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論文の内容の要旨
Abstract of thesis

（目的　Purpose）

Stylissatin A (SA) is a proline-rich cyclic peptide isolated from the Pupa New Guinean marine sponge *Stylissa massa*, and it has been reported with anti-inflammation activity against macrophage. However, the detailed mechanisms of this natural chemical compound have not yet been investigated. In this doctoral dissertation, the author focuses on identification of pharmacophore of SA by analysis of structure activity relationship and clarifying the mechanisms of the anti-inflammation activity as well as adipogenesis inhibitory function. Furthermore, the author successfully carried out identification of SA-binding target molecule for its signal transduction mechanism.

（対象と方法　Materials and Methods）

All experimental materials and methods used in this proposed project~~s~~ are summarized as follows:

1. Solid-phase peptide synthesis (SPPS) and their spectroscopic data analyses
   All SA derivatives were synthesized by PetiSyzer Shaking System Description (Hipep Laboratories) and 5 mL LibraTube with filter (Hipep Laboratories) according to the Fmoc/*t*Bu strategy. All crude materials were purified by an ODS column chromatography under each condition. All structures were determined by use of 1D and 2D NMR spectroscopic analysis, Marfey's methods, and MS/MS analysis.

2. Qualitative testing
   Both Kiser and Chloranil tests were performed.
3. All biochemical techniques such as cell viability assay, cell lysate preparation, immunochemical pull-down assay, Western blotting, ELISA, gel electrophoresis and its silver staining, were performed by each standard protocol.
4. Cell bioactivity assay
   Both murine macrophage RAW264.7 and murine preadipocyte 3T3-L1 cells were used for anti-inflammation activity and adipogenesis inhibitory function analysis, respectively.

（結果 Results）

First, in order to synthesize SA, the author selected Pro as C-terminal residues for synthetic route of linear peptide (cyclization precursor of SA) for solid-phase peptide synthesis (SPPS), to prevent epimerization during cyclization. Next, Phe rather than Ile was selected as N-terminal in order to reduce steric hindrance during cyclization. Thus, a cyclization site between Pro^5-Phe^6 was chosen. Then, in order to get cyclization precursor 2, the hydroxyl group of tyrosine was protected by *tert*-butyl (*t*Bu) group and Barlos resin (L-proline-supported 2-chlorotrityl resin) was used under mild acid conditions. After getting cyclization precursor 2, cyclic peptide (*t*BuSA) was successfully synthesized and *t*Bu group was removed by TFA. NMR data of the synthetic SA showed confirmation of the desired compound obtained for further bioassay.

Secondly, the author synthesized two different kinds of SA derivatives, namely modification of tyrosine residue and replacement with D-amino acids to *t*BuSA, for analysis of structure activity relationship. The results showed all derivatives with ether functional group including *t*Bu-, propargyl-, methyl- and benzyl-group potent NO production inhibitory activity than SA. However, acetate and glucose derivatives did not show anti-inflammatory activity. In the case of replacement with D-amino acids to *t*BuSA, SA D-amino acid derivatives, which contain one or two D-amino acids, including D-Tyr^1, D-Phe^2, D-Pro^3, D-*allo*-Ile^4, D-Pro^5, D-Phe^6, D-*allo*-Ile^7 and D-$Pro^{3,5}$ analogs, were evaluated using same LPS-stimulated RAW264.7 cells assay. Intriguingly, D-Pro^5derivative showed 4 times higher potency and the highest value of selectivity index and most desirable anti-inflammatory activity among all of the synthetic SA derivatives. Indeed, the D-Tyr^1-*t*BuSA was found to inhibit nitric oxide (NO) and to suppress inducible nitric oxide synthase (iNOS) expression. Further experiments showed inhibitory effect of IL-6 and TNFα by the D-Tyr^1-*t*BuSA.

Thirdly, the author investigated any possible link between inflammation and obesity, since obesity is thought to be accompanied with the chronic inflammation of adipose tissue, which is closely associated with metabolic disorders such as obesity, insulin resistance, and type 2 diabetes. Using the murine 3T3-L1 fibroblasts, the author found that fat accumulations were reduced in SA and *t*BuSA treated cells, and both SA and *t*BuSA potently inhibit the differentiation of murine 3T3-L1 preadipocytes. Interestingly, the D-Tyr^1-*t*BuSA showed most potent adipogenesis inhibitory activity with highest selectivity index. Furthermore, the D-Tyr^1-*t*BuSA was found to suppress the expression of transcription factors, such as PPARγ (Peroxisome Proliferator-Activate Receptor γ) and the C/EBPα (CCAAT-Enhancer-Binding Protein α) in dose-dependent manner.

Finally, the author identified the ACADL (acyl-CoA dehydrogenase, long chain) as a target protein specific for the SA biotin probe. Functional assay for ACADL enzyme activity clearly showed the upregulation in SA and D-Tyr^1-*t*BuSA treated RAW264.7 cells.

（考察 Discussion）

The author showed that an anti-inflammatory marine proline-rich cyclic peptide, Stylissatin A (SA), and its derivatives potently inhibit the differentiation of preadipocytes, and identified ACADL as the molecular target specifically binding to D-Tyr^1-*t*BuSA.

For anti-inflammation function, upregulation of fatty acids by SA analogs might activate transcription factors

PPARγ, and PPARγ activates IκBα by directly binding to IκBα promoter, which inhibits the NF-κB function and suppresses the IL-6 and TNF-α production. Thus, SA analogs were considered to inhibit ACADL activity.

On the other hand, for adipogenesis inhibitory function, protein expression level of PPARγ was downregulated by D-Tyr[1]-*t*BuSA and adipocyte differentiation and lipid droplet accumulation were also suppressed. However, although ACADL is also identified as target molecule in 3T3-L1 cells, unlike the proposed mechanisms that when ACADL was inhibited by SA analogs, increased fatty acids would activate PPARγ, the results of PPARγ expression levels with SA derivatives did not match this proposed hypothesis.

Taken together with all experimental data including structure activity relationship and biological assay by using a series of SA derivatives suggested that ACADL is indeed one of SA binding targets, but that the D-Tyr[1]-*t*BuSA probe is not the same as natural SA, and that improving SA probe might contribute to discovering a new molecular target in order to understand different signal transduction mechanisms.

審査の結果の要旨
Abstract of assessment result

（批評　General Comments）

The applicant successfully synthesized a series of SA derivatives and identified a molecular target, ACADL specific for SA biotin probe. Furthermore, the applicant clearly demonstrated functional studies using two different cell lines for anti-inflammation and adipogenesis inhibitory function with analysis of structure-activity relationships of SA derivatives. All experimental data are well characterized and reasonably discussed among entire proposed projects. Moreover, the applicant could master double major including chemical synthesis and molecular biological techniques.

（最終試験の結果　Assessment）

The final examination committee conducted a meeting as a final examination on 8th January, 2021. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

（結論　Conclusion）

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.