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Baohuoside-1 inhibits activated T cell proliferation at G_1–S phase transition

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Abstract

Background: The effect of baohuoside-1 (B1), a novel flavonoid, on cell proliferation and the cell cycle was evaluated in this study.
Methods: The antiproliferative properties of B1 were evaluated by proliferation assay. Western blotting and flow cytometric analysis were employed to investigate the expression of cyclins and cyclin-dependent kinase proteins.
Results: The major findings were (1) B1 effectively inhibited the cell proliferation activated by mitogenic antigen, with a 50% inhibitory concentration in low μM and in a dose- and time-dependent manner. (2) B1 resulted in G_1–S phase cells arrest. (3) It down-regulated the expression of cyclin A, D and p33 cyclin-dependent kinase-2 (p33cdk2) proteins. (4) B1 suppressed the growth of several tumor cell lines. (5) B1 prevented rat heart allograft rejection in vivo.
Conclusions: B1 immunosuppression of mitogen-activated T cell proliferation occurs in G_1–S transition. It may be associated with the expression of cyclin A, D and p33cdk2 proteins. B1 prevents rat heart allograft rejection in vivo. The mechanism of B1 is different from tacrolimus and sirolimus.

Keywords: Baohuoside-1; Cell cycle; Immunosuppressant; Tacrolimus

1. Introduction

Immunosuppressive agents that bind immunophilins, such as cyclosporin A (CsA), tacrolimus (FK506), and sirolimus (RAPA), have been proven not only to contribute in the fields of organ transplantation and some autoimmune diseases, but also represent useful molecular tools for dissecting the intracellular signal transduction pathway involved in T cell activation [1]. However, despite their widespread utility in the treatment of acute graft rejection, they are not very potent in reducing chronic rejection. In addition, these immunosuppressants have deleterious side-effects, particularly renal toxicity, and are frequently associated with B cell lymphomas [2]. Thus, current strategies have encouraged the identification and characterization of new immunosuppressive agents that could serve as combined therapy [3]. A crucial aspect in the selection of these new compounds is that they should have different mechanisms of action and cellular targets than currently-used immunosuppressants.

Abbreviations: APCs, antigen presenting cells; B1, baohuoside-1; CsA, cyclosporin A; Con A, concanavalin A; cdks, cyclin-dependent kinases; EBV, Epstein–Barr virus; IC_{50}, 50% inhibitory concentration; FK506, tacrolimus; FACS, fluorescence activated cell sorting; Ig, immunoglobulin; LPS, lipopolysaccharide; MACS, magnetic activated cell sorting; PI, propidium iodide; PMA, Phorbol 12-myristate 13-acetate; PBMCs, peripheral blood mononuclear cells; pRb, retinoblastoma protein; RAPA, rapamycin; SAC, *Staphylococcus aureus* cowan I bacteria.

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The herb *Epimedium davidii* (Berberidaceae), also named *YinYangHuo*, grows in Sichuan, China. One of many herbs in herbal remedies from ancient Chinese pharmacopeia, it was commonly applied in traditional Chinese medicine for the treatment of nephritis, infertility, rheumatism and cancer. A series of flavonoids isolated from *Epimedium davidii* [4] were shown to exert different immunopharmacological properties in humoral and cellular immunity [5]. Baohuoside-1 (B1), a member of the family with the $C_{27}H_{30}O_{10}$ chemical structure and molecular weight of 514 Da, is novel and completely distinct from CsA, FK506 and RAPA [6].

Our previous study has described the B1 immunosuppression of cell proliferation in vitro, and its prolongation of allograft survival in vivo [7]. The compound is encouraging since it efficiently inhibits the proliferation of T and B cells induced by mitogens and mixed lymphocyte reactions (MLRs) in rats and humans, in a dose- and time-dependent manner. This inhibition is not simply due to toxic effects, with recovery upon B1 removal. In the Ca^{2+}-independent and -dependent antigen stimulation pathways, although B1 and FK506 have different inhibitive modes on CD69 expression stimulated by Phorbol 12-myristate 13-acetate (PMA) or Ca^{2+} ionophore, they both block T cell proliferation and reduce CD69 expression induced by anti-CD3/CD28. CD69 expression is also markedly down-regulated through synergy between B1 and FK506. We also found that additional immunosuppression by B1 with FK506 combination therapy in a rat heart transplantation model has a different mechanism of action compared to CsA, FK506, and RAPA.

The aims of this study were to evaluate the effect of B1 on mitogen-activated cell proliferation, cell cycle progression and expression of cyclins and cyclin-dependent kinase (cdk) proteins in vitro. Whether B1 was also able to its ability to inhibit the growth of malignant cells was also investigated.

2. Materials and methods

2.1. Reagents

B1 was obtained from ImmuneTech Development Inc., Montreal, Canada. Its purity (98%) was confirmed by mass spectrometry analysis. B1 and FK506 were prepared as 10^{-2} M stock solution in 50% ethanol. The final ethanol concentration (<0.05%) did not affect either maximal responses or baseline values in any assay [7,8]. Cyclin A, cyclin D, p33cdk2, CD3 and CD28 monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA).

2.2. Animals

Adult male Lewis (LEW; $RT1^l$) and Brown Norway (BN; $RT1^n$) rats were purchased from Charles River (St. Constant, QC) and were maintained in controlled light/dark cycles pathogen free environment and allowed free access to water and chew.

2.3. Cell lines

Human T lymphoma Jurkat, human promyelocytic leukemia HL-60, Burkitt's lymphoma Raji, human lung carcinoma A549 and human cervical carcinoma Hela cell lines were purchased the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured and maintained in complete RPMI 1640 at 37 °C in a 5% CO_2 incubator.

2.4. Cell isolation and purification by magnetic activated cell sorting (MACS)

Human PBMCs were incubated with CD2 antibody-conjugated magnetic microbeads, loaded and isolated by MACS in a depletion ferromagnetic matrix column set within a magnetic field. The purity and viability of T and B cells were confirmed by FACS and trypan blue exclusion (98%).

2.5. Cell proliferation assays

Human T cells were stimulated respectively by Con A (2 μg/ml), anti-CD3 (1 μg/ml)/anti-CD28 (1 μg/ml), PMA (10 ng/ml)/inonmycin (1 μg/ml) in the presence or absence of indicated concentrations (10^{-12} to 10^{-4} M) of B1, or FK506. B cells were stimulated by LPS (10 μg/ml), SAC (1:30,000 dilutions) and anti-IgM (10 μg/ml)/anti-CD40 (10 μg/ml) antibodies, respectively, in the presence or absence of B1, or FK506. After 56 h, the cultures were labeled with 1 μCi of ^{3}H-thymidine for 16 h, and harvested on glass filters. The percentage of inhibition was calculated according to the mean of triplicates from the counts per minute (CPM) of each sample. The IC50 was defined as the drug concentration required to inhibit the proliferation responses of cells by 50%.

2.6. Intracellular fluorescence analyses

Lymphocytes were treated with different concentrations of B1 (10^{-5} M), FK506 (10^{-8} M) and RAPA (10^{-8} M), respectively, and then stimulated with Con A (2 μg/ml) for 72 h. The cells were then fixed and permeabilized thoroughly by adding Cytofix/Cytoperm solution, and stained intracellularly with anti-cyclin A, anti-cyclin D and anti-cdk2 antibodies, respectively. Fluorescence analysis was performed by FACScan (Becton Dickson, CA).

2.7. Measurement of cdk and cyclin proteins

Lymphocytes were treated with of B1 (10^{-5} M), FK506 (10^{-8} M) and RAPA (10^{-8} M), respectively, in the presence of Con A (2 μg/ml) for 36 h. Protein extracts were electrophoresed on separate 12% SDS–polyacrylamide gels and transferred overnight to nitrocellulose membranes. Filters were blocked in PBS containing 0.1% Tween 20 for 1 h, followed by overnight incubation with anti-cyclin A antibody (1 μg/ml), anti-cyclin D antibody (1 μg/ml) and anti-p33cdk2 antibody (1/2500 dilution), respectively. The filters were then washed and incubated with goat anti-rabbit IgG conjugated to peroxidase for 1 h; signals were detected by a chemiluminescence detection system (Bio-Rad, CA) and exposed to Kodak XAR films. These experiments were repeated separately 3 times.