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Isoeleutherin and eleutherinol, naturally occurring selective modulators of Th cell-mediated immune responses

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ABSTRACT

Natural compounds possessing naphthopyran moiety have been attracted by their anti-bacterial, anti-fungal, and anti-viral activities, as well as anti-tumor activities. Although chemical structures were critical for the potential biological activities, the detailed functional mechanisms remained unclear. Here, we have studied the effects of naphthopyran derivatives (eleutherin, isoeleutherin, and eleutherinol) on T helper cell-mediated immune responses to understand the mechanisms of their anti-microbial and anti-tumor activities. The study revealed that isoeleutherin, which has 1,4-naphthoquinone ring with α-methyl group, selectively and specifically stimulated IFNγ production through the activation of T-bet gene transcription, thus enhancing Th1-mediated immune responses. However, a natural naphthopyran-4-one, eleutherinol dramatically inhibited both IFNγ and IL-2 productions during Th cell activation by suppressing the gene transcriptions of cytokines. Therefore, we suggest that the chemical modification and chirality of naphthopyran moiety in isoeleutherin and eleutherinol may be critical for the selective modulation of T helper cell-mediated immune responses.

Pyranonaphthoquinones are a diverse compound family of naphthopyran derivatives, which are naturally occurring naphtho[2,3-c]pyran-5,10-diones widespread in bacteria, fungi, aphides, and higher plants [1–6]. They have been known to have important biological activities such as anti-fungal, anti-viral, and anti-tumor activity as well as antibiotics [3,5,7–9]. A variety of naphthopyran derivatives have been isolated and identified as natural phytochemicals [1,3–5]. Eleutherin and isoeleutherin are the simplest and major naphthopyran possessing 1,4-naphthoquinone moiety isolated from *Eleutherine bulbosa* [6] and *Eleutherine americana* (Iridaceae) [10]. Numerous numbers of pyranonaphthoquinones including eleutherin, isoeleutherin, elecanicin, eleutherol, and eleutherinone possessing 1,4-naphthoquinone moiety and eleutherinol, a natural naphthopyrone are known as natural products in Eleutherine genus in Iridaceae family [3,5,6,11]. Plants classified to Eleutherine have been used as a folk medicine for treating heart diseases such as angina pectoris and intestinal infections [12,13] and reported to show inhibitory activity against HIV infection [14]. In addition, Krishnan and Bastow [15] described anti-tumor activity of eleutherin by the inhibition of topoisomerase II with stereospecific and selective inhibitory activity. The studies on chemistry of constituents in Eleutherine plants are well established and support therapeutic potentials as anti-infectious and anti-tumor drugs, however, no relation could be established between the chemical constituent and potential mechanisms for biological activities.

Activation of CD4+ T helper (Th) cells and differentiation into effector Th1 cells are critically required for anti-bacterial, anti-viral, and anti-fungal activities as well as inflammation [16,17]. TCR engagement of CD4+ Th cells polarizes into two major subsets of effector Th cells, such as Th1 and Th2 cells [18–20]. Two subsets, Th1 and Th2 cells, are distinguished from the master cytokines they produce. While Th2 cells produce signature cytokines such as IL-4, IL-5, and IL-13 and are involved in antibody-mediated humoral immune responses, Th1 cells activate IFNγ expression and thus are responsible for the subsequent activation of macrophages and cytotoxic CD8+ T cells to kill microbes [21]. Cell lineage commitment into either Th1 or Th2 cells is regulated by environmental cytokine milieu and also specific transcription factors. GATA-3 is a well-known Th2-specific transcription factor, which exclusively expressed in Th2 cells and strongly activates gene transcription of Th2 cytokines [22]. On the other hand, T-bet is a master regulator of Th1 cells. The facts that the ectopic expression or knockout of T-bet gene dramatically increased or impaired IFNγ productions [23,24] convinced T-bet is critically required for both IFNγ production and Th1 cell differentiation.

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Here, we studied the effects of two representative pyranonaphthoquinones, eleutherin and isoeleutherin, and a naphthopyrone, eleutherinol, on the activation and differentiation of Th cells. We observed that isoeleutherin, a major constituent of *E. americana* significantly stimulated IFNγ production and induced Th1 cell differentiation, which may contribute to anti-microbial and anti-tumor activities. Moreover, we found that a natural naphthopyran-4-one, eleutherinol inhibited Th cell activation, suggesting that the chemical modification and chirality of naphthopyran moiety may be critical for the selective modulation of T helper cell-mediated immune responses.

Materials and methods

Isolation and purification of eleutherin, isoeleutherin, and eleutherinol. The bulbs of *E.* americana Merr. Et Heyne (Iridaceae) were collected at Batu Herba Medica Centre, East Java, Indonesia, in May 2005. Eleutherin, isoeleutherin, and eleutherinol were purified from the methanol extracts by column chromatography and subsequent semi-preparative HPLC separation with the 98% purity. The structures were confirmed by NMR and presented in Fig. 1A.

Reagents. Recombinant human IL-2, anti-CD3, and anti-CD28 antibodies and PE-conjugated annexin V and IFNγ antibodies were purchased from BD Pharmingen (San Diego, CA). All cytokines and antibodies for ELISA were from BD Pharmingen. Monensine was obtained from Sigma-Aldrich Inc. (St. Louis, MO).

Mice. Wild type C57BL6 and T-bet knockout/BL6 mice were housed in specific pathogen-free conditions at Ewha Womans University. All mice handling and experiments were done in accordance with Institutional Animal Care and Use Committee guidelines.

Isolation and activation of CD4+ Th cells in vitro. Single cells were isolated form lymph node and spleen of wild type mice and incubated with mouse CD4 micro beads for 30 min according to the manufacturer's instruction (Miltenyi Biotech., Auburn, CA). Single cell suspensions of CD4+ Th cells were isolated and stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). Recombinant human IL-2 (100 U/ml) was added up to enhance T cell activation and proliferation.

Intracellular cytokine staining and annexin V staining. Cells were fixed with 4% paraformaldehyde solution, rinsed with permeabilization buffer (0.1% saponin, 0.1% sodium azide, 1% FBS in PBS), and incubated with either PE-conjugated anti-IFNγ Ab or PE-conjugated annexin V. Cells were washed twice with FACS buffer

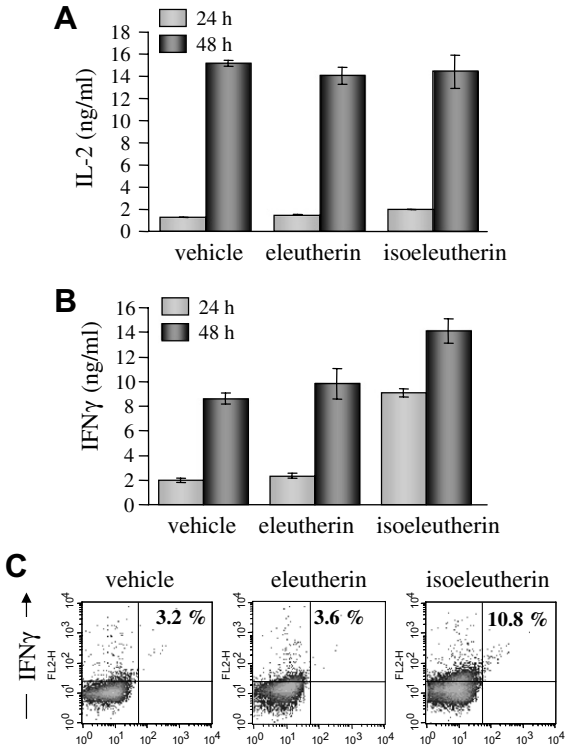

Fig. 2. Stimulation of IFNγ production from Th cells by isoeleutherin. Isolated CD4+ Th cells were incubated with 10 μM concentration of either eleutherin or isoeleutherin concomitantly with TCR stimulation. Supernatants were collected from 24 to 48 h stimulated Th cells for measuring cytokines, IL-2 (A) and IFNγ (B). (C) Monensine was added to the activated Th cells 2 h prior to harvest and collected for intracellular cytokine staining. Activated Th cells were incubated with PE-anti-IFNγ Ab followed by flow cytometric analysis.

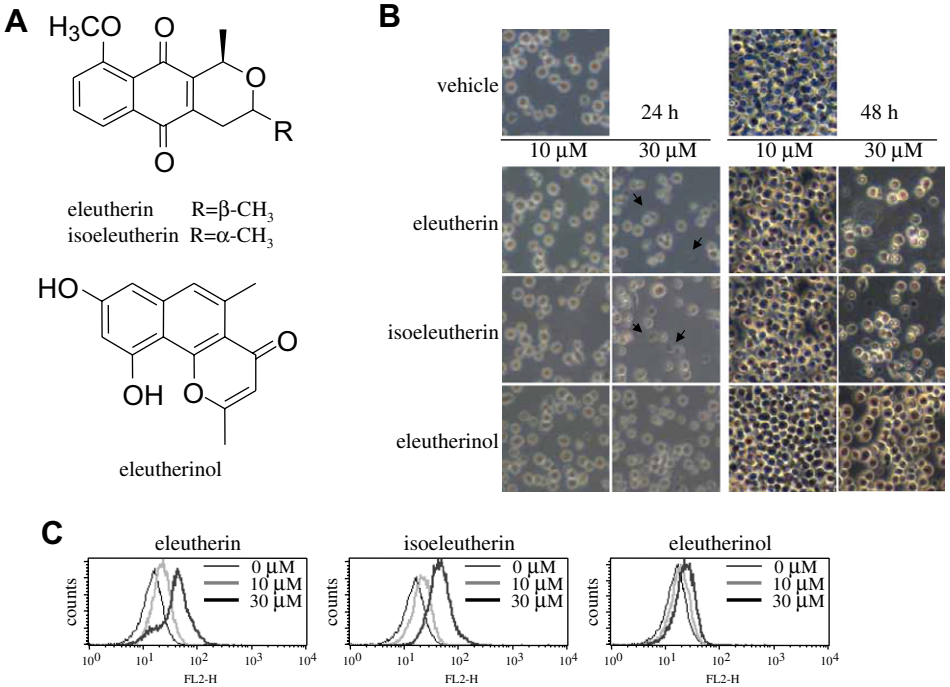

Fig. 1. Effects of naphthopyran derivatives on Th cell activation upon TCR stimulation. (A) Structures of naphthopyran derivatives, eleutherin, isoeleutherin, and eleutherinol isolated from *E. americana*. (B) Cell morphologies of Th cells upon TCR stimulation in the presence of pyranonaphthoquinones. CD4+ Th cells isolated from lymph node and spleen were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) for 24 or 48 h with different concentrations of the compounds as indicated. (C) Apoptosis assay by annexin V staining. Different doses of compounds were treated in Th cells triggered by TCR for 48 h. Activated Th cells were incubated with PE-annexin V and analyzed by flow cytometric analysis.