Form 3

Abstract of Thesis

Name （Md. Imran Hossain）	
Title	Synthesis and Immuno-stimulatory Effects of Heavy Atom-Labeled Lipidic Ligands to Investigate Precise Ligand Recognition Mechanism of Antigen-presenting Protein CD1d (抗原提示タンパク質CD1dと脂質性リガンドの結合機構解明のための重原子標識体の化学合成と免疫活性化作用)

Antigen presenting protein CD1d regulates the stimulation of natural killer T (NKT) cells through the presentation of endogenous and exogenous lipidic antigens, glycolipids and phospholipids, to the T cell receptors (TCR). CD1d has deep hydrophobic binding pockets, A' and F' to accommodate long alkyl chains. The head group of lipidic ligands interact with the TCR to form a ternary complex, CD1d-lipid-TCR. Such recognition induces the secretion of various cytokines comprising TH1 type such as IFN-γ, IL-12 and GM-CSF, and TH2 type such as IL-4, 5, 10 and 12. Due to the pro- and anti-inflammatory effects of IFN-γ and IL-4, respectively, polarized cytokine production has been a target to develop immuno-therapeutics. The responsible factors for the regulation of the cytokine production remain poorly understood. However, structural modification of the antigen can tune the cytokine production. A precise interaction study of the antigen-protein complex is necessary to reveal unknown mechanism in secreting the antigen-mediated cytokines.

In this study, I prepared analogues of glycolipid, α-GalCer (KRN7000) that were labeled with a heavy atom at an acyl chain to examine their precise binding modes in the lipid-protein complex (Fig. **1A**). In addition, self-antigen lyso-phosphatidylethanolamine (eLPE) derivatives having p-fluorobenzyl groups are synthesized to study antigen-protein interactions (Fig. **2A**). KRN7000 is a congener of marine natural product agelasphin, potent anticancer agent, which binds specifically to CD1d. Two alkyl chains, acyl and phytosphingosine, of the ceramide part fit into the A' and F' hydrophobic binding groves of CD1d and galactose moiety remains on the surface of the protein to be presented toward TCR. Due to low electron density and flexibility of the alkyl chains, precise binding modes of the molecule, especially for alkyl chains, could not be determined in atomic resolution.

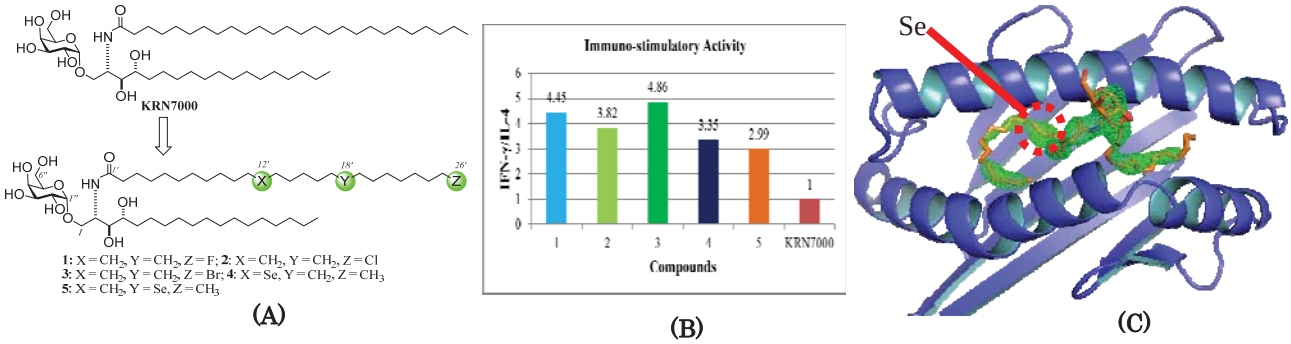

Fig. 1. (A) Structures of KRN7000 and labelled analogues. **(B)** TH1/TH2 selectivity of the analogues of GalCers in cytokine production. **(C)** Electron density map (F_o-F_c; 3σ) of the 12'Se-GC (**4**) in hCD1d at 2.4 Å resolution. The high electron density of Se is marked by the red circle.

I have introduced Se and Br at the 12' or 18' and 26' positions, respectively, of KRN7000 to increase electron density of the fatty acid chain. To synthesis the molecules, first galactose fluoride was prepared, then connected with appropriately protected phytosphingosine acceptor in α-glyosidic linkage. The heavy atom labeled fatty acids were synthesized before loading them to the amine group of phytosphingosine. Immuno-stimulatory activates, cytokine (IFN-γ and IL-4) production, of the α-GalCer analogs were evaluated using murine spleen cells. All of the synthesized derivatives exhibited similar potency to the positive control KRN7000 in IFN-γ secretion, whereas lower potent in IL-4 production. Results suggest the efficient trimetric

complex formation between the synthesized α-GalCers and CD1d-TCR. One of the α-GalCer derivatives, 12'SeGC (**4**), was co-crystalized with hCD1d and structure of the complex was successfully solved by collaborators at higher resolution 2.4 Å (Fig. **1C**). The high electron density due to the Se atom can be detected by the enhanced electron density map. Thus, using heavy atom labeled α-GalCer analog the location of carbon atom at 12' position can be determined precisely.

CD1d participates in presenting intrinsic ligands, usually as a weaker activator of NKT cells. Due to the lower potency, it is difficult to find high affinity intrinsic ligands using cell based cytokine production assays. For the next step, I developed an alternative *in-vitro* assay using SPR technique to determine direct binding affinity of antigens towards CD1d for further structural studies. To analyze binding affinities, p-flurobenzyl substituted and other related analogs were prepared as analogues of an intrinsic ligand lyso-phosphatidylethanolamine (Fig. **2A**), which were known to more or less bind to CD1d. For the SPR experiments, human CD1d was immobilized on the sensor chip using the amine-coupling method. Samples were diluted in 10 mM PBS (137 mM NaCl, 27 mM KCl) pH 7.4 running buffer in 10-1000 nM concentration before running them though the protein immobilized surface. Series of dose dependent SPR sensorgrams obtained were fitted into theoretical curves using 1:1 binding models to calculate the dissociation constants (K_D).

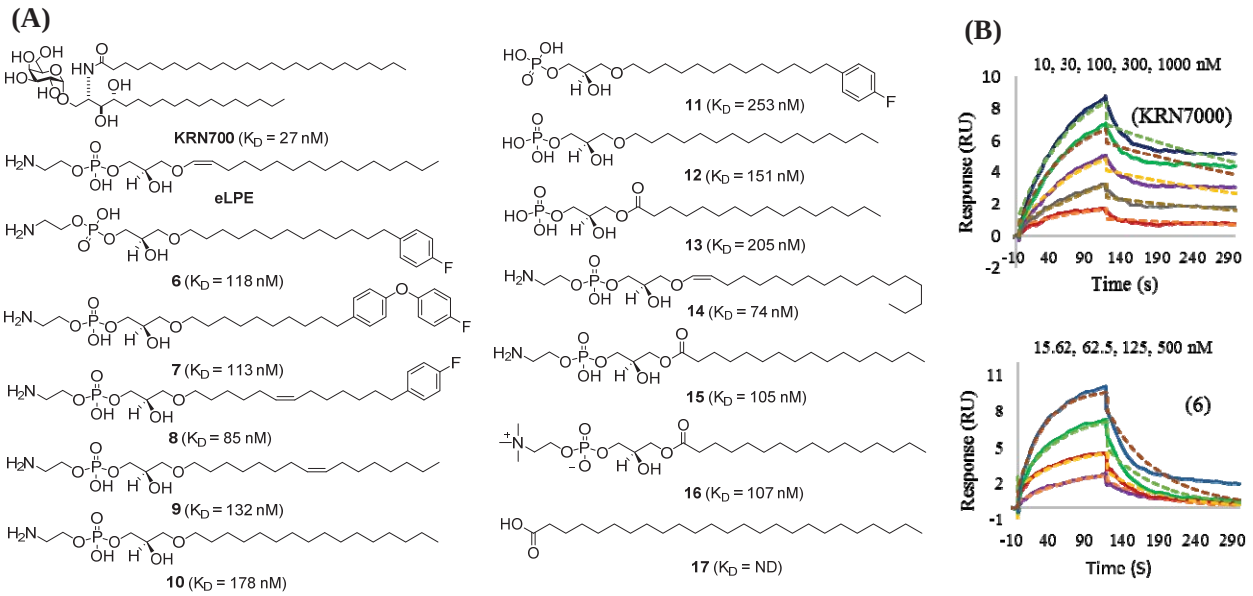

Fig. 2. **(A)** Structures of the compounds and dissociation constants (K_D). **(B)** Representative SPR sensorgrams. As a result, KRN7000 showed the higher binding affinity to CD1d than any analogues of lyso-phosphatidylethanolamine (eLPE). Compounds having p-fluorobenzyl groups and/or cis double bonds exhibited higher binding affinities compared to those with unsubstituted or saturated alkyl chains. Linkage between head and tail group has no significant effect on binding affinity. However, ethanolamine at the head of the lipids affect the lipid protein interactions.

In conclusion, I have prepared heavy atom labeled GalCer and p-fluro-benzyl containing eLPE analogs. The cytokine production assay of the GalCers showed the efficient ligand protein complexation. Precise recognition of 12'SeGC (**4**) by CD1d was determined by X-ray crystallography. Direct CD1d-binding affinity of intrinsic ligands bearing low biological activitieswere successfully evaluated using SPR technique.

Published paper: Synthesis and Th1-immunostimulatory activity of α-galactosylceramide analogues bearing a halogen-containing or selenium-containing acyl chain. Md. Imran Hossain, Shinya Hanashima, Takuto Nomura, Sébastian Lethu, Hiroshi Tsuchikawa, Michio Murata, Hiroki Kusaka, Shunsuke Kita, and Katsumi Maenaka. *Bioorg. Med. Chem.* **24**, 3687-3695 (2016).

様式 7

論文審査の結果の要旨及び担当者

氏　名	（ Md. Imran Hossain ）

	（職）	氏　名
論文審査担当者	主査　教授	村田　道雄
	副査　教授	久保　孝史
	副査　教授	北條　裕信

論文審査の結果の要旨

免疫に重要な役割を果たす抗原提示細胞は、抗原に対する受容体の一種である CD1d を発現している。CD1d 受容体は脂質性リガンドの結合によってナチュラルキラーT 細胞の免疫応答を促し、リガンドの種類により Th1 型または Th2 型細胞に分化することが知られているが、その詳細な分子メカニズムは分かっていない。研究の障害の一つとなっているのは、X 線結晶解析においてリガンド CD1d 受容体に結合したリガンドの構造が明確でない点が挙げられる。そこで Hossain 氏は、まず CD1d に結合することによって免疫応答を誘起することが知られているα－ガラクトシルセラミド（α-Gal-Cer）誘導体に関して、セレン等の高い原子密度を有する原子を導入することによって受容体への結合部位を正確に特定することを第一の目的とした。アルキル鎖の中間にセレン原子、末端に臭素原子を導入したα-Gal-Cer 誘導体を新規に設計し、それらの合成に成功した。得られた化合物は標準物質と比較して同等の活性および若干の Th1 選択性を示したが、化合物の脂肪酸メチレン基やメイル基をそれぞれセレン原子や臭素原子に置換したことによる生物活性への影響は比較的軽微なものであった。また、北海道大学との共同研究による結晶X線構造に基づき、重要な脂肪鎖上のセレン原子の位置を明確に決定することに成功した。これは、CD1d リガンドのアルキル炭素の位置を正確に決定しることを可能にした。

一方で、CD1d に結合して Th2 選択性を示すとされているグレセリルエーテル型のリゾリン脂質である pLPE の誘導体についても、その受容体への結合部位を正確に特定するべく、様々な誘導体の合成を試みた。各種の誘導体の CD1d に対する親和性を測定するために、表面プラズモン共鳴法の適応を試みた結果、基盤に固定化して CD1d にリガンドを吸着させることによって、リガンド結合による僅かな差を観測することに成功した。その結果、pLPE やα-Gal-Cer などの脂溶性リガンドの CD1d への親和性をある程度見積もることができた。これによって、各種の pLPE 誘導体の親和性をα-Gal-Cer と比較して測定した結果、フルオロフェニル基を末端に持つ pLPE 誘導体が結合位置の解析に有用である可能性を示した。

以上のように、Md. Imran Hossain 氏は、博士論文研究として構造解析に応用できる Th1 および Th2 選択性を持つ種々の CD1d リガンドの化学合成およびその生物活性の測定を行った。また、Hossain 氏は、重原子置換したリガンドを合成して、共同研究による結晶X線構造解析を成功に導き、生物活性に重要な脂肪鎖の位置を明確に決定することに成功した。これらの知見は、今後、選択的免疫誘導のメカニズム解析や医薬品への応用研究に役立つと期待される。したがって、本論文は博士(理学)の学位論文として十分価値あるものと認める。