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NK026680 inhibits T-cell function in an IL-2-dependent manner and prolongs cardiac allograft survival in rats

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ABSTRACT

NK026680 is a triazolopyrimidine derivative that has been shown to inhibit dendritic cell maturation and activation. Here, we examined the immunosuppressive properties of NK026680 on T-cell function and assessed its immunosuppressive efficacy in an ACI ($RT1^{av1}$ haplotype) to Lewis ($RT1^{l}$) rat heart transplantation model. The effects of NK026680 on T-cell proliferation, activation, and cytokine production were investigated *in vitro*. Heart transplant recipient rats were administered NK026680 daily for 14 days post-transplantation. In addition to graft survival time, alloimmune responses and graft histology at 4–10 days post-transplantation were assessed. NK026680 was found to inhibit proliferation, CD25 upregulation, IL-2 production, and cell cycle progression in αCD3/αCD28-stimulated murine T cells. These effects were likely due to suppression of the p38 mitogen-activated protein kinase pathway and the subsequent inhibition of p65, c-Fos, and to a lesser extent, c-Jun. Daily NK026680 treatment suppressed alloimmune responses, prevented cellular infiltration into allografts, and prolonged graft survival. The anti-rejection effects of NK026680 were enhanced by tacrolimus. In conclusion, NK026680 inhibits the activation of T cells and prolongs cardiac allograft survival in rats. These features make it a potential candidate immunosuppressant for the treatment of organ transplant patients in the future.

1. Introduction

The long-term administration of calcineurin inhibitors (CNIs) leads to an increased risk of the development of unfavorable side effects [1,2]. Recently, regimens that avoid the use of CNIs have been successfully adopted by transplant centers. There have been reports that these regimens can lead to reductions in drug-related adverse events along with equivalent outcomes in allograft survival following transplantation [3–7]. Despite this, CNIs are still key immunosuppressants in practice, and safer alternatives are limited. Thus, the development of new agents that have less drug-related side effects is essential.

Triazolopyrimidines are synthetic heterocycles with valuable bioactivity. Trapidil, a platelet-derived growth factor antagonist, is one of the major triazolopyrimidines and has a broad spectrum of biological activities. Previous studies have shown its protective effects in ischemia–reperfusion injury in some experimental models [8–12] and its clinical benefits in controlling angina pectoris and reducing angiographic in-stent restenosis [13,14]. These effects are due to trapidil's myriad of pharmacological properties that include nitroglycerine-like vasodilating action, inhibition of platelet aggregation, facilitation of the biosynthesis of prostacyclin, inhibition of thromboxane A2, and reduction of lipid peroxidation. Trapidil has also been reported to suppress IL-6, IL-12, and TNF-α production by blocking CD40 expression on monocytes and macrophages in humans [15,16].

NK026680 is a novel triazolopyrimidine derivative compound that also has very potent bioactivity. It has been reported to ameliorate mortality in acute lethal graft-versus-host disease in both MHC classes I and II disparate mice [17], prevent glomerulonephritis and perinuclear antineutrophil cytoplasmic antibody production in SCG/Kj mice [18], and prolong liver allograft survival in rats [19]. The findings in these reports suggested that such effects may be the result of impaired dendritic cell (DC) function. Here, we studied the effects of

Abbreviations: AP-1, activator protein-1; CNI, calcineurin inhibitor; DC, dendritic cell; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MLR, mixed lymphocyte reaction; MST, median survival time; NF-κB, nuclear factor-kappa B; NFAT, nuclear factor of activated T cells; SAPK, stress-activated protein kinase.

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NK026680 on T-cell function *in vitro* and the *in vivo* immunosuppressive effects of NK026680 alone or in combination with tacrolimus in a fully MHC-incompatible rat cardiac transplantation model.

2. Materials and methods

2.1. Animals

Male C57BL/6 (B6; H-2^b haplotype) and BALB/c (H-2^d) mice were purchased from Japan SLC (Shizuoka, Japan). Male Lewis ($RT1^l$ haplotype) and ACI ($RT1^{av1}$) rats were purchased from Kyudo (Fukuoka, Japan). Animals were maintained in a specific pathogen-free facility and used at 9–12 weeks of age. All experiments were approved by the Institutional Animal Care Committee and conducted under the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University.

2.2. Reagents

NK026680 (molecular weight = 419 Da) [17] was provided by Nippon Kayaku Co., Ltd. The powder form of NK026680 was dissolved in 0.05% dimethylsulfoxide (Sigma-Aldrich; St. Louis, MO) for *in vitro* assays and suspended in 0.5% carboxymethylcellulose (CMC; Shin-Etsu Chemical Industry; Tokyo, Japan) for *in vivo* experiments due to its poor water solubility. Tacrolimus powder (20%: Asteras Pharmaceutical Co.; Osaka, Japan) was dissolved in distilled water. Anti-mouse CD3 (145-2C11), CD4 (RM4-5), CD25 (7D4), and CD28 (37.51) monoclonal antibodies (mAbs) and anti-human CD3 (HIT3a) and CD28 (CD28.2) mAbs were obtained from BD Biosciences (San Jose, CA). Anti-rat CD4 (W3/25) and CD8 (OX-8) antibodies (Abs) were obtained from AbD Serotec (Oxford, UK). Abs against phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182), p38 MAPK, phospho-p44/p42 MAPK (extracellular signal-regulated kinase 1/2; ERK1/2) (Thr202/Tyr204), p44/p42 MAPK, phospho-stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK), SAPK/JNK, and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA).

2.3. Cell preparation and culture

Primary murine and rat leukocytes were isolated from spleens. After erythrocyte lysis with ACK buffer (Lonza, Walkersville, MD), T cells were enriched to greater than 90% purity by passing the cell suspension through a nylon-wool mesh column (R&D Systems, Minneapolis, MN). Human peripheral blood mononuclear cells (PBMCs) were obtained from the peripheral blood of healthy volunteers. After erythrocyte lysis, $CD4^+$ T cells were enriched to greater than 95% purity via a $CD4^+$ T-cell isolation kit and a magnetic cell separation system (Miltenyi Biotec, Auburn, CA). Complete RPMI 1640 media containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 50 μM 2-mercaptoethanol were used for all cell cultures.

2.4. Proliferation assays

2.4.1. Mouse

For the mixed lymphocyte reaction (MLR), irradiated (30Gy, 137Cs) BALB/c mouse splenocytes (5×10^5 cells/well) were co-cultured with B6 mouse splenocytes (5×10^5 cells/well) for 3 days. Purified B6 mouse T cells (5×10^5 cells/well) were stimulated with 1 μg/ml anti-CD28 (αCD28) mAb and 1 μg/ml plate-bound anti-CD3 (αCD3) mAb for 2 days.

2.4.2. Rat

Irradiated ACI rat splenocytes (2×10^5 cells/well) were co-cultured with Lewis rat lymphocytes (2×10^5 cells/well) obtained from the cervical and auxiliary lymph nodes for 5 days. Purified Lewis rat T cells (2×10^5 cells/well) were stimulated with concanavalin A (Con A; 2 μg/ml; Sigma-Aldrich) for 3 days.

2.4.3. Human

Irradiated PBMCs (1×10^5 cells/well) were co-cultured with allogeneic PBMCs (1×10^5 cells/well) for 5 days. T cells (1×10^5 cells/well) were stimulated with 1 μg/ml αCD28 mAb and 1 μg/ml plate-bound αCD3 mAb for 3 days.

2.4.4. Proliferation assay

Cells were cultured in complete RPMI 1640 media at 37 °C and 5% CO_2 plus air. Cells were pulsed with ^{3}H-thymidine (1 μCi/well) 8 or 16 h before the analysis of thymidine incorporation with a β-counter (Perkin Elmer; Boston, MA).

2.5. Flow cytometry

Cells were stained with an isotype control or specific mAbs against CD4 and CD25 and then analyzed with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences). For each analysis, 10,000 $CD4^+$ lymphocytes were acquired.

2.6. Cytokine measurement

IL-2 protein levels in culture supernatants were measured by enzyme-linked immunosorbent assay with a cytokine assay kit (R&D Systems). All measurements were performed in duplicate. The IFN-γ production of lymphocytes obtained from transplant recipients was examined by enzyme-linked immunospot assay as previously described [20].

2.7. Cell cycle analysis

Cell cycle analysis was performed using a Bromodeoxyuridine (BrdU) Flow Kit (BD Biosciences). Stimulated B6 mouse T cells were incubated with 10 μM BrdU for the final 30 min of culture, fixed, and permeabilized. DNA was digested by incubating cells with 300 μg/ml DNase at 37 °C for 60 min. Cells were stained with a fluorescein isothiocyanate-conjugated anti-BrdU Ab and 7-amino actinomycin D (7-AAD) prior to analysis by flow cytometry.

2.8. Nuclear protein extraction and quantification

Nuclear protein was harvested from 2×10^7 cells B6 mouse T cells according to the manufacturer's instructions (Nuclear Extract Kit; Activemotif, Carlsbad, CA). The levels of nuclear factor-kappa B (NF-κB), nuclear factor of activated T cells (NFAT), and activator protein-1 (AP-1) DNA binding activity were examined with the TransAM Kit (Activemotif) [21].

2.9. Immunoblotting

Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, and a protease inhibitor cocktail). Protein (30 μg) was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% dry milk and 0.1% Tween (Sigma-Aldrich) in PBS, incubated with primary Ab, and then incubated with horseradish peroxidase-conjugated secondary Ab. Bands were detected by enhanced chemiluminescence.