





 Cite this: *Chem. Commun.*, 2024, 60, 2212

 Received 28th November 2023,  
Accepted 26th January 2024

DOI: 10.1039/d3cc05807g

rsc.li/chemcomm

## A closer look at ligand specificity for cellular activation of NOD2 with synthetic muramyl dipeptide analogues†

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To further understand the specificity of muramyl dipeptide (MDP) sensing by NOD2, we evaluated the compatibility of synthetic MDP analogues for cellular uptake and NAGK phosphorylation, the pre-requisite steps of intracellular NOD2 activation. Our results revealed that these two prior steps do not confer ligand stereoselectivity; yet NAGK strictly discriminates against the disaccharide NOD2 agonists for phosphorylation *in vitro*, despite it being indispensable for the cellular NOD2-stimulating effects of these analogues, implying potential glycosidase cleavage as a novel intermediate step for cellular activation of NOD2.

Mammalian NOD2 protein is a key innate immune sensor that recognizes bacterial peptidoglycan fragments for host defense. Previous studies have established that muramyl dipeptide (MDP), the smallest conserved motif in most bacterial peptidoglycan, acts as the minimal NOD2 agonist.<sup>1,2</sup> Consisting of a monosaccharide *N*-acetyl muramic acid (MurNAc) with an *L*-Ala-*D*-Glx (Glu/Gln) dipeptide, MDP has been widely utilized as a canonical ligand for NOD2 activation. Upon stimulation, NOD2 undergoes self-oligomerization and recruits adapter proteins, which leads to the activation of transcription factor NF- $\kappa$ B for downstream inflammatory responses.<sup>3</sup> Importantly, mutations of NOD2 are associated with severe chronic inflammatory diseases such as Crohn's disease and asthma.<sup>4</sup>

Despite the biological importance of NOD2 in host immunity, detailed mechanisms underlying the ligand specificity for the cellular activation of NOD2 have remained elusive. Notably, the prevalent assay to study NOD2 stimulation utilizes reporter cells, in which mammalian cells (*i.e.* HEK293T) are transfected with the NOD2 gene as well as an NF- $\kappa$ B-driven reporter gene (Fig. 1). Such cell-based reporter assay has offered great

insights into the molecular requirements of NOD2 agonists, including the minimal activating motif, and the stringent stereospecificity to the stem dipeptide in MDP.<sup>5</sup> On the other hand, biochemical characterization of ligand-NOD2 interaction revealed that certain biologically inactive MDP analogues were still able to bind to NOD2 with comparable affinity as the natural ligand.<sup>6</sup> Such confounding observations between cell-based assay and *in vitro* binding studies warrant further investigations on the determinants of NOD2 ligand specificity in the cellular context.

Given that mammalian NOD2 is an intracellular protein, small-molecule agonists have to enter the host cell and undergo potential cellular modification/processing prior to sensing by NOD2. Recently, Hornung and co-workers discovered that MDP is phosphorylated by *N*-acetylglucosamine kinase (NAGK) to yield 6-*O*-phospho-MDP, which constitutes the *bona fide* NOD2 agonist in mammalian cells.<sup>7</sup> Remarkably, NAGK-dependent phosphorylation of peptidoglycan proves an indispensable intermediate step for the cellular activation of NOD2. With this in mind, we pondered whether the specific ligand requirements of NOD2 agonists could stem from the selectivity of cellular uptake and/or NAGK phosphorylation, the two pre-requisite steps for NOD2 activation in cell-based assays (Fig. 1).

Toward this goal, we first chemically synthesized a panel of MDP analogues. For monosaccharide muropeptides, an aryl thioglycoside starting material was synthesized in 5 steps without any chromatographic purification (Scheme 1 and ESI†). DMTMM emerged as our preferred coupling agent for lactoyl group amidation since blocking of *O*-4 and *O*-6 hydroxyls is not mandatory.<sup>8</sup> Of note, the low solubility of the coupled products enabled convenient purification by filtration. Thioglycoside removal using *N*-iodosuccinimide followed by saponification afforded the final products, albeit in modest yield. Thus, we obtained MDP(<sub>L,D</sub>), the natural isomer of peptidoglycan, as well as MDP(<sub>L,L</sub>) and MDP(<sub>D,D</sub>), the diastereoisomers of MDP. In addition, we synthesized MurNAc-A, an analogue that lacks the second amino acid, as well as MurNAc-GE and MurNAc-A(OCF<sub>3</sub>)E, which

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3cc05807g>



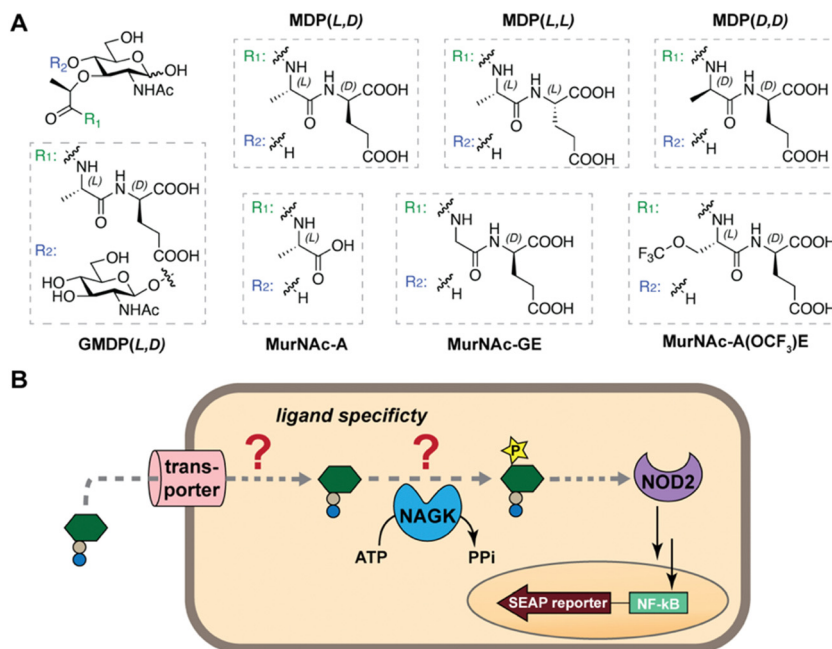
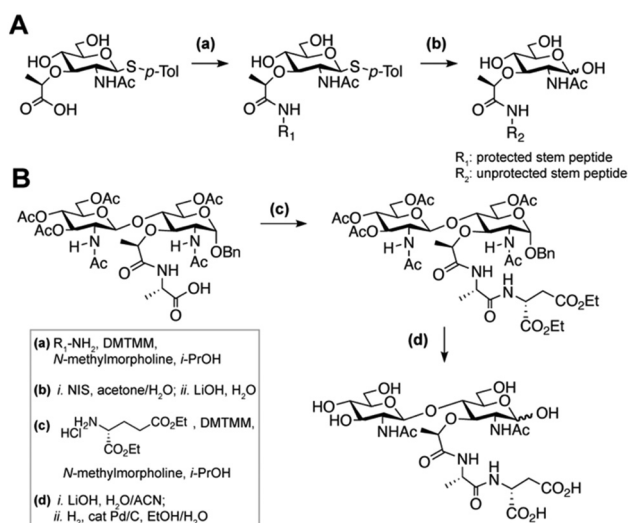


Fig. 1 The panel of synthetic muramyl dipeptide (MDP) analogues to explore the ligand specificity of cellular entry and NAGK phosphorylation in the intermediate steps of cellular activation of NOD2.



Scheme 1 Synthetic routes for MDP analogues.

manifest a smaller or bulkier substituent at the first amino acid position, respectively. We also prepared GMDP-(L,D), the disaccharide analogue of MDP. The required disaccharide precursor was obtained on a gram-scale using the reported method<sup>9</sup> and was advanced *via* DMTMM-mediated amidation and deprotection to afford GMDP-(L,D).

With synthetic MDP analogues in hand, we evaluated their NOD2-stimulating effects using HEK-Blue NOD2 reporter assay. As expected, unnatural stereoisomers MDP(L,L) and MDP(D,D) failed to activate human or mouse NOD2 even at high concentrations, in stark contrast to the strong NOD2-stimulating activity of the natural MDP(L,D) (Fig. 2 and Fig. S1, ESI<sup>†</sup>). While

our results align with the previously established stereoselectivity of NOD2, where altering the stereochemistry of either amino acid in MDP completely abrogates its cellular effects,<sup>2</sup> unfortunately, the underlying mechanisms of such selectivity are still beyond our understanding in the absence of MDP-bound NOD2 cocrystal structures up to date. In attempting to further decipher the molecular determinants, we pondered on if MDP analogue with an achiral dipeptide remains agonistic to NOD2. In particular, peptidoglycan in pathogenic bacteria *Mycobacterium leprae* and *Chlamydia trachomatis* manifests Gly instead of L-Ala as the first amino acid in stem peptide, yielding the MurNac-GE fragment as a natural muropptide.<sup>10,11</sup> Remarkably, we observed that MurNac-GE and canonical MDP(L,D) ligand manifest similar potency in the HEK-blue NOD2 reporter assay, suggesting that the lack of the first chiral center in the dipeptide of MDP analogue does not negatively impact NOD2 activation (Fig. 2). In addition, the presence of a bulkier substituent at L-Ala in the MurNac-A(OCF<sub>3</sub>)E analogue also does not interfere with NOD2 stimulation, whereas the removal of the D-Glx in MurNac-A renders it totally inactive (Fig. 2). Thus, our results revealed the

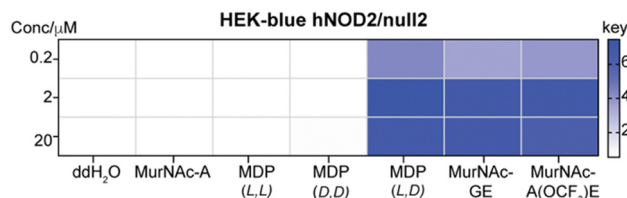


Fig. 2 Evaluation of MDP analogues in HEK-blue hNOD2 reporter assay. Heatmap of OD<sub>650nm</sub> readings that are normalized with Null2 parental cells as background. Results are of three independent experiments with biological duplicates.



broader tolerance of NOD2 towards the first amino acid in MDP analogues except when the opposite stereo-center is in place. This promiscuity may have evolved to render the ability of mammalian NOD2 to recognize and respond to particular peptidoglycan from *M. leprae* and *C. trachomatis*.<sup>12,13</sup>

To further address the ligand stereospecificity for NOD2 stimulation, we next examined the cellular uptake and NAGK-dependent phosphorylation of MDP<sub>(L,D)</sub>, the natural isomer, as well as the two unnatural stereoisomers, MDP<sub>(L,L)</sub> and MDP<sub>(D,D)</sub>. The human di/tripeptide transporter hPepT1 is a known transporter for MDP uptake in colonic epithelial cells.<sup>14</sup> Strikingly, it was previously demonstrated that natural MDP but not its inactive stereoisomers inhibited the uptake of glycylsarcosine *via* hPepT1 in a competition assay, providing indirect evidence for the stereoselective recognition of MDP analogues by hPepT1 transporter.<sup>14</sup> Since HEK293T cells express low endogenous levels of hPepT1,<sup>15</sup> we wondered if it could serve as a selective gatekeeper for MDP analogues in the cell-based NOD2 activation assay. Upon incubation of HEK293T cells with the respective MDP isomer, we harvested cell pellets to directly quantify the cellular levels of MDP using LC-HRMS (Fig. 3A). Interestingly, no intracellular MDP was detectable in cells harvested 6 h post-incubation, indicating a slow MDP uptake process in HEK293 cells; however, robust MDP signals were observed in cells collected after overnight incubation. Notably, the long duration of MDP incubation for cellular uptake

corroborates with the time period (> 16 h) required to observe a positive NF- $\kappa$ B response in HEK-blue NOD2 reporter cells. In two independent biological replicates, we found that the intracellular amounts of MDP<sub>(L,L)</sub> and MDP<sub>(D,D)</sub> were slightly lower than that of the natural isomer MDP<sub>(L,D)</sub> in HEK293T cells, although no statistical significance was obtained (Fig. 3A). However, such subtle differences in the cellular uptake of MDP stereoisomers seem unlikely to account for the complete inactivity of MDP<sub>(L,L)</sub> and MDP<sub>(L,D)</sub> for NOD2 stimulation in the reporter cells (Fig. 2). Given the low endogenous hPepT1 expression in HEK293T cells, we reasoned that MDP stereoisomers may also enter the cell *via* passive diffusion, or potentially are taken up by other transporters such as SLC46A3.<sup>16</sup> In either case, the biologically inactive MDP stereoisomers were still able to enter HEK293T cells yet exhibited no NOD2-stimulating activity. Consistently, we demonstrated that lipofectamine-facilitated delivery of the MDP stereoisomers into RAW264.7 macrophage cells still failed to trigger proinflammatory cytokines such as TNF $\alpha$  (Fig. 3B), confirming that the cellular entry of ligand is not a stereoselective determinant for NOD2 activation.

NAGK-dependent phosphorylation of MDP has recently been identified as an indispensable prerequisite for intracellular NOD2 activation.<sup>7</sup> Nevertheless, the substrate stereospecificity of NAGK phosphorylation has not been established to date. We set up the *in vitro* phosphorylation assay by incubating the respective MDP stereoisomer with recombinant NAGK and ATP for 2 h, prior to subjecting the mixture to LC-MS for detection of the desired phospho-MDP product. Interestingly, we found that NAGK readily phosphorylated both inactive MDP stereoisomers MDP<sub>(L,L)</sub> and MDP<sub>(D,D)</sub> (Fig. 3C), and concluded that NAGK does not confer stereospecificity to differentiate MDP stereoisomers. In efforts to gain molecular insights into how different diastereomers potentially interact with NOD2, we performed *in silico* docking of the NOD2-LRR domain with MDP<sub>(L,D)</sub>, <sub>(L,L)</sub> and <sub>(D,D)</sub>, respectively. Although all three ligands have similar binding energy with NOD2-LRR, MDP<sub>(L,L)</sub> and <sub>(D,D)</sub> manifest different docked poses compared to the natural isomer MDP<sub>(L,D)</sub> (Fig. S2, ESI<sup>†</sup>). Such distinct orientations may not be able to activate NOD2 for subsequent downstream signaling.

Recognizing NAGK is not stereoselective towards the stem peptide in peptidoglycan substrates, we next inquired if NAGK exhibits any regiospecific recognition for mucopeptide phosphorylation. Notably, the disaccharide analogue GMDP, a potent NOD2 agonist (Fig. 4A), bears an *N*-acetylglucosamine (GlcNAc) in addition to MurNAc, both of which sugars are potential substrates for 6-*O*-phosphorylation by NAGK. However, we were surprised to find that *in vitro* NAGK reaction with GMDP did not yield any phosphorylated product in LC-MS analysis, although NAGK readily phosphorylated GlcNAc and MDP under the same reaction conditions (Fig. 4B and Fig. S4, ESI<sup>†</sup>). To further confirm our observations, we resorted to the ADP-glo assay that quantifies the amount of ADP formed in the NAGK reaction, as a complementary approach to assess the substrate specificity of NAGK (Fig. 4C). First, in the reaction of NAGK with GlcNAc, we observed the rapid formation of ADP

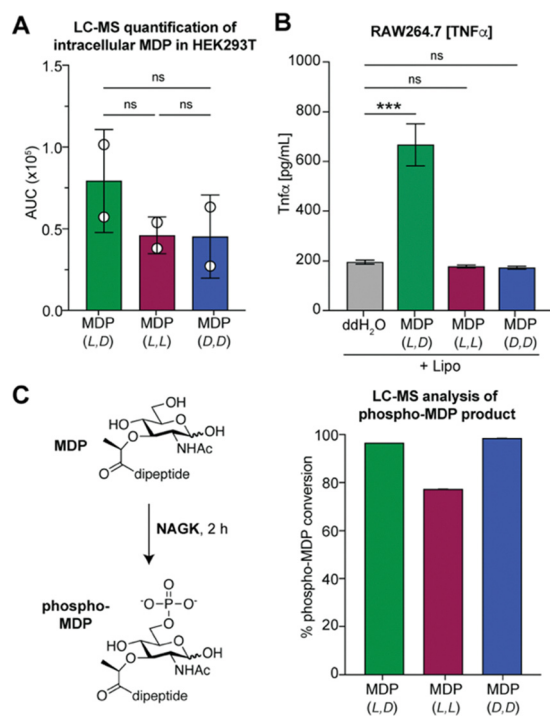
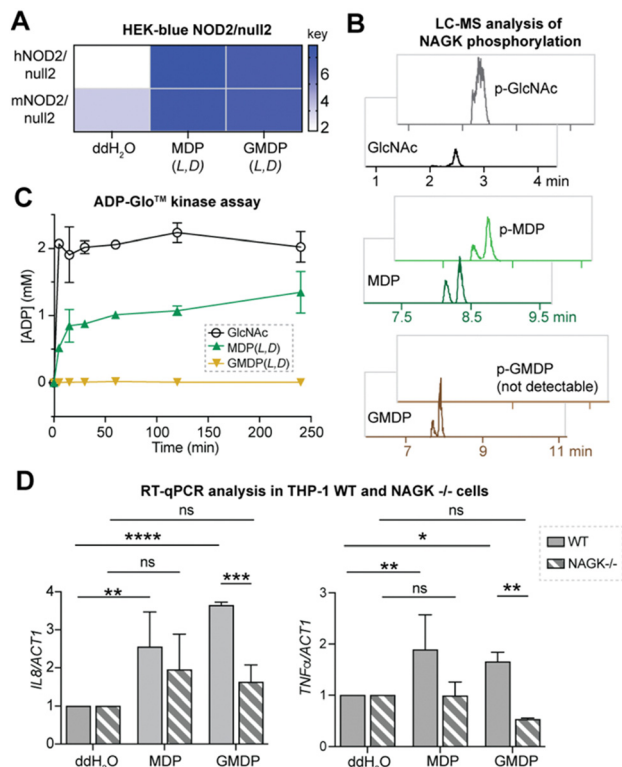


Fig. 3 The inactive MDP stereoisomers, MDP<sub>(L,L)</sub> and MDP<sub>(D,D)</sub>, exhibit similar cellular uptake (A) and (B) and NAGK phosphorylation as the natural MDP<sub>(L,D)</sub> ligand. Error bars represent replicated experiments with statistical analysis using ordinary one-way ANOVA. Results are presented as mean  $\pm$  SEM. \*\*\* $p$  < 0.005. (C) LC-MS analysis of NAGK phosphorylation of MDP analogues.





**Fig. 4** Disaccharide GMDP is not a substrate for NAGK phosphorylation *in vitro* yet its potent NOD2 agonistic activity still requires NAGK. (A) Heatmap of OD<sub>650nm</sub> readings that are normalized with Null2 parental cells as background. (B) LC-MS traces of the phosphorylated products for respective substrates upon NAGK reaction *in vitro*. (C) Time course analysis of NAGK phosphorylation with ADP-glo assay. Error bars represent triplicates. (D) RT-qPCR analysis of cytokine expression in THP-1 WT and NAGK<sup>-/-</sup> cells upon stimulation by MDP and GMDP. Error bars represent replicated experiments with statistical analysis performed using ordinary one-way ANOVA. Results are presented as mean  $\pm$  SE and calculated using the  $2^{-\Delta\Delta Ct}$  method. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.005, \*\*\*\* $p$  < 0.001.

within 5 min, indicating robust kinase activity of the recombinant NAGK *in vitro*. Similar effects was observed for MurNAc (Fig. S4A, ESI<sup>†</sup>) Next, for the NAGK reaction with MDP<sub>(L,D)</sub>, we captured a time-dependent gradual increase of ADP, which corresponds to the specific conversion of MDP<sub>(L,D)</sub> to phospho-MDP<sub>(L,D)</sub> by NAGK. On the other hand, no ADP was detected in the reaction of NAGK with GMDP over an extended incubation period, which supports the inability of NAGK to phosphorylate GMDP as observed in LC-MS analysis. In sum, the presence of the stem peptide in monosaccharide mucopeptide (*i.e.* MDP) impedes the reactivity of NAGK, although the stereochemistry of the first amino acid in MDP analogues does not affect the NAGK reaction. Nevertheless, the presence of a disaccharide backbone in mucopeptide (*i.e.* GMDP) completely abrogates NAGK phosphorylation. The exclusion of GMDP by NAGK as a potential substrate may be likely due to NAGK being unable to accommodate disaccharide molecules into its active site, as observed in our molecular docking analysis (Fig. S3, ESI<sup>†</sup>).

Remarkably, GMDP-stimulated cytokine expressions such as *tnfa*, *il6* and *il8* were significantly impaired in NAGK knockout

human monocyte THP-1 cells, supporting that NAGK is essential for NOD2 signaling (Fig. 4D). In order to reconcile the paradoxical findings that GMDP is not a NAGK substrate yet its cellular activity is dependent on NAGK, we hypothesized that GMDP may undergo glycosidase cleavage in the host cell to yield MDP for NAGK phosphorylation and subsequent NOD2 activation. Indeed, we detected MDP from GMDP-treated THP-1 cells (Fig. S5, ESI<sup>†</sup>). Thus, addressing the cleavage mechanisms of disaccharide mucopeptides in mammalian cells may shed insights into potential regulatory mechanisms for NOD2 activation. In summary, with synthetic MDP analogues, we established key molecular insights into the ligand specificity of cellular entry and NAGK phosphorylation for NOD2 activation, revealing other potential unappreciated intermediate cellular steps for NOD2 sensing of disaccharide MDP ligands.

## Conflicts of interest

There are no conflicts to declare.

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