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Flavonoids, centaurein and centaureidin, from *Bidens pilosa*, stimulate IFN-γ expression

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Abstract

Bidens pilosa is used as an ethnical medicine for bacterial infection or immune modulation in Asia, America and Africa. Here, we employed an IFN-γ promoter-driven luciferase reporter construct and T cells to characterize immunomodulatory compounds from this plant based on a bioactivity-guided isolation principle. We found that PHA, a positive control, caused a six-fold increase in IFN-γ promoter activity. In contrast, hot water crude extracts from *Bidens pilosa* and its butanol subfraction increased IFN-γ promoter activity to two- and six-fold, respectively. Finally, centaurein (EC_{50} = 75 μg/ml) and its aglycone, centaureidin (EC_{50} = 0.9 μg/ml), isolated from this butanol subfraction, augmented IFN-γ promoter activity by ∼four-fold. Consistent with the role of centaurein or its aglycone in IFN-γ regulation, we showed that centaurein induced the activity of NFAT and NFκB enhancers, located within the IFN-γ promoter, in Jurkat cells. Overall, our results showed that centaurein regulated IFN-γ transcription, probably via NFAT and NFκB in T cells.

Keywords: Centaurein; Centaureidin; Interferon gamma; Flavonoid; *Bidens pilosa*

1. Introduction

Bidens pilosa L. belongs to the Asteraceae family, the largest flowering plant family in the world. It is widely distributed in tropical areas. It is used to treat infections in South Africa and Taiwan, stomach illness in Mexico, malaria and liver disorders in Brazil and diabetes in Taiwan and South America (Marles and Farnsworth, 1995; Brandao et al., 1997; Pereira et al., 1999; Ubillas et al., 2000). Moreover, some phytochemicals purified from *Bidens pilosa* have been claimed to possess anti-inflammatory, immunosuppressive, anti-bacterial and anti-malarial functions and an inhibition for the prostaglandin synthesis pathway (Geissberger and Sequin, 1991; Jager et al., 1996; Rabe and van Staden, 1997; Chiang et al., 2005). However, these plant components are insufficient to explain the habitual use of *Bidens pilosa* as an immunomodulator or anti-infectious agents in various regions of the world. Therefore, further characterization of bioactive immunomodulatory compound(s) from this plant is required.

T cells are key players in human immunity including cellular and humoral immunity. T cells also can secrete cytokines (e.g., IFN-γ, IL-4, etc.), mediate other immune cells (macrophage, B cells, leukocytes, etc.) and maintain immunity against pathogens. T cells are normally the primary targets for immunity-related drugs. For instance, cyclosporine A can inhibit T cell activation and therefore, suppress graft rejection. Moreover, T cells can produce immunoregulatory cytokines on encountering antigens or on activation. Among these cytokines, IFN-γ is a key cytokine produced by activated T cells. Furthermore, IFN-γ expression has been reported to be regulated by many nuclear factors such as NFAT, NFκB, AP-1, T-bet and so on (Penix et al., 1996; Sica et al., 1997; Sweetser et al., 1998; Szabo et al., 2000). IFN-γ modulates a variety of immune

Abbreviations: AP-1, activator protein-1; BGFI, bioactivity-guided fractionation and isolation; IFN-γ, interferon gamma; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor-κB; PHA, phytohemagglutinin; T-bet, T-box expressed in T cells; TCR, T cell receptor

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responses including pathogen clearance, tumor eradication, T cell activation and inflammatory responses (Abbas et al., 1996; Swain, 1999). Accordingly, defects in IFN-γ pathway are associated with the susceptibility to disease caused by intracellular pathogens such as *Mycobacteria*, *Salmonella*, and some viruses (Dorman and Holland, 2000).

In this paper, we used luciferase reporter assays to identify the bioactive compounds from *Bidens pilosa*. We successfully identified two flavonoids, namely centaurein and centaureidin, from *Bidens pilosa* with the ability to boost IFN-γ transcription, using a bioactivity-guided fractionation and isolation (BGFI) procedure. Besides, we also examined the likely mechanism by which centaurein could up-regulate IFN-γ transcription.

2. Materials and methods

2.1. Extraction and fractionation of Bidens pilosa

Bidens pilosa was collected from the campus of Academia Sinica, Taiwan, in April, 2001 and a voucher specimen (no. 0211943) deposited at the Herbarium of National Taiwan University, Taiwan. The fresh whole plant of *Bidens pilosa* (1.2 kg) was grounded and extracted with boiling water (9 l × 2). The extract was evaporated in vacuo to yield a residue (66.4 g) and suspended in water (1 l). Subsequently, this extract was subjected to luciferase and MTT assays in T cells, based on a BGFI principle. The crude extract of *Bidens pilosa* was further fractionated into butanol and water subfractions using *n*-butanol (1 l × 3). Each fraction was evaporated on a vacuum rotary evaporator, under reduced pressure, to remove organic solvent, lyophilized and determined the yields. The yields of the butanol and water fractions were 10.2 and 56.2 g, respectively.

2.2. Isolation of centaurein and centaureidin

The BuOH fraction was subsequently chromatographed over RP-18 silica gel with a MeOH/H_2O gradient solvent system. Centaurein and centaureidin were isolated from this fraction and subjected to luciferase and MTT assays. The structure of centaurein was elucidated as published elsewhere (Chiang et al., 2004). The ^{1}H NMR of centaureidin was obtained from Bruker Avance 600 AV spectrometer (600 MHz, CD_3OD) and chemical shifts of ^{1}H were as follows: δ 7.64 (1H, dd, J = 8.4, 1.8 Hz), 7.60 (1H, d, J = 1.8 Hz), 7.07 (1H, d, J = 8.4 Hz), 6.48 (1H, s), 3.93, 3.86, 3.78 (each 3H, s). ^{13}C NMR data (CD_3OD) of centaureidin were in good agreement with the published data (Flamini et al., 2001). The purities of centaurein and centaureidin are >95% using ^{1}H NMR and HPLC determination.

2.3. Plasmids

Plasmid pIFN-γ-Luc was composed of a 615-bp human IFN-γ promoter (−487 to +128 bp) fused with luciferase reporter gene. Plasmids pAP-1-Luc and pNFκB-Luc, containing a septameric AP-1 binding site and a pentameric NFκB binding site upstream of a luciferase reporter gene driven by a minimal promoter, a TATA box, were purchased from Stratagene (CA, USA). Plasmid pNFAT-Luc containing a trimeric NFAT binding site linked to a luciferase reporter gene driven by a minimal promoter was a kind gift from Dr. Crabtree (Stanford University, CA, USA). pRL-TK containing thymidine kinase promoter and *Renilla* luciferase reporter gene was purchased from Promega Corp. (WI, USA).

2.4. Cell culture, transient transfection, luminometry and MTT assay

Human leukemic T cells, Jurkat cells, were grown as previously published (Yang et al., 2001). Jurkat cells (10×10^6) in 0.6 ml RPMI 1640 medium were electroporated at 975 μF and 260 V (Bio-Rad Gene Pulser) with various luciferase reporter construct (i.e., pIFN-γ-Luc, pAP-1-Luc, pNFAT-Luc or pNFκB-Luc, 10 μg) plus 1 μg pRL-TK. After a 2-h recovery, the cells were left stimulated with vehicle, PHA (1 μg/ml) or herbal fractions/compounds at the indicated concentrations for 24 h. Dual luciferase reporter assays were performed to measure promoter activity according to the manufacturer's instructions (Promega, WI, USA). The efficiency of transfection, as determined by *Renilla* luciferase activity in the lysate, was used to normalize the activity of firefly luciferase. The normalized firefly luciferase activities are presented in arbitrary units (AU) (Yang et al., 2001). Cell viability was determined using MTT test (Chang et al., 2004).

2.5. Generation of T cell stable clones

Jurkat cells were electroporated with a pIFN-γ-Luc plasmid. After a 24-h recovery, the cells were selected in RPMI 1640 medium supplemented with antibiotic G418 at 1.5 mg/ml for 3 weeks. G418-resistant clones were obtained by a series of limiting dilutions and further analyzed for IFN-γ promoter activity using luciferase reporter assays.

2.6. IFN-γ measurement

Splenocytes from DO11.10 TCR transgenic mice were activated with anti-CD3 (2 μg/ml) and anti-CD28 (3 μg/ml)-coated plates for 48 h. The resting cells were then transferred to a new plate, followed by a treatment with vehicle (mock), positive control (PHA) at 1 μg/ml, and centaurein at 5, 50 and 100 μg/ml for 24, 48 and 72 h. Supernatants from the aforementioned treatments were diluted to determine their IFN-γ concentrations using an ELISA kit (eBioscience, CA, USA).

2.7. Statistical analysis

Data from three independent experiments or more are presented as mean ± standard deviation (S.D.). Student's *t*-test was used for statistical analysis. $p(*) < 0.05$ is considered significant.

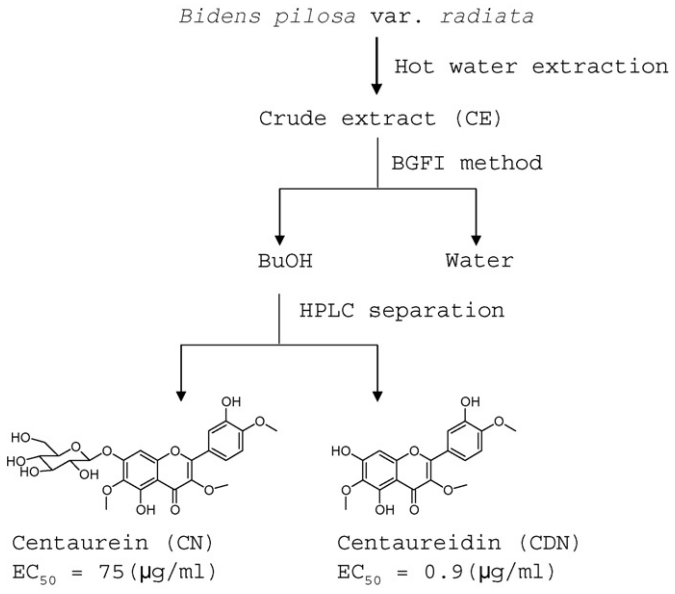

Fig. 1. Flowchart for the identification of bioactive compound(s) from plant crude extracts based on a bioactivity-guided fractionation and isolation (BGFI) method. Based on a BGFI principle, *Bidens pilosa* extract was subjected to luciferase assays in T cells. This extract was further fractionated into butanol (BuOH) and water subfractions using *n*-butanol. Two bioactives, centaurein and centaureidin, were isolated from the active butanol subfraction.

3. Results

3.1. Centaurein and centaureidin, two bioactive flavonoids isolated from Bidens pilosa, can stimulate IFN-γ transcription in a T cell line, Jurkat cells

We first wanted to identify the bioactive compounds from immunomodulatory *Bidens pilosa*. To this end, we combined an IFN-γ promoter, luciferase reporter genes, and Jurkat T cells to analyze bioactive extracts, fractions or pure compounds in a BGFI manner (Fig. 1). We found that *Bidens pilosa* hot water crude extracts at 500 μg/ml could induce a two-fold increase in IFN-γ promoter activity (Table 1). Subsequently, both butanol and water subfractions were obtained from the *Bidens pilosa* hot water crude extracts already partitioned with *n*-butanol. This butanol subfraction at 500 μg/ml increased IFN-γ promoter activity by six-fold (Table 1). This increase may be attributed to the enrichment of bioactive phytocompounds in the butanol fraction. Based on a bioactivity-guided purification, centaurein and centaureidin from the *Bidens pilosa* butanol subfraction were identified as bioactive compounds. We found that centaurein at 100 μg/ml and centaureidin at 2 μg/ml could increase IFN-γ promoter activity to around four-fold (Table 1). PHA treatment is reported to cause T cell activation as well as T cell death, known as activation-induced cell death (Chwae et al., 2002). PHA treatment at 1 μg/ml induced IFN-γ production as well as cell death in T cells (Table 1). Similarly, centaurein treatment at 100 μg/ml and centaureidin treatment at 2 μg/ml induced IFN-γ transcription as well as cell death in T cells (Table 1). In contrast, over 60% of COS cells, a fibroblast cell line, were living when they were incubated with PHA, centaurein and centaureidin at the same concentrations (data not shown), supporting the notion that PHA and the above flavonoids caused cell death in T cells possibly via T cell activation but not cytotoxicity. Overall, centaurein and centaureidin, with the ability to up-regulate IFN-γ transcription, could be identified from *Bidens pilosa* crude extract using the above T cell-based luciferase assays.

3.2. Centaurein, can up-regulate IFN-γ expression in T cell stable clones and splenocytes

We next wanted to confirm the efficacy of centaurein in IFN-γ transcription in stable clones of Jurkat cells. We demonstrated that centaurein at 100 μg/ml up-regulated IFN-γ expression in three different stable clones (Fig. 2A). Therefore, centaurein was able to stimulate IFN-γ transcription in stable clones. Interestingly, transient transfectants seemed to up-regulate IFN-γ transcription a little more than the clones obtained here after various stimulations. We also evaluated the effect of centaurein on IFN-γ production in primary T cells. To this end, we first amplified T cells from the spleens of DO11.10 TCR transgenic mice which exclusively produce $CD4^+$ T cells but not $CD8^+$ T cells. Then we used centaurein to treat these resting T cells pre-treated with anti-CD3 plus anti-CD28 antibodies. Consistent with the data in Jurkat cells, we found that centaurein increased IFN-γ production in mouse splenic T cells (Fig. 2B). These results show that centaurein can boost IFN-γ expression in primary T cells.

3.3. Centaurein up-regulates the activity of NFAT and NFκB enhancers

Transcriptional factors such as AP-1, NFAT, and NFκB are reported to bind to IFN-γ promoter and regulate IFN-γ transcrip-

Table 1
Effects of the crude extract, subfraction and flavonoids of *Bidens pilosa* and PHA on IFN-γ promoter activity and cell viability

Treatment	Dose (μg/ml)	pIFN-γ (folds)[a]	*p*-Value[b]	Cell viability[c]
Crude extract (CE)	500	1.87 ± 0.29	0.0070	81.19 ± 5.20
BuOH subfraction	500	5.91 ± 0.71	0.0003	78.29 ± 3.47
Centaurein (CN)	100	4.19 ± 0.61	0.0008	22.59 ± 0.84
Centaureidin (CDN)	2	3.64 ± 0.43	0.0004	22.14 ± 0.80
PHA[d]	1	6.02 ± 0.88	0.0006	28.65 ± 1.07

[a] IFN-γ promoter activity in folds was obtained from the ratio of the normalized firefly luciferase activity in treated cells to that in control (PBS) cells.
[b] *p*-Value was determined using Student's *t*-test.
[c] Cell viability was determined using MTT test.
[d] PHA was used as a positive control.

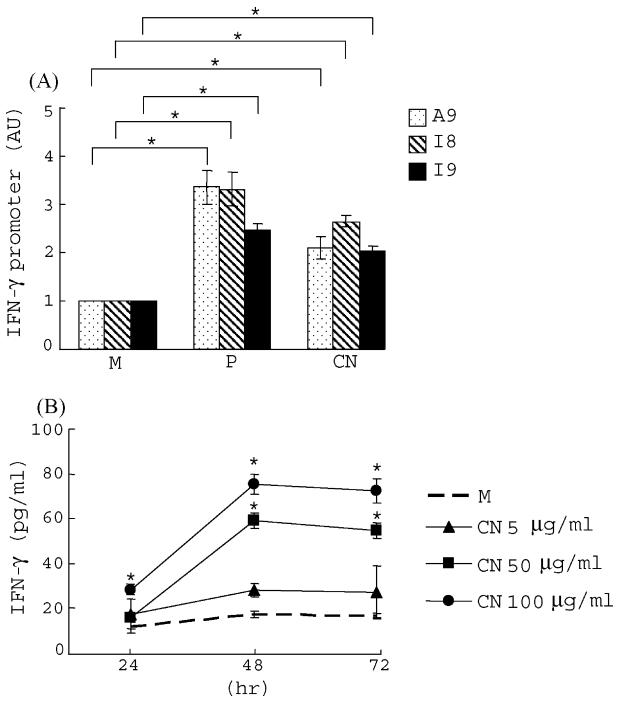

Fig. 2. Centaurein can elevate IFN-γ promoter activity in Jurkat stable clones and primary T cells. (A) Jurkat stable clones (A9, I8 and I9) were left stimulated with vehicle (M), PHA (P), or centaurein (CN, 100 μg/ml). The IFN-γ promoter activity in AU is obtained by normalizing the firefly luciferase activity of different treatments to that of the vehicle (M). (B) Mouse splenocytes from DO11.10 TCR transgenic mice were stimulated with anti-CD3 and anti-CD28 antibodies, followed by a second run of stimulation with vehicle (M) or centaurein (CN) at 5, 50 and 100 μg/ml. The concentration of IFN-γ was obtained using an ELISA kit (lower panel). Data representative of three independent experiments were expressed as mean ± S.D. and $p(*) < 0.05$ is considered statistically significant.

tion in T cells (Penix et al., 1996; Sica et al., 1997; Sweetser et al., 1998). To better understand the mechanism by which centaurein augments IFN-γ transcription, we tested whether centaurein can modulate the aforementioned transcriptional factors. As anticipated, PHA induced a 23-fold increase in NFAT enhancer activity, a 3-fold increase in NFκB enhancer activity and a 10-fold increase in AP-1 enhancer activity in Jurkat cells. In contrast, centaurein caused a four-fold increase in NFAT enhancer activity and three-fold in NFκB enhancer activity in Jurkat cells. However, centaurein had, if any, little effect on AP-1 enhancer activity in Jurkat cells. Therefore, we concluded that centaurein modulates IFN-γ expression possibly via both NFAT and NFκB.

4. Discussion and conclusion

Bidens pilosa has been claimed as an anti-infectious or immunomodulatory folk medicine. Here, we evaluated the immune efficacy of *Bidens pilosa*, as evidenced in the up-regulation of IFN-γ, a potent cytokine in many immunomodulatory aspects. Moreover, we have effectively identified two bioactive flavonoids from *Bidens pilosa* with the ability to stimulate IFN-γ expression using a BGFI method. It was reported that centaurein and centaureidin were synthesized in *Bidens pilosa* or other plants (Chiang et al., 2004). However, their biological functions remained unknown. In this paper, we, for the first time, manifested that centaurein and its aglycone, centaureidin, were able to modulate IFN-γ transcription. In this paper, centaurein was used to further study how both flavonoids could stimulate IFN-γ transcription. Our results showed that centaurein could activate the enhancer activity of NFAT and NFκB, involved in the IFN-γ transcription. Therefore, we postulated that centaurein mediated IFN-γ expression through nuclear factors such as NFAT and NFκB. However, it is still possible that centaurein up-regulate IFN-γ expression via other nuclear factors whose promoters have NFAT and NFκB enhancer elements (e.g., T-bet). Our data manifested that centaurein or centaureidin can in vitro boost IFN-γ production. Their in vivo function in immune modulation (e.g., pathogen clearance) needs to be further verified in animal models.

We here proved the concept that a combination of IFN-γ promoter, luciferase as a reporter gene, and T cells can be used to screen immunomodulatory phytochemicals from the *Bidens pilosa* plant, traditionally used as a folk medicine to improve immunity and infections. This screening method may be further improved and developed into a high throughput platform for evaluating and screening other immunomodulatory herbs, fractions and compounds. Although the use of luciferase as a reporter gene in biological assays is not a brand-new idea, yet it is relatively rapid, sensitive, cost-effective, and feasible for robotization (New et al., 2003). The effective dose of centaurein used here to stimulate the IFN-γ production is relative high (50–100 μg/ml). In contrast, centaureidin, an aglycone of centaurein, at 2 μg/ml has similar effect as centaurein at 100 μg/ml on IFN-γ stimulation. Of note, we proved that compounds with a low or high potency were able to be identified in our experimental setups.

PHA, a T cell stimulant, can activate T cells to produce cytokines like IFN-γ and then cause T cell death. This phenomenon is known as activation-induced cell death (Chwae et al., 2002). We also observed that similar to PHA, centaurein and centaureidin in some cases induced IFN-γ transcription as well as apoptosis in T cells (Table 1). However, PHA and both flavonoids at the same dose showed a marginal effect on the cell death of a non-T cell line, COS cells (data not shown), suggesting that the significant effect of the above compounds on T cell death was partially ascribed to activation-induced cell death. How both flavonoids can cause T cell activation-induced death needs to be further examined.

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