An aminooquinazoline inhibitor of the essential bacterial cell wall synthetic enzyme GlmU has a unique non-protein-kinase-like binding mode

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INTRODUCTION

Inhibition of cell wall biosynthesis has been a successful approach for developing antibiotics. Both β-lactam-containing compounds and glycopeptides exploit this mode of action and are well established in the clinic. However, the emergence of mechanism-based resistance is an ongoing challenge [1]. Resistance can be overcome in some cases by additional optimization of existing scaffolds [2] and in other cases by combinations with other drugs [3]. Inhibition of novel targets is another way of circumventing existing clinical resistance, and some examples are now entering clinical testing [1].

GlmU is a bifunctional enzyme that converts GlcN-1-P (D-glucosamine-1-phosphate) into UDP-GlcNAc (uridyldiphosphate-N-acetylglucosamine), an essential precursor in lipopolysaccharide and peptidoglycan synthesis in bacteria. Inhibition of GlmU results in a decrease in UDP-GlcNAc available for cell wall biosynthesis, resulting in a loss of cell viability [4,5]. GlmU performs two distinct reactions [6] (Figure 1). The first is the transfer of an acetyl group from acetyl-CoA to glucosamine-1-phosphate producing GlcNAc-1-P (N-acetylglucosamine-1-phosphate) and CoA [5]. This reaction occurs in the C-terminal portion of the polypeptide. Structural studies of Haemophilus influenzae, Escherichia coli and Streptococcus pneumoniae isoenzymes bound to acetyl-CoA have shown that the C-terminal domain assembles as a trimer and the catalytic sites are composed of portions of all three of the monomers at the interface of two subunits and the extended C-terminal tail of the third [7,8]. The second reaction transfers uridylyl monophosphate from UTP to form UDP-GlcNAc and a PP i (pyrophosphate ion). This reaction is contained entirely in the N-terminal domain of each GlmU monomer [5] and is the rate-limiting step of the overall catalytic reaction. Structural studies have shown that UTP and GlcNAc-1-P bind to separate regions of the binding site [7–10]. The kinetic reaction mechanism is ordered, with UTP binding first followed by GlcNAc-1-P, which triggers a conformational change that is required for product formation [10,11].

GlmU is an attractive target for antibacterial drug discovery due to its importance in bacterial cell wall biosynthesis and the absence of any comparable enzyme in humans [12]. Previous publications have shown success in targeting both domains of GlmU with small molecule inhibitors. An allosteric inhibitor of the H. influenzae isoenzyme was identified through a HTS (high-throughput screen) targeting the uridyltransferase domain [13]. There are two structures in the PDB (codes 2W0V and 2W0W) showing quinazoline compounds in the UTP-binding pocket, which are presumably UTP competitive. Inhibitors have also been identified for the acetyltransferase reaction in the C-terminal domain that were either competitive or showed mixed competition with acetyl-CoA [14]. More recently, sulfonamide containing inhibitors against the C-terminal domain of H. influenzae GlmU have been reported [15,16], and several biological methods were used to confirm binding to the acetyl-CoA-binding site. The sulfonamides with antimicrobial activity against an acrB efflux pump mutant of H. influenzae were shown to act via GlmU using strains that overexpress that target. Additionally, resistant mutants were isolated that mapped to the acetyltransferase domain of GlmU [15]. Together, these data provided validation that chemical inhibition of the GlmU enzyme can halt bacterial growth in vitro.

Key words: aminooquinazoline, cell wall biosynthesis, GlmU, high-throughput screening, inhibitor, kinase, N-acetylglucosamine-1-phosphate uridylyltransferase.

Abbreviations used: DTT, dithiothreitol; GlcNAc-1-P, N-acetylglucosamine-1-phosphate; GlcN-1-P, D-glucosamine-1-phosphate; HTS, high-throughput screen; ITC, isothermal titration calorimetry; MIC, minimum inhibitory concentration; PP i, pyrophosphate ion; RMSD, root mean square deviation; SAR, structure–activity relationship; UDP-GlcNAc, uridyldiphosphate-N-acetylglucosamine.

The atomic co-ordinates and structures factors from the present study have been deposited in the PDB under accession code 4E1K.

To whom correspondence should be addressed (email peter.doig@astraZeneca.com).
The present study describes the identification of a series of aminoquinazolines that are UTP competitive. Quinazolines are known hinge binders in kinases [17] and we anticipated analogous interactions in the UTP pocket. However, the crystal structure revealed an unexpected binding mode, with ample scope for future structure-based drug design.

**EXPERIMENTAL**

**GlmU expression and purification**

Genomic DNA and GlmU constructs were prepared from *H. influenzae* and *E. coli* as described previously [15]. The *H. influenzae* glmU was subcloned into pET30a. The *E. coli* glmU was cloned into pZT7#3.3 [18]. Both expression constructs consist of full-length native sequence, with no additional affinity tags. Both *H. influenzae* and *E. coli* GlmU enzymes were expressed in *E. coli* BL21(DE3) cells and purified as described previously [15].

**Compounds**

The compounds used in the present study were either described or synthesized according to methods described previously [19–21].

**Susceptibility testing**

MICs (minimum inhibitory concentrations) were determined using broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute [22].

**Assay conditions**

The standard conditions for compound inhibition assays were as follows: 50 mM Hepes/NaOH (pH 7.5), 5 mM DTT (dithiothreitol), 0.01 M MgCl₂ and 260 pM GlmU trimer. Substrate concentrations were previously [15]. The isoenzyme assay contained 32 μM GlcNac-1-P (Sigma) and 260 pM GlmU trimer. Quantitative UTP depletion and UDP-GlcNAc production were measured by absorbance at 260 nm. Nephelometry was used to estimate the solubility of compounds in the assay buffer [50 mM Hepes-NaOH (pH 7.5) and 2% (v/v) DMSO]. Measurements were made using a Nephelostar (BMG Labtech), and solubility was determined as the point of signal change due to light scattering.

**NMR**

*E. coli* GlmU (5.9 mg/ml) was equilibrated with NMR buffer consisting of 50 mM deuterated Tris/HCl (Cambridge Isotope Laboratories) adjusted to pH 7.6 with HCl, 5% (v/v) ²H₂O and 5 mM deuterated DTT (Cambridge Isotope Laboratories) using Micro BioSpin-6 centrifugal gel-filtration columns (Bio-Rad Laboratories). The concentration of the equilibrated sample was measured using the method of Bradford [24]. Aliquots of the protein were flash frozen on dry ice and stored at −80°C. The ¹H spectra were acquired at room temperature utilizing a Bruker DRX-500MHz spectrometer with a 5 mm room temperature broadband indirect probe. All of the samples were dissolved in 10% ²H₂O and 90% H₂O. Water suppression was accomplished with the use of an excitation sculpting pulse sequence [25,25].

**ITC (isothermal titration calorimetry)**

ITC experiments were conducted at 23°C using a Microcal VP ITC. *H. influenzae* GlmU was formulated at 20 μM in 25 mM Tris (pH 7.5), 50 mM NaCl and 2% DMSO. Approximately 1.4 ml of protein was loaded into the sample cell. Compound 1 was solubilized at 10 mM in DMSO and diluted into the same buffer to 200 μM (final DMSO 2%). A total of 24
injections of ligand were sequentially added to the sample cell, titrating the protein and producing a final compound-to-protein molar ratio of approximately 2.2:1. Thermodynamic parameters $N$ (stoichiometry), $K_0$ (association constant), $\Delta H^\circ$ (enthalpic change) and $T\Delta S$ (entropy change) were obtained using Origin ITC software (v. 6.0, Microcal Software).

**Crystallography**

Purified *H. influenzae* GlmU was crystallized as described previously [13] with minor modification. The protein was buffer exchanged into 10 mM Hepes (pH 7.5), 500 mM NaCl and 0.2 mM TCEP [tris-(2-carboxyethyl)phosphine], and concentrated to 16 mg/mL for crystallization. The compound was solubilized in DMSO to a concentration of 100 mM and diluted into protein to a final concentration of 2.5 mM giving ~8:1 molar excess of ligand to protein. Crystals were grown by vapour diffusion at room temperature. Optimal conditions were obtained in 4 $\mu$L hanging drops containing equal parts of protein and reservoir solution of 1.6–1.8 M ammonium sulfate and 0.1 M Mes (pH 6.1). Crystals appeared overnight, grew over several days and were frozen in cryosolution containing 8 $\mu$L of reservoir solution, 2 $\mu$L of ethylene glycol and 0.5 $\mu$L of the compound solubilized in 100% DMSO at a concentration of 100 mM.

Diffraction data were obtained in-house from a Rigaku FR-E + generator outfitted with a Saturn 944+ CCD (charge-coupled device) detector. Data were integrated and scaled using autoPROC software [26]. The structure was determined by molecular replacement using AMoRe [27] and refined using Refmac [27] and Buster (http://www.globalphasing.com/buster/) with rebuilding in Coot [29]. Water molecules were added to the structure using Buster and removed after visual inspection of the electron density. Final refinement statistics converged with $R_{work} = 0.181$ and $R_{free} = 0.206$.

**RESULTS**

**Hit evaluation summary**

An HTS was performed against the *H. influenzae* GlmU uridylyltransferase reaction using an inorganic pyrophosphatase-coupled Malachite green assay to monitor the release of the by-product phosphate. The screen identified an aminooquinazoline inhibitor, and the series was further defined using closely analogous compounds (Table 1). Compounds had IC$_{50}$ values against *H. influenzae* and *E. coli* GlmU ranging from 160 nM to $>200$ $\mu$M. To exclude false positives, IC$_{50}$ values against *H. influenzae* GlmU uridylyltransferase were verified with an HPLC analog to binding competition experiments were performed between compound 4 and GlcNac-1-P and UTP [31,32]. In the first experiment (Figure 2C), 10 $\mu$M (monomer concentration) E. coli GlmU was added to 50 $\mu$M GlcNac-1-P. In the one-dimensional $^1$H NMR spectrum, a reduction in intensity and broadening of the acetyl methyl proton resonance at $\delta$1.98 ppm was observed due to binding with the protein, as expected. We then added 11 $\mu$M of compound 4 to the NMR tube and observed no increase in intensity/sharpening of the acetyl resonance, indicating that the compound did not displace, and therefore does not compete with, the substrate. In the second experiment (Figure 2D), we added 5 $\mu$M GlmU to 50 $\mu$M UTP and observed broadening of the pyrimidine resonance at 87.85 ppm, demonstrating binding of UTP to GlmU. We then added 25 $\mu$M compound 4 and observed sharpening of the UTP resonances, indicating that the compound displaced UTP from the binding site. This result shows that compound 4 is competitive with UTP.

Additional kinetic characterization of the series with *E. coli* GlmU was undertaken to further confirm the nature of compound binding with respect to the binding of the substrates UTP and GlcNac-1-P. Kinetic experiments were done at several concentrations in triplicate using compound 4, and regression analysis and global fitting of the data were performed with GraFit 5.0 (http://www.eurithacus.com/grafit/). Data were fitted to models of inhibition using Lineweaver–Burk transformations. When the concentration of GlcNac-1-P was varied, compound 4 showed parallel lines in the Lineweaver–Burk plot indicative of uncompetitive inhibition (Figure 2E). In the Lineweaver–Burk plot in which UTP was varied, compound 4 showed lines intersecting on the y-axis indicative of competitive inhibition

**Characterization of binding of aminooquinazoline series to GlmU**

Several experiments were performed to characterize the mechanism of inhibition and the nature of the binding mode. The binding of compound 1 to *H. influenzae* GlmU was confirmed in solution by ITC. The $K_a$ value was determined to be 0.5 $\mu$M with $\Delta H$ of $-12.9 \pm 0.4$ kcal/mol, $T\Delta S$ of $-4.4$ kcal/mol and stoichiometry ($N$) of 1.17 $\pm$ 0.02 (Figures 2A and 2B). The measured $K_a$ value is consistent with the IC$_{50}$ value of 0.27 $\mu$M. The enthalpic contribution is generally indicative of hydrogen bond, electrostatic and van der Waals interactions, whereas the entropic contribution generally reflects hydrophobic interactions [30]. In this case, the binding is mainly enthalpically driven with an unfavourable entropic contribution.

In order to determine with which substrate(s) the aminooquinazoline series competes, one-dimensional $^1$H NMR binding competition experiments were performed between compound 4 and GlcNac-1-P and UTP [31,32]. In the first experiment (Figure 2C), 10 $\mu$M (monomer concentration) E. coli GlmU was added to 50 $\mu$M GlcNac-1-P. In the one-dimensional $^1$H NMR spectrum, a reduction in intensity and broadening of the acetyl methyl proton resonance at $\delta$1.98 ppm was observed due to binding with the protein, as expected. We then added 11 $\mu$M of compound 4 to the NMR tube and observed no increase in intensity/sharpening of the acetyl resonance, indicating that the compound did not displace, and therefore does not compete with, the substrate. In the second experiment (Figure 2D), we added 5 $\mu$M GlmU to 50 $\mu$M UTP and observed broadening of the pyrimidine resonance at 87.85 ppm, demonstrating binding of UTP to GlmU. We then added 25 $\mu$M compound 4 and observed sharpening of the UTP resonances, indicating that the compound displaced UTP from the binding site. This result shows that compound 4 is competitive with UTP.

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### Table 1 Structures and biochemical properties of aminoquinazoline inhibitors of GlmU uridylytransferase

NT, not tested.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>( H. \text{influenzae } I_{C_{50}} ) (( \mu \text{M} ))</th>
<th>( E. \text{coli } I_{C_{50}} ) (( \mu \text{M} ))</th>
<th>Nephelometry (( \mu \text{M} ))</th>
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<td>1</td>
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<td>( 1.30 \pm 0.3 ) (( n = 2 ))</td>
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<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
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<td>11 (( n = 1 ))</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>( 1.8 \pm 0.9 ) (( n = 3 ))</td>
<td>10.1 (( n = 1 ))</td>
<td>( &gt; 200 )</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>( 0.16 \pm 0.02 ) (( n = 6 ))</td>
<td>0.21 (( n = 1 ))</td>
<td>( &gt; 200 )</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>( 0.46 \pm 0.1 ) (( n = 2 ))</td>
<td>( 0.46 \pm 0.07 ) (( n = 2 ))</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>( &gt; 200 ) (( n = 1 ))</td>
<td>NT</td>
<td>200</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Structure 7" /></td>
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<tr>
<td>8</td>
<td><img src="image8.png" alt="Structure 8" /></td>
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<td>( &gt; 200 ) (( n = 1 ))</td>
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<td><img src="image11.png" alt="Structure 11" /></td>
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</table>
Structure determination of compound 1 bound to GlmU

To define the molecular interactions, crystallographic studies of compound 1 with H. influenzae GlmU were undertaken. The structure of the inhibitor bound to GlmU was determined by molecular replacement from in-house diffraction data at 2.0 Å (1 Å = 0.1 nm) using a previously reported structure (PDB code 2VD4) as a search model. Clearly defined electron density was visible in the initial difference maps for the expected inhibitor. The inhibitor was readily modelled into the density and refined (Figure 3). An additional strong difference density peak in the active site was modelled as a sulfate ion. The data collection and refinement statistics are found in Table 2.

GlmU has two distinct domains, each of which is responsible for a separate biochemical reaction (Figure 4A). The acetyltransferase reaction occurs in the C-terminal domain. This C-terminal domain assembles as a trimer to form a pedestal-like structure composed of an extended β-helix [34]. Catalysis occurs at the interface of the subunits. The uridylyltransferase reaction occurs in the N-terminal domain (Figure 4A).

The uridylyltransferase domain undergoes several changes in conformation from the apo structure (PDB code 2V0H) to the product-bound structure (PDB code 2V0I) [11,13] (Figure 4B). The apo- and UTP-bound structures have an open active-site conformation, whereas in the product-bound structure, the active site has contracted and become more closed. This closure involves a rigid body movement of the N-terminal lobe (residues 131–170) (Figure 4B). It was previously postulated that GlcNac-1-P binding induces conformational changes at this subsite in order to create the proper stereochemical environment needed for attack of the α-phosphate group of UTP [11]. This movement drives the reaction by bringing the two substrates into close proximity for catalysis. These movements are further illustrated by comparison of the compound-1-bound structure with previously reported GlmU structures (apo-, UDP- and UDP-GlcNac-bound forms) (Table 3). Determination of the RMSDs (root mean square deviations) for each comparison indicated that the uridylyltransferase domain of the compound 1 inhibitor-bound structure more closely resembles the apo- and UDP-bound forms than the product-bound GlcNac-UDP structure. In all of the cases, the acetyltransferase domains superimpose very closely. The conformational changes are even more dramatic in the S. pneumoniae isoenzyme (results not shown), with similar movements of the upper N-terminal lobe and constriction of the active site, but also additional rearrangement of the loop 184–196 to close over the active site.

Compound 1 skirts the perimeter of the GlcNac-1-P-binding pocket, with the quinazoline core occupying the binding pocket for the ribose sugar of UTP (Figure 5A), consistent with the kinetic and NMR data. The quinazoline core packs on top of Leu11 and Tyr103, where the ribose from UTP would otherwise bind. The 7-hydroxyl forms a key hydrogen bond interaction with the backbone amide of Ala13. This hydrogen bond is also seen with the 2-oxygen in the uridine ring of UTP. This is the only hydrogen bond interaction shared between substrate and inhibitor (Figure 5A). A bifurcated hydrogen bond interaction is observed between the aniline-nitrogen and the side chain of Asp90 (Figure 5A). An additional hydrogen bond is formed between the benzamide nitrogen and the backbone carbonyl of Val213 (Figure 5A). The number of key hydrogen bond interactions observed is consistent with the strong binding enthalpy measured by ITC.

With compound 1 bound, the structure of H. influenzae GlmU more closely resembles the open, apo, form of the enzyme than the closed, product-bound, form, with some notable exceptions. Loop 75–81 forms the backside of the UTP pocket. In the structure with compound 1, Gln79 is located in the pocket to form hydrogen bond bridging interactions between Gln76 and Gly81 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460405add.htm). As a result, part of a pocket that would normally have been occupied by UTP has been filled by protein residue side chains. When UTP or product is bound, Gln79 moves out of the way, allowing Gln76 to form both donor and acceptor hydrogen bond interactions with UTP (Supplementary Figure S1). Efforts to optimize compound 1 to engage Gln76 could be a possible rationale for improving the inhibitory potency of the series.

Structural rationale for the observed SAR

Some of the key observations can be explained with regard to the left-hand side aminooquinazolines ring, central aromatic linker and right-hand side benzamide. With the left-hand side aminooquinazoline, a methyl substituent at the 7-hydroxy position (compounds 1 and 2) would be expected to decrease potency of the compound since it would weaken the hydrogen bond interaction with Ala13 (Figure 5A). This is reflected in a 13-fold loss in potency of inhibition of H. influenzae GlmU. Complete removal of the quinazoline moiety (compounds 6, 7 and 8) resulted in a loss of inhibition, indicating its importance as an anchor in the active site.

No specific interactions between the protein and the central aromatic linker were observed. This is consistent with the similar inhibitory potencies of compounds containing either the 1,4-diaminophenyl core (compounds 1–3) or the 2,5-diaminopyrimidine core (compounds 4 and 5).

The right-hand side phenyl ring of the benzamide sits in a lipophilic pocket and the crystal structure suggests that some substitution there would be tolerated (Figure 5A). Substitution of this phenyl group (compound 2) with pyridine (compound 3), or addition of halogens (compounds 4 and 5), had little effect on potency. Increasing the lipophilicity with Cl/F substitutions (compound 4) slightly improved potency, with CI being preferred.
Figure 2 Characterization of the binding and mode of inhibition of compound 1 compared with GlmU

(A) Binding of compound 1 to H. influenzae GlmU measured by ITC. Compound 1 was titrated into GlmU and the heats were measured. (B) Integrated enthalpies were fit to a one-site-binding model, giving the enthalpy \( \Delta H \), binding constant \( K_a \), stoichiometry \( N \) and the entropy \( \Delta S \). (C) NMR spectra showing compound 4 is not competitive with GlcNAc-1P. The peak at \( \delta 1.98 \) ppm represents the acetyl methyl protons of GlcNAc-1P. a, buffer; b, 50 \( \mu \)M GlcNAc-1P in buffer; c, addition of 10 \( \mu \)M GlmU; and d, addition of 11 \( \mu \)M compound 4. (D) NMR spectra showing compound 4 is competitive with UTP. The doublet at \( \delta 7.85 \) ppm is the pyrimidine proton of UTP. A, 50 \( \mu \)M UTP; b, addition of 5 \( \mu \)M GlmU (monomer concentration); and c, addition of 25 \( \mu \)M compound 4. (E) Lineweaver–Burk plots. Plotting the inverse of the rates at fixed concentrations of UTP as a function of GlcNAc-1P gives parallel lines indicating that compound 4 is uncompetitive with GlcNAc-1P. (F) When plotted as a function of UTP with fixed concentrations of GlcNAc-1P, the lines intersect on the y-axis indicating that compound 4 is competitive with UTP.

at the meta (4) rather than para (5) position. Modification of the benzamide linker from an amide to a more flexible alkoxyimethylene (compound 10) results in a 10-fold loss of potency. This substitution would eliminate a hydrogen bond interaction seen from the amide nitrogen and the backbone carbonyl of Val\(^{223} \) (Figure 5A). Removal of the benzamide (compound 9) eliminates both the lipophilic interaction and the hydrogen bond to Val\(^{223} \). Not surprisingly, compound 9 is completely inactive.

DISCUSSION

The enzymes involved in biosynthesis of the bacterial cell wall are important and attractive targets in drug discovery. Inhibition of GlmU causes loss of cell viability [4,5], and the lack of corresponding enzymes in humans makes this target attractive from a safety perspective. An HTS to find inhibitors of the enzymatic activity of the N-terminal domain of GlmU identified a series of aminoquinazolines. Enzymatic and biophysical characterization showed that the compounds are competitive with UTP, but not GlcNAc. The enzyme–inhibitor co-crystal structure showed that the compound occupies part of the UTP-binding pocket, locking the catalytic site in an open conformation and blocking UTP binding.

Upon binding of the second substrate (GlcNAc-1-P), the enzyme undergoes a conformational change to a closed form (Figure 4B). This closure constrains the lipophilic pocket where the right-hand side benzamide tail sits. By occupying this pocket, compound 1 locks GlmU in the open conformation, which is incompatible with enzyme catalysis. Meanwhile, the left-hand quinazoline head group prevents binding of UTP. It is expected that by maintaining and optimizing the two sides of this lead series improved target potency can be achieved.
Table 2  Data collection and refinement statistics for GlmU bound to compound 1

<table>
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<tr>
<td>α, β, γ (°)</td>
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<tr>
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<td>I/σI</td>
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<td>Multiplicity</td>
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Refinement

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</tr>
<tr>
<td>Disallowed</td>
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</table>

| Values in parentheses are for highest-resolution shell. $R_{merge} = \frac{\sum_{hkl} \sum_{i} (|F_{i}(hkl)| - |F_{obs}(hkl)|)}{\sum_{hkl} \sum_{i} |F_{i}(hkl)|}$. $R_{work}$ is the cross-validation R factor computed for the test set of 5% of unique reflections. |

Figure 3  Electron density on the quinazoline inhibitor

Data collected in-house yielded data that were processed to 2.0 Å resolution. The quinazoline compound is highlighted in magenta. An additional strong difference density peak in the active site was modelled as a sulfate ion (arrow). Protein side chains are shown in grey and waters as red spheres. The $2F_{o} - F_{c}$ electron density map is displayed as a blue mesh and contoured at 1 s. The overall clarity of the map allowed for the unambiguous placement of the inhibitor in the active site.

Figure 4  X-ray crystal structure of the GlmU monomer from H. influenzae

(A) GlmU is a bifunctional enzyme with the acetyltransferase reaction occurring in the C-terminal domain and the uridyltransferase reaction occurring in the N-terminal domain. The bound inhibitor, compound 1, is highlighted in magenta. (B) Grey shows the compound 1 bound form of the enzyme, and green shows the product-bound form (PDB code 2V0I). The product is shown with green carbon atoms. During catalysis, the active site constricts from an open to a closed conformation. This involves movement of several loops, as indicated by the arrows. Compound 1 binds to the open form that is almost identical to the apo-enzyme.

Quinazoline-containing compounds often exhibit inhibitory activity against protein kinases by competition with ATP. The quinazoline moiety binds where the adenine base of ATP engages the enzyme. The 1-nitrogen of the quinazoline is an hydrogen bond acceptor from the backbone nitrogen of the kinase hinge motif (Figure 5B) [17]. This hinge interaction mimics that of the nucleotide. Given our kinetic and NMR data establishing the aminoquinazoline series as ATP competitive, it was anticipated that the quinazoline core would bind in the base-binding region of the UTP-binding site and mimic interactions seen by the uridine ring. Surprisingly, our crystal structure revealed that none of these interactions were made. Instead, the quinazoline ring binds in the ribose binding site of GlmU. The only interaction made in the UTP pocket with this series is the hydrogen bond between the 7-position hydroxyl and Ala13, also formed by the 2-oxygen in the uridine ring of UTP (Figure 5A).

The presence of the quinazoline in this scaffold may raise selectivity concerns due to the potential for inhibition of kinases present in humans. However, the binding mode offers scope to mitigate this concern by removal of the 1-nitrogen from the quinazoline core. This would likely eliminate kinase binding with little impact on GlmU binding.

Three inhibitor-bound structures with GlmU have been reported (PDB codes 2W0V and 2W0W, and [13]). In two of these co-crystal structures, the ligand is a quinazoline (compound 11). Interestingly, the central linker and lipophilic right-hand side of
Table 3 Comparison of the compound 1-bound structure of *H. influenzae* GlmU using RMSD analysis to the respective apo-, UDP- and UDP-GlcNAc-bound structures

<table>
<thead>
<tr>
<th></th>
<th>Apo (PDB code 2V0H)</th>
<th>UDP (PDB code 2V0K)</th>
<th>GlcNac-UDP (PDB code 2V0I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridyltransferase domain</td>
<td>0.566</td>
<td>0.563</td>
<td>1.330</td>
</tr>
<tr>
<td>Acetyltransferase domain</td>
<td>0.449</td>
<td>0.322</td>
<td>0.432</td>
</tr>
<tr>
<td>Overall</td>
<td>0.584</td>
<td>0.537</td>
<td>1.682</td>
</tr>
</tbody>
</table>

our series is replaced by a small acidic trifluoro sulfonamide or a proton at the 4-quinazoline position. When we overlay these quinazoline structures with others (Figure 5C), our ligand is shifted and flipped in the binding site relative to the previously reported compounds. The 2-position of the quinazoline ring superimposes with our 4-position. It seems that the substitution pattern at the 2- and 4-positions may dictate the binding mode of the quinazoline core.

As a result of this alternative binding mode there are a number of differences in their interactions. Although it would not be expected to form a hydrogen bond interaction at physiological pH, Asp105 is within hydrogen bond range of the 1-nitrogen of the quinazoline core in the earlier reported structures. In contrast, the nitrogen atoms in our core do not form any specific interactions (Figures 5A and 5C). Another difference is the presence of a hydrogen bond between the carbonyl of the tetrahydronapthyridinone substituent (compound 11) and the backbone amide of Gly225, whereas our compound forms the hydrogen bond to the backbone carbonyl of Val223. Finally, there is an isobutyl group of compound 11 occupying the lipophilic pocket instead of an aromatic ring. The inorganic sulfate in our crystal structure is in the same vicinity as the trifluoro sulfonamide in the previously reported structure (Figure 5C), suggesting a strategy to build further potency into the series. Substitution at this 2-position could enhance potency by accessing the GlcNAc-1-P-binding site.

An allosteric inhibitor of GlmU was reported (IC$_{50}$ = 18 μM) with a completely different binding mode than our quinazoline series (Figure 5D, compound 12) [13]. This compound binds outside both the GlcNAc and the UTP pockets. The only similarity to our compound is that the phenyl substituent binds in the lipophilic pocket with a very similar binding mode. By occupying the lipophilic pocket, the allosteric inhibitor locks the enzyme in the open conformation, thereby inhibiting the conformational changes to the closed conformation, necessary for catalysis.

Compound 1 and other members of the aminoquinazoline series inhibit isoenzymes of both Gram-negative species tested, *H. influenzae* and *E. coli*. In contrast, there was no measurable inhibition of isoenzymes of the Gram-positive species *S. pneumoniae* or *Staphylococcus aureus* (results not shown). The difference in potency between Gram-negative and Gram-positive isoenzymes is not surprising given the comparatively low sequence conservation in the N-terminal domains of GlmU between Gram-positive and Gram-negative bacteria, including some insertions and deletions that impact the tertiary conformations of various loops (Supplementary Figures S2 and S3 at http://www.BiochemJ.org/bj/446/bj4460405add.htm). The overall amino acid sequence identity between *H. influenzae* and *S. pneumoniae* GlmU is only 43 %, whereas that between *H. influenzae* and *E. coli* is 65 %. Comparison of the *S. pneumoniae* apo GlmU structure with GlmU from *H. influenzae* reveals several amino acid sequence differences that would be expected to impact binding affinity. Tyr103 forms a key

Figure 5 Details of the *H. influenzae* GlmU active site

(A) Compound 1 (magenta) superimposed on the product UDP-GlcNAc (green). Hydrogen bonds are indicated by broken lines. The quinazoline core superimposes on the ribose sugar. One hydrogen bond is conserved between the ribose sugar and the 7-hydroxyl of the quinazoline. A sulfate ion was also modelled into difference density, but is not shown in the Figure for clarity (see C and D). (B) Illustration of how quinazolines bind to the hinge region of kinases (PDB codes 2ITX and 2ITY). In this case, gefitinib (magenta) is shown bound to EGFR kinase, superimposed on where ATP (green) binds. There is one hydrogen bond from the quinazoline to the backbone of the hinge, whereas ATP forms two. (C) Compound 1 superimposed on the quinazoline (pink, compound 11) found in PDB code 2W0W. The quinazoline cores are flipped relative to each other. Arrow indicates position of the sulfate ion. (D) Compound 1 superimposed on an allosteric inhibitor (pink, compound 12) of GlmU [13]. The only region that overlaps is the benzyl ring in the lipophilic pocket. Arrow indicates position of the sulfate ion.
packing interaction in H. influenzae and this residue is an alanine residue in the S. pneumoniae structure. In addition, the lipophilic pocket in S. pneumoniae GlmU is smaller and is not expected to accommodate the benzamide side chain without further rearrangement. Therefore differences seen in the active site and current SAR of the series suggest it would be difficult to produce broad-spectrum inhibition with this scaffold, but it should be feasible to obtain more potent inhibition of both Gram-negative isoenzymes. In conclusion, the present study reports the discovery of a potent quinazoline series that inhibits GlmU. Although the compounds are UTP competitive, they possess a unique binding mode compared with structurally similar inhibitors binding to protein kinases and demonstrate that similar compounds can have significantly different action against different targets.

AUTHOR CONTRIBUTION
All of the authors participated in the writing of this paper. Peter Doig and Tory Nash carried out the biochemical studies and developed the enzyme assays. Adam Shapiro and Camil Jouban performed the NMR studies. Carolyn Blackett was responsible for performance of the HTS and analysis of screening results. Nicholas Larsen was responsible for the crystalization experiments and, with F. Ann Borack-Sjodin, was responsible for the analysis and interpretation of these results. Marshal Morningstar and Arthur Patten performed chemical evaluation and analysis of the HTS data as well as compound synthesis.

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28 Reference deleted
33 Reference deleted
SUPPLEMENTARY ONLINE DATA

An aminoquinazoline inhibitor of the essential bacterial cell wall synthetic enzyme GlmU has a unique non-protein-kinase-like binding mode

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Figure S1  Detailed structural rearrangements in the UTP pocket

In the compound 1-bound structure (magenta, grey = GlmU), GlmU resembles the open form of the enzyme. Gln79 donates a hydrogen bond to Gln76. In the UTP- and/or product-bound structure (green), Gln79 moves out of the way allowing Gln76 to form donor and acceptor hydrogen bond interactions with UTP. Arrows indicate movements, and broken lines indicate hydrogen bond interactions.

The atomic co-ordinates and structures factors from the present study have been deposited in the PDB under accession code 4E1K.

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Figure S2 ClustalW sequence alignment of H. influenzae and E. coli GlmU

Blue lettering is identical, green is similar and red is dissimilar. The N-terminal uridylyltransferase domain is 65% identical and 25% similar.

Figure S3 ClustalW sequence alignment of H. influenzae and S. pneumoniae GlmU

Blue lettering is identical, green is similar and black is dissimilar. The N-terminal uridylyltransferase domain is 43% identical and 26% similar.

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