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Simple synthetic toll-like receptor 2 ligands

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ABSTRACT

Stimulation of toll-like receptor 2 (TLR2) by bacterial lipoproteins induces fast non-specific immune responses against pathogens followed by slow but specific adaptive immune responses. Development of synthetic TLR2 agonists/antagonists would be useful in the prevention of different infectious and immunologic disorders. The current study reports synthesis and TLR2 activity of two simple TLR2 ligands, which feature minimal structural requirement for TLR2 activity (two long lipid chains) and stimulate agonistic activity at nanomolar concentration.

The innate immune system plays a critical role in the detection of invading pathogens and activation of suitable protective responses.[1,2] Immune cells are equipped with a set of receptors which rapidly detect conserved microbial products leading to acute inflammatory responses and initiation of a cascade of adaptive immune responses.[3–5] For example, several toll-like receptors (TLR) are involved in the recognition and immune stimulation caused by bacterial lipids or lipoproteins (TLR 1, 2, and 6), bacterial liposaccharides (TLR4), and microbial nucleic acids (TLR 3, 7, 8, and 9).[6] Due to the involvement of TLR2 in the recognition of bacterial lipoproteins, much interest is directed towards the development of synthetic ligands of this receptor. These efforts have contributed to our understanding of the role of TLR2 in immune responses.

The ability of TLR2 to modulate immune responses is obviously shown by the powerful adjuvant activity of synthetic di- and triacylated lipopeptides (Fig. 1; Pam_2Cys, Pam_3Cys respectively).[7] The search for new TLR ligands was supported by the success of monophosphoryl lipid A as an adjuvant in clinical trials, and its acceptance by regulatory agencies in Europe and in Australia as a component of various vaccines.[8] Numerous investigations showed that adjuvant and TLR activity of bacterial lipoproteins are greatly dependent on the lipid moiety.[9–14] It was found that the minimal structural requirement to activate TLR2 is two ester bound fatty acids of chain length longer than eight carbon atoms.[11] The amide bound fatty acid is of lesser importance. Biological activity data for lipoproteins modified at the natural amino acid *S*-(2,3-dihydroxypropyl)-L-cysteine showed the importance of the stereochemical orientation and the thioether group for activity.[10,12] Recently, X-ray crystal structures of TLR2-ligand complexes have been revealed which provide good information about the TLR2 structure as well as its binding to Pam_3Cys.[15,16] The role of the lipid chains in binding to TLR1-2 dimer was clearly demonstrated where the two ester palmitoyl groups bind to a hydrophobic pocket in the TLR2 side while the amide palmitoyl chain is inserted into a hydrophobic channel in TLR1.

Lipoamino acids are synthetic amino acids with long alkyl side chains which combine the properties of amino acids (amphoteric bifunctional moiety) and lipids. This nature makes them easily accessible building blocks to design and engineer lipopeptides which mimic natural TLR2 ligands.[17] Simple lipid moieties (**5**[18] and **6**,[19] Fig. 2) incorporating two 16 carbon containing chains are proposed as novel TLR2 ligands. The compounds feature some

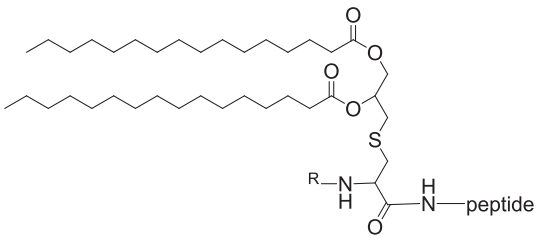

Figure 1. Structure of synthetic palmitoyl-*S*-glyceryl cysteine lipopeptides: Pam_2Cys; ^{R} = H, Pam_3Cys; ^{R} = palmitoyl.

Abbreviations: Boc, *t*-butoxycarbonyl; DMF, *N*,*N*-dimethylformamide; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEK 293, human embryonic kidney 293; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TLR, toll-like receptor.

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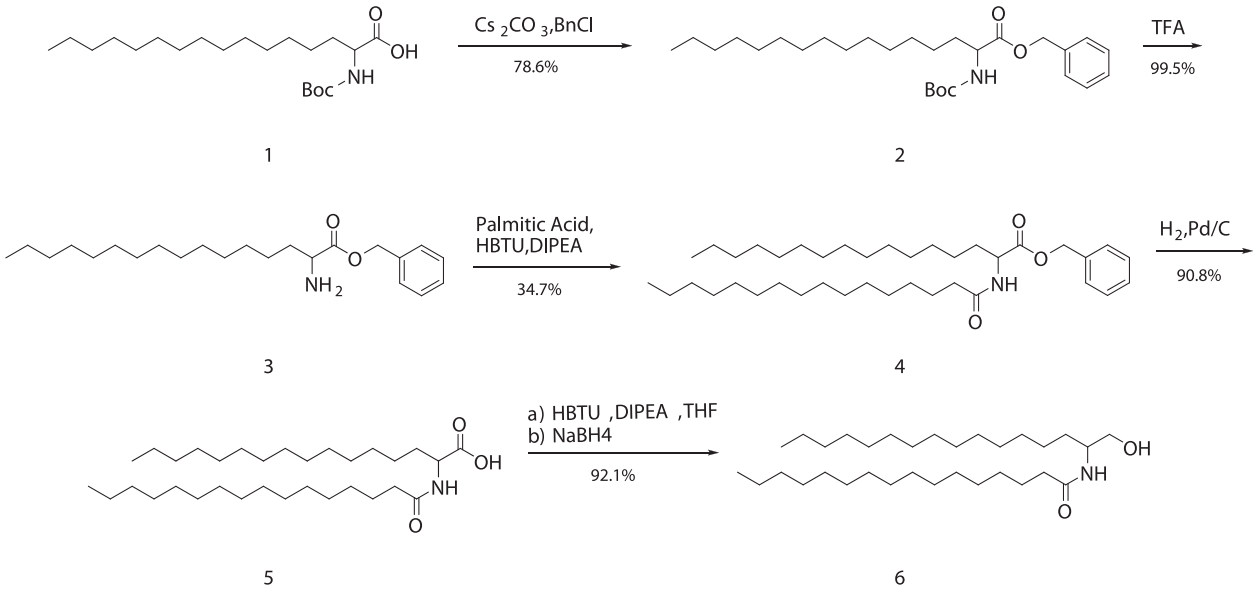

Figure 2. Synthesis of target lipoamino acid-based TLR2 ligands.

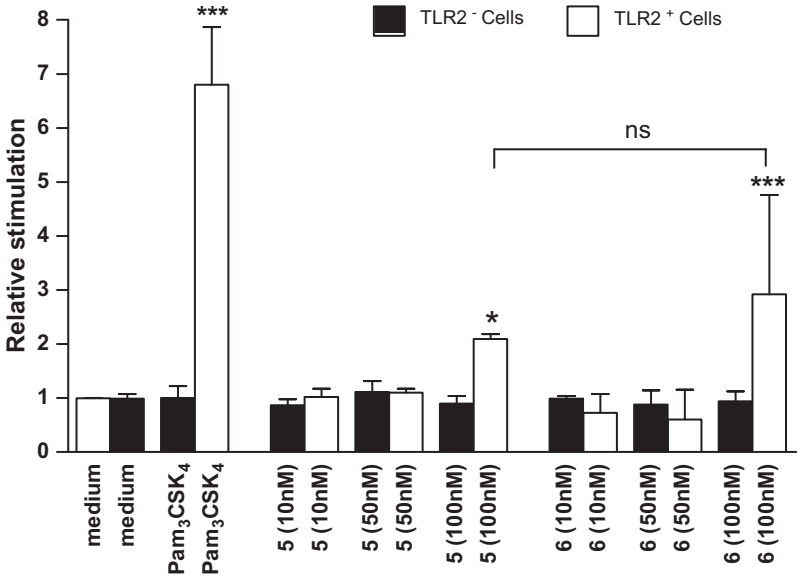

Figure 3. Data are shown as means ± SD of three-culture runs in two separate experiments. Variation between groups were analyzed using the two-tailed Student's *t* test and were regarded statistically significant if the *p* value was <0.05.

similarity to the lipid moiety of other TLR2 ligands; for example, Pam_2Cys and Pam_3Cys The proposed compounds have great advantages over Pam_2Cys and Pam_3Cys such as stability against esterases enzymes and oxidation, the ease of synthesis, and low cost production. We can also engineer the number and length of lipid chains to optimize the TLR2 activity. Racemic mixture of lipoamino acids were tested and it has been shown that chirality influences the reactivity of similar compounds.[20] However for preliminary studies presented here the focus was on the structure–activity of simple lipid moieties with two 16 carbon containing chains. Lipid moieties such as lipoamino acids can also induce apoptosis of cells.[21] As such a model cell line that is routinely used to verify activity of similar lipid moieties was used for our compounds.[11]

The *N*-Boc-protected 2-aminohexadecanoic lipoamino acid (Fig. 2, **1**) was synthesized as described by Gibbons et al.[22] followed by an O-benzylation reaction. Benzylation was performed to protect the carboxylic group during the subsequent coupling of an activated palmitic acid. Purification of the benzyl ester by column chromatography was essential as the presence of any residual benzyl chloride can lead to side products in the next reaction step. Deprotection of amino group of compound **2** was accomplished quantitatively using trifluoroacetic acid to afford benzyl ester **3**. To obtain compound **4**, coupling of the activated [HBTU/DIPEA] palmitic acid to lipoamine **3** was difficult. Using THF as a solvent led to very low yield. The reaction in DMF increased the yield and the formation of side products was greatly diminished. Silica flash column chromatography was applied for purification and after many attempts it was concluded that using pure DCM eluent achieved the highest purity with acceptable yield of compound **4** (35%).

Two subsequent reduction reactions were performed to obtain target compounds **5** and **6** respectively in high yield (>90%). First reduction was achieved by hydrogenolysis of benzyl ester **4** in THF/methanol over palladium as a catalyst to afford acid **5**. The

second reaction was carried out in THF with HBTU/DIPEA activation and $NaBH_4$ reduction. The product **6** was purified using silica gel chromatography (DCM/ether, 4:1).

Human embryonic kidney (HEK293) cells stably expressing TLR2 were used in TLR2 stimulation experiment. HEK293 cells were transiently transfected with pNF-κB-Luc Cis-reporter plasmid as a reporter gene. Negative control wells were treated with media while positive control wells were stimulated by 10 nM of Pam_3Cys analogue. Luciferase activity (NF-κB activation) of the compounds was measured, normalized against protein concentration and expressed relative to cells treated with media. Compounds **5** and **6** were tested at 10, 50, and 100 nM concentration (Fig. 3) and the results indicated that the compounds stimulated signaling through TLR2 at 100 nM concentration. These results also showed that the additional functionalities present in the Pam_3Cys [thioether linkage, *S*-(2,3-dihydroxypropyl)-L-cysteine, the ester functionality] were non-essential for TLR2 activity. Additionally, reduction of the terminal carboxyl group of compound **5** to give to alcohol **6** did not affect TLR2 activity of the constructs.

TLR 2 recognizes lipoproteins of the outer bacterial membranes. Beside native lipoproteins, synthetic bacterial lipids such as Pam_2Cys and Pam_3Cys also activated TLR2. In an on-going research to develop a TLR2-activating lipopeptides, we developed lipoamino acid-based Pam_2Cys-like analogues. Two compounds were able to activate TLR2 receptors at nanomolar level. The proposed compounds are simpler than other reported synthetic analogues of bacterial lipoproteins.

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