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The synthesis and evaluation of phenoxyacylhydroxamic acids as potential agents for *Helicobacter pylori* infections

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ABSTRACT

Two series of ω-phenoxy contained acylhydroxamic acids as novel urease inhibitors were designed and synthesized. Biological activity evaluations revealed that ω-phenoxypropinoylhydroxamic acids were more active than phenoxyacetohydroxamic acids. Out of these compounds, 3-(3,4-dichlorophenoxy)propionylhydroxamic acid **c24** showed significant potency against urease in both cell free extract (IC_{50} = 0.061 ± 0.003 μM) and intact cell (IC_{50} = 0.89 ± 0.05 μM), being over 450- and 120-fold more potent than the clinically prescribed urease inhibitor AHA, repectively. Non-linear fitting of experimental data (V-[S]) suggested a mixed-type inhibition mechanism and a dual site binding mode of these compounds.

1. Introduction

Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach, causing gastric and duodenal ulcers, and in some case leading to gastric carcinomas and lymphomas.[1,2] The Center for Disease Control and Prevention estimates that more than two-thirds of the world's population is infected with *H. pylori* bacteria.[3] A triple therapy, dual antibiotic treatment incorporating a proton pump inhibitor, is the most common used method for eradicating *H. pylori* infection. However, the emergence of antibiotic resistant *H. pylori* leads to antibiotics being increasingly ineffective, and development of new therapies is highly needed.

One of the major adaptations that allow *H. pylori* to thrive in highly acidic stomach environment is the utilization of urease (EC 3.5.1.5). This enzyme catalyzes urea degradation to produce ammonia and carbamate, creating a neutralizing environment for bacterial survival.[2,4] In addition, Mobley and his coauthors disclosed that the urease-negative mutant of *H. pylori* strain was unable to colonize the gastric mucosa under the acidic conditions of the stomach.[5,6] These observations show that *H. pylori* urease plays an important role in various gastric-related diseases, especially in pathogenesis of gastric and peptic ulcers, and is considered as one of the *H. pylori* virulence factors. Inhibition of urease might lead to a very mild evolutionary pressure for the development of resistance, and seems to be an exciting strategy for treating *H. pylori* infections. Furthermore, humans do not produce urease, and no human nickel enzymes are known, making urease an ideal therapeutic target.[7–10] In the past decades, despite thousands of urease inhibitors were reported including nickel chelators,[11] substrate analogues[12–14] and metal complexes,[15,16] effective inhibitors have not been exhaustively explored. Most of the known urease inhibitors display either only a moderate potency or unfavorable features with the failure of clinical applications. Up to date, only one urease inhibitor, acetohydroxamic acid (AHA), has been approved for the treatment of patients with infection induced staghorn renal calculi,[17] suggesting the urgent need of more in-depth investigations in urease inhibitor design and discovery.

In the past several years, our efforts have focused on the discovery of novel urease inhibitors with structural diversity. Many classes of compounds (e.g. deoxybenzoins,[18] 1,2-diarylethanes,[19] flavonoids,[20,21] flavenes,[22] 3-arylpropionylhydroxamic acid[23,24] and furan-2(5*H*)-ones[25]) were identified as urease inhibitors in our group, and 3-arylpropionylhydroxamic acid deserves to be mentioned. The known Ni-binding property of hydroxamic acid and the S-H…π forming property of aryl ring with the residue Cys321[24] are highly required elements for *H. pylori* urease inhibition, suggesting that 3-arylpropionylhydroxamic acid is a promising scaffold of urease inhibitor for the expanding survey.

In the present study, we describe the biological evaluation of

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phenoxyacylhydroxamic acids as novel *H. pylori* urease inhibitors, which were designed by replacing CHOH in 3-arylpropionylhydroxamic acids scaffold with an O atom. The successful synthesis of the designed compounds facilitated the kinetic evaluation of their potency against ureases purified from *H. pylori*, together with the *H. pylori* whole-cell urease studies.

2. Materials and methods

2.1. Biology materials

Protease inhibitors (Complete, Mini, EDTA-free) were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Brucella broth was from Becton Dickinson and Company (Sparks, MD). Sheep sterile and defibrinated blood was from Hyclone (Utah, American).

2.2. Bacteria

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was grown in Brucella broth supplemented with 10% sheep sterile and defibrinated blood for 24 h at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2), as our previously described literatures.[18,19,21,26]

2.3. Preparation of H. pylori urease

For urease inhibition assays, 50 mL broth cultures (2.0×10^8 CFU/mL) were centrifuged (5000 × *g*, 4 °C) to collect the bacteria, and after washing twice with phosphate-buffered saline (pH 7.4), the *H. pylori* precipitation was stored at −80 °C for 8 h, and then was returned to room temperature, and after addition of 3 mL of distilled water and protease inhibitors, sonication was performed for 60 s. Following centrifugation (15,000 × *g*, 4 °C), the supernatant was desalted through Sephadex G-25 column (PD-10 columns, Amersham Pharmacia Biotech, Uppsala, Sweden). The resultant crude urease solution was added to an equal volume of glycerol and stored at 4 °C until use in the experiment.

2.4. Measurement of urease inhibitory activity

The assay mixture, containing 25 mL (10U) of *H. pylori* urease which was replaced by 25 mL of cell suspension (4.0×10^7 CFU/mL) for the urease assay of intact cells and 25 mL of the test compound, was preincubated for 1.5 h at room temperature in a 96-well assay plate. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[27]

2.5. Kinetic study

Based on the indophenol method, the velocity of ammonia production (V) was measured in the presence of concentration gradients of urea ([S]) for every specific concentration of **24c** ([*I*]). Nonlinear fitting curves to data of V and [S] were used to determine the type of enzyme inhibition based on the general kinetics equation (Eq. (1)). Consequently, resulted data of *a* (Eq. (2)) or *b* (Eq. (3)) and [*I*] were treated as a linear fitting to give the inhibitory constants K_i' and K_i, which were determined from the intersection on the X-axis of the corresponding fitting line. All experiments were conducted in triplicate.

2.6. Protocol of docking study

Molecular docking of compounds **c12**, **c18** and **c24** into the structure of *H. pylori* urease complex structure was carried out using SYBYL-X version 2.1.1 software suite (Tripos, Inc., St. Louis, MO).[28] The X-ray structure of urease from *H. pylori* was downloaded from the Protein Data Bank (PDB code: 1e9y)[29] and was modified by adding hydrogen atoms and removing water as well as cocrystallized substrate (AHA). The active site was defined as all the amino acid residues confined within a 5 Å radius sphere centered about AHA, and the composite structure without original ligand was utilized as the in silico model for docking studies. Default parameters and values within the minimization dialogue were used except where otherwise mentioned. The docked conformations of ligands were evaluated and ranked using Surflex-Dock and four scoring functions implemented in the CSCORE software module within the SYBYL-X environment. The CSCORE module allowed consensus scoring that integrated multiple well-known scoring functions such as ChemScore, d-Score, G-Score and PMF-Score to evaluate docked ligand conformations.

2.7. Chemistry

All chemicals (reagent grade) used were purchased from Aldrich (U.S.A.) and Sinopharm Chemical Reagent Co., Ltd (China). Melting points (uncorrected) were determined on a XT4 MP apparatus (Taike Corp., Beijing, China). EI mass spectra were obtained on Waters GCT mass spectrometer, and ^{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were performed on a Foss Heraeus CHN-O-Rapid instrument and were within ± 0.4% of the theoretical values.

2.7.1. General procedure for the preparation of compound b

To a solution of a selected phenol (**a**, 5 mmol) in dry acetone (25 mL) was added an equivalent of anhydrous K_2CO_3, which was heated to reflux (or in dry DMSO at room temperature). Then, 4.5 mmol of ethyl bromoacetate (or ethyl 3-bromopropionate) in dry acetone or DMSO (5 mL) was added dropwise over 30 min. The reaction mixture was stirred for 3–8 h (monitored by TLC), and was poured into distilled water (20 mL). The mixture was extracted with EtOAc (3 × 50 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash silica chromatography to afford **b**.

2.7.2. General procedure for the preparation of compounds *c1–c37*

A mixture of sodium methoxide (10 mmol) and hydroxylamine hydrochloride (6 mmol) in anhydrous methanol was well stirred for 30 min. To this mixture was added compound **b** (3 mmol), and the mixture was stirred at room temperature for 1–3 h. The reaction was quenched with ice (30 g) and neutralized with dilute HCl, which was extracted with ethyl acetate (3 × 60 mL). The organic layers were collected, dried over sodium sulfate, filtered, and evaporated *in vacuo*. The residue was purified by flash silica chromatography (methanol-dichloromethane) to afford pure product (**c1–c35**) as a white solid.

*2.7.2.1. 2-Phenoxyacetohydroxamic acid (**c1**).* White powder, 48%, mp: 113–115 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.45 (s, 2H, CH_2); 6.93–6.99 (m, 3H, 2,6,4-ArH); 7.25–7.35 (m, 2H, 3,5-ArH); 8.98 (s, 1H, NH); 10.83 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 66.21 (CH_2); 115.07 (2C, 2,6-ArC); 121.59 (4-ArC); 129.91 (2C, 3,5-ArC); 158.23 (1-ArC); 164.76 (C═O); MS (EI) *m/z* 167 (M^+), 150, 77, 60; Anal. Calcd for $C_8H_9NO_3$: C 57.48; H 5.43; N 8.38; found: C 57.45; H 5.43; N 8.39.

*2.7.2.2. 2-(o-Methylphenoxy)acetohydroxamic acid (**c2**).* White powder, 52%, mp: 105–107 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.20 (s, 3H, CH_3); 4.47 (s, 2H, CH_2); 6.85 (d, J = 8.1 Hz, 1H, 6-ArH); 6.87 (t, J = 7.5 Hz, 1H, 5-ArH); 7.14 (t, J = 7.4 Hz, 1H, 4-ArH); 7.15 (d, J = 7.5 Hz, 1H, 3-ArH); 9.87 (s, 2H, NH and OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 20.49 (CH_3); 66.85 (CH_2); 113.52 (6-ArC); 120.70 (4-ArC); 125.60 (2-ArC); 126.17 (5-ArC); 134.36 (5-ArC); 158.83 (1-ArC); 163.93 (C═O); MS (EI) *m/z* 181 (M^+), 164, 91, 60; Anal. Calcd for $C_9H_{11}NO_3$: C 59.66; H 6.12; N 7.73; found: C 59.68; H 6.12; N 7.72.