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Cytopiloyne, a novel polyacetylenic glucoside from *Bidens pilosa*, functions as a T helper cell modulator

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Abstract

An extract of *Bidens pilosa*, an anti-diabetic Asteraceae plant, has recently been reported to modulate T cell differentiation and prevent the development of non-obese diabetes (NOD) in NOD mice. In this paper, a novel bioactive polyacetylenic glucoside, cytopiloyne (**1**), was identified from the *Bidens pilosa* extract using *ex vivo* T cell differentiation assays based on a bioactivity-guided fractionation and isolation procedure. Its structure was elucidated as 2β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne by various spectroscopic methods. Functional studies showed that cytopiloyne was able to inhibit the differentiation of naïve T helper (Th0) cells into type I T helper (Th1) cells but to promote the differentiation of Th0 cells into type II T helper (Th2) cell. Accordingly, cytopiloyne also suppressed IFN-γ expression and promoted IL-4 expression in mouse splenocytes *ex vivo*. These results suggest that cytopiloyne functions as a T cell modulator that may directly contribute to the ethnopharmacological effect of *Bidens pilosa* extract on preventing diabetes. Moreover, cytopiloyne can serve as an index compound for quality control of lot-to-lot extract preparations of *Bidens pilosa*.

Keywords: *Bidens pilosa*; Polyacetylenic glucoside; T cell differentiation

1. Introduction

Naïve $CD4^+$ T (Th0) cells can differentiate into two functionally distinct subsets, type I T helper cells (Th1) and type II T helper cells (Th2), as defined and categorized by their functions and cytokine profiles. Th1 cells secrete interferon gamma (IFN-γ), interleukin (IL)-2, lymphotoxin (LT) and tumor necrosis factor α (TNFα) or TNFβ. In contrast, Th2 cells secrete IL-4, IL-5, IL-13 and/or IL-10 (Mosmann and Coffman, 1989; Murphy and Reiner, 2002). Early *in vitro* studies have shown that cytokines are very effective at driving the differentiation of Th0 cells into Th1 or Th2 cells. For example, IL-12 can drive differentiation of Th0 into Th1 cells and IL-4 can drive differentiation of Th0 into Th2 cells (Abbas et al., 1996). Th1 cells and their cytokines (e.g. IFN-γ) antagonize Th2 cell generation, and Th2 cells and their cytokines (e.g. IL-4) antagonize Th1 cell generation. Th1 cells and IFN-γ can exacerbate Th1-mediated autoimmune diseases such as non-obese diabetic (NOD) diseases, rheumatoid arthritis and Crohn's disease whereas Th2 cells and IL-4 can aggravate Th2-modulated disorders such as asthma (Abbas et al., 1996).

NOD mice can spontaneously develop autoimmune diabetes accompanied by the destruction of pancreatic β cells. NOD mice with autoimmune diabetes exhibit many of the same genetic and immunopathological features as humans with insulin-dependent diabetes mellitus (IDDM or type I diabetes) and, as such, serve as an ideal mouse model for IDDM research (Castano and Eisenbarth, 1990). During the progression of non-obese diabetes, leukocytes first infiltrate into the pancreatic β islets, a process termed insulitis, and then β cells are destroyed, leading to hypoinsulinemia and hyperglycemia. In NOD mice, $CD4^+$ Th1 cells are the leukocytes that play a dominant role in this disease (Katz et al., 1995; Toyoda and Formby, 1998).

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Plant extracts or compounds that can modulate T helper cell differentiation are considered to be of great potential as therapeutic agents to treat Th cell-mediated immune diseases. *Bidens pilosa* Linn. var. *radiate* (Asteraceae) is a herbal plant widely distributed in tropical and sub-tropical regions of the world and has been popularly used as herbal tea ingredient or used in traditional medicine for various disorders, such as diabetes, inflammation, etc. A number of articles regarding to the phytochemical or bioactivity studies of the genus *Bidens* have been reported. For instance, chalcones (Redl et al., 1993; De Tommasi et al., 1998; Li et al., 2005), flavonoids (Wang et al., 1997a,b; Brandão et al., 1998; De Tommasi et al., 1998; Sarker et al., 2000; Li et al., 2005), polyacetylenes (Redl et al., 1994; Brandão et al., 1997; Chang et al., 2000; Ubillas et al., 2000; Wang et al., 2001; Li et al., 2004), neolignan glucosides (Wang et al., 2006), aurone glucosides and phenylpropanoid glucosides (Sashida et al., 1991), a diterpene (Zulueta et al., 1995), and a disubstituted acetylacetone (Kumar and Sinha, 2003) have been identified from extracts of *Bidens* spp. plants. Bioactivities of *Bidens pilosa* plant extracts, including anti-hyperglycemic (Ubillas et al., 2000), anti-hypertensive (Dimo et al., 2001; Dimo et al., 2002; Dimo et al., 2003; Nguelefack et al., 2005), anti-ulcerogenic (Tan et al., 2000), hepatoprotective (Chin et al., 1996), immunomodulatory and anti-inflammatory (Jager et al., 1996; Pereira et al., 1999; Abajo et al., 2004; Chiang et al., 2005), anti-leukemic (Chang et al., 2001), anti-malarial (Brandão et al., 1997; Andrade-Neto et al., 2004), anti-bacterial (Rabe and van Staden, 1997), anti-microbial (Khan et al., 2001; Rojas et al., 2006), anticancer and antipyretic (Sundararajan et al., 2006), anti-virus (Chiang et al., 2003), anti-oxidative (Chiang et al., 2004; Yang et al., 2006), and anti-angiogenic (Wu et al., 2004) effects have been reported.

Our previous report showed that a butanol fraction of *Bidens pilosa* can inhibit the differentiation of human Th0 cells into Th1 cells but enhance their transition into Th2 cells *ex vivo* (Chang et al., 2004). We and others also reported that this plant species had an anti-diabetic activity in NOD mice (an animal model for type I diabetes) (Chang et al., 2004), *db*/*db* mice (an animal model for type II diabetes) (Ubillas et al., 2000) and alloxan-treated mice (Alarcon-Aguilar et al., 2002). Moreover, two polyacetylenic glucosides, 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**2**) and 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**3**), that could prevent the onset of diabetes in NOD mice (Chang et al., 2004) and *db*/*db* mice (Ubillas et al., 2000) have been identified from *Bidens pilosa*. Here, we reported that another novel polyacetyelene, namely cytopiloyne (**1**), from *Bidens pilosa* could modulate T cell functions.

2. Materials and methods

2.1. Reagents and general experimental procedures

Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma (MO, USA). Goat anti-hamster IgG was purchased from Caltag (CA, USA). Anti-CD3 mAb and anti-CD28 mAb were purchased from BD Biosciences (CA, USA). Recombinant IL-2, IL-4, IL-12, anti-IL-4, anti-IL-12, FITC-conjugated anti-IFN-γ, and PE-conjugated anti-IL4 mAbs were purchased from R and D Systems (MN, USA). RPMI 1640 medium and its supplements were purchased from Gibco (CA, USA). All other chemicals and solvents used in this study were of HPLC or reagent grade. Optical rotation of cytopiloyne was measured on a JASCO DIP-1000 digital polarimeter. IR, UV, and FABMS spectra were recorded on a Perkin-Elmer 983G spectrophotometer, a Beckman DU 640 spectrometer, and a JEOL JMS-H110 mass spectrometer, respectively. ^{1}H and ^{13}C NMR spectra were measured with a Bruker DMX-500 spectrometer.

2.2. Plant materials

Bidens pilosa Linn. var. *radiata* (Asteraceae) was collected on the campus of Academia Sinica, Taiwan, in 2003. A voucher specimen (no. 11519) has been deposited at the Herbarium of the Institute of Botany, Academia Sinica, Taipei, Taiwan.

2.3. Compound isolation

Approximately, 2.8 kg of raw materials crushed from fresh whole plants were extracted twice with MeOH (10 l) at room temperature (3 days × 2). The extract was evaporated *in vacuo* to yield a residue, which was suspended in H_2O (1 l), and this was then partitioned with ethyl acetate (1 l × 3). The combined ethyl acetate layer afforded a black syrup (27 g), which was subsequently chromatographed over Si gel column (Merck 230–400 mesh, 5 cm × 30 cm) eluted with CH_2Cl_2–MeOH mixtures of increasing polarity to give a total of eight sub-fractions (fr. 1–fr. 8). Fr. 6 (eluant of 10% MeOH/CH_2Cl_2 from the open column) was identified as a bioactive fraction using *in vitro* Th cell differentiation assay. This fraction was further separated by a PR-18 preparative HPLC column [Phenomenex Luna 5 μ C18 (2), 250 mm × 10 mm] eluted with 35% MeCN in water to afford compounds **1** (15 mg), **2** (32 mg), and **3** (23 mg).

2.4. Compound identification

Cytopiloyne (2β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne, **1**): amorphous colorless solid; $[\alpha]_D^{22}$ 52.8° (CH_3OH, *c* 0.4); UV λ_{max}^{MeOH} nm (log ε): 214 (4.86), 224 (5.10), 235 (5.10) nm; IR ν_{max}^{KBr} cm^{-1}: 3438, 3271, 2930, 2886, 2231, 1365, 1106, 1082, 1051, 1026, 994 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; FABMS, *m*/*z*: 363 $[M+H]^+$; HRFABMS, *m*/*z*: found: 363.1435; calc. for $C_{19}H_{23}O_7$: 363.1444.

2.5. T cell isolation, growth, differentiation and intracellular staining

$CD4^+CD62L^{hi}$ naïve T helper (Th0) cells were purified from the lymph nodes of BALB/c mice by positive selection using a MACS column (Miltenyi, CA, USA) and grown in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol in a 5% CO_2 incubator. Th0 cells were incubated with 2 ng/ml IL-12 and 0.3 μg/ml anti-IL-4 mAb (Th1 condition) or 10 ng/ml IL-4 and 0.2 μg/ml anti-IL-12 mAb

Table 1
NMR data for cytopiloyne (500 and 125 MHz in CD_3OD, *J* in Hz)

Position	δ_H	δ_C, mult
1	3.59 (2H, m)	65.8, t
2	3.75 (1H, p, 6.8)	81.6, d
3	1.78 (2H, q, 6.8)	31.4, t
4	2.58 (2H, t, 6.8)	16.1, t
5	–	66.2, s[a]
6	–	61.8, s[a]
7	–	60.0, s[a]
8	–	60.9, s[a]
9	–	62.4, s[a]
10	–	64.9, s[a]
11	–	81.6, s
12	–	77.9, s
13	1.98 (3H, s)	3.8, q
1′	4.32 (1H, d, 7.8)	104.8, d
2′	3.19 (1H, dd, 9.1, 7.8)	75.2, d
3′	3.34 (1H, m)	77.9, d
4′	3.30 (1H, m)	71.5, d
5′	3.30 (1H, m)	77.9, d
6′	3.65 (1H, dd, 12.0, 6.5); 3.85 (1H, dd, 12.0, 1.7)	62.6, t

[a] Data could be interchanged. Data shown in parentheses are coupling constants (*J*) in Hz.

(Th2 condition) in 48-well plates pre-coated with 5 μg/ml goat anti-hamster IgG and anti-CD3 mAb (1 μg/ml) plus anti-CD28 mAb (1 μg/ml). IL-2 (8 ng/ml) was added to culture medium after 48 h during T cell culture. Cytopiloyne was added to the differentiating cells for 24 h, 3 days after cytokine and antibody additions. For intracellular cytokine staining, T cells were treated with PMA (50 ng/ml)/ionomycin (0.5 μg/ml) for 4 h, Golgiplug (BD Biosciences, CA, USA) was then added for an additional 2 h, followed by intracellular staining using FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL4 mAbs. Finally, the cells were analyzed with a fluorescence activated cell sorter (FACS).

2.6. Cell viability assay

Cell survival was determined by a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Cells (1×10^5/well) in 96-well plates treated with or without cytopiloyne at the indicated dosage and duration were examined for cell viability.

2.7. RT-PCR analysis of gene expression in mouse splenocytes

Splenocytes from BALB/c mice aged 6–8 weeks were washed with PBS and then resuspended in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. The splenocytes were incubated in 24-well plates pre-coated with 5 μg/ml goat anti-hamster IgG and anti-CD3 mAb (2 μg/ml) in the presence of cytopiloyne. Following treatment with cytopiloyne at the indicated dosage and duration, the splenocytes were harvested for RNA extraction using Trizol reagent (Invitrogen, CA, USA). Two microgram of total RNA from different samples was converted into the first-stranded cDNA products using a first-strand cDNA synthesis kit (Amersham Biosciences, NJ, USA). The cDNA products (1 μl) were used as the templates for PCR amplification. Specific PCR primer pairs were described as follows: IFN-γ sense (5′-AACGCTTACACACTGCATCTTGG-3′) and anti-sense (5′-GACTTCAAAGAGTCTGAGG-3′); IL-4 sense (5′-GAATGTACCAGGAGCAGCCATATC-3′) and anti-sense (5′-CTCAGTACTACGAGTAATCCA-3′); G3PDH sense (5′-ACCACAGTCCATGCCATCAC-3′) and anti-sense (5′-TCCACCACCCTGTTGCTGTA-3′). The PCR annealing temperature was 55 °C, and the cycle number for each study was chosen in a linear range to avoid the plateau effect. The amplified PCR products were resolved on 2% agarose gel, followed by ethidium bromide staining. The relative expression level of mouse cytokines was obtained from the ratio of the signal of each cytokine to that of the G3PDH control.

2.8. Detection of cytokines in mouse splenocytes

The splenocytes from BALB/c mice were incubated in 24-well plates pre-coated with 5 μg/ml goat anti-hamster IgG and anti-CD3 mAb (2 μg/ml) in the presence of cytopiloyne as described above. The culture supernatant was collected at 0, 6, 18, 24, 48, 72 and 96 h and the production of IFN-γ and IL-4 was determined using ELISA kits (eBioscience, CA, USA). The concentration of a specific cytokine was obtained by calculating against a calibration curve using the recombinant cytokine as the standard.

3. Results

3.1. Characterization of cytopiloyne from Bidens pilosa

Our previous data showed that a butanol fraction from hot water extract of *Bidens pilosa* could modulate the differentiation of human helper T cells and prevent autoimmune diabetes in NOD mice (Chang et al., 2004). Two polyacetylenic glucosides

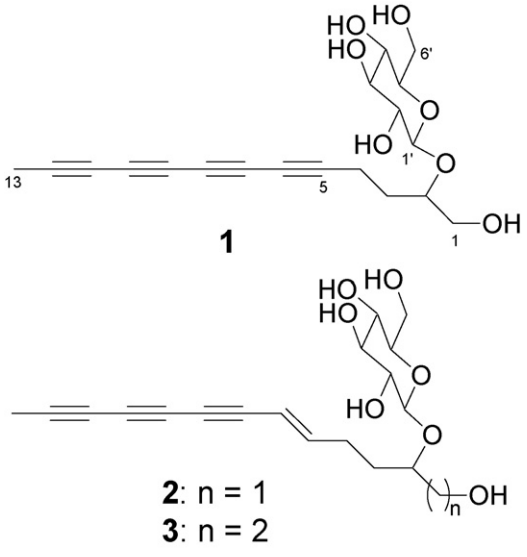

Fig. 1. Chemical structures of the three bioactive polyacetylenes (1–3) identified from plant extract of *Bidens pilosa*.

(compounds **2** and **3**) were isolated from the hot water extract of *Bidens pilosa* using a bioactivity-guided fractionation and isolation method. Polyacetylenes have been obtained as natural products and showed potent biological activities. However, they are unstable and polymerized when concentrated (Yamaguchi et al., 1995). As part of our research interest in immunomodulator and the function of polyacetylenes on immune system, we used a mild extraction condition (i.e. cold MeOH) to extract plant material to see if other bioactive but labile polyacetylenes can be isolated from this species. Based on a bioactivity-directed strategy using T cell differentiation assays (data not shown), a bioactive fraction (eluant of 10% MeOH/CH_2Cl_2 from the silica gel) was obtained from EtOAc layer of the MeOH extract. Three major compounds (**1**–**3**) were isolated from this fraction and their structures were elucidated by spectroscopic methods (Fig. 1). A new polyacetylenic glucoside, namely cytopiloyne (**1**), was isolated as an amorphous colorless solid. Its molecular formula was found to be $C_{19}H_{22}O_7$ by HRFABMS, indicating nine indices of hydrogen deficiency (IHD). Its IR spectrum showed the presence of hydroxyl groups (3438 and 3271 cm^{-1}) and acetylenic groups (2231 cm^{-1}). Its ^1H NMR spectrum (Table 1) exhibited signals for a singlet methyl group [δ_H 1.98 (s, H_3-13)], four methylene protons [δ_H 1.78 (2H, q, J = 6.8 Hz, H_2-3), and 2.58 (2H, t, J = 6.8 Hz, H_2-4)], two oxymethene protons [δ_H 3.59 (2H, m, H_2-1)], one oxymethine proton [δ_H 3.75 (1H, p, J = 6.8 Hz, H-2)], and one glucose moiety. ^{13}C NMR (Table 1) and DEPT spectra of cytopiloyne indicated one CH_3, four CH_2, six CH, and eight C, including one anomeric carbon [δ_C 104.8 (C-1′)] and seven carbons to which oxygen was attached. Cytopiloyne had similar ^1H and ^{13}C NMR data to compound **3** on the polyyne and the glucose moiety (Rucker et al., 1992). From the molecular formula and the supporting evidence obtained in this and previous studies, cytopiloyne was identified as a tetrayne with a terminal methyl group and a vicinal diol at the other terminus. 2D NMR methods (i.e. HMQC, HMBC, NOESY and COSY) confirmed the assigned structure. Therefore, cytopiloyne was elucidated as 2β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne.

3.2. *Cytopiloyne inhibits differentiation of Th0 cells into Th1 cells but promotes differentiation of Th0 cells into Th2 cells*

In our preliminary study, we observed that cytopiloyne (**1**) was able to inhibit the differentiation of human Th0 cells into

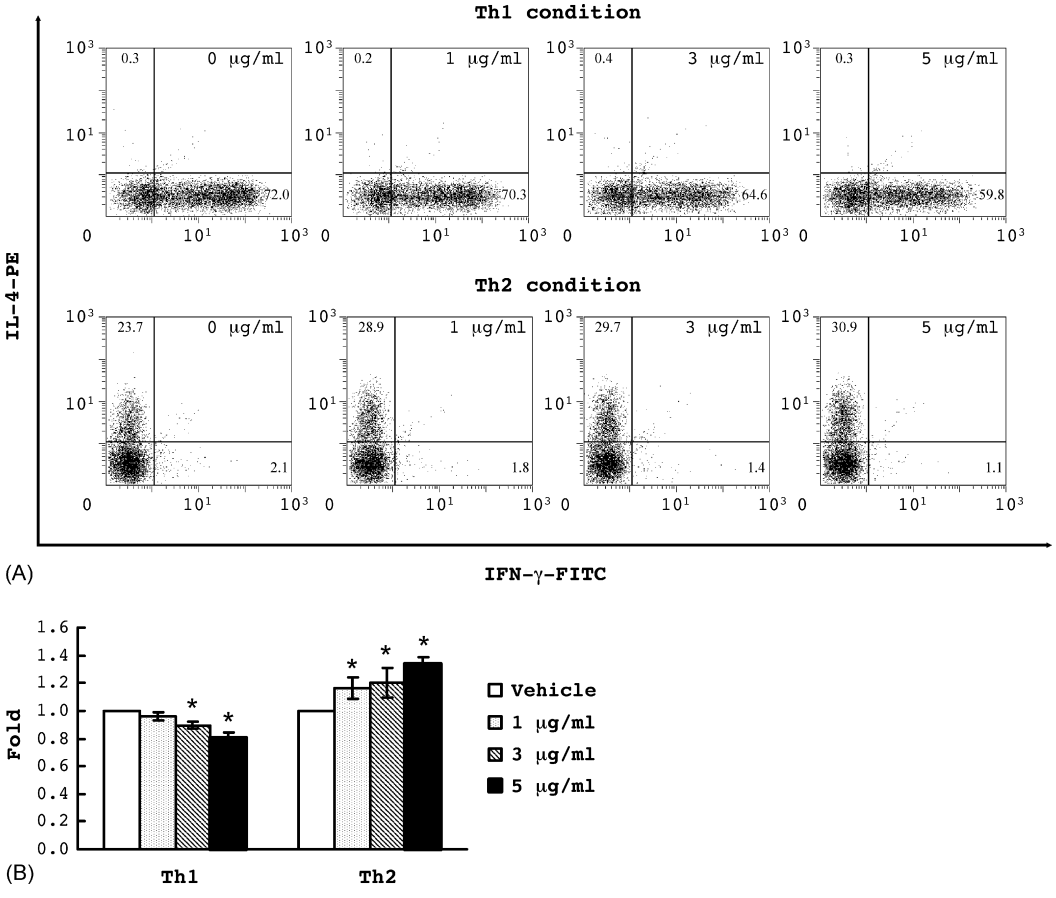

Fig. 2. Cytopiloyne inhibits differentiation of Th0 cells into Th1 cells but promotes differentiation of Th0 cells into Th2 cells. (A) $CD4^+$ T cells from BALB/c mice were cultured under Th1 (upper panel) or Th2 (lower panel) conditions in the presence of cytopiloyne at 0, 1, 3 and 5 μg/ml. T cells were stained with anti-IFN-γ or anti-IL-4 mAbs, followed by FACS analysis. The percentages of IFN-γ-producing and IL-4-producing cells were calculated, respectively. The data are representative of one of three similar experimental results. (B) Data from Fig. 2A are shown in folds. The decrease in folds of the IFN-γ producing Th1 cell population or the increase in folds of the IL-4 producing Th2 cell population was obtained from the following formula: the percentage of Th1 (Th2) cells treated with compound/the percentage of Th1 (Th2) cells treated with vehicle. Statistical analyses between vehicle control (0 dosage) and compound treatment were performed using Student's *t*-test. Significant difference is indicated by asterisk (*) with a p value <0.05.

Th1 cells in a more effective way than compounds **2** and **3** (data not shown). In the present study, we investigated in detail the effect of cytopiloyne on modulating T cell differentiation. $CD4^+$ T cells were isolated from lymph nodes of BALB/c mice for Th cell differentiation study. We found that cytopiloyne concentration-dependently (1–5 μg/ml) decreased the percentage of INF-γ-producing cells (i.e. Th1 cells) from 72.0% to 59.8% (upper panel, Fig. 2A). Since Th1 and Th2 cell differentiation is cross-regulated and mutually antagonized (Abbas et al., 1996), we next examined whether cytopiloyne could modulate Th2 cell differentiation. We found that an addition of cytopiloyne to the differentiating Th cells increased the percentage of mouse IL-4-producing cells (i.e. Th2 cells) from 23.7% to 30.9% in a concentration-dependent manner (lower panel, Fig. 2A). Cytopiloyne at these doses did not show any cytotoxicity toward the differentiating cells even after a 24 h incubation (data not shown). We concluded that cytopiloyne inhibited Th1 cell differentiation but increased Th2 cell differentiation in mouse T cells (Fig. 2B).

3.3. Cytopiloyne inhibits IFN-γ but promotes IL-4 transcription in mouse splenocytes

The Th1 cytokine, IFN-γ, promotes the differentiation of Th0 cells into Th1 cells but inhibits the differentiation of Th0 cells into Th2 cells. In marked contrast, IL-4 antagonizes the function of IFN-γ in T cell differentiation (Abbas et al., 1996). Since cytopiloyne promoted Th2 but not Th1 cell differentiation, we subsequently assessed whether cytopiloyne could modulate the transcription of IFN-γ or IL-4 in mouse splenocytes stimulated with anti-CD3 antibody. We found that splenocytic levels of IFN-γ mRNA decreased whereas IL-4 mRNA increased, both in a concentration-dependent manner, following cytopiloyne treatment (Fig. 3A). Cytopiloyne at 0.1–3 μg/ml did not show significant cytotoxicity to mouse splenocytes after a 72-h incubation and greater than 70% cell viability was detected even after a 96 h incubation with the compound (Fig. 3B).

3.4. Cytopiloyne inhibits IFN-γ but promotes IL-4 production in mouse splenocytes

The effect of cytopiloyne on the production of the aforementioned cytokines was also investigated using an ELISA kit. Similar to the effect of cytopiloyne on cytokine gene transcription, we found that cytopiloyne inhibited IFN-γ protein production in splenocytes in a concentration-dependent manner. When splenocytes were treated with 3 μg/ml cytopiloyne for 72 and 96 h, the protein level of IFN-γ decreased to 18.6% and 44.4%, respectively, of the DMSO (vehicle) treated cells (Fig. 4). In contrast, treatment with 3 μg/ml cytopiloyne for 72 and 96 h augmented IL-4 production in splenocytes to 198.5% and 247.0%, respectively, of the vehicle-treated cells (Fig. 4). The role of cytopiloyne in cytokine production is consistent with its role in cytokine gene transcription and Th cell differentiation.

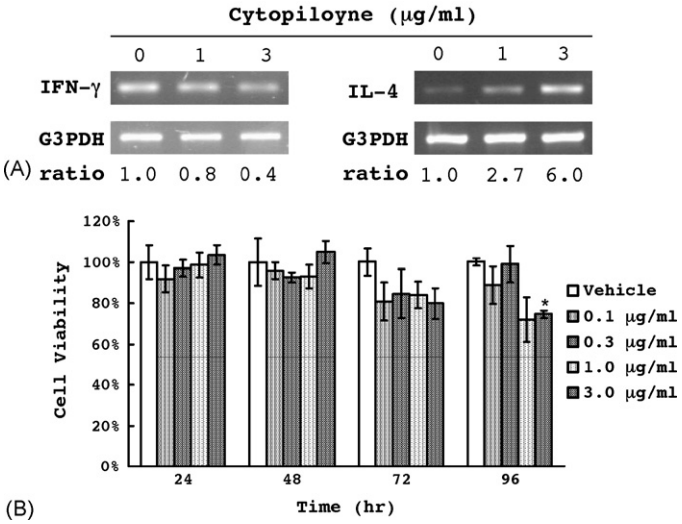

Fig. 3. Effect of cytopiloyne on the transcription of IFN-γ and IL-4 in mouse splenocytes. (A) Mouse splenocytes treated with anti-CD3 (2 μg/ml) in the presence of cytopiloyne (0, 1 and 3 μg/ml) for 48 and 72 h to detect IFN-γ and IL-4 mRNA, respectively. cDNA synthesized from total RNA (2 μg) was subjected to PCR using specific primer pairs of IFN-γ, IL-4 and G3PDH. The PCR products were resolved on 2% agarose gel and the signal intensity of each band measured by densitometric analysis. The ratio was obtained by the normalization of the signals for IFN-γ (left panel) or IL-4 (right panel) to those of G3PDH. The ratio of IFN-γ and IL-4 signals to that of G3PDH in the vehicle control was set as 1. (B) Mouse splenocytes incubated with vehicle (DMSO) or cytopiloyne (from 0.1 to 3 μg/ml) for 24, 48, 72 or 96 h. The percentage of viable cells was determined using a CCK-8 kit. The data are representative of three experiments and expressed as mean ± S.D. Statistical analyses between vehicle and cytopiloyne treatment were performed using the Student's *t*-test. $p < 0.01$ (*) was considered statistically significant.

4. Discussion

Extracts or acetylenic constituents from *Bidens pilosa* showed anti-hyperglycemic effects in mouse models (Ubillas et al., 2000; Chang et al., 2004). Our previous studies demonstrated that a butanol fraction from *Bidens pilosa* prevented non-obese diabetes mice from developing diabetes probably *via* modulation of Th cell differentiation (Chang et al., 2004). Here, we provide evidence that a novel polyacetylenic glucoside, cytopiloyne, isolated from *Bidens pilosa* can inhibit Th1 differentiation and promote Th2 differentiation. It is interesting to note that cytopiloyne is the most potent polyacetylenic glucoside to regulate T cell differentiation in *Bidens pilosa*.

One important question raised by this study is how cytopiloyne can drive the differentiation of Th0 cells into Th2 cells. It is thought that cytokines such as IL-4 and IFN-γ are the key players in the modulation of T cell differentiation. Since cytopiloyne can up-regulate IL-4 and down-regulate IFN-γ gene expressions, it is very likely that cytopiloyne promotes Th2 cell differentiation *via* its cross regulatory effect on the Th1 and Th2 cytokines *ex vivo* and *in vivo*. Another question is how polyacetylenic glycosides prevent non-obese diabetes. It is well-known that Th1 cells play a dominant role in causing non-obese diabetes, and we observed in this study that cytopiloyne favored the differentiation of Th0 cells into Th2 cells and the expression of IL-4 but not IFN-γ *ex vivo* (Figs. 2–4). Further *in vivo*

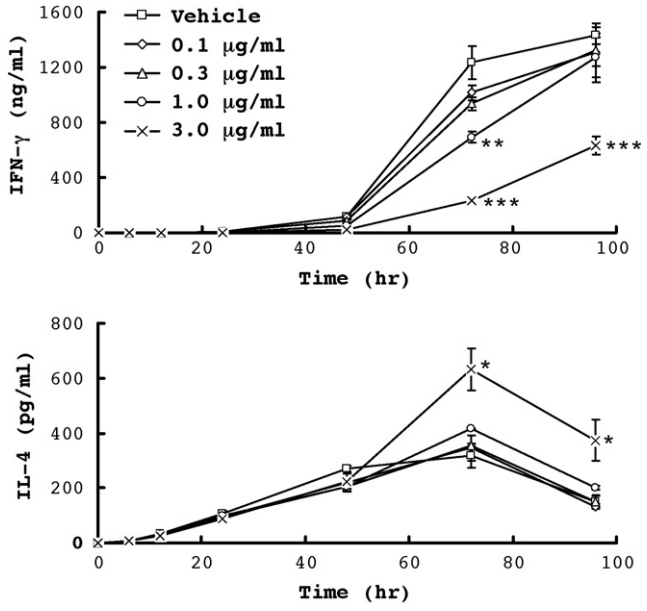

Fig. 4. Effect of cytopiloyne on IFN-γ and IL-4 production in mouse splenocytes. Splenocytes from BABL/c mice were stimulated with anti-CD3 (2 μg/ml) in the presence of DMSO or cytopiloyne (from 0.1 to 3 μg/ml). Concentrations of IFN-γ (upper panel) and IL-4 (lower panel) in medium were determined using an ELISA kit. The data are representative of three experiments and expressed as mean ± S.D. Statistical analyses between vehicle and cytopiloyne treatment at 72 and 96 h were performed using the Student's *t*-test with the Bonferroni adjustment. $p < 0.05/4$ (*), $p < 0.01/4$ (**) and $p < 0.001/4$ (***) were considered statistically significant.

evidence revealed that cytopiloyne exhibited similar regulatory effect on both cytokines in NOD mice (data not shown). It is thus very plausible that cytopiloyne protects NOD mice from diabetes development *via* the regulation of T cell differentiation as well as the secretion of Th1 and Th2 cytokines as shown in the schematic diagram proposed in Fig. 5. An overproduction of Th2 cytokines might cause Th2 cell-mediated autoimmune disorders (e.g. allergy) and antagonize Th1 cell-mediated immunity. Our recent report showed that the fraction of *Bidens pilosa* containing cytopiloyne caused a deterioration of Th2 cell-mediated airway inflammation induced by ovalbumin in BALB/c mice

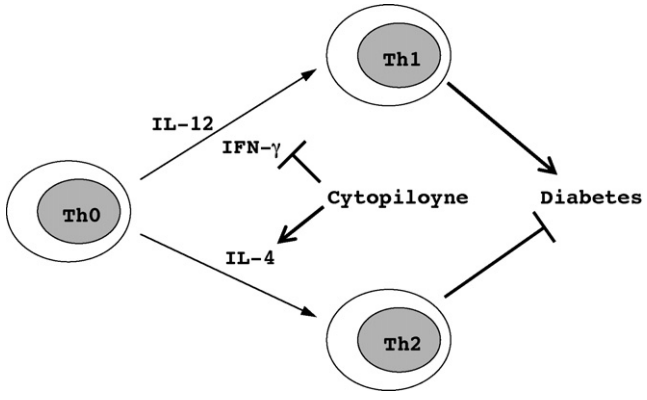

Fig. 5. A scheme for the role of cytopiloyne in modulating cytokine production. Cytopiloyne decreases IFN-γ expression and increases IL-4 expression in splenocytes and $CD4^+$ T cells. This implies the mechanism by which cytopiloyne can prevent the onset of non-obese diabetes.

(Chang et al., 2005). Therefore, the bioactive fraction of *Bidens pilosa* containing polyacetylenic glucosides might risk aggravating allergy and asthma though this fraction and its derived polyacetylenes have a beneficial effect on Th1 cell-mediated autoimmune diseases such as diabetes. Currently, there is no much therapeutics available for autoimmune disorders. Therapeutics such as cyclosporine A can shut down the immune system and lead to an elevated risk of infection and cancers. Therefore, a selective inhibitory compound for Th1 cells may provide an alternative treatment for Th1-mediated autoimmune illnesses. Moreover, the target protein of cytopiloyne might be important in modulating Th cell differentiation. Target identification using biotinylated cytopiloyne as a probe to pulldown the target protein followed by proteomic methodology was undertaken in our laboratory.

5. Conclusion

In the present report, a new polyacetylenic glucoside (referred to as cytopiloyne) was identified from *Bidens pilosa* extract that inhibits the differentiation of naïve T helper (Th0) cells into type I T helper (Th1) cells but promotes the differentiation of Th0 cells into type II T helper (Th2) cells. We observed that cytopiloyne promotes Th2 cell differentiation via its cross regulatory effect on the Th1 and Th2 cytokines. This modulatory effect on T cell differentiation of cytopiloyne may provide a possible mechanism for the ethnopharmacological effect of *Bidens pilosa* on preventing diabetes.

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