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Research paper

Structural requirements of acylated Gly-L-Ala-D-Glu analogs for activation of the innate immune receptor NOD2

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ABSTRACT

The fragment of bacterial peptidoglycan muramyl dipeptide (MDP) has long been known for its adjuvant activity, however the underlying mechanism of this action has only recently been elucidated. It is ascribed to its agonist action on the nucleotide-binding oligomerization domain-containing protein 2 (NOD2). In spite of the pressing need for novel adjuvants for human use, this discovery is hampered, by not knowing the structural requirements underlying the immunostimulatory activity. We have investigated how minor modifications of hit compound acyl Gly-L-Ala-D-Glu derivative **I** modulate the molecular recognition by NOD2. A series of novel desmuramyldipeptides has been designed and synthesized leading to the identification of compound **16**, in which the sugar moiety is replaced by a 6-phenylindole moiety, that exhibits the strongest NOD2 activation to date sans the carbohydrate moiety. The results have enabled a deeper understanding of the structural requirements of desmuramylpeptides for NOD2 activation.

1. Introduction

The innate immune mechanisms that orchestrate the initial defense response against invading pathogens rely on the specific detection of their molecular signatures by evolutionarily conserved sentinels such as pattern recognition receptors (PRRs). The nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a pivotal member of the cytosolic NOD-like receptor (NLR) family of PRRs and plays a major role in innate immunity [1]. Its expression pattern is limited, by being expressed mostly in professional immune cells and, to a minor extent, in epithelial cells of the intestine and lung and in stem cells [1–3]. NOD2 protein is characterized by a tripartite structure and that consists of: two caspase activation and recruitment domains (CARDs), a nucleotide-binding oligomerization (NOD) domain and multiple leucine-rich repeats (LRRs) [4]. Recent evidence suggests that NOD2 resides in an autoinhibited state, its LRRs being folded over the NOD domain, and undergoes specific conformational changes that promote its activation only on recognition of a distinct and conserved constituent of bacterial peptidoglycan [2,5].

Muramyl dipeptide (MDP), a motif found in the cell wall of most types of bacteria, is the smallest fragment, able to activate NOD2 [6,7]. Moreover, using both biophysical and biochemical assays, a direct interaction between MDP and NOD2 protein has been confirmed [5,8]. On recognition of MDP, NOD2 becomes activated and undergoes self-oligomerization. It then engages the serine–-threonine kinase receptor-interacting protein 2 (RIP2) that, in turn, initiates the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. These result in proinflammatory and antimicrobial responses characterized by the secretion of cytokines, induction of autophagy and production of antimicrobial peptides [9]. These downstream signaling pathways are tightly regulated by a wide variety of regulatory proteins and are also dependent on the subcellular localization of NOD2 and other components of this pathway [9].

NOD2 signaling can influence adaptive immune responses [10,11]. Further, the presence of NOD2 ligands positively amplifies the potential of other immune adjuvants such as Toll-like receptor

Abbreviations: CARD, caspase activation and recruitment domain; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; LRR, leucine-rich repeats; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; MTS, ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium); Mur*N*Ac, *N*-acetylmuramyl; NF-κB, nuclear factor κB; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domain; PRR, pattern recognition receptors; RIP2, receptor-interacting serine–threonine kinase; SAR, structure/activity relationship; SEAP, secreted embryonic alkaline phosphatase; TLR, toll-like receptor; TFA, trifluoroacetic acid.

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(TLR) ligands, this crosstalk between NLRs and TLRs being crucial for adaptive immunity [12–14]. The recent progress in our understanding of innate immunity and its potential for shaping adaptive immune responses underlines the importance of NLRs and has provided new insights for vaccine development [15]. In 1974, MDP was shown to be the minimal mycobacterial component responsible for the efficacy of Freund's complete adjuvant [16]. However, its use has since been restricted to veterinary vaccines, as it was considered too pyrogenic for clinical application [17]. In addition to their considerable potential as adjuvants, NOD2 agonists possess anti-infective properties and can also act synergistically in combination with antibiotics [15,18]. Recently, yet another important role of NOD2, namely stem cell protection by recognition of commensal microbiota was highlighted by Nigro [3].

MDP derivatives would be useful immunomodulators if certain drawbacks, including their pyrogenicity, their rapid elimination and lack of oral availability, could be overcome. Over the past decades a considerable number of studies involving synthesis have been undertaken, leading to the discovery of numerous lipophilic MDP derivatives, either chemically modified or conjugated with carriers, devoid of pyrogenicity but with retained adjuvant and immunomodulatory potential [19–27]. The early considered lipophilic derivatives (shown in Fig. 1) and lipophilic delivery carrier systems have displayed significantly increased adjuvant activity than MDP, indicating that its parent structure can be modulated to enhance the adjuvant activity [17,27–29]. Incidentally, mifamurtide (also termed MTP-PE) is one of the major lipophilic derivatives developed by pharmaceutical industries that has recently entered clinical trials as a component of an influenza vaccine [18], while murabutide has also entered numerous clinical trials [22].

Desmuramylpeptides are MDP derivatives lacking the carbohydrate moiety. We and others have shown that the sugar *N*-acetylmuramyl moiety (Mur*N*Ac) is not required for NOD2 activation [20,21,30], contrary to the previous belief that only MDP derivatives with an intact Mur*N*Ac are active. Here we investigate how minor modifications of the parent compound acyl Gly-L-Ala-D-Glu derivative **I** modulate the molecular recognition by NOD2. Our aim was to generate desmuramylpeptides with enhanced lipophilicity, and with retained efficacy as MDP. We screened the NOD2-dependent NF-κB activation capacity of the library of these synthesized analogs on HEK293 cells overexpressing the NOD2 gene. Compound **16**, in which the sugar moiety was replaced by a 6-phenylindole moiety, was identified as a compound that exhibits the strongest NOD2 activation to date sans the carbohydrate moiety. These results thus provide a further and deeper understanding of the structural requirements underlying the NOD2-stimulatory activity of desmuramylpeptides.

2. Results and discussion

2.1. Design

Multiple mechanisms have been suggested by which MDP can enter the cell cytoplasm, such as the SLC15 family of peptide transporters, outer membrane vesicles, and *via* endocytosis [31–34]. The mechanisms underlying NOD2 ligand internalization are, however, not yet fully understood. However, desmuramylpeptides have been shown to enter the cell by passive absorption, a route that depends on their lipophilicity [35]. The facts that membrane-penetrating methods of delivery and introduction of hydrophobic groups to NOD agonists strongly enhance the potency of the compounds further reinforces the notion that these compounds can cross the membrane by passive absorption, thus reaching their designated intracellular target [28,36,37]. Studies of the structure-activity relationships (SAR) of MDP derivatives have suggested that introduction of a lipophilic substituent into MDP can increase its adjuvant activity [38]. The L-Ala-D-isoGln pharmacophore has long been considered as essential for immunostimulatory activity, allowing only limited variations of amino acid type and virtually none regarding the stereochemistry. In our previous

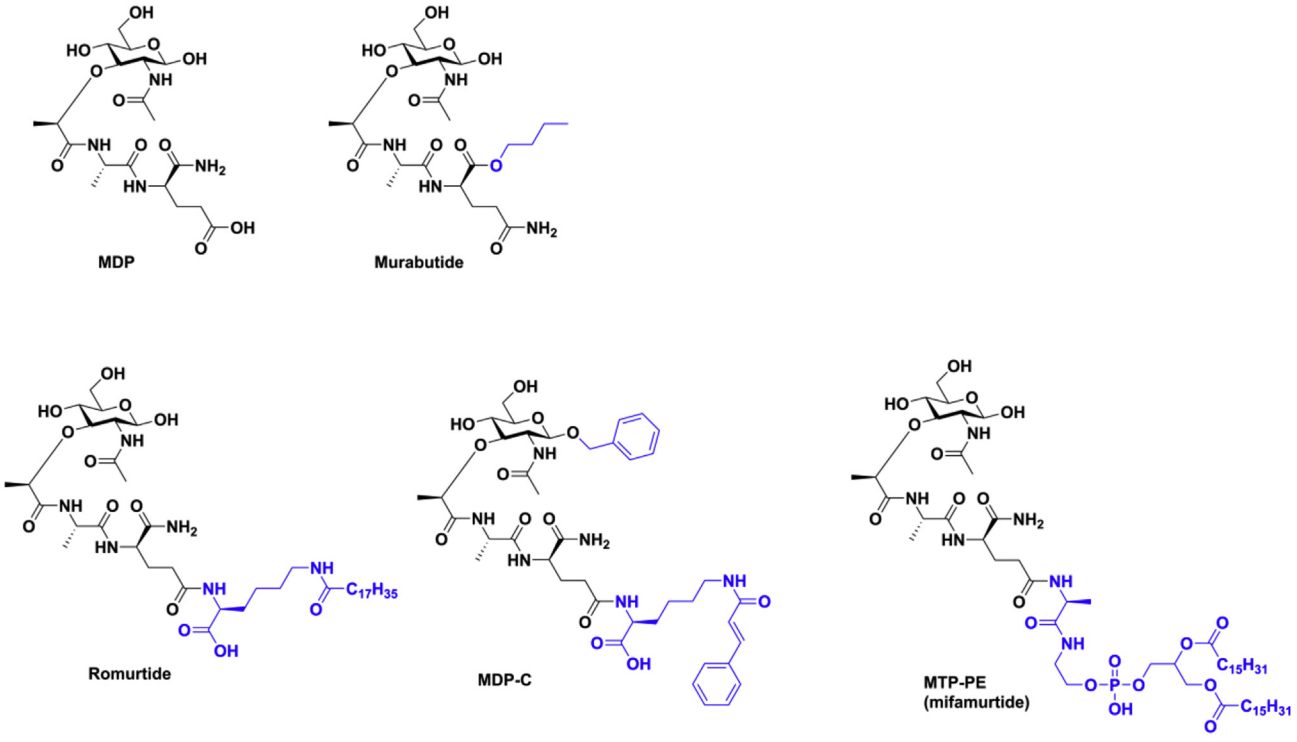

Fig. 1. Representative lipophilic MDP derivatives with an intact MDP core structure.