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Research paper

Design, synthesis and immunological evaluation of novel amphiphilic desmuramyl peptides

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ABSTRACT

Muramyl dipeptide (MDP) – an essential bacterial cell wall component – is recognized by our immune system as pathogen-associated molecular pattern (PAMP) which results in immune responses with adverse toxic effects. In order to harness the beneficial properties from the pro-inflammatory characteristics of the bacterial cell wall motif, MDP was strategically re-designed while conserving the L-D configurations of the dipeptide moiety. The muramic acid was replaced with a hydrophilic arene and lipophilic chain was introduced at peptide end to give the amphiphilic desmuramyl peptides (DMPs). The novel DMPs were found to modulate the immune response by amplifying the LPS-induced surface glycoprotein (ICAM-1) expression in THP-1 cells without showing significant toxicity. Furthermore, these compounds were able to trigger the secretion of higher levels of pro-inflammatory cytokine (TNF-α) than the well-studied NOD2 agonist, Murabutide.

1. Introduction

Innate immune system is the first line of defense against invading pathogens [1]. The system is comprised of different pattern recognition receptors (PRRs) such as Toll-like receptor (TLR), Nod-like receptor (NLR) and retinoic acid inducible gene (RIG)-like receptor [2]. PRRs are capable of detecting the invading microorganisms by recognizing the pathogen-associated molecular patterns (PAMPs) and triggering a cascade of responses against them. Common PAMPs usually encompass bacterial cell wall components like peptidoglycan (PGN), lipopolysaccharides (LPS) and bacterial DNA [3]. The innate immune response to PGN is largely mediated by NLRs such as NOD2, which recognizes muramyl dipeptide (MDP) [2]. MDP is the smallest bioactive fragment of bacterial PGN consisting of one carbohydrate and two amino acids (Fig. 1) [1]. This molecule is found both in Gram-positive and Gram-negative bacteria [1]. When mammalian cells expressing NOD2 are treated with MDP, an inflammatory response is activated triggering the expression of adhesion molecules (e.g. ICAM-1) and the production of proinflammatory cytokines such as interlukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) [1,3]. However, MDP possesses serious drawbacks such as poor penetration through cell membrane, and rapid elimination [3]. Furthermore, it induces severe local reactions similar to some bacterial infections in humans and is considered too toxic to be used in clinical applications [4]. Numerous structural modifications in MDP have been done with the intention of improving the pharmacological properties and lowering the toxicological profile [2]. These efforts led to the discovery of many useful hydrophilic derivatives such as murabutide (MB), temurtide, nor-MDP, glucosaminyl-MDP and paclitaxel-MDP [3,5–7]. Lipophilic derivatives of MDP containing an additional amino acid residue at C-terminus including mafimurtide, romurtide and muramyl tripeptide phospatidylethanolamine also reached the clinical stage of development [3]. Furthermore, a variety of carbohydrate analogs of MDP containing manno-, galacto-, xylo-, allo- and L-idomuramic acid have been synthesized [8]. D-manno- and D-galacto-type muramyl dipeptides were found as active as the D-*gluco*-type muramyl dipeptide on the induction of type IV hypersensitivity in guinea pigs [9]. Some furanoid MDP analogs containing D-glucofuranose had better immunoadjuvant activity compared to the parent MDP molecule [10],

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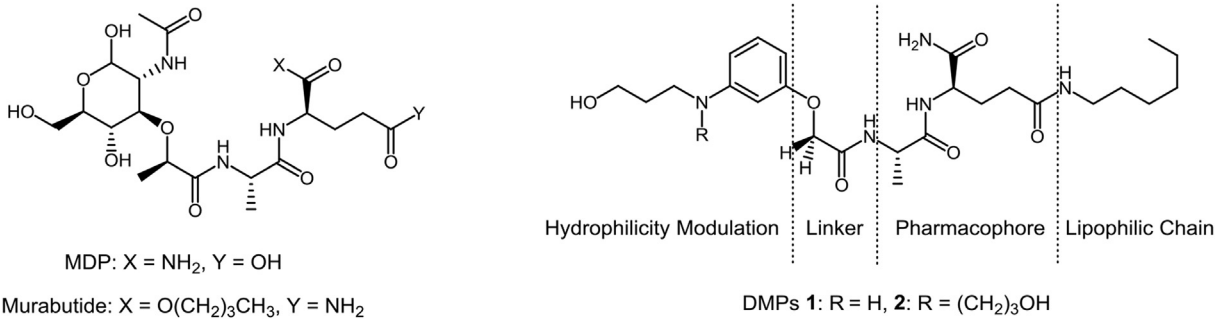

Fig. 1. Structural components of novel amphiphilic desmuramyl peptides (right).

whereas the 2-deoxy-D-arabinohexose analogue was completely inactive [11]. Synthetic PGN fragments containing di-, tetra- and octasaccharides coupled to dipeptide moiety also had definite immunostimulating activities similar to that of MDP but their tumor necrosis factor-alpha (TNF-α) inducing potencies decreased as the glycan chain became longer from di-, tetra-to octasaccharides [12].

Carbohydrate moiety is apparently not essential for the pyrogenic and immunoadjuvant activity of peptidoglycan fragments [8]. Desmuramyl peptides (DMPs), which are MDP derivatives lacking the carbohydrate moiety, have also been reported. For example, O-(L-alanyl-D-isoglutamine-L-alanyl)-glycerol-3-mycolate was found as active as MDP in stimulating mouse resistance to infection [13]. Several DMPs have shown significant immunomodulatory activity and remarkable antitumor potency [2,14,15]. For example, Gang Liu and co-workers replaced the muramic acid moiety in MDP with hydrophobic arenes. The novel DMP – paclitaxel conjugates combine chemotherapy and immunotherapy in the treatment of cancer and had dual antitumor growth with metastasis activities [14,15]. Sollner et al. synthesized 7-oxo-octanoyl group containing DMP. The new molecule was found to be an apyrogenic immunomodulator and had tumor growth delay effect [16]. Moriguchi and co-workers prepared a phthalimido-desmuramyldipeptide – another immunomodulating agent capable of restoring the interleukin-10 capacity in rodents [17]. Jakopin et al. synthesized DMPs incorporating either a pyrido-fused [1,2]-benzisothiazole moiety or an indole scaffold and studied their immunomodulatory properties [2,3]. DMPs containing carbocyclic ring have also been reported to be immunologically active [18]. Although a good number of DMPs have been reported in the literature, they lack hydrophilicity due to the elimination of muramic acid – the carbohydrate moiety of MDP. A balanced approach towards lipophilicity and hydrophilicity is important for the exhibition of interesting biological activity [19]. Murabutide (MB) is a prime example of such approach in which MDP was strategically modified by introducing a modest lipophilic chain at peptide end. The new molecule proved to be a safe immunomodulator, capable of enhancing the host's resistance against bacterial and viral infections without significant toxicity [2,16]. With traditional immunomodulatory agents exhibiting limited efficacy, the development of new multifunctional and non-toxic drugs capable of altering the immune response safely is extremely important [3]. However, immunomodulation – a therapeutic need of the new millennium – is still in its infancy [3,20].

MDP is composed of *N*-acetyl-D-glucosamine linked with two amino acids (L-alanine and D-isoglutamine) via lactic acid moiety (Fig. 1) [1]. Recognition of MDP by innate immune system is highly stereospecific of the L-D isomer. D-L diastereomer (*N*-acetylmuramyl-D-alanyl-L-isoglutamine) was found completely inactive [21] just like D-D [22] and L-L isomers [1]. Biological activity of MDP is lost, when D-isoglutamine is replaced by the L-isoglutamine enantiomer [23]. Although L-alanine can be replaced by a similar amino acid such as L-serine or L-valine, D-isoglutamine cannot be replaced by another amino acid [24]. However, peptide chain can be prolonged at carboxy-terminus [25]. It's therefore imperative to conserve L-D configuration of the dipeptide in the design of DMPs. Several studies have delineated that the intact muramyl moiety containing *N*-acetylglucosamine and lactic acid is not essential for biological activity [18,19]. However, previously reported DMPs lack hydrophilic character – an important variable towards the activation of NLRs found in cytosol [2]. A further literature search revealed that existing methods did not describe the preparation of DMPs presenting both hydrophilic and lipophilic characters in the same molecule. In our attempt to rationally modulate the hydrophilicity in DMPs, aryl amine containing hydroxylated *N*-alkyl groups was introduced (Fig. 1). The hydrophilic aromatic amines were then linked by glycolic acid linker to the N-terminus of L-alanine-D-isoglutamine dipeptide. The glycolic acid linker was employed to replace the D-lactic acid in MDP, thus further simplifying the structure by removing the chirality present in the lactic acid moiety. Furthermore, an aliphatic chain was added at the peptide end to facilitate their penetration through cell membrane.

DMPs possessing both hydrophilic and lipophilic properties have not yet been explored. In this report, we describe the synthesis of novel amphiphilic DMPs (**1**–**2**), in which the carbohydrate moiety of MDP was replaced by hydrophilic arene – an entity suitable in cytosol to target the intracellular NLRs for the activation of innate immune system. Complementary lipophilicity was achieved by introducing a modest aliphatic chain to assist cell membrane penetration. We also examined whether these novel structures, alone or in combination, can modulate the expression of cell surface glycoprotein *viz.* intercellular adhesion molecule-1 (ICAM-1) which plays an important role in immune and inflammatory processes. Tumour necrosis factor-alpha (TNF-α) is one of the major cytokines and important mediator of immunologic and inflammatory reactions [26]. Investigation using enzyme linked immunosorbent assay (ELISA) also demonstrated the production of pro-inflammatory cytokine (TNF-α) by these amphiphilic desmuramylpeptides (**1**–**2**).

2. Results and discussion

2.1. Synthesis

The convergent synthesis of amphiphilic DMPs began with the alkylation of 3-nitrophenol to give *tert*-butyl 2-(3-nitrophenoxy) acetate **3**. Reduction of nitro group in **3** via Pd/C with molecular hydrogen afforded *tert*-butyl 2-(3-aminophenoxy)acetate **4**. Refluxing **4** with 3-bromopropanol in the presence of *N,N*-diisopropylethylamine resulted in mono-alkylation of free amine to give