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Development of the Potent Anti-Rheumatoid Arthritis Compound Derived from Rosmarinic Acid and the Evaluation of the Activity in Collagen-Induced Arthritis Mouse Model

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ABSTRACT
Lck, one of the Src family protein tyrosine kinases, plays a critical role in T-cell receptor-induced signaling that leads to T-cell activation, proliferation and differentiation. Interruption of the molecular interaction through the lck SH2 domain blocks T-cell activation and subsequent proliferation. Previously, we demonstrated that rosmarinic acid (RosA), an lck SH2 domain inhibitor, suppresses T-cell function via dual mechanism such as inhibition of T-cell activation and lck-dependent apoptosis induction. RosA alleviated arthritis in Collagen-Induced Arthritis (CIA) model but a fairly high amount of RosA was required. To overcome this problem, we generated various kinds of C-terminus modified RosA analogues and found a methyl ester derivative of RosA (RosA-Me) to have the most improved inhibition activity in IL-2 promoter analysis and T-cell proliferation assay. The objective of this study was to assess the anti-arthritic activity of RosA-Me compared to the parent compound RosA and Methotrexate (MTX) in a murine CIA model. Compared to vehicle, treatment of mice with RosA-Me (50 mg kg^{-1} day^{-1}, i.p.) greatly reduced the inflammation indexes which suggests RosA-Me has better *in vivo* anti-rheumatoid arthritis activity acquired maybe from the stronger cell penetrating ability. Thus, our studies suggest that RosA-Me is a RosA derivative with a potent anti-arthritic effect in CIA, through its combined immunosuppressive and anti-inflammatory actions.

Key words: Anti-rheumatoid arthritis, rosmarinic acid, collagen-induced arthritis

INTRODUCTION

Immunosuppressive drugs disturb the activity of immune system and have been used for immunosuppressive therapy such as rejection of organ transplantation, treating autoimmune diseases (rheumatoid arthritis, multiple sclerosis and ulcerative colitis) and treating other non-autoimmune hyper-inflammatory diseases (allergic asthma) (Kovarik, 2013). For this purpose, lots of immunosuppressive agents have been developed. For example, allograft rejection is controlled by using cyclosporin A, azathioprine and corticosteroids (Snell *et al*., 2013). Among them, cyclosporin A is the most powerful and frequently used immunosuppressant (Hardinger and Brennan, 2013). Other immunosuppressive agents such as FK506 and rapamycin have been used in the treatment of rheumatoid arthritis (Drosos, 2002). However, these drugs are reported to be closely associated with a high incidence of side effects such as nephrotoxicity and/or hepatotoxicity (Naesens *et al*., 2009). Thus, it is still needed to develop the novel immunosuppressive drug that have low side-effect but high selectivity for the clinical application.

Lck, one of the Src family protein tyrosine kinases, plays a critical role in T-cell receptor-induced signaling that leads to T-cell activation, proliferation and differentiation (Billadeau, 2010). Thus, ligands against lck SH2 domain could be one of the therapeutic ways for various immune system related disorders such as autoimmune disease, leukemia and

lymphomas (Sawyer *et al*., 2002). For this reason, recently lots of studies on peptide-based antagonistic ligands against lck SH2 domain revealed that peptide sequence containing "pYEEIE" has the considerable binding affinity to lck SH2 domains (Broadbridge and Sharma, 2000; Beaulieu *et al*., 1999; Morelock *et al*., 1995; Zhou *et al*., 1993). However, it is not easy to clinically use this kind of peptide based ligands because they are weak to phosphatase and protease and have very low cell penetration ability. Therefore, natural/synthetic chemical compounds could be more reasonable options as an alternative way to develop non-phosphopeptide inhibitors for lck SH2 domains.

Previously, to select the more promising derivatives that can inhibit lck SH2 domain and block T-cell activation signaling pathway, we tested various kinds of RosA derivatives for *in vitro* binding affinity to lck SH2 domain and T-cell activation and successfully screened some potent RosA derivatives (Park *et al*., 2007; Kang *et al*., 2003). Here, among them, we evaluated the *in vivo* anti-rheumatoid arthritis activity of methyl ester form (RosA-Me) and the parent compound (RosA) by adapting collagen-induced arthritis mouse model, a kind of the conventional of *in vivo* tests for T-cell mediated diseases.

MATERIALS AND METHODS

Synthesis of RosA-Me from RosA: Rosmarinic acid (0.1 g, 0.277 mmol, 1 equiv) was, respectively, suspended in methanol and to the solution was added drop-wisely thionyl chloride (0.2 mL, 2.77 mmol, 10 equiv) at 0 ice bath. Each reaction mixture was equally stirred for 18 h under N2 gas. Then the reaction mixture was diluted with ethyl acetate (10 mL) and sequentially washed with 1 N aqueous HCl, 10% aqueous $NaHCO_3$, distilled water and brine. The organic layer was dried over $MgSO_4$ and the crude product was purified by silica gel column chromatography (normal phase, eluting sequentially with 5:4:1, n-hexane/ethyl acetate/methanol) to afford the 85% yield. And the crude product was purified by preparative HPLC by using (0.1% TFA H_2O/acetonitrile) solvent system. The yield was 93%. 1 H NMR (DMSO-d6/TMS, 400 MHz): The 2.96 (br, 2H), 3.64 (s, 3H), 5.11-5.12 (m, 1H), 6.28 (d, J = 16 Hz, 1H), 6.51 (d, J = 8 Hz, 1 H), 6.63-6.65 (m, 2 H), 6.78 (d, J = 8 Hz, 1 H), 7.02-7.07 (m, 2 H), 7.50 (d, J = 16 Hz, 1 H), 8.76 (s, 1 H), 8.8 (s, 1 H), 9.17 (s, 1 H), 9.68 (s, 1 H). 13C NMR (DMSO-d6/TMS, 400 MHz): The 30.7, 52.0, 72.8, 112.8, 114.9, 115.4, 115.7, 116.7, 121.7, 125.3, 126.6, 134.9, 144.1, 145.0, 145.6, 146.4, 148.7, 165.9, 169.9. ESI MS: (M+H)+ calcd m/e 375.10, obsd m/e 374.79.

T-cell proliferation assay: Splenocytes (2×10^5 cells/well) were incubated with RosA or RosA-Me for 2 h prior to activation with immobilized anti-mouse CD3 antibody (200 ng mL^{-1}, 2C11, PharMingen) and kept incubated with RosA or RosA-Me throughout the whole process. After 3 days of incubation at 37 in a 5% CO_2 incubator, the cells were pulsed with 0.5 μ Ci of [methyl-3H] thymidine (Amersham) per well for 16 h at 37 and harvested onto filter paper with a Skatron harvesting apparatus (Autowash 2000, Skatron, Lier, Norway). Thymidine incorporation was determined by a Wallac 1450 Microbeta Trilux g-scintillation counter (Wallac, EG&G, Milton Keynes, GB) and counts per minute (cpm) values from triplicate wells were expressed as Mean± Standard Errors (SE).

IL-2 promoter assay: Jurkat T-cells (1×10^6) were transfected with IL-2-luc reporter plasmid (pGL3/IL-2-Luc) using Superfect™ according to the manufacturer's instructions (Qiagen Inc., CA). After 24 h incubation, cells were treated with RosA or RosA-Me for 30 min and then activated by incubation with immobilized anti-human CD3 antibody (5 μg mL^{-1}) for 16 h. The luciferase activity was determined with a Berthold luminometer LB953 (EG&G Berthold, Bad Wildbad, Germany).

CIA mouse experiment: The 100 μg bovine type II collagen (C II) and CFA emulsion was injected hypodermically into the tail base of DBA/1Lac J mice (male, 8 weeks) to induce collagen-induced arthritis. After 2 weeks, booster immunization was carried out with 50 μg C II/IFA. From the 3rd week after primary immunization, reagents were repeatedly injected into peritoneal cavities of 6 mice per group for 15 days. The control groups were injected with 5% ethanol-olive oil mixture by 100 μL day^{-1} dosage every day in total 15 times. Meanwhile, the RosA and RosA-Me were melted in ethanol and then emulsified in olive oil, in which the final concentration of the compounds was adjusted to 5% which was injected to experimental group by 50 mg kg^{-1} day^{-1} dosage everyday in total 15 times. From the 3rd week after primary immunization, the extent of edema and swelling of the joint was observed everyday to determine arthritic points. The criterions of arthritis index point "0" about each leg of the mouse means "No swelling and boil". The criterions of arthritis index point "1" about each leg of the mouse means "A mild swelling and reddened localized in mid foot (tarsal bone) or angle joint". The criterions of arthritis index point "2" about each leg of the mouse means "A mild swelling and reddened foot from angle joint to mid foot". The criterions of arthritis index point "3" about each leg of the mouse means "A medium swelling and reddened foot from angle joint to metatarsal bone". The criterions of arthritis index point "4" about each leg of the mouse means "A swelling and reddened foot covering from angle to toe". By summing up this standard point, arthritis inhibition effect of each compound was also presented by the Mean±Standard deviation with the arthritis index of other mice after adding up the arthritis index of each legs of a mouse ([maximum arthritis index 4/leg] ×[4 legs/mouse] = maximum arthritis index/mouse 16).

RESULTS

RosA-Me inhibits TCR-induced splenocytic T-cell proliferation and blocks TCR-induced IL-2 promoter activation: To compare *in vitro* anti T-cell proliferation

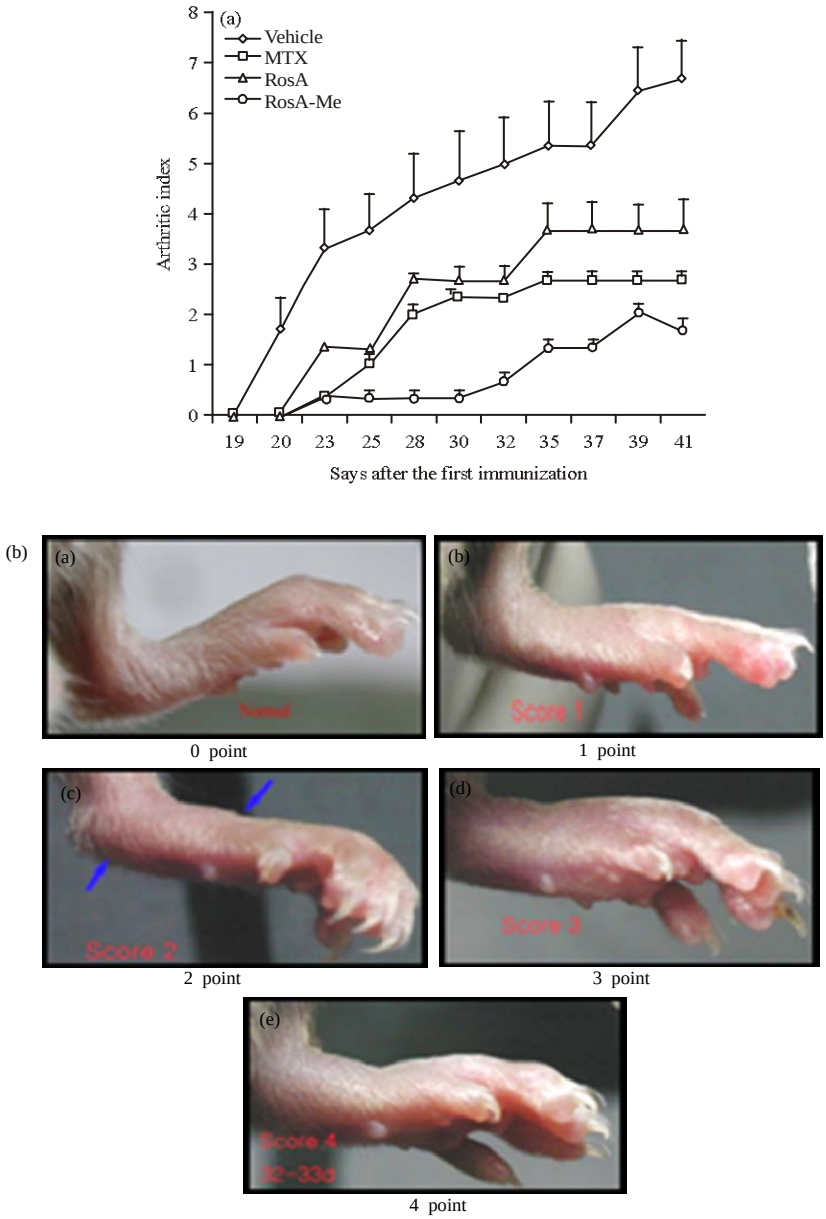
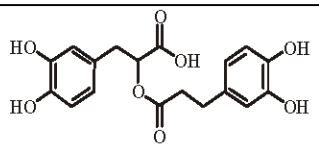
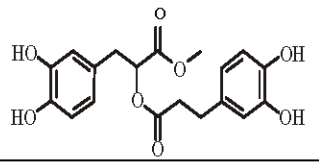


Fig. 1(a-e): Modulation of CIA in the mice by the RosA and RosA-Me. Mice were treated daily i.p. with Vehicle (ETOH/Cremophore/Distilled water), MTX (Methotrexate, 2 mg kg^{-1} day^{-1}, as positive control), RosA, or RosA-Me (50 mg kg^{-1} day^{-1}) from day 21 to day 41 after the first immunization with collagen. During the development of the arthritis, mice paws were individually scored. Data are expressed as Means±Standard Error (SEM) (n = 7 per group)

activity of RosA and RosA-Me, Splenocytes were preincubated with RosA and RosA-Me (25 μM) for 2 h, activated with immobilized anti-CD3 antibody for 3 days and pulsed with [^{3}H] thymidine for last 16 h of incubation. cpm values from triplicate wells were expressed as Mean±Standard Error (SE). As shown in Table 1, RosA-Me more potently inhibited splenocyte proliferation than RosA. Subsequently, we also assessed whether RosA-Me could block IL-2 promoter activation under the same condition the above assay. Jurkat cells were transfected with the IL-2-luc reporter, treated with indicated amount of RosA or RosA-Me (25 μM) for 30 min and activated with immobilized anti-CD3 antibody for 16 h. IL-2 promoter assays were performed in duplicate and representatives of three independent experiments are shown. RosA-Me could inhibit the expression of IL-2 gene much stronger than RosA did (Table 1).

RosA-Me inhibits collagen-induced arthritis *in vivo*: As shown in Fig. 1, compared to vehicle or RosA, treatment of mice with RosA-Me (50 mg kg^{-1} day^{-1}, i.p.) greatly reduced

Table 1: Chemical structure of RosA and RosA-Me and inhibition ability (%) for T-cell proliferation and IL-2 promoter assay

Name	Structure	T-cell (%)[a]	IL-2 (%)[b]
RosA		45±5	50±4
RosA-Me		10±2	8±2

(a) Splenocytes were preincubated with RosA and RosA-Me (25 μM) for 2 h, activated with immobilized anti-CD3 antibody for 3 days and pulsed with [^{3}H] thymidine for last 16 h of incubation, (b) Jurkat cells were transfected with the IL-2-luc reporter, treated with indicated amount of RosA or RosA-Me (25 μM) for 30 min and activated with immobilized anti-CD3 antibody for 16 h

the inflammation indexes which suggests RosA-Me has better *in vivo* anti-rheumatoid arthritis activity acquired maybe from the stronger cell penetrating ability. Methotrexate (2 mg kg^{-1} day^{-1}, MTX) was used for the positive control.

DISCUSSION

Rosmarinic acid (α-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid; RosA) is a natural compound from Labiataeherbs, such as rosemary, sweet basil and perilla. RosA is known to have a broad range of immune-modulating activity by regulating oxidative stress (Al-Sereiti *et al*., 1999; Zheng and Wang, 2001; Van Kessel *et al*., 1986). Recently a few studies reported that RosA has an anti-inflammatory activity can be used to treat various inflammatory disorders via blocking complement activation and inhibiting lipoxygenase and cyclooxygenase activity (Sahu *et al*., 1999; Kimura *et al*., 1987; Kelm *et al*., 2000). Also, some *in vivo* studies have shown that RosA inhibits several complement-dependent inflammatory processes including cobra venom factor-induced paw edema (Englberger *et al*., 1988). However, RosA has pretty many high hydrophilic chemical groups in its structure such as 4 hydroxyl and one carboxyl acid groups that make RosA not considered for the real application to human. So in this study, to improve the cell penetrating ability, we prepared new derivative that has more hydrophobicity than RosA as an anti-inflammatory agent that will help inhibition of TCR-signaling and subsequent T-cell proliferation.

Rheumatoid Arthritis (RA) is a chronic inflammatory disease that has specific symptom such as synovitis and joint damage. There have been known about lots of genetic and environmental causes of RA. And the pathogenesis of RA is pretty complex including synovial cell proliferation, lymphocyte infiltration and cartilage and bone destruction. Many kind of animal experimental models have been adapted to research about the molecular mechanisms in RA development (Youn, 2005). Also, these animal models have been taken to develop the novel therapeutic way to treat RA. Among these models, CIA is one of the most commonly used models to study the pathogenesis of RA since the clinical aspects of CIA are characterized by the pathogenesis including proliferation of synovial cells, infiltration of lymphocytes and formation of pannus which are generally similar to that of RA (Brand *et al*., 2007). In this report, among these models, by using Collagen-Induced Arthritis (CIA) mouse model, we describe the development of the potent anti-rheumatoid arthritis compound derived from rosmarinic acid and the evaluation of the activity. As explained before, we demonstrated that RosA, an lck SH2 inhibitor, suppresses T-cell function via dual mechanism such as inhibition of T-cell activation and lck-dependent apoptosis induction (Won *et al*., 2003). RosA-Me inhibits the activity of lck-SH2 domain and the expression of IL-2 gene inducing immune response, leading to the inhibition of immune response *in vivo* and *in vitro*. Therefore, RosA-Me can be effectively used for inhibiting lck SH2 domain which can be applied to suppressing graft rejection, autoimmune diseases and inflammatory diseases. In conclusion, RosA-Me has sufficient high activity even at low dosages than the parent RosA for CIA mouse model, so RosA-Me can be used as novel treatment agents for arthritis diseases.

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