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ortho-Substituted lipidated Brartemicin derivative shows promising Mincle-mediated adjuvant activity†

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The macrophage inducible C-type lectin (Mincle) is a pathogen recognition receptor (PRR) that is a promising target for the development of Th1-polarising vaccine adjuvants. We recently reported on the synthesis and evaluation of lipidated Brartemicin analogues that showed Mincle agonist activity, with our lead agonist exhibiting potent Th1 adjuvant activity that was greater than that of trehalose dibehenate (TDB). Herein, we report on the efficient synthesis and subsequent biological evaluation of additional lipidated Brartemicin analogues that were designed to determine the structural requirements for optimal Mincle signalling. While all the Brartemicin analogues retained their ability to signal through Mincle and induce a functional response, the *o*-substituted and *m*,*m*-disubstituted derivatives (**5a** and **5d**, respectively) induced a potent inflammatory response when using cells of both murine and human origin, with this response being the greatest observed thus far. As the inflammatory response elicited by **5a** was slightly better than that induced by **5d**, our findings point to *o*-substituted Brartemicin analogues as the preferred scaffold for further adjuvant development.

Introduction

The macrophage inducible C-type lectin (Mincle, Clec4e, ClecSf9) is a pattern recognition receptor commonly expressed on the cell surface of innate immune cells such as macrophages, dendritic cells (DCs), and neutrophils.[1,2] Mincle plays a pivotal role in innate immunity through the recognition of a vast array of both endogenous and exogenous amphiphilic compounds, many of which incorporate a lipid-chain.[3,4] The activation of Mincle by an appropriate ligand leads to phosphorylation of the FcRγ adaptor protein, which in turn triggers the recruitment of spleen tyrosine kinase (syk) to the cell membrane and activation of the Card9-Bcl10-Malt-1 signalling cascade.[2,5,6] Translocation of the nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) to the cell nucleus then promotes the expression of inflammatory cytokines and chemokines, such as interleukin (IL)-1β, macrophage inflammatory protein (MIP)-2, and IL-6.

Since the discovery of Mincle, significant effort has been directed toward the identification of self and foreign Mincle ligands.[3,4] Trehalose glycolipids (TGLs), such as the *Mycobacterium tuberculosis* cell wall glycolipid, trehalose dimycolate (TDM, **1**, Fig. 1), and its synthetic analogue, trehalose dibehenate (TDB, **2**), were among the first described Mincle ligands with the capacity to trigger a potent inflammatory response.[5–7] Given the ability of TGLs to induce an inflamma-

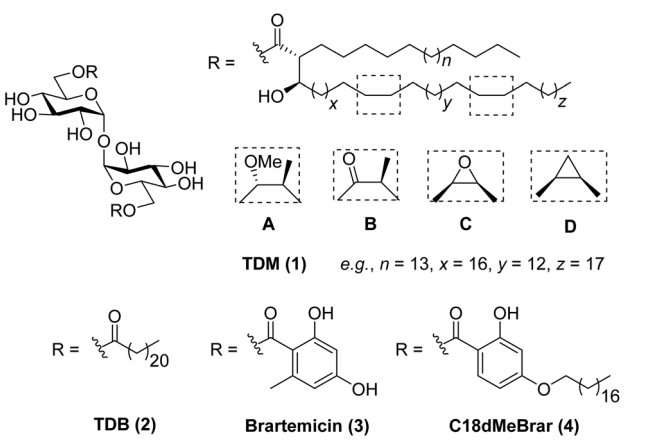

Fig. 1 Representative trehalose glycolipids investigated for their Mincle agonist activity.

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tory immune response, there has been substantial interest in harnessing the inherent immunostimulatory properties of TGLs for use as vaccine adjuvants. As such, TDB (**2**) has been formulated into a variety of dimethyldioctadecylammonium (DDA)-containing liposomes, including liposomes comprised of DDA and TDB (DDA:TDB, termed CAF01) as well as DDA: TDB liposomes supplemented with the TLR-3 ligand, poly-IC (CAF05),[8] or the TLR-4 ligand, monophosphoryl lipid (MPL)-A (CAF06).[9] All three formulations (CAF01, CAF05, and CAF06) have been found to induce a strong T cell response,[8–10] with further evaluation of CAF01 highlighting promising adjuvant activity in tuberculosis,[11–15] malaria,[15] influenza,[16] and chlamydia[15] vaccination models. CAF01 has been progressed to human clinical trials, whereby healthy volunteers were vaccinated with the tuberculosis antigen Ag86B-ESAT-6 adjuvanted with CAF01.[14,17] During these trials, vaccination was found to produce a potent and persisting T cell response and the vaccine was found to be 'safe and tolerable'.

In addition to TDM (**1**) and TDB (**2**), a vast array of lipid-containing glucose-,[18–23] mannose-,[20,24] glycerol-,[18,25] and trehalose-derived ligands[26–34] have been identified as Mincle agonists. Mincle recognises carbohydrate-derived ligands through its carbohydrate recognition domain (CRD), which contains the Glu-Pro-Asn (EPN) motif that mediates binding to glucose- and mannose-containing structures.[28,29] Here, Ca^{2+} found within the EPN is thought to bind to the 3- and 4-hydroxyls of one glucose-residue, while an adjacent hydrophobic groove is thought to be able to accommodate the α-branched or linear lipid portions commonly found in Mincle ligands.[28,29,35] Additionally, it has been proposed that within bovine Mincle (bMincle), Arg182 is suitably placed to interact with the hydroxyls of treahalose.[29]

In 2015, the natural product Brartemicin (**3**) was determined to be a high-affinity ligand for bovine Mincle through the use of competition binding assays and molecular simulation.[36] Furthermore, a crystal structure obtained of Brartemicin (**3**) binding to bMincle suggested that, much like trehalose, Brartemicin (**3**) binds the CRD of Mincle.[35] Therein, one of the aromatic esters of Brartemicin (**3**) was located near Arg182, which suggests that π–cation interactions may be important for the binding of aromatic Mincle ligands.[35] Following these studies, we reported on the synthesis and biological evaluation of a variety of Brartemicin analogues and demonstrated that Brartemicin analogues incorporating long (C_{18}) lipid chains, but not Brartemicin itself, were potent Mincle agonists.[37] Moreover, Arg183 (hMincle) was deemed crucial for ligand binding of the C_{18}-alkylated desmethyl-brartemicin analogue (C18dMeBrar, **4**), with **4** subsequently being found to possess superior adjuvant activity to TDB in a delayed-type hypersensitivity (DHT) immunisation assay where OVA was used as the model antigen.

While lipidated Brartemicin analogues are known to be potent Mincle agonists, the exact structural requirements for Mincle signalling remain unknown. To this end, we sought to further explore the structure–activity relationships (SAR) of a library of Brartemicin analogues. Here, analogues containing a

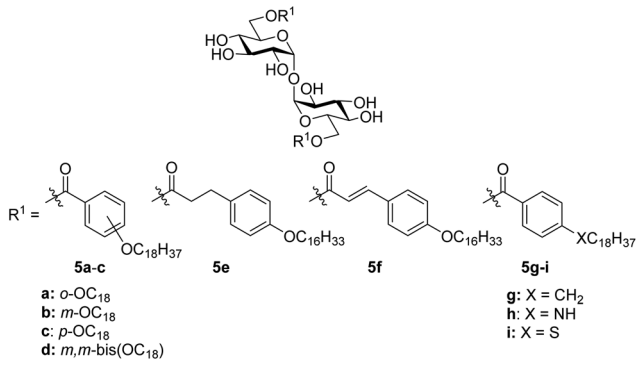


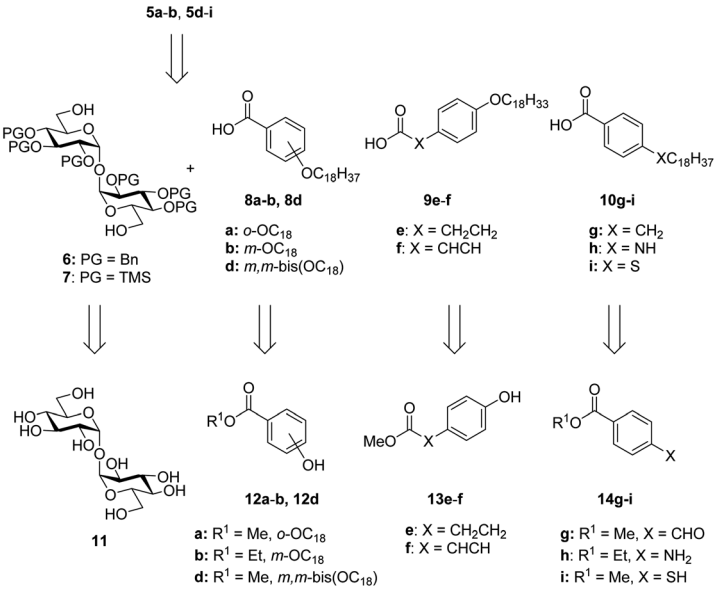
Fig. 2 Target lipidated Brartemicin analogues.

C_{18}-lipid chain at the phenyl *ortho* (*o*)- and *meta* (*m*)-positions (**5a** and **5b**, respectively, Fig. 2) were proposed so that their immunostimulatory activity could be compared to the previously described *para* (*p*)-alkylated analogue **5c** (*p*-OC_{18}), which in our earlier studies was found to exhibit similar agonist activity to C18dMeBrar (**4**).[37] Given the potent Mincle agonist activity of branched trehalose glycolipids,[7,19,33,38] we also proposed that the increased lipophilicity of the *m*,*m*-bis (OC_{18}) Brartemicin derivative **5d** might enhance Mincle adjuvant activity. As the interaction between C18dMeBrar (**4**) and Arg183 is crucial for ligand binding and cellular activation,[37] Brartemicin analogues that integrated either an ethylene (**5e**) or an ethynyl (**5f**) spacer between the trehalose ester and the aromatic scaffold were proposed so that further insight in to the positioning of the aromatic moiety and Arg183 could be gained. For these adducts, a shorter (C_{16}) lipid chain would be included to maintain the overall lipid length. Finally, carbon-linked (**5f**), nitrogen-linked (**5h**), and sulfur-linked (**5i**) derivatives would be prepared so as to investigate the importance of the linkage between the aromatic scaffold and the lipid-chain. Herein, we report on the synthesis of these derivatives and their ability to activate Mincle expressing nuclear factor of activated T cell (NFAT)-green fluorescent protein (GFP) reporter cells,[2,39] bone marrow derived macrophages of murine origin,[40] and human peripheral blood monocytes.[41]

Results and discussion

Synthesis of Brartemicin analogues

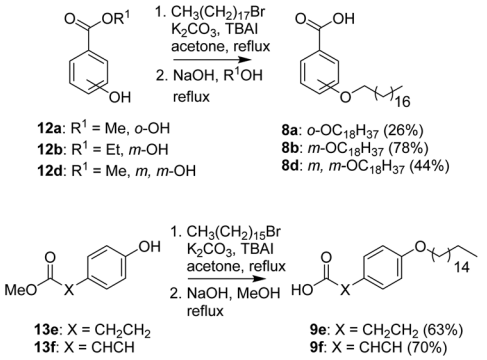
To synthesise the desired lipidated Brartemicin analogues, we envisioned a strategy whereby the target analogues **5a–b** and **5d–i** could be accessed *via* the esterification of a suitably protected trehalose derivative (**6** or **7**) with a range of appropriately functionalised carboxylic acids (**8a–b**, **8d**, **9e–f**, and **10g–i**, Scheme 1).[26] Here, it was proposed that benzylated trehalose **6**, which is readily accessible from α,α′-D-trehalose (**11**) in three steps,[26] could be esterified with acids **8a–b**, **8d**, **9e**, and **10g–i** to give the corresponding protected glycolipids, which after debenzylation would provide target analogues **5a–b**, **5d–e**, and **5g–i**. For those target glycolipids that would not tolerate the



Scheme 1 Retrosynthetic analysis of the target lipidated Brartemicin analogues.

hydrogenolysis conditions required for global debenzylation, namely **5f** and **5i**, it was proposed that the silyl ether protected trehalose derivative **7**, which is prepared from α,α′-D-trehalose (**11**) in a single synthetic step,[42] could be used for esterification with acids **9f** and **10i** to give the corresponding protected glycolipids. Subsequent removal of the TMS protecting groups would yield the desired glycolipids **5f** and **5i**. Acids **8a–b**, **8d**, **9e–f** and **10i** were envisioned to be prepared from commercially available esters (**12a–b**, **12d**, **13e–f**, and **14i**, respectively) *via* alkylation with either 1-bromooctadecane[37] or 1-bromohexadecane[37,43] and subsequent base-catalysed hydrolysis of the ester.[37] The carbon-linked benzoic acid **10g** could be prepared from aldehyde **14g** through a Wittig reaction[44] using octadecyltriphenylphosphonium bromide, which is readily accessible from 1-bromooctadecane,[45] and subsequent hydrolysis of the methyl ester.[37] Finally, we proposed that alkylamino benzoic acid **10h** could be prepared from ethyl ester **14h** through reductive amination with octadecanal followed by base-mediated hydrolysis of the ester.[37,46] Octadecanal is, in turn, prepared through the oxidation of 1-octadecanol.[47]

With the restrosynthetic strategy in place, the syntheses of carboxylic acids containing ether-linked lipids were undertaken (Scheme 2). Here, methyl salicylate (**12a**), ethyl 3-hydroxybenzoate (**12b**), and methyl 3,5-dihydroxybenzoate (**12d**) were alkylated with 1-bromooctadecane in the presence of tetrabutylammonium iodide (TBAI) to give the corresponding lipophilic esters. Treatment of the lipophilic esters with sodium hydroxide in the appropriate alcohol then yielded the target benzoic acids (**8a**, **8b**, and **8d**) in modest (26%) to excellent (78%) yield over two steps. To prepare the carboxylic acids incorporating the ethylene and ethenyl spacers (**9e** and **9f**, respectively), dihydrocinnamate **13e** and cinnamate **13f** were first treated with 1-bromohexadecane to install the slightly truncated lipid-chain. Subsequent hydrolysis of the methyl

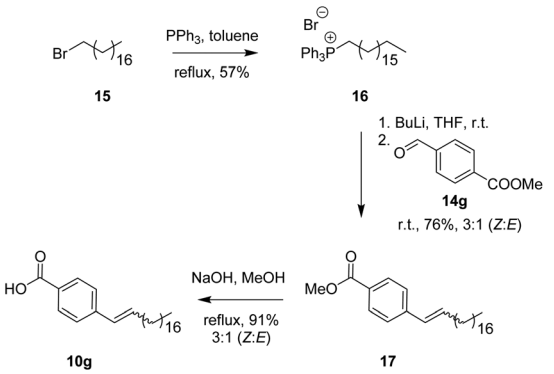


Scheme 2 Synthesis of acids **8a–b**, **8d**, and **9e–f**.

esters then afforded the desired acids **9e** and **9f** in good yields (63–70% over two steps).

Next, we embarked on the synthesis of the carbon-linked glycolipid (**5f**), whereby 1-bromooctadecane (**15**) was treated with triphenylphosphine in toluene to give the corresponding phosphonium bromide **16**, according to a literature procedure (Scheme 3).[45] Subsequent reaction of phosphonium bromide **16** with BuLi afforded the corresponding ylide, which was immediatly subjected to a Wittig reaction with methyl 4-formylbenzoate (**14g**) to give methyl benzoates **17** as a 3 : 1 mixture of the *Z*- and *E*-alkenes in good (76%) yield. Subjection of benzoates **17** to 5 M sodium hydroxide in MeOH then gave the desired benzoic acids **10g** as an isomeric mixture. As hydrogenation of the alkene was to occur at a later stage in the synthesis, no attempt was made to separate the *E*- and *Z*-isomers of **10g**.

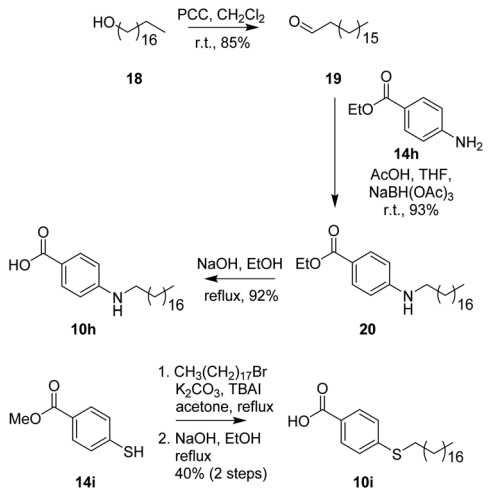
To prepare the alkylamino benzoic acid **18**, octadecanol **19** was first converted to the corresponding aldehyde **19** using a pyridinium chlorochromate mediated oxidation to yield octa-



Scheme 3 Synthesis of acid **10g**.

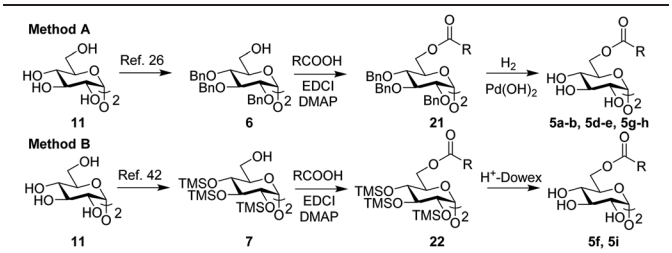
decanal (**19**) in excellent (85%) yield (Scheme 4).[47] Reductive amination of aldehyde **16** with ethyl 4-aminobenzoate in the presence of sodium triacetoxyborohydride then gave the nitrogen-linked lipophilic ester **20**, which was hydrolysed in the presence of sodium hydroxide and ethanol to give target alkylamino benzoate **10h** in excellent (92%) yield. Finally, the sulfur-linked acid **10i** was prepared *via* the alkylation of methyl 4-mercaptobenzoate **14i** with 1-bromooctadecane, followed by the base-mediated hydrolysis of the methyl ester to give the target acid **10i** in modest yield (40% over two steps). Here, the reduced solubility of acid **10i** in typical organic solvents (*e.g.* EtOAc and CH_2Cl_2) necessitated the use of hot solvent to extract the target compound during work-up. In this way, the desired compound was isolated in excellent yield (quant.).

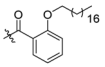
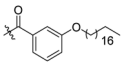
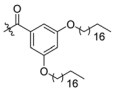
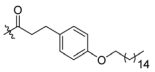
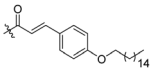
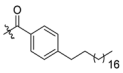
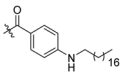
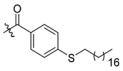
With the carboxylic acids in hand, the target lipidated Brartemicin derivatives were prepared *via* the esterification of hexa-*O*-Bn-trehalose (**6**, Method A) or hexa-*O*-TMS-trehalose (**7**, Method B) with the previously prepared carboxylic acids (Table 1). Accordingly, α,α′-D-trehalose (**11**) was tritylated at the 6- and 6′-hydroxyls and per-benzylated, followed by removal of



Scheme 4 Synthesis of benzoic acids **10h** and **14i**.

Table 1 Assembly of lipidated Brartemicin analogues



Entry	Diester	Method	Yield (%) 6 → 21 or 7 → 22	Yield (%) 21 → 5 or 22 → 5
1		A	**21a**, 84	**5a**, 57
2		A	**21b**, 88	**5b**, 67
3		A	**21d**, 61	**5d**, 54
4		A	**21e**, 44	**5e**, 64
5		B	**22f**, 34	**5f**, 40
6		A	**21g**, 34	**5g**, 58
7		A	**21h**, 38	**5h**, 28
8		B	**22i**, 77	**5i**, 52

the trityl groups according to a literature procedure (Method A).[26] Benzylated trehalose **6** was then subjected to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-(dimethylamino)pyridine (DMAP) mediated esterifications with acids **8a–b**, **8d**, **9e**, and **10g–h** to give the protected glycolipids (**21a–b**, **21d–e**, and **21g–i**) in modest (34%) to excellent (88%) yields. Here, Heteronuclear Multiple Bond Correlations (HMBCs) between the carbonyl carbons of the lipophilic esters and H-6a and H-6b of the trehalose scaffold confirmed that the aromatic lipids had been successfully installed. Hydrogenolysis of the benzylated glycolipids (**21a–b**, **21d–e**, and **21g–i**) using Pearlman's catalyst followed by silica-gel column chromatography provided the target glycolipids in 28–67% yield. To prepare those target derivatives that are not compatible with hydrogenolysis conditions, the synthetic strategy reported by Toubiana *et al.*[42] was employed to prepare 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-Trehalose (**7**) from α,α′-D-trehalose (**11**) in a single synthetic step (Method B). More specifically, α,α′-D-trehalose (**11**) was treated with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and tetra-*N*-butylammoniuim fluoride (TBAF) to give the per-silylated adduct, which was subsequently treated with pot-

assium carbonate and methanol to selectively remove the primary TMS ethers. Esterification of the TMS-protected derivative **7** with cinnamic acid **9f** and sulfur-linked acid **10i** yielded the TMS-protected glycolipids **22f** and **22i** in modest (34%) to good (77%) yield. Global TMS-ether deprotection using H^+-Dowex furnished the remaining target analogues (**5f** and **5i**) in modest (40–52%) yields.

Structure-activity relationship of lipidated Brartemicin analogues

With the target lipidated Brartemicin analogues **5a–5i** in hand, the ability of these analogues to signal through Mincle was assessed using NFAT-GFP reporter cells.[2,39] Here, plate-bound TDB (**2**) and analogues **5a–i** were employed to stimulate NFAT-GFP reporter cells expressing murine Mincle (mMincle) + FcRγ (Fig. 3a), human Mincle (hMincle) + FcRγ (Fig. 3b), or FcRγ only (Fig. 3). Mincle binding and cellular activation was monitored by the expression of GFP, as detected by flow cytometry. Consistent with our previous findings,[37] the lipidated Brartemicin analogues **5a–i** all strongly activated mMincle and hMincle expressing reporter cells, with the newly synthesised analogues (**5a–b**, **5d–i**) inducing the expression of comparable quantities of GFP to TDB (**2**) and the previously described lipidated Brartemicin analogue **5c** (p-OC_{18}) (Fig. 3).[37] At the concentration of 0.1 nmol per well, **5a** (o-OC_{18}) and **5b** (m-OC_{18}) best activated the hMincle and mMincle reporter cells, with treatment with **5a** (o-OC_{18}) producing approximately double the proportion of GFP positive cells as the p-OC_{18} analogue, **5c**. These data suggest that while Mincle can accommodate o-, m-, and p-substituted Brartemicin analogues (**5a**, **5b**, and **5c**, respectively), ligands substituted at the o-hydroxyl provide a powerful scaffold for the generation of potent Mincle ligands. At the higher ligand concentration (1 nmol per well) however, the response induced by **5a** (o-OC_{18}) decreased, particularly in the hMincle reporter assay. We recently determined that glycolipid presentation, which is influenced by ligand solubility, could affect Mincle agonist activity.[48] Thus, changes in the concentration of **5a** (o-OC_{18}) may have subtly affected the manner in which it was presented to Mincle. Of the remaining Brartemicin analogues, **5d** (m,m-bis[OC_{18}]), **5e** (p-DHCinOC_{16}), **5f** (p-CinOC_{16}), **5g** (p-CC_{18}), **5h** (p-NHC_{18}), and **5i** (p-SC_{18}), all induced the mMincle and hMincle reporter cells to express approximately equal quantities of GFP as TDB (**2**) and **5c** (p-OC_{18}). This further exemplifies the tolerance of Mincle and its ability to accommodate alterations to the lipid structure in Brartemicin derivatives, including differences in the lipid-position, lipid-number, aromatic position, and linkage type. Moreover, when comparing this data to that obtained from our first generation library of lipidated-brartemicin derivatives,[37] it can be seen that **5a** exhibits similar mMincle- and hMincle-mediated signalling to that of C18dMeBrar **4** (see Fig. 1, ESI†).

While the reporter cells are a convenient tool for the identification of new Mincle ligands, we wanted to assess the ability of the Brartemicin analogues to induce an immune response

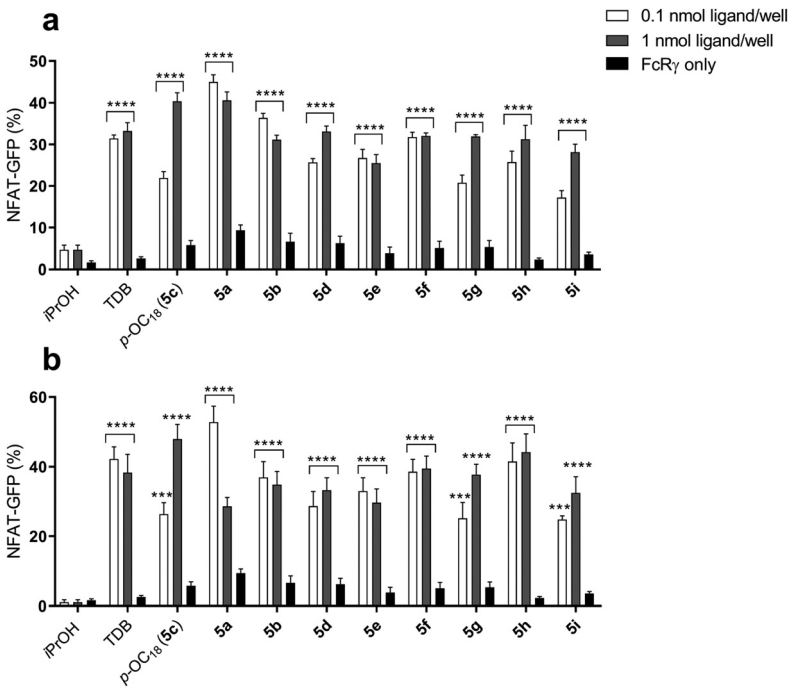


Fig. 3 Lipidated Brartemicin analogues bind and signal through mMincle and hMincle. NFAT-GFP 2B4 reporter cells expressing mMincle + FcRγ (a), hMincle + FcRγ (b), or FcRγ-only were stimulated using ligand-coated plates (0.1 or 1 nmol per well) for 20 hours. The 2B4 cells were then harvested and examined for NFAT-GFP expression using flow cytometry. Data represent the mean of two independent experiments performed in duplicate (mean ± SEM). Statistical significance was calculated in comparison to iPrOH only using 2-way ANOVA (Dunnett's multiple comparison test), $^{****}P \leq 0.0001$, $^{***}P \leq 0.001$.

in a system more akin to a physiological environment. Accordingly, the Brartemicin derivatives were screened for their ability to activate BMDMs to produce the pro- and anti-inflammatory cytokine IL-6, chemokine MIP-2, and the pro-inflammatory cytokine IL-1β. Here, the Brartemicin analogues (**5a–i**) stimulated BMDMs to produce appreciable quantities of IL-6, MIP-2, and IL-1β, with all Brartemicin derivatives (**5a–i**) and TDB (**2**) producing approximately equal amounts of IL-6 and MIP-2 (Fig. 4). At the concentration of 0.1 nmol per well however, subtle differences were observed in the relative levels of IL-6 produced by BMDMs following stimulation with the newly prepared Brartemicin analogues. For example, **5d** (*m*,*m*-bis[OC_{18}]) induced the BMDMs to produce comparatively more IL-6 than TDB (**2**) or **5c** (*p*-OC_{18}), while **5e** (*p*-$DHCinOC_{16}$) and **5f** (*p*-$CinOC_{16}$) induced the production of less IL-6 than TDB (**2**) and **5c** (*p*-OC_{18}). In addition to IL-6 and MIP-2, the lipidated Brartemicin analogues (**5a–i**) all stimulated the BMDMs to produce significant quantities of IL-1β, with **5b** (*m*-OC_{18}), **5e** (*p*-$DHCinOC_{16}$), **5f** (*p*-$CinOC_{16}$), **5g** (*p*-CC_{18}), and **5i** (*p*-SC_{18}) stimulating similar levels of IL-1β production to both TDB (**2**) and **5c** (*p*-OC_{18}). Moreover, **5a** (*o*-OC_{18}) and, to a lesser extent **5d** (*m*,*m*-bis[OC_{18}]) and **5h** (*p*-NHC_{18}), exhibited stronger agonist activity than C18dMeBrar **4** (see Fig. 2, ESI†). Remarkably, at the concentration of 1 nmol per well, **5a** (*o*-OC_{18}), **5d** (*m*,*m*-bis[OC_{18}]), and **5h** (*p*-NHC_{18}) induced approximately a 2–3-fold increase in IL-1β production, as compared to TDB (**2**) and **5c** (*p*-OC_{18}) (Fig. 4). This striking increase in IL-1β production by BMDMs stimulated with **5a**, **5d**, and **5h** suggests that these Brartemicin derivatives, and in particular **5a** (*o*-OC_{18}), possess great promise as vaccine adjuvants.

In addition to BMDMs, we also wanted to assess the ability of the lipidated Brartemicin analogues to stimulate human peripheral blood monocytes to produce the inflammatory cytokine, IL-8 (Fig. 5).[49] Thus, human monocytes, which have previously been shown to express Mincle,[41,50,51] were isolated from whole blood and incubated in plates coated with 0.1 or 1 nmol per well of TDB (**2**), *p*-OC_{18} (**5c**), C18dMeBrar, or lipidated analogues **5a–b** and **5d–i**. For comparison, the ability

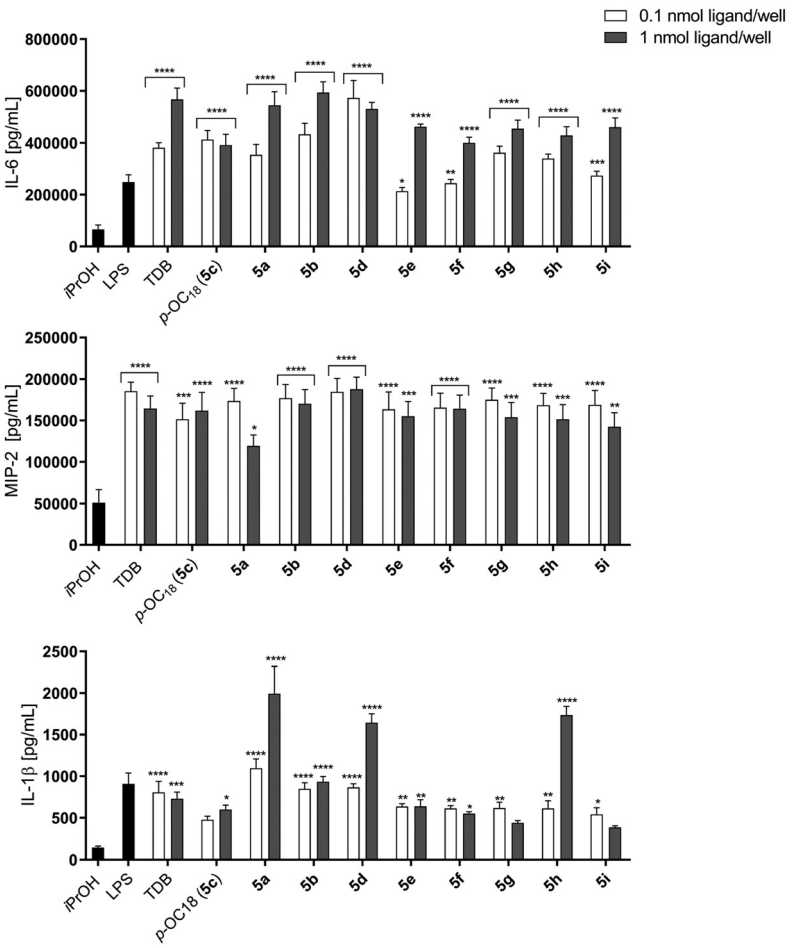


Fig. 4 Lipidated Brartemicin analogues induce inflammatory cytokine and chemokine production by BMDMs. Harvested WT BMDMs were incubated in plates coated with Brartemicin derivatives (**5a–5i**, 0.1 and 1 nmol ligand per well), iPrOH, LPS (100 ng mL^{-1}) or TDB (**2**, 0.1 or 1 nmol ligand per well). IL-6, MIP-2, and IL-1β production was measured by ELISA of the supernatant collected after 24 hours. Data represents the mean of two (IL-6) or three (MIP-2, IL-1β) experiments performed in duplicate (mean ± SEM). Statistical significance was calculated in comparison to iPrOH only using 2-way ANOVA (Dunnett's multiple comparison test), ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

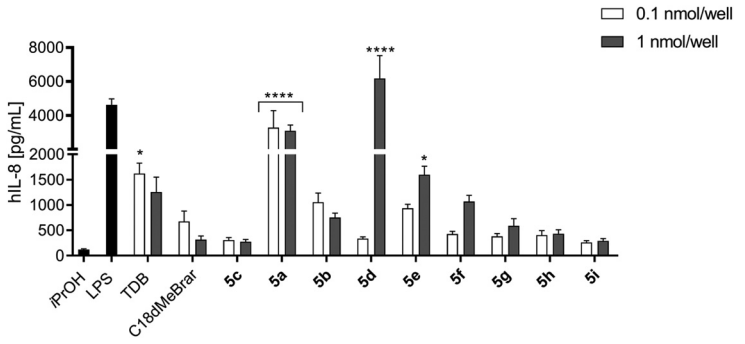


Fig. 5 Lipidated Brartemicin analogues induce IL-8 production by human monocytes. Human monocytes negatively enriched from whole blood were incubated in plates coated with Brartemicin derivatives (**5a–5i**, 0.1 and 1 nmol ligand per well), iPrOH, LPS (100 ng mL^{-1}) or TDB (**2**, 0.1 or 1 nmol ligand per well). After 24 hours, the supernatant was collected and IL-8 levels were determined by ELISA. Data represents the mean of three independent experiments performed in triplicate (mean ± SEM). Statistical significance was calculated in comparison to iPrOH only using 2-way ANOVA (Dunnett's multiple comparison test), $****P \leq 0.0001$, $***P \leq 0.001$, $**P \leq 0.01$, $*P \leq 0.05$.

of C18dMeBrar (**4**)[37] to activate human monocytes was investigated as this data had not been previously obtained. Herein, the *o*- and *m*,*m*-substituted analogues (**5a** and **5d**) strongly activated the monocytes to produce appreciable quantities of IL-8, as compared to TDB (**2**), **5c** (*p*-OC_{18}), and C18dMeBrar (**4**). In contrast, **5b** (*m*-OC_{18}), **5e** (*p*-DHCinOC_{16}), **5f** (*p*-CinOC_{16}), **5g** (*p*-CC_{18}), **5h** (*p*-NHC_{18}), and **5i** (*p*-SC_{18}) induced the production of modest levels of IL-8. While we cannot conclusively say that the production of IL-8 by human monocytes in response to TDB or **5a** and **5d** is solely dependent on Mincle signalling, the data from the reporter cell assays where Mincle-dependence was observed indicates that IL-8 production, at least in part, is due to the engagement of Mincle.

Taken as a whole, our findings further support the growing body of evidence suggesting that trehalose diesters incorporating long-chain lipids are potent Mincle agonists that induce an inflammatory immune response by antigen presenting cells.[18,26,33,34,52] Furthermore, we have demonstrated that both mMincle and hMincle readily accommodate changes to the lipid structure of Brartemicin derivatives, with analogues of **5c** (*p*-OC_{18}) that incorporate modifications to the position of the lipid-chain and aromatic ring, the number of lipid-chains, and the type of linkage between the aromatic ring and the lipid-chain retaining their ability to signal through both mMincle and hMincle (Fig. 3). Herein, the positioning of the lipid on the Brartemicin scaffold appears to have more influence on Mincle agonist activity than the type of lipid-linker used, as illustrated by the ability of the Brartemicin analogue substituted at the *ortho*-hydroxyl (**5a** [*o*-OC_{18}]) to activate the reporter cells at low concentrations (Fig. 3) and induced a strong immune response in both BMDMs and human monocytes (Fig. 4 and 5). To explain this observation, attempts to dock **5a** (*o*-OC_{18}) into the CRD of Mincle were made.[37] However, a poor docking score was observed. By considering our own work,[37] and that of others,[36] it has been previously demonstrated that Mincle binding does not directly correlate to a functional immune response. Thus, it is possible that **5a** (*o*-OC_{18}) binds to a site other than the CRD on Mincle,[3] or that Mincle undergoes a conformational change upon ligand binding and, by doing so, is better able to accommodate ligands. Notwithstanding, the strong adjuvant activity elicited by **5a** (*o*-OC_{18}) is noteworthy. We also observed that the addition of a second C_{18}-lipid onto each of the aromatic diesters (*i.e.* **5d**, *m*,*m*-bis[OC_{18}]) led to an increase in IL-1β and IL-8 production by BMDMs and human monocytes, respectively.

Taken together, these results further corroborate previous studies that determined that alterations to the lipid-structure of trehalose diesters can influence ligand activity,[33,34,37,38,53] however, to date, it is difficult to predict how such subtle changes will influence Mincle-mediated agonist activity.[3] Herein, we found that more pronounced differences in the immunomodulatory activity of the lipidated-brartemicin derivatives were observed when changes were made to the orientation of the lipid chain, as illustrated by **5a** (*o*-OC_{18}) and **5d** (*m*,*m*-bis[OC_{18}]), which are strong agonists of both mMincle and hMincle and are also able to induce inflammatory responses in *in vitro* assays using cells of murine and human origin. Moreover, the potential of species-specific differences in the agonist activity of Mincle ligands,[3] and the importance of employing a number of cellular assays so as to best investigate the structure–activity relationships of Mincle ligands[40,48] is further illustrated in our work. As **5a** (*o*-OC_{18}) elicits a more potent immune response when using both murine and human *in vitro* assays, these findings cement *o*-substituted Brartemicin analogues as the preferred scaffold for further adjuvant development.

Conclusions

At present, TDB is the preferred Mincle agonist for adjuvant development due to its structural simplicity and the promising results achieved with the CAF01 liposome system. Due to the success of TDB, there has been much interest in the development of additional Mincle agonists that have enhanced activity. We recently reported on the synthesis of several lipi-

dated Brartemicin derivatives (*e.g.*, **4** [C18dMeBrar] and **5c** [*p*-OC_{18}]) that possess potent Mincle agonist activity and are available in five steps from commercially available materials. In addition, our lead agonist, **4** (C18dMeBrar), demonstrated potent Th1 adjuvant activity, which was greater than that of TDB. In this work, we have prepared an additional series of lipidated Brartemicin analogues that feature structural modifications such as alterations to the positioning of the lipophilic chain on the aromatic diesters, the total number of lipophilic chains, the position of the aromatic group on the lipid-chain, and the type of linkage incorporated between the aromatic group and the lipid-tail. The target glycolipids were efficiently prepared from commercially available materials (4–5 steps, longest linear sequence) and in overall yields of 5–46%. While all the synthesised ligands demonstrated an ability to signal through mMincle and hMincle, the *o*-substituted derivative **5a** (*o*-OC_{18}) and, to a lesser extent, the *m*,*m*-substituted derivative **5d** (*m*,*m*-bis[OC_{18}]) induced potent inflammatory responses in both BMDMs and human monocytes and are therefore, potent Mincle ligands that possess superior activity to TDB, **5c** (*p*-OC_{18}), and C18dMeBrar (**4**).

Conflicts of interest

There are no conflicts of interest to declare.

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All experimental mice were housed in the animal facility at the Malaghan Institute of Medical Research, Wellington, New Zealand, and all murine experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals (protocol nr 22371). The use of human leukocyte from healthy donors with written informed consent was approved by New Zealand Northern A Health and Disability Ethics Committee (approval number 15/NTA/178).

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