Immunosuppressive effect of compound K on islet transplantation in a STZ-induced diabetic mouse model

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Abstract

Islet transplantation is a therapeutic option for type 1 diabetes but its long-term success is limited by islet allograft survival. Many factors imperil islet survival, especially the adverse effects and toxicity due to clinical immunosuppressants. Compound K (Cpd K) is a synthesized analog of highly unsaturated fatty acids from *Isatis tinctoria L. (Cruciferae)*. Here we investigated the therapeutic effect of Cpd K in diabetic mice and found that it significantly prolonged islet allograft survival with minimal adverse effects after 10 days. Furthermore, it reduced the proportion of CD4$^+$ and CD8$^+$ T cells in spleen and lymph nodes, inhibited lymphocyte infiltration in allografts, suppressed serum IL-2 and IFN-γ secretion, and increased TGF-β and Foxp3 mRNA expression. Surprisingly, Cpd K and rapamycin (Rapa) had a synergistic effect. Cpd K suppressed proliferation of naive T cells by inducing T cell anergy and promoting the generation of regulatory T cells (Tregs). In addition, NF-κB signaling was also blocked. Taken together, these findings indicate that Cpd K may have a potential immunosuppressant effective on islet transplantation.

**Key words**: islet transplantation, Cpd K, Tregs, anergy, NF-κB

Diabetes mellitus is a fatal disease and has become a worldwide health issue. In 2012, more than 371 million people had diabetes (1). People with diabetes suffer devastating neural and vascular complications, such as neuropathy, metabolic syndrome, cardiovascular disease, and retinopathy (2,3). Type I diabetes mellitus is a chronic autoimmune disease resulting from destruction of insulin-secreting β cells in
the islets of Langerhans (4). For most type 1 diabetics, insulin therapy may be sufficient to maintain glycemic control, but hypoglycemia is a potentially lethal side effect of insulin treatment. Pancreas transplantation reduces insulin dependence, but it may also increase risk of major surgery (5). Since the Edmonton protocol was developed in 2000 (6), islet transplantation had become a more widespread therapy for type 1 diabetics. However, it has been reported that rapamycin (Rapa) and tacrolimus (FK506) inhibit β cell regeneration and results in nephrotoxicity following chronic treatment (7). Meanwhile, the high risk of sensitization after failed islet transplantation due to the production of donor specific antibodies (DSA) raises great concerns (8). Therefore, development of new immunosuppressants with improved safety and effectiveness for islet transplantation is needed.

Natural products and their derivatives have played an extraordinary role in preventing and curing human diseases (9-11). It is thought that more than half of all clinically used drugs are of natural product origin (12). Several immunosuppressants (cyclosporin A, tacrolimus and rapamycin) and immunomodulators (fingolimod) currently in clinical use were discovered or developed from natural sources (13-16). *Isatis tinctoria* L. (Cruciferae) is an ancient Chinese medicinal plant in which the leaf and root are used. Others have shown that lipophilic extracts from Cruciferae have anti-inflammatory and antiallergic activities in vivo (17,18), but their immunosuppressive activity remains unclear. In our previous studies, we isolated a highly unsaturated fatty acid from the Cruciferae root and synthesized dozens of analogs, (19). Cpd K, with a molecular weight of 546.51, is one of these analogs. In
addition, compound J, another analogue with a molecular weight of 461.32, has been shown to reverse multidrug resistance in tongue cancer (19).

In this study, we investigated the immunosuppressive effect and mechanism of action of Cpd K after islet transplantation in streptozocin (STZ)-induced diabetic mice. We first examined islet allograft survival after treatment with Cpd K alone or in combination with sub-therapeutic dose of rapamycin (sub Rapa). Immunorejection after allotransplantation is mediated mainly by T lymphocytes. Thus, we examined the activation, anergy, and apoptosis of T cells and T-lymphocyte subsets, especially Tregs, following Cpd K treatment. Our results revealed the immunosuppressive effects of Cpd K are mediated through T cell inhibition, T cell anergy, and increasing the proportion of Tregs. Moreover, we demonstrate Cpd K has a strong synergistic effect with Rapa. Together, these findings indicate that Cpd K may be a potential effective immunosuppressant for islet transplantation.

**Research Design and Methods**

**Preparation of Cpd K.** Cpd K, an analog of a highly unsaturated fatty acid, was synthesized at the College of Pharmacy, Guangxi Medical University (Guangxi, China). Its identity was confirmed by spectrum analysis, with a purity of more than 99.5% based on high performance liquid chromatography (HPLC) analysis. Cpd K stock solutions were prepared by dissolving in absolute ethanol to final concentrations of 10 and 20 mg/mL, followed by sterile filtration (pore size: 0.22 µm).

**Chemicals and antibodies.** Rapa was obtained from LC Laboratories (Woburn, MA,
USA) and dissolved in phosphate-buffered saline (PBS, 0.01 M, pH 7.2) to a final concentration of 0.02 mg/ml. Arsenic Trioxide (As$_2$O$_3$) was obtained from SL Pharmaceutical Co Ltd., and concanavalin A (Con A, C0412) from Sigma. Recombinant Murine IL-2 was from Perprotech. Anti-β-Actin (N21) (SC130656) was from Santa Cruz. Antibody to IκBα phosphorylated at Ser32-Ser36 (5A5) (#9246), to NF-κB p65 phosphorylated at Ser536 (93H1) (#3033), to JNK phosphorylated at Ser473 (D9E) (#4060) and to p38 phosphorylated at Thr180-Tyr182 (#9211) were from Cell Signaling Technology. FITC anti-mouse CD4 (RM4-5) and PE anti-mouse Foxp3 (FJK-16s) and their isotype controls were from eBioscience. PE/Cy5 anti-mouse CD8α (53-6.7) and its isotype control were from Biolegend.

**Experimental animals.** All animals were purchased from Slac Laboratory Animal Co. Ltd (Shanghai, China). Female C57BL/6 (H-2K$^b$) and BALB/c (H-2K$^d$) mice (8–12 weeks old) were used as graft recipients and donors, respectively. All animals were maintained and bred in specific pathogen-free facilities, following NIH ‘Principles of Laboratory Animal Care’.

**Experimental diabetic mice.** Mice were fasted overnight and then injected intraperitoneally with 180–220 mg/kg of STZ dissolved in 0.1 M sodium citrate buffer (pH 4.4). Blood glucose was measured using a One Touch glucose analyzer (FreeStyle, Abbott, Abbott Park, IL, USA). Diabetes onset was defined as two consecutive daily blood glucose measurements of > 16.7 mM.

**Experimental treatment of diabetic mice.** Five treatment groups were defined: normal saline treatment as control, sub-therapeutic Rapa (0.1 mg/kg.d) treatment (sub
Rapa), Rapa treatment (0.2 mg/kg.d) (Rapa), Cpd K (20 mg/kg.d) treatment (Cpd K), combination of Cpd K (20 mg/kg.d) and Rapa (0.1 mg/kg.d) treatment (Cpd K + sub Rapa). Cpd K and Rapa were administered orally and intraperitoneally over 0-9 days after transplantation, respectively. The number of animals in each group was 6 (n = 6).

**Islet isolation, purification, and transplantation.** Donor islets were isolated and transplanted by kidney subcapsular injection as previously described (20,21). Islet transplants were considered functional with two consecutive blood glucose measurements < 8 mM. The time of islet graft rejection was defined as the first day of two consecutive blood glucose measurements >11.1 mM.

**Cell culture**

**Pancreas islets.** Isolated Islets were dissociated into single cell suspension by incubating with 0.25% trypsin-EDTA and 25 U/ml DNase I for 15 minutes at 37°C. Islet cells were maintained in RPMI 1640 medium supplemented with 20% (vol/vol) FBS.

**T cells.** T cells were isolated from spleen by negative isolation with nylon wool columns (Wako, Richmond, VA, USA), and the purity was about 90-95% (data not shown), then maintained in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, penicillin, and streptomycin. All cells were cultured in a humidified chamber with 5% CO₂ at 37°C.

**Apoptosis assay.** Cells were treated with different concentrations of Cpd K or 4 µM As₂O₃ for 24 h at 37°C and then washed twice with pre-chilled PBS (0.01 M, pH 7.2). Apoptosis assays were performed using a PE-Annexin V detection kit (BD, Franklin
Lakes, NJ, USA) following the manufacturer's protocol. All samples were analyzed by flow cytometry with FACScan (BD).

**Insulin secretion assay.** Ten isolated islets were incubated with different concentrations of Cpd K and 5.6 mM glucose for 1 h at 37°C in DMEM (Gibco, Grand Island, NY, USA), and the medium replaced with fresh DMEM containing 16.7 mM glucose. Supernatant was collected after incubating for 1 h and measured using a Rat/Mouse Insulin 96-Well Plate Assay Kit (Millipore, Billerica, MA, USA).

**Histopathology analysis.** Kidney islet grafts were removed from recipient mice at day 10 post-transplantation, fixed in 4% paraformaldehyde fixative (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), and embedded in paraffin. Grafts were cut into 5 µm sections and stained with hematoxylin and eosin (H&E), and finally examined by microscopy.

**Immunohistochemistry analysis (IHC).** IHC was performed with Polink-2 plus Polymer HRP Detection System (GBI Labs, Mukilteo, WA, USA) following the manufacturer's protocol. Rabbit anti-insulin mAb (#3014) was from Cell Signaling Technology (Danvers, MA, USA). Samples were visualized with a DAB detection kit (Maixin-Bio, Fuzhou, China).

**Intraperitoneal Glucose Tolerance Test (IPGTT).** Recipient mice were subjected to an IPGTT test at day 8 post-transplantation. Mice were fasted for 10 h by removal to a clean cage without food at the end of their dark feeding cycle, and weighed. A fasting glucose level was obtained from tail vein. D-Glucose (1 mg/g bodyweight) was injected intraperitoneally. Blood glucose values were measured at 0, 5, 10, 15, 30, 60,
and 120 min. We assessed the area under the curve (AUC) for glucose using the trapezoidal rule and the area above baseline.

**Mixed lymphocyte reaction (MLR) assay.** Splenic T cells, which purity was about 90-95% (data not shown), were isolated from recipient mice using nylon wool columns (Wako, Richmond, VA, USA) and used as responder cells. BALB/c splenocytes were used as stimulator cells and which were pre-treated with mitomycin C (40 µg/ml, Amresco, Solon, OH, USA). Responder cells (5×10^5 cells) were co-cultured in 96-well plates with stimulator cells (5×10^4 cells) in 200 µl RPMI 1640 supplemented with 10% (vol/vol) FBS, 1% penicillin and streptomycin, and incubated at 37°C in 5% CO₂ humidified atmosphere for 72 h. Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA). OD values were measured by a microplate reader (Model 680 reader, Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm (reference wavelength at 690 nm) with the measurements performed in triplicate.

**Flow cytometry analysis.** 1×10^6 lymphocytes from spleen and lymph nodes of recipient mice were suspended in 100 µl PBS. Following incubation with FITC anti-mouse CD4 (RM4-5), PE/Cy5 anti-mouse CD8α (53-6.7) and isotype controls at 4°C for 30 min, Tregs were labeled using the Mouse Regulatory T cell staining kit (eBioscience) following the manufacturer's protocol. Stained cells were detected on the FACScan and the data analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

**Enzyme-linked immunosorbent assay (ELISA).** ELISAs were performed using
commercially available kits (NeoBioscience Technology Co. Ltd., Shenzhen, China) to detect the quantify IL-2, IFN-γ, IL-4 and TGF-β in cultured T cell supernatants and the sera of recipient mice, according to the manufacturer's instructions. Each reaction was carried out in triplicate.

**CFSE (Carboxyfluorescein diacetate succinimidyl ester) assay.** Splenic T lymphocytes were stained with CFSE (Sigma), and cultured in 96-well plates at a concentration of 1.5×10^6 cells/ml. Cells were exposed to different concentrations of Cpd K and 2.5 µg/ml Con A (Sigma) in RPMI 1640 supplemented with 10% (vol/vol) FBS, and 1% penicillin and streptomycin. Lymphocyte proliferation was analyzed by flow cytometry after 48 h.

**Quantitative real-time PCR (qRT-PCR) analysis.** Total RNA was isolated from islet grafts and cultured T lymphocytes with Trizol (Life Technologies, Carlsbad, CA, USA). Reverse transcription and qRT-PCR were performed using commercially available reagents (Toyobo, Osaka, Japan). The StepOne Real-Time PCR System (ABI, Foster City, CA, USA) was employed to detect IL-2, IFN-γ, IL-4, TGF-β and Foxp3. β-actin served as a control. The following primer sequences were used for qRT-PCR:

- **β-actin:** forward 5′-CATCCGTAAGACCTCTATGCAAC-3′, and reverse 5′-ATGGAGCCACCGATCCACA-3′;
- **IL-2:** forward 5′-GGAGCAGCTGGATGGGATCCAC-3′, and reverse 5′-AATCCGACGCTGGCTCCAG-3′;
- **IFN-γ:** forward 5′-CGGCACAGTCATTGAAAAGCCTA-3′, and reverse
5'-GTTGCTGATGGCCTGATTGTCU3';
IL-4: forward 5'-TCTCGAATGTACCAGGAGCCATATCU3’, and reverse 5'-AGCACCTTGGAAGCCCTACAGA-3’;
TGF-β: forward: 5’-TGACGTCACTGGAGTTGTACGGU3’, and reverse 5’-GGTTGCATGTGAGGATGGGTGC-3’;
Foxp3: forward 5’-CAGCTCTGCTGGCGAAAGTGU3’, and reverse 5’-TCGTCGAAGGCAGGATCAGG-3’.

**Immunoblot analysis.** Cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with protease inhibitor ‘cocktail’ (Roche Diagnostics). Extract protein concentrations were determined by BCA assay (Pierce, Rockford, IL, USA). Immunoblot analysis was performed as previously described (22).

**Statistical analysis.** The data are expressed as mean ± SEM. The median survival times (MSTs) of the five groups were calculated and compared by the Kaplan–Meier method. One-way analysis of variance (ANOVA) was employed to evaluate the significance of multiple comparisons and a Bonferroni correction was calculated and applied. Differences were considered to be statistically significant at $P < 0.05$. All analyses were performed using the GraphPad Prism® software (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**Cpd K is non-toxic to islet and has low nephrotoxicity.** To determine Cpd K
cytotoxicity on islet cells, we purified BALB/c islet cells, and treated them with 0, 40, 80, 100, and 120 µg/ml Cpd K for 24 h. A PE-Annexin V/7-AAD kit was employed to assay cellular apoptosis. But we did not observe any at the concentrations tested (Fig. 1A). In addition, insulin secretion analysis showed that Cpd K is non-toxic to islets in vitro (Fig. 1B). We also determined the effect of Cpd K on mouse kidney function (Fig. 1C and D), but did not observe any obvious changes in sera creatinine and urea nitrogen following treatment with normal saline, Cpd K (20mg/kg.d) or Cpd K (40mg/kg.d). Together, these results indicate that Cpd K is non-toxic to islets and has low nephrotoxicity.

**Effect of Cpd K on islet allografts in STZ-induced diabetic mice.** We next investigated the effect of Cpd K on islet allograft survival time in STZ-induce diabetic mice. Cpd K (20 mg/kg.d) administered orally for 10 days significantly prolonged survival time compared with controls (Fig. 2A, P=0.0007). Immunohistochemistry analysis detected insulin secretion from islet allografts, with insulin levels in the Cpd K treated group higher than those in controls (Fig. 2B). We nephrectomized mice receiving the combined therapeutics at day 120 post-transplantation, and observed elevated glucose levels (Fig. 2C). This result indicates that islet grafts maintain normoglycemia. IPGTT result from day 8 post-transplantation suggests Cpd K may improve glucose intolerance in diabetic mice (Fig. 2D and E).

**Cpd K effects on inflammatory response in STZ-induced diabetes after islet transplantation.** Grafts were dissociated from recipient mice at day 10 post-transplantation and processed for histology. Grafts from control mice exhibited
islet damage and were filled with infiltrating inflammatory cells. However, Cpd K treated mice showed less islet damage and fewer infiltrating inflammatory cells (Fig. 3A). Next, graft and sera expression of inflammatory cytokines was analyzed by qRT-PCR and ELISA (Fig. 3B and C). IL-2 and IFN-γ in the Cpd K group were effectively down regulated at both the mRNA and protein levels, whereas there was no significant influence on IL-4. MLR test results suggest recipient splenic T cells from Cpd K treated mice showed a reduced proliferative response when stimulated with mitomycin C treated donor splenocytes compared with controls (Fig. 4A). Moreover, Cpd K significantly down-regulated IL-2 and IFN-γ expression as determined by MLR tests, but had no effect on IL-4 (Fig. 4B). These results suggest that Cpd K may suppress the function of Th1 cells.

**Effects of Cpd K on CD4⁺, CD8⁺ and regulatory T cells (Tregs) in STZ-induced diabetic mice.** Flow cytometry was employed to investigate Cpd K effects on splenic and lymph node CD4⁺, CD8⁺ T cells and Tregs of recipient mice at day 10 post-transplantation. Cpd K administered orally for 10 days effectively decreased the proportion of splenic and lymph node CD4⁺ and CD8⁺ T cells (Fig. 5), whereas the proportion of Tregs increased (Fig. 6A). It’s known that TGF-β induces the generation of Tregs, and Foxp3 is a key transcriptional regulator in Tregs. We next examined TGF-β and Foxp3 expression in islet grafts and sera of recipient mice. Cpd K increased graft TGF-β and Foxp3 mRNA levels (Fig. 6B) and sera TGF-β concentrations (Fig. 6C) compared to control.

**Synergistic effects of Cpd K and Rapa in STZ-induced diabetic mice.** To
investigate the synergistic effects of Cpd K and Rapa, Cpd K (20 mg/kg.d) and sub-therapeutic rapamycin (sub Rapa, 0.1 mg/kg.d) were administrated orally and intraperitoneally, respectively, at day 0-9 post-transplantation. We found that this combination therapeutic schedule resulted in longer survival times (Fig. 2A), elevated insulin levels (Fig. 2B), and resulted in better glucose tolerance (Fig. 2D and E) than Cpd K or sub-Rapa treatment alone. Moreover, the combination of Cpd K and sub-Rapa inhibits the inflammatory reaction (Fig. 3) and immune response (Fig. 4), effectively reducing the proportion of CD4$^+$ and CD8$^+$ T cells (Fig. 5). It also increases splenic and lymph node Tregs (Fig. 6) of recipient mice compared with Cpd K or sub-Rapa treatment alone. The effects of Cpd K plus sub-Rapa treatment on diabetic mice was similar to full-dose Rapa treatment.

**Effects of Cpd K on the T cell proliferation, anergy, apoptosis, and activation in vitro.** Con A stimulated T cell proliferation was suppressed by Cpd K in a dose-dependent manner (Fig. 7A and B). Nevertheless, 100 U/ml exogenous IL-2 reversed suppression (Fig. 7C). However, T cell apoptosis was not observed at all Cpd K treatment concentrations (Fig. 7D). We next assayed potential Cpd K target proteins in mice using the INVDOCK approach, and found 13 candidates, including Rel A (Fig. 8A), a key transcription factor regulating T cell activation. Thus, it appears we detect the effects of Cpd K on the expression of T cell activation-related signaling molecules. As shown in Fig. 8B, Cpd K reversed increased phosphorylated NF-kb (p65-Ser536) protein expression, whereas expression of phosphorylated I-kbα, phosphorylated p38 and phosphorylated JNK were not influenced. Notably, we found Cpd K inhibited
IL-2 and IFN-γ expression in cultured Con A stimulated primary T cells (Fig. 8C). These results suggest that Cpd K inhibits T cell proliferation by inducing T cell anergy and blocking NF-κB signaling.

**Discussion**

We have demonstrated for the first time, to the best of our knowledge, that Cpd K alleviates the alloimmune response and maintains islet function after islet transplantation in a STZ-induced diabetic mouse model. We also provide in vitro evidence that Cpd K represses T lymphocyte proliferation through enhancing T cell anergy and blockage of NF-κB signaling, which plays an important role in the immune system (23-25).

Diabetic patients often suffer from severe immune rejection after islet transplantation. T lymphocytes have been shown to be the main mediators during allo- and autoimmune responses (26,27). The current immunosuppressants used in clinical islet transplantation, such as FK506 and Rapa, show their immunosuppressive effects by inhibiting the activation, proliferation, and survival of T lymphocytes (28-32). However, a number of publications have shown that severe side effects are manifested during clinical use of these immunosuppressants, including nephrotoxicity, neurotoxicity, and inhibition of β cell regeneration (7,33,34). These side effects limit the clinical application of immunosuppressants by affecting survival of transplanted organs. Therefore, development of new effective and safe immunosuppressants has become urgent. Natural products, especially some agents in traditional Chinese medicine, are getting more attention. A number of derivative compounds, made from
such medicines, had been applied in the clinic and have played important roles in preventing and curing human diseases. FTY720 and arsenic trioxide, which are from traditional Chinese medicine, suppress the immune response (35-37). In this study, Cpd K, a compound synthesized from Cruciferae, has been examined in-depth in mouse islet transplantation models and shown to prolong islet graft survival in recipients. Furthermore, Cpd K exhibited low-level toxicity to islets and low nephrotoxicity. Nevertheless, Synergistic effects of Cpd K and Rapa were observed in STZ-induced diabetic mice, suggesting that a combination therapy may minimize the adverse effects of Rapa in the clinic. To investigate the mechanism by which Cpd K suppresses the immune response, we examined Cpd K effects on the proliferation, differentiation, anergy, apoptosis, and activation of T cell subsets and Tregs in vivo and in vitro.

CD4\(^+\) T cells play a key role in the immune system, including stimulation of antibody production by B cells and enhancement of CD8\(^+\) T cell responses, which play important roles in islet transplantation (38). Furthermore, various functions are acquired by differentiation of naive CD4\(^+\) T cells stimulated by special antigens, which become effector or memory cells. Th1 and Th2 cells are two important lineages differentiated from naive CD4\(^+\) T cells. IL-12 and IFN-\(\gamma\) play important roles in the differentiation of Th1 cells by secreting IFN-\(\gamma\) and promoting cell-mediated immune responses (39,40). IL-4 is essential for the induction of Th2 cells, of which IL-4 is a signature cytokine, and Th2 cells promote humoral immune responses (41,42). To investigate which T cells were impaired after treatment with Cpd K in recipient mice,
we examined the proportion of splenic and lymph node CD4$^+$ and CD8$^+$ T cells. The results showed that both CD4$^+$ and CD8$^+$ T cells decreased following Cpd K treatment. In addition, Cpd K decreased IL-2 and IFN-γ expression, with no change in IL-4 expression. These results suggest that Cpd K protects islet allografts by inhibiting the Th1-mediated immune response.

Induction of immunological tolerance to transplants would avoid rejection and eventually wean transplant recipients off immunosuppressive drugs (43,44). Joffre et al. (45) have shown that Tregs protect skin and cardiac allografts from acute and chronic rejection. Tregs differentiate from naive CD4$^+$ T cells, and are dependent on T cell activation in the presence of TGF-β and IL-2 (46-48). Furthermore, Foxp3 is reported to be a key transcriptional regulator of Tregs. A recent study demonstrated that Foxp3$^+$ Tregs exert their regulatory function by the initiation of long-term tolerance, whereas T regulatory type 1 (Tr1) cells regulate maintenance (49). Here, we found that Cpd K induces Tregs during early stage transplantation, whereas we did not observe Treg expansion in long-term recipients (data not shown).

T cell anergy occurs when T cells encounter antigens presented by chemically fixed antigen presenting celles (APCs), which are unable to upregulate co-stimulatory ligands. In addition, poor proliferation and decreased IL-2 production is observed in anergic T cells. However, the anergic status is reversible by the addition of exogenous IL-2 (50), and we also found that poor T cell proliferation was reversible by adding exogenous IL-2 (100 U/ml). We thus analyzed T cell apoptosis following Cpd K treatment since apoptosis induction is an important mechanism for some
immunosuppressive agents, such as arsenic trioxide, which promote apoptosis of mouse T cells by activating JNK and p38 signaling pathways (35). However, we did not find significant induction of T cell apoptosis by Cpd K. These results indicate that Cpd K may induce anergy.

Previous studies have shown immunosuppressive agents such as cyclosporin A and FK506 inhibit the immune response and protect allografts from rejection after organ transplantation by suppressing TCR/CD28-mediated T cell activation (28). To investigate whether Cpd K affects T cells activation, we measured T cell proliferation after Cpd K treatment. The results show that Cpd K inhibits T cell proliferation in a dose-dependent manner. Surprisingly, the results of inverse docking (INVDOCK) analysis suggested that Cpd K binds to Rel A (p65), a NF-κB family member that regulates cytokine expression. Furthermore, the NF-κB, NFAT, and MAPK signaling pathways play key roles in T cell activation and the immune response. We found that Cpd K reversed the increase in p65 phosphorylation, but did not influence increased phosphorylated p38 and JNK in cultured primary T cells. In contrast, phosphorylation of IκBα, an important inhibitor of NF-κB, did not decrease following Cpd K treatment. These results suggest a NF-κB-related signaling is involved in the immunosuppressive activity of Cpd K.

In conclusion, Cpd K suppresses T cell-mediated immune responses and prolongs islet graft survival in STZ-induced diabetic mice. NF-κB-related signaling and anergy appear to be involved in the immunosuppressive effects mediated by Cpd K. Moreover, Cpd K decreased Th1 IFN-γ expression and increased production of Tregs,
which play important roles in the induction of transplant tolerance. However, the mechanism should be revealed in the future. We also found that Cpd K exhibits low toxicity to islets and appears to have synergistic effects with Rapa. Our findings suggest that Cpd K may be a potential therapeutic option for diabetic patients treated with islet transplantation.

Acknowledgments

This work was supported by grants from the Major State Scientific Research Program of China (No. 2012CBA01303), the National Natural Science Foundation of China (No. 81302546), and the Natural Science Foundation of Fujian Province (No. 2011J01253).

The authors do not have any conflicts of interest relevant to this article.

P.F.M. designed experiments, performed research, analyzed data, contributed to discussions, and wrote the manuscript. J.J. contributed to discussions, and edited the manuscript. C.G., P.P.C. and J.L.L. performed research and analyzed data. X.H. contributed to discussions. Y.Y.L., Q.L., Y.Z.P. and S.H. performed research. M.C.C. and W.S. analyzed data. Q.Z. contributed to discussions. Q.Q. prepared Cpd K. J.J.X. designed experiments, performed research, analyzed data, and contributed to discussions. Z.Q.Q. designed research, contributed to discussions, and edited the manuscript. J.J.X. and Z.Q.Q. are the guarantors and take full responsibility to the article and its originality.

The authors thank Xiao-Hong Huang, Hua-Xiu Sui and Bao-Ying Xie, Medical
College, Xiamen University, for their technical assistance.

References

5. Jamiołkowski RM, Guo LY, Li YR, Shaffer SM, Naji A. Islet Transplantation in Type I Diabetes Mellitus. The Yale journal of biology and medicine 2012;85:7
38. Baumann EE, Buckingham F, Herold KC. Intrathymic transplantation of islet antigen affects CD8+ diabetogenic T-cells resulting in tolerance to autoimmune IDDM. Diabetes 1995;44:871-877
43. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation--how much of the promise has been realized? Nature medicine 2005;11:605-613


Figure legends

FIG. 1. Cpd K is non-toxic to islet cells and has low nephrotoxicity. A: Islet cells were purified and treated with 0, 40, 80, 120, or 160 µg/mL Cpd K for 24 h, and apoptosis assayed. The numbers are for representative data of three independent experiments. B: Insulin secretion from isolated mouse islets was detected in the presence of 5.6 and 16.7 mM glucose. Islets were treated with various concentrations of Cpd K plus 5.6 mM glucose. C and D: C57BL6 mice were administrated orally with normal saline, 20 mg/kg.d and 40 mg/kg.d Cpd K for 10 and 20 days, and sera creatinine (CRE) and urea nitrogen (BUN) measured by an automated biochemical analyzer (Beckman Coulter, Brea, CA, USA). Data are presented as mean ± SEM of three independent experiments. ***P < 0.001 vs. 5.6 mM glucose.

FIG. 2. Effects of Cpd K on islet graft survival, insulin secretion, and glucose intolerance in diabetic mice. Islet graft survival (A) and blood glucose levels (C) in recipient mice with different treatments. Graft survival was calculated by the Kaplan–Meier method and compared by a log-rank test (n=6). B: Insulin IHC assays were carried out at day 10 post-transplantation. IPGTT was performed on day 8, and blood glucose levels were measured (D) and the area under curve (AUC) calculated (E). Data are presented as mean ± SEM of three independent experiments. *P < 0.05 vs.
control group; #\(P < 0.05\) vs. sub Rapa group; +\(P < 0.05\) vs. control group.

**FIG. 3.** Cpd K effect on inflammatory cell infiltration and cytokine expression in diabetic mice. Grafts were removed from recipient mice at day 10 post-transplantation and sera prepared. A: Pathological analysis of islet grafts was performed by H&E staining, there are inflammatory cells both around and within the islet grafts. Graft IL-2, IFN-\(\gamma\), and IL-4 mRNA levels were quantified by qRT-PCR (B) and protein concentrations in recipient mouse sera were determined by ELISA (C). Each reaction was carried out in triplicate. Data are presented as mean ± SEM of three independent experiments. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. control group; #\(P < 0.05\), ##\(P < 0.01\), ###\(P < 0.001\) vs. sub Rapa group; +\(P < 0.05\), ++\(P < 0.01\), +++\(P < 0.001\) vs. Cpd K group.

**FIG. 4.** Cpd K effect on T lymphocyte-mediated immune response. MLR assays were used to test the proliferative response of recipient splenic T cells to donor BALB/c (A), and IL-2, IFN-\(\gamma\), and IL-4 supernatant concentrations were tested by ELISA (B). Each reaction was carried out in triplicate. Data are presented as mean ± SEM of three independent experiments. &&&\(P < 0.001\) vs. Naïve group; *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. control group; #\(P < 0.05\), ##\(P < 0.01\), ###\(P < 0.001\) vs. sub Rapa group; +++\(P < 0.001\) vs. sub Cpd K group.

**FIG. 5.** Cpd K effects on the generation of CD4\(^+\) and CD8\(^+\) T cells. Proportion of splenic and lymph node CD4\(^+\) and CD8\(^+\) T cells were analyzed by flow cytometry at day 10 post-transplantation (n=3 mice per group). The numbers are for representative data of three independent experiments.
**FIG. 6.** Cpd K effect on Tregs induction. Proportions of splenic and lymph node CD4\(^+\) Foxp3\(^+\) Treg cells were analyzed by flow cytometry at day 10 post-transplantation (n=3 mice per group) (A). Graft TGF-β and Foxp3 mRNA levels were quantified by qRT-PCR (B) and recipient mouse sera TGF-β concentrations were quantified by ELISA (C). A: The numbers are for representative data of three independent experiments. B, C: Data are presented as mean ± SEM of three independent experiments. \(*P < 0.05, **P < 0.01\) vs. control group; \(#P < 0.05, ##P < 0.01\) vs. sub Rapa group; \(^+P < 0.05, ^{++}P < 0.01, ^{+++}P < 0.001\) vs. Cpd K group.

**FIG. 7.** Cpd K effect on T lymphocytes in vitro. The proliferation, anergy, and apoptosis of Con A stimulated cultured primary T cells was assayed. T cells were treated with different concentrations of Cpd K for 48 h and proliferation analyzed by BrdU (A) and CFSE (B) assays. T cells were first treated with different concentrations of Cpd K for 24 h and then with 0, 50, and 100 U/ml exogenous IL-2 for another 48 h. BrdU was used to analyze T cell anergy (C). T cell apoptosis was evaluated by flow cytometry with PE-Annexin V/7-AAD after treatment with different concentrations of Cpd K for 24 h (D). Data in A and C are presented as mean ± SEM of three independent experiments. \(*P < 0.05, **P < 0.01\) vs. group with treatment of Con A alone; \(^#P < 0.05, ^{##}P < 0.01\) vs. group with treatment of 100 or 120 µg/ml Cpd K alone. B, D: The numbers are representative data of three independent experiments.

**FIG. 8.** Cpd K effects on T cell activation-related signaling molecules. Potential Cpd K target proteins were analyzed by INVDOCK (A). Primary T cells were cultured with Con A and different concentrations of Cpd K for 24 h, and expression of T cell
receptor-related signaling molecules (phosphorylated NF-κb p65, phosphorylated Iκbα, phosphorylated JNK, and phosphorylated p38) were determined by western blotting (B). After 48 h treatment with Con A and various concentrations of Cpd K, primary T cells were collected and RNA isolated. IL-2 (C) and IFN-γ (D) mRNA levels were quantified by qRT-PCR. B: Representative images of three independent experiments are shown. Data in C and D are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. group with treatment of Con A alone.
FIG. 1. Cpd K is non-toxic to islet cells and has low nephrotoxicity. A: Islet cells were purified and treated with 0, 40, 80, 120, 160 µg/mL Cpd K for 24 h, and apoptosis tests performed. The numbers are for representative data of three independent experiments. B: Insulin secretion from isolated mice islets was detected in the presence of 5.6 and 16.7 mM glucose. The islets were only treated with various concentrations of Cpd K in the medium including 5.6 mM glucose. C and D: The C57BL6 mice were administrated orally with normal saline, 20 mg/kg.d and 40 mg/kg.d Cpd K for 10 and 20 days, the creatinine (CRE) and urea nitrogen (BUN) in sera were measured by automatic biochemical analyzer (Beckman Coulter, CA, USA). Data are presented as mean ± SEM of three independent experiments. ***P < 0.001 vs. 5.6 mM Glucose group.

134x99mm (300 x 300 DPI)
FIG. 2. Effects of Cpd K on islet graft survival, insulin secretion, and glucose intolerance in diabetic mice. Islet graft survival (A) and blood glucose levels (C) in recipient mice with different treatments. Graft survival was calculated by the Kaplan–Meier method and compared by a log-rank test (n=6). B: Insulin IHC assays were carried out at day 10 post-transplantation. IPGTT was performed on day 8, and blood glucose levels were measured (D) and the area under curve (AUC) calculated (E). Data are presented as mean ± SEM of three independent experiments. *P < 0.05 vs. control group; #P < 0.05 vs. sub Rapa group; +P < 0.05 vs. control group.

226x284mm (300 x 300 DPI)
FIG. 3. Cpd K effect on inflammatory cell infiltration and cytokine expression in diabetic mice. Grafts were removed from recipient mice at day 10 post-transplantation and sera prepared. A: Pathological analysis of islet grafts was performed by H&E staining, there are inflammatory cells both around and within the islet grafts. Graft IL-2, IFN-γ, and IL-4 mRNA levels were quantified by qRT-PCR (B) and protein concentrations in recipient mouse sera were determined by ELISA (C). Each reaction was carried out in triplicate. Data are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. sub Rapa group; +P < 0.05, ++P < 0.01, +++P < 0.001 vs. Cpd K group.

138x106mm (300 x 300 DPI)
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126x181mm (300 x 300 DPI)
FIG. 5. Cpd K effects on the generation of CD4+ and CD8+ T cells. Proportion of splenic and lymph node CD4+ and CD8+ T cells were analyzed by flow cytometry at day 10 post-transplantation (n=3 mice per group). The numbers are for representative data of three independent experiments.

80x35mm (300 x 300 DPI)
FIG. 6. Cpd K effect on Tregs induction. Proportions of splenic and lymph node CD4+ Foxp3+ Treg cells were analyzed by flow cytometry at day 10 post-transplantation (n=3 mice per group) (A). Graft TGF-β and Foxp3 mRNA levels were quantified by qRT-PCR (B) and recipient mouse sera TGF-β concentrations were quantified by ELISA (C). A: The numbers are for representative data of three independent experiments. B, C: Data are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. sub Rapa group; +P < 0.05, ++P < 0.01, +++P < 0.001 vs. Cpd K group.

146x119mm (300 x 300 DPI)
FIG. 7. Cpd K effect on T lymphocytes in vitro. The proliferation, anergy, and apoptosis of Con A stimulated cultured primary T cells was assayed. T cells were treated with different concentrations of Cpd K for 48 h and proliferation analyzed by BrdU (A) and CFSE (B) assays. T cells were first treated with different concentrations of Cpd K for 24 h and then with 0, 50, and 100 U/ml exogenous IL-2 for another 48 h. BrdU was used to analyze T cell anergy (C). T cell apoptosis was evaluated by flow cytometry with PE-Annexin V/7-AAD after treatment with different concentrations of Cpd K for 24 h (D). Data in A and C are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 vs. group with treatment of Con A alone; #P < 0.05, ##P < 0.01 vs. group with treatment of 100 or 120 µg/ml Cpd K alone. B, D: The numbers are representative data of three independent experiments.
FIG. 8. Cpd K effects on T cell activation-related signaling molecules. Potential Cpd K target proteins were analyzed by INVDOCK (A). Primary T cells were cultured with Con A and different concentrations of Cpd K for 24 h, and expression of T cell receptor-related signaling molecules (phosphorylated NFκκb p65, phosphorylated IkBα, phosphorylated JNK, and phosphorylated p38) were determined by western blotting (B). After 48 h treatment with Con A and various concentrations of Cpd K, primary T cells were collected and RNA isolated. IL-2 (C) and IFN-γ (D) mRNA levels were quantified by qRT-PCR. B: Representative images of three independent experiments are shown. Data in C and D are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. group with treatment of Con A alone.