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# Chelerythrine ameliorates acute cardiac allograft rejection in mice

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ABSTRACT

The improvement in graft survival over the past decade has been mainly due to calcineurin inhibitors, which interfere with the calcium-mediated pathway. Recently, other pathways such as those mediated by protein kinase C (PKC) are coming into view. The purpose of this study was to assess the immunosuppressive properties of chelerythrine, a specific PKC inhibitor, in preventing acute rejection in murine heterotopic heart transplantation. Mice were randomly divided into control and chelerythrine treated group. The control group received PBS while the chelerythrine treated group was given intraperitoneal injection doses (1, 5, 10 mg/kg) of chelerythrine from day 0 to day 14 after heart transplantation. Six days after transplantation, cardiac allografts were harvested for further tests. The mean survival time (MST) of the cardiac allograft in untreated animals was 8 days while graft MSTs observed in chelerythrine treated group was 13 and 23 days at 5 and 10 mg/kg treatment doses, respectively ($P < 0.05$). Histologic assessment of the allograft in chelerythrine group showed a significant decline in histologic rejection score, as well as CD4+ and CD8+ T cell infiltration and ICAM-1+ endothelial cell activation. Down-regulation of Th1/Th2 cytokine expression was observed in chelerythrine treatment group. Meanwhile, chelerythrine was also found to inhibit the dephosphorylation of phosphorylated nuclear factor of activated T cells (NFAT) protein 1 and 4.



## 1. Introduction

Over the last two decades, the administration of calcineurin inhibitors (CNIs), the well-known immunosuppressive drugs, has contributed to the success of solid organ transplantation, which has led to the reduction in rates of acute rejection episodes and prolonged graft survival [1, 2]. Nevertheless, the clinical utilization of CNIs is restricted by their side effects, such as nephrotoxicity, neurotoxicity, and new-onset diabetogenicity [3]. Furthermore, CNIs are not useful in the prevention of chronic allogenic rejection and the induction of tolerance. Hence, immunosuppressant that possesses a novel mode of action offering less side effect profiles would be worth searching for.

A rising immunoregulatory agent targets the inhibition of protein kinase C (PKC), an important signaling kinase in T cell activation [4–6]. Chelerythrine, a benzophenanthridine alkaloid, is a specific inhibitor of PKC [7]. The biological actions of chelerythrine have been recognized for over 20 years. This interesting plant alkaloid has diverse intracellular effects: it inhibits selective PKC isoenzymes, targets PPAR gamma, binds to adenosine and interacts with cyclic nucleotide phosphodiesterases. It also non-competitively inhibits ATP hydrolysis and alters $Ca^{++}$ homeostasis. It has been investigated for its potential therapeutic roles in oncology, bipolar disorder, fungal infections, as well as in dental products [8–15]. The current article extends this action to examine its potential in moderating graft rejection and prolonging survival in murine cardiac transplant models.

Also, recurrence of the primary tumor is one of the major obstacles in clinical organ transplantation when treating patients with malignancies, such as liver transplantation for hepatocellular carcinoma. Agents with both anti-rejection and anti-tumor effects are particularly valuable

*Abbreviations:* PKC, protein kinase C; NFAT, nuclear factor of activated T cells; MST, mean survival time; CNIs, calcineurin inhibitors; SEM, standard error of the mean; ANOVA, analysis of variance; IL, interleukin; TNF, tumor necrosis factor; PCR, polymerase chain reaction; MHC, main histocompatibility complex; mAb, monoclonal antibody; IFN, interferon.

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for these recipients. Chelerythrine is not only a good candidate for chemotherapeutic regimen but may also contribute to the development of successful immune therapy for some carcinomas due to its apoptotic effect [16]. Chelerythrine has the potential to offer more benefits to cancer patients as it blocks the early T- and B-cell activation with less whole body involvement and side effects [17,18].

## 2. Materials and methods

### 2.1. Animals and experimental design

Male BALB/C (H-$2^d$) and C57BL/6 (H-$2^b$) mice at 10 to 12 weeks of age, weighing 20 g to 25 g, were obtained from the Institute of Organ Transplantation, the First Affiliated Hospital, Medicine College, Zhejiang University (Hangzhou, PR China). Mice were housed under a standard SPF environment with a 12 h dark-light cycle and free access to water and food. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Review Committees of Zhejiang University.

Heterotopic heart transplantations were performed among fully MHC mismatched mice strains from BALB/C to C57BL/6. The mice were randomly divided into control group and chelerythrine treated group. The control group received PBS, and the chelerythrine treated group received an intraperitoneal injection of chelerythrine (1, 5, 10 mg/kg) from days 0 to 14 after heart transplantation. Chelerythrine (purchased from Yixin Biotech Co. Ltd., Shanghai, China) was immediately dissolved in their corresponding PBS volume before usage.

### 2.2. Murine cardiac transplantation

Survival analysis was done with six animals in each group. All surgical procedures were under clean but not sterile conditions with an operating microscope (S6D Leica, Germany) at 6× to 12.5× magnification. Isoflurane was administrated as a general inhalation anesthesia in all cases. The heterotopic cardiac transplantation was performed as previously described [19]. Graft survival was examined by daily abdominal wall palpation in the recipients. The completion of graft rejection was defined as a cessation of graft palpitation, confirmed by laparotomy. Another six animals in 5 mg/kg chelerythrine and control groups were euthanized with a high concentration of $CO_2$ on post-transplantation day 6 for further experiments.

### 2.3. Graft histology

The transplanted hearts were removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Each specimen was sliced at 4 μm before staining with hematoxylin and eosin (H&E). Two pathologists blindly assessed the rejection level of grafts by a scoring system based on the modified International Society for Heart and Lung Transplantation 2004 method [20].

### 2.4. Immunohistochemistry

For immunopathological evaluation, paraffin sections were incubated with monoclonal antibodies. Primary antibodies were rat anti-CD4 mAb (GK 1.5; R&D), rat anti-CD8a mAb (53-6.7; R&D) and hamster anti-ICAM-1 mAb (BD Biosciences Pharmingen). Secondary antibodies were detected with horseradish peroxidase-conjugated goat anti-rat antibodies and goat anti-hamster immunoglobulin G and then visualized with DAB. Digital scanning with high-power field magnification of 400× was used in counting positive immunostained CD4, CD8 T cells and ICAM-1 endothelial cells of 20 fields.

### 2.5. Real-time PCR

Total RNA was isolated from small pieces of each cardiac graft tissue (50 to 100 mg) in 5 mg/kg chelerythrine and untreated control group on post-transplantation day 6 using the TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA,) according to the manufacturer's instructions. Absorbance was measured at 260 and 280 nm, to verify the integrity and purity of the isolated RNA. The extracted RNA was stored at −80 °C until use for PCR. Reverse transcription was performed on 3 μg of RNA with an oligo (dT) primer and M-MuL virus reverse transcriptase (Promega Corporation, Madison, WI, USA).

SYBR Green (TAKARA Company, China) was used for the quantification of PCR reactions. The prepared cDNA was subjected to different PCR in the presence of 5′ and 3′ murine IL-2, IL-4, IL-10, IL-17, IFN-γ, and TNF-α primer pairs (IDT Company, USA). The sequences of the primers are described in Table 1. Mouse β-actin was used as an endogenous control to standardize the amount of cDNA added to the reaction. The PCR amplification was placed in an ABI PRISM 7500 Real-Time PCR System (ABI/PE, Foster City, CA, USA). Steps used in the PCR reaction were: 1) initial denaturation at 94 °C for 3 min; 2) 40 cycles of denaturation at 94 °C for 20 s and annealing extension at 60 °C for 45 s. The data were analyzed using $2^{-\Delta\Delta CT}$ method. The ΔCt value was defined as the absolute value of the difference between the Ct value of the target gene and β-actin for each sample. ΔΔCt was defined as the relative value of the difference between the treated and sham operation group. The fold change of the target gene was calculated using the equation: quantity of target gene $= 2^{-\Delta\Delta CT}$.

### 2.6. Western blot analysis

To investigate the signaling pathway of chelerythrine, total protein fractions were purified from allografts in the control and 5 mg/kg chelerythrine-treated groups ($n = 6$ for each group). Anti-nuclear factor of activated T cells (NFAT)1, anti-phosphorylated NFAT1, anti-NFAT2, anti-NFAT4, anti-phosphorylated NFAT4, anti-AP1 (Abcam, USA) and anti-GAPDH (Dawen Biotec, Hangzhou, China) antibody were used for western blot analysis. Equal amounts of protein (40 μg/lane) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking of non-specific binding sites, membranes were incubated overnight at 4 °C with primary antibody (1:500–2000), and anti-GAPDH antibody (1:4000) followed by the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000; Dawen Biotec).Then the membranes were developed in the ECL Western detection reagents (Amersham–Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's protocol. The gray value of each band was analyzed and auto-calculated by ImageLab software (Bio-Rad, USA).

**Table 1**
Sequences of the primers for IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ and β-actin used in the present study.

| Gene | Primer | Sequence (5′-3′) | Size (bp) |
|---|---|---|---|
| IL-2 | Forward | GCGGCATGTTCTGGATTTGACTC | 152 |
| | Reverse | CCACCACAGTTGCTGACTCATC | |
| IL-4 | Forward | ATCATCGGCATTTTGAACGAGGTC | 125 |
| | Reverse | ACCTTGGAAGCCCTACAGACGA | |
| IL-10 | Forward | CGGGAAGACAATAACTGCACCC | 130 |
| | Reverse | CGGTTAGCAGTATGTTGTCCAGC | |
| IL-17 | Forward | CAGACTACCTCAACCGTTCCAC | 130 |
| | Reverse | TCCAGCTTTCCCTCCGCATTGA | |
| TNF-α | Forward | TCTCATCAGTTCTATGGCCC | 212 |
| | Reverse | GGGAGTAGACAAGGTACAAC | |
| IFN-γ | Forward | ACTGGCAAAAGGATGGTGACA | 214 |
| | Reverse | TGGACCTGTGGGTTGTTGAC | |
| β-Actin | Forward | TGACAGGATGCAGAAGGAGA | 131 |
| | Reverse | GCTGGAAGGTGGACAGTGAG | |

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