ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2019, **55**, 711

Received 9th September 2018,
Accepted 8th December 2018

DOI: 10.1039/c8cc07322h

rsc.li/chemcomm

Synthesis of glycerolipids containing simple linear acyl chains or aromatic rings and evaluation of their Mincle signaling activity†

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Mincle, expressed in activated phagocytes, recognizes the lipid ligand to activate the innate immune system. We have synthesized glycerol derivatives possessing simple alkyl chains or aromatic rings and elucidated their structure–activity relationships using a Mincle-mediated signaling assay. The activity depends on the length of the simple acyl chains of the glycerol derivatives.

Pattern recognition receptors (PRRs), which are expressed in phagocytes such as macrophages and dendritic cells, play key roles in the innate immune system. PRRs include Toll-like receptors (TLRs),[1,2] Nucleotide-binding Oligomerization Domain (NOD)-like receptors (NLRs),[3,4] and C-type lectin receptors (CLRs), which possess calcium ions in a carbohydrate recognition domain (CRD).[5,6] Mincle (also called Clec4e and Clecsf9) is one of the CLRs expressed in activated phagocytes.[7] Mincle has been known to recognize dead cells through the binding to SAP130[8] and trehalose dimycolate (TDM) as an agonist.[9] Because TDM is one of the glycolipids in the cell wall of *Mycobacterium tuberculosis*, Mincle is being assessed to serve as a sensor of *M. tuberculosis*. The recognition of ligands such as TDM leads to the activation of NF-κB *via* SYK-Card9-Bcl10-MALT1 signaling through ITAM-containing adaptor molecule FcRγ.[10] NF-κB induces the transcription of cytokines to shape the development of T cells into various effector helper T cells such as TH1 and TH17 subtypes.

To date, several Mincle ligands have been reported.[11,12] Typical Mincle ligands are trehalose esters such as TDM and trehalose dibehenate (TDB).[9,10,13] Trehalose dicorynomycolates (TDCMs), whose acyl moieties consist of corynomycolic acid from *Corynebacteria*[14] and brartemicin and its derivatives,[15,16] are also known as Mincle agonists. Other glycolipids such as glucose monoesters[14,17,18] and mannose monoesters[18] also exhibit Mincle agonist activity. Additionally, glycerolipids can exhibit Mincle-mediated signaling activity. Glycerol monobehenate (GroMB)[19] and glycerol monocorynomycolate (GroMCM)[14] are glycerolipids of the typical Mincle ligands whose acyl moieties consist of behenic acid and mycolic acid, respectively. Of note, glycerolipids exhibit only human Mincle-mediated signaling activity, while other glycolipid types of Mincle ligands including TDM demonstrate both human and murine Mincle signaling activity. However, in contrast to glycolipids, the molecular mechanism of the Mincle-mediated signaling of glycerolipids is largely unknown. In this study, we focused on glycerolipids as Mincle ligands and elucidated the structure–activity relationships between the acyl moiety of glycerolipids and Mincle agonist activity. We designed glycerol monoesters whose acyl chain lengths are from C_{22} to C_{30}. Glycerol monoacyl ester (C_{22}) is glycerol monobehenate (GroMB), and glycerol monoacyl ester (C_{30}) is reported as a natural product found in wheat bran[20] and Abaca (*Musa textilis*) leaf fibers.[21] Meanwhile, we also designed other glycerolipids associated with aromatic rings based on brartemicin, which is a trehalose-containing natural compound that has signaling activity (Fig. 1).

First, we focused on the length of the acyl chain of glycerolipids. Previously, we have demonstrated that the length of the acyl chain in trehalose monoesters (C_8, C_{10}, C_{12}) influences the binding activity of Mincle using surface plasmon resonance (SPR) and demonstrated that a minimum lipid length of C_{10} was required for the binding.[22] The lipid structure–activity relationships of various Mincle ligands such as trehalose diesters,[23] trehalose monoesters,[24] iso-branched trehalose diesters,[25] β-gentiobiosyl diacylglycerides[26] and glucose mono-corynomycolate[27] have been reported, but the lipid length–signaling activity relationship of glycerolipids is still unclear. In order to investigate the lipid length of glycerolipids–signaling activity relationship, we prepared glycerolipids possessing C_{22} (GroMB), C_{24}, C_{26}, C_{28} and C_{30} acyl chains. The appropriate carboxylic acid was converted to acid chloride and subjected to isopropylidene glycerol to obtain acylated isopropylidene glycerol. The glycerolipids were obtained

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8cc07322h

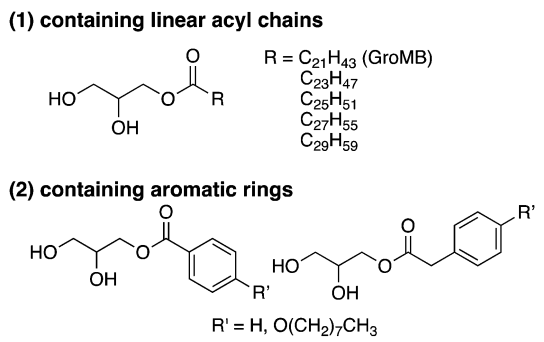

Fig. 1 The structures of glycerolipids containing linear acyl chains or aromatic rings.

by the deprotection of the acetal group (Fig. 2A, method A). Another method is condensation with the appropriate carboxylic acid and isopropylidene glycerol, following the deprotection of isopropylidene to provide glycerolipids (Fig. 2A, method B). Unfortunately, these synthetic methods gave a mixture of the desired compound with the migrated 2-acyl compound in each glycerolipid. The proportions of the 2-acyl compound in each inseparable mixture are approximately 4.1–13.8% detected by ^{1}H NMR.

Next, we designed novel glycerolipids with aromatic rings instead of an acyl chain to increase the affinity with human Mincle. Actually, brartemicin, a natural product isolated from *Nonomuraea* sp., is a trehalose conjugated with two aromatic rings (Fig. 2B),[28] which bound to Mincle with high affinity.[15] A crystallographic study of the brartemicin complex of bovine Mincle[29] revealed that one of the aromatic rings interacts with the hydrophobic groove composed of Leu172, Val173, Val194, Phe197 and Phe198 (corresponding to residues Ile173, Ala174, Val195, Phe198 and Leu199 in human Mincle), while the trehalose moiety is located at the calcium ion binding site. Thus, the benzoyl and phenyl acetic acid types of lipid moieties expected to interact with the hydrophobic groove were prepared. Moreover, we synthesized glycerolipids possessing aromatic rings with additional alkyl chains. These compounds were prepared by the same method as in Fig. 2A and C.

Mincle-mediated signaling activity was evaluated using the nuclear factor of activated T cells (NFAT)-green fluorescent protein (GFP) reporter assay, which has been widely used for the functional evaluation of Mincle ligands.[9,14,16,19,27,30–33] The reporter cells expressing human or murine Mincle with FcRγ were stimulated with plate-coated derivatives. The signaling activity was determined by measuring GFP production. The positive control, TDM, induced the activation of both human and murine Mincles in a dose-dependent manner. Regarding the series of glycerol derivatives possessing different lengths of acyl chains, the signaling intensity increased with the length of acyl chains. Thus, the lengths of the acyl chains of the glycerolipids have a vast impact on the signaling activity among acyl chains from C_{22} to C_{30} (Fig. 3A). Compared to 2′*S*-GroMCM, which is known as the best Mincle agonist of glycerol derivatives with chirality at the 2′-position and corynomycolate as the acyl moiety, GroC30 also demonstrated potent Mincle-mediated signaling activity.

On the other hand, glycerolipids containing an aromatic ring did not show any detectable signaling activity (Fig. 3B). Recently, lipidated brartemicins possessing trehalose as a head group and various lipid moieties containing aromatic rings were synthesized and exhibited Mincle-mediated signaling activity.[16] The lipid moiety of compound **5b** is similar (less than one carbon in the alkyl chain) to the lipid moiety of brartemicin derivatives but did not exhibit signaling activity. A previous study showed that one of the two aromatic rings of brartemicin is located in a hydrophobic pocket and the other interacts with calcium ion neighboring Arg182 (corresponding to Arg183 in human Mincle) π–cation interactions.[15] Our derivatives have only one aromatic ring and thus possibly could not create

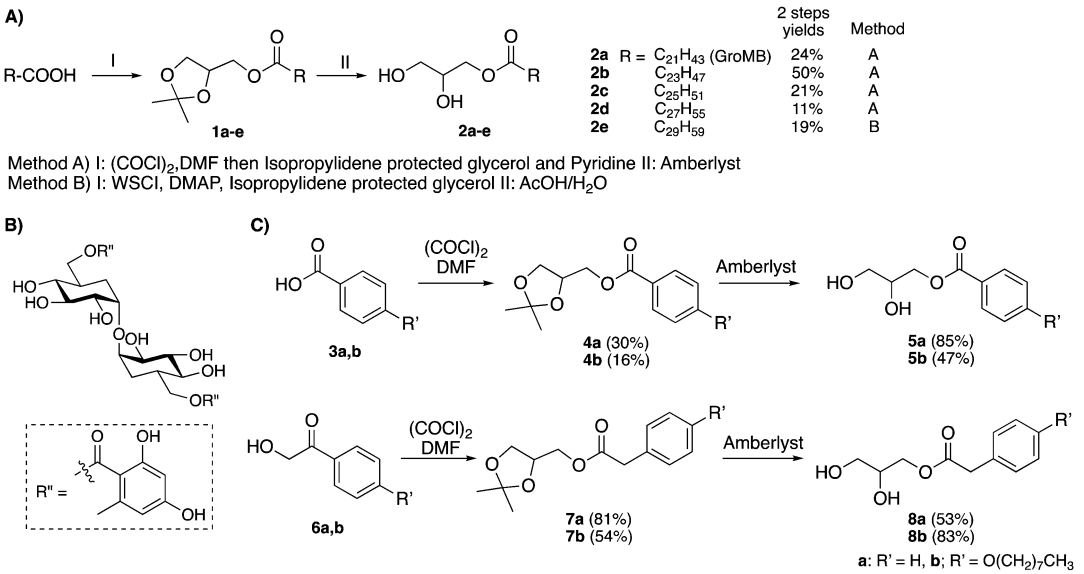

Fig. 2 (A) Synthesis of **2a–2e**, (B) the structure of brartemicin, and (C) synthesis of **5a** and **b** and **8a** and **b**.

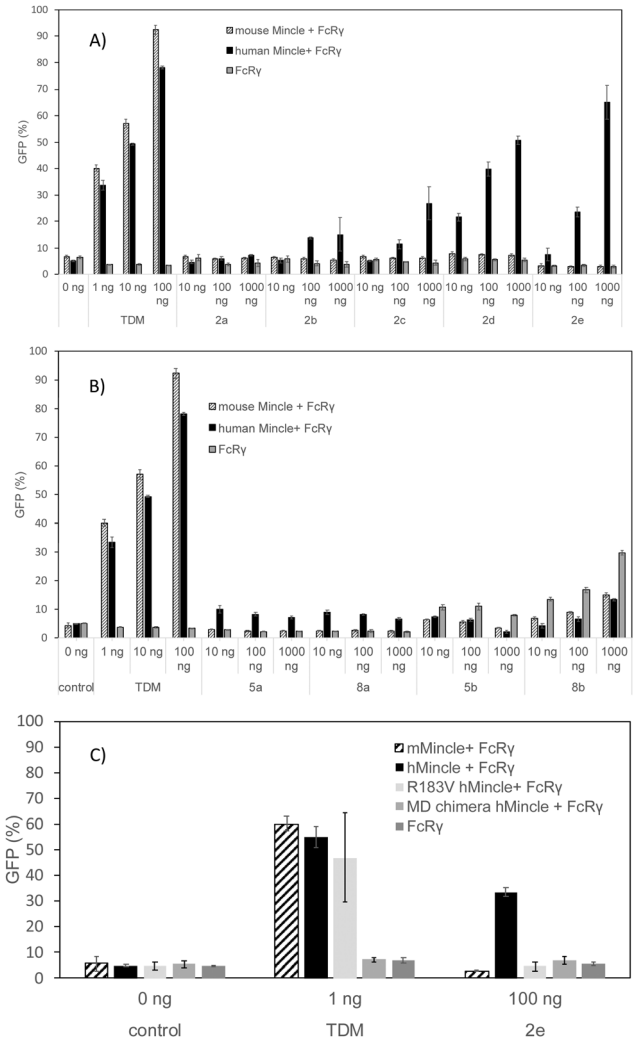

Fig. 3 The evaluation of (A) monoacyl glycerolipids, and (B) the glycerolipids containing aromatic rings using NFAT-GFP reporter cells expressing human or murine Mincle, or FcRγ alone. (C) The evaluation of **2e** using reporter cells expressing mutant Mincles.

π–cation interactions with Arg, resulting in the lack of signaling activity.

In order to understand the interaction between Mincle and glycerol derivatives, we successfully built complex models with the glycerol derivatives including glycerol C_{30} using the crystal structure of bovine Mincle bound to trehalose (Fig. 4, details in the ESI†). The model showed that the ligands could be set up for their glycerol moiety to interact with calcium ions and for the part of their acyl chains neighboring glycerol to accommodate into a hydrophobic groove consisting of Ile173, Ala174, Phe198 and Leu199, consistent with previous reports.[16,18] However, the hydrophobic groove only has sufficient space to accommodate C_{12} carbons of the acyl chain at most. The distal portion of the lipid moiety may be exposed to the outside and/or weakly fit onto somewhat hydrophobic patches of Mincle. On the other hand, Arg183 could interact with aromatic rings next to or around the glycerol moiety in the case of ligands possessing them.

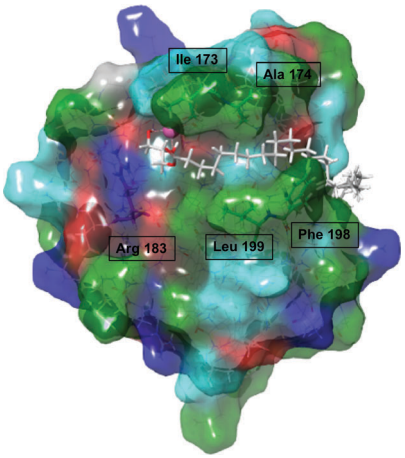

Fig. 4 Model of the glycerolipid C_{30}–Mincle complex.

To examine the lipid recognition site, we evaluated the Mincle-mediated signaling activity for glycerolipid C_{30} using reporter cells expressing either the hMincle R183V mutant or the hMincle MD chimera whose hydrophobic region (residues 195–202) is replaced by the corresponding region of Dectin-2 (residues 192–199).[22] **2e** exhibited reduced NFAT signaling activity for both mutants as glycolipids we previously reported (Fig. 3C). Thus, this result suggests that glycerolipids interact with the hydrophobic groove similarly to glycolipids, consistent with the model of the glycerolipid C_{30}–Mincle complex (Fig. 4).

In conclusion, we have synthesized two types of glycerolipids possessing simple linear acyl chains or aromatic rings and evaluated their Mincle-mediated signaling activity. The glycerolipids having an aromatic ring with an alkoxyl chain do not exhibit signaling activity, in contrast to lipidated brartemicin derivatives with similar lipid moieties. On the other hand, glycerolipids possessing simple acyl chains harbor the relationships of the signaling activity with C_{22}–C_{30} acyl chain length. To the best of our knowledge, this is the first report on the relationships of the length of the lipid moiety of glycerolipids and the signaling activity. In particular, glycerol C_{30} showed potent signaling activity. This agonist can be one of the best templates that can be easily utilized in and expanded to various areas of chemical biology research and drug discovery. Currently, the structural study of the binding modes of human and murine Mincles with lipid moieties of conjugated lipids is underway.

This research was partially supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number 18am0101093j and the Middle Molecule Project from AMED under Grant Number 18ae0101047h, Hokkaido University, the Global Facility Center (GFC), the Pharma Science Open Unit (PSOU), funded by MEXT under the "Support Program for Implementation of New Equipment Sharing System", the Hokkaido University Biosurface project, the Takeda Science Foundation, the Akiyama Life Science Foundation and a Grant-in-Aid for Scientific Research

from Japan Society of the Promotion Science under Grant number JP17H02207. We also thank Mr D. Yoshidome for three-dimensional structure analysis.

Conflicts of interest

There are no conflicts of interest to declare.

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