Structure-based discovery of inhibitors of *Helicobacter pylori* urease: abridged secondary publication

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KEY MESSAGES

1. Structure-based screening was performed by docking >270 000 ligands to the crystal structures of UreG using the program DOCK.
2. Ranked by the calculated binding energies and a clustering analysis, 73 ligands were selected for *in-vitro* enzyme assay.
3. The inhibition against UreG was further improved by screening structural analogues of initial hits. The best inhibitors have IC_{50} values in the range of 20-30 μM.
4. UreG inhibitors identified, like the urease inhibitor acetohydroxamic acid, could suppress the survival of *Helicobacter pylori* in acid medium, suggesting that inhibiting the urease maturation could be a novel target for inhibiting the growth of *H pylori* in acid environment.

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Introduction

Survival of *Helicobacter pylori* in acid stomach is dependent on the urease activity that produces the neutralising ammonia from the urea.[1] For example, *H pylori* cannot survive in acid medium without the substrate urea or in the presence of urease inhibitors such as acetohydroxamic acid. The biosynthesis of active urease requires a post-translational carbamylation of an active-site lysine residue and insertion of two nickel ions to its active site.[2] This maturation process is assisted by four urease maturation factors (or urease accessory proteins), namely UreE, UreF, UreG, and UreH. Our research group has determined the crystal structures of the UreG/UreF/UreH complex, which shed insights into the mechanism of urease maturation.[2] In particular, we demonstrated how the GTP hydrolysis may promote dissociation of the UreG dimer, resulting in the release of nickel.

As the survival of *H pylori* in acid stomach requires the biosynthesis of active urease, we argue that inhibitors of UreG, a GTPase essential to the urease maturation process could be a novel strategy to treat *H pylori* infection. We proposed to use structure-based screening to identify potential inhibitors of UreG, and to screen top-ranked ligand experimentally using an in-vitro enzyme assay. We then tested if the UreG inhibitors identified can inhibit the growth of *H pylori* in an acid medium.

Methods

Structure-based screening

The DOCK 6.5 (http://dock.compbio.ucsf.edu) was used to perform the docking simulations. Five independent simulations were performed: four targeting the active site of UreG of the dimeric or the monomeric form of UreG in complex with GDP or GMPPNP, and one targeting the binding surface of UreG/UreF. For each simulation, we docked 273 477 ligands in the SPECS library of the ZINC database. For each simulation, 20 docked ligands were selected for further functional assay by two criteria. First, the docked ligands were ranked according to the binding energies calculated by the DOCK, and the top 10 ranked ligands were selected. Second, the top 1% of the ranked ligands were clustered according to their structural similarity using the ChemMine Tools (http://chemmine.ucr.edu/). One representative ligand, with the strongest binding energy, from each of the 10 largest clusters was selected. After checking redundancy and commercial availability, 73 ligands were purchased from SPECS (http://www.specs.net) for wet-lab characterisation.

In-vitro enzyme assay

Expression of *H pylori* UreG was performed as described previously.[2] Zero to 250 μM of selected ligands were added to the enzyme-mix solution (5 μM UreG, 2.5 μM $NiSO_4$, 5 mM $NaHCO_3$, 150 mM KCl, 2 mM $MgSO_4$, 1 mM TCEP, 100 mM Tris-HCl buffer at pH 7.5), and were pre-incubated at 37°C for 15 min. Enzyme reaction was started by addition of 0.3 mM GTP substrate and stopped by addition of malachite green solution (3% ammonium molybdate, 3.46 mM malachite green, 2.5 M sulfuric acid). Rate of phosphate release was measured by absorbance at 630 nm. The IC_{50} values were determined by fitting the data to a 2-parameters logistic model.

TABLE 1. Ligands selected for *in-vitro* enzyme assay

Compound No.	ZINC ID	Compound No.	ZINC ID	Compound No.	ZINC ID
1	ZINC08449002	26	ZINC02162718	51	ZINC13807353
2	ZINC08439400	27	ZINC08443800	52	ZINC00648688
3	ZINC08453762	28	ZINC08437875	53	ZINC08398604
4	ZINC08456291	29	ZINC08441228	54	ZINC19923341
5	ZINC04577554	30	ZINC02189737	55	ZINC20219862
6	ZINC08427581	31	ZINC08383771	56	ZINC20219859
7	ZINC08492481	32	ZINC05918826	57	ZINC02133508
8	ZINC08396600	33	ZINC04065337	58	ZINC20219389
9	ZINC08425959	34	ZINC01811794	59	ZINC08453144
10	ZINC08386335	35	ZINC08440845	60	ZINC02860696
11	ZINC08431251	36	ZINC08449015	61	ZINC16228240
12	ZINC08400008	37	ZINC00702974	62	ZINC13571381
13	ZINC08398283	38	ZINC06195955	63	ZINC09096557
14	ZINC08492375	39	ZINC08383742	64	ZINC08425693
15	ZINC08451538	40	ZINC19938372	65	ZINC19938388
16	ZINC08452640	41	ZINC08426112	66	ZINC05360685
17	ZINC08424865	42	ZINC08438744	67	ZINC08695208
18	ZINC08417256	43	ZINC48696926	68	ZINC00657433
19	ZINC08383253	44	ZINC19938425	69	ZINC00863679
20	ZINC08426140	45	ZINC04061246	70	ZINC13945995
21	ZINC06197239	46	ZINC19938462	71	ZINC04664681
22	ZINC08454888	47	ZINC00623301	72	ZINC19872272
23	ZINC08401405	48	ZINC19923356	73	ZINC04114176
24	ZINC02049630	49	ZINC20264072		
25	ZINC08452470	50	ZINC19909927		

Effect of ligands on the survival of *H pylori* in acid medium

The acid resistance test was used to test the effect of selected UreG inhibitors on the survival of *H pylori* in acid medium.[1] In brief, *H pylori* SS1 strain was cultured in 3 mL Brucella broth (Becton, Dickinson & Co.) with 5% foetal bovine serum (Gibco) with or without 100 μM ligands in a microaerobic environment at 37°C for 2 days. 0.5 mL of *H pylori* culture at OD600 =1 was centrifuged at 2000 g for 10 min and the cell pellet was then resuspended in pH 1 acid saline (100 mM HCl, 50 mM NaCl, 100 μM $NiCl_2$) with or without 5 mM urea and 100 μM ligands. After incubation at 37°C for 30 min, the survived colonies of *H pylori* were grown in BBL agar plates and were counted.

Results

Structure-based screening of UreG inhibitors and in-vitro enzyme assay

We have previously reported the crystal structure of UreG/UreF/UreH complex. UreG, a GTPase essential for urease maturation, forms a dimer when in complex with GTP and nickel.[2] We argued that any ligands that can block the active-site of UreG or its dimerisation should inhibit the GTPase activity. To this end, we have performed five independent docking simulations, ~270,000 ligands from the SPECS library of ZINC database were screened *in-silico*. Among the ligand candidates selected for *in-vitro* enzyme assay (Table 1), ligand 2, 4, 5, and 37 showed inhibitory effect with < 50% at 100 μM.

Analogues of ligand 37 showed improvement in inhibition against UreG activity

Among the ligands that showed inhibition towards UreG, we selected ligand 37 for further characterisation because (1) a number of structural analogues are available commercially in the SPECS library; and (2) its chemical/physical properties are in closer agreement with the drug-likeness predicted by the Lipinski's rule of five (M.W. <~500, hydrogen bond acceptor <5 and acceptors <10, LogP <5).[3] We identified 9 analogues of ligand 37 that shared a common 'central rings' structural motif, but differed in the R1 and R2 groups (Fig). To test the effect of different R1/R2 groups to the inhibitory effect on UreG activity, we measured the relative activity of UreG in the presence of 0-250 μM of ligand 37 and its analogues, and compared their IC_{50} values (Fig). Noteworthy substitution of R1 group in ligand 37a resulted in a compound that did not inhibit UreG activity, suggesting the imidazole group in R1 is important. The IC_{50} for ligand 37 was 59±3 μM. Substitution of R2 group resulted in IC_{50} values in ligand 37b to 37i ranging from 21 to 85 μM (Fig). It is likely that ligand forms specific interaction with UreG, which is dependent on the chemical structure of R2. In particular, the best analogue, 37e, has a >2-fold decrease in the IC_{50} values.

UreG inhibitors suppressed the survival of *H pylori* in acid medium

The best two inhibitors of UreG, 37e and 37h, were tested to determine if they affected the urease-dependent survival of *H pylori* in acid medium using an established method.[1] Consistent with previous observations, in the presence of 5 mM urea, *H pylori* survived in pH 1 for 30 min (Table 2). In contrast, survival of *H pylori* was greatly reduced when 100 μM of acetohydroxamic acid was added or when urea was absence. These observations suggest that the survival of *H pylori* in acid medium depends on the urease activity (Table 2). Interestingly, the survival of *H pylori* was suppressed to a level comparable to that in the absence of urea when 100 μM of UreG inhibitors 37e or 37h were added (Table 2). Taken together, our results suggest that these UreG inhibitors suppressed the survival of *H pylori* by abolishing the biosynthesis of active urease.

Discussion

In this study, we have performed structure-based screening to identify inhibitors of UreG. Based on the crystal structures determined in our laboratory, ~270 000 ligands in the SPECS library of the ZINC

database were screened using the DOCK (http://dock.compbio.ucsf.edu), and 73 top ranked ligands were selected to assay experimentally for their inhibition against UreG. After improvement by screening structural analogues, the best UreG inhibitors have IC_{50} values in the range of 20-30 μM.

Survival of *H pylori* in acidic environment is dependent on the urease activity that releases the neutralising ammonia from urea. *H pylori* cannot survive in acid medium without the substrate urea or in the presence of a urease inhibitor (Table 2).[1] Since the biosynthesis of active urease requires the GTPase activity of UreG, we anticipated that inhibitors of UreG should reduce the survivability of *H pylori* in acid medium. In fact, inhibitors 37e and 37h suppressed the survival of *H pylori* in acid medium (Table 2). The result is encouraging because blocking the biosynthesis of active urease can kill *H pylori* in acid medium, like what was observed for the urease inhibitor acetohydroxamic acid (Table 2).[1] Moreover, the growth of *H pylori* at neutral pH was not affected by these ligands. Taken together, our results suggest that the inhibition of *H pylori* growth in acid medium was a result of blocking the urease maturation pathway.

We have identified UreG inhibitors that can kill *H pylori* in acid medium. This suggests that blocking the urease maturation could be a novel target for *H pylori* infection. Nonetheless, there is still a long research/development process before any drugs of this kind are demonstrated clinically useful. UreG inhibitors 37e and 37h were tested for their *in-vivo* efficacy in an *H pylori*–infected mouse model.[4] Only 1/10 in the 37h-treatment group showed negative results on the urease test. Although histological examination revealed a somewhat reduced infection of *H pylori* in the mouse stomach, the pathogen was not eradicated by the treatment of 37e or 37h. This result suggests that the inhibitors identified in this study still need further optimisation. One can use combinatorial chemistry or structure-based rational design to further improve the existing inhibitors of UreG. Another feasible strategy to improve efficacy is to combine the UreG inhibitors with urease inhibitors in the treatment of *H pylori*. Using a weak urease inhibitor such as acetohydroxamic acid in treating *H pylori* infection probably requires a high dose that may lead to other side-effects.[5] We argue that UreG inhibitors should work synergistically with urease inhibitors because the required dose of urease inhibitors could be lower because the pathogen can no longer produce large amounts of active urease.

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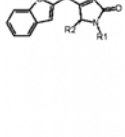
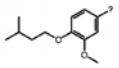
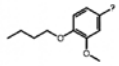
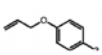
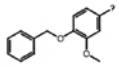
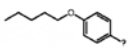
	ZINC ID	Central Rings	R1	R2	IC_{50} (μM)
37	ZINC00702974				59±3
37a	ZINC08441340				>250
37b	ZINC08897821				85±3
37c	ZINC04019664				54±4
37d	ZINC00702964				85±4
37e	ZINC00702966				21±2
37f	ZINC08441292				57±3
37g	ZINC08441299				34±2
37h	ZINC08441301				31±2
37i	ZINC00702980				39±2

FIG. IC_{50} values of ligand 37 and its analogues (37a – 37i) in inhibiting GTPase activity of UreG

TABLE 2. Survival of *Helicobacter pylori* in acid medium is suppressed by UreG inhibitors

Treatment	Mean±SD colony count after acid shock, 10^3 cfu/mL	Survival rate, %
-Urea	0.2±0.2	0.2
+Urea	117±11	100
+Urea +AHA	1.1±1.4	1.2
+Urea +ligand 37e	0.4±0.2	0.3
+Urea +ligand 37h	0.0±0.1	0.0

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