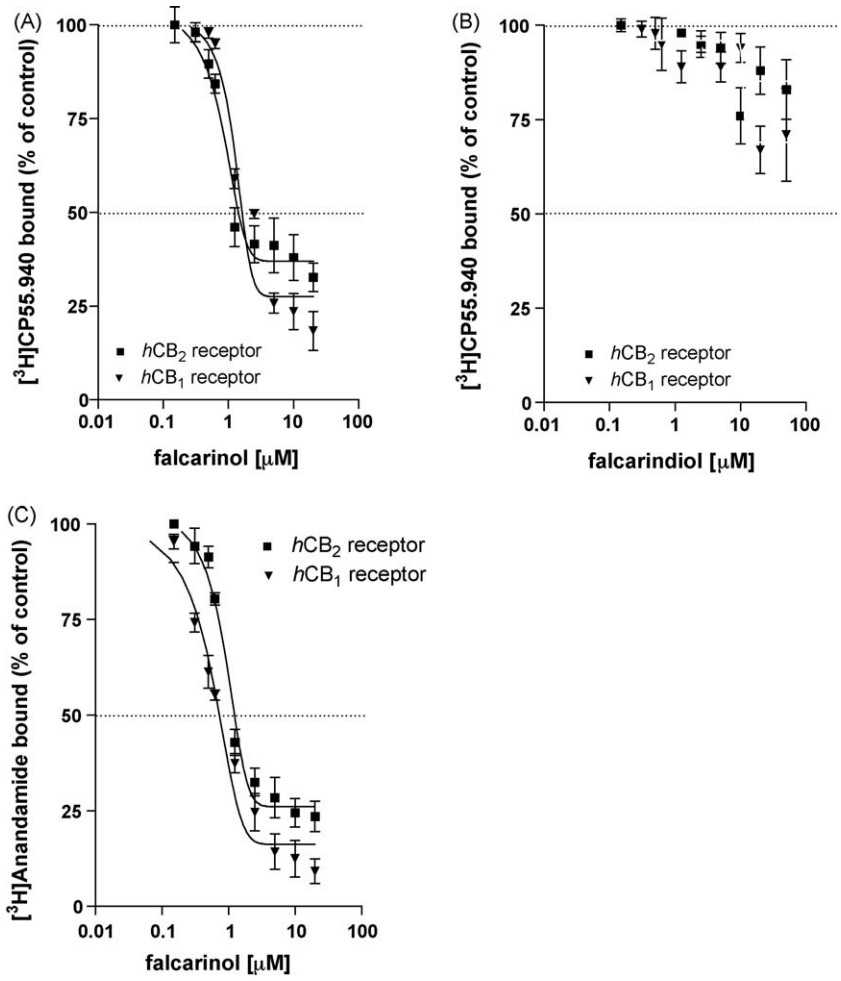

Fig. 3. CB receptor binding affinities of falcarinol. Falcarinol (A) but not falcarindiol (B) displaces [^{3}H]CP-55,940 from hCB$_1$ and hCB$_2$ receptors expressed in HEK293 cells. Sigmoidal displacement curves ($R^2 > 0.95$ for CB$_2$ and CB$_1$) showing overall displacement (65% and 75%) of [^{3}H]CP55940 with K_i values of 2.36 μM and 3.78 μM, respectively. Using [^{3}H]anandamide as the radioligand the K_i values were determined as 594 nM (CB$_1$) and 2.1 μM (CB$_2$), respectively. Data show mean values of six measurements from three independent experiments.

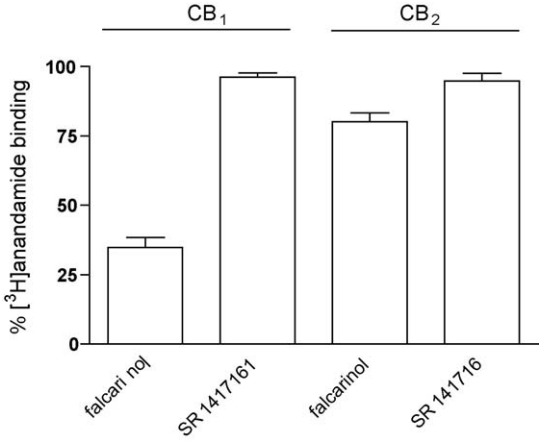

Fig. 4. 20 μM of falcarinol and rimonabant (SR141716) were incubated with CB$_1$ or CB$_2$ receptor membrane preparations for 3 h at 37 °C. After thorough washing of the membranes with cold buffer (50 mM Tris–HCl, pH 7.4) the membranes were saturated with high concentrations of [^{3}H]anandamide (50 nM) prior to a final washing. The %[^{3}H]anandamide binding relative to vehicle control is 34% in CB$_1$ versus 81% in CB$_2$ for falcarinol and 97% in CB$_1$ and 95% in CB$_2$ for rimonabant. Data are mean values ± SD of three independent experiments.

was significantly less pronounced (Fig. 4), thus suggesting a selective blocking of the anandamide binding site in the CB$_1$ receptor. Dixon analyses further indicated that falcarinol competitively interacts with the anandamide binding site in the CB$_1$ receptor (Supplementary Fig. 3). Although not a final proof, these results together with the relatively potent *in vivo* effects (*vide infra*) strongly suggest covalent and potentially irreversible interaction with the CB$_1$ receptor. Notably, falcarindiol, which has an additional hydroxyl group at carbon 8 did not displace [^{3}H]CP55,940 from CB receptors (Fig. 3). Moreover, the introduction of a hydroxyl group at position 8 (Fig. 1) led to loss of CB receptor affinity and also compound **3** (Fig. 1) with a C4–C5 double bond instead of the electron rich triple bond and oxidation of the secondary alcohol to the ketone (Fig. 1) did not bind to the CB receptors (not shown).

3.5. Falcarinol is a CB$_1$ receptor-selective inverse agonist

The significant displacement of [^{3}H]CP55,940 and [^{3}H]anandamide by falcarinol was a clear indication that the compound might be able to either activate or block CB receptors in general and the CB$_1$ receptor in particular. We next investigated whether falcarinol was able to modulate cAMP levels in CB$_1$ and CB$_2$ transfected CHO cell lines. When cells were incubated with

falcarinol, the equilibrium of constitutive cAMP was shifted towards increased cAMP levels in a concentration-dependent manner only in CB_1-transfected cells (Fig. 5). In order to compare the potency of this effect to a known control we used the CB_1 receptor-selective inverse agonist rimonabant. Noteworthy, while rimonabant gave a full response at low nM concentrations, falcarinol gave a comparable response at higher nM concentrations, reflecting its approximately 100-fold weaker binding affinity, with an almost identical response curve shifted to the dextralateral side by two log units (Fig. 5). Since inverse agonists typically act as antagonists we co-incubated the potent CB receptor agonist WIN55,212-2 (CB_1 $K_i < 20$ nM) with falcarinol under conditions in which the adenylate cylcase is stimulated by forskolin. In this assay setup, low concentrations of falcarinol did not block the inhibitory effect of WIN55,212-2 (Fig. 5). On the other hand, 5–20 μM falcarinol potently inhibited the effect of WIN55,212-2 (Fig. 5), thus providing further evidence of the CB_1 receptor antagonizing nature of this natural product. By contrast, falcarinol did not interfere with constitutive (Fig. 5) or forskoline-stimulated cAMP (not shown) in CB_2-transfected cells, thus suggesting that falcarinol is not a functional ligand at the CB_2 receptor, or alternatively, that it does not signal via cAMP. Because full CB_2 receptor agonists typically lead to intracellular calcium transients in HL60 cells (which can be blocked by the CB_2 receptor selective antagonist SR144528 [18]), we also tested falcarinol in HL60 cells. As shown in Fig. 5, falcarinol led to weak calcium responses, which could be blocked by SR144528. Therefore, falcarinol may also act as a very weak partial agonist at the CB_2 receptor acting exclusively via Go or yet unknown signals, similar to the CB_2 receptor-active *N*-alkylamides from *Echinacea* previously reported [18].

3.6. Expression of CB receptors in keratinocytes and potent modulation of chemokines IL-8 and CCL2/MCP-1 by falcarinol

Falcarinol has previously been reported to cause pro-allergic effects in human skin [10,11,19] and the CB_1 receptor has been shown to be critically involved in experimental dermatitis in mouse

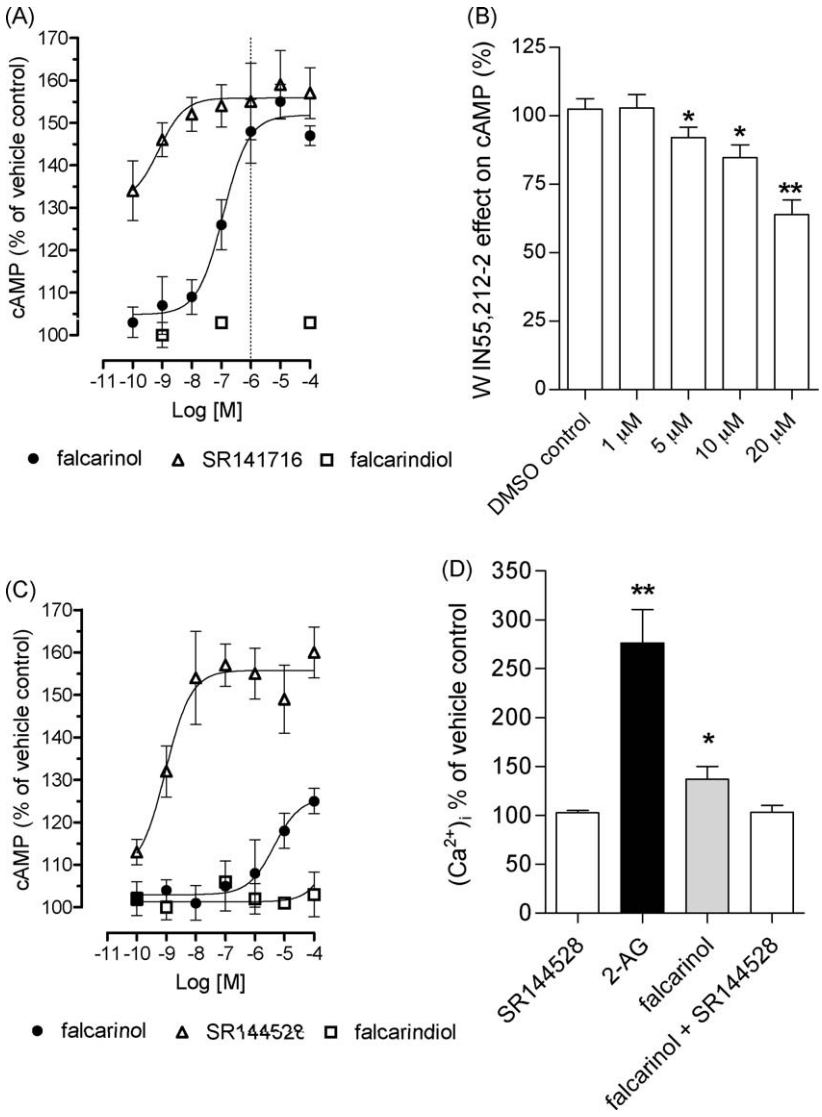

Fig. 5. Functional effects in CB_1/CB_2 receptor transfected CHO and HL60 cell lines. (A) Concentration-dependent effect of falcarinol and falcarindiol on cAMP in CB_1 receptor transfected CHO cells. Rimonabant (SR141716) is shown as positive control (B) Blockage of cAMP inhibition by WIN55,212-2 (0.5 μM) in forskolin-stimulated CHO cells by falcarinol (1–20 μM). (C) Concentration-dependent effect of falcarinol and falcarindiol on cAMP in CB_2 receptor transfected CHO cells. The inverse agonist SR144528 is shown as positive control. (D) Effect of falcarinol (10 μM) on intracellular Ca^{2+} in CB receptor expressing HL60 cells. SR144528 (1 μM) and 2-AG (10 μM) were used as controls. 2-AG induces 256% and falcarinol induces 137%. Data show mean values ± SEM of at least 3 independent experiments. $^*p < 0.05$; $^{**}p < 0.01$.

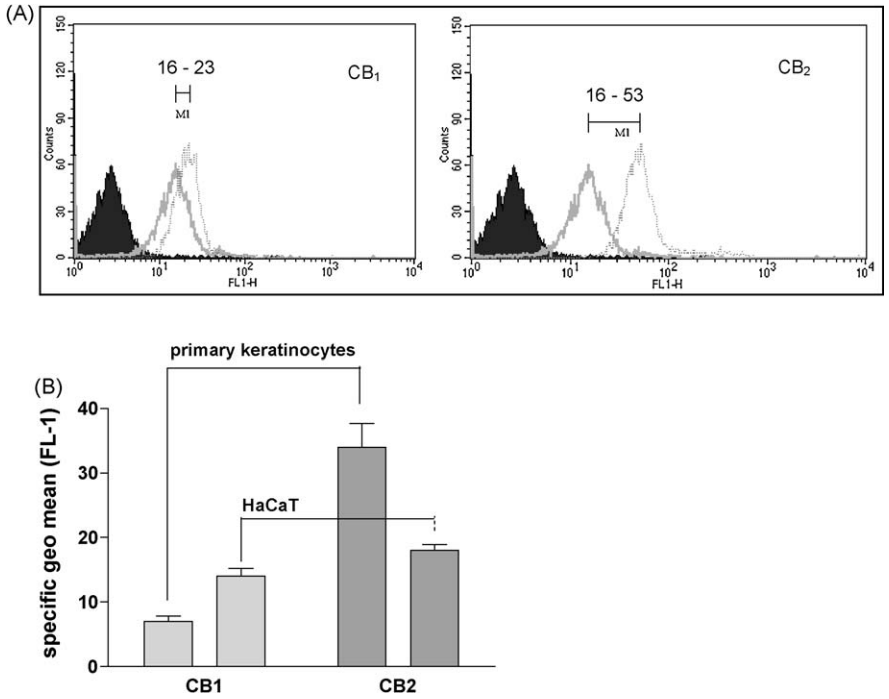

Fig. 6. Quantification of CB_1 and CB_2 receptor surface expression in primary human and HaCaT keratinocytes. (A) FL-1 shows autofluorescence (black population), non-specific binding of secondary antibody (dark grey population) and specific immunofluorescence obtanied with CB_1 (Ab3558) and CB_2 (Ab3561) receptor antibodies, respectively (light grey population). Histogram shows representative measurement (5000 cells counted). (B) Differences in CB receptor expression between primary and HaCaT keratinocytes, showing makred increased expression of CB_2 in primary keratinocytes (geo mean corrected for non-specific binding of secondary Ab). Data show mean values ± SEM obtained with two human primary keratinocyte samples and HaCaT cells, each measured three times.

skin (see Section 1). We therefore tested the hypothesis that the pro-allergic natural product falcarinol was able to modulate chemokine expression in keratinocytes (Fig. 6). This hypothesis was based on the assumption that falcarinol may act via CB receptors present in keratinocytes, which is the most abundant cell type in skin. We performed immunofluorescence FACS experiments on both primary human keratinocytes and HaCaT cells to measure CB receptor surface expression. Significant CB_1 and CB_2 receptor surface expressions were found in primary keratinocytes and in In HaCaT epidermal keratinocytes (Fig. 6). CB_2 receptor surface expression was stronger in the primary keratinocytes than HaCaT cells. Notably, the amount of receptor may not necessarily provide information about the number of fully functional receptors. We next incubated falcarinol (5 μM) with HaCaT cells (10^6 cells) for 8 h and analyzed the release of the chemokines IL-8 and CCL2/MCP-1 into cell culture media as these chemokines play crucial roles in skin inflammation [20,21]. While IL-8 was strongly up-regulated by falcarinol (~750 pg/ml), CCL2/MCP-1 was only weakly induced (~35 pg/ml), most likely reflecting the relative degrees of expression in these cells induced upon stimulation with TNF-alpha (Fig. 7). The same was observed with rimonabant, which was used as positive control (Fig. 7A). Thus, falcarinol and rimonabant stimulated the expression of chemokines from HaCaT keratinocytes to a similar degree as the pro-inflammatory cytokine TNF-alpha, albeit significantly less potently. The concentration-dependent increase of IL-8 and CCL2/MCP-1 by falcarinol could be partially reversed by co-incubation of excess anandamide (Fig. 7B), thus clearly suggesting a CB_1 receptor-dependent mechanism underlying the modulation of the chemokines IL-8 and CCL2/MCP-1.

3.7. Falcarinol reverses the inhibitory effect of anandamide on TNF-alpha induced chemokine expression

Based on previous reports that cannabinoids are able to inhibit the expression of chemokines upon exposure to proinflammatory stimuli [22,23] and that anandamide modulates skin development specifically via CB_1 signalling and inhibition of the transcription factor AP-1 [8], we studied the effect of falcarinol on the action of anandamide. As shown in Fig. 8, anandamide (10 μM) reduced the effect of TNF-alpha on chemokine expression by approximately 70%. The anti-inflammatory effect of anandamide (i.e. inhibition of chemokine expression induced by 50 ng/ml of TNF-alpha) was significantly reversed (blocked) by falcarinol and rimonabant, but not by the CB receptor-inactive falcarindiol (Fig. 8), again providing strong evidence for the antagonistic effects of falcarinol at the CB_1 receptor.

3.8. Falcarinol aggravates histamine-induced oedema reactions in human skin

Since exposure to falcarinol from vegetables and plant sources may easily yield topical concentrations in the μM range we exposed human skin to falcarinol and rimonabant as the positive control (0.5 mg) (Fig. 9). In four independent skin exposures using prick tests on the inner forearm of healthy individuals neither falcarinol nor rimonabant produced signs of spontaneous skin irritation (Fig. 9). In two cases the exposure was repeated after 1 week and did not result in skin irritation *per se* (not shown). On the other hand, exposure of compounds (0.5 mg) prior to skin prick (wealing) tests with histamine (0.05 mg) resulted in significantly aggravation of histamine-triggered oedema formation (Fig. 9). We conclude that this effect may be based on the synergisitc irritant effect of IL-8 and histamine previously reported [20,24].

4. Discussion

As shown in this study, the polyacetylene alcohol falcarinol possesses a molecular scaffold which does not differentiate between CB_1 and CB_2 receptors, similar to Δ^9-THC, endocanna-

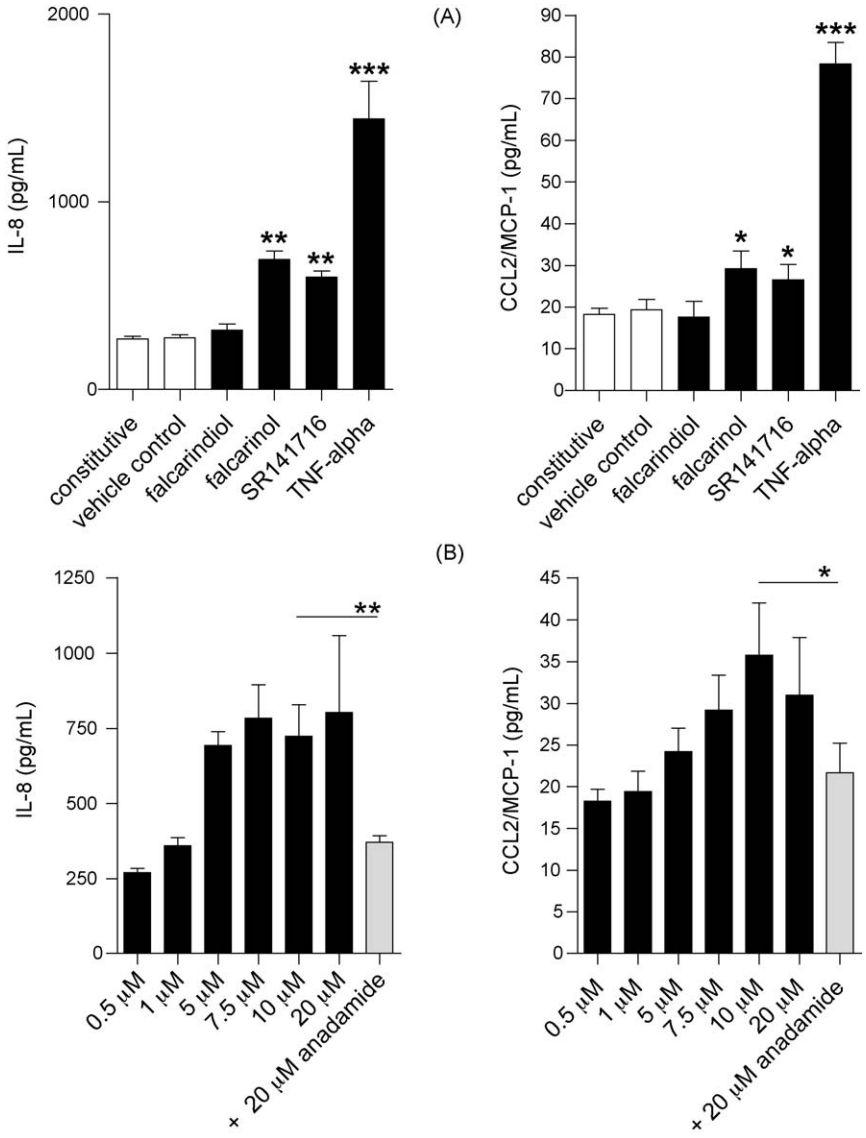

Fig. 7. Effect of falcarinol, rimonabant (SR1441716) and anandamide on IL-8 and CCL2/MCP-1 expression in HaCaT keratinocytes (10^6 cells). (A) falcarinol (5 μM) and rimonabant (5 μM) stimulate IL-8 and CCL2/MCPL-1 expression from HaCaT cells (10^6 cells). TNF-alpha (50 ng/ml) was used a positive control. (B) Concentration-dependent effects of falcarinol on IL-8 and CCL2/MCPL-1 stimulation from HaCaT cells (10^6 cells) are blocked by anandamide (20 μM). Data show mean values ± SEM of at least 3 independent experiments. $^*p < 0.05$; $^{**}p < 0.01$.

binoids and some synthetic polyacetylenes (ketoalkenes) [25]. The endocannabinoids anandamide and 2-AG, as well as the cannabimimetic plant secondary metabolites *N*-alkylamides are derivatives of the fatty acid pathway or derived from polyketides, respectively [26,27]. Only few pharmacological studies have been conducted on plant polyacetylenic lipids so far [27,28]. Here we report that the relatively widespread natural product falcarinol binds to the anandamide binding site in the CB_1 receptor and potently inhibits the action of anandamide by acting as covalent and potentially irreversible inverse agonist (i.e. antagonist).

Falcarinol, the most bioactive of the C_{17}-polyacetylenes [13], has previously been identified as the main compound responsible for the allergenic skin reactions caused by species of the Araliaceae [10] and Apiaceae [19]. Falcarinol is also a common phytoalexin and was isolated for the first time from *Panax ginseng* C. A. Meyer by Takashi & Yoshikura and named panaxynol [29]. Unaware of the contribution by their Japanese colleagues, Bohlmann et al. [30] reported the same compound from *Falcaria vulgaris* L. (Apiaceae) and named it falcarinol in coherence with the oxidation product falcarinone discussed previously [31]. A number of common vegetables were screened for toxic effects against *Daphnia magna* Straus by Crosby and Aharonson [32] and they found that the extract of carrots was among the most potent. Carotatoxin was isolated as the active principle [32] and later found to be identical with falcarinol and panaxynol. Falcarinol and polyacetylenes in general are known for their instability towards atmospheric conditions, especially towards sunlight [33–35]. We found that only freshly purified falcarinol exerted significant CB receptor binding affinity and that the K_i value increased accordingly with ongoing degradation. In agreement with the results obtained by Hansen et al. [10] we found that purified falcarinol was highly instable. Once adverted to the problem of decomposition we attempted to characterize the major degradation products of falcarinol. The 4,5-dihydrofalcarinol (ginsenoyne J) was found in freezer-stored falcarinol and may be spontaneously generated by dismutation reactions. The TLC control of sunlight-exposed and previously freezer-stored falcarinol revealed substantial amounts of its oxidation products falcarinone and *E*-heptadeca-1,8-diene-4,6-diyne-3,10-diol while 4,5-dihydrofalcarinol was not detectable anymore.

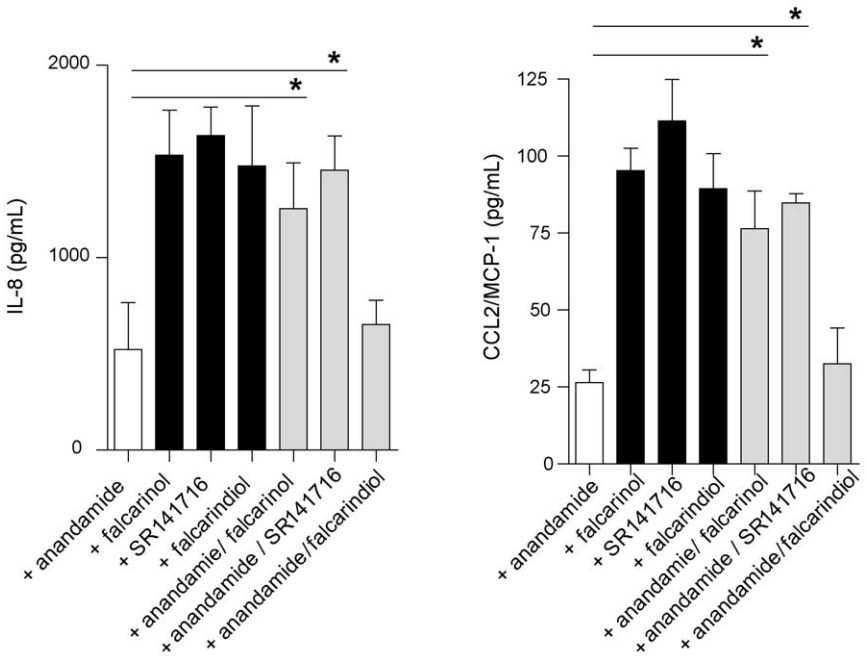

Fig. 8. Falcarinol (10 μM) and rimonabant (10 μM) reverse anandamide-induced blockage of TNF-alpha (50 ng/ml) stimulated IL-8 and CCL2/MCP-1 expression from HaCaT cells (10^6 cells). Falcarindiol (10 μM) had no effect. Data show mean values ± SEM of at least three independent experiments. $^*p < 0.05$.

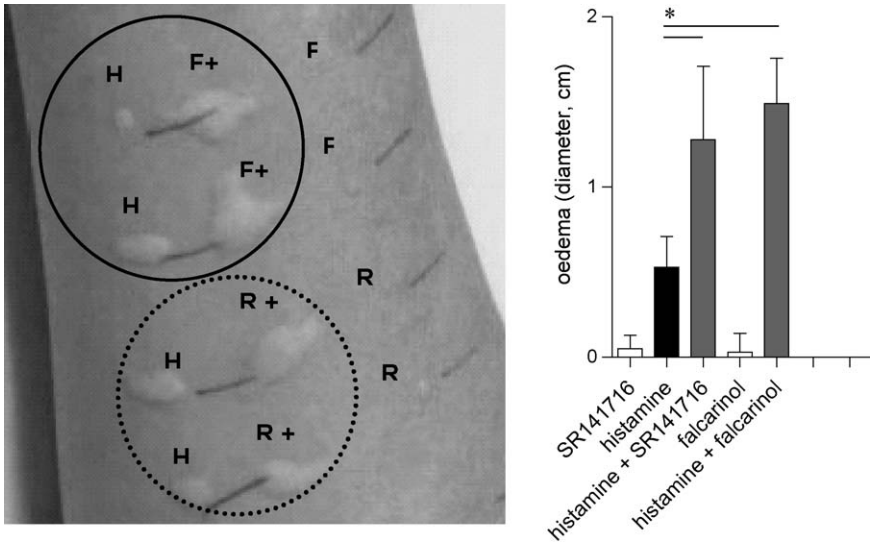

Fig. 9. *In vivo* effects of falcarinol, rimonabant and histamine on induction of oedema in human forearm skin after prick. 0.5 mg of falcarinol (F), rimonabant (R), and 0.05 mg histamine hydrochloride (H) were applied alone or in combination for skin prick test analysis. In the combination experiments the size of the weal (oedema) was clearly increased from approximately 0.6 cm to approximately 1.2 cm with both falcarinol (circle) and rimonabant (dashed circle). Neither falcarinol nor rimonabant showed reproducible wealing when applied alone. The image shown is the nicest example but representative of experiments obtained with 4 different individuals (see quantification showing mean values ± SD, $^*p < 0.05$).

Somewhat surprisingly, most previous studies on the biological activities of purified falcarinol do not address the pronounced instability of this natural product. However, notable exceptions are the studies by Hansen et al. [10], Hausen et al. [11] and Gafner et al. [36], where also closely related oxidation products (falcarindiol, falcarinone, 16,17-dehydrofalcarinol, 11,12-16,17-didehydrofalcarinol and 11,12-dehydrofalcarinol) were screened in patch and guinea pig maximization tests. Conclusively, their results showed that only falcarinol elicited strong skin reactions (dermatitis), while didehydrofalcarinol and 11,12-dehydrofalcarinol showed very weak allergenic potential and the other oxidized forms were inactive [10,11,36].

Notably, most individuals showing positive patch tests to carrot are able to eat falcarinol-containing vegetables without signs of allergic reactions [19]. In particular, the falcarinol (and falcarindiol) containing vegetables carrot (*Daucus carota* L.), celery (*Apium graveolens* L.), fennel (*Foeniculum vulgare* Mill.), and parsnip (*Pastinaca sativa* L.) account for a considerable uptake and exposure to this phytochemical in daily life [16]. It has previously been suggested that the allergenicity of falcarinol might be due to its ability to form a carbocation, thereby acting as a reactive alkylating agent towards mercapto and amino groups in proteins [10]. Others have proposed that this putative mechanism might explain the bioactivity of falcarinol in general [35]. However, no

experimental evidence has been published to verify this hypothesis. In the present study, we provide preliminary experimental evidence of a selective covalent and potentially irreversible binding interaction of falcarinol with the CB_1 receptor. We postulate an alternative, non mechanism to explain the skin irritant effects of falcarinol, which is based on its functional interaction with the CB_1 receptor and blockage of anadamide signaling.

Our data show that falcarinol and rimonabant *in vitro* lead to pro-allergic chemokine expression (IL-8 and CCL2/MCP-1) from keratinocytes in an apparently CB_1 receptor-dependent manner. *In vivo*, up-regulation of chemokine expression may be responsible for the synergistic action with histamine. Interestingly, rimonabant, which is significantly more potent *in vitro*, did not produce stronger irritation than falcarinol, despite its stronger binding affinity at the CB_1 receptor. This may be explained by the covalent and therefore potentially irreversible interaction of falcarinol with the CB_1 receptor in skin, although the exact underlying receptor pharmacology remains to be elucidated. Alternatively, falcarinol may also target other receptors that lead to additional aggravation of the effect.

It has been shown that human skin partakes in the peripheral ECS primarily through the keratinocytes, which have been shown to express both CB_1 and CB_2 receptors [6,8]. Anandamide expression has previously been shown in mouse skin [37]. CB receptor expression was further reported in epithelial cells of hair follicles, sebocytes and eccrine sweat glands [9]. A marked increase in allergic response was detectable in mice lacking both cannabinoid CB_1 and CB_2 receptors compared with wild-type animals after a challenge with the contact allergen 2,4-dinitrofluorobenzene [6]. Intriguingly, mice treated with the CB_1 receptor antagonist rimonabant showed a stronger response towards an experimental induction of cutaneous contact hypersensitivity (CHS), the mouse model for ACD in humans, while the CB_2 specific agonist HU-308 applied subcutaneous or topically showed no effect or also increased the allergic response [6]. In contrast, subcutaneously administered CB_1 receptor agonist Δ^9-THC and pharmacological inhibition of anandamide degradation significantly decreased induced CHS inflammation [6].

Other studies have shown that intraperitoneally administrated rimonabant dose-dependently provoked scratching and head-twitching in naïve mice, while Δ^9-THC was capable of attenuating the rimonabant-induced head-twiching at higher dosages [38] and that the administration of the non-selective cannabinoid receptor agonist HU210 significantly reduced histamin induced inflammation and pruritus in human volunteers [39]. This is in excellent agreement with our data. We show that both falcarinol and rimonabant block the effects of anandamide at the CB_1 receptor and thereby modulate the expression of the chemokines IL-8 and CCL2/MCP-1, which are involved in contact dermatitis. IL-8 is a chemokine with potent neutrophil chemotactic and activating properties and it is active in inflammatory conditions. E.g. IL-8 has been identified in human inflammatory skin conditions where it is likely to be responsible for neutrophil recruitment from the circulation [40]. Since keratinocytes are involved in the aetiology of dermatitis and IL-8 is also known to act synergistically with histamine [20,24], our data suggest that the pro-allergic effects previously reported with falcarinol may be related to the inverse agonistic (i.e. antagonistic) effects of falcarinol at the CB_1 receptor in keratinocytes. Although no biochemical characterization of skin was carried out, e.g. determination of IL-8 levels upon exposure of falcarinol and rimonabant, we could further corroborate our findings *in vivo* using histamine prick tests. Both rimonabant and falcarinol aggravated histamine-induced oedema formation in humans, thus being indicative of a CB_1 receptor-mediated mechanism. Although the exposure times of falcarinol and rimonabant were significantly shorter than applied in the cellular assays, chemokine and anandamide expression may be more rapid in skin. Therefore, our data suggest that falcarinol may facilitate sensitization to other allergens, by blocking the ECS, rather than being an allergen itself, thus providing a rational explanation for the skin irritation caused by topical falcarinol. Moreover, this study also confirms previous data on the role of the CB_1 receptor in skin inflammation. Further studies are needed to elucidate the molecular mechanism (thiol trapping) of the covalent interaction of falcarinol with the CB_1 receptor and whether other potentially reversible Michael-type conjugations, e.g. with glutathione, may influence this reaction differentially in different tissues such as skin and gastrointestinal tract.

Acknowledgements

The HaCaT keratinocytes were kindly provided by Prof. Dr. Michael Detmar, IPW, ETH Zürich, Switzerland. Primary keratinocytes were also obtained from Dr. Tiffany Florence, MaryKay Inc., USA. We are thankful to Dr. Michaela Begala (Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari), Dr. Pierluigi Caboni and Dr. Giorgia Sarais (Dipartimento di Tossicologia, Università di Cagliari) for the MS analyses and the library service provided by Giovanni Pilo (Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari). JG would like to thank Prof. Dr. Giovanni Appendino (Università del Piemonte Orientale) for interesting discussions. This work was supported by the Swiss National Science Foundation (SNF) and grant of the MIUR (progetto "rientro cervelli").

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.02.015.

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