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The effect of NAG–thiazoline on morphology and surface hydrophobicity of *Escherichia coli*

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Abstract

The β-hexosaminidase inhibitor and structural analog of the putative oxazolium reaction intermediate of lytic transglycosylases, *N*-acetylglucosamine thiazoline (NAG–thiazoline), was synthesized in 46% overall yield and tested as an inhibitor of *Escherichia coli* growth. NAG–thiazoline, at concentrations up to 1 mg/ml, was not found to affect the viability of *E. coli* DH5α. However, the compound did induce morphological changes to the cells. Growth of cells in the presence of NAG–thiazoline caused an apparent inhibition of the biosynthesis of the cylindrical regions of the cells such that they became much shorter in length. The surface of these shorter cells was found to be much less hydrophobic compared to untreated cells as determined by the bacterial adhesion to hydrocarbon (BATH) assay. In addition, the co-administration of NAG–thiazoline with $1.7 \times$ MIC concentrations of ampicillin prevented cell lysis suggesting that the compound inhibited autolytic enzymes, in particular the lytic transglycosylases.

Keywords: *Escherichia coli*; Morphology; NAG–thiazoline; Lytic transglycosylase; Penicillin-induced lysis; Autolysin

1. Introduction

The generally accepted model for the biosynthesis of peptidoglycan in *Escherichia coli* invokes enzyme complexes comprised of both transferases, a collection of penicillin-binding proteins (PBPs), and the lytic transglycosylases (LTs) (recently reviewed by Young [1]). The high-molecular weight PBPs catalyse the incorporation of newly synthesized and translocated peptidoglycan precursor molecule, Lipid II, into the existing sacculus at sites made available through the action of the LTs. Inhibition of the transglycosylase or transpeptidase activities of the PBPs by moenomycin and the β-lactam antibiotics, respectively, leads to autolysis through the continued action of the LTs (reviewed in [2]). In addition, mutant strains deficient in either of the two classes of enzymes are characterized by significantly altered cell morphologies [1,3].

While much effort has been made to understand the function and mechanism of action of the PBPs with the aim to extend their usefulness as a target for antibacterial therapy, the LTs have attracted considerably less attention [4]. LTs function to cleave the same bond in peptidoglycan as the lysozymes, specifically the β-$(1 \rightarrow 4)$-glycosidic bond between the MurNAc and GlcNAc residues [5,6]. However, the LTs are catalytically distinct from the hydrolytic lysozymes because they are not hydrolases but cleave peptidoglycan with the concomitant formation of 1,6-anhydro-MurNAc residues (Fig. 1) [5].

LTs appear to be ubiquitous in the eubacteria that produce peptidoglycan (viz., all but the cell wall-less

Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; NAG–thiazoline, *N*-acetylglucosamine-thiazoline; LT, lytic transglycosylase; PBP, penicillin-binding protein; SEM, scanning electron microscopy.

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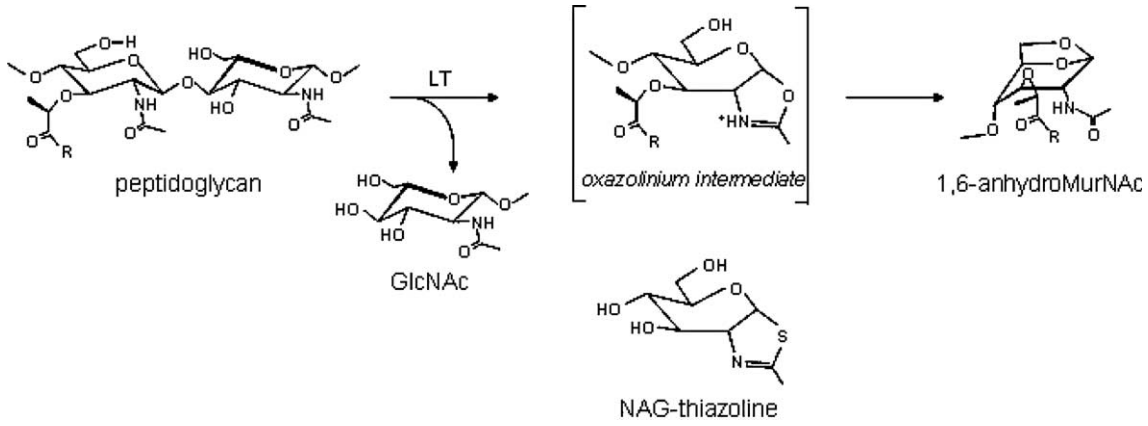

Fig. 1. Proposed reaction pathway of lytic transglycosylases (LT) and structure of NAG–thiazoline, an analogue of the oxazolium intermediate. R denotes the stem peptide of muramoyl residues involved in cross-linking of the glycan strands of peptidoglycan which are comprised of alternating GlcNAc and MurNAc residues.

mycoplasmas), and even in some lytic bacteriophages [7]. Given the apparent importance of these enzymes to the growth of the bacteria, the search for inhibitors of the LTs may prove to be valuable for the development of a new class of antibiotics with broad specificity. Based on both X-ray crystallographic and inhibition studies with bulgecin [8–10], it has been suggested that the LTs use substrate-assisted catalysis which proceeds through the formation of an oxazolium intermediate (Fig. 1) involving the muramyl residue at the site of cleavage in peptidoglycan. Recent investigations in our laboratory [11] have shown that purified LTs are inhibited by the β-hexosaminidase inhibitor and structural analog of the putative oxazolium intermediate, *N*-acetylglucosamine thiazoline (NAG–thiazoline) [12,13] (Fig. 1). In this study, we show that NAG–thiazoline causes changes to both morphology and cell surface hydrophobicity in growing *E. coli* cells, while also inhibiting β-lactam-induced autolysis.

2. Materials and methods

2.1. Synthesis of NAG–thiazoline

NAG–Thiazoline was prepared in 46% overall yield from peracetylated GlcNAc by treatment with Lawesson's reagent according to the procedure of Knapp et al. [13] with modifications. Thus, a solution of 1.03 g (2.66 mmol) of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (Toronto Research Chemicals, Toronto, Ont.) in 10 ml of dry toluene was treated with 0.68 g (1.68 mmol) of Lawesson's reagent (Sigma–Aldrich, St. Loius, MO), and the reaction mixture was heated to 60 °C for 1.5 h. The reaction mixture was then cooled to room temperature, washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated. The resulting yellow syrup containing the crude thiazoline triacetate was chromatographed on silica gel with ethyl acetate/hexane (7:1) as the solvent to give 0.42 g (46%) of purified peracetate.

A portion of the thiazoline triacetate (0.42 g) was treated with 0.2 M methanolic sodium methoxide (10 ml) with stirring for 10 min. After de-ionization with Bio-Rad 50W-X4 (H^+ form; Bio-Rad Laboratories) cation-exchange resin, the reaction mixture was filtered and the filtrate was concentrated to give 0.27 g NAG–thiazoline as a hygroscopic brownish solid. Purity and identity to the known NAG–thiazoline [13] was confirmed by TLC and NMR analysis. R_f 0.21 (2:1:1:1 ethyl acetate/isopropanol/H_2O/acetic acid); ^{1}H NMR (400 MHz, CD_3OD) δ 6.37 (d, 1H, $J = 7.0$ Hz, H-1), 5.60 (d, 0.1H, $J = 4.7$ Hz, residual GlcNAc H-1) 4.30 (m, 1H, H-2), 4.19 (t, 1H, $J = 4.0$ Hz, H-3), 3.78 (dd,1H, $J = 2.6$, 12 Hz, H-6^1), 3.65 (dd, 1H, $J = 6.1$, 12 Hz, H-6), 3.62 (ddd, 1H, $J = 1.0$, 3.8, 9.1 Hz, H-4), 3.28 (m, 1H, H-5), 2.22 (d, 3H, $J = 1.6$ Hz, Thiazoline CH_3). ^{13}C NMR (100 MHz, CD_3OD) δ 170.0 (S**C**=N), 90.80, 80.65, 76.40, 74.23, 71.43, 63.45, 24.25.

2.2. Growth studies

Growth of *E. coli* DH5α (Clonetech) cultures in the presence and absence of NAG–thiazoline was examined by culturing one colony in Luria–Bertani (LB) broth (1% tryptone peptone, 1% NaCl, 0.5% yeast extract) at 37 °C with shaking. After 16 h, a 1/50 dilution of the culture was performed in triplicate into fresh LB broth containing NAG–thiazoline and growth was monitored turbidometrically (OD_{600}). Identical dilutions of the starter culture were inoculated into fresh LB broth alone to serve as controls.

In other experiments to examine the effect of NAG–thiazoline on penicillin-induced autolysis, cultures of *E. coli* DH5α were grown in LB broth at 37 °C to a cell density of OD_{600} of 0.18 (early exponential phase) and

divided into three aliquots. Each aliquot was then supplemented with either nothing (to serve as controls), 5 μg/ml (1.7 × MIC) ampicillin, or 5 μg/ml ampicillin and 300 μg/ml NAG–thiazoline. Subsequent growth at 37 °C was monitored turbidometrically for 5 h while samples were withdrawn periodically for microscopic examination.

2.3. Morphological effect of NAG–thiazoline on E. coli

Phase contrast microscopy of unstained wet mounts of cells was conducted using Nomarski optics on a Leica microscope with differential interference contrast. Images were captured and then processed with Adobe Photoshop software.

For scanning electron microscopy, cells were harvested from 1 ml samples of cultures by centrifugation at 8000*g* for 10 min at 4 °C. The bacterial cell pellet was washed in phosphate buffered saline to remove any residual broth. The pellet was then suspended in 70 mM Sorensen's phosphate, pH 6.8, placed on a 0.2 μm polycarbonate membrane filter (Poetics Corp. Livermore, CA), and fixed with 2% gluteraldehyde in phosphate-buffered saline for 1 h and post-fixed with OsO_4. The samples were then rinsed in several changes of buffer, dehydrated through a series of ethanol washes, critical point dried (Ladd Industries) using carbon dioxide, and sputter coated with 20 nm of gold/palladium in a Hummer VII sputter coater (Anatech Corp. Alexandria, VA). To visualize the organisms, the filters were scanned using a Hitachi S-570 SEM (Tokyo, Japan), and images were collected directly from the SEM using Quartz PCI software (Quartz Imaging Corp. Vancouver, BC).

2.4. Affect of NAG–thiazoline on LPS profile of E. coli

Cultures of *E. coli* DH5α grown for 6 h (late exponential phase) in LB containing 0 or 600 μg/ml NAG–thiazoline (each in triplicate) were harvested by centrifugation (6000*g*, 10 min, 4 °C) and the cells were resuspended in 100 μl of NuPAGE® (Invitrogen Life Technologies Inc.) sample buffer for analysis of their LPS profiles [14]. After incubation at 100 °C for 10 min, 50 μg of Proteinase K was added and incubated at 60 °C for 1h. Samples were then boiled at 100 °C for 10 min and diluted 1/10 before loading onto a NuPAGE® 4–12% gradient electrophoresis gel. Samples were subjected to electrophoresis for 80 min at 120 V and the LPS was detected by silver staining.

2.5. Cell-surface hydrophobicity assay

Surface hydrophobicity of cells grown in the presence and absence of NAG–thiazoline was assessed using the bacterial adhesion to hydrocarbon (BATH) assay of Rosenberg et al. [15]. Cells grown to late exponential phase in LB broth containing 0, 100, 300, or 600 μg/ml of NAG–thiazoline were harvested by centrifugation and washed with 3 ml of 150 mM phosphate buffer, pH 7.1 containing 30 mM urea and 0.8 mM $MgSO_4$. Triplicate samples of the washed cells were resuspended in the same buffer to an OD_{400} of 1.0, transferred to glass test tubes, and treated with 0.1 ml hexadecane. Following incubation for 15 min at 37 °C with shaking, the samples were vortexed for 2 min and the phases were allowed to separate for 10 min. The OD_{400} of each of the aqueous phases was measured and expressed as a percentage of the difference between the adhesion to the glass and adhesion to hexadecane.

3. Results and discussion

3.1. Growth inhibition studies

The addition of up to 600 μg/ml NAG–thiazoline did not affect *E. coli* cultures grown in LB broth as compared to cultures supplemented with equal concentrations of GlcNAc, the parent compound of NAG–thiazoline (data not shown). To further examine for any inhibitory effect of NAG–thiazoline on cell growth, determination of its minimum inhibitory concentration was attempted. Thus, doubling dilutions of the compound were added to LB broth and growth was monitored turbidometrically following inoculation and incubation at 37 °C for 8 h. As with the growth curve experiments, all cultures were found to be viable and NAG–thiazoline was not found to inhibit growth at concentrations as high as 1 mg/ml.

3.2. Morphological effect of NAG–thiazoline on E. coli

Bulgecin (a natural product comprised of a 4-*O*-sulfonyl-GlcNAc residue linked with a 4-hydroxy-5-(hydroxymethyl)-L-proline), in combination with sub-lethal concentrations of β-lactam antibiotics, has been shown to cause prominent bulges in the middle of elongated *E. coli* cells without being inhibitory to cell growth [9]. Subsequent in vitro studies indicated that the compound inhibits only the family 1 *E. coli* lytic transglycosylase, Slt70 at low concentrations [8,9] suggesting a specific role for this enzyme in the maintenance of cell shape and peptidoglycan metabolism. Given that this selective lytic transglycosylase inhibitor does not inhibit growth but rather affects the cell morphology of *E. coli*, cells grown in the presence and absence of NAG–thiazoline were prepared for examination by phase contrast microscopy. Cells grown at 37 °C in LB broth for 3 h in the presence of 600 μg/ml NAG–thiazoline displayed an unusual rounded up or "stubby" morphology (Fig. 2). This change occurred in all cells. In contrast, cells treated

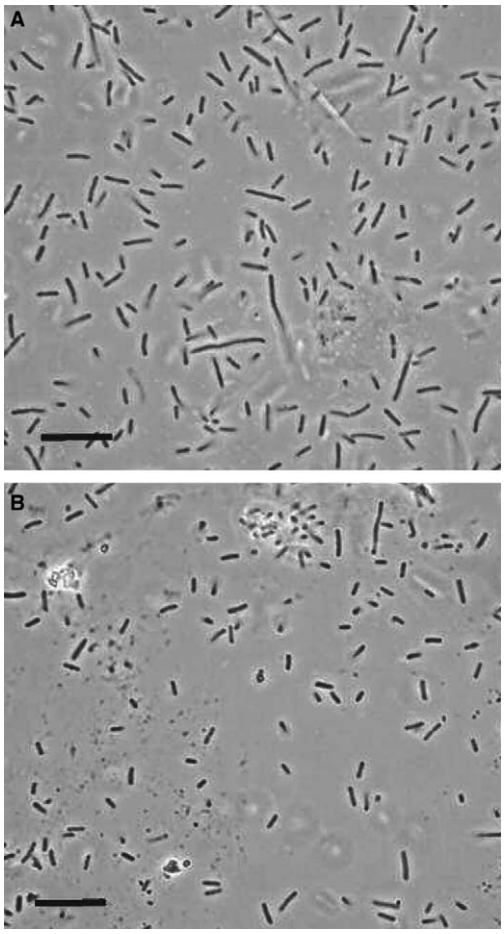

Fig. 2. Phase contrast micrographs of *E. coli* DH5α cells grown in LB broth at 37 °C and the (A) absence and (B) presence of 600 μg/ml NAG–thiazoline. Bar denotes 5 μm.

with equivalent concentrations of GlcNAc showed no such alteration in morphology. It should be noted that, unlike the situation with bulgecin, the observed change in cell morphology caused by NAG–thiazoline did not require the co-administration of sub-lethal quantities of β-lactams suggesting that, in this case, more than one type of LT may have been affected by exposure to NAG–thiazoline. Indeed, the non-lethal affect of NAG–thiazoline on cell morphology is more analogous to the observations made with mutant strains of *E. coli* in which the genes of all six known membrane-bound lytic transglycosylases have been disrupted [3]. These deletion mutants showed growth in chains and were slightly coccoid in shape (viz., a reduction in cylindrical cell shape). However, while this interpretation is consistent with the observations, it is also conceivable that NAG–thiazoline may inhibit a different class of enzyme associated with cell morphology.

Scanning electron microscopy (SEM) was used in an effort to provide both further details and a statistical analysis of the morphological changes to *E. coli* resulting from exposure to NAG–thiazoline. Initial experiments involved the examination of *E. coli* cells treated with 300 μg/ml NAG–thiazoline which showed only a 15% decrease in cell length compared to the control samples incubated in the absence of the compound(data not shown). However, it became apparent that the SEM images did not represent the entire cell population because the absolute total of treated cells observed in the SEM fields was considerably lower compared to that from control cultures. Thus, only a sub-population of the treated cells were fixed to the polycarbonate membranes negating the opportunity to observe and measure the smaller ones seen by phase microscopy.

3.3. Analysis of cell surface properties

The finding that the shorter, treated *E. coli* cells did not adhere to SEM grids suggested the surface properties of these cells was altered, likely as an indirect result of the morphological changes. A preliminary investigation of this possibility was conducted by analysing both the LPS profiles and cell surface hydrophobicities of the cells. The LPS profiles of cells grown in the presence of NAG–thiazoline, as judged by gel electrophoresis, were not significantly different to control cells (data not shown). This result was not unexpected given that *E. coli* DH5α is a rough strain thus producing LPS molecules comprised of lipid A, core oligosaccharide, and no more than one O-chain subunit. However, the overall hydrophobicity of the cell surface was greatly influenced by growth in the presence of NAG–thiazoline. As indicated in Table 1, the surface of treated cells was clearly more hydrophilic in nature as very few cells partitioned into the organic solvent compared to those from control cultures. Moreover, this alteration in cell surface hydrophobicity appeared to be dependant on NAG–thiazoline concentration. Similar alterations in cell surface hydrophobicity have been observed with different pathogenic bacteria following antibiotic treatments and can result in modified adhesion propensity and thereby their pathogenicity (e.g., [16] and references therein).

3.4. Inhibition of penicillin-induced lysis by NAG–thiazoline

Changes to bacterial cell morphology have to involve alterations, either directly or indirectly, to the metabo-

Table 1
Cell surface hydrophobicities of *E. coli* DH5α

Growth conditions	BATH (%)[a]
Control	31.1 ± 2.76
+100 μg/ml NAG thiazoline	22.6 ± 3.57
+300 μg/ml NAG thiazoline	9.64 ± 0.772
+600 μg/ml NAG thiazoline	2.86 ± 0.316

[a] Mean ± SD ($n = 3$).

lism of the rigid cell wall polymer peptidoglycan. NAG–thiazoline has been used previously to investigate the reaction mechanism of a bacterial β-hexosaminidase [12,13,17]. NAG–thiazoline–lipid II hybrids have also been synthesized as potential inhibitors of transglycosylases involved in peptidoglycan biosynthesis [18]. Recent studies in our laboratory have shown NAG–thiazoline to be an inhibitor of the lytic transglycosylases [11]. Thus, the morphological changes accompanying the treatment of cells with NAG–thiazoline observed in this study may have resulted from the inhibition of one or more autolytic enzymes, including the lytic transglycosylases. This view was supported by observations that the co-administration of this compound with ampicillin prevented the cell autolysis that normally accompanies exposure to β-lactam antibiotics. As shown in Fig. 3, the addition of $1.7 \times$ MIC (viz. 5 μg/ml) of ampicillin to growing cultures of *E. coli* resulted in both the cessation of growth and a time-dependent loss in turbidity consistent with the onset of autolysis. This lysis was confirmed by microscopic examination of the cultures. Inclusion of 300 μg/ml NAG–thiazoline together with the β-lactam did not prevent the antibiotic-induced cessation of growth, but in contrast to the control cultures, these cells did not undergo autolysis. Moreover, the extremely elongated cell morphology that results from treatment of *E. coli* with ampicillin prior to their autolysis was not observed with cells co-treated with NAG–thiazoline (data not shown). Given these observations, together with the in vitro studies made with purified lytic transglycosylase [11], it is tempting to speculate that NAG–thiazoline inhibits the lytic transglycosylases that comprise the complexes responsible for the biosynthesis of peptidoglycan in the cylindrical regions of the sacculus. Clearly, however, more detailed studies will be required to support this view.

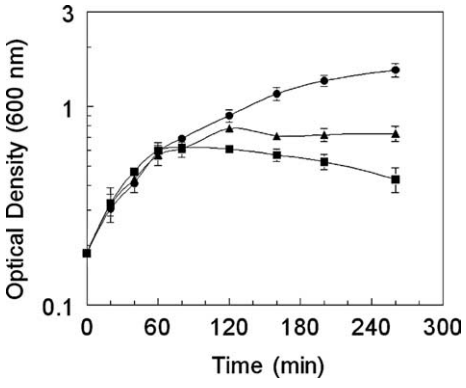

Fig. 3. Inhibition of ampicillin-induced lysis by NAG–thiazoline. Cultures of *E. coli* DH5α were grown in LB broth at 37 °C to a cell density of OD_{600} of 0.18 (early exponential phase), divided into three aliquots, and then supplemented with (●) nothing (control), (■) 5 μg/ml ($1.7 \times$ MIC) ampicillin, or (▲) 5 μg/ml ampicillin and 300 μg/ml NAG–thiazoline. The error bars denote standard deviation ($n = 3$).

3.5. Concluding remarks

By virtue of its unique chemical structure in nature and its importance to bacterial cell viability, the metabolism of peptidoglycan has been exploited as a target for antibacterial drug development (recently reviewed by Koch [19]). Unfortunately, some of the important antibiotics developed are quickly becoming irrelevant for clinical use as drug resistance levels increase in both human and animal pathogens. For example, the prevalence of β-lactamases among a variety of important pathogens has seriously compromised the general therapeutic use of the β-lactams (monobactams, penicillins, cephalosporins), compounds that are functional analogs of the stem peptide portion of the peptidoglycan-repeating unit. Using a different mode of resistance, vancomycin-resistance in enterococci (VRE) develops when alterations arise in the stem peptides of their peptidoglycan resulting in a decreased affinity for the antibiotic [20]. Despite these alarming trends, however, the peptidoglycan sacculus still contains other potential targets for drug development [21], including the lytic transglycosylases. The apparent ubiquity of these enzymes amongst the bacteria [7] further supports their usefulness as an antibacterial target. The ability of NAG–thiazoline to cause changes to the hydrophobicity of the *E. coli* cell surface, a factor associated with adherence of pathogens to host cells, together with the inhibition of peptidoglycan biosynthetic complexes responsible for cell morphology and autolysis demonstrated in this study provides further impetus to continue investigating the potential of the lytic transglycosylases as an alternative target for the development of a new class of antibiotics.

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