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A Picomolar Transition State Analogue Inhibitor of MTAN as a Specific Antibiotic for *Helicobacter pylori*

Shanzhi Wang,[†] Antti M. Haapalainen,[†] Funing Yan,[†] Quan Du,[†] Peter C. Tyler,[‡] Gary B. Evans,[‡] Agnes Rinaldo-Matthis,[†,∥] Rosemary L. Brown,[§] Gillian E. Norris,[§] Steven C. Almo,[†] and Vern L. Schramm*[,†]

[†]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461, United States

[‡]Carbohydrate Chemistry Team, Industrial Research Ltd., Lower Hutt, New Zealand

[§]Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

Supporting Information

ABSTRACT: *Campylobacter* and *Helicobacter* species express a 6-amino-6-deoxyfutalosine *N*-ribosylhydrolase (*Hp*MTAN) proposed to function in menaquinone synthesis. BuT-DADMe-ImmA is a 36 pM transition state analogue of *Hp*MTAN, and the crystal structure of the enzyme−inhibitor complex reveals the mechanism of inhibition. BuT-DADMe-ImmA has a MIC_{90} value of <8 ng/mL for *Helicobacter pylori* growth but does not cause growth arrest in other common clinical pathogens, thus demonstrating potential as an *H. pylori*-specific antibiotic.

Helicobacter pylori is a Gram-negative bacterium and lives microaerophilically in the gastric mucosa of its human host. It is related to 85% of gastric and 95% of duodenal ulcers.[1] After fewer than 30 years of antibiotic use in the patient population, drug resistance makes it increasingly difficult to eradicate *H. pylori* using a combination of two antibiotics with 2 weeks of therapy.[2] Antibiotics with new targets and mechanisms of action are needed to treat *H. pylori* infections.

Gram-negative bacteria are dependent on menaquinone as electron transporters and have an essential biosynthetic pathways for these critical metabolites.[3] In contrast, humans lack menaquinone synthesis, and targeting the menaquinone pathway may provide an antibacterial drug design approach. Recently, a menaquinone synthetic pathway was proposed in *Campylobacter* and *Helicobacter* that differs from that in most Gram-negative bacteria.[4,5] In these unusual organisms, 6-amino-6-deoxyfutalosine is synthesized by MqnA and cleaved at the N-ribosidic bond by a 5′-methylthioadenosine/*S*-adenosylhomocysteine hydrolase (MTAN) with specificity for 5′-methylthioadenosine and adenosylhomocysteine as well as 6-amino-6-deoxyfutalosine. *Hp*MTAN converts 6-amino-6-deoxyfutalosine to adenine and dehypoxanthine futalosine, the latter being used as the precursor of menaquinone synthesis (Figure 1A). The early reactions of this pathway do not exist in the normal bacterial flora of humans, making the enzymes catalyzing these reactions appealing drug targets.

*Hp*MTAN is closely related to the MTANs found in other bacteria. In most bacteria, MTANs are associated with quorum sensing and *S*-adenosylmethionine recycling are not involved in manzquinone synthesis, and are not essential for bacterial growth.[6] Transition state analogue inhibitors of picomolar to femtomolar affinity have been developed to interrupt bacterial functions associated with quorum sensing.[6,7] We tested MTAN transition state analogues against *Hp*MTAN to see if inhibition extended to this class of enzyme and to test if blocking *Hp*MTAN would block growth of *H. pylori*.

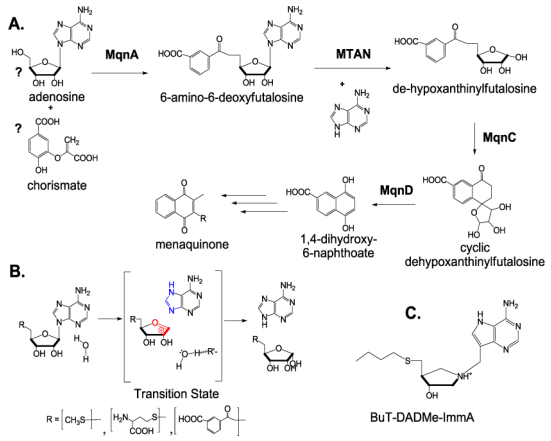

Figure 1. Proposed menaquinone pathway, reactions catalyzed by MTANs, their proposed transition states and a transition state analogue inhibitor. (A) Proposed early steps of the menaquinone pathway in *H. pylori*[4]. The proposed substrates of the MqnA reaction are labeled with question marks. (B) Reaction catalyzed by *Hp*MTAN and the proposed transition state. R′ is a proton abstracting group leading to the formation of hydroxide from water after the transition state is passed. Candidates are Glu13 and Glu175 (Figure 2). (C) Features of the transition state (blue and red) are represented in the BuT-DADMe-ImmA inhibitor.

Transition states of several bacterial MTANs have been shown to have ribocationic character with minimal participation of the attacking water nucleophile and a N7-protonated, neutral adenine leaving group.[8−10] We assumed that the transition state of *Hp*MTAN would be similar based on its homology to other MTANs (Figure 1B).[4] Transition state analogues are known to bind tightly to their cognate enzymes[11,12] by converting the

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dynamic protein motion involved in catalysis to a more stable thermodynamic state.[13]

BuT-DADMe-ImmA was previously characterized as a transition state analogue inhibitor of *Ec*MTAN (Figure 1C).[8] Here, we performed inhibition assays using BuT-DADMe-ImmA against recombinant *Hp*MTAN with 5′-methylthioadenosine as the substrate. Purified *Hp*MTAN uses both 5′-methylthioadenosine and 6-amino-6-deoxyfutalosine as facile substrates. The enzyme exhibits low K_m values for both substrates of 0.6 ± 0.3 and 0.8 ± 0.3 μM and k_{cat} values of 12.1 ± 2.3 and 4.3 ± 0.9 s^{-1}, respectively. This gives high catalytic efficiency values (k_{cat}/K_m) of 2.0 × 10^7 M^{-1} s^{-1} for 5′-methylthioadenosine and 5.4 × 10^6 M^{-1} s^{-1} for 6-amino-6-deoxyfutalosine. BuT-DADMe-ImmA is a slow-onset tight-binding inhibitor with an initial inhibition constant (K_i) of 0.8 nM and, following a slow onset of inhibition, an equilibrium dissociation constant ($K_i^* = K_d$) of 36 pM (see the Supporting Information). With a K_m value of 0.8 μM for 6-amino-6-deoxyfutalosine as the substrate, the K_m/K_d ratio is 22200 for this substrate. The low K_d value supports the proposal that BuT-DADMe-ImmA is a transition state analogue inhibitor for *Hp*MTAN. A comparison of the structures of the 5′-methylthioadenosine or *S*-adenosylhomocysteine substrates for *Hp*MTAN with their transition states (Figure 1B) shows three features of BuT-DADMe-ImmA that mimic the transition states: a hydroxyl-pyrrolidine moiety, a methylene bridge between the base and sugar, and 9-deazaadenine (Figure 1B,C). The nitrogen of the hydroxypyrrolidine moiety has a pK_a value of 9 and thus mimics the positive charge of a ribocation at the transition state. The methylene bridge extends the distance between the sugar and the purine base leaving group, as this distance is near 3 Å at the transition state. The 9-deazaadenine alters conjugation in the purine ring, causing an elevated pK_a and protonation of N7, resembling the N7-protonated adenine leaving group at the transition state.

Catalytic site features involved in tight binding of BuT-DADMe-ImmA were established from the crystal structure of *Hp*MTAN in complex with BuT-DADMe-ImmA (Figure 2). Like other MTANs, *Hp*MTAN is a homodimer belonging to the superfamily of purine and uridine phosphorylases with active sites located at the dimer interface (Figure S1 of the Supporting Information). Adenine binding is stabilized by hydrogen bonds between N7 and OG2 of Asp198 and between N1 and the main chain NH group of Val154. The hydrophobic group at the 5′-ribosyl position binding site is not tightly constrained but is surrounded by a hydrophobic environment, allowing variation at this position (Figure 2A and Figure S1). Direct interactions between BuT-DADMe-ImmA and *Hp*MTAN include five hydrogen bonds and a large number of hydrophobic interactions (Figure 2). The transition state analogue complexes of MTANs include the nucleophilic water molecule in crystal structures and in complexes detected by mass spectrometry in the gas phase.[14,15] In *Hp*MTAN, the nucleophilic water molecule is 2.6 Å from the cationic hydroxypyrrolidine nitrogen, the site of water attack in the ribocation transition state (Figure 2). The water molecule is stabilized in *Hp*MTAN by four hydrogen bonds from protein with two from bound BuT-DADMe-ImmA, contacts clearly contributing to the high affinity of the inhibitor complex.

We tested the effects of BuT-DADMe-ImmA on *H. pylori* growing on 5% horse blood agar. At 6 ng/mL, slight growth was detected, and at 8 ng/mL, no growth was detected; therefore, the MIC_{90} value for inhibition of *H. pylori* growth is <8 ng/mL (Figure 3A). The MIC_{90} value of 8 ng/mL corresponds to a chemical concentration of 23 nM, sufficient to saturate *Hp*MTAN with its K_d value of 36 pM.

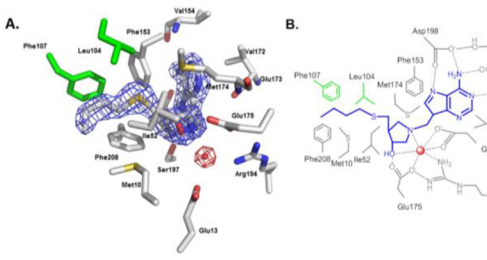

Figure 2. Active site of *Hp*MTAN in complex with BuT-DADMe-ImmA. (A) Crystal structure of the active site of *Hp*MTAN with bound BuT-DADMe-ImmA. The figure shows a $2F_o - F_c$ map around the inhibitor (blue grid) and catalytic water molecule (red grid) contoured at 1.5σ. The graph was generated using Pymol. (B) Schematic representation of interactions among BuT-DADMe-ImmA (blue), a water molecule (red sphere), and residues of *Hp*MTAN. The residues colored green belong to the neighboring monomer of the *Hp*MTAN dimer. Dashed lines represent hydrogen bonds. All hydrogen bonds are ≤3 Å in length except for three shown to water (3′-OH, 3.1 Å; Glu175, 3.3 Å; Glu13, 3.2 Å).

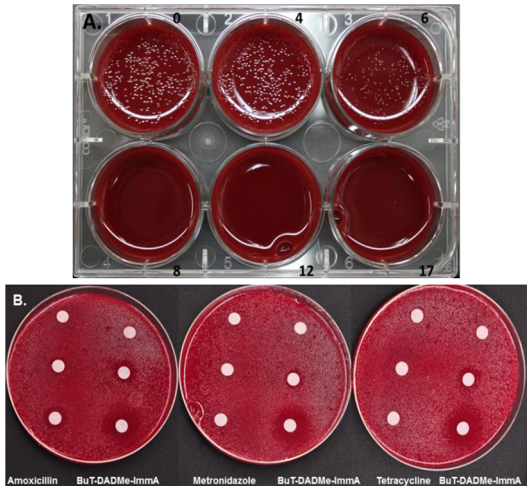

Figure 3. Effects of BuT-DADMe-ImmA on *H. pylori* growth. (A) BuT-DADMe-ImmA (ng/mL) inhibits growth on blood agar (one of five experiments in triplicate). (B) Inhibitory effects of BuT-DADMe-ImmA compared with amoxicillin, metronidazole, and tetracyclin in zone of inhibition studies. Drug quantities per disk were 0 (top disk), 10 ng (middle disk), and 20 ng (bottom disk). Each specified antibiotic was applied to the disk in the same manner. Small zones of inhibition were seen with 10 ng of BuT-DADMe-ImmA (middle right) and large zones at 20 ng (bottom right).

Commonly used antibiotics in *H. pylori* infections include amoxicillin, metronidazole, and tetracycline. We compared the anti-*H. pylori* effects of BuT-DADMe-ImmA to those of common antibiotics. The zones of inhibition for BuT-DADMe-ImmA are larger than those for any of the other antibiotics (Figure 3B). Equivalent amounts of amoxicillin gave a smaller zone of growth inhibition than BuT-DADMe-ImmA, and equivalent amounts of metronidazole or tetracycline gave no growth inhibition. Thus, BuT-DADMe-ImmA is more efficient at inhibition of *H. pylori* growth than antibiotics commonly used in ulcer therapy.[2]

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In most bacteria, MTANs catalyze the hydrolysis of the N-ribosidic bonds of 5′- methylthioadenosine and *S*-adenosylhomocysteine. These reactions are involved in bacterial quorum sensing, sulfur recycling via *S*-adenosylmethionine, and polyamine synthesis;[16] however, most bacterial MTANs are not essential for bacterial proliferation as judged by planktonic growth conditions. Thus, BuT-DADMe-ImmA did not affect the growth of *Escherichia coli* or *Vibrio cholerae*, although MTAN activity was totally abolished.[6] Likewise, *mtn* gene deletion in *E. coli* does not affect growth on rich medium but creates biotin auxotrophs.[6,17] We also tested the effects BuT-DADMe-ImmA on the growth of other clinically common pathogens, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Salmonella enterica*, and *Pseudomonas aeruginosa*. At culture concentrations of 5 μg/mL BuT-DADMe-ImmA, no growth inhibition was observed for those bacteria, consistent with a nonessential role for their MTANs. Because of the species specificity for this rare menaquinone pathway, treatment of *H. pylori* infections with BuT-DADMe-ImmA would be unlikely to generate antibiotic resistance in off-target bacteria.

Bacterial genome analysis predicts the *Hp*MTAN-mediated pathway for menaquinone biosynthesis to be rare, but also to be present in *Campylobacter* species.[4] *Campylobacter jejuni* is the world's leading cause of bacterial gastroenteritis.[18]

The action of *Hp*MTAN is proposed to be in the hydrolysis of 6-amino-6-deoxyfutalosine, and we specifically tested the enzyme for this function. The enzyme shows robust catalytic activity on this substrate with a catalytic efficiency of 5.4×10^6 M^{-1} s^{-1}. The effects of BuT-DADMe-ImmA on the enzyme and growth of *H. pylori* demonstrate a critical role of *Hp*MTAN in *H. pylori* and support the proposed pathway of an essential menaquinone biosynthetic pathway for its electron transfer chain or other function.[4,19]

Drug resistance has developed quickly in *H. pylori*, and currently, ∼30% of *H. pylori* infections are resistant to single-agent first-line drugs.[20] As a result, the current approach commonly uses triple-agent therapy for *H. pylori* infections and includes two antibiotics with different mechanisms of action. Even with triple-agent therapy, >20% of *H. pylori* infections are not readily eradicated.[2] Resistance in the *H. pylori* population is no doubt partially due to exposing *H. pylori* to broad spectrum antibiotics during the treatment of other bacterial infections. In addition, current eradication of *H. pylori* requires antibiotics for ≥2 weeks and increases the probability of resistance development if treatment is interrupted. The results with BuT-DADMe-ImmA indicate potential as a narrow spectrum antibiotic, with the opportunity for trials as a single agent or in drug combinations. The drugability of BuT-DADMe-ImmA has yet to be established. However, a similar compound with a thiomethyl rather than a thiobutyl substituent is orally available and shows low toxicity in mice.[21]

The other pathogens (*Campylobacter* species) in which MTAN also appears to be essential are currently treated clinically with ciprofloxacin, erythromycin, or azithromycin. BuT-DADMe-ImmA is a more powerful antibiotic for its target in *H. pylori* than other antibiotics are for their targets. Thus, BuT-DADMe-ImmA could also be a candidate for *Campylobacter* infections. Thus, BuT-DADMe-ImmA has potential as a specific antibiotic in organisms using MTANs in any essential biosynthetic step. Drug combinations using BuT-DADMe-ImmA may also address current issues of antibiotic resistance.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The crystal structure of the *Hp*MTAN−BuT-DADMe-ImmA complex is deposited in the Protein Data Bank as entry 4FFS.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: vern.schramm@einstein.yu.edu. Phone: (718) 430-2813.

Present Address

∥Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm, Sweden.

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Notes

V.L.S., P.C.T., and G.B.E. declare the following financial interest. The Albert Einstein College of Medicine and Industrial Research Ltd. own joint patents in regard to the synthesis and use of BuT-DADMe-ImmA. The patent owners are seeking commercial licenses for development of this technology.

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