Role for PPARγ in IL-2 inhibition in T cells by Echinacea-derived undeca-2E-ene-8,10-diynoic acid isobutylamide

By: Kevin Spelman, Katrina Iiams-Hauser, Nadja B. Cech, Ethan Will Taylor, Nicholas Smirnoff, and Cynthia A. Wenner

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Abstract:

Certain fatty acid amides from *Echinacea* spp. have demonstrated moderate to high cannabinoid activity. As a result, CB2 activation is currently hypothesized to be the basis of activity for immunomodulation by *Echinacea* spp. PPARγ, an orphan nuclear receptor and lipid sensor, is known to inhibit IL-2 production and be activated by fatty acid derivatives such as the endocannabinoids. In these investigations, we demonstrate that undeca-2E-ene-8,10-diynoic acid, an *Echinacea angustifolia*-derived alkylamide lacking affinity for the CB2 receptor, inhibits IL-2 secretion in Jurkat T cells through PPARγ activity at low micromolar concentrations (330 ng/mL). The IL-2 inhibition is reversed by the addition of the selective PPARγ antagonist T0070907. Additionally, we show that that undeca-2-ene-8,10-diynoic acid stimulates 3T3-L1 differentiation, a process dependent on PPARγ activity. These experiments demonstrate that PPARγ is involved in T cell IL-2 inhibition by undeca-2-ene-8,10-diynoic acid and suggest that cytokine modulation by the alkylamides is due to polyvalent activity.

Keywords: *Echinacea* | Alkylamides | Cytokines | Undeca-2E-ene-8,10-diynoic acid isobutylamide | PPAR | IL-2

Article:

1. Introduction

Peroxisome proliferator activated receptor gamma (PPARγ) is an orphan nuclear receptor that regulates transcription of target genes in response to the binding of small lipophilic ligands [1]. It was originally described as a compulsory target in differentiating adipocytes [2]. Later it was identified in other tissues [3] and a role in macrophage [4], dendritic cell [5] and T cell [6] activity was observed. Intriguingly, recent results suggest that endogenous cannabinoids such as anandamide and 2-arachidonoylglycerol are agonists of PPARγ [7], [8].

Recent work has demonstrated that several of the unsaturated alkylamides of *Echinacea* spp. exhibit significant affinity for the cannabinoid-2-receptor (CB2) [9] and that in the presence of these alkylamides, LPS-induced TNF secretion is down-regulated, a function of alkylamides targeting CB2 in macrophages [10]. Raduner et al., [11] confirmed the high CB2 affinity of the 2,4-diene unsaturated isobutylamides (olefinic alkylamides). Moreover, this group also reported that the selective CB2 antagonist SR144528 is incapable of fully inhibiting increases in total cellular concentrations of Ca^{2+} in HL60 cells induced by 2-arachidonoylglycerol (2-AG) and alkylamides, suggesting the possibility of the involvement of a second receptor. *Echinacea* spp.-derived alkylamides have been shown to inhibit IL-2 secretion by human T cells [12], but the basis of alkylamide-induced IL-2 inhibition has not yet been reported.

PPARγ activation has recently been found to be responsible for IL-2 inhibition induced by the endocannabinoids [7], [8]. In addition, Christensen et al. [13] recently demonstrated that various fatty acids and alkylamides from the flowers of *E. purpurea* activated PPARγ at high concentrations (40–100 μM). Considering the structural similarity between 2-AG and alkylamides, we investigated the possibility of induction of PPARγ activity by the alkylamide undeca-2E-ene-8,10-diynoic acid isobutylamide (hitherto referred to as undecaenediynoic acid isobutylamide) found in *E. angustifolia* and *Spilanthes acmella* (Fig. 1) [14]. This 2-ene alkylamide, containing a diacetylinic tail, has previously been found to have negligible affinity for CB2 [11]. Utilizing the previously established models of IL-2 inhibition and 3T3-L1 adipogenesis, we investigated the possibility that PPARγ is a target of undecaenediynoic acid isobutylamide, and that interaction of this alkylamide with PPARγ contributes to alkylamide-induced IL-2 inhibition and adipocyte differentiation.

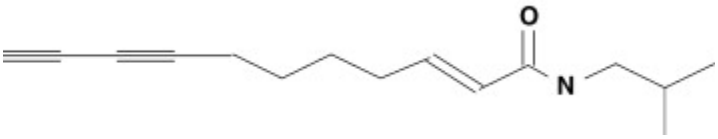

Fig. 1. Undeca-2E-ene-8,10-diynoic acid isobutylamide (undecaenediynoic acid isobutylamide).

2. Materials and methods

2.1. Reagents

The following materials, chemicals and reagents were used. All reagents were from Sigma-Aldrich (St. Louis, MO) except the following: undeca-2E-ene-8,10-diynoic acid isobutylamide (MW 231.34; certificate of analysis verified identity by NMR and HPLC, and purity of ≥ 99% by HPLC lot #21235-501; (Chromadex Inc., Santa Anna, CA); troglitazone (MW 441.5; Rezulin, abbreviated as TZD, gift from Ron Morrison, UNCG, Department of Nutrition); T0070907 (MW 277.7; Cayman Chemicals, Ann Arbor, MI); ethanol (AAPER, Shelbyville, KY); nanopure water (Nanopure Diamond D11931, Barnstead International, Thermolyne, Dubuque, IA); calf serum (Colorado Serum Co., Denver, CO); human IL-2 Duo Set ELISA Kit-DY202 (R&D Systems, Minneapolis, MN); Jurkat E6.1 cells (ATCC, Manassas, VA); 3T3-L1 preadipocytes (a gift from Ron Morrison); nitro-cellulose membranes (Bio-Rad, Hercules, CA); anti-human PPAR γ primary antibody (Aviva Systems Biology, San Diego, CA); and goat anti-rabbit conjugated to horse radish peroxidase (US Biological, Swampscott, MA).

2.2. Immunodectection of PPARγ protein in Jurkat cells by Western blotting

Cells were harvested (2×10^6 cells) and lysed by boiling for 5 min. Nuclear extracts were analyzed in a denaturing 10% polyacrylamide gel, electrotransferred to a supported nitro-cellulose membrane, and immunoblotted with the PPAR γ primary antibody (Aviva Systems ARP32880_T100). The membranes were soaked in blocking buffer [5% nonfat dry milk diluted in Tris-buffered saline — 0.1% Tween-20 (TBS-T)] for 1 h at room temperature with the indicated primary antibody (1:10,000). After washing, membranes were developed with horseradish peroxidase-conjugated secondary antibodies and visualized with a chemiluminescent detection system (GE Healthcare/Amersham Biosciences, Buckinghamshire, England). A double band is the expected image resulting from this antibody.

2.3. Fibroblast cell culture and differentiation

3T3-L1 cells were cultured in DMEM plus 10% calf serum, 4 mM l-glutamine and 1 mM Na pyruvate. Cells were plated in 6 well plates in a total of 1.5 mL of medium and grown to confluence over 4–5 days. 48 h after confluence was achieved (day 0), insulin (10 μg/mL) was added to all wells except the insulin free control. On day 2, media was changed to contain FBS and the following treatment conditions, which were added to separate triplicate wells: undecaenediynoic acid isobutylamide (5.0 μg/mL, 7.5 μg/mL and 10 μg/mL); TZD positive control (10 μM); insulin negative control (10 μg/mL); and vehicle control (EtOH:DMSO 0.4%:0.1%) were added. Every 2 days, medium and all reagents, including treatments, were replenished after a PBS wash. Experiment was halted on day 5, at which time adipogenesis was determined by photomicroscopy by morphology and the presence of the obvious prominent lipid vacuoles. Images were taken with a SPOT digital camera mounted on an Olympus BX60 fluorescence microscope.

2.4. Jurkat cell culture and IL-2 ELISA

Human E6.1 Jurkat T cells were cultured in RPMI 1640 with 10% FBS, 2 mM l-glutamine and 1 mM Na pyruvate. After serum starvation for 7 h, Jurkat cells were plated in 96 well culture plates at 1.25×10^5 cells/mL in RPMI 1640 (without phenol red) with 10% FBS. Cells were treated with PMA (1.25 ng/mL) and PHA (0.25 μg/mL) and the selective antagonist T0070907 or DMSO vehicle was added to appropriate wells and incubated at room temperature for 15 min after which alkylamides and TZD were added. Plates were then incubated for 18 h. Cell supernatants were collected (100 μL) and assayed for IL-2 by ELISA (Human IL-2 Duo Set ELISA Kit). All test conditions were assayed in triplicate and verified with repeated experiments three times.

2.5. Cell survival by XTT assay

The cytotoxicity of undecaenediynoic acid isobutylamide was measured by the XTT colorimetric assay [15], which was performed on the same plated cells cultured for the IL-2 cytokine testing in dual ELISA/XTT assays. For the cell viability standard curve, unstimulated cells were plated in triplicate at 2.5×10^4, 2×10^4, 1.5×10^4, 7.5×10^3, 5.0×10^3, and 2.5×10^3 cells/well. After removal of 100 μL supernatant from all wells, 100 μL of 1 mg/mL of 2,3-bis(2-methoxy-4-nitro-

5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) plus 0.02 mM phenazine methosulfate (PMS), in medium was added. After 5 h incubation, ODs at 450 nm (Teacan Sunrise plate reader, Grödig, Austria) were measured and cell concentrations extrapolated from known cell concentrations in standard curve. Test conditions were assayed in triplicate and repeated in two experiments.

2.6. Statistical analysis

All data are expressed as means ± SE of experiments conducted in triplicate. Statistical analysis was performed using Student's *t*-test and analysis of variance (one-way ANOVA). The accepted level of significance was $p < 0.05$.

3. Results

3.1. Presence of PPARγ receptor in Jurkat cells

Jurkat cells have previously been demonstrated to express the PPARγ nuclear hormone receptor [16]. The presence of the PPARγ protein in the Jurkat E6.1 strain of cells used for these experiments was confirmed by Western blot (see supplemental information). Nuclear extracts of untreated Jurkats were incubated with polycolonal PPARγ antibodies from 1 μg/mL to 4 μg/mL following standard Western blot procedures. Analysis of Jurkat cell nuclear extracts confirmed the expression of PPARγ receptor in Jurkat E6.1 cells. A signal of 56 kDa is seen at 1 μg/mL that grows in intensity with increasing PPARγ antibody concentration. These results demonstrate that PPARγ protein is expressed in the E6.1 strain of Jurkat cells used in this study.

3.2. The PPARγ selective antagonist T0070907 attenuates the IL-2 inhibition induced by undecaenediynoic acid isobutylamide at suboptimal Jurkat stimulation

To assess the response of T cells to undecaenediynoic acid isobutylamide (Fig. 1), suboptimal IL-2 stimulation of Jurkats by PMA/PHA (PMA 1.25 ng/mL & PHA 0.25 μg/mL) was used. Previous work has used this model system to demonstrate that PPARγ mediates IL-2 inhibition in T cells [7], [8], [16]. Cells were serum starved for 7 h, plated and then exposed to vehicle control (EtOH/DMSO), positive control (TZD-a selective PPARγ agonist), positive control with antagonist (TZD/T0070907), undecaenediynoic acid isobutylamide or undecaenediynoic acid isobutylamide with antagonist (UDA/T0070907) at increasing concentrations. T0070907 was held at constant concentration (2.5 μM). After 18 h incubation, ELISA of the supernatants collected from wells treated with the various conditions was performed. Results demonstrate that undecaenediynoic acid isobutylamide dose-dependently inhibits IL-2 production beginning at 0.33 μg/mL (1.4 μM) (Fig. 2). When the selective PPARγ antagonist T0070907 is added with undecaenediynoic acid isobutylamide, a dose-dependent attenuation of the IL-2 inhibition is seen starting at 0.33 μg/mL. At this concentration, the IL-2 level returns to baseline in the wells treated with T0070907. However, at higher levels of undecaenediynoic acid isobutylamide treatment, the antagonistic effect of T0070907 on IL-2 secretion is lost, as would be expected in increasing concentrations of a competitive agonist and a fixed concentration of antagonist.

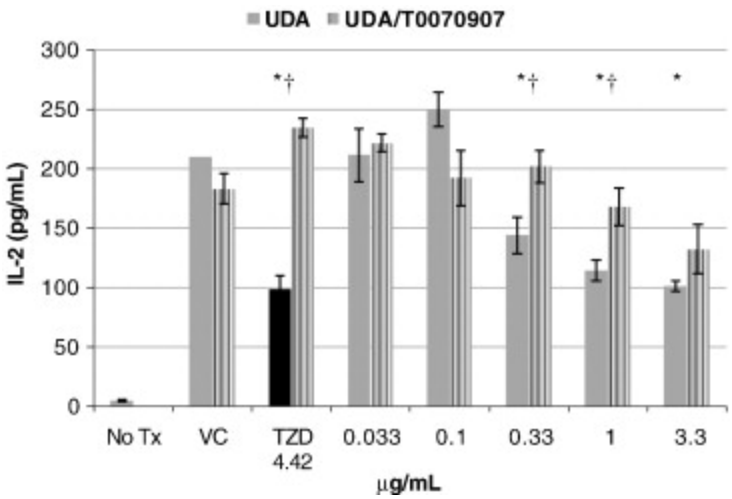

Fig. 2. PPAR-γ antagonist T0070907 attenuated the undecaenediynoic acid isobutylamide (UDA) induced inhibition of IL-2 secretion by suboptimally stimulated Jurkat E6.1 cells. Cells were subjected to suboptimal mitogenic stimulation (PMA 1.25 ng/mL, PHA 0.25 μg/mL) after serum starvation, for induction of IL-2 in all cases except in the no treatment group (No Tx). The selective agonist troglitazone (TZD) represents a positive control (black bar). Stripped bars represents the combination of 2.5 μM of T0070907 with the matched treatments (vehicle-VC, undecaenediynoic acid isobutylamide, TZD) shown in the left bar of each pair. Results show a dose dependent response of IL-2 by undecaenediynoic acid isobutylamide and the positive control TZD. The T0070907 treatments dose-dependently attenuate the IL-2 inhibition (up to 1 μg/mL undecaenediynoic acid isobutylamide) that occurs upon treatment of Jurkat cells with increasing doses of undecaenediynoic acid isobutylamide (grey bars). The positive control (TZD black bar), also shows significant inhibition of IL-2 which is blocked by T0070907. Values are mean + S.E. of experiments performed in triplicate. One-way ANOVA was used to determine statistical significance between the vehicle (VC) versus the various treatments (undecaenediynoic acid isobutylamide or TZD) treated groups (*$p < 0.05$) and the undecaenediynoic acid isobutylamide versus the undecaenediynoic acid isobutylamide/T0070907 treated groups (†$p < 0.05$).

3.3. Cell survival of Jurkats after treatment with undecaenediynoic acid isobutylamide and undecaenediynoic acid isobutylamide/T0070907 does not account for IL-2 modulation

We next determined if the IL-2 changes seen in Jurkats treated in the experimental conditions were due to the effects of undecaenediynoic acid isobutylamide and undecaenediynoic acid isobutylamide/T0070907 and not due to significant changes in cell number under these treatment conditions. Cells from the same treatment conditions in the same experiment in which supernatants were harvested for IL-2 ELISA were assayed for cell viability by the XTT assay. A statistically significant ($p < 0.05$) proliferative effect of undecaenediynoic acid isobutylamide on the Jurkat E6.1 cells was observed (Fig. **3**). Thus, the IL-2 inhibition induced by undecaenediynoic acid isobutylamide is not due to cell death, as IL-2 inhibition occurs despite the increase in cell number. In addition, these data demonstrate that the increase in IL-2 concentration observed in wells of Jurkat cells treated with the PPARγ selective antagonist T0070907 are not due to an increase in cell number.

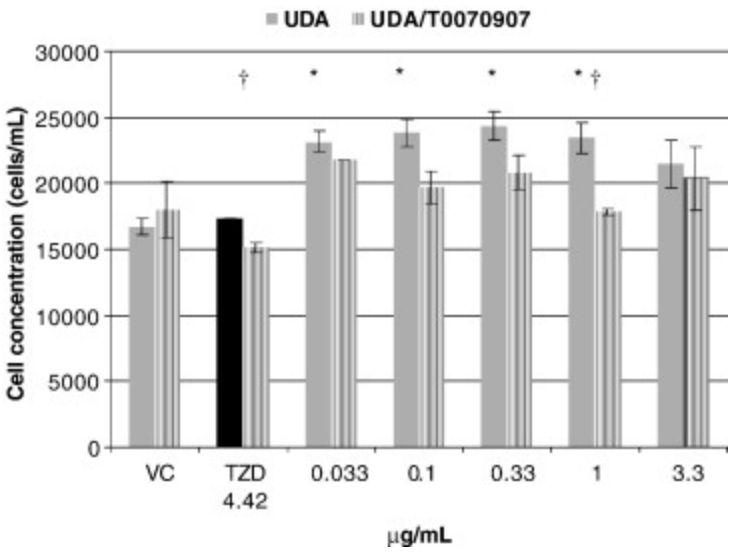

Fig. 3. Cell survival assay (XTT assay) of Jurkat E6.1 cells treated with undecaenediynoic acid isobutylamide (UDA) and T0070907. Cells were subjected to identical conditions as the IL-2 assays. The selective agonist troglitazone (TZD) represents a positive control (black bar). Stripped bars represent the combination of 2.5 μM of T0070907 with the matched treatments (vehicle-VC; undecaenediynoic acid isobutylamide; or TZD) shown in the left bar of each pair. A proliferative effect by undecaenediynoic acid isobutylamide (grey bars) on Jurkat cells is observed. A trend showing inhibition of proliferative effects is observed with the addition of the selective PPARγ antagonist T0070907. The positive control, selective PPARγ agonist TZD combined with T0070907, shows a statistically significant inhibition of proliferation as compared to TZD alone (black bar). One-way ANOVA was used to determine statistical significance between the vehicle (VC) versus the undecaenediynoic acid isobutylamide — or TZD-treated groups (*$p < 0.05$), and the undecaenediynoic acid isobutylamide versus the undecaenediynoic acid isobutylamide/T0070907 treated groups (†$p < 0.05$).

3.4. Treatment of 3T3-L1 preadipocytes with undecaenediynoic acid isobutylamide indicates PPARγ involvement

The 3T3-L1 cell line, which is PPARγ dependent for differentiation into adipocytes, has been widely established as a model to probe for PPARγ activity [17], [18]. To verify PPARγ involvement in the inhibition of IL-2 by undecadiynoic acid in T cells, 3T3-L1 differentiation in the presence of controls or undecaenediynoic acid isobutylamide was analyzed to confirm that this alkylamide is able to induce PPARγ-dependent preadipocyte differentiation.

3T3-L1 cells were grown to confluence and then exposed to experimental conditions including the TZD positive control (10 μM), insulin negative control (10 μg/mL), vehicle control, or treatment with undecaenediynoic acid isobutylamide (+ insulin 10 μg/mL) at increasing concentrations (5.0 μg/mL, 7.5 μg/mL and 10 μg/mL).

Differentiation was assessed morphologically by the relatively round shape of the adipocytes and by the presence of the obvious prominent lipid vacuoles seen by microscopy. The vehicle control (VC) and the insulin control exhibit no differentiation, while undecaenediynoic acid isobutylamide induced a dose dependent adipogenesis in 3T3-L1 cells that was morphologically

indistinguishable from that induced by the selective PPARγ agonist positive control (TZD) (Fig. 4).

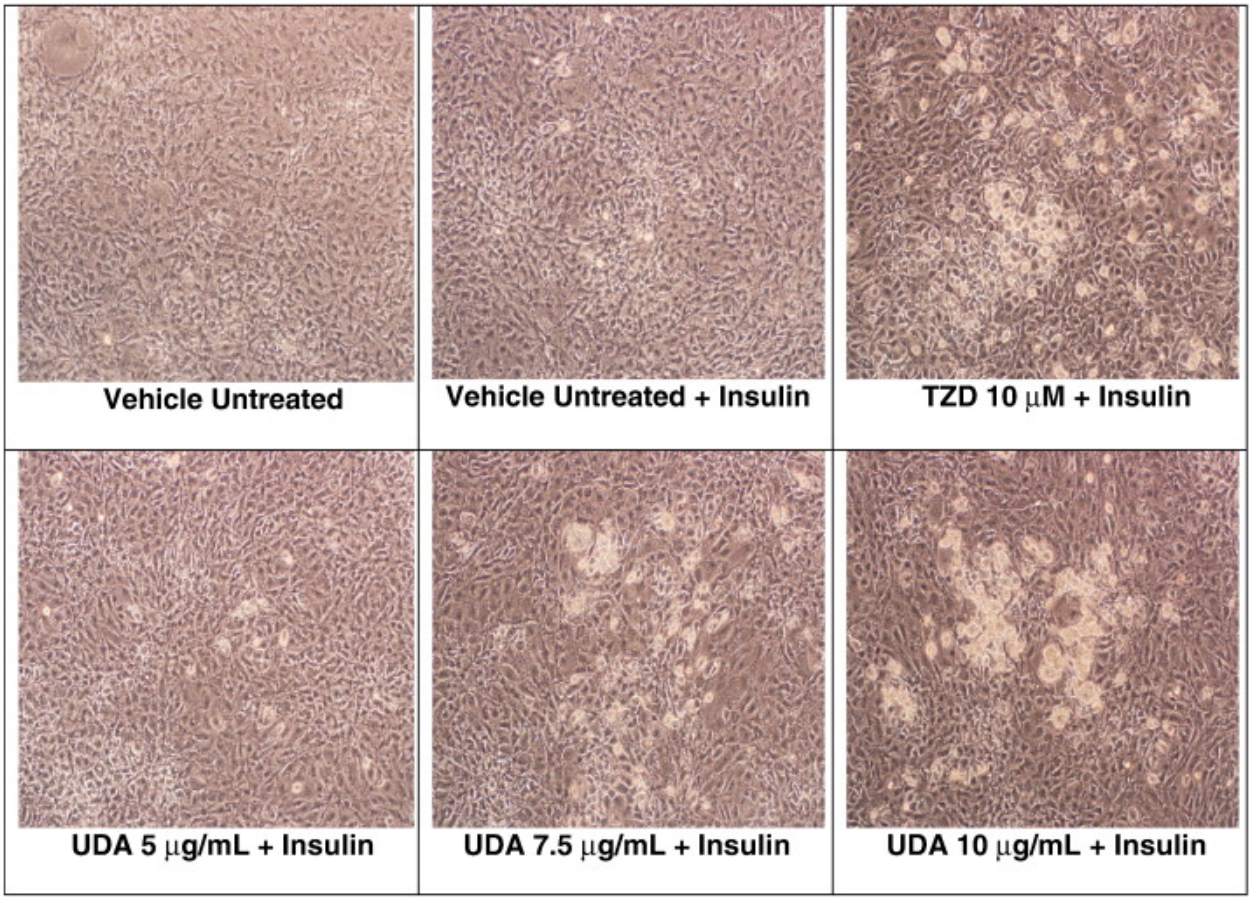

Fig. 4. Dose-dependent response of 3T3-L1 cell differentiation by undeca-2E-ene-8,10-diynoic acid (UDA). Increasing concentrations of UDA enhance 3T3-L1 differentiation, as observed by microscopy. 3T3-L1 cells were plated in 6 well plates with 1.5 mL of medium and grown to confluence over 5 days. After confluence was achieved, UDA, insulin positive control (10 μg/mL), TZD (10 μM), or vehicle control (EtOH/DMSO) were added. Medium and treatment conditions were replenished every 2 days after PBS wash. Photomicrographs were taken using an Olympus inverted microscope at day 5. Results shown are representative of triplicate experiments.

4. Discussion

PPARγ plays a role in a variety of diseases including diabetes, atherosclerosis, inflammation, cancer and autoimmune disorders [19], [20], [21], [22], [23]. Thus, novel PPARγ ligands may offer further therapeutic options for a wide array of diseases. PPARγ ligands include fatty acid derivatives, as well as the selective PPARγ agonists known as the thiazolidinediones (TZDs). Both classes of compounds have been shown to reduce IL-2 levels in T cells [6], [16], [24], [25], [26]. This effect has been shown via reporter assays to be due to the PPARγ nuclear receptor [8]. Moreover, endocannabinoids, which share common structure with some of the unsaturated alkylamides, have been shown to reduce IL-2 levels via PPARγ [7], [8].

While cannabinoid 2 receptor (CB2) activity has been shown for the 2,4-diene olefinic alkylamides, the 2-ene alkylamide undecaenediynoic acid isobutylamide (Fig. 1) has shown relatively no affinity for CB2 [11]. With this in mind, we probed for PPARγ activity with undecaenediynoic acid isobutylamide. The effect of undecaenediynoic acid isobutylamide treatment on PPARγ activation was assessed using two well established PPARγ mediated biological responses, IL-2 production and PPARγ-dependent adipogenesis in 3T3-L1 cells.

As shown in Fig. 2, the selective PPARγ antagonist T0070907 at 2.5 μM is effective in attenuating the IL-2 inhibitory effects of undecaenediynoic acid isobutylamide at alkylamide concentrations between 330 ng/mL and 1 μg/mL. This is in range of relevant physiological alkylamide concentrations based on *in vivo* human pharmacokinetic data [27]. T0070907 appears to lose its ability to attenuate the IL-2 inhibition at higher concentrations of undecaenediynoic acid isobutylamide. This apparent loss of antagonist activity is an expected observation of competitive agonism. The fixed dose of T0070907 (2.5 μM) appears to be overwhelmed by increasing concentrations of undecaenediynoic acid isobutylamide ranging two orders of magnitude. It is also possible that at high undecaenediynoic acid isobutylamide concentrations, IL-2 inhibition occurs independently of PPARγ activity. Alternatively, recent data suggests that the PPARγ receptor, which presents a particularly large binding cavity as compared to other nuclear receptors, may bind more than one ligand at a time[26]. For example, rosiglitazone, a selective PPARγ agonist structurally similar to the agonist used in these studies, occupies only about 40% of the ligand-binding site in the ternary complex of PPARγ, leaving adequate room for other ligands [28]. Crystal structures of PPARγ demonstrate that PPARγ can bind two 9-(*S*)-hydroxyoctadecadienoic acid molecules concurrently [29]. Thus a more involved explanation of the loss of the antagonism by T0070907 at higher undecaenediynoic acid isobutylamide concentrations may be that PPARγ is binding both T0070907 and undecaenediynoic acid isobutylamide at the same time and this may result in an overall IL-2 inhibitory effect. It is also possible these results could be explained by undecaenediynoic acid isobutylamide acting as a partial agonist, as most recently suggested for the alkylamide hexadeca-2E,9Z,12Z,14E-tetraenoic acid isobutylamide in a fibroblast *in vitro* model [13].

Undecaenediynoic acid isobutylamide, is considered a 2-ene alkylamide, which commonly contain diacetylinic tails, and are more characteristic of the alkylamides found in *E. angustifolia.* This is opposed to the 2,4-diene alkylamides, more commonly found in *E. purpurea,* which are more commonly olefinic alkylamides. However, both *Echinacea* species contain olefinic and acetylinic classes of alkylamides [30]. The olefinic 2,4-dienes have demonstrated moderate to high CB2 affinity, while the diacetylinic 2-enes have thus far, with few exceptions, demonstrated negligible CB2 affinity [11]. Considering Christensen's et al. [13] recent work demonstrating activation of PPARγ in fibroblasts with olefinic alkylamides at high concentrations (40–100 μM) and our results showing PPARγ activation at much lower concentrations (1.4–14 μM) with a diacetylinic alkylamide, it is possible that other diacetylinic alkylamides which have lower affinities for CB2 than the olefinic alkylamides, may activate PPARγ.

Accordingly, it may be that *E. angustifolia* and *E. purpurea* differ in the degree of PPARγ and CB2 mediated immunomodulation. Previous studies report that the alkylamide investigated here, undecaenediynoic acid isobutylamide*,* comprises 5% of the alkylamide content of *E. angustifolia*

root [31]. Furthermore, up to 55% of *E. angustifolia* alkylamides possess a diacetylenic tail like that of undecaenediynoic acid isobutylamide [32]. This is in contrast to *E. purpurea* root, which does not contain undecaenediynoic acid isobutylamide in detectable concentrations [30], and contains less than 45% diacetylenic alkylamides overall [31]. Of interest, previous reports by the eclectic physicians of the early-mid 19th to mid 20th century, who brought *Echinacea* into clinical practice, suggested that the two species differed in effect [33]. Further research is needed to evaluate how *E. angustifolia* and *E. purpurea* vary in their effects, possibly due to differential actions on pathways such as PPARγ and CB2.

Our results should be interpreted carefully. There are other proteins known to directly influence adipocyte differentiation other than PPARγ. This includes CCAAT/enhancer binding proteins (C/EBP), and the basic helix-loop-helix-leucine zipper transcription factor sterol regulator element-binding-protein-1c [29]. Furthermore, activation of C/EBP is known to engage the PPARγ pathways in both 3T3-L1 cells [34] and T cells [35]. Of particular relevance, a recent gene expression study using microarrays suggested C/EBPβ is an upstream activation node for many of the pathways activated by *Echinacea* [29]. It remains to be determined whether C/EBPβ plays a role in the PPARγ dependent alkylamide interactions reported here.

In summary, these experiments illustrate the involvement of PPARγ in the inhibition of IL-2 secretion by T cells in response to undeca-2-ene-8,10-diynoic acid isobutylamide. We demonstrate a decrease in IL-2 levels in this model system starting at 330 ng/mL of undeca-2E-ene-8,10-diynoic acid isobutylamide, which is reversed by the addition of a PPARγ selective antagonist. However, the possibility that other targets are involved in this effect is not ruled out by these data. Thus, it is possible that there is a combination of effects due to PPARγ and other targets that inhibit IL-2 production and induce adipocyte differentiation. These results, coupled with previous results demonstrating cannabinoid activity of the alkylamides, suggest that the immunomodulatory potential of the alkylamides is likely due to polyvalent actions. Further investigations are needed to elucidate the role of PPARγ and other potential alkylamide targets in the IL-2 inhibitory response of T cells to undecaenediynoic acid isobutylamide.

Acknowledgements

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Supplemental Information

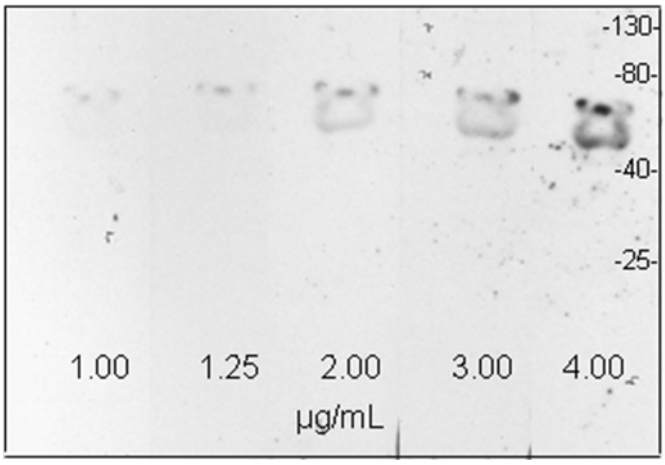

Figure 1.

Immunodetection of PPAR-γ in Jurkat cells by Western blotting. Nuclear extracts of untreated Jurkat E6.1 cells were treated with rabbit polyclonal PPARγ antibody from 1 µg/mL to 4 µg/mL. A band at 55 kDa, which increases in intensity with increasing PPAR- γ antibody, confirms the expression of the PPAR-γ nuclear protein in these cells. Sizes of the marker proteins are shown on the right hand side of the blot.