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Advances in Desmuramyl Peptide Research

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THIS PAPER IS DEDICATED TO PROF. KATA MLINARIĆ-MAJERSKI ON THE OCCASION OF HER 70TH BIRTHDAY

Abstract: Immune adjuvants are added to vaccines in order to enhance the immune response to an antigen. Muramyl dipeptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, is the smallest structural unit of peptidoglycans showing the immunostimulating activity. Muramyl dipeptide analogues without the hydrophilic *N*-acetylmuramyl moiety are called desmuramyl peptides. Here, we provide review of desmuramyl peptides which were synthesized in order to improve the pharmacological properties of parent muramyl dipeptide, including our results regarding adamantane containing derivatives. Approach for future design of novel immunostimulators based on multiple pathogen recognition receptor activation was also considered.

Keywords: adjuvant, desmuramyl peptides, immunostimulation, pathogen recognition receptors.

INTRODUCTION

IMMUNIZATION helps to inhibit the spread of infectious and cancer diseases. Vaccines can be used prophylactically in order to prevent the effect of infection, and therapeutically such as vaccines against cancer. Prophylactic vaccines stimulate immune systems to respond rapidly to pathogens and eliminate them.[1] Vaccines are manufactured on the basis of well characterized antigens, but their immune response is often weak, largely because of the inability of the antigens to induce maturation of dendritic cells (DCs), the primary antigen-presenting cells (APCs) that react to foreign pathogens and trigger the immune response.[1] Since the early 20th century, the concept of vaccine adjuvant was introduced. Immune adjuvant is a part of a vaccine that enhances the immune response to an antigen, either by the ability to form a depot which extends antigen exposure to APCs or by direct interaction with APCs.[1,2] Mineral compounds (aluminium based adjuvants), bacterial products, oil-based emulsions and liposomes have been evaluated as adjuvants. Adjuvants can be classified according to different criteria. Based on their mechanism of action they can be divided into two groups; delivery system (particulate adjuvants) and immune potentiators (immunostimulatory adjuvants). Delivery systems include mineral salts, lipid particles and microparticles.[3]

Aluminium based adjuvants are the most widespread and the most preferred for human applications. Besides aluminium salts, FDA has licensed oil-in water emulsions (MF59 and AS03), AS04 (3-*O*-deacylated monophosphoryl lipid A (MPL) plus aluminum salts), CpG ODN and AS01 (MPL and saponin QS-21 formulated in liposomes) for human vaccines.[4] Freund's adjuvants are one of the most powerful and widely used oil-water emulsion adjuvants in experimental immunology. Freund's complete adjuvant (FCA) contains heat-killed mycobacteria (Mycobacterium tuberculosis) and Freund's incomplete adjuvant is oil-water emulsion without mycobacteria added. They are extensively used in animal experimental models due to their high efficacy.[5] It was discovered that peptidoglycan, a bacterial cell wall polymeric component, is active part of the FCA.

In 1974 it was shown that muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine) is the smallest peptidoglycan fragment (Figure 1) capable of replacing whole mycobacterium in FCA.[6] MDP acts as an immune potentiator which activates innate immune system.

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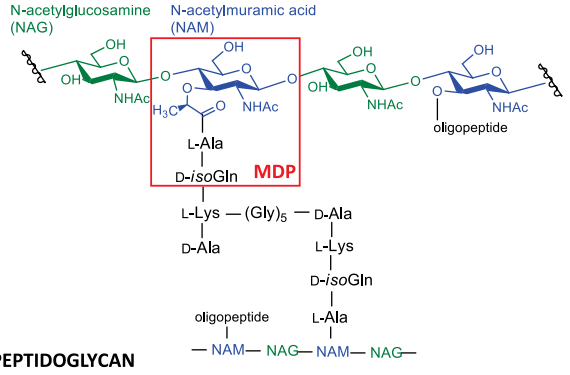

Figure 1. MDP, the smallest peptidoglycan fragments with immunostimulating properties.

Immune potentiators in general activate immune responses through pattern-recognition receptors (PRRs) or directly, for example by cytokines. PRRs are classified into: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs).[7] Muropeptides, breakdown products of polymeric peptidoglycan, act as agonists of PRRs and therefore exhibit immunostimulatory properties.[8–10] They activate innate immune responses and contribute to the development of adaptive immunity.

Numerous MDP analogues and derivatives were synthesized, in order to improve the pharmacological properties of MDP, such as pyrogenicity, poor cellular penetration, rapid elimination, the toxicological profile, for possible clinical application.[11–13] Mifamurtide (liposomal muramyl tripeptide phosphatidyl ethanolamine, L-MTP-PE, Figure 2) is used in clinics for treatment of osteocarcinoma. L-MTP-PE activates the immune response and in addition to standard chemotherapy it enables the statistically significant reduction in the risk of death from osteosarcoma.[14] Another example of safe MDP derivative is murabutide (Figure 2). Murabutide interacts with cells of innate and adaptive immune system and enhance the resistance against microbial infections.[15]

MDP analogues without the *N*-acetylmuramyl moiety are called desmuramyl peptides (DMP). Structure-activity studies of the MDP derivatives and analogues suggest that L-Ala-D-isoGln pharmacophore is essential for the immunostimulatory properties and that the introduction of lipophilic substituent into MDP analogues can increase its adjuvant activity.[11] The desmuramyl dipeptides were shown to enter the cell by passive absorption, which is directly dependent on their lipophilicity.[16] The absence of structural data for the binding site of MDP untill 2016 has made the design of novel DMP immunostimulators difficult.[17] Till then the structure-activity relationship studies of known compounds with immunostimulation properties were the only route for rational drug design of novel adjuvants. In this article is given an overview of the most important DMP structural scaffolds.

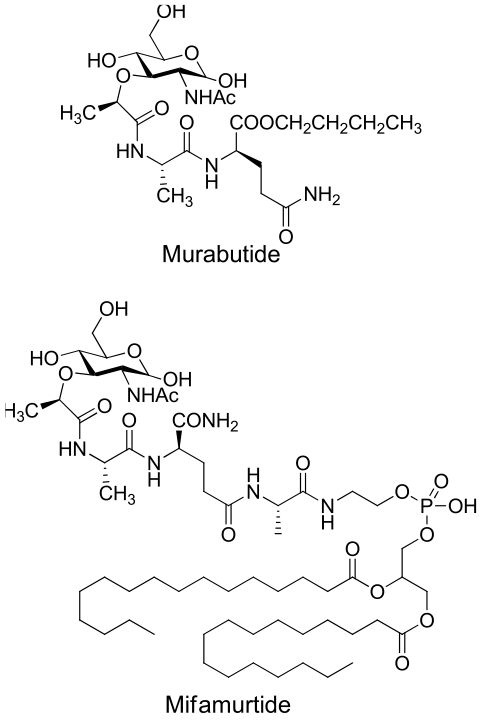

Figure 2. MDP derivatives used in clinics.

MECHANISM OF ACTION

MDP triggers an immune response by activating the mammalian NOD-like receptor, nucleotide binding oligomerization domain-containing protein 2 (NOD2). NOD2, also known as NLRC2 or CARD15, is an intracellular protein (1040 residues, 110 kD) involved in signalling response to peptidoglycan fragment which is recognized as a pathogen-associated molecular pattern (PAMP). NOD2 belongs to signal transduction ATPases associated with numerous domains (STAND) and is composed of two N-terminal caspase recruitment domains (CARDs), a central nucleotide oligomerization domain (NOD) and leucine-rich repeat domain (LRR) which is believed to act as MDP sensor.[18] Maekawa *et al* have determined crystal structure of ADP-bound NOD2. They confirmed that LRR domain of NOD2 consists of ten LRR and that previously proposed MDP-binding site is on the concave surface of the LRR domain.[17] The NOD domain contains several subdomains; a nucleotide-binding domain (NBD), a winged-helix domain (WHD) and two helix domains (HD1 and HD2). Closed and inactive form of NOD2 is characterized by ADP-mediated packed conformation which is stabilized by interaction between WHD, and NBD and HD1. Hydrophobic pocket

suitable for accommodating glycan or peptide part of MDP, formed by amino acid residues highly conserved among different species of NOD2, was also determined.[19]

Lauro with coworkers[19] have defined, using surface plasmon resonance methodology, the critical residues responsible for NOD2-MDP interaction, namely, Arg857, Trp911 and Ser913. They concluded that the sugar and peptide portions of MDP contribute to the binding and that aromatic Trp911 residue is involved in carbohydrate-binding site.[19]

Upon MDP binding, HD2 mediates conformational changes of the NBD, WH, and HD1 to allow ADP-ATP exchange, oligomerization which induces a signalling complex named nodosome and downstream signalling. After interaction between the LRR domain and MDP (Figure 3), a complex conformational changes appear that result in protein oligomerization and then further the effector CARDs mediation of intracellular signalling *via* the nuclear factor-kappa B (NF-*κ*B) pathway to activate innate immunity through macrophage response as well as adaptive immunity through the production of antigen-specific T-cells.[20]

MDP binding to NOD2 was also proven by biophysical and biochemical assays.[18,21] Grimes and coworkers have shown that the binding between MDP and NOD2 is pH dependent and that it occurs in acidic pH (optimal pH range is from 5.0 to 6.5).[18] To support this, Lauro and others demonstrated in lysis experiment that NOD2 forms insoluble pellets between pH 5 to 6 because of binding to peptidoglycan fragments and by pH increasing over pH 8.5 it became soluble, suggesting that NOD2 is no longer bound to peptidoglycan fragments.[19]

Upon MDP recognition, NOD2 undergoes self-oligomerization and activates the serine-threonine kinase receptor-interacting protein 2 (RIP2). Active RIP2 leads to ubiquitination of NF-*κ*B. Subsequently, the NF-*κ*B and mitogen-activated protein kinase (MAPK) signaling pathways are initiated, resulting in the production of cytokines and certain antimicrobial peptides and the induction of additional processes, which characterize a proinflammatory/antimicrobial response.[22]

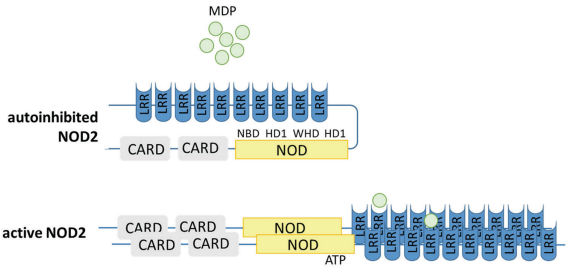

Figure 3. NOD2 changes upon MDP binding.

SYNTHETIC DESMURAMYL PEPTIDES

DMP derivatives described in the literature mostly comprise a lipophilic moiety linked to the dipeptide core structure in order to improve pharmacological properties. In general, the most active ones have cyclic substituents.[23] First desmuramyl peptides showing common activities like MDP were carbocyclic MDP analogs **I** and **II** (Figure 4) which also exhibited antitumor activity.[24,25] A carbocyclic MDP analogue **II** with *trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]-acetyl group and D-isoglutamine instead of D-glutamic acid, in contrast to MDP, was apyrogenic and nontoxic.[25]

N-Acetylmuramic acid (Mur*N*Ac) was in the continuation of DMP research replaced by phthalimido, LK423, and adamantyl substituents, LEK415 and LEK517 derivatives, shown in Figure 5. LK423 upregulated interleukin-10 (IL-10) gene expression in the spleen of cyclophosphamide treated mice.[26] Additionally, phtalimido desmuramyl dipeptide LK423 showed potential as therapeutic agent for inflammatory bowel disease. Treatment of mice with dextran sulfate sodium-induced colitis by subcutaneous low dose LK423 injections resulted in delaying the progression of inflammation in the colon.[27] It was also demonstrated that colon-specific delivery of LK-423 by microcapsules composed of pectin cores enhances the therapeutic effect of the drug in treating ulcerative colitis in rats.[28] Furthermore, LK423 stimulated the production of tumor necrosis factor (TNF) *in vitro* at phorbol 12-myristate 13-acetate (PMA) and ionomycin-stimulated cultures of human peripheral blood mononuclear cells (PBMCs).[29]

Phthalimido-desmuramyl dipeptide LK423 was additionally modified giving the phosphono analogues.[30,31] Derivative LK415[32] is adamantane analogue of LK423, while LK517 is phosphonate analogue of LK415, as shown at Figure 5. LK423, LK415 and LK517 are strong regulators of IL-12 and interferon IFN-γ synthesis. Furthermore, introduction of the phosphonate moiety in LK415 showed to be important for the increase of T-cell cytokine production.[23,31]

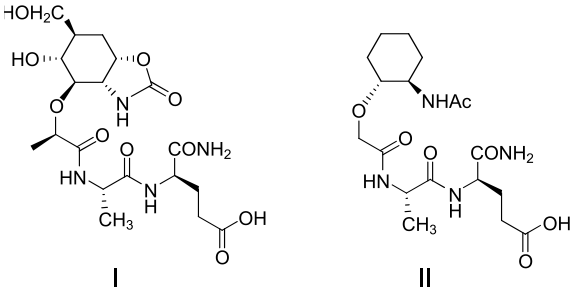

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Figure 4. Carbocyclic desmuramyl peptides.

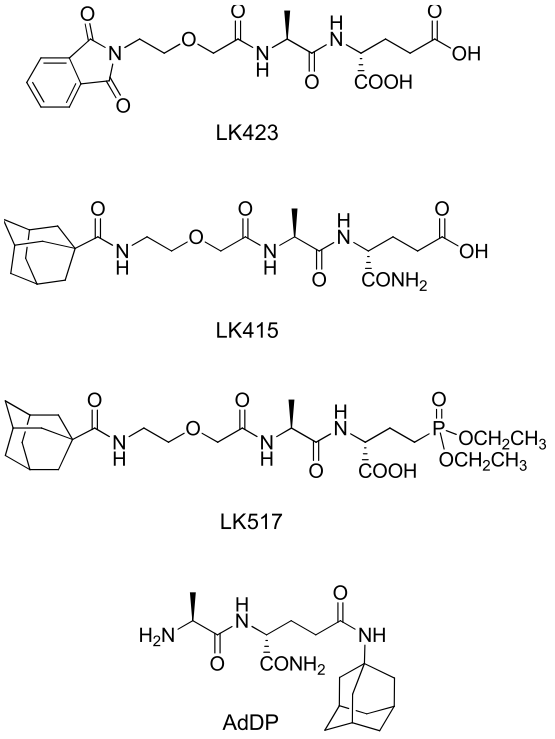

Figure 5. Phthalimido and adamantyl containing desmuramyl dipeptides.

Mašek with coworkers[33] have synthesized another adamantane substituted desmuramyldipeptide, adamantylamide dipeptide (AdDP) which contains 1-adamantamine, known antiviral agent, linked to carboxylic group of D-isoglutamine. AdDP was shown to be an effective and non-toxic immunoadjuvant.[33,34] In BALB/c mice using ovalbumin (OVA) as an antigen, dose–response relationship was observed on the anti-OVA antibody production, when AdDP was used by the intraperitoneal or oral administration route. The IgG isotype analysis points out that AdDP stimulates enhancement of IgG1 antibodies which is associated with a dominant Th2 response.[34]

Furthermore, attachment of adamantyl group to DMP core structures was also used for the preparation of immunostimulating adamantyl tripeptides in which the *N*-acetylmuramic portion of MDP was replaced with adamantyl glycine. Diastereoisomers of adamant-1-yl tripeptides **IIIa** and **IIIb**[35] and adamant-2-yl tripeptides **IVa** and **IVb**[36] (Figure 6) were synthesized and shown to exhibit adjuvant (immunostimulating) activities *in vivo*, in the mouse model. All tested adamantyl tripeptides did not show a tendency to modulate the OVA-specific response towards more pronounced Th1 or Th2 type of immune response, even though pronounced difference in IgG1 response was observed, similarly as with AdDP derivative.

Researchers from Faculty of Pharmacy, University of Ljubljana have investigated DMPs with saccharine, indole and pyrrolo/pyrido-fused[1,2]benzisothiazol moieties.[37–39] Different heteroaromatic ring systems were considerd as π-electronic substititutes for the glucosamine residue of MDP. Initial screening of their immunomodulatory properties were performed *in vitro*, evaluating their effect on lipopolysaccharide (LPS)-induced cytokine release in human monocytic cell line THP-1. Selected compounds and murabutide as reference compound were further investigated using LPS and PMA/ionomycin-stimulated human PBMCs. Indole scaffold-based compounds **V** and **VI** (Figure 7) proved to be effective down-modulators of proinflammatory cytokine synthesis and desmuramyldipeptide **VII** with a pyrido-fused [1,2]-benzisothiazole moiety and ethyl ester functionality, significantly enhanced the production of multiple proinflammatory cytokines (TNF-α, IL-6, IL-1b and IL-8).[37] Based on these results second generation of lipophilic DMPs with heteroaromatic scaffolds were synthesized and biologically evaluated.[38] In order to increase lipophilicity furthermore, carboxylic groups of dipeptide were replaced by ethyl ester. Their Nod2-agonistic activity were screened using Nod2-transfected HEK-Blue Nod2 cells, and subsequently their effect on the proinflammatory cytokine production of PMA/ionomycin-stimulated human PBMCs was evaluated relative to that of murabutide.[38] The most active were DMPs **VIII** and **IX** so they were candidates for further studies (Figure 7).

Indol moiety proved to be an appropriate surrogate for the Mur*N*Ac of MDP and introduction of ester functionality has allowed better internalization of compounds into cell. Therefore, minor modifications of hit compound acyl Gly-L-Ala-D-Glu derivative **VII** was performed and compound **X** (Figure 8) with a 6-phenylindole moiety, was identified as the strongest NOD2 agonist in this class of indole-scaffold based compounds, enabling a deeper understanding of the structural requirements of DMPs for NOD2 activation.[39]

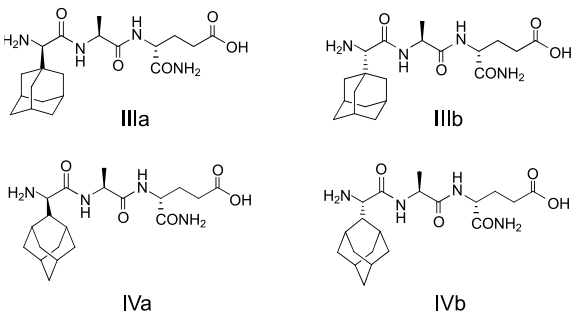

Figure 6. Adamantyl tripeptides.

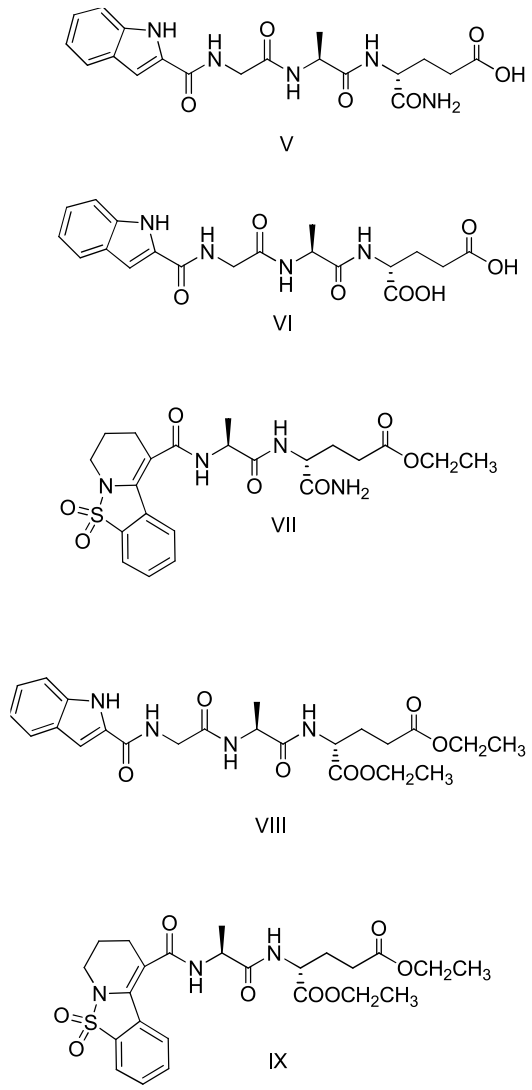

Figure 7. Indole scaffold and pyrido-fused [1,2]-benziso-thiazole based desmuramyl peptides.

Further improvement of aromatic DMPs based on cinnamoylglycine as an Mur*N*Ac surrogate, lead to the next generation of acyl tripeptides which NOD2-dependet NF-κB activation capacity was determined on human embryonic kidney 293 cells (HEK293) overexpressing the NOD2 gene.[40] Compound **XI** was identified as the most potent NOD2 agonist of the DMP class, so far. Results indicate that cinnamoylglycine is an efficient surrogate of Mur*N*Ac and that increase of lipophilicity, by introduced ethyl ester functionality and addition of lipophilic L-Ala substituent, stimulate the immune responce. It also augmented LPS induced pro-inflammatory cytokine release from PBMCs as well as the increase of OVA-specific IgG titers in a mouse model. These findings indicate that **XI** can induce both innate and adaptive immune responses.[40]

Liu and coworkers[41] have also prepared aromatic DMP, twenty DMP derivatives in which benzoic acid with different substituents in the positions 2, 4 and 5 of the phenyl ring was attached to the L-Ala-D-isoGln core peptide structure. They exhibited strong adjuvant activity and have enhanced the antigenicity of hepatitis B surface antigen (IPQSLDSWWTSL).[41] Beside described lipophilic DMP derivatives several amphiphilic derivatives were described in the literature (Figure 9). Our group has prepared mannosylated adamantyl tripeptides with immuno-stimulating activity.[42] Mannose was attached to the desmuramyl dipeptide because it may contribute to the recognition of the adjuvant compound by specific mannose receptors expressed at immune cells. It is known that mannosylated drug delivery systems enhance uptake and activation of dendritic cells and increase T cell prolife-ration.[43–46] Mannosylation of the adamantylated des-muramyl peptides amplified the adjuvant activity in experiments *in vivo*.[42,46] Different isomers of mannosyl-ated adamantyl tripeptides, regarding the chiral centers introduced at adamantyl glycine and spacer connecting the sugar part to the adamantyl tripeptide were synthesized and biologically evaluated.[42,46] Introduction of mannose has significantly stimulated the immune response. We assume that mannose effects the immune response by mannose receptors family, group I CLRs, present on immunocompetent cells (such as macrophages and dendritic cells).[47,48] The most active ManAdTP derivative (Figure 9) which have a D-configuration at the (adamant-1-yl)glycine moiety and (*R*)-configuration on hydroxy-isobutyryl linker was used as a hit compound for the design of new ones. Two series of compounds were prepared: (i) derivatives containing glycolyl linker between mannose and dipeptide, and (ii) derivatives containing (*R*)-hydroxy-isobutyryl linker. In both series, positions of adamantane

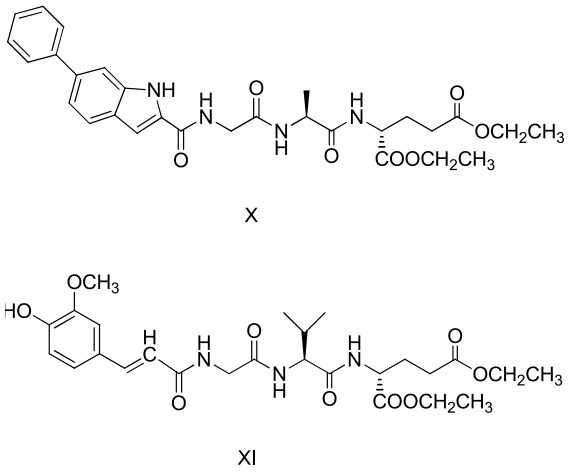

Figure 8. The most active lipophilic aryl desmuramyl peptides.

binding (to N- or C-terminus) were altered in comparison with derivatives lacking adamantane moiety.[49] Glycolic linker was introduced because it was demonstrated that *N*-glycolyl muramyl peptides induce significantly higher activation of NOD2 than MDP.[50,51] Immunostimulating properties of synthesized derivatives were assessed *in vivo* using ovalbumin as an antigen. All mannosylated peptides with glycolyl linker exhibited higher adjuvant activity than analogues with hydroxyisobutyryl linker. It was observed that the most suitable position of adamantane in this class of compounds is at peptide N-terminus. Compound **XII** was identified as the most potent adjuvant in this class of mannosylated desmuramyl peptides, so far.[49]

Jiang with coworkers[52] have prepared amphiphilic DMP derivatives in which muramic acid was replaced with a hydrophilic arene and lipophilic chain was introduced to the peptide. The hydrophilic aromatic amines were connected by glycolic acid linker to the N-terminus of L-alanine-D-isoglutamine dipeptide. The glycolic acid linker was added at the peptide end in order to facilitate their penetration through cell membrane. The **XIII** and **XIV** DMPs

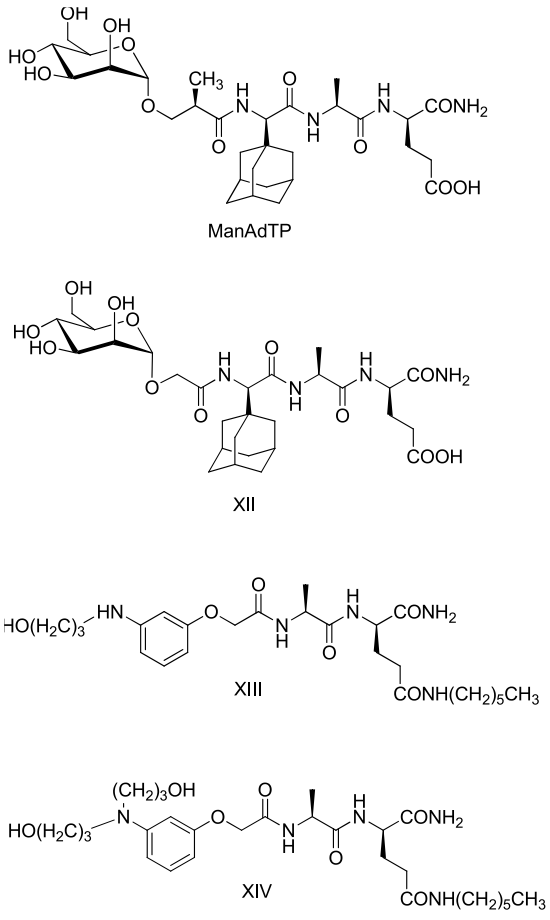

Figure 9. Perspective amphiphilic DMP derivatives.

(Figure 9) 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 murabutide.[52] It can be concluded that balanced lipophilicity and hydrophilicity is important for the exhibition of biological activity of DMP.

FUTURE PERSPECTIVE

Development of new adjuvants in future will be focused on safe adjuvants which will generate multifaceted immune responses and consequently enable tuning of disease-specific immune responses. Therefore, the PRR pathways represents an attractive source of novel adjuvants for vaccines. Triggering of certain PRR can induce diverse immune responses through complex signaling processes which depend on crosstalk with other PRRs. Application of multi-PRR activation approaches can increase immunity significantly.[53] For example, NOD2 agonists act synergistically and enhance the adjuvant activity of TLR ligands.[54–56] Furthermore, a combination of NLR/TLR ligands induce Th1-polarized response. Another example of dual adjuvant system represents CLR/TLR ligands.[57] Glycan structures build up tumor antigens and regulate immune reaction by specific binding to CLRs. Therefore, compounds with expressed CLR agonist or antagonist properties could also be considered as potential agents for cancer immunotherapy.[58,59] Desmuramyl peptide-based agents can be used for immunotherapy in the treatment of cancer, for example DMP paclitaxel conjugates.[60,61]

Preparation of appropriate formulations acting as delivery systems represents another approach for the design of efficient adjuvants. Strength and mechanisms of immunostimulation induced by nanocarrier vaccines depend on chemical composition, particle size and homogeneity, charge, nature and location of antigens and/or adjuvants within the delivery systems. Liposomes are often used carrier systems. Liposome characteristics can be adjusted in order to achieve desired properties. Water-soluble compounds can be entrapped within the aqueous inner space, whereas lipophilic ones can intercalate into the lipid bilayer. Modifications of liposomes can additionally broaden their application. Modifications can be performed by attachment of antigens and/or adjuvants on liposome surface either by adsorption or stable chemical linking.[62] In several studies reported earlier, adamantane-based DMP derivatives were incorporated into liposomes.[63–65] Studies have confirmed that liposomal formulations can improve biological activity and that adamantyl moiety incorporates into the lipid core of the bilayer due to its lipophilic properties.[66]

CONCLUSIONS

Design of novel efficient adjuvants is based on knowledge of their mechanisms of action and previously performed structure-activity relationship studies. Muramyl dipeptide-based immunostimulators are important class of compounds for the development of novel adjuvants. Modification of desmuramyl dipeptide core with lipophilic substituents based on aromatic and adamantane structures, and preparation of amphiphilic desmuramyl peptide derivatives represents a perspective approach for the development of novel adjuvants.

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