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The flavonoid kurarinone inhibits clinical progression of EAE through inhibiting Th1 and Th17 cell differentiation and proliferation

Liang Xie[a], Wei Gong[a], Jin Chen[b], Hong-wu Xie[c], Man Wang[a], Xiao-ping Yin[a], Wei Wu[a,*]

[a] Department of Neurology, the Second Affiliated Hospital of Nanchang University, Nanchang, China
[b] Department of Neurology, the First Affiliated Hospital of Nanchang University, Nanchang, China
[c] Neuroscience Research Institute, Peking University, Beijing, China

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ABSTRACT

Introduction: The flavonoid kurarinone suppresses CD4+ T-cell-mediated chronic inflammatory dermatitis. However, kurarinone's effects upon autoimmune central nervous system (CNS) disease remain unknown. We investigated the potential therapeutic effects and molecular mechanism(s) of kurarinone in an experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis (MS).

Materials and methods: Myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide-induced EAE was constructed in wild-type mice. Effects of kurarinone (100 mg/kg/day) upon clinical scores were assessed based on physical traits and signs. Spinal cord sections were extracted to assess inflammation, demyelination, and mRNA expression of key pro-inflammatory cytokines and chemokines. CNS-infiltrating mononuclear cells (MNCs) and splenocytes were harvested; flow cytometry was then applied to determine CD4+ and CD8+ T-cell percentages as well as Th1/Th2/Th17 subset percentages. Purified naïve CD4+ T-cells underwent in vitro T-cell polarization and proliferation to assess kurarinone's effects.

Results: Prophylactic and treatment regimens of kurarinone significantly improved clinical scores in the MOG_{35-55} peptide-induced EAE model ($P < 0.05$). Kurarinone significantly lowered CNS inflammation and demyelination (61% and 83% decreases, respectively; $P < 0.05$), significantly decreased MNC infiltration into CNS tissue (42% decrease; $P < 0.05$), and significantly inhibited levels of several pro-inflammatory cytokines and chemokines ($P < 0.05$). Kurarinone significantly lowered CD4+ and CD8+ CNS T-cell counts (51% and 80% decreases, respectively; $P < 0.05$) and significantly reduced CNS Th1 and Th17 cell percentages (24% and 44% decreases, respectively; $P < 0.05$). Kurarinone significantly inhibited in vitro Th1, Th2, and Th17 cell differentiation and proliferation ($P < 0.05$).

Conclusions: Kurarinone significantly inhibits the clinical progression of EAE through the inhibition of Th1 and Th17 cell differentiation and proliferation. Kurarinone may show promise as an immunomodulatory therapeutic agent in treating MS.

1. Introduction

Multiple sclerosis (MS) is a serious central nervous system (CNS) disorder characterized by lymphocyte-driven inflammation, microglial activation, demyelination, and axonal degeneration [1]. Immune-mediated animal models of demyelination have been important tools for investigating the pathogenesis of MS [2]. The most popular murine model of MS – myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) [3] – can be constructed through injecting a MOG peptide (MOG_{35-55}), which activates myelin-specific T-cell-mediated inflammatory signaling cascades, leading to demyelination and axonal degeneration [4]. In particular, the earliest phase of MOG-induced EAE (preceding observable disease onset) is characterized by a brain cell-targeting autoimmune response as evidenced by pronounced downregulation of neuronal and oligodendrocyte marker gene expression [5]. Moreover, as the disease course progresses in severity, the expression of inflammatory markers progressively rises [5], which highlights the importance of measuring

Abbreviations: Th1, T helper cells 1; Th17, T helper cells 17; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; $MOG_{35–55}$, Myelin oligodendrocyte glycoprotein; MNCs, mononuclear cells

* Corresponding authors at: Department of Neurology, The Second Affiliated Hospital of Nanchang University, No. 1 Minde Road, Nanchang, Jiangxi 330006, China.
E-mail address: wuwei0791@163.com (W. Wu).

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inflammation marker expression to assess disease progression in EAE. As the effector CD4 + T-cell subsets Th1 and Th17 are primarily responsible for mediating the initial autoimmune response in EAE [6], an improved understanding of the role of Th1 and Th17 cells in EAE is needed to develop novel MS therapies that can specifically target these CD4 + T-cell subsets.

Flavonoids are naturally-occurring botanical polyphenolic compounds with a core C6-C3-C6 carbon skeleton made of two phenyl rings buttressing a heterocyclic pyrane ring; thus, the classification of flavonoids (e.g., flavonols, flavones, and flavanones) is based on the hydroxylation pattern of the phenyl rings and the structural features of the pyrane ring [7, 8]. Remarkably, flavonoids as a group have been shown to display pronounced immunomodulatory properties via affecting Th1/Th2/Th17 cytokine balance [7]. More specifically, several flavanones (e.g., alpinetin, farrerol, liquiritin, liquiritigenin, and naringin) have been shown to positively affect Th1/Th2/Th17 cytokine balance, suppress airway hyperresponsiveness, and reduce pro-inflammatory NF-κB signal transduction in murine models of asthma [7].

Therefore, novel flavanones may serve as promising candidate therapies for MS via modulating the Th1/Th2/Th17 cytokine balance. Notably, kurarinone is a lavandulylated flavanone derived from the root of the shrub *Sophora flavescens* [9], which has been shown to suppress CD4 + T-cell differentiation and repress the development of chronic inflammatory dermatitis [10]. Despite these previous findings, kurarinone's effects upon autoimmune CNS disease and Th1/Th2/Th17 cell balance remain unknown. Therefore, here we investigated the potential therapeutic effects and molecular mechanism(s) of kurarinone in a well-established murine model of EAE.

2. Materials and methods

2.1. Ethics approval

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Nanchang University (Nanchang, China). The animal procedures followed in this study were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (US National Research Council, 2010).

2.2. Chemicals

Kurarinone (> 99% purity) was purchased from WuXi PharmaTech (Shanghai, China). Stock solutions were made by dissolving kurarinone in DMSO, and these were diluted with PBS prior to injection to obtain the desired final concentration. Myelin oligodendrocyte glycoprotein 35–55 peptide (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK) was purchased from GL Biochem (Shanghai, China). Freund's Complete Adjuvant H37Ra was purchased from Difco (Detroit, MI, USA). Pertussis toxin was purchased from List Biological Lab (Epsom, England). Ionomycin and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and GolgiPlug was purchased from BD Biosciences (San Jose, CA, USA). TRIzol reagent was purchased from Tiangen (Beijing, China).

2.3. In vivo kurarinone toxicity testing

Prior to construction of the EAE model, we first tested for kurarinone toxicity in healthy wild-type C57BL/6 mice (females, 8–10 weeks old) obtained from our university's Animal Center. Various doses of kurarinone (0, 10, 50, 100, and 200 mg/kg/day) were injected daily via i.p. injection over the course of 30 days. There were no detectable overt toxic effects from kurarinone therapy, as kurarinone at all doses produced no deaths or significant weight changes in the treated animals. Moreover, to rule out any possible lymphocyte cytotoxicity from kurarinone therapy, non-terminal blood draws into sodium EDTA tubes were performed by saphenous venipuncture every three days during the 30-day course. Lymphocyte counts were then tabulated by counting cells with a hemocytometer; no significant changes in lymphocyte counts were detected at any kurarinone dose across the 30-day treatment period (Supplemental Fig. 1A).

2.4. EAE Induction and Kurarinone Treatment

Wild-type C57BL/6 mice (females, 8–10 weeks old) were obtained from our university's Animal Center for use in this study. Mice were immunized for active EAE induction through subcutaneous (s.c.) injection on the back as previously described by Zhang et al. [11]. In brief, immunization of mice was achieved by injection of MOG_{35-55} (200 μg) emulsified in Freund's Complete Adjuvant H37Ra (Difco, Detroit, MI, USA), which contains *Mycobacterium tuberculosis* H37Ra at a concentration of 4 mg/ml. Post-immunization (p.i.), days 0 and 2, a dose of pertussis toxin (200 ng) was given to mice via intraperitoneal (i.p.) injection. Clinical scores ranging from 0 to 5 were calculated blindly by two researchers each day according to values assigned to specific physical traits and signs. From greatest to least severe, these scores are: grade 5, death; grade 4.5, near death, moribund; grade 4, complete paralysis of two limbs; grade 3, complete paralysis of a single limb; grade 2.5, partial limb paralysis and ataxia; grade 2, dysfunctional gait with limp tail and ataxia; and grade 1, dysfunctional gait with tail tonicity or limp tail. A kurarinone dose optimization study was first performed to determine the proper dosage (i.e., 100 mg/kg/day). Then, this dosage was injected daily via i.p. injection. Daily injections were started on the day of immunization (day 0 p.i.) or on the day of disease onset (day 10 p.i.) to test for prophylaxis or treatment, respectively.

2.5. Histopathology

To assess the histopathology of the spinal cord (hereafter referred to as the CNS), mice were transcardially perfused with PBS, followed by perfusion with 4% paraformaldehyde, and rinsing of the spinal cord tissue with ethanol and xylene. Paraffin-embedded lumbar level 3 (L3) sections (5 μm) were then stained with hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB) to assess inflammation and demyelination, respectively. Pathological assessment was performed blindly using a 0–3 scale as previously described by Zhang et al. [11].

2.6. Preparation of CNS-infiltrating MNCs

Infiltrating mononuclear cells (MNCs) were harvested from the CNS via perfusion with 30 ml PBS through the heart to remove peripheral blood. Suspensions of single cells were generated, and MNCs were separated by the use of a 70/37% Percoll gradient (GE Healthcare, Piscataway, NJ, USA). Trypan blue stain (0.4%) was subsequently used to count viable cells.

2.7. Flow cytometry

Expression of cell surface markers was measured by flow cytometry (FACSAria, BD Biosciences) using fluorophore-conjugated antibodies at the recommended dilution relative to isotype control antibodies as previously described by Zhang et al. [11]. Briefly, CNS-infiltrating MNCs or splenocytes were harvested on day 18 p.i. from EAE mice under the treatment regimen (i.e., treatment starting on day 10 p.i.). From staining with primary antibodies against CD4 and CD8, flow cytometry was applied to determine the CD4 + and CD8 + T-cell percentages from the lymphocyte gates of the CNS-infiltrating MNCs and splenocytes. The cells were then stimulated with MOG_{35-55} for 24 h (CNS cells, 10 μg/ml) or for 72 h (spleen cells, 25 μg/ml), followed by a 5-h stimulation with ionomycin (500 ng/ml) and PMA (50 ng/ml) plus GolgiPlug. After washing, cells were then fixed and permeabilized with Perm/Fix solution (eBioscences, San Diego, CA, USA). Cells were then