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Specific inhibition effects of *N*-pentafluorobenzyl-1-deoxynojirimycin on human CD4+ T cells

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Abstract—We first synthesized *N*-pentafluorobenzyl-1-deoxynojirimycin (5F-DNM), one new derivative of 1-deoxynojirimycin (DNM). Effects on human peripheral blood mononuclear cells (PMBC) and secretion of cytokines from human PBMC by 5F-DNM were investigated. It was first found that 5F-DNM remarkably inhibited the secretion of interleukin-4 (IL-4) and had a specific inhibition on the expression of CD4 molecules. 5F-DNM, much less toxic than cyclosporin A, might be used as a new candidate of immunosuppressant for specifically treating Th2-mediated immune diseases.

Many critical interactions among cells of the immune system are controlled by soluble mediators, cytokines. Cytokines play important roles in immune and inflammatory responses. IL-4 is a glycoprotein cytokine secreted mainly from activated Th2 cells, mast cells and NK cells. IL-4 is known to play a role in allergic diseases by promoting IgE production. IFN-γ secretion is a hallmark of Th1 lymphocytes. It is also secreted by nearly all CD8+ T cells and NK cells. IFN-γ is an important immunoregulatory cytokine and has found clinical application as an immunostimulator in chronic granulomatous disease and other disorders.

DNM, an inhibitor of glucosidase I and II, inhibits the first steps in the processing of the N-linked glycan precursor, Glc3Man9GlcNAc2. It is also a potential inhibitor of tumor metastasis and viral replication, including that of the human immunodeficiency virus (the causative agent of acquired immunodeficiency syndrome, AIDS).[1–3] DNM could have many kinds of beneficial effects as therapeutic agents that are usually low toxic to the body and have been widely used as therapeutic agents.[4–7] However the effects of the inhibitor on immune system are not well known.[8–10]

In this study, we first synthesized 5F-DNM, a new derivative of DNM. 5F-DNM was synthesized by the procedure shown in Figure 1. *N*-Pentafluorobenzyl and DNM were mixed in DMF solution in the presence of Cs_2CO_3. After 18 h, crude 5F-DNM was obtained and further purified by C-18 column. The purified 5F-DNM was obtained in the yield of 67%. Purification of 5F-DNM was performed by our novel method (Fig. 1).[11] We designed F instead of H in DNM. We succeeded in

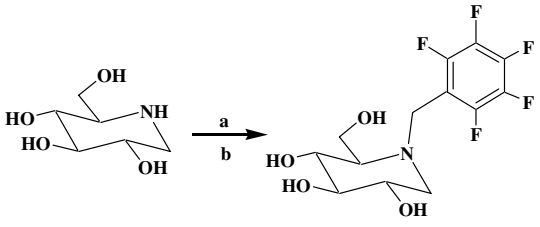

Figure 1. Synthesis of 5F-DNM. Reagents and conditions: (a) *N*-pentafluorobenzyl, Cs_2CO_3, DMF, 18 h; (b) C18, isopropanol/H_2O/$NH_3 \cdot H_2O$ = 190/10/1, 79%.

Keywords: Immunosuppressant; CD4+ T; Inhibitor.
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purifying it by C-18 column, which could remove small molecule salts and retained starting material.

Fresh peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors and separated by Ficoll–Hypaque density gradients. In order to assay the effects of DNM and 5F-DNM on the secretion of cytokines from PBMC, the cells were pretreated with 10 μg/mL concanavalin A (type IV) (Promega) for 4 h and incubated with different concentration of DNM, 5F-DNM, or cyclosporin A (CyA), which is a well known immunosuppression drug specifically inhibiting CD4+ T cells, respectively, for 72 h at 37 °C in medium containing 10% newborn bovine serum (NBS) and 5% CO_2. The secretion of IL-4 was detected from the supernatant of cells by the use of human ELISA kit. 5F-DNM at concentration of 10 μM decreased the secreted IL-4 level about 6-fold lower than that of the control cells (Fig. 2). The inhibition ability to the IL-4 secretion by 5F-DNM is significantly stronger than that of the DNM and is similar as that of CyA. Inhibition efficiency by the compounds was in a dose-dependent manner. All data are the mean of three independent experiments ± SD, $P < 0.01$.

The assay of the secretion of IFN-γ from human PBMCs was similar as the assay of IL-4. Supernatant of cells was detected by human IFN-γ ELISA kit. The level of IFN-γ secretion was obviously decreased by 5F-DNM, when its concentration was more than 30 mM. The inhibition ability of 5F-DNM to the IFN-γ secretion is weakest among those of DNM and CyA (Fig. 3). The inhibition efficiency of the compounds was in a dose-dependent manner.

There are two major types of lymphocytes, T cells, and B cells in human system. T cells give rise to cellular immunity and B cells give rise to humoral immunity, that involves production of immunoglobulin based on the stimulation by antigen. T cells contain two classes of

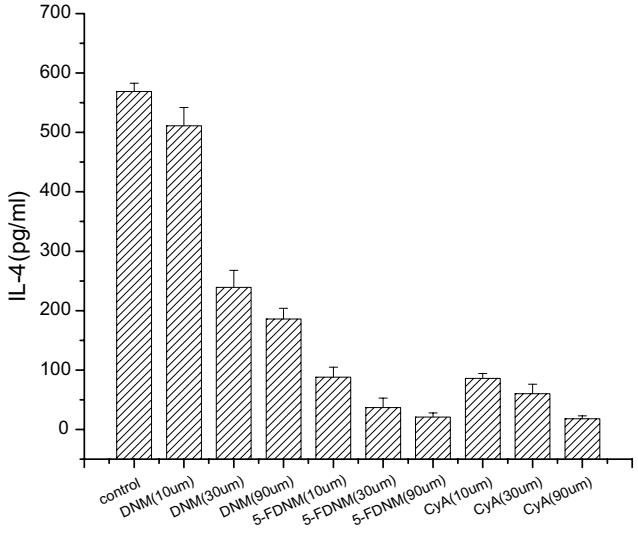

Figure 2. The effects of 5F-DNM, DNM, and CyA on the secretion of IL-4 from human PBMCs.

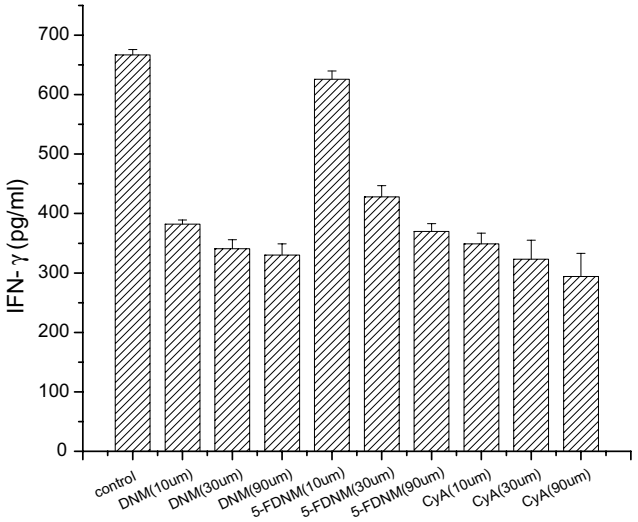

Figure 3. The effects of 5F-DNM, DNM, and CyA on the secretion of IFN-γ from human PBMCs.

T lymphocytes, T helper cells and T cytotoxicity cells, and have specific T cell receptor (CTR) and CD3 molecules on the T cells surfaces. T helper (Th) cells have two major types, Th1 and Th2, express CD4 and provide help for B cell growth and differentiation. T Cytotoxicity (Tc) cells express CD8 and recognize viral antigens presented on the surface of infected cells, and kill these cells.

Human PBMCs pretreated with 10 μg/mL concanavalin A (type IV) for 4 h were incubated with different concentrations of DNM, 5F-DNM, or CyA, respectively, at 37 °C for 72 h in the medium containing 10% newborn bovine serum (NBS) and 5% CO_2. Briefly, 10 μL of FITC conjugated mouse anti-human CD4 antibody and 10 μL of PE conjugated mouse anti-human CD8 or CD19 antibody (Caltag laboratories) were incubated with 1×10^6 PBMCs in the dark for 20 min at room temperature. The stained cells were analyzed with a Beckman Coulter EPICS ALTRA II flow cytometer. FITC or PE conjugated mouse IgG1 isotype controls were used as isotype matched negative controls. It was first found that 5F-DNM had a specific inhibition on the expression of CD4, which are markers of CD4+ T cells (Fig. 4A), minor inhibition on CD19, which are markers of B cells (Fig. 4B), and minor increase effects on CD8, which are markers of CD8+ T cells (Fig. 4C). The reasons of those difference are not well known. We speculate that two factors maybe contribute to the differences. Firstly, the difference is due to different numbers of N-glycans expressed on CD4 and CD8. It is known that human CD8 has only one N-glycosylation site while human CD4 has two N-glycosylation sites. The more N-glycosylation sites there are, the stronger inhibition effects of the glycoprotein processing inhibitors maybe have; Secondly, in different immune molecules, N-glycans are located in different structural regions of glycoprotein, which are quite fundamental for the maturation, function, and expression of the glycoprotein. Human CD19 has six potential N-glycans, however the inhibition on CD19 is milder than that of

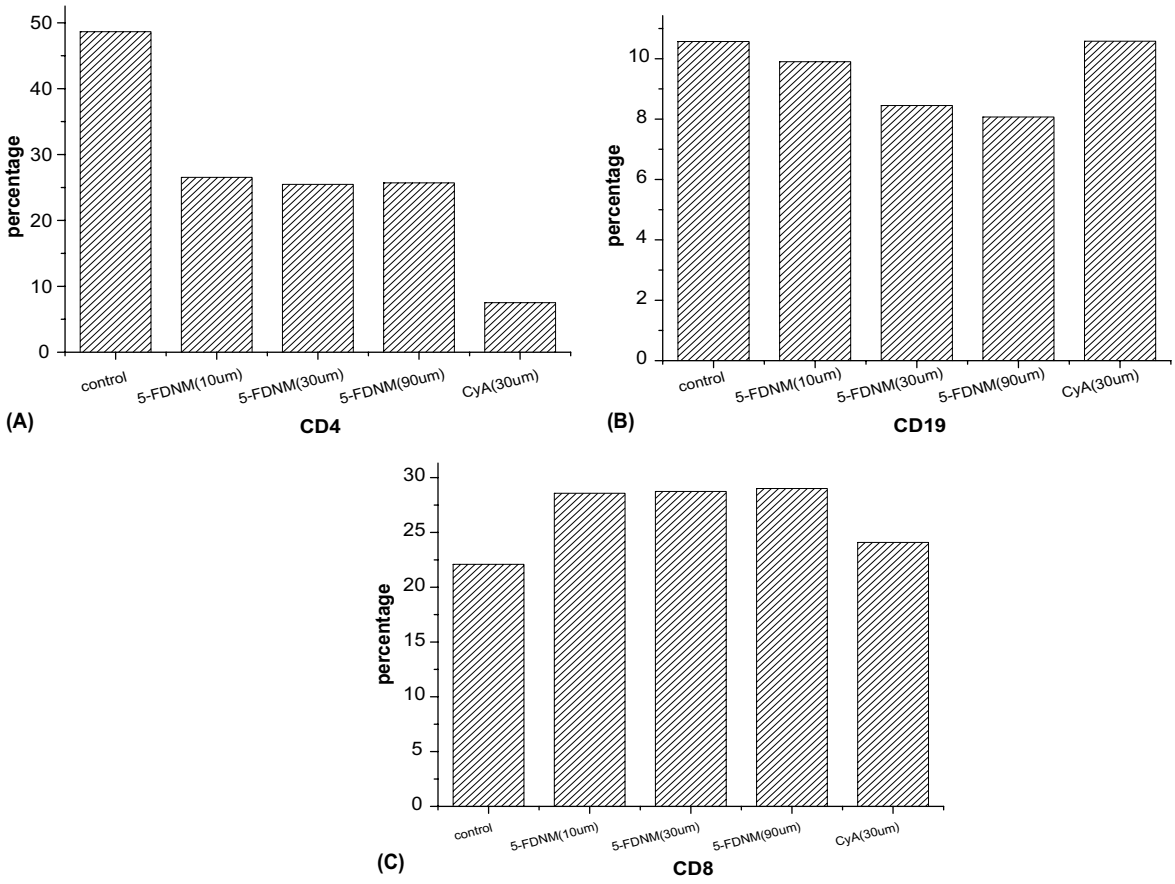

Figure 4. Effects of 5F-DNM and CyA on the expression of CD4 (A), CD19 (B), and CD8 (C) in human PBMCs.

CD4, the reason is maybe due to different structural location of the N-glycans. Not well known of how the sugar chain contributes to glycoprotein function and less known of the specific effects of these inhibitors limit our explaining the differences.

Two subsets of Th lymphocytes, Th1 and Th2, differ in their cytokine secretion patterns.[12] The Th1 subset of CD4+ T cells secretes cytokines usually associated with inflammation such as interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β), and induces cell-mediated immune responses. The Th2 subset produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and is associated with humoral immune responses. Th1 responses predominate in organ-specific autoimmune disorders, acute allograft rejection, and in some chronic inflammatory disorders. In contrast, Th2 responses mainly in Omenn's syndrome, transplantation tolerance, chronic graft-*verse*-host disease, systemic sclerosis, and allergic diseases.[4]

The 50% inhibitory concentrations (IC_{50})[12] were determined by the tetrazolium chlorimetric reduction assay (MTT assay), which measures the mitochondrial dehydrogenase activity of surviving cells. IC_{50} of 5F-DNM, DNM and CyA to human PBMCs proliferation by 72 h MTT assay was 20.18, 25.62, and 8.97 μM, respectively. These results showed that DNM and 5F-DNM were much less toxic than CyA to human PBMCs. Our study first found that 5F-DNM significantly decreased the secretion of Th2 type cytokine IL-4 (Fig. 2) and had a specific inhibition effect on the expression of CD4, which are markers of CD4+ T cells, and a minor inhibition effect on CD19 of B cells. 5F-DNM at low concentration of 10 μM did not affect >95% of IFN-γ secretion, while CyA and DNM at concentration of 10 μM affected about 50% of IFN-γ secretion. 5F-DNM, even at high concentration of 90 μM, did not affect >50% of IFN-γ secretion (Fig. 3). Considering that IL-4 and IFN-γ is a hallmark of the Th2 and Th1 subset, respectively, 5F-DNM might hold more effects on Th2 cells and less effects on Th1 cells than CyA and DNM. The data present here demonstrate that the new compound, 5F-DNM, might have a possible therapeutic use in the Th2-mediated immune diseases such as chronic graft-*verse*-host disease and systemic sclerosis. Few drugs are now available as immunotherapy drugs in Th2-mediated immune diseases.[13,14] The new drug, 5F-DNM, we synthesized, could be used as a new candidate of immunotherapy drug in Th2-mediated immune diseases and as an agent for the treatment of chronic severe asthma, chronic granulomatous disease, and against intracellular bacterial and viral infections.

DNM and the 5F-DNM might be used as immunosuppressants. Importantly, we found the new compound, 5F-DNM, might be used as an immunosuppressant for Th2 cells, especially when at lower

concentration, and might hold potential as a new specific immunosuppressive drug.

Acknowledgements

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