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Effects of Isoflavonoids on Mouse Lymphocyte Proliferation *In Vitro*

Soon Young Namgoong[1], Chang Hee Lee[2] and Hyun Pyo Kim[1]

[1]*College of Pharmacy, and* [2]*Dept. of Chemistry, Kangweon National University, Chuncheon 200-701, Korea*

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The suppressive activity of isoflavonoids against lymphocyte proliferation *in vitro* was examined. Isoflavonoid derivatives tested were isoflavones isolated from *Pueraria radix* and synthesized 7-O-substituted biochanin A derivatives. The certain isoflavones such as biochanin A and 2-carbethoxybiochanin A were found to possess the suppressive activity against concanavaline A (Con A)-induced lymphocyte proliferation from mouse spleen. Against mixed lymphocyte culture reaction, biochanin A, 2-carbethoxybiochanin A, daidzein, formononetin, genistein and 7-O-isopropylbiochanin A showed the suppressive activity at 10^{-5} M. However, all isoflavones tested did not show the suppressive activity against lymphocyte proliferation induced by B-cell mitogen, lipopolysaccharide (LPS). In general, isoflavones were revealed to be less active than flavones/flavonols.

Key words: Isoflavonoid, Lymphocyte proliferation, Structure-activity relationship, *Pueraria radix*, Biochanin A, Daidzein, Genistein

INTRODUCTION

Flavonoids have been reported to possess the immunomodulatory activities *in vivo* and *in vitro* (Middleton and Kandaswami, 1992; Berg and Daniel, 1988). Several authors demonstrated that the certain flavonoid derivatives (quercetin, tangeretin, etc.) showed the suppressive activity against mitogen-induced lymphocyte proliferation (Mookerjee *et al.*, 1986; Pignol *et al.*, 1988; Hirano *et al.*, 1989). In order to establish the structure-activity relationship of flavonoids, we have examined the effects of 34 structurally diverse flavonoid aglycones and glycosides commercially available or isolated from various medicinal plants on lymphocyte proliferation, and mainly flavone and flavonol derivatives having a C-ring 2,3-double bond were found to show the suppressive activity against concanavalin A (Con A)-induced lymphocyte proliferation and mixed lymphocyte culture reaction (MLC) at higher than 10^{-6} M (Namgoong *et al.*, 1994). It was also found that various glycosidic substitution(s) to A- and/or C-ring of flavonoid aglycones eliminated the suppressive activity of their aglycones, regardless of sugar compositions and positions of substitutions. It is worthy of studying the suppressive activity of isoflavonoids against lymphocyte proliferation because isoflavonoids are also widely distributed in plant kingdom and especially in medicinal plants. For this purpose, we have isolated the several isoflavones from *Pueraria radix* and synthesized the 7-O-substituted biochanin A derivatives (Lee *et al.*, 1994). In this investigation, the suppressive activity of isoflavonoids against lymphocyte proliferation was examined and the structure-activity relationship was discussed.

Correspondence to: Hyun Pyo Kim, College of Pharmacy, Kangwon National University, Chuncheon, 200-701, Korea

MATERIALS AND METHODS

Animals and Reagents

Male C57BL/6 (H-2^b) and C3H/N (H-2^k) mice were kept in our animal facility under the conditions of 22± 1℃, 12 hr/12 hr (L/D) cycle and sacrificed as a spleen source at 6-7 weeks of age. Biochanin A, 2-carbethoxybiochanin A and apigenin were purchased from Aldrich Chem. Co. (USA). The 7-O-substituted derivatives of biochanin A such as 5-O-methyl-, ethyl-, isopropyl-, isobutyl-, allyl- and 5,7-O-dimethyl biochanin A were chemically synthesized from biochanin A. Daidzein, formononetin, puerarol, genistein, daidzin, puerarin and PG-3 were isolated from the methanol extract of *Pueraria radix*. The synthetic and isolating procedures of these derivatives were described elsewhere (Lee *et al.*, 1994). The chemical structures of isoflavonoids used

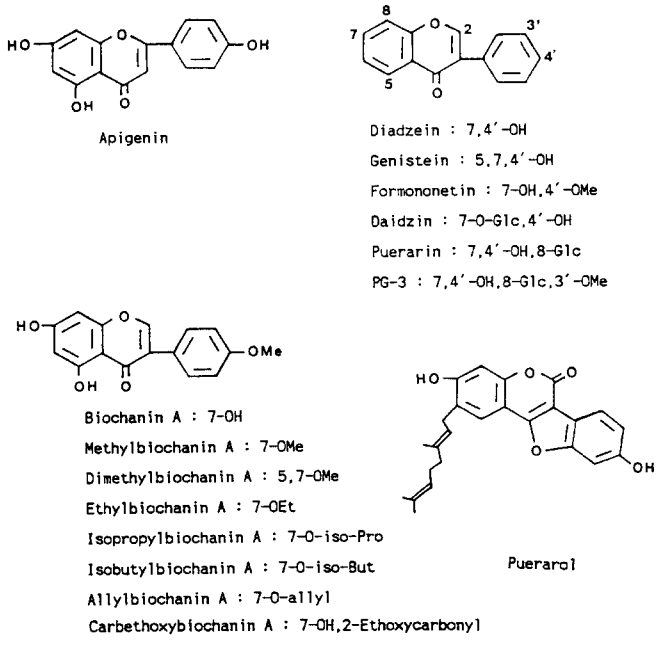

Fig. 1. Chemical structures of flavonoids.

in this study were shown in Fig. 1. Stock solutions of flavonoids were prepared by dissolving in DMSO and diluted with RPMI 1640. Concanavaline A (Con A) was purchased from Pharmacia (Sweden) and lipopolysaccharide (LPS, *E. coli* 0127 : B8) was from Sigma Chem. Co. RPMI 1640, phosphate buffered saline (PBS), glutamine, antibiotic solution and fetal calf serum (FCS) were obtained from Gibco Lab. (USA). [^{3}H]-Thymidine (6.7 Ci/mmol) was purchased from NEN. All the other reagents used were of the highest grades available.

Suppression of Isoflavones Against Lymphocyte Proliferation *In Vitro*

All detailed procedures were same as previously described (Namgoong *et al.*, 1994). Briefly, splenocytes from C57BL/6 mice were used and an optimal concentration of a mitogen used was 2.5 μg/ml for Con A or 75 μg/ml for LPS. Lymphocytes (2×10^5 cells/200 μl/well) were incubated in 96-well cell culture plate (Nunc) using RPMI 1640 (10% FCS) media. Total incubation time was 72 hrs at 37℃ in a 5% CO_2 incubator and [^{3}H]-thymidine (0.2 μCi/well) was added to each well 18 hrs before harvesting the cultures with multiple cell havestor (Skatron). Radioactivity of the dried filter was counted on a liquid scintillation counter (Pharmacia).

For one way mixed lymphocyte culture reaction (MLC), mitomycin C-treated splenocytes from C3H mice were used as target cells and splenocytes from C57BL mice were used as responder cells (ratio of target to responder cells = 2 : 1). Total incubation time was 96 hrs and the other procedures were exactly same as the procedures of Con A-induced lymphocyte proliferation.

The flavonoids were added simutanuously with addition of a mitogen or target cells at the initiation of culture. All lymphocyte cultures were performed in triplicates and the data are given as arithmatic mean and standard deviation (DPM ± SD) of [^{3}H]-thymidine uptake. Student t-test was employed for evaluating the statistical significance. Stimulation Index (S.I.) was calculated as follows;

$$\text{S.I.} = \frac{\text{DPM of tested group} - \text{DPM of control group without mitogen}}{\text{DPM of mitogen-treated group} - \text{DPM of control group without mitogen}}$$

RESULTS AND DISCUSSION

We have previously found that the several flavone and flavonol derivatives having a C-2,3-double bond showed more or less the suppressive activity against lymphocyte proliferation induced by T-cell mitogen, or alloantigenic stimulation (IC_{50} = 1 − 10 μM). For comparing with flavones/flavonols, the suppressive activity of isoflavones (having a C-2,3-double bond) against lymphocyte proliferation was examined.

As shown in Fig. 2a and 2b, certain isoflavones were found to be active at 10^{-5} M against lymphocyte proliferation induced by T-cell mitogen (Con A) or alloantigenic stimulation (MLC). Fig. 2a has shown that biochanin A, carbethoxybiochanin A and puerarol were significantly active at 10^{-5} M (72%, 58% and 62% suppression, respectively) against lymphocyte proliferation induced by T-cell mitogen (Con A). The other isoflavones isolated from *Pueraria radix* or biochanin A derivatives synthesized did not show the suppressive activity. Against one way mixed lymphocyte culture reaction, the several derivatives including biochanin A, carbethoxybiochanin A, daidzein, genistein, formononetin, puerarol and 7-O-isopropylbiochanin A showed the suppressive activity (Fig. 2b). The most active one was biochanin A. However, all isoflavones tested did not show the significant suppression against lymphocyte proliferation induced by B-cell mitogen (LPS) up to the concentrations of 10^{-5} M (Fig. 2c). All isoflavone glycosides obtained from *Pueraria radix* were not active at 10^{-5} M against Con A/LPS-induced lymphocyte proliferation or MLC. Fig. 3 demonstrated the dose-dependent suppressions of biochanin A and apigenin (flavone) against mitogen-induced lymphocyte prolife-

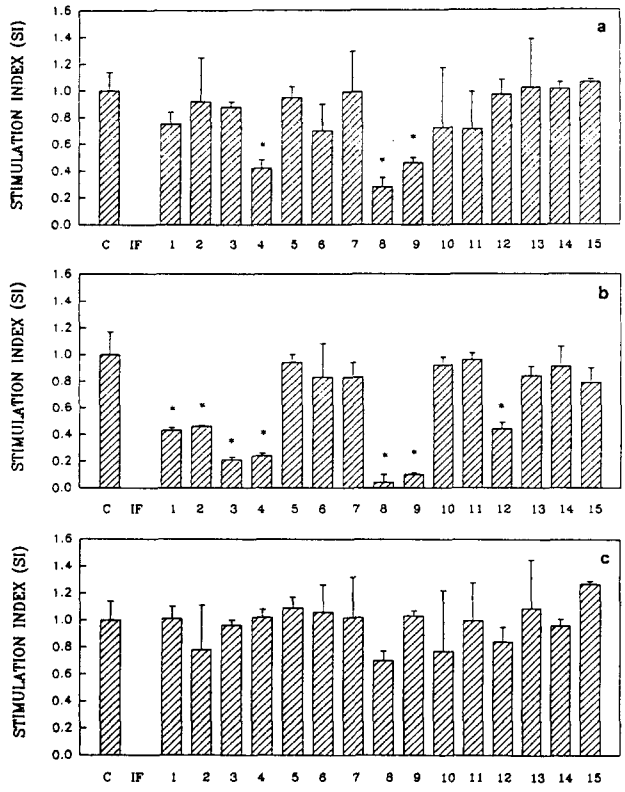

Fig. 2. Effects of isoflavones on mitogen-induced lymphocyte proliferation and mixed lymphocyte culture reation. All compounds were incubated at 10^{-5} M. *: $P<0.001$, significantly different from control. Control (C), daidzein (IF-1), formononetin (2), genistein (3), puerarol (4), daidzin (5), puerarin (6), PG-3 (7), biochanin A (8), carbethoxybiochanin A (9), 7-O-methyl-biochanin A (10), 7-O-ethylbiochanin A (11), 7-O-isopropyl-biochanin A (12), 7-O-isobutylbiochanin A (13), 7-O-allylbiochanin A (14), 5,7-O-dimethylbiochanin A (15).
(a) Con A-induced lymphocyte proliferation, C: Con A-treated control without isoflavones (37,480± 5,395 DPM).
(b) Mixed lymphocyte culture, C: Alloantigen-treated control without isoflavones (18,975± 3,238 DPM).
(c) LPS-induced lymphocyte proliferation, C: LPS-treated control without isoflavonoids (8,377± 1,462 DPM).

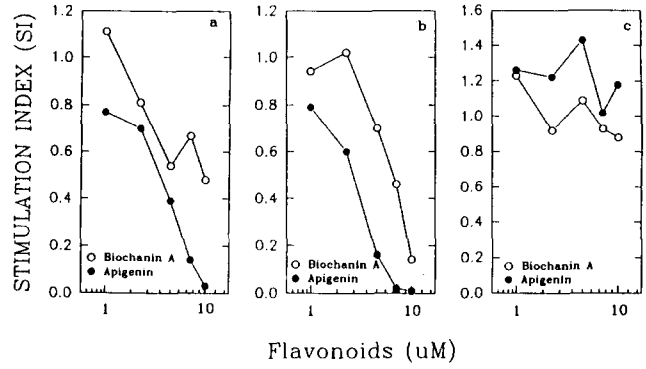

Fig. 3. Dose-dependent suppression of biochanin A and apigenin against lymphocyte proliferation.
(a) Con A-induced lymphocyte proliferation
(b) Mixed lymphocyte culture reaction
(c) LPS-induced lymphocyte proliferation

ration and MLC. IC_{50} values of biochanin A were found to be 5-10 μM for the suppression of Con A-induced lymphocyte proliferation and MLC. Biochanin A was revealed to be less active than apigenin.

Because Con A is a T-cell mitogen and T-lymphocytes (Tcyt) are believed to play an important role in MLC reaction, it is suggested that isoflavones may affect T-cell proliferation rather than B-cell proliferation. We observed the same phenomenon with flavones/flavonols (Namgoong *et al.*, 1994). Although Hirano *et al.* (1989) reported that daidzein showed the suppressive activity against Con A-induced lymphocyte proliferation (IC_{50}=approximately 4 μM), daidzein and genistein did not showed the suppressive activity against Con A-induced lymphocyte proliferation (IC_{50}>10 μM) in our experiment. This difference in potency by isoflavones may be partly explained by the different culture conditions used including sources of lymphocytes (i.e., human peripheral lymphocytes vs murine splenocytes). Generally, isoflavones were revealed to be less active than flavones/flavonols. It was interesting to note that genistein, a recently discovered protein tyrosine kinase (PTK) inhibitor (Akiyama *et al.*, 1987; Punt *et al.*, 1989), did not show the potent suppression of lymphocyte proliferation.

When the cytotoxicity of isoflavones were checked using trypan blue exclusion test, isoflavones such as biochanin A and daidzein at 10^{-5} M did not reduce the viability of splenocytes without a mitogen for 24 hrs (Data not shown). These results indicated that the suppressive activity of isoflavones against lymphocyte proliferation was not due to the direct cytotoxic effects of isoflavones.

From this study, the following structure-activity relationship could be deduced: 1. Isoflavones were generally less active than flavones/flavonols against lymphocyte proliferation induced by T-mitogen (Con A) or alloantigenic stimulation (i.e., apigenin vs genistein/biochanin A)., 2. Isoflavones showed the relative selectivity of suppression against T-lymphocyte proliferation rather than B-lymphocyte proliferation., 3. Glycosylation to isoflavone aglycones eliminated the suppressive activity of their parent aglycones (i.e., daidzein vs puerarin/daidzin/PG-3)., 4. The various alkyl substitutions at A-ring 7-OH of biochanin A reduced (or eliminated) the suppressive activity (i.e., biochanin A vs synthesized biochanin A derivatives)., 5. 5-Hydroxyl group at A-ring may be important to show the suppression of lymphocyte proliferation (i.e., biochanin A vs formononetin).

In conclusion, certain isoflavones having a C-2,3-double bond showed relatively T-cell specific suppression against lymphocyte proliferation induced by mito-

gen/alloantigen *in vitro*, although isoflavones were generally less active than flavones/flavonols. This effect may possibly contribute to the known anti-inflammatory and immunomodulatory actions of flavonoids *in vivo*.

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