Next generation Glucose-1-phosphate thymidylyltransferase (RmlA) inhibitors: An extended SAR study to direct future design

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ABSTRACT

The monosaccharide L-rhamnose is an important component of bacterial cell walls. The first step in the L-rhamnose biosynthetic pathway is catalysed by glucose-1-phosphate thymidylyltransferase (RmlA), which condenses glucose-1-phosphate (Glu-1-P) with deoxythymidine triphosphate (dTTP) to yield dTDP-α-glucose. In addition to the active site where catalysis of this reaction occurs, RmlA has an allosteric site that is significant for its function. Building on previous reports, SAR studies have explored further the allosteric site, leading to the identification of very potent P. aeruginosa RmlA inhibitors. Modification at the C6-NH2 of the inhibitor’s pyrimidinedione core structure was tolerated. X-ray crystallographic analysis of the complexes of P. aeruginosa RmlA with the novel analogues revealed that C6-alkyl substituents can be used to position a modifiable amine just outside the allosteric pocket. This opens up the possibility of linking a siderophore to this class of inhibitor with the goal of enhancing bacterial cell wall permeability.

1. Introduction

The continued global emergence of multi-drug resistant bacteria is a major health concern. The time taken for resistance to new drugs to arise is often rapid and the pace of antibiotic discovery has slowed since the golden era of the 1940–60s.1 The development of novel antimicrobials that avoid the existing mechanisms of resistance and target new pathways is recognised as a high priority for research.

The outer membrane (OM) protects gram-negative bacteria from antibiotic attack and is essential for survival.2 The OM is composed of lipopolysaccharides (LPS) that in many, but not all, bacteria contain L-rhamnose, a C6 sugar unit. For example, in P. aeruginosa L-rhamnose is a component of the LPS and deletion of one of the genes responsible for its biosynthesis results in a bacterium that has much lower virulence in a mouse model.3 In M. tuberculosis the arabinogalactan unit in the cell wall is linked to the peptidoglycan by a disaccharide phosphodiester linker that has a L-rhamnose component (a decaprenyl-diphospho-N-acetylglucosamine rhamnosyl molecule).4 The enzymes involved in the biosynthesis of L-rhamnose are therefore potential anti-tuberculosis drug targets.5–6 The L-rhamnose biosynthetic pathway involves four enzymes, RmlA-RmlD, which catalyse the conversion of glucose-1-phosphate (Glu-1-P) to the L-rhamnose precursor deoxythymidine diphosphate-L-rhamnose (dTDP-L-rhamnose 7, Scheme 1).6 Since this biosynthetic pathway is not found in eukaryotes, these enzymes are attractive targets for the development of novel selective antibiotics. Small molecule inhibitors of RmlA 7–9 and RmlC 10–11 have already been reported.

RmlA, a glucose-1-phosphate thymidylyltransferase, is the first enzyme in the pathway and catalyses the condensation of Glu-1-P with deoxythymidine triphosphate (dTTP) to give dTDP-α-glucose (Scheme 1).5,7,7–14 The inhibition of RmlA by dTDP-L-rhamnose (the final product of the four step reaction sequence) has been reported7,13 and it would therefore appear that, in bacteria, RmlA is the point of control for flux through the biosynthetic pathway. RmlA exists as a dimer of dimers and is functional as a tetramer. As a consequence of its structure, the active sites cluster at the dimer-dimer interface and the allosteric (or regulatory) sites cluster at the monomer–monomer interface within each dimer.11,14

We have previously reported a series of potent novel small molecule...
allosteric inhibitors of *P. aeruginosa* RmlA\(^7\) (for example **Compound 8a** in Figure 1A, compound numbering taken from the original report\(^7\)). In the previous work\(^7\), examples of our *in vitro* RmlA inhibitors were tested for their ability to inhibit the growth of *M. tuberculosis* (H37Rv) in which RmlA has been shown to be essential.\(^{12}\) Even though sequence alignment of RmlA from *P. aeruginosa* and *M. tuberculosis* showed the two proteins are highly conserved, the selected compounds demonstrated only weak activity against *M. tuberculosis* bacteria (MIC\(_{100}\) values > 25 µg/mL). For example, the potent *in vitro* *P. aeruginosa* RmlA inhibitor 8a (IC\(_{50}\) = 0.073 ± 0.001 µM\(^7\)) was shown to have only a weak effect on *M. tuberculosis* (MIC\(_{100}\) = 100 µg/mL). Apart from off-target effects and minor sequence differences in the RmlA homologues from the two bacteria, another possible reason for the poor effect on live bacteria is the inability of the tested analogues to penetrate the bacterial cells. The cell envelope of mycobacteria, comprised of polysaccharides and lipids, functions as a natural shield that is effective at blocking the entry of small molecules into the protoplasm.\(^{16-17}\)

Re-examination of the RmlA-8a complex [PDB 4ASJ] revealed a hydrophobic pocket that was only partly occupied by the N\(^1\)-substituent (Figures 1B and 1C). In an initial attempt to explore further the impact of structural changes on the binding of compound 8a, we chose to vary the R\(_1\) substituent (Figure 1A). However, the main focus of this report builds on the observation that the C6-NH\(_2\) group of 8a points out of the allosteric binding pocket based on our analysis of the RmlA-8a complex (Figure 1C). It was decided to assess whether substitution of one of the NH bonds in the C6-NH\(_2\) group with an extended alkyl chain (represented by R\(_2\) in Figure 1A) could be tolerated by *P. aeruginosa* RmlA as this could provide a vector out of the allosteric pocket to an open space whilst retaining the *in vitro* inhibitory activity of the current series of inhibitors.

**Scheme 1.** \(\alpha\)-Rhamnose biosynthetic pathway involving 4 enzymes which catalyse the conversion of Glu-1-P to dTDP-\(\alpha\)-rhamnose.
analogaues. If successful this could provide the foundation for the development of a new series of RmlA inhibitors. For example, the attachment of a bacterial cell wall permeabilizer could be achieved via the newly incorporated linker unit at the C6-NH$_2$ position.

2. Results and discussion

Our previous studies focused on structure activity relationship (SAR) analyses involving the sulfonamide and N$^2$-alky substituents in 8a (Figure 1A). However, no attempts to optimize the substituent at the N$^1$-position were made. This current study started with examination of the reported structure of the RmlA-8a complex [PDB 4A5J] and revealed that the N$^1$-substituent pocket was formed from both main- and side-chain atoms of residues Leu45, His119, Glu120, Ile256, Arg259 and Gln260 (Figure 1B). Visual inspection showed that this binding pocket was not ideally filled by the unsubstituted N$^1$-benzyl group in 8a and therefore it was proposed that alternative N$^1$-substituents could improve target binding. A pilot SAR study was performed to explore this hypothesis (see linked Data in Brief report for a detailed discussion). In summary, it was concluded that the key driver in increasing the potency was the presence of a substituent at the 4-position of the N$^1$-benzyl group. It was found that a para-bromobenzyl substituent (compound 1a in Table 1) was optimal (see linked Data in Brief report and PDB codes 5FU1, 5FYE, 5FU0, 5FTS, 5FTV, 5FU8). Consequently, all R$_2$-modified analogues were prepared in the N$^1$-p-bromobenzyl series with one exception (compound 1f, Scheme 2 and Table 1).

The X-ray crystallographic analysis of the RmlA-1a complex (Figure 2, PDB 5FTV) confirmed that in the p-bromobenzyl series, as well as for 8a, substitution at the C6-NH$_2$ position should enable positioning of a modifiable functional group in proximity to the mouth of the allosteric site (Figure S1A). If this could be achieved, not only would the only remaining position available for modification in this inhibitor series have been explored, but future efforts to prepare analogues with enhanced bacterial cell wall permeability would also be facilitated (Figures S1B and S1C). Preliminary molecular modelling studies predicted that the C6-NH$_2$ modified analogue 1b (Table 1 and Scheme 2 for structure) binds in the allosteric site of the enzyme in a similar configuration to the parent analogue 1a (Figure S2). In addition, the extended C6-amainoalyl chain was predicted to reach out towards the mouth of the allosteric pocket, as designed. Analogues 1b and 1c with n-propyl and ethyl-containing linkers were therefore synthesised (Scheme 2).

It was decided to incorporate the extended C6-amainoalyl chains of 1b and 1c early in the reaction sequence (Scheme 2). Selective N$^1$-alkylation of the starting material 6-chlorouracil (2) with 4-bromo-benzyl chloride under basic conditions enabled isolation of the N$^1$-benzylated product to give 3a,$^{19-20}$ 6-Aminouracils 4b and 4c were then synthesized by reaction of 3a with the corresponding amines 5b (2 × CH$_2$) and 5c (3 × CH$_2$) in moderate yield. The remaining steps – bromination (to give 6b and 6c), addition of methylamine (to give 7b and 7c) and sulfonamide formation were based on our previous report (see linked Data in Brief report for additional examples of this reaction sequence) and enabled the successful conversion of 4b and 4c to the N-Boc protected versions (8b and 8c) of the final compounds. Subsequent Boc deprotection of 8b and 8c using TFA gave 1b and 1c respectively as the TFA salts (Scheme 2).

The introduction of the ethyl C6 aminoaalkyl chain in 1c led to a complete loss of activity$^{13}$ against P. aeruginosa RmlA, whereas incorporation of the n-propyl linker in 1b retained activity (IC$_{50}$ of 0.86 μM, Table 1, entries 2 and 3, see Figures S3 and S4 legends for a discussion on the lack of activity of 1c). X-ray crystallographic analysis of the complex of RmlA with 1b [PDB 6TQG] showed that 1b was bound in the allosteric site of RmlA as expected. Compared with the C6-NH$_2$ unsubstituted analogue 1a (Table 1, entry 1), most of the ligand–protein interactions were retained in the RmlA-1b complex (Figure S5), however, some differences were observed. For example, whereas the C6-NH$_2$ group in 1a showed hydrogen bonding to the protein backbone (Gly115 and His119) through the interaction with two different molecules of water, 1b retained the interaction with Gly115 but lost the water-mediated hydrogen bond to His119 (as expected, Figure S3). Consistent with the docking studies, the extended aminoaalkyl chain in 1b pointed out of the allosteric pocket and the distance between the nitrogen of the newly introduced terminal methylamine in 1b to the C-terminal Tyr293 residue was 3.8 Å. The terminal NH in 1b interacted with a network of water molecules ultimately linking to the C-terminal Tyr293 (Figure S3).

Based on the initial success with 1b being a sub-micromolar inhibitor of P. aeruginosa RmlA, it was decided to extend the length of the linker unit from n-propyl in 1b to n-butyl in 1d and n-pentyl in 1e (Scheme 2) in an attempt to position the terminus of the C6-amainoalyl chain outside the allosteric pocket. In the case of 1d and 1e, a primary amine was incorporated at the end of the chain (see Figure S5 legend for more discussions). There was a risk that the more extended and flexible alkyl chains in 1d and 1e may undergo hydrophobic collapse. Therefore a heteroaromatic ring was incorporated into the linker unit in an attempt to minimise the chances of this occurring. The triazole-containing compound 1f was therefore synthesised (Scheme 2). In the case of 1f, the para-Br in the N$^1$-benzyl moiety was also removed to provide additional room for the triazole group in 1f to adjust its position in the allosteric site. The synthesis of the additional analogues 1d and 1e was achieved in an analogous manner to the synthesis of 1b and 1c (Scheme 2). The synthesis of 1f required incorporation of an azide functional group at the terminus of the C6-linker unit through formation of 4f (n = 3, R$_2$ = N$_3$, Scheme 2). The azide group was compatible with the subsequent steps enabling 4f to be successfully converted to 8f. The copper-catalysed azide-alkyne click (CuAAC) reaction of 8f with propargylamine gave 1f although the unoptimized yields over the final two steps in the sequence were low (Scheme 2). If analogue 1f was found to retain activity against P. aeruginosa RmlA, future work should enable the relatively easy incorporation of a bacterial cell wall permeabilizer using this CuAAC approach.

The increased length of the linker chain in analogues 1d and 1e compared to 1b led to a 2.5-fold increase in potency with 1d and 1e having IC$_{50}$ values of 0.303 ± 0.026 μM and 0.316 ± 0.023 μM respectively (Table 1, entries 4 and 5 vs. entry 2). The structure of the RmlA-1d complex [PDB 6T38] confirmed that instead of interacting with any protein residues, the terminal amine of the C6-amainoalyl chain in 1d was positioned out of the pocket, approximately equidistant between His119 and Tyr293 (Figures 3A and 3B).

Table 1

| Entry | Compd.$^{[a]}$ | R$_1$ | R$_2$ | % Inhibition at 10 μM | IC$_{50}$ (μM) |$^{[b]}$ |
|-------|----------------|-------|-------|-----------------------|-----------------|
| 1     | 1a             | 4-    | H     | 100                   | 0.034 ±        |
| 2     | 1b             | 4-    | (CH$_2$)$_2$NHCH$_3$ | 100              | 0.002          |
| 3     | 1c             | 4-    | (CH$_2$)$_2$NHCH$_3$ | 0                | 0.096          |
| 4     | 1d             | 4-    | (CH$_2$)$_2$NH$_3$  | 100              | 0.303 ±        |
| 5     | 1e             | 4-    | (CH$_2$)$_2$NH$_2$  | 100              | 0.026          |
| 6     | 1f             | Ph    | (CH$_2$)$_2$N$_3$   | 100              | 2.470 ±        |

[a] The following PDB codes are assigned to structures of the complexes of RmlA bound to 1b (6TQG), 1d (6T38), 1f (6T37); [b] SD, standard deviation (n = 3).
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T37] revealed that the triazole moiety of inhibit containing analogue [38: NH₂CH₂NCH₃(Boc)], 5e: NH₂(CH₂)₃NHBoc, 5f: NH₂(CH₂)₃N₂, EtOH, 100 °C, sealed tube, 3 hrs; 4b = 45%, 4c = 45%, 4d = 50%, 4e = 65%, 4f = 78%; (iii) N-Bromo-succinimide, MeOH, 25 °C, 10 min; 6b = 85%; 6c = 85%; 6d = 86%; 6e = 87%; 6f = 81%; (iv) 40% w.w. aq. MeNH₂, 70 °C, 1 h; 7b = 56%, 7c = 42%; 7d = 67%, 7e = 80%; 7f = 94%; (v) benzensulfonyl chloride, pyridine, DCM, 25 °C, 18 hrs; (vi) tri-fluoroacetic acid, DCM, 25 °C, overnight; 1b = 50%; 1c = 46%; 1d = 48%; 1e = 38%; yields are after two steps (v and vi); (vii) ascorbic acid, CuSO₄·5H₂O, propargylamine, BuOH/H₂O, 25 °C, 3 hrs; 1f = 10%; the yield is after two steps (v and vii).

Scheme 2. Synthesis of C6-NH₂ analogues. Reagents and conditions: (i) benzyl chloride or 4-bromo-benzyl chloride, K₂CO₃, DMSO, 65 °C, 30 min; 3a = 38%, 3b = 45%; (ii) for 4b-4f: required amine (5b: NH₂(CH₂)₃NCH₃(Boc)), 5e: NH₂(CH₂)₃NHBoc, 5f: NH₂(CH₂)₃N₂, EtOH, 100 °C, sealed tube, 3 hrs; 4b = 45%, 4c = 45%, 4d = 50%, 4e = 65%, 4f = 78%; (iii) N-Bromo-succinimide, MeOH, 25 °C, 10 min; 6b = 85%; 6c = 85%; 6d = 86%; 6e = 87%; 6f = 81%; (iv) 40% w.w. aq. MeNH₂, 70 °C, 1 h; 7b = 56%, 7c = 42%; 7d = 67%, 7e = 80%; 7f = 94%; (v) benzensulfonyl chloride, pyridine, DCM, 25 °C, 18 hrs; (vi) tri-fluoroacetic acid, DCM, 25 °C, overnight; 1b = 50%; 1c = 46%; 1d = 48%; 1e = 38%; yields are after two steps (v and vi); (vii) ascorbic acid, CuSO₄·5H₂O, propargylamine, BuOH/H₂O, 25 °C, 3 hrs; 1f = 10%; the yield is after two steps (v and vii).

3. Conclusions

A significant extension of our previous studies on the inhibition of P. aeruginosa RmA, the first enzyme in the L-rhamnose biosynthetic pathway, by pyrimidinedione-based compounds is reported. A pilot SAR study involving modifications at the N³ position of the heterocyclic core led to the identification of a number of potent inhibitors of P. aeruginosa RmA including the p-bromo-benzyl substituted analogue 1a. Subsequent modification at the C6-NH₂ position showed that different linker lengths at this position were tolerated. Throughout these studies detailed analysis of the binding modes of the compounds has been possible by X-ray crystallographic analysis of a large number of RmA-inhibitor complexes. One highlight from this structural study is the demonstration that for inhibitors 1d and 1f the terminus of the newly incorporated linker unit sits outside the allosteric binding pocket. This provides a real opportunity, in future work, to attach a siderophore to the end of the linker unit with the goal of potentially increasing the bacterial cell wall permeability of this class of inhibitors through the ability of the siderophore to be sequestered by the bacterium. }
4. Experimental section

4.1. Synthesis of analogues

All intermediates and final compounds were prepared according to the protocols supplied in the Supporting Information and Data in Brief. Examples of the synthesis of 1d (intermediates and final compound) and the synthesis of 1b, 1c, 1e and 1f (final compounds) are shown below.

4.2. 1-Benzyl-6-chloropyrimidine-2,4(1H,3H)-dione (3a)

A mixture of 6-chlorouracil 2 (5.0 g, 34.2 mmol, 1.0 eq.), 4-bromo-benzyl chloride (10.5 g, 51.3 mmol, 1.5 eq.), and K$_2$CO$_3$ (2.3 g, 17.1 mmol, 0.5 eq.) in DMSO (100.0 mL, 3.0 mL/mmol) was stirred at 65 °C for 30 min. 10% aqueous solution of NaOH (100.0 mL, 3.0 mL/mmol) was added to the hot reaction mixture with stirring. The reaction mixture was acidified with conc. aqueous HCl to pH = 2. The resulting aqueous mixture was kept in a refrigerator, and the resulting precipitate was collected by filtration, washed with water (60.0 mL, 2.0 mL/mmol), and dried. 3a was obtained as a white solid (4.4 g, 13.9 mmol, 38%). Mp. 183–187 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.77 (1H, s, NH), 7.57 (2H, d, $J=8.4$ Hz, H3′, H5′), 7.25 (2H, d, $J=8.4$ Hz, H2′, H6′), 6.02 (1H, s, H5), 5.12 (2H, s, CH2), $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 161.5 (C4), 151.0 (C6), 136.2 (C1′), 132.0 (C3′ and C5′), 129.3 (C2′ and C6′), 121.0 (C4′), 103.0 (C5), 48.1 (NCH$_2$). HRMS (ES$^+$) m/z calculated for C$_{11}$H$_7$BrClN$_2$O$_2$ [M + H]$^+$: 314.9536; found: 314.9537.

4.3. Tert-butyl (4-((3-(4-bromobenzyl)-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)amino)butyl)carbamate (4d)

To a stirred solution of 1-benzyl-6-chlorouracil 3a (4.0 g, 12.7 mmol, 1.0 eq.) in ethanol (38.0 mL, 3.0 mL/mmol), tert-butyl (4-amino-butyric acid) carbamate 5d (4.8 g, 25.4 mmol, 2.0 eq.) was added. The resulting yellow solution was stirred at 100 °C in a sealed tube for 3 h. The solvent was evaporated in vacuo, and the crude product was purified by column chromatography (50% EtOAc in petroleum ether). 4d was obtained as a yellow solid (3.0 g, 6.3 mmol, 50%). Mp. 286–288 °C. $\nu_{max}$ cm$^{-1}$ 3342 (N–H), 2983 (C–H), 1665 (C=O), 1605 (N–H), 1530 (N–H), 1447 (C=C), 1387 (N–C), 1163 (C–(O)-O), 777 (Ar–C=H), 669 (C–Br). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 10.05 (1H, s, H3), 7.44 (2H, d, $J=7.9$ Hz, H3′ and H5′), 7.13 (2H, d, $J=8.1$ Hz, H2′ and H6′), 5.59 (1H, s, H1′), 5.17 (2H, s, NCH$_2$), 4.86 (1H, s, H6′), 4.76 (1H, s, H5), 3.02 (2H, t, $J=6.0$ Hz, H2), 2.95 (2H, t, $J=6.0$ Hz, H5′), 1.57 – 1.46 (m, 2H, H4′), 1.43 (s, 9H, 3 × CH$_3$), 1.34 – 1.28 (m, 2H, H3′).
H3′). 13C NMR (126 MHz, CDCl3) δ 164.1 (C4), 156.5 (C–O–O), 154.7 (C2), 151.6 (C6), 134.4 (C′1), 132.1 (C3′ and C5′), 128.2 (C′2 and C6′), 121.8 (C′4), 79.5 (O–C–O), 75.5 (C5), 43.9 (NCH3), 43.3 (C′3), 39.6 (C5′), 28.4 (3 × CH3), 27.8 (C′3′), 24.4 (C′4′). HRMS (ES′) m/z calculated for C29H38N2BrNO4 [M + H]+: 546.1703; found: 546.1705.

4.4. Tert-buty 1-(1-(4-bromobenzyl)-6-((2-(methylamino)ethyl)amino)-2,4-

tetrahydropryvinidin-5-yl)-N-methylbenzenesulfonamid e (1b)

To a stirred solution of the amine 7b (150.0 mg, 0.3 mmol, 1.0 eq.) in dry DCM (2.0 mL, 7.0 mL/mmol) was added pyridine (0.1 mL, 1.5 mmol, 5.0 eq.) followed by sulfon chloride (80.0 mg, 0.5 mmol, 1.5 eq.). The resulting yellow solution was stirred at rt for 18 h. The solvent was removed in vacuo and water (2.0 mL, 7.0 mL/mmol) was added to the residue followed by 1 M HCl to reach acidic pH to get the crude of 8d. To a solution of 8d in DCM (1.5 mL, 5.0 mL/mmol) was added trifluoroacetic acid (0.3 mL, 1.0 mL/mmol). The solution was allowed to stir at room temperature overnight. The mixture was basified with ammonia solution (1.0 mL, 3.0 mL/mmol) and was extracted with DCM three times (5.0 mL × 3, 15.0 mL/mmol). The combined organic phases were washed with saturated aqueous NaCl (5.0 mL,15.0 mL/mmol), dried over anhydrous Na2SO4 and concentrated in vacuo. The crude product was purified by column chromatography (50% acetone in petroleum ether with 1% Et3N).

4.5. Tert-buty 1-(1-(4-bromobenzyl)-5-(methylamino)-2,6-
dioxo-1,2,3,6-tetrahydropryvinidin-4-yl)(amino)butyl)carbamate (7d)

To a stirred solution of the amine 7d (150.0 mg, 0.3 mmol, 1.0 eq.) in dry DCM (2.0 mL, 7.0 mL/mmol) was added pyridine (0.1 mL, 1.5 mmol, 5.0 eq.) followed by sulfon chloride (80.0 mg, 0.5 mmol, 1.5 eq.). The resulting yellow solution was stirred at rt for 18 h. The solvent was removed in vacuo and water (2.0 mL, 7.0 mL/mmol) was added to the residue followed by 1 M HCl to reach acidic pH to get the crude of 8d. To a solution of 8d in DCM (1.5 mL, 5.0 mL/mmol) was added trifluoroacetic acid (0.3 mL, 1.0 mL/mmol). The solution was allowed to stir at room temperature overnight. The mixture was basified with ammonia solution (1.0 mL, 3.0 mL/mmol) and was extracted with DCM three times (5.0 mL × 3, 15.0 mL/mmol). The combined organic phases were washed with saturated aqueous NaCl (5.0 mL,15.0 mL/mmol), dried over anhydrous Na2SO4 and concentrated in vacuo. The crude product was purified by column chromatography (50% acetone in petroleum ether with 1% Et3N).
a solution of 8c in DCM (1.0 mL, 5.0 µmol/ml) was added and the mixture was basified with ammonia solution (0.6 mL, 3.0 µmol/ml) and was extracted with DCM three times (5.0 mL × 3). The combined organic phases were washed with saturated aqueous NaCl (3.0 mL, 15.0 µmol/ml), dried over anhydrous Na2SO4 and concentrated in vacuo. The crude product was purified by column chromatography (50% acetone in petroleum ether with 1% Et3N). 1H was obtained as a yellow solid (51.0 µg, 0.1 mmol, 46%) via 8c. Mp. 283–286 °C. 1H NMR (400 MHz, CDCl3) δ 150.16 (C6), 131.53 (C4), 129.29 (C3), 127.5 (C′), 126.4 (C″), 124.6 (C′′′), 120.4 (C′′″), 109.1 (C′′), 107.1 (C′″), 105.4 (C′″″), 54.7 (NCH3). HRMS (MS) m/z calculated for C21H25BrN2O3S [M + H]+: 522.0611; found: 522.0622.

4.9. N-(6-((5-aminopyrimidin-2-ylamino)-1-(4-bromobenzyl)-2,4-dioxo-1,2,3,4-tetrahydroprimidin-5-yl)-N-methylbenzenesulfonamide (1e)

To a stirred solution of the amine 7e (150.0 mg, 0.3 mmol, 1.0 eq.) in dry DCM (2.0 mL, 7.0 µmol/ml) was added pyridine (0.1 mL, 1.5 mmol, 5.0 eq.) followed by sulfonyl chloride (80.0 mg, 0.5 mmol, 1.5 eq.). The resulting yellow solution was stirred at rt for 18 h. The solvent was removed in vacuo and water (2.0 mL, 7.0 µmol/ml) was added to the residue followed by 1 M HCl to reach acid pH to get the crude of 8e. To a solution of 8e in DCM (1.5 mL, 5.0 µmol/ml) was added trifluoroacetic acid (0.3 mL, 1.0 µmol/ml). The solution was allowed to stir at room temperature overnight. The mixture was basified with saturated aqueous NaCl (4.5 mL, 15.0 µmol/ml) and was extracted with DCM three times (5.0 mL × 3). The combined organic phases were washed with saturated aqueous NaCl (4.5 mL, 15.0 µmol/ml), dried over anhydrous Na2SO4 and concentrated in vacuo. The crude product was purified by column chromatography (50% acetone in petroleum ether with 1% Et3N). 1H was obtained as a yellow solid (58.2 mg, 0.1 mmol, 36%) via 8e. Mp. 317–319 °C, 1H NMR (400 MHz, CDCl3) δ 150.16 (C6), 131.53 (C4), 129.29 (C3), 127.5 (C′), 126.4 (C″), 124.6 (C′′′), 120.4 (C′′″), 109.1 (C′′), 107.1 (C′″), 105.4 (C′″″), 54.7 (NCH3). HRMS (ES) m/z calculated for C32H35BrN2O3S [M + H]+: 552.0611; found: 552.0622.

4.11. Cloning, expression and purification

P. aeruginosa RmlA was cloned, expressed and purified based on protocols previously reported.32

4.12. In vitro biological assays

Each assay was performed in a 101 µL reaction volume containing 50 mM Tris (pH 7.4), 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0), 0.05% NP-40, 15 nM recombinant RmlA, 0.8 µg/ml pyrophosphatase, 5 µM dTTP and 5 µM G-1-P. The inhibitor was added to the plate (20 µL) followed by RmlA (30 µL). The reaction was initiated with the addition of dTTP (25 µL), 5 µM G-1-P and pyrophosphatase (1 µL) as a mixture in one charge (51 µL total) and the assay was allowed to run for 30 min at room temperature. The reaction was quenched with addition of BIOMOL® Green reagent (100 µL) and was allowed to develop before the absorbance of each well was measured at 620 nm.

4.13. Protein Crystallization, co-Crystallization and soaking

Crystals were grown by the sitting drop vapour diffusion method as previously described.33 Drops contained 1 µL of protein (10 mg/ml) mixed with 1 µL precipitant (4–12% PEG 6000, 0.1–0.15 M MES pH 6.0, 0.05–0.1 M MgCl2, 0.1–0.15 M NaBr, 1% β-mercaptoethanol). Crystals grew overnight to dimensions of 0.2 × 0.2 × 0.1 mm. Complexes of RmlA with inhibitor were prepared by soaking or co-crystallization. For soaking, solid compound was added to drops containing crystals and allowed to incube for between 2 and 24 h prior to data collection. For co-crystallization, solid compound was incubated with protein in solution for 1 h prior to setting up sitting drops.

4.14. Data collection

Data were collected at the Diamond Light Source synchrotron or in-house using a Rigaku MicroMax007HFm X-ray generator. Data were processed with iMOSFLM34 or XIA235 incorporating XDS. Each structure was solved using MOLREP36 with 4AS1 as the search model with the inhibitor removed. REFMAC37 was used to refine the models.
with model building in COOT [28] and ligands built with PRODRG [29]. Structural figures were prepared using Pymol [30] and CCP4MG [31].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The coordinates of the RmlA complexes have been deposited in the Protein Data Bank (pdb codes 5FUH, 5FYE, 5FU0, 5FTS, 5FTV, 5FU8, 6TQG, 6T38, 6T37).

Raw data files can be found at DOI: 10.6084/m9.figshare.16657876.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116477.

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