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SPECIAL FEATURE REVIEW

Translation of peptidoglycan metabolites into immunotherapeutics

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Abstract

The discovery of defined peptidoglycan metabolites that activate host immunity and their specific receptors has revealed fundamental insights into host–microbe recognition and afforded new opportunities for therapeutic development against infection and cancer. In this review, we summarise the discovery of two key peptidoglycan metabolites, γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) and muramyl dipeptide and their respective receptors, Nod1 and Nod2, and review progress towards translating these findings into therapeutic agents. Notably, synthetic derivatives of peptidoglycan metabolites have already yielded approved drugs for chemotherapy-induced leukopenia and paediatric osteosarcoma; however, the broad effects of peptidoglycan metabolites on host immunity suggest additional translational opportunities for new therapeutics towards other cancers, microbial infections and inflammatory diseases.

Keywords: adjuvant, cancer, infection, microbiota, pattern recognition receptor, peptidoglycan

INTRODUCTION

The immune system provides crucial defences against pathogens to protect the host from disease.[1] At a molecular level, immune signalling is mediated through direct receptor binding to a variety of microbial factors, known collectively as microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively). In turn, recognition of MAMPs by cell-surface or intracellular pattern recognition receptors (PRRs) stimulates transcriptional and cellular programmes to produce an immune response. The activation of these immune programmes mediates the rapid and direct clearance of microbes and primes adaptive responses to prevent subsequent infections.[1] Conversely, hyperactivation of PRRs can lead to chronic inflammation.[2] As such, MAMPs present attractive therapeutic leads to modulate host immunity during microbial infections, autoimmune diseases and even failures of normal immunosurveillance such as cancer.

One major source of MAMPs is peptidoglycan (PG), a rigid, mesh-like glycopeptide polymer found in nearly all species of bacteria as protection from osmotic shock.[3,4] PG is composed of a polysaccharide of repeating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues with a variable peptide stem attached to the 3-*O*-lactoyl group of MurNAc (Figure 1).[5] The peptide chains of the PG monomers are then crosslinked during cell wall biosynthesis through bridging amino acid residues to provide further structural rigidity. Gram-positive and Gram-negative bacteria differ both in the amount and in chemical composition of their PG. Gram-positive

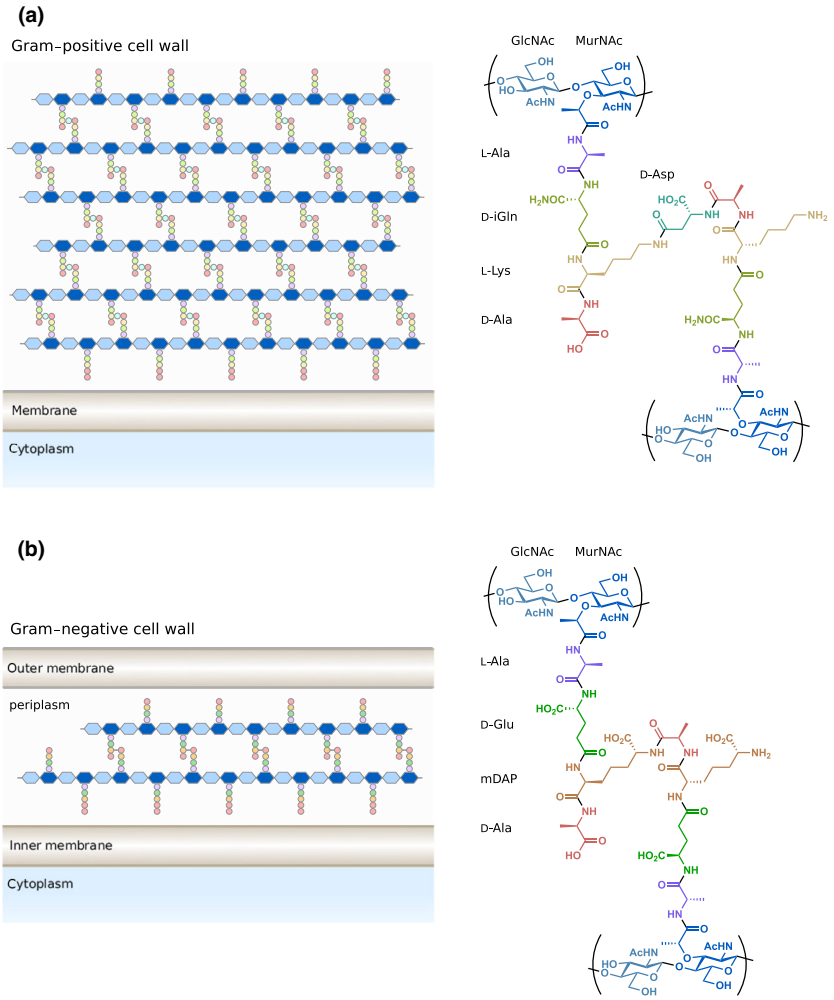

Figure 1. Structure of Gram-positive and Gram-negative peptidoglycan. **(a)** Gram-positive bacteria like *Enterococcus faecium* contain a thick layer of peptidoglycan outside of their single membrane. Gram-positive bacteria usually contain D-isoglutamine (D-iGln) and L-lysine at the second and third positions of the peptide stem. **(b)** Gram-negative bacteria such as *Escherichia coli* contain a thin layer of peptidoglycan within their periplasm. Gram-negative bacteria generally utilise D-glutamate (D-Glu) and *meso*-diaminopimelic acid (mDAP) at the second and third positions of the peptide stem. The location and composition of the crosslink between peptide stems vary between species. Ac, acetyl.

bacteria contain a thick PG layer up to 80 nm in diameter on their outer surface and generally possess a lysine residue at the third position of the peptide stem (Figure 1a). Conversely, Gram-negative cells carry a much thinner layer (5–10 nm) between their inner and outer phospholipid membranes and utilise *meso*-diaminopimelic acid (DAP) within their peptide stems (Figure 1b). Species-specific differences are also observed in the composition and length of the crosslinking peptide bridge as well as chemical modifications to the polysaccharide backbone.[5] Importantly, the degradation of PG by bacterial and host hydrolytic enzymes gives rise to numerous PG metabolites that can then be recognised by the host.[6,7]

HOST RECOGNITION OF PG METABOLITES

The immunomodulatory activity of bacterial PG was first described at the molecular level in 1974[8] through fractionation studies of complete Freund's adjuvant (CFA), an emulsion of heat-inactivated mycobacterial cells, a surfactant and mineral oil first published in 1937.[9] Here, isolated water-soluble, low molecular weight PG fragments were demonstrated as the minimal components of CFA necessary for its adjuvant effects.[8] However, the first evidence of host receptors for these metabolites came much later in the early 2000s with the discovery of the

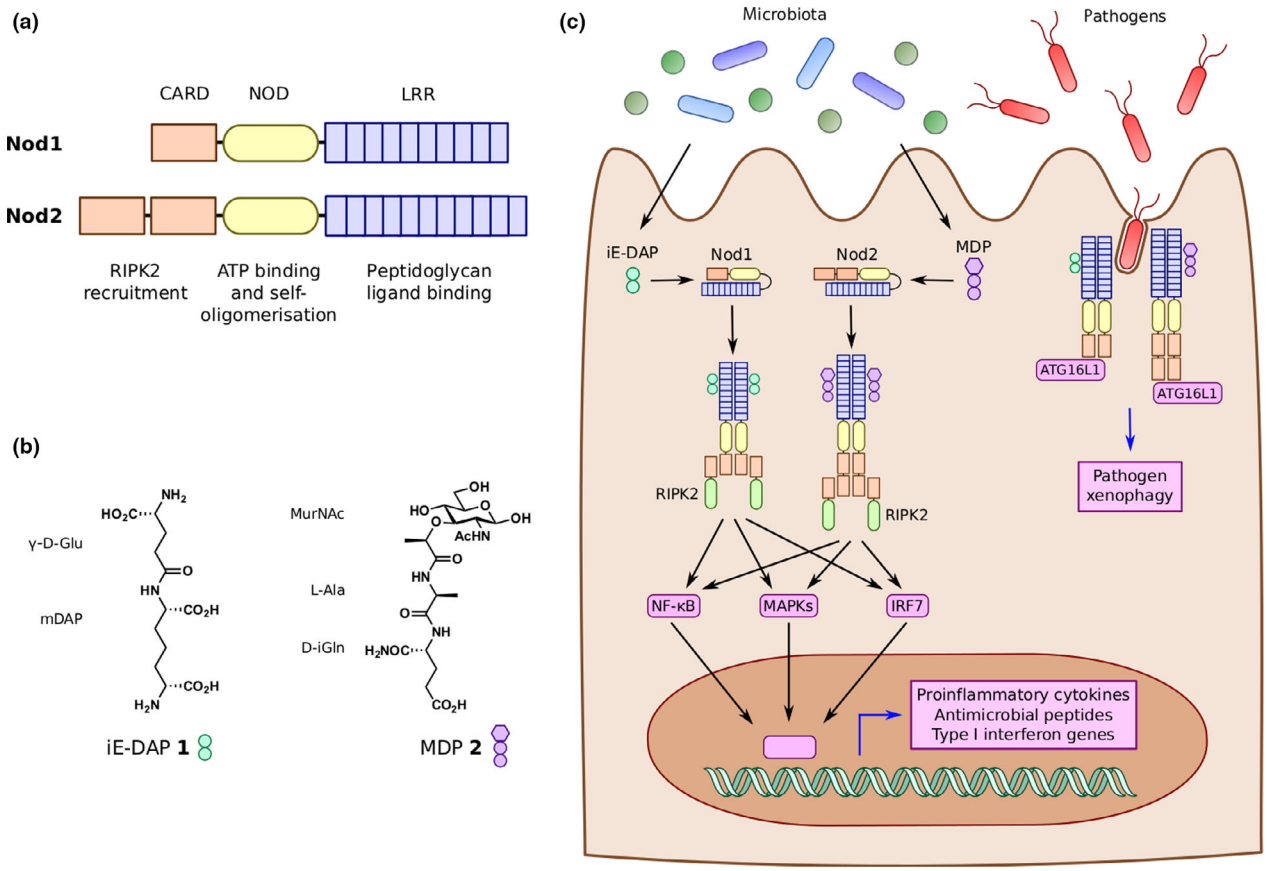

Figure 2. Peptidoglycan pattern recognition receptors. **(a)** Nucleotide-binding oligomerisation domains 1 and 2 (Nod1 and Nod2) are conserved pattern recognition receptors of peptidoglycan fragments. Both proteins contain 1 or 2 caspase activation and recruitment domains (CARD), a NOD, and 10 or 11 leucine-rich repeat (LRR) domains. **(b)** Nod1 recognises peptidoglycan fragments containing mDAP, the smallest of which is iE-DAP **1**. Conversely, Nod2 recognises muropeptides, with MDP **2** as the minimal active unit. **(c)** iE-DAP and MDP derived from local bacteria or pathogens bind to Nod1 and Nod2, respectively. Activated Nod receptors oligomerise and recruit RIPK2. Through downstream adapter proteins and signalling cascades, Nod receptors activate NF-κB, MAPK and IRF7 pathways to elicit expression of proinflammatory cytokines, antimicrobial peptides and type I interferon genes. Nod receptors also localise to sites of bacterial invasion to recruit ATG16L1 and induce autophagy pathways against the invading bacteria, known as xenophagy.

cytosolic nucleotide-binding oligomerisation domain (NOD) proteins Nod1 and Nod2 (Figure 2a).[10–13] These PRRs share a similar domain architecture consisting of 1 or 2 caspase activation and recruitment domains (CARD), a NOD, and 10 or 11 leucine-rich repeat (LRR) domains in Nod1 and Nod2, respectively.[14–16] Nod1 is expressed in a variety of different cell types and tissues and is activated by PG fragments containing DAP mostly from Gram-negative bacteria.[10,11] The fragment γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP, **1**, Figure 2b) is often cited as the minimal motif necessary for binding to Nod1[10,17]; however, murine Nod1 requires the presence of an additional D-Ala residue for activation.[18] The activity of DAP alone is controversial as conflicting reports regarding its activity have been documented.[17,19,20] Conversely, Nod2 expression is restricted to hematopoietic cells and the epithelium of barrier tissues such as the skin, lungs and gastrointestinal tract, including $Lgr5^{+}$ stem cells[21] and Paneth cells[22] of the intestinal crypts. Nod2 recognises MurNAc-containing PG fragments with *N*-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP, **2**, Figure 2b) as the minimal unit for Nod2 activation.[12,13] In addition to indirect, loss-of-function studies using genetic knockout, subsequent pulldown, surface plasmon resonance and photo-crosslinking experiments established that the Nod receptors directly interact with these PG metabolites.[23–26] More recently, members of

the membrane-bound ADP ribosylation factor (Arf) family of proteins were also shown to directly interact with MDP.[26] Arf6 and to a lesser extent Arf1 and Arf4 were found to associate with MDP in a Nod2-dependent manner, suggesting that these Arf-family GTPase may directly modulate MDP-Nod2 signalling. The importance of membrane association for Nod signalling is further supported by the discovery of Nod1 and Nod2 S-palmitoylation, which was critical for their downstream signalling.[27]

In addition to the Nods, other proteins have been implicated in PG metabolite sensing. Toll-like receptor 2 (TLR2) had previously been shown to respond to PG stimulation with a preference for DAP-containing fragments.[28,29] However, this finding has been controversial as other reports suggested that TLR2 activation was due instead to contaminating lipoproteins and lipoteichoic acids (LTAs) within the PG preparations.[30] Hexokinase (HK) has also been demonstrated to sense PG through binding to GlcNAc derived from PG after phagosomal degradation.[31] GlcNAc inhibited HK activity and led to its dissociation from the mitochondrial outer membrane, which in turn was sufficient to activate cytokine processing and secretion via the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome.

Upon binding to their respective PG metabolites, both Nod1 and Nod2 are believed to shift from an auto-inhibited form to an active conformation that then can self-oligomerise (Figure 2c).[14–16] The oligomeric Nod complexes then recruit receptor interacting serine/threonine kinase 2 (RIPK2) via CARD-CARD interactions.[32,33] In turn, the Nod/RIPK2 complex activates mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and interferon transcription factor (IRF) pathways, which promotes the transcription of proinflammatory cytokines, antimicrobial peptides (AMPs) and type I interferon genes.[14–16] Complex formation, stability and signal transduction are tightly controlled through numerous other proteins reviewed elsewhere.[16] These regulatory mechanisms include the newly discovered role of heat-shock protein B8 (HspB8), which directly binds to both Nod1 and Nod2, prevents their aggregation and may enhance the assembly of active Nod1/2 oligomers.[34] In epithelial cells, Nod1/2 signalling leads to the cellular and tissue restriction of pathogens through induction of pathogen-directed autophagy, or xenophagy,[35] and the production of barrier-promoting proteins and AMPs, respectively (Figures 2 and 3).[36,37] Moreover, activation of macrophages and other phagocytic myeloid cells elicits the direct killing of microbes and the clearance of host cells that present non-self signals from either internal pathogens or cancer-related mutations (Figures 2 and 3).[1]

In addition to rapid clearance of microbes, PG fragments have been implicated in priming longer term immune responses. For example, Nod2 activation trains monocytes via epigenetic reprogramming to better respond to subsequent microbial challenges.[38,39] Similarly, sensing of Nod1-activating PG fragments by circulating monocytes is necessary to enhance systemic immune priming prior to infection.[40,41] PG-mediated activation of Nods also promotes adaptive immune responses. Stimulation of either Nod1 or Nod2 leads to Th2 responses and the production of antigen-specific antibodies.[42,43] Moreover, co-stimulation with Nod1 or Nod2 agonists and other MAMPs that engage TLRs produces a combination of Th1, Th2 and Th17 responses.[42–44]

Through these small molecule–protein interactions, PG metabolites coordinate both the priming and activation of host immune responses to maintain health and combat infection. Therefore, iE-DAP, MDP and their derivatives may function as possible drug candidates to improve immune activation before and during disease. In this review, we summarise efforts towards improved synthetic analogs of Nod1 and Nod2 agonists. We focus on chemical trends that have led to more potent derivatives and how these molecules have been employed to modulate a variety of host immune processes against infection and cancer.

CHEMICAL DERIVATIVES OF PG METABOLITES

Due to their wide-ranging biological activities, the therapeutic application of PG metabolites has garnered significant attention since their initial discoveries. However, these molecules have many properties that complicate if not completely preclude them from direct clinical use. Both iE-DAP and MDP are hydrophilic molecules that can easily be excreted from the body. MDP is more than 50% cleared by the kidneys in 30 min following intravenous or subcutaneous injection

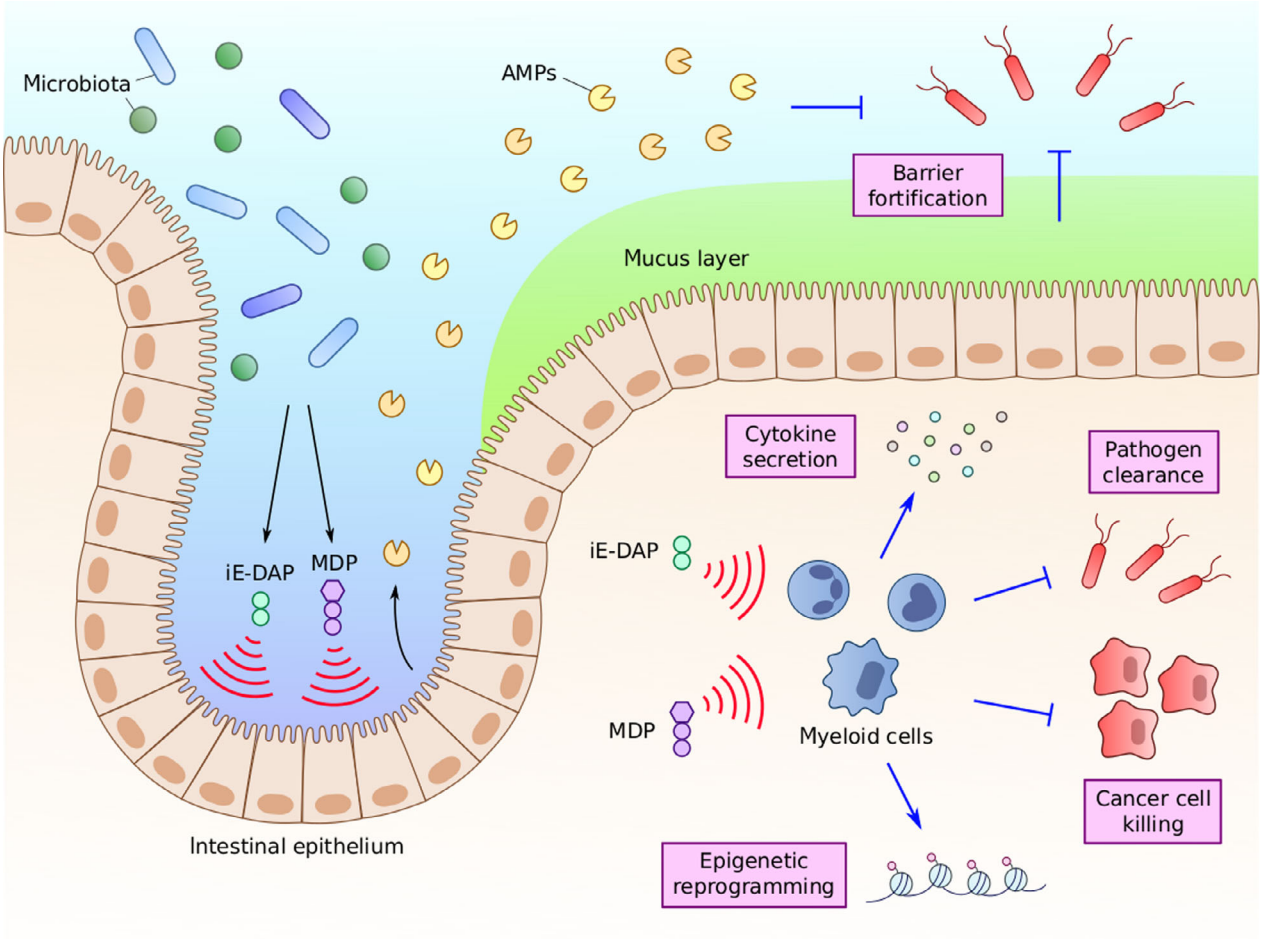

Figure 3. Peptidoglycan activation leads to diverse immune responses**.** Peptidoglycan fragments iE-DAP and MDP exhibit a wide array of activities through the activation of numerous cell types. Activation of intestinal epithelial cells leads to the increased production of mucins and antimicrobial peptides (AMPs) to improve the intestinal epithelial barrier and prevent infection. Activation of different myeloid cell populations can lead to increased cytokine secretion, epigenetic reprogramming, and the direct killing of pathogens and other foreign-presenting cells such as cancer cells.

in mice and more than 90% by 2 h.[45] Although both molecules contain chemical motifs that are uncommon or not present in animals such as the γ-linkage and D-isomer of Glu/Gln, PG derivatives may be subject to host-mediated hydrolysis in circulation. For instance, rat serum was found to degrade MDP into its monomeric components, although the timescale of this process was much slower than excretion.[46] Finally, the broad immune modulatory activity of these molecules may lead to undesired effects. MDP in particular has been demonstrated to be both pyrogenic (fever-inducing)[47,48] and somnogenic (sleep-inducing).[49,50] Therefore, chemical optimisation of the iE-DAP and MDP scaffolds has focused not only on improved potency *in vitro* but also enhanced bioavailability and reduced side effects *in vivo*.

Although iE-DAP has been described as the minimal active structure to stimulate Nod1, other naturally occurring, DAP-containing compounds based on larger PG fragments retain activity. One of the earliest discovered immune active PG metabolites containing DAP was FK-156 (**3**, Figure 4), the lactoyl-conjugated tetrapeptide D-Lac-L-Ala-γ-D-Glu-mDAP-L-Gly.[51] Isolated in 1982 from Gram-positive *Streptomyces olivaceogriseus* and *S. violaceus* strains,[52] FK-156 was found to induce proliferation of murine splenocytes, protect against lethal challenge with *Escherichia coli* and improve carbon clearance from the blood, an early assay for phagocytic activity

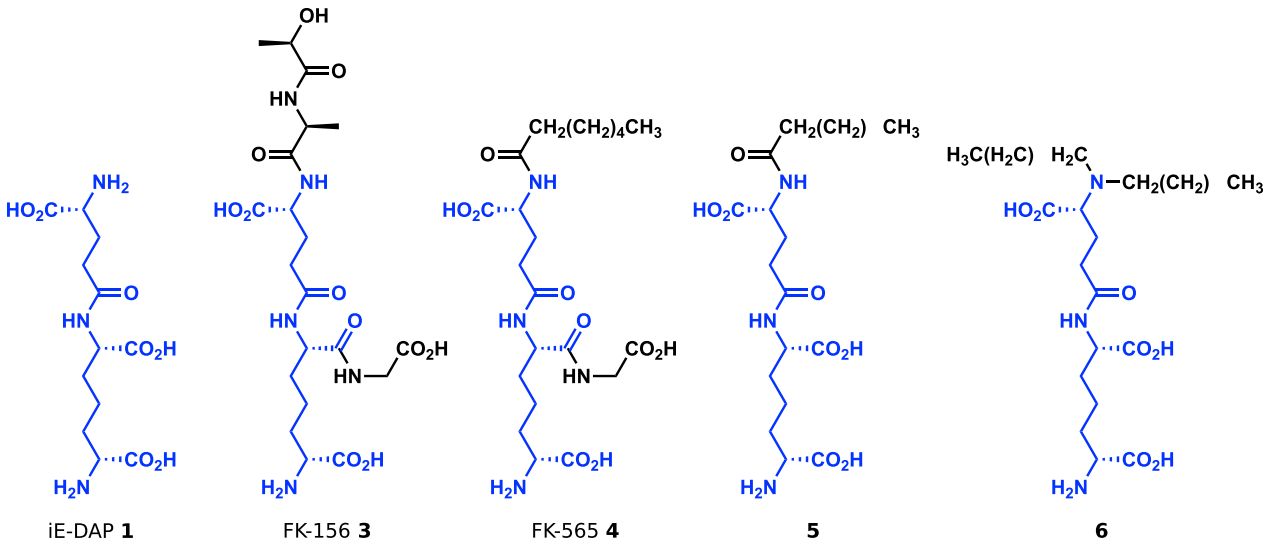

Figure 4. Representative iE-DAP-derived Nod1 agonists. The chemical structures of iE-DAP and representative Nod1 agonists are shown. In all structures, the iE-DAP scaffold is highlighted in blue. In general, a trend towards higher lipophilicity has resulted in increased biological activity.

in vivo.[53] Further synthetic studies based on the FK-156 scaffold yielded a number of other immune active analogs, the most widely used being the heptanoyl tripeptide FK-565 (Hep-γ-D-Glu-mDAP-L-Gly, **4**).[54] Testing of these compounds including FK-565 uncovered both that the D-Lac-L-Ala residues of FK-156 were not required for activity and that long-chain fatty acid acylation improved overall activity, mirroring contemporary synthetic studies with MDP (see below). Using a reductivist approach, it was also found that γ-D-Glu-mDAP or iE-DAP was the smallest derivative of FK-156 that could elicit activity,[55] revealing the minimal Nod1 ligand over 20 years before the discovery of its receptor.

The elucidation of Nod1 as the iE-DAP receptor in 2003[10,11] allowed for synthetic studies to focus more directly on receptor activation as a primary readout (Figure 2c). Using an *in vitro* NF-κB reporter assay, Wolfert *et al.*[56] tested a panel of six synthetic iE-DAP analogs, showing that amidation of γ-D-Glu (referred to as isoglutamine, iGln) abrogated Nod1 activation. As iGln is commonly found across different species, these findings revealed a Nod1-dependent mechanism for immune evasion through endogenous PG modification. Similar to *N*-acylated FK-565, Hasegawa *et al.*[57] showed that *N*-acyl derivatisation of the iE-DAP dipeptide was well tolerated by Nod1 using NF-κB reporter and monocyte chemokine secretion assays, with *N*-myristoyl (C14) iE-DAP (**5**) exhibiting more than 100-fold higher potency than unmodified iE-DAP. Subsequently, Agnihotri *et al.*[58] reported a thorough structure–activity relationship study examining modification of all terminal heteroatoms on iE-DAP against commercial *N*-lauroyl (C12) iE-DAP as a standard. As expected, loss of either the terminal carboxylic acid or amine of DAP abolished Nod1 activation. No large changes in EC_{50} values were observed after esterification of any carboxylic acid of iE-DAP; however, amidation of γ-D-Glu or the terminal amine of DAP with caused a significant drop in activity. Interestingly, dealkylation of the γ-D-Glu amine with lauryl groups provided an agonist over 10x more potent than C12-iE-DAP (**6**, EC_{50} = 0.0015 nM using the NF-κB reporter assay), but this increase in activity did not extend to other *N*,*N*-dialkyl compounds. Jakopin *et al.*[3] also examined constraining the orientation of the flexible sp carbon backbone of DAP through the introduction of an alkene.[59] Although no differences in activity were observed for the four derivatives at a single concentration via an NF-κB reporter assay, one compound based on the *N*,*N*-dialkyl modification of Agnihotri *et al.* with an alkene between the γ and δ carbons showed higher activity than its counterpart with a β-γ alkene in a cytokine secretion assay. However, this compound was not directly tested against the alkane derivative, so it is unclear whether

conformational rigidity of the DAP moiety improves Nod1 activation or is simply tolerated.

Because MDP was found to be biologically active over 35 years ago, it is unsurprising that extensive efforts have been made to produce variants that improve upon its potency. As with iE-DAP, these efforts have focused broadly on elucidating the necessary structural components, examining natural variants and improving bioavailability. Many early studies focused on the structural flexibility of three monomeric residues of MDP. For example, screening of L-amino acids to replace L-Ala found that the position was somewhat tolerant of other side groups, with L-Val, L-Ser and L-α-aminobutyric acid able to slightly improve biological activity.[60] The D-iGln residue was less amenable to substitution, with L-iGln or D-Asp causing loss of activity.[47,61] Interestingly, D-iGln could be substituted for D-Glu to maintain adjuvant and anti-infective activity, while causing a loss of pyrogenicity.[62] Methyl esterification of D-Glu showed similar activity as the diacid form of D-Glu, whereas *N*-methyl amides of D-iGln caused inactivation.[62] Most early modifications to MurNAc led to a decrease or loss of activity. For example, reduction to the sugar alcohol yielded an inactive molecule, whereas removal of the methyl group from the lactyl group of MurNAc showed a decrease in adjuvant activity as well.[61]

Analogs that mirror natural PG structures have also been thoroughly studied. Both GlcNAc-MDP structural isomers containing the disaccharide repeating unit of PG were produced either chemoenzymatically or synthetically and demonstrated similar or higher adjuvant activity as MDP.[63,64] More recently, Wang *et al.*[65] synthesised longer tetrasaccharide fragments and found 10-fold higher activation of Nod2 when treated with MurNAc(L-Ala-D-iGln)-GlcNAc-MurNAc(L-Ala-D-iGln)-GlcNAc compared to the isomer with GlcNAc at the nonreducing end of the tetrasaccharide. Extension of the peptide stem showed residue-specific effects. For example, inclusion of DAP to mimic most Gram-negative PG fragments showed no Nod2 activation; however, the addition of L-Lys or L-ornithine to MDP had little detrimental effect on potency using an NF-κB reporter assay.[17] Further elongation of the peptide stem to include one or two naturally occurring D-Ala residues led to a concomitant decrease in activity via cytokine secretion assay.[66] Substitution of the *N*-acetyl group of MurNAc with an *N*-glycolyl group, a modification found in some *Actinomycetes* species including mycobacteria, showed higher Nod2 activation, supporting previous observations of the highly immunogenic potential of mycobacterial PG.[67]

As with iE-DAP, the addition of acyl groups to MDP has been explored as a method to improve bioavailability presumably through both longer half-life *in vivo* and better cellular uptake. Acylation of the MDP scaffold has been profiled both at the 6-*O* position of MurNAc and at the C terminus of the peptide stem. For example, a series of 6-*O*-acyl derivatives with a range of lengths from acetyl (C2) to triacontanoyl (C30) was screened for protection of mice against lethal challenge with *E. coli*, where the 6-*O*-stearoyl (C18) modification **7** (Figure 5) showed the highest overall survival.[68] Further extension of the 6-*O*-acyl group with linear or branched fatty acids up to 48 carbon atoms in total produced the 6-*O*-(2-(tetradecyl)hexadecanoyl) variant B30-MDP **8**, which elicited heightened immunoadjuvant activity and increased serum antibody levels.[69] Structural optimisation of these acyl MDP compounds led to the discovery of MDP-Lys(L18) **9** also known as romurtide or muroctasin, in which ε-*N*-stearoyl-L-Lys was attached to the MDP scaffold.[70] Romurtide has been shown to stimulate macrophages to release cytokines including colony-stimulating factors that in turn can increase white blood cell and platelet counts,[71,72] and the molecule is currently in use in Japan to treat leukopenia after chemotherapy. Other acylpeptide MDP derivatives have also been reported including butyl ester-containing murabutide, MurNAc-L-Ala-D-Gln(*O*-*n*Bu) **10**.[73] Similar to the MDP analog containing the dimethyl ester of D-Glu, murabutide retains its adjuvant and anti-infective properties without any pyrogenic side effects even at high doses.[73,74]

Although removal of MurNAc from the dipeptide stem prevents Nod2 stimulation,[17] the MurNAc moiety can be replaced with noncarbohydrate structures while retaining Nod2 activity. Termed 'desmuramyl' peptides, these aglycon structures generally utilise hydrophobic groups to replace MurNAc. For example, some early studies described the immune activity of a number of dipeptide structures conjugated to alkyl groups such as octadecanoic acid,[75] 7-oxooctanoic acid,[76] glyceryl mycolate[77] and adamantane.[78] More recently, substituted

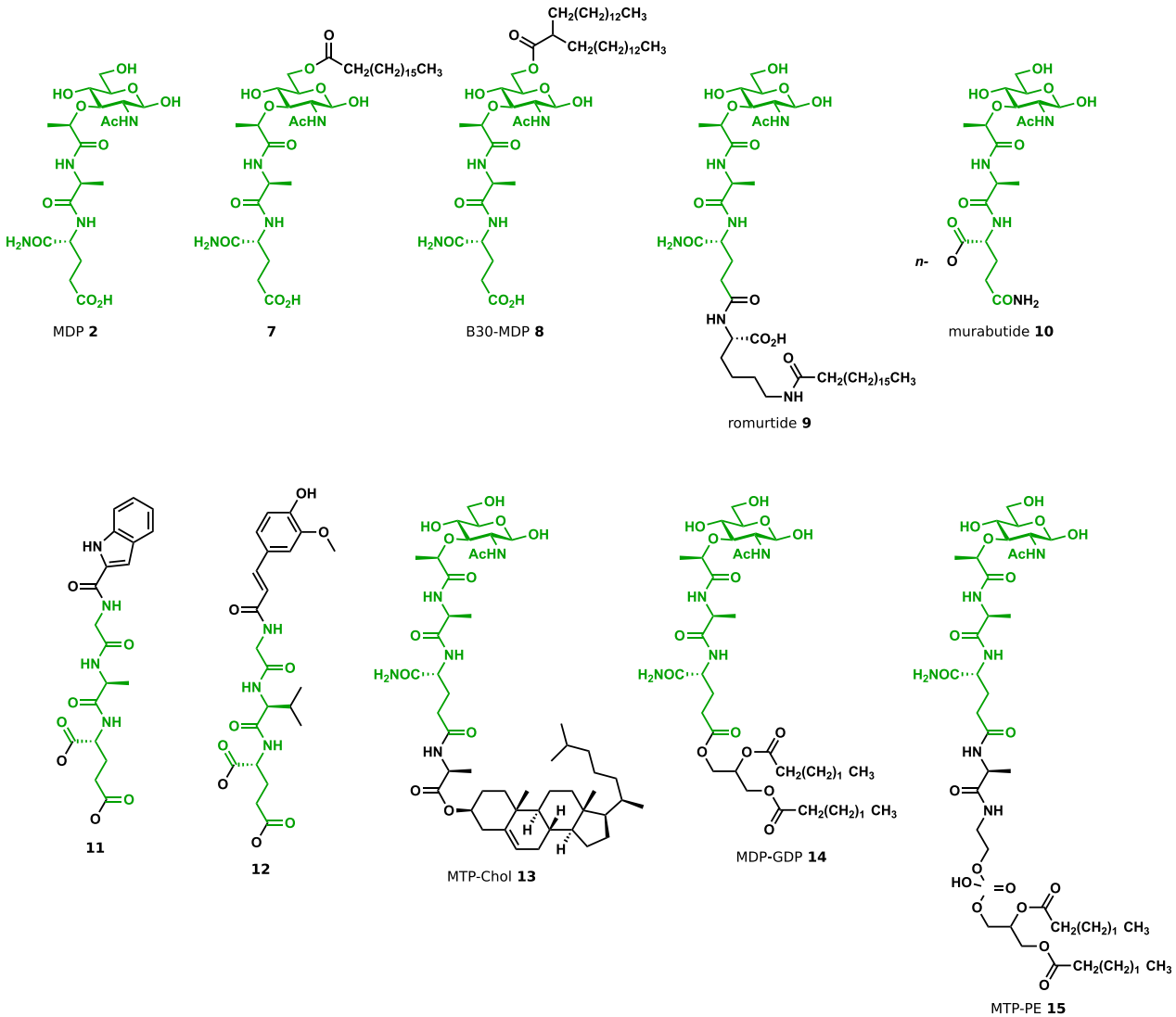

Figure 5. Representative MDP-derived Nod2 agonists. The chemical structures of MDP and representative Nod2 agonists are shown. In all structures, the MDP scaffold is highlighted in green. As seen with Nod1-targeted molecules, an increase in lipophilicity has resulted in more potent agonists. Modifications such as esterification also have decreased side effects such as pyrogenicity observed with the parental MDP molecule. Moreover, departure from the MurNAc monosaccharide has yielded compounds with similar efficacy. *n*-Bu, *n*-butyl; Et, ethyl.

aromatic rings have also been explored to produce potent Nod2 agonists. The MDP derivative **11** in which MurNAc was replaced with an indole-2-ylcarboxamido group showed similar activity as murabutide in both NF-κB reporter and cytokine secretion assays.[79] The related *trans*-feruloyl analog **12** exhibited low nanomolar activation (EC_{50} = 46 nM), providing functional MDP analogs with equivalent or even more potent activity without the hydrolysable reducing sugar.[80]

PEPTIDOGLYCAN METABOLITES AND INFECTION RESPONSE

Nod activation by PG fragments is an important determinant of host defences against many microbial infections. Indeed, Nod signalling via NF-κB has been observed upon *in vitro* infection by a variety of Gram-negative bacteria such as *Campylobacter jejuni*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* and Gram-positive bacteria including *Clostridium difficile* and *Staphylococcus*

aureus as well as mycobacteria, as reviewed elsewhere.[16,81] In nonhematopoietic cells such as epithelial cells, PG metabolite sensing is thought to aid in the establishment and maintenance of the barrier between the host and environment. In the gut, this occurs through cell-intrinsic xenophagy-mediated clearance, the physical exclusion of pathogens by the production of mucins and direct killing via AMP secretion (Figures 2 and 3).[36,37] For example, Nod1 has been correlated with the expression of the mucin protein Muc2 and the AMPs α- and β-defensins.[82,83] Early studies indicated that Nod2 also altered the expression of α-defensins produced by Paneth cells *in vivo*[84]; however, subsequent work did not corroborate this observation.[85]

Although the metabolites sensed by Nod1 and Nod2 may be derived from invading pathogens, associated commensal species may also contribute active PG fragments. Recently, the Gram-positive species *Enterococcus faecium* was found to increase host tolerance to infection by both *Salmonella enterica* serovar Typhimurium and *C. difficile*.[86,87] Resistance to infection after *E. faecium* colonisation was lost in *Nod2*$^{-/-}$ animals, and the protective activity was associated with the production of Muc2 and AMPs as well as normalisation of the intestinal epithelium. The protective phenotype was linked to the secreted PG endopeptidase, SagA, which was sufficient to promote protection when heterologously expressed in the nonprotective species *Enterococcus faecalis*. Structural and biochemical characterisation of its catalytic domain revealed that SagA cleaves PG fragments to release the disaccharide-dipeptide GlcNAc-MDP (GMDP), which was shown to activate Nod2 *in vitro*.[88] Probiotic *Lactobacillus plantarum* engineered to express wild-type SagA recapitulated its protective phenotype against *C. difficile*, whereas no protective effect was observed upon colonisation with bacteria expressing mutant SagA variants in which the catalytic residues or secretion signal was ablated. Therefore, the delivery of active PG metabolites to the gut may be achieved not only through small molecules but also by genetically engineered probiotics.

Beyond the epithelial line of defence, myeloid cells initiate diverse immune pathways upon sensing PG metabolites (Figure 3). Phagocytosis along with the secretion of reactive oxygen species and proteases provides a direct and rapid means for certain myeloid cells to kill and clear invading pathogens. The activation of cytotoxic pathways particularly in macrophages after stimulation with synthetic Nod agonists has been examined extensively in terms of cancer cell killing (see next section). As an additional acute response, PG sensing can elicit the secretion of multiple cytokines. For example, stimulation of human monocyte-derived macrophages with murabutide **10** led to an increase in expression and secretion of numerous cytokines including IL-1β, IL-6, TNFα, RANTES, IL-8 and MIP-1β.[89] Additionally, stimulation of macrophages with romurtide **9** led to increased secretion of colony-stimulating factors to promote hematopoiesis.[71,72]

PEPTIDOGLYCAN METABOLITES AND ADJUVANTICITY

In addition to native host sensing of PG metabolites during acute infections, noninfectious PG sources have been applied as adjuvants to prevent or combat disease for nearly 100 years. Through iterative passaging of the bacterium *Mycobacterium bovis*, Albert Calmette and Camille Guerin produced the weakened mycobacterial strain now known as Bacillus Calmette-Guérin (BCG).[90] First used in humans in 1921, BCG found success as a vaccine against tuberculosis,[91] and derivatives of the BCG vaccine are still in use today in countries with prevalent tuberculosis infection rates. In 1937, Jules Freund utilised a dried, inactivated form of the related strain *Mycobacterium tuberculosis* to produce CFA,[9] which was found to elicit a Th1 response and delayed-type hypersensitivity.[92] As described above, studies in the 1970s found that MDP **2** was the minimal bioactive component of CFA,[8] paving the way for synthetic PG metabolites to be used as adjuvants.

Muramyl dipeptide derivatives have proven useful in numerous preclinical models of vaccination. Owl monkeys were successfully vaccinated against *Plasmodium falciparum*, the parasite responsible for malaria in humans, by co-injection with 6-*O*-stearoyl-MDP **7** in liposomes.[93] Co-administration of romurtide **9** with a temperature-sensitive, live *Salmonella enteritidis* vaccine (Ts-O) in mice led to an increase in response as demonstrated by footpad delayed-type hypersensitivity.[94] Interestingly, administration of romurtide either 48 h prior to or immediately after vaccination both augmented its efficacy. Combination of the inactivated B-1 vaccine of

hantavirus with either romurtide or B30-MDP **8** improved delayed-type hypersensitivity upon challenge seven days postinjection.[95] B30-MDP also increased immunisation in mice using X-irradiated L5178Y-ML25 lymphoma cells and prevented subsequent metastases better than vaccination with the irradiated cells alone.[96] More recently, liposomal-encapsulated desmuramyl compound **12** showed an increase in serum levels of antigen-specific IgG upon OVA peptide vaccination; yet, its effects were less pronounced than MDP.[80] Unfortunately, these preclinical observations have not directly translated to the clinic. The muramyl tripeptide-phosphatidylethanolamine conjugate MTP-PE **15** had been explored in Phase I clinical trials as a vaccine adjuvant for both influenza and human immunodeficiency virus type 1 (HIV-1), but its addition to the vaccines demonstrated significantly increased adverse side effects with little to no improvement of immunogenicity.[97–99]

Along with vaccine adjuvant activity, MDP-based molecules demonstrate nonspecific, therapeutic adjuvanticity. This general anti-infective effect was first observed in children treated with the BCG vaccine during the early 20th century, where the mortality rate was notably reduced compared to unvaccinated children.[100] The molecular basis of this epidemiological observation was discovered in 2012, when BCG vaccination was found to induce epigenetic reprogramming of monocytes in a Nod2-dependent manner.[38] This activity enhanced the response of innate immune cells against subsequent challenges in a memory-like but nonspecific manner, a process now known as trained immunity.[39] This phenotype was demonstrated in peripheral blood mononuclear cells isolated from healthy adults treated with the BCG vaccine, where cells exhibited increased cytokine production upon stimulation with not only the vaccine-specific pathogen *M. tuberculosis* but also the unrelated bacterium *S. aureus* and even the yeast *Candida albicans*.[38] Nevertheless, synthetic studies to identify more potent MDP analogs had previously demonstrated that these molecules exhibited nonspecific, anti-infective activity when administered prior to pathogen challenge. For example, both 6-*O*-stearoyl-MDP **7** and romurtide **9** were developed using a protection assay in a murine model of *E. coli*-mediated sepsis.[68,70] The two molecules were also found to effectively limit infection of the opportunistic pathogen *Cornyebacterium kutscheri* in a cortisone-mediated model of immunosuppression.[101] Similarly, 6-*O*-stearoyl-MDP enhanced host resistance against both *E. coli* and *C. albicans* infection in immunocompromised mice treated with either X-ray irradiation or cyclophosphamide[102] and limited *P. aeruginosa*-associated pneumonia in immunocompromised guinea pigs.[103] Nonspecific protection was observed as well during viral infections. Romurtide and B30-MDP **8** were found to limit infection by herpes simplex virus type 1,[104] herpes simplex virus type 2[105] and vaccinia virus.[105] Moreover, murabutide **10** has been shown to limit HIV-1 viral loads in a humanised murine model.[106] However, it remains uncertain whether these effects are truly via long-term epigenetic reprogramming as many of the assays were conducted soon after adjuvant administration.

In addition to Nod2 agonists, Nod1 stimulation has yielded nonspecific adjuvants to combat infection. For instance, oral or subcutaneous dosing with Nod1 agonists FK-156 **3** and FK-565 **4** was shown to enhance host tolerance of subcutaneous *S. aureus* infection as well as systemic infection by a variety of Gram-positive and Gram-negative bacteria including *E. coli*, *P. aeruginosa* and *Listeria monocytogenes*.[107] Nod1 agonists from endogenous sources have also been correlated with systemic, nonspecific immune priming. Neutrophils harvested from either germ-free or antibiotic-treated mice showed deficient killing of *Streptococcus pneumoniae* and *S. aureus*.[40] Using knockout animal models, this deficiency was traced to Nod1 expression. Interestingly, neutrophils from animals prestimulated with heat-killed, Gram-negative *Haemophilus influenzae*, which produces mDAP-containing PG, showed enhanced cytotoxic activity against both pathogens compared to unstimulated animals. The results were recapitulated by stimulation with a MurNAc-tripeptide fragment containing mDAP. Although distinct from canonical trained immunity, these findings suggest that PG metabolites from persistent, nonpathogenic sources such as the gut microbiota can also mediate the systemic priming and increased activity of immune cells.

PEPTIDOGLYCAN METABOLITES AND CANCER TREATMENT

Peptidoglycan metabolites, and specifically muropeptides, have been shown for nearly 40 years to improve clearance and survival in

animal models of cancer. In fact, evidence for the antitumoral activity of PG fragments was indirectly demonstrated even prior to the discovery of MDP as the minimal active component of CFA. In these experiments, direct, intratumoral injection of live BCG was shown to decrease tumor size and prevent metastases in a guinea pig model of cancer. However, BCG immunotherapy in humans led to numerous detrimental side effects, including persistent infection, fever and liver disease.[108,109] To alleviate these issues, considerable efforts were made towards identifying nonviable replacements for BCG. Among the studies, it was identified that MDP acylated at the 6-*O* position with mycolic acid, a lipid found in *Mycobacterium* species, could suppress fibrosarcoma growth in mice when co-injected intradermally.[110] Notably, tumor growth was only limited when the acyl MDP was pre-incubated with oil droplets, whereas buffer suspension of the molecule showed no effect. MDP derivatives that were acylated or that had replaced L-Ala with certain bulkier amino acids also showed tumoricidal activity after intralesional injection into a tumor model in guinea pigs.[111] Similarly, this activity depended on the formation of mineral oil emulsions containing trehalose dimycolate, with either component alone showing no effect.

Although these studies suggested that MDP derivatives in combination with other mycobacterial-derived compounds could effectively treat tumors, it remained unclear whether MDP alone would prove an effective antitumor agent. At the same time, MDP was demonstrated to potently activate macrophages and lead to tumor cell killing *in vitro*,[112,113] and the effects were potentiated by encapsulation of MDP into multilamellar vesicles or liposomes.[114] Accordingly, repeated intravenous injection of liposome-encapsulated MDP in mice treated with a metastatic model of melanoma led to an overall decrease in pulmonary metastases and partial survival of the cohort.[115] Here, free MDP at 40x the concentration of liposomal MDP did not prove effective, suggesting that soluble MDP may be cleared too rapidly from the animals and that liposome encapsulation improved endocytic uptake of the small molecule. Moreover, this process did not depend on indirect, T cell-mediated activation of macrophages as tumoricidal activity was still observed *in vivo* using UV-irradiated, thymectomised and X-ray irradiated, or athymic nude mice.[116]

Based on these observations, new MDP derivatives were produced in which a muropeptide was conjugated to a variety of lipophilic molecules to improve liposome incorporation or produce micelles as a single agent. For example, liposomes containing MDP-L-alanyl-cholesterol (MTP-Chol, **13**) developed in 1985 were shown to be eightfold more effective than liposome-encapsulated MDP in inducing macrophage cytotoxic activity, putatively due in part to leakage of water-soluble MDP from the liposome.[117] Subsequent *in vivo* studies in which multiple doses of MTP-Chol-containing liposomes were administered intravenously showed that early MTP-Chol treatment could prevent liver metastases after tail vein injection with the M5076 histiocytosarcoma cell line.[118] Moreover, intragastric administrations of MTP-Chol liposomes along with a prophylactic dose two days prior tumor cell injection also moderately limited hepatic metastases. Numerous other lipophilic derivatives of MDP were analysed as well, with similar antitumoral activities reported *in vivo*.[119,120]

Of the many lipophilic MDP compounds, two formulations reached clinical trial stage. One molecule, which was developed in 1985, was produced by conjugating the muropeptide moiety to an analog of the dilipid tail of phospholipids to produce MDP-glyceryldipalmitate (MDP-GDP, **14**).[117] Similar to MTP-Chol, liposomes containing MDP-GDP were shown to be 10-fold more effective than MDP-containing liposomes in activating macrophage-mediated cell killing of tumor cells. Starting three days after tail vein injection of the B16-BL6 melanoma cell line, intravenous injections of MDP-GDP-containing liposomes led to a decrease in the number of pulmonary metastases. Interestingly, this treatment regimen led to the eradication of smaller metastases but appeared to have little effect on larger nodules. In a separate study using the B16-F1 model of liver metastasis, it was seen that either a single prophylactic injection or multiple therapeutic injections of liposomal MDP-GDP could effectively decrease the number of hepatic tumors.[121] Similarly, using the H-59 model of liver metastases, therapeutic activity of liposomal MDP-GDP against hepatic tumor formation inversely correlated with the number of administered tumor cells, and pretreatment of the animals showed a similar enhancement of drug efficacy.[122] Driven by preclinical results, MDP-GDP

was developed into the disaccharide-tripeptide drug candidate ImmTher, which showed low toxicity in a Phase I clinical trial.[123] Two Phase II clinical trials were established in 1997 and 1998, respectively, to examine the usefulness of ImmTher administration after chemotherapy and surgical resection in patients with high-risk Ewing's sarcoma,[124,125] a rare bone cancer found in children and young adults that most commonly metastasises to the lungs. The first trial (NCT00038142) was officially terminated in March 2016 due to low accrual.[124] The second trial (NCT00003667) completed recruitment in August 2000,[125] but no public results on the trial outcomes are available on clinicaltrials.gov as of November 2019.

Another structurally similar MDP derivative MTP-PE **15** was developed in 1982 via conjugation of MDP-L-alanine to the phospholipid dipalmitoylphosphatidylethanolamine.[126] MTP-PE administration via liposome encapsulation showed increased survival in both a UV-induced skin cancer model[127] and spontaneous and resection-based B16-BL6 metastasis models in mice,[128] with the best responses observed with more frequent and earlier therapeutic intervention. MTP-PE could also be formulated as a micellar single agent, and oral administration of MTP-PE micelles showed body-wide distribution via radiotracing experiments and decreased the number of pulmonary and lymph node metastases in the B16/BL6 amputation model.[129] However, oral MTP-PE treatment did not show efficacy in the treatment of larger, established metastases, suggesting that MDP-based activation of macrophages was only effective to inhibit rather than treat metastases. In further experiments, treatment of spontaneous canine osteosarcoma after limb amputation with liposomal MTP-PE led to an overall increase in disease-free and overall survival.[130] Liposomal MTP-PE, renamed mifamurtide and later Junovan (IDM Pharma) and now Mepact (Takeda), began Phase I and Phase II clinical trial enrolment in the mid-1980s, where it showed no major toxicity in adults with refractory metastatic cancer and led to increased circulating TNFα and IL-6 in patients with relapsed osteosarcoma, respectively.[131,132] These successes led to a large-scale Phase III clinical trial started by the Children's Cancer Group and Pediatric Oncology Group, later merged as the Children's Oncology Group, in 1993.[133–135] Enrolling patients under the age of 30 with newly diagnosed osteosarcoma, the INT 0133 study was conducted with a 2x2 factorial design to determine whether the addition of the alkylating agent ifosfamide and/or liposomal MTP-PE to standard chemotherapy would improve event-free and overall survival. Although results from the trial showed an overall increase in event-free and overall survival upon administration of liposomal MTP-PE from pooled groups with and without ifosfamide treatment,[134] the trial received criticism due to the factorial study design and the possible interaction between ifosfamide and liposomal MTP-PE.[136] These concerns ultimately led to the rejection of liposomal MTP-PE as a new osteosarcoma treatment by the US Food and Drug Administration. However, the drug is currently approved in the European Union for the treatment of nonmetastatic osteosarcoma after resection along with multidrug chemotherapy.

In addition to its well-studied effects on macrophages, MDP and its derivatives have also been implicated to limit tumor growth through the activation of other myeloid populations including dendritic cells (DCs). For example, various 6-*O*-acylated MDP derivatives were found to improve expression of mature cell-surface markers such as CD80, CD83 and CD86 and increased TNFα production in monocyte-derived DCs (moDCs).[137] However, these results were ablated using TLR2/4 blocking antibodies or genetic knockout, which suggested that this process occurred independent of Nod2. Later studies showed that treatment of moDCs with romurtide **9** in combination with IFNβ, a cytokine that leads to DC-mediated cross-presentation to $CD8^+$ T cells *in vitro*,[138] also led to increased phenotypic maturation as shown by flow cytometry and cytokine production.[139] *In vitro* treatment of purified, allogeneic T cells with moDCs activated with IFNβ and romurtide showed a significant increase in IFNγ production. Furthermore, adjacent, intradermal co-injection of IFNβ and varying concentrations of romurtide led to a significant decrease in primary tumor size and improved lymphocytic infiltration using a subcutaneous B16-F10 melanoma model in mice. Similar activation results upon MDP treatment were also observed in human DCs differentiated *in vitro* using mononuclear bone marrow cells purified from acute leukaemia patients.[140] More broadly, MDP was shown to elicit the migration of $Ly6C^{high}$ monocytes from the bone marrow to the lungs[141] and to convert inflammatory, $Ly6C^{high}$ monocytes into $Ly6C^{low}$ monocytes that exhibit patrolling properties,[142] highlighting the complex

and likely simultaneous activities of MDP in systemic circulation on myeloid cell populations.

Nod1 agonists FK-156 **3** and FK-565 **4** have demonstrated antitumoral activity in preclinical models as well. Direct intratumoral injection of either compound into established P388 leukaemia subcutaneous tumors led to a significant decrease in tumor growth.[143] Distal, nuchal administration also modestly slowed tumor development. In addition, FK-565 was shown to enhance cytotoxic activity of macrophages against B16 melanoma cells *in vitro* and *in vivo*.[144] Although a clinical trial for FK-565 was initiated based on these promising results, the trial was ended in 1995 without further progression. More recently, FK-565 was shown to act on $CD8\alpha^+$ DCs to promote cross-presentation.[145] Treatment of DC and OT-I T-cell co-cultures with the OVA antigen and FK-565 led to increased T-cell proliferation. Treatment of intradermal, OVA-expressing E.G7 lymphoma tumors with FK-565 alone showed no difference in tumor growth, but co-administration of FK-565 and the OVA peptide significantly impeded tumor growth and increased OVA-specific $CD8^+$ T cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the elucidation of their chemical structures over 30 years ago, hundreds of MDP and iE-DAP derivatives have been synthesised. However, only two of these molecules, romurtide **9** and mifamurtide **10**, have successfully entered the clinic, highlighting the disconnect between preclinical activity and clinical implementation. Nevertheless, synthetic studies have yielded a number of molecules that have greatly improved activity over the natural precursors. Molecules such as *N,N*-dilauryl-iE-DAP **6** and murabutide not only provide useful tools to interrogate the outcomes of Nod agonism *in vitro* but also allow for the direct testing of Nod activity in diverse *in vivo* contexts. Moreover, the development of desmuramyl compounds **11** and **12** underscores the essentially untapped chemical potential for Nod agonism found by moving away from the natural PG architecture.

A clear synthetic theme among MDP and iE-DAP analogs is the marked increase in lipophilicity. This is somewhat unsurprising as both MDP and iE-DAP are relatively hydrophilic molecules, and the addition of lipophilic tails as well as the formation of liposomes likely improve cellular uptake. Yet, these advances suggest that the enhanced activity from many current, 'best-in-class' molecules results largely from improved bioavailability rather than increased binding affinity towards the Nod proteins. The desmuramyl compounds **11** and **12** demonstrate that Nod2 agonism can be achieved without the complete glycopeptide scaffold, opening up the possibility for chemically distinct, small molecule Nod agonists. Therefore, approaches such as traditional, high-throughput screening methods may help to identify Nod agonists with higher 'on-target' binding affinity. The development of more 'synthetic' Nod agonists may also subvert other PG processing enzymes expressed in the host and microbiota that could interfere with naturally occurring peptide-based PG ligands. Bacteria have evolved numerous modifications to their PG structure including glycan *O*-acetylation and *N*-deacetylation to avoid immune surveillance.[146] Thus, defining the chemical diversity of bacterial PG may offer an alternative, native screen for structure–activity relationships to augment the development of Nod agonists. Unfortunately, no structure of either Nod protein in its active conformation has been solved, limiting *in silico* screening for Nod agonists. Although the recently solved apoprotein structure of rabbit Nod2[147] has allowed for preliminary docking studies of the possible MDP binding site, it remains unclear whether Nods undergo significant structural changes upon binding to reveal altered or possibly new sites to chemically target. Therefore, the structural elucidation of active Nod1 and Nod2 with bound PG ligands could greatly facilitate progress towards next-generation Nod agonists.

Finally, the broader success of Nod targeting in the clinic remains unclear. Complementary efforts to define the chemical determinants and biological activity of Nod agonists have yielded enticing preclinical results for the treatment of infection, cancer and even obesity-induced insulin resistance.[148] Through this iterative process, current Nod agonists have overcome a number of *in vivo* challenges faced by MDP and iE-DAP. The addition of lipophilic tails has provided molecules that remain in the body much longer than the quickly excreted native structures. Moreover, murabutide and other analogs have demonstrated similar efficacy as MDP without pyrogenic side effects. Nevertheless, very few adjuvants overall have received clinical approval, underscoring the

difficulty in selectively controlling immune responses from molecules with such multifaceted activities. For cancer treatment in particular, the use of Nod agonists must overcome lingering questions. Liposomal MTP-PE **15** has received approval for use against paediatric osteosarcoma after both resection and multidrug chemotherapy. This indication reflects preclinical data in which MDP derivatives were successful only when administered prior to or at the onset of metastases. Thus, the strategic implementation of Nod agonists in combination with other therapies may provide the most effective path forward to improve patient outcomes.

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CONFLICT OF INTEREST

MEG and HCH are inventors on a patent filed by The Rockefeller University for the use of SagA towards the treatment of cancer and infection. Rise Therapeutics has licensed the patent on SagA for probiotic development.

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