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Discovery of new Gram-negative antivirulence drugs: Structure and properties of novel *E. coli* WaaC inhibitors

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ABSTRACT

Heptosyltransferases such as WaaC represent promising and attractive targets for the discovery of new Gram-negative antibacterial drugs based on antivirulence mechanisms. We report herein our approach to the identification of the first micromolar inhibitors of WaaC and the preliminary SAR generated from this family of 2-aryl-5-methyl-4-(5-aryl-furan-2-yl-methylene)-2,4-dihydro-pyrazol-3-ones identified by virtual screening.

The global emergence of resistance to antimicrobial agents is increasingly limiting the effectiveness of current drugs, and there is a medical and social need for new anti-infective therapies, particularly for the treatment of nosocomial infections and the life threatening conditions due to septicemia.

Bacterial resistance to antibiotics is a serious public health problem.[1–3] Two factors contribute to this phenomenon: the emergence of resistant bacteria consequential to the selection induced by massive use of antibiotics in human and veterinary medicine, and diffusion of resistant bacteria in various ecosystems.

Although resistant Gram-positive bacteria such as MRSA have become the most frequently isolated pathogens among patients with nosocomial infections, the prevalence of resistant Gram-negative bacteria such as the ESBL Enterobacteriaceae (e.g., *E. coli, K. pneumoniae*) and the multidrug-resistant (MDR) *P. aeruginosa* and *A. baumanii* has also been increasing, the majority of these Gram-negative pathogens being now resistant to several commonly prescribed antibiotics. In order to treat infections due to MDR bacteria, new molecules with new mechanisms of action are urgently needed.

One innovative therapeutic concept that has been recently introduced to find new antimicrobial agents is antivirulence.[4] This concept is based on interfering with the host/pathogen interactions and is different from the classical antibiotics in two key ways. First, rather than targeting genes that are essential for basic metabolism in vitro, antivirulence focuses on mechanisms that are essential for host/pathogen interactions especially those allowing the bacteria to escape the innate immune system of the host. Secondly, because the antivirulence approach targets specifically the invasive bacteria (i.e., activity only against bacteria that are causing pathogenesis via dissemination in the host) there is no growth inhibition of bacteria constitutive of the normal flora such as gut or skin. This approach by saving bacterial growth in natural sites preserves the efficiency of antibiotics while also decreasing the selection of resistant bacteria, the selective pressure being limited to the compartments where innate immunity is active.

Lipopolysaccharide (LPS, Scheme 1) is a key element of the outer membrane of Gram-negative bacteria. It constitutes the outer leaflet of the outer membrane and is essential for cell survival[5] although an LPS-deficient *N. meningitidis* mutant as well as an engineered *E. coli* mutant expressing only the Lipid A instead of the Kdo_2–Lipid A have recently been described.[6,7] LPS biosynthesis occurs in the cytoplasm, the heptose molecules being sequentially added to the Kdo_2–Lipid A module (Kdo: 3-deoxy-D-*manno*-oct-2-ulosonic acid) by two heptosyltransferases called WaaC (Scheme 2), WaaF and WaaQ if a third heptose molecule is present.[8] Gram-negative bacteria that lack heptose display the deep-rough phenotype[9] and show a reduction in outer membrane protein content, an

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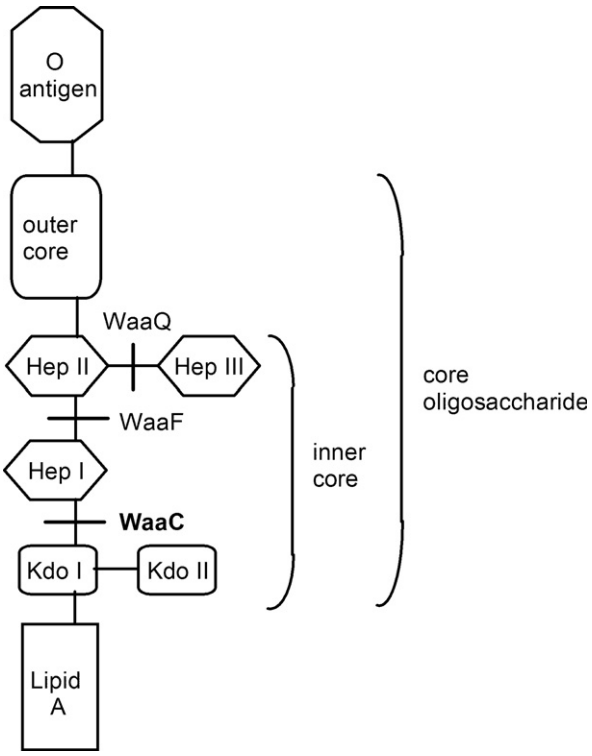


Scheme 1. Schematic representation of *E. coli* LPS (Hep, heptose).

increased sensitivity towards detergents or hydrophobic antibiotics and are more susceptible to phagocytosis by macrophages.[10]

In addition, a *N. meningitidis* deep-rough mutant has been shown to present an attenuated virulence in an infant rat model[11] and we have recently demonstrated that the inner core LPS which constitutes the outer leaflet of Gram-negative bacteria outer membrane is necessary to protect *E. coli* from complement killing in vitro and for virulence in vivo but is dispensable for gut colonization.[12]

The heptosyltransferases such as WaaC which are conserved in Gram-negative bacteria, represent promising and attractive targets for the discovery of new antibacterial drugs based on antivirulence mechanisms.

We report herein our approach to the identification of WaaC inhibitors and the preliminary SAR generated from a family of 2-aryl-5-methyl-4-(5-aryl-furan-2-yl-methylene)-2,4-dihydro-pyrazol-3-ones identified by virtual screening and further optimized.

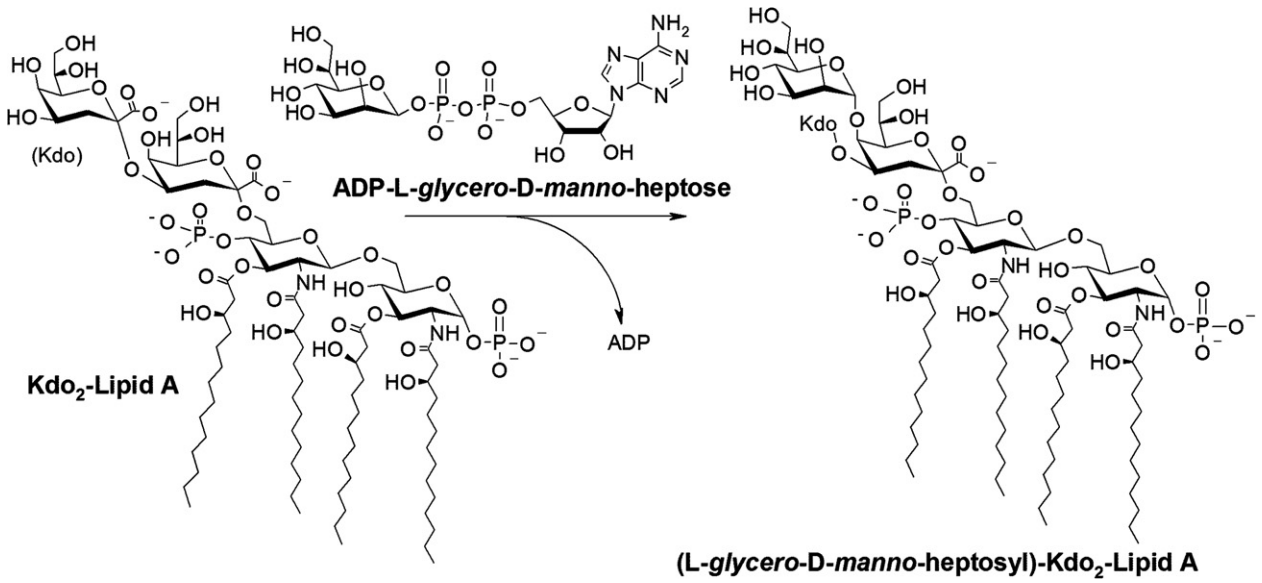
First, a biochemical assay was set up using a synthetic ADP-L-*glycero*-β-D-*manno*-heptose sugar donor[13] and a commercial Re-LPS (from *S. minnesota*) as the acceptor substrate dissolved in TritonX100 micelles. WaaC activity was monitored by a coupled assay involving pyruvate kinase (PK) and luciferase or lactate dehydrogenase as coupling enzymes to detect the product ADP.

On the other hand we have used the crystal structure of WaaC solved at 1.9 Å resolution in complex with ADP and a substrate analogue[14] to identify the active site and binding cavities of the enzyme to carry out a virtual screening. In particular, a small hydrophilic and charged cavity very close to the activated heptose was identified as a potential binding site for the acceptor Kdo of the LPS substrate. Using this model, 5 million of commercially available compounds were electronically/virtually screened by targeting the acceptor binding site.[15] Two hundred thirty-seven hits were selected according to docking score, Lipinski violations, chemical stability and diversity, and tested. One compound, **1a** (Table 1) was identified as a potent inhibitor of WaaC, with an IC_{50} of 2 μM. According to the generated model of the Kdo site, the phenyl furyl moiety of compound **1a** binds the protein partly out of the small Kdo pocket while intruding deeply the second phenyl bearing a carboxylate group in the hydrophilic pocket to make an electrostatic contact with the Arg 120 or Arg 143 and non polar contacts with Ile 287, His 139 and Ala 140 (Fig. 1).

A series of analogues of **1a** was then synthesized to explore the structure–activity relationship and the mechanism of action.

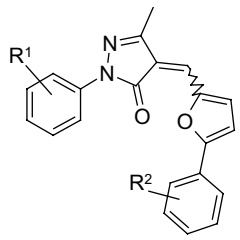
The 2-aryl-5-methyl-4-(5-aryl-furan-2-yl-methylene)-2,4-dihydro-pyrazol-3-one analogues **1a–n** were readily synthesized by condensation of 2-aryl-5-methyl-2,4-dihydro-pyrazol-3-one derivatives **2** with 5-aryl-2-furaldehyde derivatives **3** (Scheme 3).[16]

The 2-aryl-5-methyl-2,4-dihydro-pyrazol-3-ones **2** were either commercially available or prepared in high yields by refluxing the corresponding aryl-hydrazines with ethyl acetoacetate in acetic acid.[17,18] 5-Aryl-furaldehydes **3** were either commercially available or prepared by Suzuki coupling of aryl boronic acids with 5-bromo-furaldehyde in moderate to high yields (21–90%). In some



Scheme 2. Addition of the first heptose molecule to a 3-deoxy-D-*manno*-oct-2-ulosonic acid residue of the Kdo_2–Lipid A molecule catalyzed by WaaC.

Table 1
Inhibition of WaaC by compounds **1a**–**1n**



Compound	R1	R2	IC_{50}[a] (μM)
1a	*m*-CO_2H	*m*-CO_2H	2
1b	*m*-CO_2H	*m*-CO_2H, *p*-Cl	1
1c	*m*-CO_2H	*m*-Me, *p*-CO_2H	31
1d	*m*-CO_2H	*m*-CO_2Me	49
1e	*m*-CO_2H	*m*-COMe	15
1f	*m*-CO_2H	*p*-CO_2H	3
1g	*p*-CO_2H	*m*-CO_2H, *p*-Cl	3.1
1h	*p*-CO_2H	*p*-CO_2H	51
1i	*p*-CO_2H	*m*-$CONH_2$	18
1j	*p*-CO_2H	None	55
1k	*m*-CO_2H	*p*-SO_2NH_2	>100
1l	None	*m*-CO_2H, *p*-Cl	>100
1m	*m*-CO_2Me	*m*-CO_2H	>100
1n	*m*-Me	*m*-CO_2H	>100

[a] Values are means of three experiments.

cases, anhydrous conditions were preferred in order to avoid saponification of the ester functionality.

Reaction of **2** with **3** in refluxing acetic acid afforded the desired 2-aryl-5-methyl-4-(5-aryl-furan-2-yl-methylene)-2,4-dihydro-pyrazol-3-ones **1a–n** in moderate to good yields (26–96%) as a mixture of *E*/*Z* isomers.[19]

Although separation of the isomers was possible by preparative HPLC, each isolated isomer rapidly equilibrated to give back the original mixture of isomers. The Z isomer was the major product as determined by NOESY NMR analysis of a mixture of isomers.[20] Compounds **1a–n** were thus tested as a mixture of isomers.

Acrylamide derivatives **4** were also synthesized as analogues of **1a** (Scheme 4). Compounds **4** were prepared from meta- and para-amino-benzoates by reductive amination with formaldehyde or acetone (30–70%) followed by acylation[21] to afford phosphonates **5** (85–99%). Horner–Wadsworth–Emmons olefination of **5** with furaldehydes **3** using Masamune–Roush conditions[22] (40–86%) and saponification (50–95%) led to the *E* isomers of acrylamide derivatives **4**.

The SAR generated was in clear agreement with the docking model and the expected mechanism of action (Table 1). The carboxylic acid of the aryl pyrazolone moiety was shown to be essential for the activity, as it can be placed in the Kdo pocket in close polar interaction with the Arg 120 (*m*-CO_2H, compounds **1a**–**1f**) or Arg 143 residues (*p*-CO_2H, compounds **1g**–**1j**). Compounds **1l**–**1n** lacking this functionality displayed no activity. The most potent compounds **1a**, **1b**, **1f** and **1g** all have two CO_2H groups with at least one in meta position to enable a close electrostatic interaction with Arg 120. In the case where R1 was *m*-CO_2H (compounds **1a**–**1f**), R2 in meta or para position could afford a suitable interaction with Arg 120 (Fig. 1). However when R1 and R2 were *p*-CO_2H (**1h**), the *p*-CO_2H group of R2 was shifted away by up to 1.5 Å and was no longer able to interact with Arg 120 (Fig. 2), resulting in a 20-fold loss of activity. Therefore when R1 was *p*-CO_2H, the conformation of the molecule required R2 to be in meta position to properly interact with Arg 120 as exemplified by **1g**.

The aromatic ring linked to the furan moiety seems to be important for the positioning of a second acceptor group, the order of potency being CO_2H (**1a**) > COMe (**1e**) = $CONH_2$ (**1i**) > CO_2Me (**1d**). The introduction of a sulfonamide group (**1k**) was deleterious for the activity.

While the introduction of a lipophilic substituent in the meta position (**1c**) had a detrimental effect, in the para position (**1b**) it afforded a gain in potency.

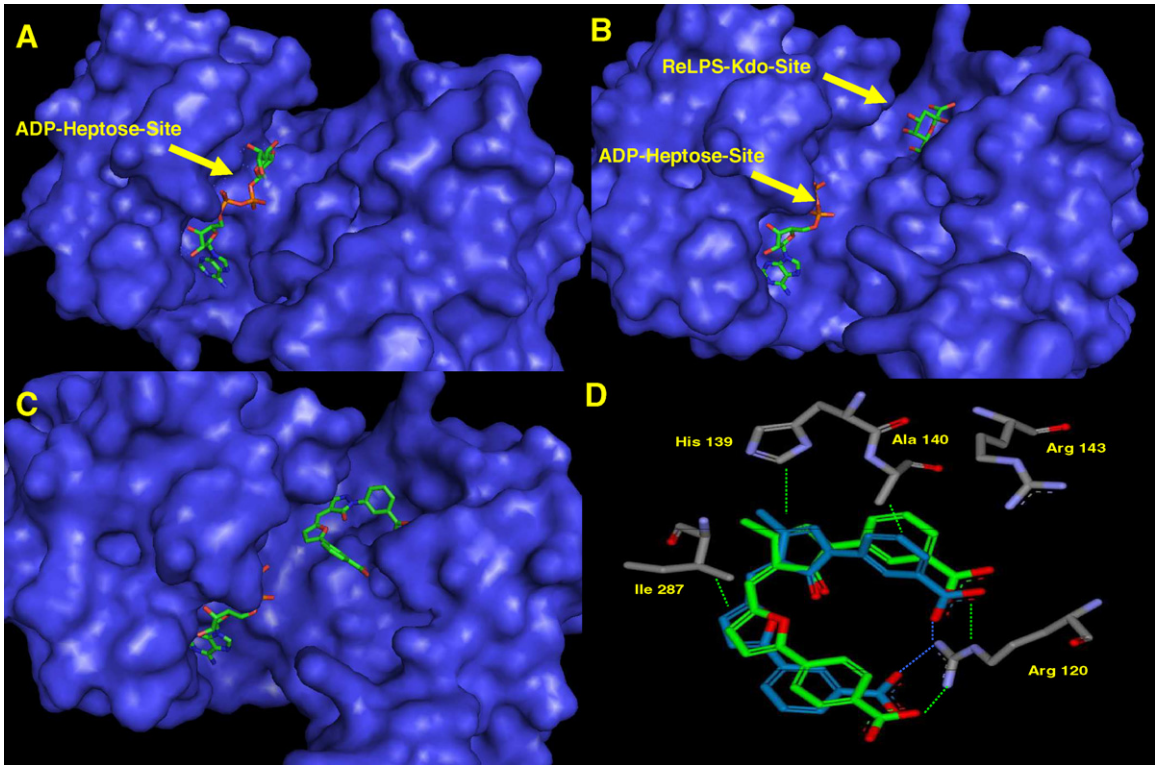
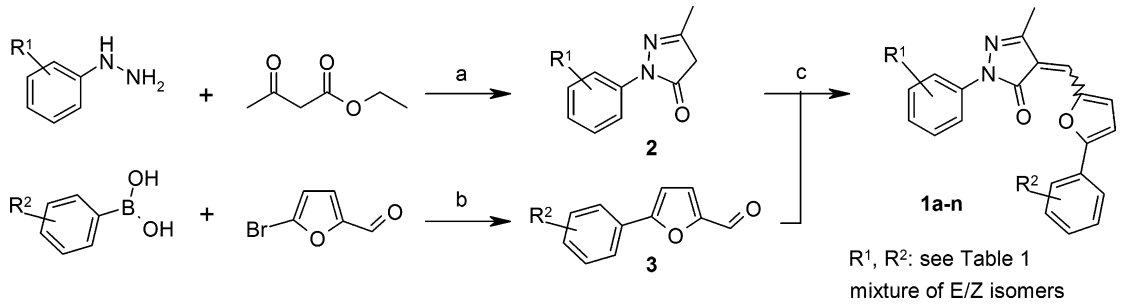
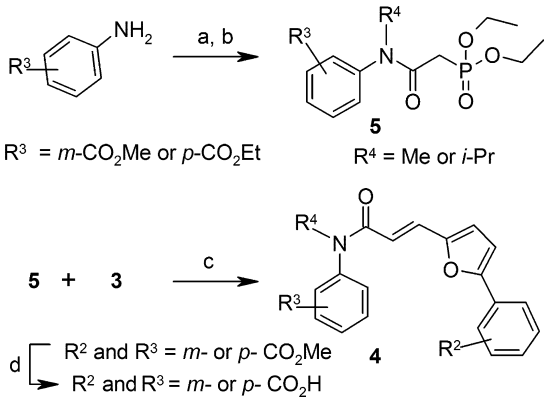


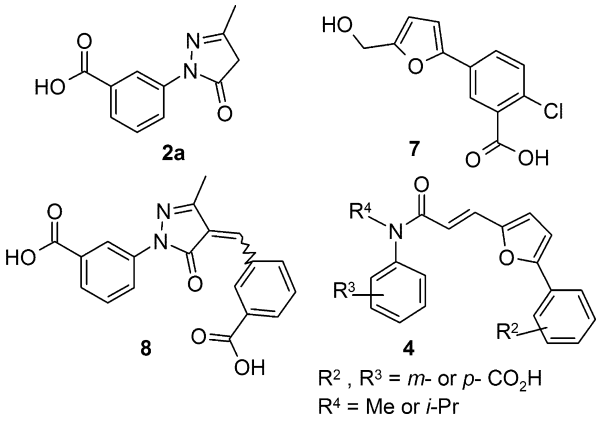
Figure 1. (A) Structure of F-ADP-heptose bound to WaaC. (B) Identification of acceptor Re-LPS site and docking of Kdo. (C) Docking of **1a** in the Re-LPS site and Kdo pocket. (D) Interactions of **1a** (blue) and **1f** (green) with key residues of WaaC.



Scheme 3. Reagents and conditions: (a) acetic acid, reflux, 3 h; (b) $Pd(PPh_3)_4$, Na_2CO_3 aqueous 2 M, toluene, methanol, reflux, 3 h; or $Pd(PPh_3)_4$, CsF, toluene, methanol, 60 °C, overnight; (c) acetic acid, sodium acetate, reflux, 3 h.



Scheme 4. Reagents and conditions: (a) formaldehyde 37% aqueous or acetone, $NaBH_3CN$ 1 M in THF, acetic acid, THF, rt, overnight; (b) (diethoxy)phosphorylacetic acid, 2-chloro-1-methyl-pyridinium iodide, TEA, dichloromethane, rt, overnight; (c) LiCl, DIPEA, acetonitrile, rt, overnight; (d) LiOH, THF, water, rt, overnight.



Scheme 5. Fragments and analogues of compounds **1a**–**1n**.

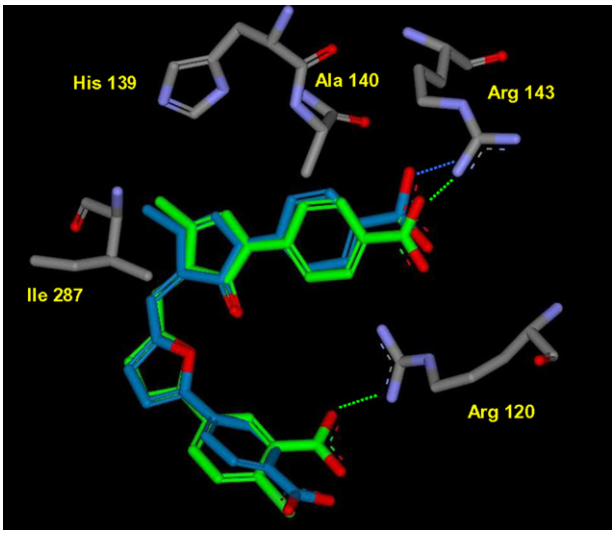


Figure 2. Lack of interactions of **1h** (blue) with Arg 120 in contrast to **1g** (green).

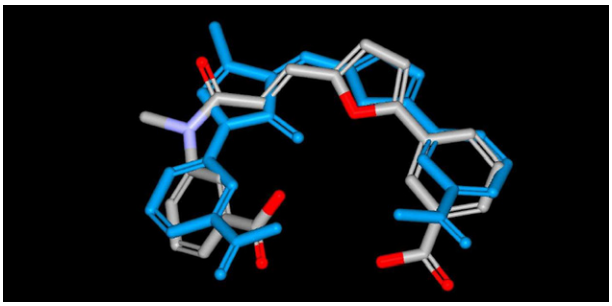


Figure 3. Overlay of **1a** with **4**.

Heterocyclic parts **2a** and **7** of the best compound **1b** were inactive, which demonstrated that the full length molecule was necessary for inhibiting the enzyme (Scheme 5). Analogues **8**[23] and **4** were also explored but displayed no activity, although acrylamide derivatives **4** positioned the two essential phenyl carboxylate groups similarly to **1a** in molecular modeling experiments (Fig. 3). Therefore we finally assessed that the furanyl-methylene-pyrazolone arrangement was essential for the best fit of our inhibitors in the Kdo pocket of the WaaC enzyme.

Mechanistic studies carried out with compound **1g** demonstrated that the inhibitors of the series were competitive with the Re-LPS substrate in agreement with the in silico binding site close to the Kdo site (Fig. 4). The inhibition by **1g** does not depend on WaaC concentration, nor on the order of addition of enzyme, substrates and inhibitor. It is not affected by the concentration of the donor substrate ADP-heptose. The inhibition kinetics are linear.

Extra-intestinal pathogenic *E. coli* (ExPEC) represent the most common cause of Gram-negative sepsis. Because WaaC defective mutant of the pathogenic *E. coli* K1 strain are significantly less virulent in mice compared to the wild type strain, the heptosyltransferase WaaC is a good target for antibacterial drugs based on the new antivirulence concept.

We have identified for the first time small molecules able to inhibit this new class of antimicrobial target.

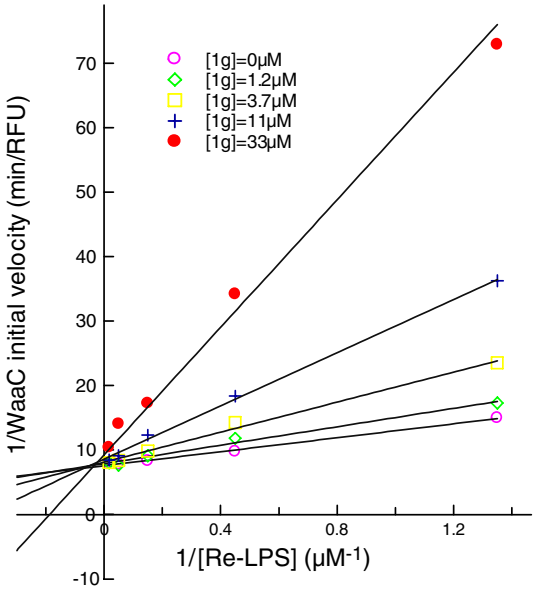


Figure 4. Competition between **1g** and Re-LPS: double reciprocal plot showing intersecting lines on *y*-axis at $1/V_{max}$.

The crystal structure of WaaC alone or in complex with ADP was used to identify the active site and binding cavities and to carry out a virtual screening of commercially available libraries. We have shown that the activity of WaaC can be easily assayed by using a synthetic ADP-heptose substrate. The 2-aryl-5-methyl-4-(5-aryl-furan-2-yl-methylene)-2,4-dihydro-pyrazol-3-one **1a** was discovered as a micromolar inhibitor and a series of analogues was synthesized to explore the SAR. All the synthetic modifications as well as the docking model and biochemical experiments are in agreement with a binding close to the acceptor site of the Re-LPS, likely in the Kdo pocket for this new class of inhibitors of WaaC.

The level of inhibition of **1b** and the preliminary SAR clearly demonstrate that this family constitutes a promising lead series for further optimization as antivirulence drug.

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19. Data for **1a** obtained as a mixture of E/Z isomers: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.38 (s, 3H, major isomer), 2.78 (s, 3H, minor isomer), 7.56–7.60 (m, 2H of both isomers and 1H of minor isomer), 7.66–7.70 (m, 1H, both isomers), 7.76 (d, *J* = 8.0 Hz, 1H, both isomers), 7.83 (d, *J* = 4.0 Hz, 1H, minor isomer), 7.86 (s, 1H, major isomer), 8.02 (d, *J* = 8.0 Hz, 1H, major isomer), 8.15 (d, *J* = 8.0 Hz, 1H, minor isomer), 8.21 (d, *J* = 8.0 Hz, 2H, both isomers), 8.47 (s, 1H, minor isomer), 8.50 (s, 1H, major isomer), 8.52 (s, 1H, minor isomer), 8.56 (s, 1H, major isomer), 8.74 (d, *J* = 4.0 Hz, 1H, major isomer). ESI-MS *m*/*z*: 417 $(M+H)^+$.
20. For instance the ratio (*Z*:*E*) for **1a** is (3.5:1) and for **1g** (3.4:1). NOESY NMR analysis (DMSO-d_6, 400 MHz) for **1a** displayed interaction between the methyl on the pyrazolone (2.38 ppm) and the olefinic proton (7.86 ppm) in the case of the Z isomer, and interaction between the methyl on the pyrazolone (2.78 ppm) and a proton of the phenyl (8.15 ppm) linked to the furan ring for the E isomer. Similar observations were made for **1g**.
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23. Compound **8** was prepared by condensation of aryl-pyrazolone **2a** with 3-formyl benzoic acid according to the same procedure as in Scheme 2.