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Screening-based discovery of the first novel ATP competitive inhibitors of the *Staphylococcus aureus* essential enzyme UMP kinase

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ABSTRACT

UMP kinase (PyrH) is an essential enzyme found only in bacteria, making it ideal as a target for the discovery of antibacterials. To identify inhibitors of PyrH, an assay employing *Staphylococcus aureus* PyrH coupled to pyruvate kinase/lactate dehydrogenase was developed and was used to perform a high throughput screen. A validated aminopyrimidine series was identified from screening. Kinetic characterization of this aminopyrimidine indicated it was a competitive inhibitor of ATP. We have shown that HTS can be used to identify potential leads for this novel target, the first ATP competitive inhibitor of PyrH reported.

1. Introduction

Nucleoside monophosphate (NMP) kinases are required for the biosynthesis of nucleic acids and various key nucleotidyl intermediates and for energy metabolism. These enzymes catalyze the transfer of the γ-phosphate of ATP to the β-position of the nucleoside monophosphate.

One member of the NMP kinase family, UMP kinase (PyrH) has a number of properties that make it an ideal target for intervention with a antimicrobial agent. Firstly, this enzyme has been shown to be essential in bacteria [1–4]. Secondly, it is ubiquitous; being found in all major pathogens, including Mycoplasma spp., thus having the potential as a target for broad-spectrum inhibitors. Thirdly, this UMP kinase is unique to bacteria, having low sequence homology and unique substrate specificity compared to that of eukaryotes [5].

Bacterial UMP kinases, unlike what has been observed with human UMP/CMP kinase, are homohexamers (UMP/CMP kinase is a monomer) that exhibit allosteric regulation [6,7]. Enzyme activity is modulated by a number of NXPs with the extent and type of regulation (positive or negative) being dependant on the particular isozyme. In general GTP is the most potent activator, whereas UTP can inhibit enzyme activity completely [6,7]. The mechanism by which these GTP and UTP modulate the activity of UMP kinase is not completely understood, but recent structural and kinetic studies of the *Escherichia coli* enzyme have shed some light on these effectors [8,9]. It is known that the presence of or the binding of both UTP and GTP increase the solubility of the enzyme and, in the case of *Streptococcus pneumoniae*, affects the dissociation constants of the subunits to favor the hexameric rather than monomeric/dimeric state of the enzyme [10]. GTP has been shown to bind to the subunit interface causing structural changes that may account for the change in the apparent affinity of the enzyme for ATP [9,11–13]. In contrast, UTP may be binding to the active site, at the ATP pocket, or additionally the UMP pocket, resulting in inhibition of substrate binding directly [8,14]. UMP kinases derived from Gram-positive bacteria exhibit considerable allosteric activation by GTP, while, generally, those from Gram-negative species are not as highly affected [6–8]. This difference in regulation might be indicative of fundamental differences between the two groups of bacteria and highlight the possibility that inhibitors active against only one group of pathogens may be identified.

Whereas considerable structural and mechanistic characterization of UMP kinase from *E. coli* has been carried out [6–9,11,14–18], UMP kinases of Gram-positive origin, especially the important pathogen *Staphylococcus aureus,* have received less attention [4,8,12,19]. Only one non-substrate analogue-based inhibitor of this enzyme has been reported to date, and that report did not demonstrate specificity or characterize the putative inhibitor [20]. Here we describe the characterization of the UMP kinase from *S. aureus* and identify, for the first time, a specific, ATP competitive, small molecule inhibitor of this enzyme discovered using high throughput screening (HTS).

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2. Materials and methods

2.1. Cloning of S. aureus UMP kinase (pyrH)

S. aureus pyrH was cloned from strain 601055. Primers INF 5174 (5′) (CCCATATGGCTCAAATTTCTAAATATAAACG) and INF 5183 (3′) (CGGGATCCATTATTTTGTAATTAACGTACCTATC) were used to obtain the *S. aureus pyrH* PCR product from a cell lysate of *S. aureus* 601055. The PCR product was purified using the Qiagen purification kit and ligated into the TOPO Invitrogen cloning vector. The ligated product was transformed into TOPO cells according to the manufacturers recommended method. Transformants were selected and the plasmid recovered using the Qiagen miniprep kit, according to the recommended method. Presence of the correct size DNA insert was verified by digestion with BamH1 and Nde1 restriction enzymes. Clones with the correct size insert, containing *S. aureus pyrH*, were selected and DNA prepared using the Qiagen Maxiprep kit. Presence of the insert was rechecked by BamH1/Nde1 digestion. The insert was sequenced to assure its identity.

The *S. aureus pyrH* insert was cloned into *E. coli* pT73.3 and transformed into BL21 (DE3). These cells were grown in LB media at 30 °C, containing tetracycline (10 μg/mL) and expression induced by addition of IPTG to a final concentration of 1 mM. Induced cells expressed a protein with an apparent molecular mass of 26,000 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), consistent with the size expected for the polypeptide. Cell paste was collected by centrifugation and stored at −20 °C until use.

2.2. Purification of S. aureus UMP kinase

The cell paste was suspended in 50 mL of Lysis Buffer (50 mM Tris–HCl, pH 8.5, 2 mM EDTA, 2 mM DTT, 2 mM UTP). Cells were disrupted by passing them twice through a French press operated at 18,000 psi, and the crude extract was centrifuged at 20,000 rpm (45Ti rotor, Beckman) for 30 min at 4 °C. The supernatant was loaded onto a 20 mL Q-Sepharose HP (HR16/10) column (Pharmacia) pre-equilibrated with Buffer A (50 mM Tris–HCl, pH 8.5, 2 mM EDTA, 2 mM DTT, 2 mM UTP). The column was then washed with Buffer A, and the protein was eluted by a linear gradient from 0 to 1 M NaCl in Buffer A. Fractions containing UMP kinase were pooled, and solid $(NH_4)_2SO_4$ (0.4 g/mL) was added to precipitate the proteins. The sample was centrifuged at 11,000 rpm for 30 min at 4 °C (JA12 rotor, Beckman), the pellet was then dissolved in 5 mL of Buffer A. The 5 mL sample was applied to a 320 mL Sephacryl S-300 (HR 26/60) (Pharmacia) pre-equilibrated with 50 mM Tris–HCl, pH 8.5, 2 mM EDTA, 2 mM DTT, 2 mM UTP, 150 mM NaCl. The fractions containing UMP kinase were identified by SDS–PAGE, were pooled and dialyzed against 1 l 50 mM Tris–HCl, pH 8.5, 0.1 mM EDTA, 1 mM UTP, 150 mM NaCl, 2 mM DTT, 20% (vol/vol) Glycerol. The protein was characterized by SDS–PAGE analysis and integration of the band intensity indicated it to be 90% pure. Analytical LC-MS was used to determine mass of the protein and indicated that the N-terminal methionine of the polypeptide predicted from the DNA sequence was not present [observed MW = 26013.0 Da (-Met), expected = 26014.4 Da (-Met)]. The protein was flash frozen in aliquots using liquid nitrogen and stored at −80 °C.

2.3. Assay for S. aureus UMP kinase

The assay was optimized to yield maximum specific activity within the constraints of desirable kinetic parameters. Assay parameters investigated included enzyme handling, substrate concentrations, salt requirements and effects, pH and buffer selection, temperature, stability of the reaction over time, linearity of the reaction over time, response of the assay to DMSO, coupler enzyme concentration and activity, and robustness of the assay.

To perform the assay, a 1.67X assay buffer stock solution was prepared such that the reaction would contain final concentrations of 50 mM Hepes pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 6.6 units/mL PK/LDH, 1 mM phosphoenolpyruvate, 0.4 mM ATP, 0.2 mM NADH, 1.29 nM *S. aureus* UMP kinase, 0.5 mM GTP and 0.001% (wt./vol) Brij-35. Prior to addition to this mixture, a 10X enzyme working stock solution containing 12.9 nM solution of *S. aureus* UMP kinase in 50 mM Hepes pH 8.5, 100 mM KCl, 5 mM GTP, 0.01% (wt/vol) Brij-35 was prepared and incubated for 15 min at room temperature. Assay plates were prepared by dispensing an appropriate volume of the 1.67X assay buffer stock to reaction vessels, adding compound DMSO stock solutions and incubating for at least 15 min at room temperature to allow equilibration of reagents before initiating reaction with a 3.33X UMP stock solution. For example, for a reaction volume of 100 μL, 2 μL of 50× compound in DMSO was added to 60 μL 1.67× buffer/enzyme mixture and mixed either by rotary shaking or pipetting. After an appropriate incubation time, the reaction was initiated with the addition of 40 μL 0.67 mM UMP (final concentration was 0.2 mM). Controls used were 2% DMSO (no inhibition) and 10 mM EDTA (100% inhibition). Blank reads were performed and reaction progress was monitored by following the decrease in NADH absorbance or fluorescence over time by either endpoint or continuous read methods. To facilitate testing large numbers of compounds, in some instances, reactions were quenched after 30 min with 10 mM EDTA and the final spectrophotometric measurements made up to several hours later.

2.4. High throughput screening

The assay was formatted to run in 384 well clear plates and subjected to screening validation. Briefly, to wells with added compounds (5 μL) or controls (100% inhibited value: 50 mM EDTA; 0% inhibited value: 0.1% (vol/vol) final DMSO final concentrations), 30 μL of assay buffer stock solution was added and incubated for 15 min at room temperature to allow equilibration of reagents before initiating the reaction with the initiation solution (15 μL). After an incubation of 60 min at room temperature, the absorbance of each well was determined at 340 nm.

The final concentration of compounds in the screening buffer was 10 μM with a final DMSO concentration of 0.1 (vol/vol) DMSO. Prior to screening the AstraZeneca compound collection, the assay was used to screen a compound validation set (9984 compounds). This set was run in duplicate (reverse order of plates) and examined for plate effects (well position on the plate and plate position within the screening run) and to estimate an expected hit rate. Calculation of Z' was used to assess the performance of the assay in the validation set and during the HTS against the complete compound library.

2.5. Follow up assays

An HPLC based assay was developed for confirmation of HTS actives. The UMP kinase assay was run as described above, without addition of PK/LDH coupling reagents or NADH. The reaction was quenched by 1:1 addition of 50 mM NaH_2PO_4/Na_2HPO_4 and pH adjustment to pH 2.8 with acetic acid. 10 μL was injected onto a Vydac 3021IC4.6 ion-exchange column (4.6 mm by 100 mm), running at a flow rate of 2 mL/min at 22 °C, equilibrated with 25 mM NaH_2PO_4/Na_2HPO_4, adjusted to pH 2.8 with acetic acid (Buffer A). An elution gradient of 125 mM NaH_2PO_4/Na_2HPO_4, adjusted to pH 2.9 with acetic acid (Buffer B), was run. The gradient was as follows: 2 min at 0%B, then linear from 0% to 100%B in 8 min, hold

100% for 2 min and return to 0%B in 1 min. Quantitative UDP and ADP production were monitored at 260 nm.

2.6. PK/LDH coupler artifact assay

Compounds were tested for their ability to inhibit the PK/LDH coupler by generating a 7 point dose response curve (final compound concentration of: 0.1 μM–100 μM). The DMSO concentration was not equalized throughout the separate concentration points when the compound solution (in 100% DMSO (vol/vol)) was diluted in water to generate the response curve and ranged from 0.001% to 1.0% (vol/vol).

Assay plates were prepared by adding appropriate diluted compound solutions (5 μL), dispensing an appropriate volume of the assay buffer stock to each well (30 μL of 83.3 mM Hepes pH 7.5, 83.3 mM KCl, 3.33 mM $MgCl_2$, 0.4 units/mL PK/LDH, 0.33 mM NADH, 1.7 mM phosphoenolpyruvate, and 0.0017% Brij-35), and incubating for at least 15 min at room temperature to allow equilibration of reagents before initiating the reaction with an initiation solution (15 μL of 0.33 mM ADP). After an incubation of 60 min at room temperature the absorbance of each well was determined at 340 nm.

2.7. Kinetic characterization of S. aureus UMP kinase

The coupled PK/LDH assay, as described above, was used for all kinetic measurements. Full kinetic characterization was performed by collecting initial velocity data for an 8 by 12 matrix of substrate concentrations (ATP and UMP respectively), where each substrate was varied. All data were taken in triplicate. Nonlinear regression analysis was performed with GraFit 5.0 [21]. The data were fit using nonlinear regression analysis according to the steady-state equations for Bi-Bi reactant systems. Data fitting was initiated using a matrix of estimated values for each parameter. Goodness of fit was determined by examination of the χ^2 for each curve and employing an *F*-test to assess statistical differences between the fits.

2.8. Mode of inhibition studies

Using the assay format for the kinetic analyses described above, inhibitor was added to each plate set at a fixed concentration (6.25, 12.5, 25 and 50 μM). At each concentration of inhibitor, data was collected varying either the UMP concentration while the ATP was fixed at 2.0 mM or the ATP concentration while the UMP was fixed at 0.7 mM.

Initial velocity data were collected in triplicate. Data were analyzed by global fit analyses to models of competitive, mixed, and uncompetitive inhibition. Data fitting was initiated using a matrix of estimated values for each parameter and goodness of fit determined. Goodness of fit was determined by examination of the Chi^2 for each curve and employing an *F*-test to assess statistical differences between the fits.

3. Results and discussion

3.1. Characterization of S. aureus UMP kinase

S. aureus was cloned and expressed well under the conditions described. However, during purification, the enzyme tended to aggregate at higher concentrations and analysis using analytical ultracentrifugation revealed a considerable heterogeneity in the enzyme form (monomer to many multimers). Addition of UTP to the purification buffers and storage solutions resulted in a near homogeneous enzyme form as measured by analytical ultracentrifugation and DLS, consistent with a hexameric state (data not shown). It was found that the protein was stable when stored at −80 °C for several months.

Here, a robust HTS assay was developed for screening *S. aureus* UMP kinase. UMP kinase catalyzes the transfer of the γ-phosphate of ATP to UMP, resulting in the formation of two products, ADP and UDP (Fig. 1). This activity was monitored using a pyruvate kinase/lactate dehydrogenase (PK/LDH) coupling reaction or HPLC. *S. aureus* UMP kinase gave linear progress curves when measured in the coupled PK/LDH assay or the HPLC assay. Specific activity was unchanged by the concentration of the enzyme in the assay and activity was independent of the detection method employed. The detection of turnover using the coupled assay allowed higher throughput and was thus more amenable for HTS. Since both UDP and ADP are substrates for pyruvate kinase, the consumption of one mole of UMP resulted in the production of two moles of NAD^+, thus amplifying the signal two fold for every turnover event, a further benefit of this detection (Fig. 1).

Characterization of *S. aureus* UMP kinase revealed a requirement for GTP to be present in the assay for maximum activation. Kinetic characterization of *S. aureus* UMP kinase was carried out under conditions of saturating GTP. Under these conditions, no cooperativity in ATP binding was observed, similar to what has been reported for *S. pneumoniae* [4,8]. This simplified the characterization and analysis of the enzyme and resulted in more robust conditions for the screening assay.

Steady-state analyses of the enzyme were performed to determine the K_m and V_{max}. The data were fit to several BiBi mechanistic equations for steady state ordered (including substrate inhibition for dead-end inhibition by substrate b of substrate a), and random (including substrate inhibition for dead-end inhibition by substrate b, random BiBi competitive inhibition and random BiBi competition with the activator). Models for simple ordered or random BiBi mechanism provided the best fits and were undistinguishable, giving similar kinetic constants (Fig. 2A and B). However, calculated K_ms for the substrates differed two fold between the models. At the time of the HTS the mechanism was not further resolved, and the kinetic parameters for the random mechanism were used for optimization. These parameters represented a more stringent situation on which to base assay substrate concentrations (i.e. limit substrate concentrations for the detection of competitive inhibitors).

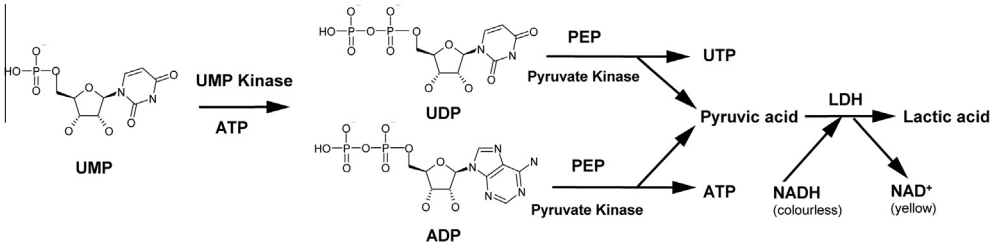
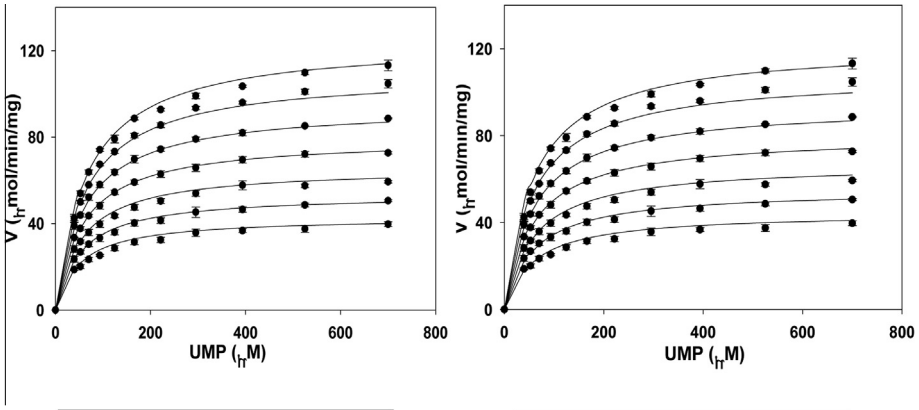


Fig. 1. Reaction of UMP kinase and detection by the coupled pyruvate kinase/lactate dehydrogenase assay.



Random BiBi	
K_m UMP	43±4
K_m ATP	742±102
V_{max}	219±6
α	2.0±0.4

Ordered BiBi	
K_m UMP	86±7
K_m ATP	1485±68
V_{max}	219±6
K_{ia}	43±4

Fig. 2. Velocity curves for *S. aureus* UMP kinase fit to a steady state random mechanism (A) or ordered mechanism (B). Data fit both models equally well.

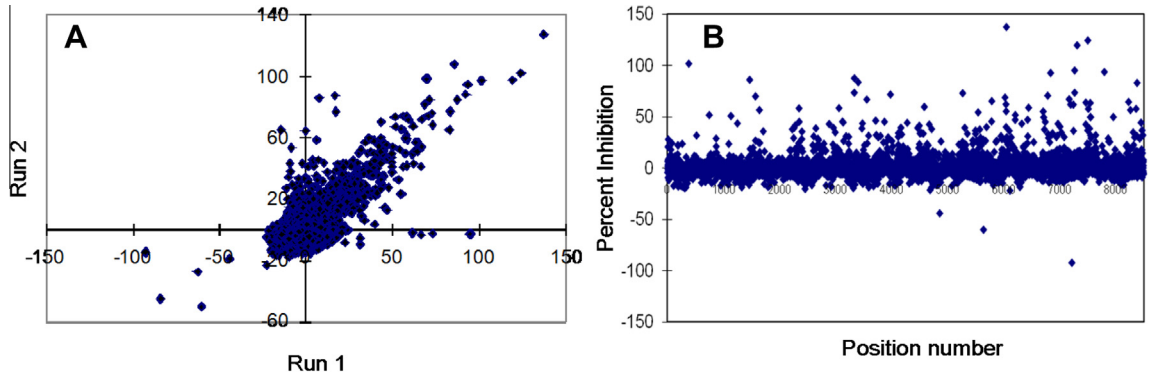


Fig. 3. Assay performance using a compound validation set. The optimized assay was validated using a compound set (9984 compounds) run in duplicate (reverse order of plates). The HTS assay did not exhibit position in run effects, showing good intra run correlation (A). In addition there was no observed effect on position in run with respect to actives (B).

3.2. Assay development and optimization

The *S. aureus* UMP kinase PK/LDH assay was optimized for a number of parameters including, buffer, salts, coupler concentration, temperature effects, and stability. These results are summarized in the Supplementary materials.

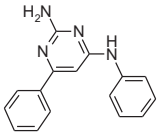
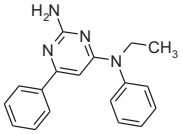
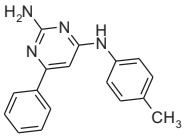
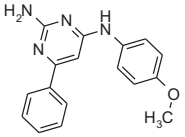
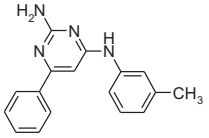
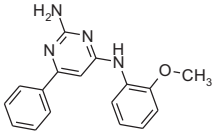
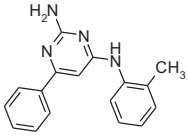
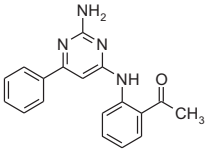
The HTS was not background corrected in the primary screen and as such compounds that interfered at 340 nm (i.e. absorption from the compound or light scattering due to poor solubility) would potentially affect the observed signal similar to actual inhibition of the enzyme. To minimize this effect in the primary screen, the signal change was maximized. In this way, small effects would not be observed as actives. In order to maximize this signal change, a bias was introduced in the screen. Whereas the concentration of ATP was set at less than 0.5 K_m, that of UMP was set at 2.3–4.6 fold of the K_m. This would result in a screen that was more sensitive in detecting ATP competitive inhibitors than for those of UMP.

3.3. HTS and identification of the UMP kinase hit

The optimized assay was validated using a compound set (9984 compounds) run in duplicate (reverse order of plates). There was high correlation between the results of the two runs (Fig. 3A). The assay did not exhibit plate or position in the run effects (Fig 3B).

The assay proceeded to full HTS. The screen had a hit rate of 1.1% based on statistically significant inhibition compared to controls (outside 2 interquartile ranges) and had a Z' ranging from

Table 1
Activity of aminopyrimidine series compounds against *S. aureus* PyrH.

Compound	Structure	*S. aureus* IC50 (μM)
1		15.6
2		>25
3		>100
4		>200
5		>100
6		32.9
7		53.8
8		4.6

0.64–0.82. Actives from the primary screen were selected for retesting in a dose response to determine the IC_{50}. Actives with an IC_{50} less than 100 μM, and a Hill slope between 0.5 and 2.0 were selected for quality control analysis (>80 purity) and subsequently tested in the PK/LDH coupler control assay to remove assay artifact actives. Actives passing these criteria were tested in the orthogonal detection HPLC based assay.

Despite the maximized signal change, a high false positive rate was observed, linked to interference at 340 nm. These false positives were generally eliminated when run in follow up dose response assays where background correction was employed. In addition, the HPLC-based assay specifically eliminated such artifact compounds that remained. Few compounds were found as false positives due to inhibition of the coupler enzymes.

3.4. Inhibitor characterization

From the analysis of the HTS data and follow up, the aminopyrimidine series was identified (Table 1) and selected for further characterization. This aminopyrimidine was found to have an IC_{50} of 16 μM in the *S. aureus* UMP kinase assay. The compound was not time dependent (observed from 0.5 to 120 min) (data not shown). Preincubation of the compound with UMP kinase, followed by rapid dilution of the enzyme into the assay mixture, restored near control levels of activity (data not shown). These data suggest that the compound is rapidly reversible and is likely in rapid equilibrium under the assay conditions.

The mode of inhibition of UMP kinase by compound 1 was examined. The best fit model was for competitive inhibition of ATP with a competitive inhibition constant K_i of 29.6 ± 2.0 (Fig. 4B) and mixed inhibition with UMP with a 5 fold difference between the competitive (K_{ic}) and uncompetitive (K_{icu}) inhibition constants (K_{ic} 18.7 ± 2.9, and K_{iuc} 92.6 ± 10.7) (Fig. 4A). The bias introduced into the design of the HTS assay had favored the identification of ATP competitive inhibitors over a UMP competitive inhibitor as discussed above.

Limited investigation of structure activity relationships was performed by testing structurally related analogues of compound 1 (Table 1). Substitution on the aniline nitrogen was not tolerated (compound 2). In addition, relatively small methyl (compound 3) and methoxy (compound 4) substitutions at para position and methyl (compound 5) substitution at meta position of the phenyl group were not tolerated. Both methyl (compound 6) and methoxy (compound 7) substitutions were tolerated at the ortho position, but with a loss of potency. However, a acetyl (compound 8) substitution at the ortho position of this ring resulted in increased potency. Taken together, these data further support the specific binding of the series to PyrH and suggest areas for further optimization.

Compounds were also tested for activity against UMP kinases derived from *E. coli* and *S. pneumoniae* in a PK/LDH coupled assay

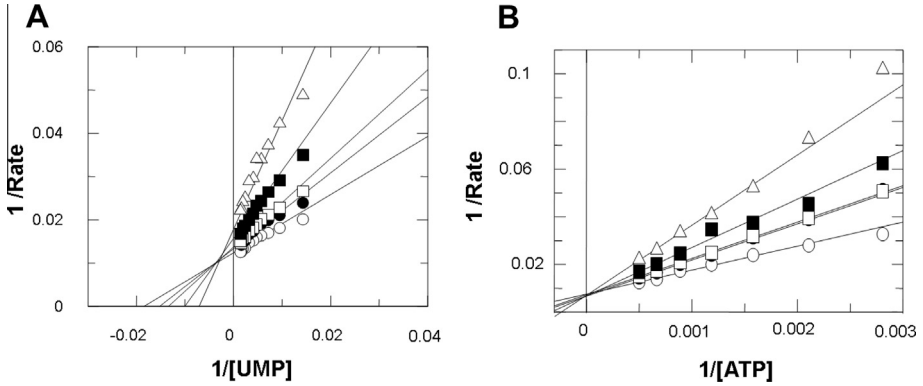


Fig. 4. Lineweaver Burk transforms of *S. aureus* UMP kinase velocity curves inhibited by increasing concentrations of compound 1. (A) UMP concentrations were varied while the ATP was fixed at 2.0 mM. (B) ATP concentrations were varied while the UMP was fixed at 0.7 mM. The concentration of compound one used was 0 (○), 6.25 (●), 12.5 (□), 25 (■), 50 (△) μM.

(data not shown). All compounds tested had IC_{50}s > 30 μM. Compounds were also tested for antibacterial activity in representative strains of *E. coli, S. aureus* and *S. pneumoniae*. Not surprisingly, no significant activity was observed against the panel of bacteria tested ($\geqslant$32 μg/mL). We believe that compounds with greater target affinity would have a better chance to show cellular activity.

We have shown that HTS can be used to identify potential leads for this novel target. The high throughput screen of UMP kinase resulted in the identification of a novel, small molecule inhibitor that is competitive with ATP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.060.

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