School of Integrative and Global Majors
Ph.D. Program in Human Biology (HBP)

論文概要

Dissertation Abstract

Title of Doctor Dissertation:

Anti-inflammation and adipogenesis inhibition functions of stylissatin A
（Stylissatin A の抗炎症および抗肥満活性に関する研究）

Last or Family Name	First	Middle
Zhang	Menghua	

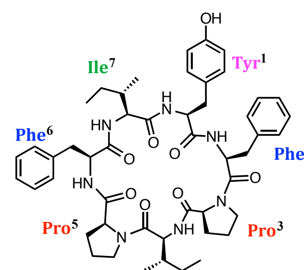
Student Number: 201535006

Primary Academic Advisors
Affiliation: Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA)
Name: Akira Shibuya

Abstract

Background:

Stylissatin A (SA, **1**) is a cyclic peptide isolated from the sponge *Stylissa massa*, which was collected on Loroata Island, Papua New Guinea. Many bioactive cyclic peptides contain modified amino acids such as *N*-methyl amino acids and D-amino acids, but SA is unique in that it is composed of only unmodified L-amino acids: isoleucine (Ile), phenylalanine (Phe), proline (Pro), and tyrosine (Tyr). SA is known for its nitric oxide (NO) inhibition function against lipopolysaccharide-(LPS) stimulated murine RAW264.7 macrophage cells (EC_{50} = 87 μM). Excessive NO production will result in the formation of peroxynitrites, which causes inflammation and cell damage. Therefore, anti-inflammation function of SA may contribute to the development of new anti-inflammatory agents. However, the target biomolecules of SA and their mechanism of action have not been studied so far.



Stylissatin A (SA, **1**)

Purpose:

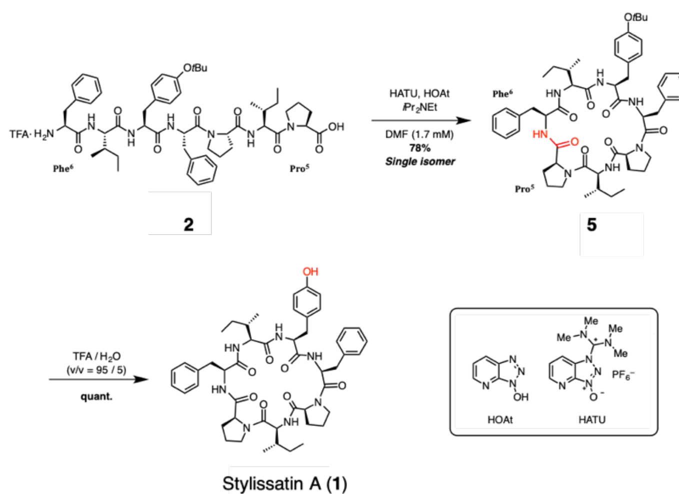
This study is aimed at identifying the pharmacophore of SA by structure-activity relationship study and clarifying the mechanism of stylissatin A analogues' anti-inflammation function. What's more, several stylissatin A derivatives were found to have adipogenesis inhibition function against murine fibroblast 3T3-L1 cells in this study, another purpose of this research is to identify the target molecule of SA analogues in

both macrophage and preadipocyte cells and establish their new mechanisms which influence both inflammation response and adipogenesis.

Results:

1. Chemical synthesis of stylissatin A

To prepare stylissatin A (**1**), linear heptapeptide **2** was synthesized in solid phase (Scheme 1). In order to reduce steric hindrance during the cyclization and prevent epimerization, proline residue was chosen as *C*-terminal residue of the cyclization precursor. Intramolecular condensation between Pro^5 and Phe^6 in **2** and removal of *t*Bu group in **5** afforded **1**.



Scheme **1**. Synthesis of stylissatin A (**1**)

2. Structure-activity relationship study of stylissatin A – Anti-inflammation activity

On the structure-activity relationship (SAR) studies of **1**, two derivatization approaches were examined. The first one is modification of tyrosine hydroxy group. By Griess assay and MTT assay, NO inhibitory activity (EC_{50}) and cytotoxicity (IC_{50}) was measured. As a result, L-Tyr^1-*t*BuSA (**5**) was found to exhibit more potent NO inhibitory activity (EC_{50}) than SA (**1**) (EC_{50} = 13 μM), while the cytotoxicity was also increased (IC_{50} = 15 μM). The second approach is introduction of D-amino acids to L-Tyr^1-*t*BuSA (**5**). By replacing with one or more D-amino acid(s), conformation of the cyclic skeleton was expected to change significantly, and the activity would be also changed. As a consequence, D-Tyr^1-*t*BuSA (**5a**) was found to show the most potent inhibitory activity of NO production with neglectable cytotoxicity (EC_{50} = 12 μM, IC_{50} > 200 μM) among 16 synthetic derivatives.

3. Effect of D-Tyr^1-*t*BuSA (**5a**) in LPS-treated RAW264.7 cells

iNOS (inducible nitric oxide synthase) is an enzyme induced by LPS to generate NO during inflammation response. On this study, it was found that D-Tyr^1-*t*BuSA (**5a**) significantly inhibited iNOS expression at 20 μM. One of the pathways that influence iNOS expression is the mitogen-activated protein kinase (MAPK) pathway, and tumor necrosis factor alpha (TNF-α) is known to activate this pathway, which proceeds to activate nuclear factor kappa-B (NF-κB). To further clarify the anti-inflammatory mechanism of SA, expression levels of TNF-α and interleukin-6 (IL-6), two inflammatory cytokines increased by NF-κB activation, were evaluated by enzyme-linked immunosorbent assay (ELISA). It was found that **5a** significantly decreased the concentrations of both IL-6 and TNF-α in a dose-dependent manner (EC_{50} = 1.4 and 5.9 μM, respectively), which inferred that anti-inflammation function of **5a** might be due to the inhibition of NF-κB signaling pathway.

4. Structure-activity relationship study of SA – Adipogenesis inhibition function

It is known that obesity is a disease closely linked to inflammation. Obesity is not just an increase in body weight and body fat percentage, but also the chronic inflammation of adipose tissue. Since SA and its derivatives showed potent anti-inflammation activities, it was thought that these compounds might also have effects on adipocyte differentiation. In fact, both SA (**1**) and L-Tyr[1]-*t*BuSA (**5**) inhibited the accumulation of fat on murine 3T3-L1 preadipocytes (EC_{50} = 9.1 and 1.9 μM, respectively). Furthermore, D-Tyr[1]-*t*BuSA (**5a**) had the most potent inhibitory effect among the 16 compounds tested (EC_{50} = 440 nM). Comparing with the results of anti-inflammation SAR study, D-Tyr[1]-*t*BuSA (**5a**) might specifically regulate intracellular signaling pathways that is common to inflammation and adipogenesis.

5. Expressions of PPARγ and C/EBPα in 3T3-L1 cell lysate treated with **5a**

PPARγ and the C/EBPα are transcription factors that control adipocyte differentiation. To further clarify the mechanism, their cellular expression levels were examined by Western blotting analysis after the sample treatment for 48 h. As the result, **5a** potently suppressed the expressions of PPARγ and C/EBPα at 20 and 60 μM, which proved the adipogenesis inhibition function of **5a**.

6. Target identification of SA analogs in macrophage cells and preadipocyte cells

To identify the target biomolecules of SA, SA-biotin probe **12** was synthesized. By the pull-down assay with RAW264.7 and 3T3-L1 cell lysates, a specific target protein at 45 kDa was affinity-purified from both cells, which was identified as acyl-CoA dehydrogenase, long chain (ACADL). ACADL catalyzes the β-oxidation of long chain fatty acids, in which acyl-CoA is converted to enoyl-CoA in the presence of FAD (flavin adenine dinucleotide), a cofactor for redox reactions. In general, when the β-oxidation is promoted, the amount of FAD^+ decrease, and when the β-oxidation is inhibited, the amount of FAD^+ increase. Thus, FAD levels are often used as a proxy for β-oxidation. It was found that FAD^+ levels were increased to 1.2 and 1.4 times compared to the control by the treatments with both **1** and **5a** at 60 μM, which means the β-oxidation level was suppressed and the activity of ACADL was inhibited.

Discussion:

From the results of SAR study of SA (**1**), D-Tyr[1]-*t*BuSA (**5a**) was found to show the strongest anti-inflammation and adipogenesis inhibition functions among all derivatives, and ACADL was identified as a common target protein in both RAW264.7 and 3T3-L1 cells. In addition, both SA (**1**) and D-Tyr[1]-*t*BuSA (**5a**) inhibited ACADL activity. Based on these results, mechanisms of action of SA and its derivatives in two cells were considered (Figure 1). For anti-inflammation function, by adding SA analogs, the amounts of liberated fatty acids would increase, which might activate transcription factor

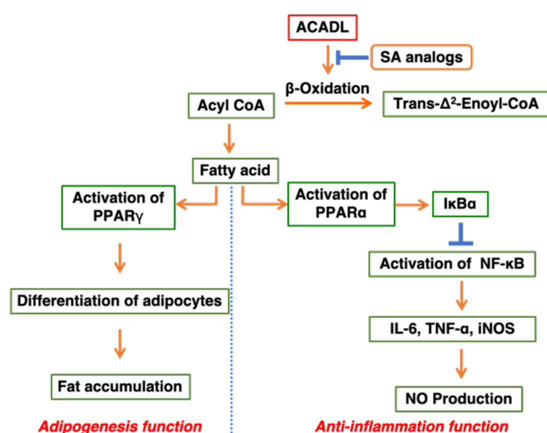


Figure 1. Proposed Mechanism

PPARα (peroxisome proliferator-activated receptor α). PPARα activates IκBα by directly binding to the IkBα promoter, which inhibits the function of NF-κB and suppresses the production of IL-6 and TNF-α and the expression level of iNOS, leading to anti-inflammatory activity. Therefore, it is considered that ACADL could be a new target molecule for anti-inflammation activity, which had not been received much attention until now. Meanwhile, ACADL was also identified as target molecule in 3T3-L1 cells. According to the proposed mechanism, when ACADL was inhibited by SA analogs, increased fatty acids would activate PPARγ. However, this hypothesis did not match to the results of cellular expression levels of PPARγ treated with SA derivatives as described above. In the above proposed mechanism, activated PPARγ would induce cell differentiation of preadipocytes and result in fat accumulation. However, SA and its derivatives inhibited them and reduced triacylglycerol accumulation levels in mature adipocytes. Thus, there may be other targets besides ACADL for the adipogenesis inhibition function of SA and its analogs. To further clarify the mechanisms of both anti-inflammation and adipogenesis inhibition functions, experiments such as DNA microarray analysis will be performed to evaluate the effect of compounds in gene level.