Small Molecule Inhibitors of a Glycoside Hydrolase Attenuate Inducible AmpC-mediated β-Lactam Resistance*

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The increasing spread of plasmid-borne ampC-ampR operons is of considerable medical importance, since the AmpC β-lactamase that they encode confer high level resistance to many third generation cephalosporins. Induction of AmpC β-lactamase from endogenous or plasmid-borne ampC-ampR operons is mediated by a catabolic inducer molecule, 1,6-anhydro-β-N-acetylmuramic acid (MurNAc) tripeptide, an intermediate of the cell wall recycling pathway derived from the peptidoglycan. Here we describe a strategy for attenuating the antibiotic resistance associated with the ampC-ampR operon by blocking the formation of the inducer molecule using small molecule inhibitors of NagZ, the glycoside hydrolase catalyzing the formation of this inducer molecule. The structure of the NagZ-inhibitor complex provides insight into the molecular basis for inhibition and enables the development of inhibitors with 100-fold selectivity for NagZ over functionally related human enzymes. These PUGNAc-derived inhibitors reduce the minimal inhibitory concentration (MIC) values for several clinically relevant cephalosporins in both wild-type and AmpC-hyperproducing strains lacking functional AmpD.

The expression of inducible chromosomal AmpC β-lactamases (1, 2) is one increasingly problematic resistance mechanism seen in many Gram-negative bacteria, including clinically opportunistic pathogens, such as Pseudomonas aeruginosa and Citrobacter freundii. These AmpC β-lactamases inactivate a broad range of β-lactam antibiotics, thereby conferring resistance to clinically important cephapemycins, cephalosporins, and even monobactams, next generation β-lactams designed to be stable against β-lactamases (3). One alarming development resulting from continued β-lactam use is the spread of genetically diverse, plasmid-borne ampC, including those that are under inducible control. Indeed, the movement of ampC genes onto plasmids (4 – 8) as well as other transposable elements has greatly increased the prevalence of this type of resistance mechanism in Gram-negative organisms (9). The regulation of ampC gene expression can vary between genera of Gram-negative bacteria. In some, chromosomal ampC is expressed constitutively, although high level expression has been suggested to decrease bacterial fitness and virulence, at least in Salmonella enterica (10). Often, however, chromosomal ampC β-lactamase is inducible as found in various clinically relevant opportunistic pathogens, such as Enterobacter spp., and P. aeruginosa (11, 12).

A major achievement in the field of antibiotic research has been the discovery of β-lactamase inhibitors. These inhibitors have been shown, in some cases, to reverse antibiotic resistance mediated by certain classes of β-lactamase. There are currently three main β-lactamase inhibitors available on the market, each having the same β-lactam core found in β-lactam antibiotics. One of these inhibitors, clavulanic acid, however has been found to be of limited use against AmpC (13, 14). The other two, sulbactam and tazobactam, demonstrate effectiveness against certain bacteria, but their general utility against AmpC remains unclear (13, 15–19). Accordingly, the development of new strategies to cope with the growing problem of AmpC β-lactamases are of considerable interest and importance.

The mechanism by which inducible AmpC β-lactamase expression is controlled is directly related to the integrity of the murein cell wall. This regulatory mechanism elegantly links antibiotic resistance to the dynamic and continuously changing balance between peptidoglycan biosynthesis and degradation (Fig. 1). These two metabolic activities must be strictly controlled by bacteria to enable cell growth yet avoid autolysis (20). During cell division, a considerable amount of the murein sacculus is degraded by various autolysins and recycled (21, 22).

The GlcNAc-1,6-anhydro-MurNAc2 peptide degradation products liberated by these autolysins are transported into the cytosol (2), where, upon arrival, the nonreducing GlcNAc residue is removed by a family 3 β-glucosaminidase known as NagZ. The resulting products are N-acetyl-d-glucosamine and a series of 1,6-anhydro-MurNAc tri-, tetra-, and pentapeptides (Fig. 2) (23, 24). These structurally unusual 1,6-anhydro-MurNAc catabolic fragments activate the transcription of inducible ampC. Acting in an antagonistic manner, UDP-MurNAc pentapeptide, a biosynthetic building block of the cell wall-derived

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The atomic coordinates and structure factors (code 2OXN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: MurNAc, N-acetylmuramic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MIC, minimal inhibitory concentration.
from these catabolic products, is involved in transcriptional repression of ampC. The dynamic balance between the concentrations of these catabolic and anabolic molecules is how these bacteria sense β-lactams and accordingly regulate transcription of ampC.

The nexus linking these two molecules with the transcription of ampC is AmpR (25), a member of the large family of LysR transcriptional regulators (26). ampC and ampR are together found in a divergent operon in which ampR is located directly upstream of ampC. The promoters for these two genes overlap, and AmpR binds to this region, thereby regulating transcription of ampC (27). In the absence of β-lactams, UDP-MurNAc pentapeptide is believed to bind to AmpR and promote a conformation in the protein that prevents transcription of ampC (20, 28). When β-lactams are present, murein biosynthesis is disrupted, the delicate balance between the inducer and repressor molecules is perturbed (21, 29), and the 1,6-anhydro-MurNAc oligopeptides accumulate in the cytoplasm. One of these compounds, believed to be 1,6-anhydro-MurNAc tripeptide,3 is thought to competitively displace UDP-MurNAc pentapeptide from AmpR and thereby induce an alternate conformation that now activates transcription of ampC (20).

To avoid overproduction of AmpC, bacteria limit the intracellular concentration of the inducer molecule by degrading and recycling it via the action of AmpD, a cytoplasmic N-acetylmuramyl-1,-alanine amidase (30, 31) that cleaves the stem peptide off of both the GlcNAc-1,6-anhydro-MurNAc tripeptide as well as the inducer 1,6-anhydro-MurNAc tripeptide (30, 31). Clinical resistance to extended spectrum (third generation) β-lactams seen in many strains of Gram-negative bacteria most commonly arises from loss-of-function mutations in the ampD gene (32–35). These mutations dramatically increase the cytosolic concentration of 1,6-anhydro-MurNAc oligopeptides and lead to constitutive AmpC expression (36).

The presence of NagZ activity in a common Gram-negative bacterium, *Escherichia coli*, was first reported over 20 years ago (37), although it has only recently been shown that deletion of the nagZ gene from bacteria harboring the ampC-ampR operon increases their susceptibility to β-lactam antibiotics (23, 24). These observations suggest a strategy to attenuate β-lactam resistance within pathogens harboring ampC-ampR. By blocking formation of the inducer molecule through inhibition of NagZ, induction of AmpC β-lactamase should be prevented, with the net effect being enhanced efficacy of β-lactam antibiotics. Accordingly, inhibition of NagZ during treatment of certain resistant Gram-negative bacterial infections could rescue many classes of β-lactam antibiotics. An effective strategy would be of particular importance for treating immunocompromised patients suffering from chronic infection by opportunistic Gram-negative pathogens as well as for certain infections in immunocompetent patients. *P. aeruginosa*, for example, is known to rapidly develop resistance to β-lactams through high level production of an inducible chromosomally encoded AmpC β-lactamase and typically requires aggressive treatments using aminoglycoside or quinolone antibiotics that are somewhat toxic.

**EXPERIMENTAL PROCEDURES**

Reagents, Enzymes, Chemical Techniques, and Bacterial Strains—All media components were obtained from Biohop. *Pfu* DNA polymerase, deoxynucleoside triphosphates, and restriction endonucleases were obtained from Fermentas. T4 DNA ligase was purchased from New England Biolabs. *E. coli* ultracompetent XL-10 Gold and XL-10 Gold Kan® cells were purchased from Stratagene. *E. coli* Tuner (ADE3) cells were purchased from Novagen. HisTrap FF columns were purchased from Amersham Biosciences. DNA fragment purification and plasmid purification kits were obtained from Qiagen. Synthesis

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3 There is some conjecture as to what 1,6-anhydro-MurNAc oligopeptide is involved in the induction process. The 1,6-anhydro-MurNAc tripeptide and the 1,6-anhydro-MurNAc pentapeptide have both been thought to induce ampC transcription (2).
of oligonucleotide primers for the C. freundii ampR-ampC operon cloning was performed by Sigma and, in the case of the V. cholerae NagZ, Invitrogen. DNA sequencing was performed by the Nucleic Acids and Peptide Service facility, University of British Columbia. All bacterial strains used in the antibiotic testing were provided by the NARA Institute of Science and Technology and the E. coli Genetic Stock Center. All solvents were dried prior to use. Synthetic reactions were monitored by TLC using Merck Kieselgel 60 F254 aluminum-backed sheets. Compounds were detected by charring with a 10% concentrated sulfuric acid in ethanol solution and heating. Flash chromatography under a positive pressure was performed with Merck Kieselgel 60 (230–400 mesh) using specified eluants. \(^{1}H\) and \(^{13}C\) NMR spectra were recorded on a Varian AS500 Unity Innova spectrometer at 500 MHz (125 MHz for \(^{13}C\)) (chemical shifts quoted relative to CD\(_3\)OD where appropriate). Elemental analyses of all synthesized compounds used in enzyme assays were performed at the Simon Fraser University Analytical Facility.

Cloning and Expression Vector Construction for NagZ V. cholerae—The V. cholerae NagZ gene (GenBank\textsuperscript{TM} AE004155) was amplified by the PCR using VENT polymerase from a V. cholerae subgenomic clone (ATCC 638129). The sense primer 5′-GATATCATATGGGACCCTTTGGTGGATG-3′ and reverse primer 5′-GATATAGGATCCTAA-TGATGGTGATGTTGATGATGATGATGATGATGATGATGATGGAGAAGCTGCTGCGGAGG-3′ were used to introduce an in-frame C-terminal His\(_{10}\) purification tag and 5′-Ndel and 3′-BamHI restriction sites (shown in bold face type) onto the PCR ampiclon. The ampiclon was restricted with Ndel and BamHI restriction endonucleases, ligated into a modified version of pET28 (Novagen) (containing a reduced multicloning site composed of Ndel/Spel/BamHI only) using T4 DNA ligase. The ligation reaction was transformed into chemically competent E. coli strain BL21 (ADE3) GOLD HTE cells (Stratagene). Recombinant plasmid was isolated from a single kanamycin-resistant transformant and verified by restriction analysis and DNA sequencing to confirm the successful construction and correct sequence of the C-terminal His\(_{10}\)-tagged VcNagZ expression construct pVcNagZ.

Protein Expression and Purification of V. cholerae NagZ—E. coli BL21 (ADE3) Gold cells harboring pVcNagZ were grown to log phase (\(A_{600} = 0.5\)) in a shaker incubator at 37 °C in 2 \(\times\) 500 ml of Luria-Bertani medium supplemented with 35 \(\mu\)g/ml kanamycin. Expression of recombinant NagZ from log phase cultures was induced by the addition of 1 mM isopropyl-\(\beta\)-D-galactopyranoside followed by a 4-h incubation at 28 °C with shaking. Cells were pelleted by centrifugation and stored at −80 °C. The induced pellets were thawed in 20 ml of ice-cold lysis buffer (1 M NaCl, 20 mM Tris-HCl, pH 8, 1 mM phenylmethylsulfonyl fluoride, 200 \(\mu\)l of protease inhibitor mixture for His-tagged proteins (Sigma) and lysed by French press. The lysate was clarified by centrifugation and loaded onto a Ni\(_{2+}\)-nitrilotriacetic acid affinity column (Qiagen) pre-equilibrated with binding buffer (1 M NaCl and 20 mM Tris-HCl, pH 8). The column was washed with 10 column volumes of binding buffer followed by two additional washes of binding buffer supplemented with 25 mM imidazole, pH 7.4, and 50 mM imidazole, pH 7.4, respectively. The NagZ protein was eluted from the column using binding buffer supplemented with 250 mM imidazole, pH 7.4. The eluate was then dialyzed overnight against 150 mM NaCl, 20 mM BisTris, pH 6.5, containing 10% glycerol. All centrifugations, chromatographic steps, and dialysis were conducted at 4 °C.

Crystallization and Cryogenic Preservation for V. cholerae—Purified VcNagZ was concentrated to 6.5 mg/ml using an Amicon Ultra centricron spin cartridge and crystallized by hanging drop vapor diffusion within 1 week using mother liquor condi-

FIGURE 2. A, NagZ-catalyzed hydrolysis of murein cell wall fragments releases two monosaccharide fragments, \(N\)-acetyl-\(\beta\)-glucosamine and the series of 1,6-anhydro-MurNAc tri-, tetra-, and pentapeptide inducer molecules that activate transcription of \(ampC\) expression. B, structures of the inhibitors studied here, C, the putative transition state of the NagZ-catalyzed hydrolysis of \(N\)-acetylglucosaminides (denoted by a double dagger).
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Synthesis of Inhibitors—The synthesis of the various 2-N-acyl PUGNAC analogues is described elsewhere (47). The representative preparation of EtBuPUG (8 in Scheme 1) followed similar methodologies.

\( \text{O-\(\text{O}\)-Deoxy-2-N-2-ethylbutyryl-\(\text{\alpha}\)-glucopyranosylidene)amino N-Phenylcarbamate 8} \)–TLC (EtOAc/MeOH: 1:19, \( \nu/\nu \)) \( R_f = 0.4; \)

\( ^{1}H \) NMR (500 MHz, CD\(_3\)OD): \( \delta 7.43-7.42 \) (m, 2H, Ar), 7.31-7.29 (m, 2H, Ar), 7.00-7.08 (m, 1H, Ar), 4.58 (m, 1H, H-2), 4.02-3.96 (m, 2H, H-5/H-6), 3.87 (dd, \( \nu = 4.0, 12.5 \) Hz, 1H, H-6), 3.79-3.74 (m, 2H, H-3/H-4), 2.15 (m, 1H, COCH), 1.63 (m, 2H, \( \text{CH}_2\text{CH}_2 \)), 1.50 (m, 2H, \( \text{CH}_2\text{CH}_2\text{CH}_2 \)), 0.96 (m, 6H, \( \text{CH}_3 \)); \( ^{13}C \) NMR (125 MHz, CD\(_3\)OD): \( \delta 178.03 \) (C = O), 158.22 (CONHPh), 153.43 (C-1), 137.93, 128.78, 123.65, 119.20 (4C, Ar), 82.81, 73.25, 68.79 (3C, C-3/C-4/C-5), 60.48 (C-6), 51.58 (C-2), 50.68 (CH), 25.77, 25.63 (2C, \( \text{CH}_2 \)), 11.40, 11.26 (2C, CH\(_3\)); Anal. (Calcd, found for C\(_{17}\)H\(_{23}\)N\(_3\)O\(_7\)): C (55.74, 55.52) H (6.65; 6.39).

Kinetic Analysis of NagZ from V. cholerae—All assays were carried out in triplicate at 37 °C using a continuous assay procedure by following the linear rate of liberation of 4-nitrophenolate as determined by absorption measurements at 400 nm. Reactions (80 \( \mu l \)) were initiated by the addition, via syringe, of enzyme (5 \( \mu l \)) and monitored for 4 min. Time-dependent assay of NagZ revealed that the enzyme was stable in the buffer over the period of the assay: 50 mM NaPi, 100 mM NaCl, 0.1% bovine serum albumin, pH 6.5. VcNagZ was used at a concentration of 0.075 \( \mu l \) with \( pN\)-GlcNAc as a substrate at a concentration of 0.5 mM. All inhibitors were tested at seven concentrations ranging from 3 times to \( 1/3 \) \( K_i \). Values were determined by linear regression of data from Dixon plots.

Cloning of \( \text{ampC} \text{-ampR Operon from C. freundii} \)—Genomic DNA from \( \text{C. freundii} \) was used as the template for cloning of \( \text{ampC} \text{-ampR operon}. \) The PCR was accomplished using the following primers 5′-GGGTATAAGCTTCTGAGCAGCAGCCGGCAGAA-3′ (HindIII cut site shown in boldface type) and 5′-GGGTATGTCGACCTAGCCGTTGTAACGCGGCTATG-3′ (SalI cut site shown in boldface type). The amplicon was restricted with Ndel and BamH1 restriction endonucleases, ligated into a modified version of pET28 (Novagen) containing a reduced multicloning site composed of Ndel/SpeI/BamH1 only) using T4 DNA ligase. The ligation mixture was transformed directly into \( \text{E. coli} \) ultracompetent cells Kan\(_{\text{R}}\), using plates containing 30 \( \mu g/mL \) of chloramphenicol. Recombinant plasmid was isolated from a single chloramphenicol-resistant transformant and verified by restriction analysis and DNA sequencing to confirm the successful construction and correct sequence of the \( \text{ampC} \text{-ampR operon} \).

Determination of the Minimal Inhibitory Concentration of β-Lactams— Cultures were prepared by inoculating 5 mL of Mueller-Hinton broth with a small amount of the desired glyceral stock of bacteria and allowed to grow at 37 °C to an \( A_{600} \) of \( -0.5 \). 96-Well plates containing a range of concentrations of β-lactam antibiotics varying by factors of 2 were prepared containing 80 \( \mu l \) of the appropriate concentration of antibiotic in Mueller-Hinton Broth. The volume was made up to 100 \( \mu l \) by the addition of either 20 \( \mu l \) of EtBuPUG (5 mm in H\(_2\)O) or 20 \( \mu l \) of H\(_2\)O. These broth were inoculated with 100 \( \mu l \) of the desired culture and allowed to incubate at 37 °C for 18 h. The optical

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density measured at 595 nm was measured for all cultures, and the MIC was determined from the concentration of antibiotic at which no growth was observed. All MIC determinations were performed in triplicate.

Agar Diffusion Tests—The appropriate bacterial culture was prepared by inoculating 5 ml of Mueller-Hinton Broth with the appropriate glycerol stock, and the culture was allowed to grow at 37 °C until the A600 reached ~0.5. The cells were harvested by centrifugation (Eppendorf 5417C) for 3 min at 13,000 r.p.m. The cells were then resuspended in 2 ml of Mueller-Hinton broth, and 500 µl of this suspension was used to inoculate the appropriate mixtures of inhibitor and Mueller-Hinton broth. Culture (a) contained 500 µl of Mueller-Hinton broth and 500 µl of 3 mM EtBuPUG in Mueller-Hinton broth. Culture (b) contained 1000 µl of Mueller-Hinton broth. These mixtures were then cultured for 60 min at 37 °C. Mueller-Hinton broth agar plates (1.5% agar) were streaked with the bacterial culture. Antibiotic discs (6-mm diameter) previously loaded with 10 µl of EtBuPUG (3 mM) or H2O alone were placed on the agar plates. After incubation overnight at 37 °C, the diameter of the inhibition zone was measured. All determinations were performed in triplicate.

β-Lactamase Assays—Bacterial culture were prepared by inoculating 8 ml of Mueller-Hinton broth with a small amount of the desired glycerol stock of bacteria, and the resultant culture was allowed to grow at 37 °C to an A600 of ~0.5. An aliquot of cells (4 ml) was used to inoculate the appropriate Mueller-Hinton broth medium mixture (4 ml) containing EtBuPUG (2 mM) (type a) or no inhibitor (type b). These mixtures were then cultured for 60 min at 37 °C. An aliquot (4 ml) was diluted using the appropriate Mueller-Hinton broth mixture (4 ml) containing EtBuPUG (2 mM) and 5 µg/ml cefoxitin or the appropriate control mixtures. Aliquots of each culture (1.5 ml) were removed at 0, 15, 30, 45, and 60 min, and cells were harvested by centrifugation (Eppendorf 5417C) for 3 min at 13,000 r.p.m. The pellet was resuspended in 200 µl of PBS buffer (50 mM NaPi, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4) and centrifuged for 3 min at 13,000 r.p.m., after which the supernatant was discarded, and the pellets were stored at ~80 °C. To determine the β-lactamase activity, the pellet was resuspended in phosphate-buffered saline buffer (200 µl) and then sonicated (4 × 15 s at 20% power; model 500; Fisher). The cell debris was then removed by centrifugation at 15,000 r.p.m. for 60 min, and the supernatant was used directly in the assay. All enzymatic assays were carried out a triplicate at 37 °C using a continuous assay procedure by following the linear rate of liberation of 2,4-dinitrophenolate from nitrocefin (initial concentration 100 µM) as determined by absorption measurements at 485 nm. Reactions (80 µl) were initiated by the addition, via syringe, of supernatant (1 µl) and monitored for 5 min. Protein concentrations of the supernatant were quantified by Bradford assay using bovine serum albumin as a standard.

RESULTS

NagZ and sequence-related glucosaminidases are members of CAZy family 3 (48) of glycoside hydrolases that also comprise glycosidases active on a variety of other saccharides. We therefore cloned and produced recombinant V. cholerae NagZ and assayed it using pNP-GlcNAc as a substrate. As we anticipated, on the basis of sequence similarity to other NagZ homologues within the family, the enzyme had significant glucosaminidase activity. As with other members of CAZy family 3, this enzyme is predicted to use a two-step, double displacement catalytic mechanism involving a covalent glycosyl-enzyme intermediate and highly dissociative oxocarbonium ion-like transition states (49). Inhibitors designed to resemble such putative transition states have been synthesized and tested with enzymes from several other families of glycoside hydrolases found in CAZy that are similar mechanistically. Few inhibitors of NagZ are known, and those that are known bear geometric resemblance to this proposed oxocarbonium ion-like transition state (37, 50). Two of the most potent inhibitors of glycosidases for several families of enzymes are the tetrahydroimidazopyridines and the 2-acetamido-glucopyranosyl hydroximolactones (51). O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate, PUGNAc (52, 53), N-acetylglucosaminonono-1,5-lactone oxime, LOGNAc, 8-acetamido-5,6,7,8-tetrahydro-6,7-dihydroxy-5-(hydroxymethyl)imidazo[1,2-a]pyridine-2-acetic acid, nagstatin (54), and a related analogue gluco-nagstatin (58) (Fig. 2) have been shown to potentiate inhibit the family 84 human O-GlcNAcase (55, 56) and the family 20 human β-glucosaminidases (52, 54, 57–61). These two human enzymes are functionally related to NagZ, since they also cleave GlcNAc off from the nonreducing termini of glycoconjugates, but they use a different catalytic mechanism and are of unrelated sequence (62). Little is known about the inhibitory susceptibility of NagZ to these various inhibitors, but we expected, however, that at least one of PUGNac, LOGNac, or gluco-nagstatin should be a potent inhibitor of NagZ, since the glucose analogues have been shown to effectively inhibit mechanistically related β-glucosidases from other families of glycoside hydrolases (51, 58).

Initial Inhibition Studies—We assayed PUGNac, LOGNac, and gluco-nagstatin as inhibitors of NagZ from V. cholerae. All three compounds were found to be good inhibitors (Table 1), but LOGNac was less potent than PUGNac, and, surprisingly, gluco-nagstatin is still less potent although it is highly effective against β-N-acetylglucosaminidases from CAZy family 20 of glycoside hydrolases (58). These findings focused our efforts on PUGNac, which unfortunately is also a potent inhibitor of the human family 84 O-GlcNAcase (55, 63) and family 20 lysosomal β-glucosaminidases (52, 57). This lack of inhibitory selectivity presents a significant challenge in developing a useful inhibitor for blocking the function of NagZ, since in a clinical setting concomitant inhibition of these human enzymes would be undesirable. We expected, however, that using PUGNac as a scaffold, a potent and selective NagZ inhibitor could be generated by simple structural variation. To facilitate the design of selective inhibitors, we determined the three-dimensional structure of NagZ in complex with PUGNac by x-ray crystallography.

Structural Studies of PUGNac in Complex with the NagZ from V. cholerae—V. cholerae NagZ is a close homologue of E. coli NagZ (56% amino acid sequence identity). Therefore, it is an excellent model for structure-guided design of inhibitors of the E. coli homolog and other related NagZ enzymes. Using
TABLE 1
Inhibition constants and selectivity of inhibitors for O-GlcNAcase, β-hexosaminidase, and NagZ

| Compound       | O-GlcNAcase  $K_i$ (µM) | β-Hexosaminidase  $K_i$ (µM) | VcNagZ  $K_i$ (µM) | Selectivity ratio ($K_{iO-GlcNAcase}/K_{iVcNagZ}$) |
|----------------|------------------------|-----------------------------|-------------------|---------------------------------------------------|
| gluco-Nagstatin| 0.43$^a$               | 0.0052$^a$                  | 15 ± 1            | 0.03                                              |
| LOGNAc         | 6.4                    | ND$^b$                      | 0.22 ± 0.03       | 29                                                |
| PUGNAc         | 0.046$^c$              | 0.036$^d$                   | 0.048 ± 0.004     | 1.0                                               |
| 2              | 1.2$^e$                | 1.2$^e$                     | 0.16 ± 0.02       | 7.5                                               |
| 3              | 2.5$^e$                | 2.6$^e$                     | 0.26 ± 0.03       | 9.6                                               |
| 4              | 40°                    | 220°                        | 0.33 ± 0.03       | 121                                              |
| 5              | 220°                   | >900°                       | 6.5 ± 1           | 33                                                |
| 6              | 9.0°                   | 20°                         | 0.51 ± 0.02       | 18                                                |
| 7              | 190°                   | >1200°                      | 15 ± 1            | 13                                                |
| EtBuPUG 8      | 33°                    | >3000°                      | 3.1 ± 0.2         | 109                                               |

$^a$ Results determined previously (56).
$^b$ IC$_{50}$ value determined for the Bos Taurus homologue (58).
$^c$ Not determined.
$^d$ Results determined previously (74).
$^e$ Results determined previously (47).

deposited coordinates from the Protein Data Bank (1TR9)$^a$ as a search model for molecular replacement, we were able to determine the x-ray crystal structure of VcNagZ in complex with PUGNAc. NagZ adopts a TIM-barrel fold with the active site mine the x-ray crystal structure of VcNagZ in complex with

TABLE 2
Crystalllographic statistics

| Parameters                        | Values            |
|-----------------------------------|-------------------|
| Crystal Information               |                   |
| Space group                       | P2$_1$/2$_1$/2$_1$|
| Solvent content (%)               | 34.91             |
| Data collection$^a$               |                   |
| Unit cell dimensions (Å)          | $a = 48.734, b = 67.850, c = 96.894$ |
| Unit cell dimensions (degrees)    | $\alpha = 90, \beta = 90, \gamma = 90$ |
| Wavelength (Å)                    | 1.54              |
| Resolution range (Å)              | 30.657–1.7        |
| High resolution bin (Å)            | 1.79–1.70         |
| Total observations                | 240,552 (23,593)  |
| Unique reflections                | 35,833 (4915)     |
| $R_{work}$                        | 0.197             |
| $R_{free}$                        | 0.213             |
| Completeness (%)                  | 99.3 (95.4)       |
| $R_{sym}$                         | 0.055 (0.144)     |

$^a$ Values in parentheses refer to the high resolution shell.
$^b$ $R_{work} = \sum_h ||F(h)|| - |\langle F(h) \rangle|/\sum_h |\langle F(h) \rangle|$, where $|\langle F(h) \rangle|$ is the weighted mean of all measurements of $|F(h)|$.
$^c$ $R_{free} = \sum_h ||F(h)_{obs}|| - |\langle F(h)_{obs} \rangle|/\sum_h |\langle F(h)_{obs} \rangle|$, for reflections in the working and test sets (5% of all data), respectively.
$^d$ Regions defined by PROCHECK from the CCP4 package.

PUGNAC I within the active site, the required rearrangement does not appear to allow 100% occupancy of the inhibitor. Because the conflict could not be resolved by increasing PUGNAC concentrations during co-crystallization, the apparent occupancy was estimated at 70%, and two Met$_{246}$ conformations were modeled with the expectation that binding of PUGNAC causes Met$_{246}$ to become somewhat disordered (Fig. 4).

Despite the mobility of this phenyl ring, several key points can be made from this structure. First, every amino acid residue within 4 Å of the inhibitor is conserved between the V. cholerae and E. coli enzymes, again confirming the validity of this model. Second, we noted immediately that directly beneath the 2-acetamido methyl group there exists a large open pocket occupied by only a single water molecule (Fig. 3). This large pocket

$^a$ The Protein Data Bank entry giving the coordinates of V. cholerae NagZ was recently deposited by J. Gorman and L. Shapiro, but details of the structure have not been reported in the literature.
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A, schematic diagram of the NagZ-PUGNac complex showing PUGNac (drawn as sticks) bound within the NagZ active site. NagZ adopt a TIM-barrel fold with the active site housed within loops extending from the C-terminal end of the strands comprising the α/β barrel. B, PUGNac bound to VcNagZ. ω-A-weighted [Fo − Fc] difference map contoured at 2.5σ. The omit map was calculated using a NagZ model from which PUGNac was omitted prior to refinement, and Met246 was modeled in both possible conformations. The PUGNac model was replaced after refinement for illustration purposes (drawn using PyMOL (72)). C, an electrostatic potential surface map of the active site region of the NagZ-PUGNac complex calculated using APBS (73) and rendered using PyMOL (72). The NagZ model shows only the Met246(A) conformation adopted upon binding PUGNac (stick model with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue). The open area under the 2-acetamido group reveals the region most likely occupied by the selective N-acyl analogues.

FIGURE 3. A, schematic diagram of the NagZ-PUGNac complex showing PUGNac (drawn as sticks) bound within the NagZ active site. NagZ adopts a TIM-barrel fold with the active site housed within loops extending from the C-terminal end of the strands comprising the α/β barrel. B, PUGNac bound to VcNagZ. ω-A-weighted [Fo − Fc] difference map contoured at 2.5σ. The omit map was calculated using a NagZ model from which PUGNac was omitted prior to refinement, and Met246 was modeled in both possible conformations. The PUGNac model was replaced after refinement for illustration purposes (drawn using PyMOL (72)). C, an electrostatic potential surface map of the active site region of the NagZ-PUGNac complex calculated using APBS (73) and rendered using PyMOL (72). The NagZ model shows only the Met246(A) conformation adopted upon binding PUGNac (stick model with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue). The open area under the 2-acetamido group reveals the region most likely occupied by the selective N-acyl analogues.

directly contrasts with the active site architectures of family 20 hexosaminidases (64), family 56 hyaluronidases, (65) and family 84 O-GlcNACases (66, 67), where the active sites of these enzymes envelope the entire 2-acetamido group and thereby promote its participation as a nucleophile during substrate-assisted catalysis. This structural difference in active site architecture between family 3 β-glucosaminidases and those of families 20, 56, and 84 suggested to us that extensions off the 2-acetamido group of PUGNAC 1 would confer high inhibitor selectivity toward family 3 β-glucosaminidases over those other functionally related enzymes.

Synthesis and Evaluation of the Selectivity of PUGNac Analogues—Accordingly, we elected to synthesize a panel of PUGNac analogues bearing different N-acyl groups (47) (Scheme 1). With this panel of inhibitors in hand, we found them to be potent inhibitors of the NagZ from V. cholerae (Table 1). Increasing the N-acyl chain length leads to greater increases in K_I values for both human O-GlcNAcase and β-hexosaminidase (47), as compared with NagZ (Table 1). Increases in chain length are much better tolerated by NagZ as compared with the two human enzymes, probably as a consequence of the more spacious active site. Branching of the aliphatic chain also does not abrogate binding to NagZ, since compound 6 is still a good inhibitor (K_I = 0.51 μM). We find that the selectivity ratio improves as the chain length increases, as illustrated for EtBu-PUG 8, which shows ~100-fold selectivity for NagZ over both human enzymes. Given the success of the relatively simple modifications made here and the availability of the three-dimensional structure, it seems feasible that much greater selectivities and potencies could be realized by further refinement of the inhibitor structure. We selected EtBuPUG to proceed with further studies, since this inhibitor retains good potency and shows the optimal selectivity.

Evaluation of Selective Inhibitors on Antibiotic Susceptibility—Several model systems have been used to evaluate the contributions of various genes and gene products, leading to induction of AmpC and consequent resistance to β-lactams. Arguably, the best characterized model is E. coli harboring a plasmid encoding the ampC-ampR divergon (25). We selected this model, since it is genetically well defined, and knockouts of various genes within E. coli are readily available. To proceed, we first amplified the ampC-ampR divergon from the ATCC8090 strain of C. freundii and cloned it into the low copy number vector PACYC184 to make pCF1. This operon from a related strain of C. freundii has been shown by Normark and co-workers (25) to provide overexpression of AmpC that is greatly induced in the presence of various β-lactams. As mentioned above, one common mechanism some Gram-negative bacteria use to develop high level resistance to β-lactams within clinics is the spontaneous deactivation of the amidase AmpD, which results in hyperproduction of AmpC β-lactamase (36). We therefore investigated the effect of NagZ inhibition on antibiotic resistance in both E. coli strain BW25113 (a generous gift from the E. coli Genetic Stock Centre) and the same strain lacking functional AmpD (a generous gift from the NARA Institute of Science and Technology, Japan).

To evaluate whether the inhibitors themselves inhibit bacterial growth, both wild-type and the ampD^- strains were incu-
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Bated in media containing EtBuPUG (409 \( \mu g \) ml\(^{-1} \), 1 mM) but no antibiotics. We found that growth rates of both the inhibitor-treated and the control cultures were identical (data not shown), indicating that the inhibitor, on its own, is not antibacterial. To evaluate the effect of the inhibitor on the susceptibility of bacteria harboring \( ampC \)-ampR (transformed using pCFI) to various \( \beta \)-lactams, we determined the MIC of certain \( \beta \)-lactam antibiotics, the second generation antibiotic Cefoxitin, and the extended spectrum (third generation) cephalosporins, Cefotaxime, Ceftriaxone, and Ceftazidime, were chosen, since they are commonly used in clinical antibiotic susceptibility experiments. Cultures treated with EtBuPUG are more susceptible to these \( \beta \)-lactam antibiotics (Table 3). Together, these results strongly suggest that EtBuPUG is able to gain access to the cytosol, where it inhibits NagZ, 1,4-\( N \)-acetylglucosaminidase. The cellular effect is most likely a decrease in the intracellular concentrations of the 1,6-anhydro-MurNAc tripeptide that results in reduced AmpC \( \beta \)-lactamase expression.

To support this proposed mechanism of action, we monitored the induction of AmpC \( \beta \)-lactamase in cultures of both wild-type and \( ampD^− \) E. coli harboring pCFI in the presence and absence of EtBuPUG. After we induced AmpC \( \beta \)-lactamase expression by treating the cultures with 2.5 \( \mu g \) ml cefoxitin, we evaluated AmpC \( \beta \)-lactamase activity from aliquots of the culture taken every 15 min using the chromogenic \( \beta \)-lactamase substrate nitrocefin (Fig. 5). In the absence of Cefoxitin induction, EtBuPUG has no effect on the observed rate of hydrolysis of nitrocefin (\( \epsilon = 20,500 \, M^{−1} \cdot cm^{−1} \) at 486 nm), indicating that it is not acting to induce AmpC production. Consistent with the mode of action we propose, we find that NagZ inhibition using EtBuPUG reduces production of the \( \beta \)-lactamase. The blockade, however, is incomplete, with expression of AmpC being reduced by 30–40%. Attenuation, rather than outright prevention, of AmpC expression is entirely consistent with the decreases in MIC values and the disk-clearing assays (Table 3). In those studies, notable increases in \( \beta \)-lactam susceptibility are observed on incubation with EtBuPUG but do not reach the baseline level of E. coli lacking the \( ampC \)-ampR operon. We speculate that NagZ inhibitors with improved potency may result in complete reversion.

**DISCUSSION**

Much effort directed toward controlling AmpC \( \beta \)-lactamase-mediated antibiotic resistance has focused on developing more stable \( \beta \)-lactam antibiotics or on developing small molecule inhibitors of AmpC \( \beta \)-lactamase that can work in a synergistic combination with

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**TABLE 3**

**Susceptibility of bacterial strains against various \( \beta \)-lactam antibiotics**

| Strain\(^a\) | Antibiotic (MIC)\(^b\) | Clearing radius\(^c\) |
|--------------|-----------------|-----------------|
|              | Cefoxitin | Cefotaxime | Ceftriaxone | Ceftazidime | Cefoxitin | Cefotaxime |
| Wild type    | 1        | 1        | <1          | 1          | 9.3      | 10.3     |
| Wild type, EtBuPUG | 64 | 64 | 32          | 64         | 10.9     | 12.9     |
| Wild type, pCFI | 32 | 16 | 16          | 32         | 8.8      | 7.1      |
| Wild type, pCFI, EtBuPUG | 2 | 2 | 2           | 2          | 10.8     | 9.0      |
| \( ampD^− \) | 512     | 128      | 128         | 256        | 8.8      | 7.1      |
| \( ampD^− \), pCFI, EtBuPUG | 256 | 64 | 32          | 64         | 10.8     | 9.0      |

\(^a\) Transformed with pCFI and treated with the EtBuPUG.

\(^b\) MIC determined by standard serial dilution.

\(^c\) Susceptibility determined in an agar diffusion assay using 6-mm filter disks loaded with 30 \( \mu g \) of cefoxitin or cefotaxime with or without EtBuPUG. The zone of clearance was measured after incubation overnight.
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FIGURE 5. β-lactamase induction in E. coli strains carrying the ampC-ampD operon from C. freundii. β-Lactamase activity was determined from clarified supernatants obtained from sonicated cultures by monitoring the rate of hydrolysis of the chromogenic β-lactam nitrocefin at 486 nm (ε = 20,500 M⁻¹ cm⁻¹). a, E. coli strains and treatment was wild type/pCF1/ EtBuPUG-induced (●), wild type/pCF1/EtBuPUG-uninduced (○), or wild type/pCF1-EtBuPUG-induced (△). b, ampD'/pCF1/EtBuPUG-induced (●), ampD'/pCF1/ EtBuPUG-uninduced (○), ampD'/pCF1-uninduced (●), ampD'/pCF1-uninduced (○).

β-lactam antibiotics. One problem associated with developing AmpC inhibitors using the β-lactam scaffold is that these molecules can induce increased β-lactamase expression (68), although more recent studies hold greater promise (69, 70).

Given the constant and rapid evolution of resistance mechanisms, there is a continuing and urgent need for development of various approaches to surmount ampC-ampR-mediated antibiotic resistance. Here we have shown that small molecule inhibition of NagZ, which acts to produce the inducer molecule, blocks the production of AmpC and thereby reduces cellular β-lactamase activity. This strategy shows promise for reversing β-lactam antibiotic resistance, since bacteria cannot rapidly develop resistance to the inhibitors by exploiting the high mutational frequency of ampC or ampD. Further, mutations in nagZ that decrease binding affinity of sugar-based inhibitors would probably also seriously compromise the catalytic efficiency of the enzyme, so production of the inducer molecule would be decreased and induction of AmpC would still be hindered.

Notably, this strategy may eventually be useful against clinically relevant strains of bacteria containing loss-of-function mutations in ampD (32–35); these mutations dramatically increase resistance in many strains of Gram-negative bacteria to many β-lactam antibiotics. Hanson and co-workers have shown that the most common mechanism by which P. aeruginosa, an opportunistic pathogen, develops β-lactam resistance is by inactivation of AmpD (32, 34, 35). The data we present reveal that EtBuPUG is also effective at reducing the β-lactam resistance of ampD⁻ strains of bacteria.

Our approach to designing a potent and selective inhibitor of NagZ, circumventing concomitant inhibition of mechanically related human enzymes, was facilitated by obtaining the three-dimensional structure of NagZ (from V. cholerae) in complex with PUGNAc using x-ray crystallography. This structure enabled the successful design of a first generation of selective and potent inhibitors but has further potential in facilitating the development of still better inhibitors. As well, structure-guided design of potent inhibitors having different scaffolds is also made possible by this structure. The design of such second generation NagZ inhibitors requires some thought, however, since not all potent inhibitors of NagZ in vitro will have the same efficacy in vivo. Indeed, MIC assays using PUGNAc, LOGNAc, and gluco-nagstatin in combination with β-lactam antibiotics revealed significant differences between each other. Incubation with PUGNAc results in effects similar to those obtained on incubation with EtBuPUG, but LOGNAc and gluco-nagstatin show no effect on MIC values (data not shown). This result is interesting, since LOGNAc has a $K_I$ value that is only 10-fold lower than that of EtBuPUG (Table 1) and suggests that some inhibitors, such as LOGNAc and gluco-nagstatin, may not gain access to the bacterial cytosol, perhaps due to the barrier presented by the outer membrane of Gram-negative bacteria.

Given that our inhibitors only have $K_I$ values in the single digit micromolar range and only partial blockage of AmpC production is observed, we envision that considerable improvement in inhibitor potency is possible. We anticipate that further structure-guided refinement of the inhibitor will lead to candidates with potential for testing in vivo. Further, recent evidence suggests that, within P. aeruginosa, AmpR acts globally to regulate antibiotic resistance and the production of various virulence factors (71). Accordingly, it is possible that inhibition of NagZ in vivo may disrupt multiple processes regulated by AmpR, thereby significantly attenuating pathogenesis.

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