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Fluoroquinolones as Urease Inhibitors: Anti-*Proteus mirabilis* Activity and Molecular Docking Studies

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Abstract The anti-*Proteus mirabilis* activity and MIC of levofloxacin and ciprofloxacin were investigated in comparison with the known urease inhibitor acetohydroxamic acid using Well Diffusion method. Also, their inhibitory effect on urease was determined by measuring ammonia production as an indicator of urease activity using the indophenol method as described by Weatherburn. AHA showed a weak anti-*Proteus mirabilis* activity the (MIC = 614.8 µM) than the two tested fluoroquinolones (MIC for levofloxacin = 3.2 µM and for ciprofloxacin = 15.62 µM). The tested fluoroquinolones experienced excellent urease inhibitory activity IC_{50} for levofloxacin = 2.9 µM and for ciprofloxacin = 3.5 µM). However, the results were supported by molecular docking studies to gain insights into the binding conformations as well as the inhibition mode of urease and showed coordination binding with the two Ni ion in the active site which are essential for urea breakdown.

Keywords: *Flurorquionolones, Urease inhibition, Anti-Proteus mirabilis, Docking*

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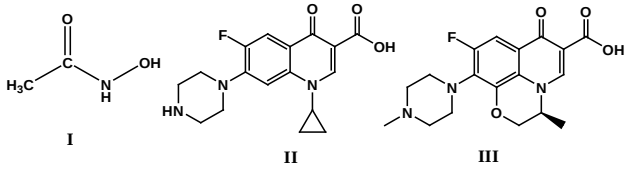
1. Introduction

Urease enzyme or aminohydrolase is a multi-subunit, nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea into carbamate and ammonia which is the final step of urea catabolism. [1] It is widely distributed among different types of bacteria like *Helicobacter pylori* and *Proteus mirabilis*. Bacterial ureases act as a virulence factor, including the formation of infection stones, pyelonephritis, peptic ulceration and hepatic encephalopathy and other diseases. [2] Furthermore, the generated ammonia causes severe metabolic disorders in addition to severe damage to the GIT epithelium by its interaction with the immune system of human being. [3,4,5] By that time, crystallographic structures for a limited number of bacterial urease has been reported. However, amino acid sequences of all investigated ureases are analogically maintained, which shows the presence of two Ni ions connected with the carboxylate group of the carbamylated lysine and coordinated by some surrounding histidine and aspartic residues [Hisα249 and Hisα275 for Ni (3001) and Hisα137, Hisα139, and Aspα363 for Ni (3002) that implies a common catalytic pathway. [6] Although, a variety of urease inhibitors have been investigated in the past, such as hydroxamic acid derivatives [7] imidazoles [8] and phosphorodiamidates, [9] a lot of them are banned from using in vivo because of their toxicity or instability and another part of them had severe side effects. So, it is crucial to synthesize new potent urease inhibitors with good stability, bioavailability, and low toxicity. Although acetohydroxamic acid (AHA) is a known urease inhibitor with a weak antibacterial activity. [10] It is a potent urease inhibitor with a Ki value of 5 mm at 25°C. [11] Stoichiometrically, two moles of hydroxamic acid can inhibit one mole of urease. [12] Moreover, AHA shows a clinically significant growth inhibition of struvite stones in the short term in patients infected with urea-splitting bacteria. [13] Acetohydroxamic acid (I) was approved by the FDA in 1983 to treat chronic urea-splitting urinary infections under the name of Lithostat®; in Europe, it was introduced as Uronefrex®. [14]

On the other hand, fluoroquinolones are a very important class of synthetic antibacterial agents against various bacterial infections including urinary tract, [15] GIT and respiratory infections. It was reported that norfloxacin can inhibit urease activity in *Proteus Vulgaris* and *Proteus mirabilis* utilizing sub-inhibitory concentrations. [16]



Based on the above findings, the aim of this research is testing the anti-*Proteus mirabilis* activity and urease inhibitory activity of the known fluoroquinolones, ciprofloxacin (II), and levofloxacin (III). In addition, we aim to prove the binding of the selected compounds with

the active site of the urease enzyme through molecular modeling studies.

2. Results and Discussion

2.1. Biological Activity

2.1.1. Antibacterial Screening

In this work, *Proteus mirabilis* strain was isolated from the urine of patient suffering from urinary tract infection. *Proteus mirabilis* strain was negative for hemolysis, motile and Urease positive. The antibacterial activity of the tested compounds against *Proteus mirabilis* showed that levofloxacin experienced the highest antibacterial activity (MIC = 3.2 µM), followed by ciprofloxacin (MIC = 15.62 µM) while other compounds showed lower activity.

Table 1. Minimum inhibitory concentration of the tested compounds against *Proteus mirabilis*

Compound	MIC (µM)
Levofloxacin	3.2
Ciprofloxacin	15.62
AHA	614.8

2.1.2. Urease Inhibitory Activity

It was found that the tested compounds and the standard urease inhibitor AHA had the ability to inhibit the urease production at sub–MIC concentrations (screening test). Change in pH value due to the production of ammonia from urea was determined at 2 h intervals for 18 h. *Proteus mirabilis* incubated with urea showed an increase in the pH value ranged from 7.3 to 9. In the presence of levofloxacin and (1/32 X MIC –1/2 MIC), A slight decrease in the pH value was observed (7.3- 8) by the increase in concentration. *N*-acetyl Ciprofloxacin and ciprofloxacin showed a change in pH value from7.3 to 7 but 2 decreased the pH value from 7.3 to 6 by the increase in the concentration.

The Urease inhibitory activity and IC_{50} of the tested compounds at different concentrations were evaluated. The results obtained were compared to that obtained by the reference AHA. It was found that the inhibitory activity of the tested compounds in comparison to a positive control (without inhibitor) as following: urease inhibition ranged from 82 to 98% for ciprofloxacin, 94 to 96.9% for *N*-acetyl Ciprofloxacin. Levofloxacin inhibited Urease enzyme in the range of 82.8- 92%. AHA showed inhibitory activity ranged from 90-98% in comparison to control (without inhibitor). IC_{50} of the tested compounds was shown in Table 2.

Table 2. IC_{50} of Urease Inhibition of the tested compounds

Compounds	IC_{50} (µM)	% of inhibition
Levofloxacin	2.9±0.2	82.8 – 92
Ciprofloxacin	3.5±0.051	90 - 96.9
AHA	120±0.06	90 - 98

2.2. Molecular Docking Studies Using MOE

The MOE® Docking program was used for the molecular docking of the newly synthesized compounds. All synthesized compounds docked into the binding pocket of the active site of urease (PDB: 1E9Y) to investigate the docking fitness scores of bioactive conformations and their specificity for urease enzyme. The docking reliability was validated using the known X-ray structure of *Helicobacter pylori* urease in complex with AHA. The ligand AHA was extracted from the complex and re-docked to the binding site of *Helicobacter pylori* urease (Figure 1). The top-ranked conformation of each compound was selected on the basis of docking score (S). The docking scores of the studied compounds are shown in Table 3.

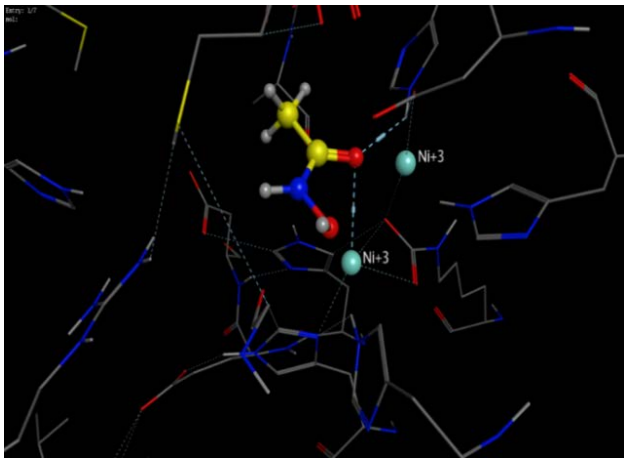


Figure 1. 3D of AHA Docked into *Helicobacter pylori* urease

The molecular docking studies showed that all the tested compounds interact with the bi-nickel center of the urease enzyme. The binding score ranges from -125 to -32 proposing that the binding differs as the functional group is varied. As compound AHA gives the lowest score, i.e. -125.530 it suggests that this is the strongest binding inhibitor.

Table 3. Docking Scores of testing compounds

Compound	Docking Score/Ki
Levofloxacin	-119.300
Ciprofloxacin	-32.627
AHA	-163.499

The binding mode of levofloxacin (Figure 2) shows two coordination bonds with both nickel atoms. The Ni3001 coordinates with the oxygen of hydroxyl group otherwise; Ni3002 coordinates with the oxygen of carbonyl group. Furthermore, Hydrogen bond is shown with His138 and a hydrophobic bond with His221.

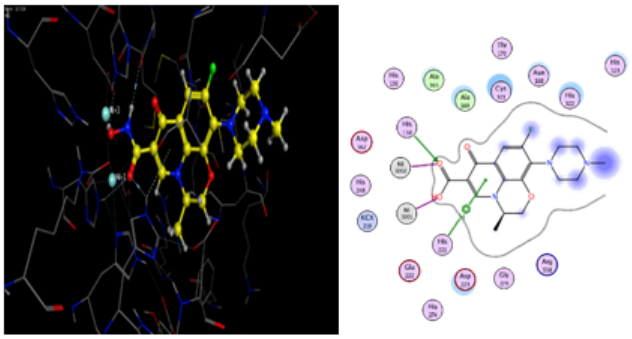


Figure 2. 2D and 3D docking modes of levofloxacin with *Helicobacter pylori* urease

In addition, ciprofloxacin (Figure 3) form two coordinate bonds with both Ni ions and one only hydrophobic bond with His221 and a hydrogen bonding with His274.

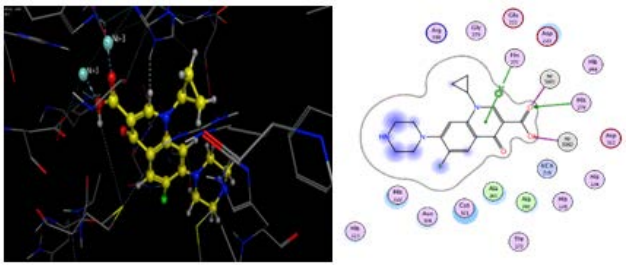


Figure 3. 2D and 3D docking modes of ciprofloxacin with *Helicobacter pylori u*rease

3. Conclusion

Levofloxacin and ciprofloxacin experienced excellent urease enzyme inhibitory activity with remarkable activity against *Proteus mirabilis*. Consequently, the two tested compounds bind with the two Ni ions of the active site of *Helibacter pylori*. It is clear that the levofloxacin and ciprofloxacin could be beneficial urease inhibitors to prevent the formation of kidney stones associated with urinary tract infection.

4. Experimental

4.1. Materials

AHA was purchased from Sigma-Aldrich. Ciprofloxacin and levofloxacin were purchased from Medical Union Pharmaceutical (MUP) Cairo, Egypt.

4.2. Biological Activity

Bacterial strains, chemicals, and media: A *Proteus mirabilis* strain was isolated from a urine sample obtained from a patient suffering from urinary tract infection. Isolation and identification were performed according to standard procedures. [15,16] The isolate was cultured on trypticase soy agar (TSA, Difco) slants for daily use and stored in a trypticase soy broth medium (TSB, Difco) along with 15% glycerol, at –80°C for subsequent uses.

4.2.1. Evaluation of the Antibacterial Effect of the Tested Compounds and the Determination of Their MIC Using Broth Microdilution Method:

The antimicrobial activity the tested compounds against *Proteus mirabilis* was performed using a microdilution method according to procedures recommended by the Clinical Laboratory Standards Institute (CLSI, 2011). Briefly, 2-fold serial dilutions of the compounds were prepared in sterile Mueller-Hinton Broth (MHB, Oxoid) for a testing concentration range of 0.244 -500µM while the standard Urease inhibitor (AHA) was tested at concentrations of (0.0032-1mM). Then 100 µL of each dilution was transferred into the well of a microtiter plate and inoculated with 5 µL of standardized (1.5 × 107CFU/ml) cell suspension. Plates were incubated at 37°C overnight, and the lowest concentration of the tested compounds that prevented visible growth was recorded as the MIC.

4.2.2. Screening for the Effect of the Tested Compounds on Urease Production:

The tested compounds were diluted in Christensen medium with urea and a phenol red as a pH-indicator. The tested concentrations were in the range of 0.244 -250µM and its biological activity were assessed after 24 h incubation at 37°C. After incubation, concentrations that inhibited the activity of urease (no change of Christensen medium color) were determined. [17]

Also, we determined the change of the pH value of TSB containing 500 mm of urea (pH adjusted to 7.3) in the presence of different concentrations (1/32, 1/16, 1/8, 1/4, 1/2 X MIC) of the tested agents. [18] Media containing urea were inoculated with 5 × 105 CFU/ml. *Proteus mirabilis* was considered as positive control while uninoculated media were used as negative control. The pH values of TSB containing urea (with the tested agents and control) were screened at 2 h intervals for 18 h along the experiment using a digital pH - meter (Elmetron CP-215). The increase in pH indicates the activity of Urease enzyme.

4.2.3. Determination of Urease Enzyme Concentration in the Bacterial Lysate and IC_{50} of the Tested Compound:

A total of 50 µL of an overnight culture of *Proteus mirabilis* in MHB were transferred into 10 ml sterile MHB and additionally incubated 18 h at 37°C with constant shaking. The cells were pelleted by centrifugation at 1258 g for 15 min (4°C). The pellet was washed three times with a 10 mm K_2HPO_4 solution and re-suspended in 2 ml of the same solution. Thereafter, to release the urease, bacteria were sonicated for 90 seconds with 0.5 cycles at 100% amplitude using an ultrasonicator (UP200H, Hielscher Ultrasonics, Teltow, Germany) in an ice container. The resulting bacterial lysate was used for the urease activity assay.

4.2.4. Determination of the Urease Activity and the Concentration of the Enzyme in the Bacterial Lysate:

Urease activity assay was performed in a microtiter plate using the phenol red colorimetric method in a mixture containing 10 mM K2HPO4 solution (pH 6.2), 0.002% phenol red and 500 mM urea (assay reagent). The increase in absorbance at 570 nm was recorded using a microplate reader (BioTek, USA). [19], [20] To determine the concentration of urease enzyme in the lysate, the lysate was centrifuged for 3 min. then the absorbance (A) of the upper solution was determined in λ = 278 nm. By using following equation, $A = \lambda bc$, where c is the concentration of solution (mol/L), b the Length of the UV cell [21,22].

4.2.5. Determination of IC_{50} of the Tested Compounds:

In vitro inhibition studies on urease were determined using Indophenols method, which measures the liberation of ammonia from the reaction. [23] The assay mixture, containing 50 µl (2 mg/ml) of enzyme and 100 µl of different concentration of the tested agents, was added to 850 µl of 25mM urea and pre-incubated for 0.5 h in water bath at 37°C. The urease reaction was stopped after 30 min incubation by the following procedure. After pre-incubation, 500 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 500 µl of alkali reagent (1% w/v NaOH and 0.075% active chloride

NaOCl) were added to 100 µl of incubation mixture and kept at 37°C for 30 min. The absorbance was measured at 625 nm. All experiments were performed in triplicate in a final volume of 1 ml, and AHA was used as a standard urease inhibitor. Percentage of inhibitions were calculated using the formula (100 – (OD sample / OD control) × 100). The concentration that provokes an inhibition halfway between the minimum and maximum response of each compound (relative IC50) was determined by monitoring the inhibition effect of various concentrations of compounds in the assay.

5. Protocol of Docking Studies

The automated docking simulation study is performed using Molecular Operating Environment (MOE®) version 2014.09, at Assiut University Faculty of Pharmacy, Chemical Computing Group Inc., and Montreal, Canada. The X-ray crystallographic structure of the target urease (1E9Y) was obtained from Protein data bank. The target compounds were constructed into a 3D model using the builder interface of the MOE program. After checking their structures and the formal charges on atoms by 2D depiction, the following steps were carried out:

- The target compounds were subjected to a conformational search.
- All conformers were subjected to energy minimization, all the minimizations were performed with MOE until an RMSD gradient of 0.01 Kcal/mole and an RMS distance of 0.1 Å with MMFF94X force-field and the partial charges were automatically calculated.

The enzyme was prepared for docking studies by:

- Hydrogen atoms were added to the system with their standard geometry.
- The atoms connection and type were checked for any errors with automatic correction.
- Selection of the receptor and its atoms potential were fixed.
- The MOE® Alpha Site Finder was used for the active site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha Spheres.

Conflict of Interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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