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Diamide inhibitors of the *Bacillus subtilis* N-acetylglucosaminidase LytG that exhibit anti-bacterial activity

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Abstract

N-acetylglucosaminidases (GlcNAcases) play an important role in the remodeling and recycling of bacterial peptidoglycan by degrading the polysaccharide backbone. Genetic deletions of autolysins can impair cell division and growth, suggesting an opportunity for using small molecule autolysin inhibitors as both tools for studying the chemical biology of autolysins and also serving as anti-bacterial agents. We report here the synthesis and evaluation of a panel of diamides that inhibit the growth of *Bacillus subtilis*. Two compounds, **fgkc** (**5**) and **fgka** (**21**), were found to be potent inhibitors (MIC 3.8 ± 1.0 and 21.3 ± 0.1 μ M respectively). These compounds inhibit the *B. subtilis* family 73 glycosyl hydrolase LytG, an *exo* GlcNAcase. Phenotypic analysis of **fgkc** (**21**)-treated cells demonstrate a propensity for cells to form linked chains, suggesting impaired cell growth and division.

Graphical Abstract

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Supporting Information

Experimental details of diamide synthesis and characterization; crystal structure information, additional details of anti-bacterial activity assays. This information is available free of charge via the internet at <http://pubs.acs.org>.

Keywords

peptidoglycan; autolysin; *N*-acetylglucosaminidase; inhibitor; diamide

Peptidoglycan (PG) is the mesh-like polymer that surrounds the cells of most types of bacteria, and is the major structural component of the cell that confers strength, support and shape.¹ This heteropolymer is composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by a $\beta 1 \rightarrow 4$ glycosidic linkage (Figure 1). Adjacent polysaccharide strands are cross-linked via stem peptides attached to the C-3 lactyl moiety of MurNAc.¹ Many clinically relevant antibiotics target various steps in the biosynthesis and assembly of PG.^{2,3} Controlled degradation of PG is also required for cell maintenance and division, and this tightly regulated process is suggested to involve the interplay between synthetic and degradative enzymes.⁴⁻⁷ The degradative enzymes are collectively referred to as autolysins, and fall into 4 broad classes based on their activity: lytic transglycosylases, *N*-acetylglucosaminidases (GlcNAcases) and muramidases, *L*-alanine amidases, and endopeptidases.

The genome of the Gram-positive Firmicute *Bacillus subtilis* encodes at least 35 differentially regulated autolysins, several of which have been characterized genetically and/or biochemically.^{8,9} Peptidoglycan metabolism in *B. subtilis* has been well characterized, and is a model organism for Gram-positive bacteria due to its genetic tractability. Despite the ease of genetic manipulation, the functional redundancy of many autolysins means that genetic deletions alone have not always been sufficient to identify the role of a specific autolysin of interest.¹⁰ Among this suite of autolysins in *B. subtilis* there are two GlcNAcases that are known to act on PG during vegetative growth: LytG, an *exo*-acting enzyme, and LytD, its *endo*-acting counterpart (Figure 1).^{10,11} Both LytG and LytD are members of glycosyl hydrolase family 73 (GH73). Recently, a comprehensive bioinformatics analysis of the GH73 family by Lipski and coworkers¹² identified five clusters within the GH73 family, each of which are distinguished by proposed differences in their mechanism of action. LytG is a member of cluster 2, while LytD belongs to cluster 4. Enzymes in the latter cluster employ anchimeric assistance from the *N*-acetyl group to assist

cleavage of the glycosidic bond, while cluster 2 enzymes utilize a mix of anchimeric assistance and inverting mechanisms (Figure S1).

We have previously reported that glycosyl triazoles demonstrate bacteriostatic activity in *B. subtilis*, identifying the compound **BL.fgba** (Figure 1 inset) as an inhibitor with 63 μ M potency.¹³ Preliminary studies suggested that the bacterial target of **BL.fgba** was an *exo* acting GlcNAcase, and cells treated with **BL.fgba** exhibited the elongated and linked phenotype characteristic of disrupted autolysin activity.^{4,8,14,15,16} Our preliminary studies indicated that the aglycone of **BL.fgba** possessed only a 2-fold weaker minimum inhibitory concentration (MIC) (125 μ M) (data not shown). Based on these findings we prepared a second generation of compounds that were designed and synthesized using the aglycone of **BL.fgba** as the lead compound. The identification of two diamides from this library with micromolar activity against *B. subtilis* demonstrates the applicability of targeting the overlooked autolysins as a potential antibacterial target.

Results and Discussion

In an effort to deconvolute the roles of the GlcNAc residue and the aglycone unit of **BL.fgba**, we examined the antibacterial activity of the galactose analog of **BL.fgba**¹³ as well as the aglycone alone (**fgba**, **1**, see Figure 2). The galacto-derivative did not exhibit any antimicrobial activity, while the aglycone (**fgba**, **1**) exhibited a MIC (125 μ M) that was comparable to **BL.fgba**. Based on this observation, we hypothesized that a panel of diamides based on **fgba** (**1**) as a lead compound might afford lower molecular weight inhibitors of equal or greater potency. A panel of 21 compounds (Figure 2) was synthesized using the Ugi reaction, with the diamides obtained in yields ranging from 23% – 92%. Analogs of **BL.fgba** in which the iodine was replaced with bromine, chlorine, or hydrogen did not show any antibacterial activity (data not shown), so we elected to retain the *o*-iodobenzoic acid substituent in all of the diamides.

The diamides were examined for their ability to inhibit the growth of *B. subtilis*. Solution cultures of *B. subtilis* were incubated in the presence of diamides (at 250 μ M) for four hours, followed by the addition of resazurin, a metabolically responsive dye that changes color from blue to red in the presence of viable bacteria.¹⁷ Three compounds (**fgoa** (**8**), **fgka** (**5**), and **fgkc** (**21**)) exhibited potent inhibition of *B. subtilis* growth in this initial screen (Figure S2). These three compounds were selected for further study and their MICs were determined using the serial dilution method. While **fgoa** (**8**) exhibited a MIC greater than 200 μ M, the diamides **fgka** (**5**) and **fgkc** (**21**) were more potent, with MIC values of 21.3 ± 0.1 and 3.8 ± 1.0 μ M respectively. The discrepancy between the initial screen and follow-up assays with **fgoa** (**8**) is likely due to non-specific inhibition of *B. subtilis* growth at the high concentrations used in the preliminary screen.

While we were encouraged by the 20-fold increase in potency from **BL.fgba** (MIC 63 μ M) we noted the extremely hydrophobic nature of the two most potent diamides, **fgka** (**5**) and **fgkc** (**21**). As a result of the structure of the original lead **BL.fgba**, all of the diamides are highly hydrophobic, with ClogP values ranging from 4.7 to 8.8, values that are higher than heptane (ClogP 4.4).¹⁸ We solved the crystal structure for **fgka** (**5**), which corroborated the

highly hydrophobic nature of the molecule (Figure 3). The molecule adopts a conformation that buries most of the polar functionality in the interior. The molecule contains two carbonyl groups that can function as hydrogen bond acceptors and one amide that can serve as a hydrogen bond donor. The secondary amide forms an intramolecular hydrogen bond with the carbonyl group of the tertiary amide, resulting in a 'folded' conformation for the molecule in which most of the heteroatoms are encapsulated by the four hydrophobic side chains. The carbonyl group of the tertiary amide is the only polar functionality that is exposed. The iodobenzene ring is distorted out of coplanarity with the amide carbonyl, and is positioned in an almost perpendicular orientation. While the conformation observed in the solid-state may not reflect the conformation in solution or the enzyme-bound conformer, it does indicate the possibility that **fgka** (**5**) and similar molecules can present large amounts of hydrophobic surface area for intermolecular interactions. Several GH73 enzymes have been crystallized, and modeling of the PG substrate in the active site^{16,19} suggests that there are several hydrophobic patches in the PG binding site. Bioinformatic analysis of cluster 2 GH73 enzymes shows a highly conserved hydrophobic region in the vicinity of the general acid catalyst (Figure S3) in the peptidoglycan binding site.

Our previous findings indicated that **BL.fgba** inhibited *p*NP-GlcNAc hydrolysis in *B. subtilis* cells, and cells grown in the presence of **BL.fgba** exhibited the elongated linked phenotype characteristic of impaired autolysin activity.¹³ We suggested at the time that the likely target of the glycosyl triazole was an *exo*- acting GlcNAcase, most likely LytG, the major active autolytic GlcNAcase from *B. subtilis*.¹¹ Using a PG hydrolysis assay, we have found that the diamides **fgka** (**5**) and **fgkc** (**21**) inhibit LytG activity. LytG was expressed in *E. coli* BL21 (DE3) and purified following the procedure described by Horsburgh and coworkers.¹¹ PG hydrolysis by LytG was monitored using turbidometry.^{11,20,21} In brief, insoluble PG was prepared from *Micrococcus luteus* using the procedure described by Atrih *et al*²² and incubated with purified LytG in the presence or absence of **fgka** (**5**) or **fgkc** (**21**). Experiments with both *B. subtilis* and *M. luteus* PG showed no difference in LytG activity. PG hydrolysis was followed by monitoring the decrease in solution turbidity over 15 minutes. Both **fgka** (**5**) and **fgkc** (**21**) reduced the rate of turbidity clearing, and **fgkc** (**21**) exhibited a 30% reduction in enzymatic activity at diamide concentrations as high as 83 μ M (Figure S4). Precise IC₅₀ values could not be determined due to the apparent aggregation and poor solubility of the inhibitors at high concentration in aqueous solution. These observations raised the possibility that **fgka** (**5**) and **fgkc** (**21**) might be functioning as 'promiscuous inhibitors'²³, which are compounds that exhibit potency in an assay through non-specific binding of aggregated inhibitors to the target protein of interest. A standard test for determining promiscuous inhibition involves conducting the assay in the presence of low concentrations of detergent, which breaks up the loose molecular aggregates. Observation of lower potency in the presence of detergent is consistent with the compound of interest functioning as a promiscuous inhibitor. However, we observed the opposite behavior – increased potency in the presence of detergent. When the PG hydrolysis assays were repeated in the presence of 0.01% Triton X-100, reliable IC₅₀ values were able to be obtained (**fgkc** (**21**): 48 ± 11 μ M; **fgka** (**8**): 88 ± 10 μ M) (Figure 4A and S5). We believe that the diamides **fgka** and **fgkc** do form aggregates in solution at high concentrations, but that the active species is actually the monomeric form of the compound.

Given the discrepancy in IC₅₀ values in the absence and presence of Triton X-100 for **fgka** (5) and **fgkc** (21) in vitro, we decided to investigate this phenomenon in the whole cell assays. The MIC assays were repeated with increasing concentrations of Triton X-100. While the inclusion of 0.01% Triton X-100 deleteriously affected bacterial growth rates, no such change was seen in the presence of 0.001% detergent (Figure S6) **fgkc** (21) and **fgka** (5) exhibited no change in MIC in the presence of 0.001% Triton X-100 (Figure 4B). While the MIC assays in the presence of 0.001% Triton X-100 do not conclusively rule out inhibition via aggregation since this is below the cmc of Triton X-100 (0.014% (v/v)), we further assessed the diamides for non-specific binding. Non-specific binding was assessed by incorporating bovine serum albumin (BSA) in the medium in the MIC assay.²⁴ MIC values were measured in the presence of increasing concentrations of BSA. If the inhibitors act through non-specific binding a linear increase in the MIC values should be observed as a function of BSA concentration. When MICs for **fgkc** (21) were determined over a concentration range of BSA from 0–10 μM, no effect on the MIC was observed except for a slight increase (2-fold) at the higher concentrations of BSA (Figure 4C). Additionally, no inhibition of lysozyme activity by **fgkc** (21) was observed (Figure S7), further corroborating that **fgkc** (21) inhibition of LytG is specific.

In order to further corroborate that **fgkc** (21) inhibits autolytic activity, *B. subtilis* cells were treated with sub-MIC concentrations of **fgkc** (21), and subsequently examined by light microscopy. As with the glycosyl triazoles previously reported, cells treated with **fgkc** (21) demonstrated an aberrant cell phenotype, with a preponderance of unseparated cells in linked chains (Figure 4D and S8). This phenotype has been seen with the knockouts of the LytG homologs AcmA/D (*Lactococcus lactis*) and LytB (*Streptococcus pneumoniae*), as well as the *B. subtilis* lytC/lytD/lytF triple mutant, and is a hallmark of impaired autolysin activity.^{8,14,15,16} The observed phenotype with **fgkc** (21) is not indicative of disruption of the plasma membrane in *B. subtilis*.²⁵ It has been reported that LytG is maximally expressed during early to mid-exponential phase growth.¹¹ Growth phase experiments with **fgkc** (21)/**fgka** (5) during lag phase, early/mid exponential, and late exponential/early stationary phase were performed (Figure S9). These experiments demonstrate that **fgkc** (21)/**fgka** (5) inhibit actively growing cells during the growth phases when LytG is maximally expressed. Analysis of cell viability upon treatment with up to two-fold the MIC of **fgkc** (21) indicate that it is bacteriostatic (Figure S10). We screened **fgkc** (21) against *B. subtilis* strains lacking the other major characterized autolysins (*lytC*,amidase; *lytD*, endoGlcNAcase; and *lytF*, endopeptidase).⁸ If **fgkc** (21) inhibits any of these enzymes, the MIC values against the corresponding mutants should increase in the absence of a putative target. In all cases, lower MIC values were observed (Figure 4E), indicating that the mutants were more sensitive to the diamide and that LytC, LytD, or LytF are not the primary targets of **fgkc** (21). The observation of modestly lower MIC values for the mutants vs. the wildtype may arise because LytC, LytD, and/or LytF represent additional secondary targets for the diamides, or because the activity or expression of the remaining autolysins, including LytG, has been altered in the mutants, sensitizing them further to **fgkc** (21).

The scarcity of new antimicrobial compounds in the drug discovery pipeline is one of the main hurdles to combatting the current antibiotic resistance crisis.^{29,30} The bacterial cell

wall, and in particular peptidoglycan has historically provided a treasure trove of antimicrobial targets. Recently there have been reports of new compounds that target cell wall synthesis, e.g. teixobactin.^{2,31} While cell wall synthesis is a well validated target, less effort has been directed towards inhibition of cell wall recycling.^{32–36}

Biophysical studies on cell wall growth in Gram-positives by Misra and coworkers provide evidence that cell wall growth is turnover-driven via hydrolysis, opening the potential for targeting autolysin activity for antibiotic development.³⁷ Genetic approaches to untangle the roles of autolysins has provided a patchwork of results in both Gram-negative and Gram-positive organisms. Target validation is complicated by observations that deletion of a single or multiple autolysins is rarely if ever lethal,^{9,11,15,16, 38–41} and is probably due to the ability of other autolysins to partially compensate for the lost activity. Although genetic inactivation of *LytG* is not lethal, differential phenotypes between chemical inhibition and genetic inactivation have been observed previously for bacteriostatic agents as well as anti-fungal agents.^{42,43} Genetic and biochemical data have demonstrated a multitude of protein-protein interactions between PG synthases, hydrolases, regulatory proteins, and cytoskeletal elements in organisms such as *B. subtilis*, *S. aureus*, *E. coli* and *S. pneumoniae*.^{44–46} The bacteriostatic activity observed in the presence of a *LytG* inhibitor could then arise as a consequence of the differential time scales involved in chemical inhibition vs. genetic deletion – the former presumably reduces *LytG* activity more rapidly before compensatory modifications in the expression and activity of other autolysins and the accompanying protein-protein interactions can be established. Detailed studies of autolysin expression profiles and PG metabolism in the presence of **fgkc (21)** could provide additional insights into this phenomenon, and are the subjects of on-going studies.

Koch and Doyle⁴⁷ proposed an inside-to-outside model for cell wall growth in *B. subtilis* that highlight the role of autolysins. In this model new glycan strands are incorporated on the inner face of the cell wall. As new material is continually incorporated, the wall moves outward and bears the stress due to hydrostatic pressure. In order for the cell to grow, tension must be released in the outer peptidoglycan layers to allow for growth along the long axis of the cell, a function carried out by autolysins. Recent studies on peptidoglycan architecture in *B. subtilis*⁴⁸ and cell wall stiffness in autolysin deletion mutants in *Staphylococcus aureus*⁴⁹ support this theory. The results observed with our diamide inhibitors in *B. subtilis* appear to support this hydrolysis driven model of cell wall growth. This model could explain the discrepancy between whole cell and in vitro assay results as new material cannot be incorporated into the stress bearing layers without the action of autolysins. Inhibition of *LytG* appears to result in an impaired ability to release tension in the outermost layers of peptidoglycan, thus preventing new cell wall material from being pushed into the stress bearing layers resulting in the impaired division phenotype observed. The results presented here demonstrate that bacterial autolysins, in particular GlcNAcases, can serve as a viable target for the development of new antibacterial agents. *B. subtilis* *LytG* is a member of cluster 2 of GH73 to which include members from pathogens such as *Listeria monocytogenes*, *Clostridioides difficile*, and *Enterococcus faecalis* suggesting that these diamides could find use in clinically relevant organisms. Finally, these diamide inhibitors can complement current genetic strategies by serving as tools to study PG metabolism.

Methods

Strains and growth conditions

B. subtilis strains were grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl), LB agar plates containing 1.5% Bacto agar, or LB plates or broth containing 0.5 M NaCl at 37 °C. When appropriate, antibiotics were included at the following concentrations: 10 µg/mL tetracycline (tet), 5 µg/mL kanamycin (kan), and 25 µg/mL lincomycin (mls). Mutants in *B. subtilis* autolysins were provided by Dr. D. Kearns at the University of Indiana. The His-tagged LytG construct was kindly provided by Dr. S. Foster at the University of Sheffield.

MIC assays

MIC values were determined using the rezasurin method.¹⁶ Briefly, second passage cells of *B. subtilis* 11774 were grown in LB media and standardized to an OD_{600 nm} = 1.0. Diamides were analyzed via serial dilution into LB media in microtitre plates. Microtitre plates were inoculated with a 1/20 dilution of the OD_{600 nm} = 1.0 cell culture. Cultures were grown statically for 4 h at 37 °C, followed by addition of 30 µL of a 0.01% (m/v) solution of rezasurin. The plates were allowed to incubate for 15 min to allow stabilization of color production. MICs were read directly off the plate or via fluorescence (λ_{ex} 560 nm, λ_{em} 590 nm). MICs were recorded as the lowest concentration that inhibited growth.

Enzyme assays

B. subtilis LytG was purified to apparent homogeneity following the procedure described by Horsburgh *et al.*¹¹ Protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue stain and concentration determined via A₂₈₀ ($\epsilon_{280 \text{ nm}}$ 48 360 M⁻¹ cm⁻¹). Turbidometric assays were carried out in 10 mM sodium citrate buffer (pH 5.6) containing 10 mM MgCl₂ with 0.01% (v/v) Triton X-100 and purified PG from *M. luteus* at a final concentration of 0.19 mg/mL. Reactions were initiated by the addition of LytG at a final concentration of 20 µg/mL. The decrease in turbidity was monitored at 600 nm on a Shimadzu UV-2600 UV-Vis spectrophotometer over the course of 15 min. IC₅₀ values were determined by monitoring the decrease in the rate of clearing at varying concentrations of diamide inhibitor. IC₅₀ values were computed by calculating initial rates and plotting as percentage activity relative to uninhibited control reactions as a function of inhibitor concentration.⁵⁰ All data was collected in biological and technical triplicate and the data analyzed using GraphPad Prism 5. IC₅₀ values were calculated using non-linear regression analysis.

Cell-based screen for aggregation associated inhibition

Compounds showing antimicrobial activity were screened for promiscuous behavior using the method adapted by Feng *et al.*²³ for the identification of aggregation-dependent inhibition. Briefly, the MIC assay was performed as described above in the presence of 0%, 0.001%, and 0.01% (v/v) Triton X-100. MICs were recorded as the lowest concentration that inhibited growth. Assays were performed in biological and technical triplicate. MIC values are reported as the average of all trials.

Cell-based screen for non-specific binding

Inhibition due to non-specific binding was carried out in a manner similar to that described by Roychoudhury *et al.*²⁴ Briefly, MIC assays were carried out as described above in the presence of increasing amounts of BSA ranging from 0 – 10 μ M. Assays were performed in biological and technical triplicate. MIC values are reported as the average of all trials.

Screen for alternate autolysin targets in *B. subtilis*

Active compounds identified in the MIC screen and did not show promiscuous behavior were screened against a series of autolysin mutants.⁸ MIC assays were run as described above using strains 3610 (parent strain), DS108 (*lytD::mIs*), DS1447 (*lytF::tet*), DS3819 (*lytC::kan*) grown in LB broth containing 0.5 M NaCl to reduce pellicle formation. MIC values were taken as the lowest concentration at which growth was inhibited. Assays were performed in biological and technical duplicate. MIC values are reported as the average of all trials.

Morphological studies of B. subtilis. Cultures were prepared from second passage of *B. subtilis* ATCC 11774 as previously described for the MIC determination. Cultures were chemically fixed in a 1:10 mixture (v:v) culture media and buffer (20 mM HEPES pH 6.8 containing 1% formaldehyde).²⁷ Samples were fixed overnight at 4 °C in order to limit *de novo* cell wall biosynthesis during fixation. Samples were stained with 0.1% methylene blue (solution in 20% ethanol). Samples were gently heated to 60 °C for 15–20 min to bring cells to a common focal plane. Samples were visualized using bright-field microscopy with a Zeiss Primo Star microscope at 1000 $\times$ magnification. Micrographs were acquired using an Axiocam ERc5s camera and Zen lite software.

Crystallization of fgka—A 10:1 hexanes/DCM solvent system was used for crystal growth. 10 mg of **fgka** was placed in a small vial and dissolved in 100 μ L DCM. A scintillation vial was filled with 1 mL hexanes, and the small uncapped vial was placed inside the scintillation vial, which was then capped and placed in the fridge for three days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BSA	bovine serum albumin
DCM	dichlormethane

GlcNAc	<i>N</i> -acetylglucosamine
GlcNAcase	<i>N</i> -acetylglucosaminidase
kan	kanamycin
LB	Luria-Bertani
MIC	minimum inhibitory concentration
mls	lincomycin
MurNAc	<i>N</i> -acetylmuramic acid
OD	optical density
PG	peptidoglycan
tet	tetracycline

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Figure 1. Structure of peptidoglycan showing the cleavage sites of the major characterized autolysins in *B. subtilis*. *Inset* - Structure of **BI.fgba**, an inhibitor of *B. subtilis* LytG.

Figure 2.
Structures of diamides synthesized and tested against *B. subtilis*.

Figure 3.
Crystal structure of the diamide **fgka** (5)

Figure 4.

Characterization of diamide inhibitors **fgkc** (21) and **fgka** (5) against *B. subtilis*. (A) Plot of LytG residual activity against log[**fgkc**]. Assay performed using purified PG and enzyme activity monitored using a turbidometric assay at 600 nm. Non-linear regression was used to calculate the IC₅₀ of **fgkc** (21). Assays were run in biological and technical triplicate. (B) Promiscuous inhibitor screen for inhibitor aggregation – MIC values were determined in the presence or absence of 0.001% (v/v) Triton X-100. (C) Role of non-specific binding in **fgkc** (21) mode of action. MIC values were determined in the presence of varying [BSA]. (D)

Microscopy of *B. subtilis* 11774 in the presence of sub-MIC (0.8×) concentration of **fgkc** (21). (E) MIC values for **fgkc** (21) against various *B. subtilis* autolysin mutants.

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