Anti-Inflammatory Compounds

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Exploring the Binding Proteins of Glycolipids with Bifunctional Chemical Probes

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Dedicated to Prof. Samuel J. Danishefsky on the occasion of his 80th birthday

Abstract: *Glycolipids are important structural components of biological membranes and perform crucial functions in living systems, including signaling transduction and interaction with extracellular environment. However, the mechanistic exploration of glycolipids in vivo is challenging because they are not genetically encoded. Herein, we designed and synthesized a series of bifunctional monogalactosyldiacylglycerol (MGDG) probes as a model by introducing diazirine and terminal alkyne moieties on an aliphatic chain. In combination with proteome profiling and molecular modeling, we have demonstrated that MGDG alleviates inflammation by antagonizing TLR4.*

Glycolipids are a class of lipids which contain a carbohydrate component and contribute to many (patho)physiological processes. Besides exerting structural effects on membranes, numerous investigations have revealed that glycolipids participate in cellular recognition, cell signaling transduction, and modulation of protein activities.[1] For example, glycosphingolipids are the most common glycolipids in mammalian cell membranes, playing important roles in cell–cell signaling, pathogen–cell surface interactions, and in cellular regulatory functions.[2] Lipopolysaccharide, an endotoxin of Gram-negative bacteria, can trigger pro-inflammatory response by its lipid A unit.[3] Eritoran, an analogue of lipid A, was developed as an antagonist of LPS (Figure 1).[4] Fatty acid esters of monogalactosyldiacylglycerol (MGDG), categorized as glycolipids, are essential components of the thylakoid membrane in chloroplasts, accounting for 80% of the membrane lipids found in green plant tissues. MGDG share a common galactosyl glycerol moiety attached with two fatty acid chains. Crude MGDG extracts have been shown to possess anti-viral,[5a] anti-tumor,[5b] anti-proliferative[5c] and anti-inflammatory activities.[5d] In particular, dilinolenoyl MGDG has also been reported to show anti-inflammatory effects in human peripheral blood neutrophils.[6] However, the exact modes of action remain elusive. Thus, designing probes to comprehensively dissect the mechanism of glycolipids is of great importance.

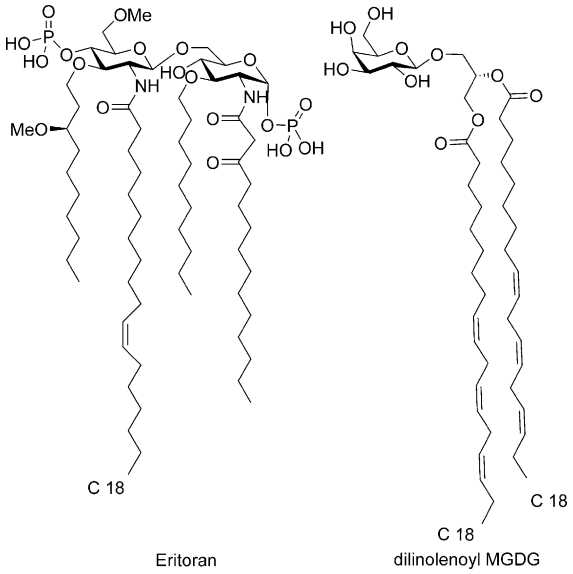

Figure 1. Representative examples of glycolipids.

A better understanding of cellular mechanisms and processes mediated by glycolipids not only contributes to biochemical studies but also guides therapeutic development. Glycolipid chemical probe analogues are undoubtedly valuable tools for understanding their behaviors in physiologically relevant contexts. Recently, a variety of chemical probes have emerged for the study of lipid–protein interactions in their native environment.[7–11] For example, Bertozzi and coworkers prepared a series of glycophosphatidylinositol (GPI) fluorescent probes and unraveled the biological function of the GPI anchor.[12] In pioneering work, the Kohler research group developed photoreactive probes that can be used to study transient glycan-mediated interactions.[13,14] In most of these probes' designs, besides a "recognition" group and "tag" group, "photoreactive trap" group was additionally

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incorporated to strengthen the interaction between glycolipids and their interacting protein targets.[1a]

Despite diverse methods developed for studying the behaviors of lipids, care must be taken to ensure that the chemical probes do not compromise the original activity of parent compounds.[15] In light of this requirement, the Yao group developed a series of remarkable minimalist linkers characterized by incorporation of a diazirine and terminal alkyne on a single aliphatic chain.[16] Haberkant and coworkers reported a 15 carbon long bifunctional fatty acid which can be effectively used for the global profiling of protein lipid interactions in living cells.[17] Furthermore, the Hang group demonstrated a bifunctional fatty acid chemical reporter applied to the analysis of protein–protein interactions of S-palmitoylated membrane receptors.[18] Inspired by these discoveries, we propose that a bifunctional fatty acid modified probe equipped with a diazirine and terminal alkyne may be useful for mechanistic studies of complex glycolipids. As described in Figure 2, we firstly prepared glycolipid probes by replacing one fatty acid chain with the bifunctional chain, which can be crosslinked to its protein binding partner after UV irradiation. Click chemistry is then used to label the alkyne group in the bifunctional chain with biotin-N_3 or fluorescein-N_3 (Fluo-N_3), allowing the respective identification or visualization of the crosslinked protein–lipid complex.

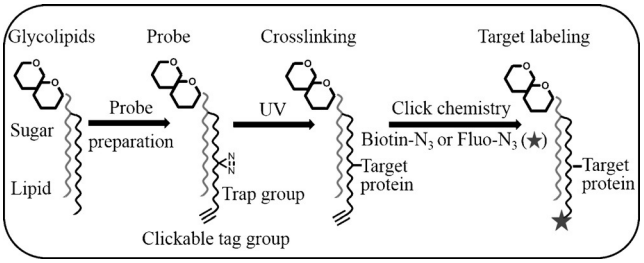
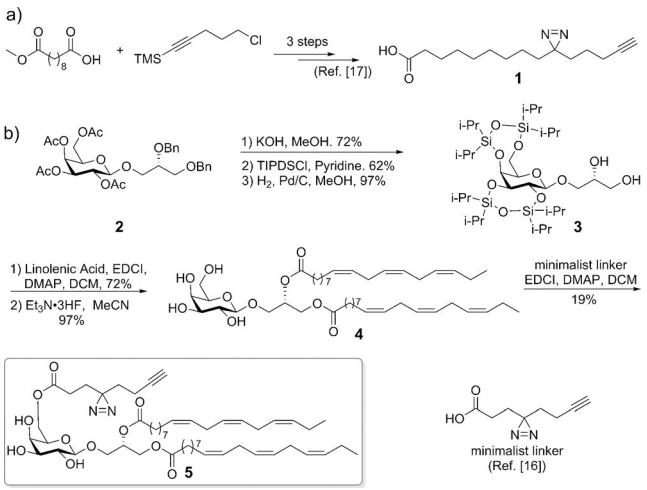


Figure 2. General scheme for the profiling of cellular target(s) of glycolipids.

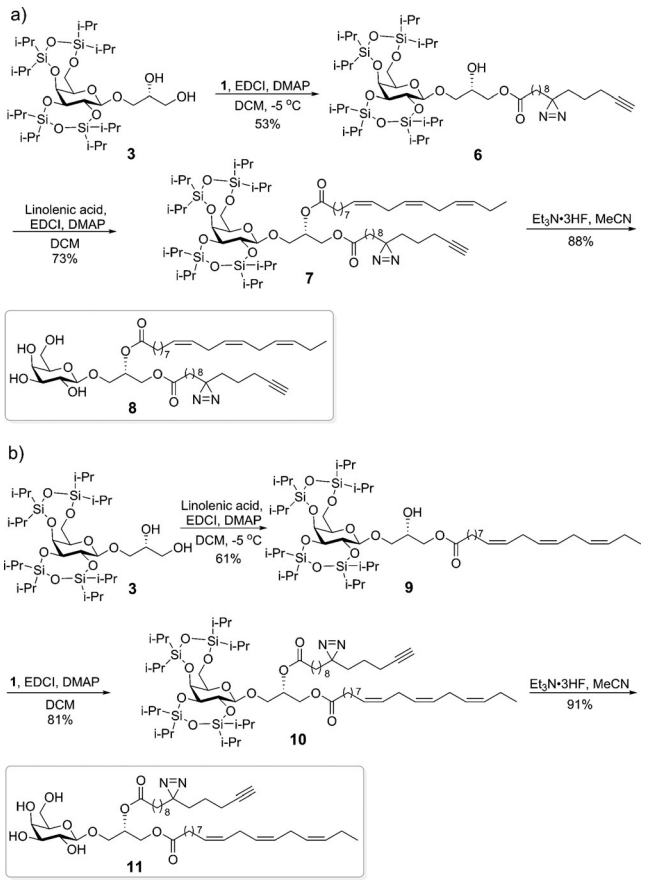
Dilinolenoyl MGDG attracted our attention for its impressive anti-inflammatory activity both in vitro and in vivo.[6,19] We expected that by incorporating the bifunctional fatty acid into MGDG, we could explore the mechanism for its anti-inflammatory activity. We envisioned that the replacement of one of the linolenoyl chains would not significantly change its size and properties. As a proof of concept, three chemical probes of MGDG were synthesized by either direct modification on the galactosyl moiety with the minimalist linker or by individual replacement of the fatty acids with the designed bifunctional fatty acid. To our knowledge, this is the first time this kind of bifunctional fatty acid has been incorporated into therapeutically important complex glycolipids for target identification.

In order to obtain the above mentioned chemical probes, we firstly synthesized the bifunctional fatty acid according to the reported procedure.[17] The bifunctional fatty acid **1** was synthesized from the acid and the TMS-protected alkyne in three steps (Scheme 1 a). Then we tried to synthesize MGDG. As an important glycolipid, MGDG has attracted much



Scheme 1. Syntheses of dilinolenoyl MGDG and its minimalist probe.

attention and several synthetic routes have been described.[20] As shown in Scheme 1 b, our synthesis of MGDG commenced with the acetate deprotection of **2**, followed by TIPDSCl protection and catalytic hydrogenation to give the diol **3**.[20e] Coupling of linolenic acid to the diol **3** afforded the silylated MGDG in 72% yield, which was then deprotected by $Et_3N{\cdot}3HF$ to yield the dilinolenoyl MGDG **4** in 97% yield. Coupling of the minimalist linker to the primary alcohol gave the minimalist probe **5**.



Scheme 2. Syntheses of bifunctional MGDG probes.

We then set out to synthesize MGDG probes modified with the bifunctional fatty acid chain. The bifunctional fatty acid **1** was coupled to the *sn*-1 position of the diol **3**, followed by a second esterification with linolenic acid to yield the silylated MGDG probe **7**. After deprotection of the compound with $Et_3N{\cdot}3HF$, we could get the probe **8** in 88% yield (Scheme 2a). In a like manner, another probe with the bifunctional fatty acid at the *sn*-2 position (probe **11**) was obtained (Scheme 2b).

After we synthesized the MGDG probes, we initially estimated the anti-inflammatory activities by using the commercially available ELISA kit which allows the determination of phospho-p38, total p38 and cell number in the same assay. Consistent with a literature result,[5d] MGDG shows strong inhibition of p38 phosphorylation without influencing total cell number. Probe **11** proved to be a more potent inhibitor of p38 phosphorylation than MGDG, while probe **8** showed a decreased inhibitory effect. However, the minimalist probe **5** was biologically inactive, thereby indicating the galactosyl moiety is essential for the target recognition (Figure 3a). Furthermore, when we re-measured the p38 inhibition effect of probes **8** and **11** after washing, we found that probe **8** totally lost its activity, which indicated probe **8** could not photo cross-link to its target protein (Figure S1 in the Supporting Information). Overall, probe **11** could be applied as a satisfactory chemical tool of MGDG for target ID.

We next performed endogenous proteome labeling followed by pull-down/LC-MS/MS aiming to identify the functional target of MGDG. We incubated human chondrocytes with probe **5** (negative control) or **11** for 1 h and then irradiated with UV light for 20 min. Subsequently the cells were lysed and subjected to Cu^I-catalyzed click reaction conditions with biotinylated azide. The resulting lysates were then incubated with streptavidin-labeled beads. After washing the beads, the precipitated proteins were resolved by SDS-PAGE and stained with silver. As shown in Figure 3b, we cut down the gel bands preferentially pulled down by probe **11** rather than **5** and then sent them for MS analysis. As shown in Table S1, we excluded the proteins present in both the **11**-treated group and the **5**-treated group in the mass analysis, and finally focused on two potential candidates: Toll-like receptor 4 (TLR4) and KTN-1 (Figure 3b). With these two potential targets in mind, we further blotted the elution with KTN-1 antibody and TLR4 antibody, respectively. As shown in Figure 3c, only TLR4 was validated to exist in the precipitate, thus demonstrating that TLR4 may be the target of MGDG. Meanwhile, when we incubated human chondrocytes with 20 μM MGDG, and probe **11** and then exposed to UV irridation for 0 or 20 min, the immunoprecipitated TLR4 protein conjugates were subjected to Western blot analysis. We found that the interaction between TLR4 and probe **11** is both UV- and CuAAC-dependent. In the presence of equal concentrations of MGDG and probe **11**, the quantity of recombinant TLR4 pulled down by **11** was reduced (Figure S2), confirming that MGDG directly interacts with TLR4. Furthermore, MGDG could antagonize LPS-mediated TLR4 activation in a dose-responsive manner by using Human TLR4 luciferase-(LUCPorter™) stable reporter cell line (Figure 3d). However, metabolites of MGDG did not show inhibitory effect (Figure S3).

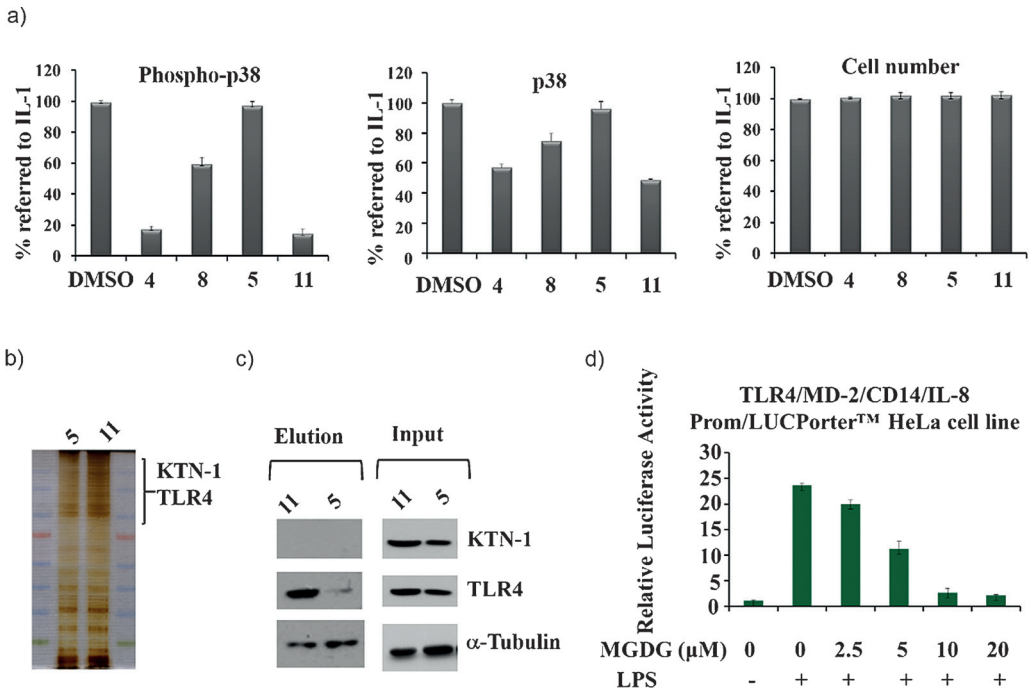


Figure 3. a) Human chondrocytes were treated with 100 U mL^{-1} IL-1α in the presence of 20 μM indicated compounds for 60 min and then phospho-p38 was measured by using p38 kit. b) Human chondrocytes cell lysates were incubated with 20 μM probe **11** or **5** for 1 h and then exposed to UV irradiation, followed by conjugating with biotin tag through Cu-catalyzed azide/alkyne cycloaddition (CuAAC) reaction, finally the lysates were used for pull-down with streptavidin-agarose beads, and the precipitates resolved by SDS-PAGE were stained by silver. The indicated bands were analyzed by mass spectrometry. c) The elution was blotted with KTN-1 and TLR4 antibody, respectively. d) MGDG inhibited LPS-mediated TLR4 activation in a dose-dependent manner by using the TLR4/MD-2/CD14/IL-8 Prom/LUCPorter™ reporter cell line.

Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition molecules, which have been implicated in the pathobiology of inflammation.[21] As the most extensively characterized Toll-like receptor, TLR4 is critical to innate immunity responses stimulated by LPS binding, which lead to the activation of

downstream signaling pathways, including the nuclear factor-κB (NF-κB) and the p38 MAPK (mitogen activated protein kinase) pathways, finally resulting in the induction of systemic inflammation.[22–26] Consequently, the anti-inflammatory effect of MGDG may be reasonably explained by TLR4 antagonism.

Next, we further explored the binding mode of MGDG to TLR4. Previous structural research indicated that the heterodimer TLR4/MD-2 is essential for the recognition of its natural ligands LPS or synthetic antagonists.[27] Eritoran is an analog of LPS and inhibits its activity by binding to the TLR4/MD-2 complex.[4] In 2007, Lee and co-workers demonstrated crystallographically that Eritoran binds to a large internal pocket in MD-2.[28] Thus, we modeled the binding pose of MGDG with TLR4/MD-2 complex. As shown in Figure 4a,b, MGDG shared a similar binding mode with Eritoran. Both two fatty chains of MGDG inserted into the pocket of MD-2. To gain experimental evidence to support this modeling, we found that both Eritoran and MGDG could outcompete the interaction between **11** and TLR4/MD-2. (Figure 4c). In contrast to Eritoran, the binding mode between MGDG and TLR4/MD-2 shows that fatty chain on *sn*-2 position forms favorable hydrophobic interactions with hTLR4 residues including F440, F463, L444, K388, Q436, which can well explain why probe **11** could form a covalent bond with TLR4 but probe **8** could not (Figure S4). In order to evaluate which of them is critical for the binding between MGDG and TLR4, we individually mutated these five residues of TLR4 into alanine. Among these mutants, both TLR4^{F440A}/MD-2 and TLR4^{F463A}/MD-2 largely decreased the ability to interact with MGDG. The other three mutants retained the binding affinity for MGDG in a manner similar to wild-type hTLR4, indicating that F440 and F463 are essential for its binding to MGDG (Figure 4d). Previous functional studies have identified that both F440 and F463 are required for cell activation by LPS.[29,30] Thus, besides binding like Eritoran (the same binding mode with Eritoran), we could expect that MGDG would antagonize TLR4 in a more effective way by precluding LPS induction. Since Eritoran entered a phase III clinical trial for the treatment of severe sepsis, an excessive inflammatory response to infection, MGDG is promising for inflammation therapy.[31] Taken together, these results establish that MGDG acts as an antagonist to exert anti-inflammatory activity by targeting the receptor complex of TLR4 and MD-2.

In conclusion, we have designed and developed a new type of bifunctional glycolipid probe which could be used effectively for mechanistic studies, especially for target identification. This probe design was demonstrated with MGDG, and among the synthesized probes, probe **11**, modified with bifunctional fatty acid chain in the *sn*-2 position retained anti-inflammatory activity. Further biochemical studies revealed for the first time that the anti-inflammatory activity of MGDG was achieved by direct antagonism of TLR4. Thus, we expect the reported strategy of using bifunctional glycolipid probes to elucidate complex protein–glycolipid interactions will find more applications in biomedical research.

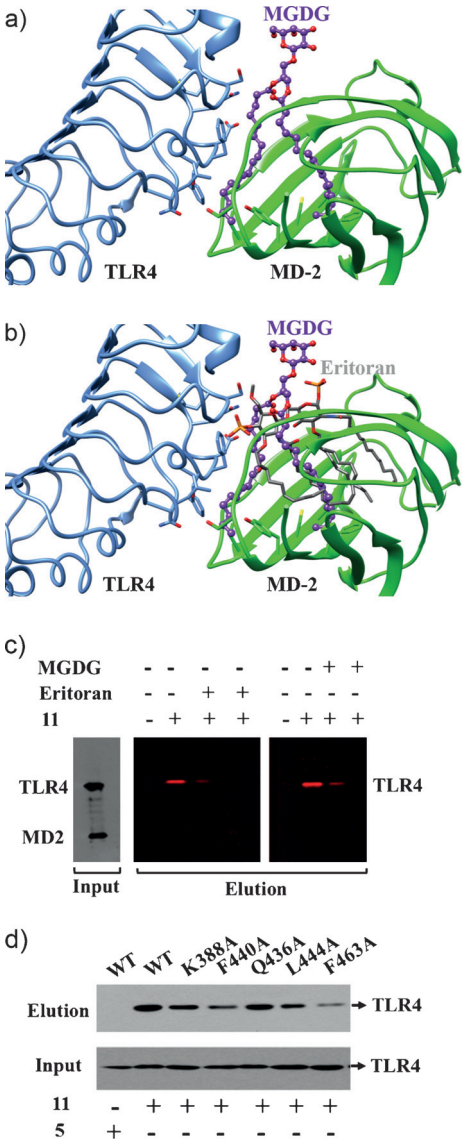


Figure 4. a) Modeled binding pose of MGDG with TLR4/MD-2 complex. b) Comparison of the binding poses between MGDG (purple) and Eritoran (gray) (PDB ID: 2Z65). c) We pre-incubated human TLR4/MD-2 recombinant with MGDG or Eritoran for 1 h and then added probe, 1 h later, the protein mixture was exposed to UV irradiation for 20 min and then subjected to Cu^{I}-catalyzed click reaction condition with Cyanine5 azide. Direct in-gel fluorescence detection of the modified proteins was performed. d) HEK293 cells were transiently transfected with the indicated expression plasmids, and then were pre-incubated with 20 or 40 μM of MGDG or Eritoran for 1 h. We isolated the TLR4 proteins by immunoprecipitation and further incubated with probe **11** for 3 h. The protein mixture was subjected to photo-crosslinking and CuAAC click chemistry. The resulting lysates were then incubated with streptavidin-labeled beads, resolved by SDS-PAGE, and analyzed by Western blot.

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