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Molecular modeling-based antioxidant arylidene barbiturates as urease inhibitors

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ABSTRACT

Previously we have reported arylidene barbiturates **1**–**18** as a novel class of antioxidants; however, their urease inhibitory potential has not yet been explored. In this communication, molecular docking studies were used to predict the potential ligands from compounds **1**–**18** which culminated in the identification of certain new urease inhibitors. Ligands were screened *in vitro* for their urease inhibitory potential. Compound **1**, as deduced from modeling studies, was found to be the most active urease inhibitor (13.0 ± 1.2 μM), when compared with the standard thiourea ($IC_{50} = 21.1 \pm 0.3$ μM). All of the compounds were found to be nontoxic to *Artemia salina* in brine shrimp lethality bioassay.

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) is an enzyme that catalyzes the hydrolysis of urea to carbon dioxide and ammonia. Many microorganisms utilize urea as a source of nitrogen for augmentation. Urease plays a pivotal role in nitrogen metabolism of plant during the germination process [1,2]. Unfortunately, excessive level of soil urease degrades urea more rapidly and results in phytopathic effects and loss of volatilized ammonia [3], while urease is a virulence factor in certain human and animal ailments. It contributes in the development of kidney stones, pyelonephritis, peptic ulcers leading to gastric cancers, and other diseases [4]. The obvious remedy for treating bacterial infection with antimicrobials, however, often proved to be unsuccessful [5]. Currently the gastric cancer [6,7] is the fourth most common cancer and the second most common cause of cancer-related deaths worldwide [8].

At present, it is accepted that *Helicobacter pylori* infection in gastrointestinal tract plays a prominent causative role [9]. Sequential changes in the gastric mucosa take place before neoplasia develops [10]. This includes low level of inflammation and ulceration of gastrointestinal membrane. Research has focused on the possibility that "oxidative stress" due to chronic inflammation may be a key step in the chain of pre-neoplastic events [11]. *H. pylori* infection leads to increased expression of inducible nitric oxide synthase and production of nitric oxide [12]. Reactive nitrogen oxides species cause damage to DNA and changes in the epithelial cell cycle [13]. Antioxidant enzymes such as catalase and superoxide dismutase may prevent the cellular damage induced by the oxidative stress caused by *H. pylori*-related inflammation [12]. A clinical trial in China reported decline in gastric cancer mortality in subjects taking antioxidant supplements [14]. A 6-year trial in Colombia tested the effect of anti-*H. pylori* treatment along with dietary supplementation with antioxidants [15]. Eradication of the infection and/or dietary supplementation with β-carotene, ascorbic acid, or both agents independently resulted in a significant regression of peptic lesions. These results support the hypothesis that oxidative stress may represent the final common path of *Helicobacter pylori*-related carcinogenesis.

Barbituric acid and its derivatives exhibited a wide range of biological activities such as antibacterial, hypotensive, and tranquilizing [16]. Furthermore, arylidene barbiturates are commonly used as precursors for the synthesis of bioactive molecules [17]

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while their derivatives are also very important intermediates in organic reactions [18].

Barbiturates played an important role throughout the history, including their traditional use as sedative and hypnotic agents, their use for schizophrenic patients in so-called "sleep cures", the application of phenobarbital and their use in the treatment of epilepsy, as well as the introduction of thiobarbiturates in intravenous anesthesia are also known [19].

Recently we have reported arylidene barbiturates **1**–**18** as a novel class of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavengers (antioxidants) [20]; however, their urease inhibitory potential was not yet been explored. Through molecular modeling simulations, performed on compounds **1**–**18**, certain potential ligands for urease binding were identified and screened *in vitro*. Current study resulted in the recognition of new urease inhibitors which have already proved their efficacy as antioxidant compounds. Urease inhibitors with antioxidant properties may be proved as hallmark for the development of new antiulcer drugs which will not only clear the *H. Pylori* infection but also the induced oxidative stress be redressed.

2. Methodology

2.1. Experimental general

2.1.1. Molecular docking simulations

In silico predictions of potential ligands from a series of compounds (**1**–**18**) in the active site of *Bacillus pasteurii* urease were performed by using GOLD program [21]. The docking protocol, the method for preparation of protein and compounds structures was used as mentioned previously [22].

2.1.2. Urease inhibition assay (In vitro)

The assays were performed at pH 8.2 (0.01 M $K_2HPO_4{\cdot}3H_2O$, 1 mM EDTA and 0.01 M LiCl). Percentage inhibitions were calculated by the formula:

$$100 - \left(\frac{OD_{testwell}}{OD_{control}}\right) \times 100; \quad \text{where} \quad OD = \text{optical density}$$

Thiourea was used as the standard inhibitor of urease.

Reaction mixtures comprising 1 unit of urease (*Bacillus pasteurii*) solution and 55 μL of buffers containing 100 mM urea were incubated with 5 μL of test compounds (1 mM concentration) at 30 °C for 15 min in 96-well plates. Urease enzymatic activity was determined by measuring ammonia production by using the indophenol method, as described by Weatherburn [23]. Briefly, 45 μL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 μL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min by using a microplate reader (Spectra Max, Molecular Devices, CA, USA). All reactions were performed in triplicate in a final volume of 200 μL.

2.1.3. Brine shrimp lethality bioassay

20 mg of the test sample was dissolved in the respective organic solvent. From this stock solution, 5, 50, 500 μL were transferred to vials (3 vials/concentration). The final concentration was 10, 100, 1000 μg/mL, respectively. The solvent was evaporated over night.

Brine shrimp (genus; *Artemia*: specie; *Artemia salina*: Linnaeus, 1758) eggs were hatched and after two days, when shrimp larvae were ready, 1 mL of seawater and 10 shrimps were added to each vial (30 shrimps/dilution), and the volume was adjusted with seawater to 5 mL per vial. After 24 h the number of survivors was counted and data were analyzed by Finney computer program to determine the LD_{50} [24,25].

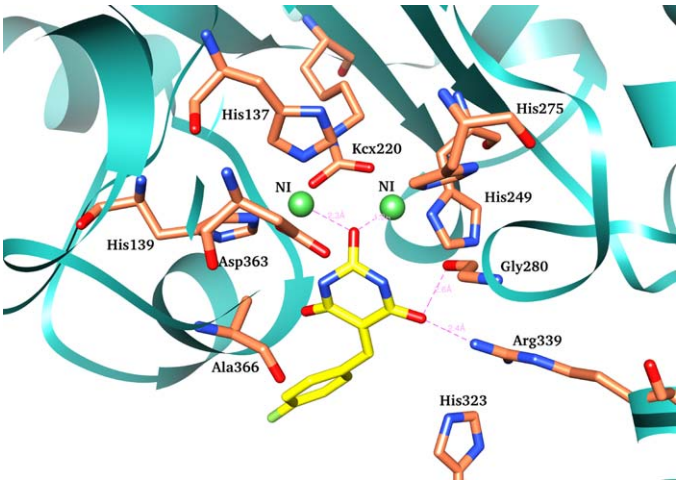


Fig. 1. Predicted binding mode of compound 1 (carbon atoms depicted in yellow) to urease. The carbon atoms in several key residues in urease are colored gold, while nitrogen and oxygen atoms are colored in blue and red, respectively.

3. Results and discussion

Previously we have reported the antioxidant potential of arylidene barbiturates [20]. In this report, the urease inhibitory potential of **1**–**18** is presented. Molecular modeling studies predicted certain compounds as potential ligands such as **1**, **6**, **7**, **11**, **13**, **15**–**17** and **18**. These compounds displayed a varying degree of urease inhibitory potential, when compared with the standard inhibitor of urease, thiourea ($IC_{50} = 21.1 \pm 0.3$ μM). Compounds **1** ($IC_{50} = 13.0 \pm 1.2$ μM), **11** ($IC_{50} = 17.6 \pm 1.3$ μM), and **13** ($IC_{50} = 19.1 \pm 1.6$ μM) showed an excellent urease inhibitory potential, while compounds **18** and **6** demonstrated a comparable inhibition potential ($IC_{50} = 23.5 \pm 1.0$, and 25.9 ± 1.0 μM, respectively) as compared to thiourea. Compound **7** was found to be the less potent among the active ligands with IC_{50} value of 49.3 ± 0.7 μM. Additionally; compounds **2**–**5**, **8**–**10**, **12** and **14** showed less than 50% inhibition, and thus their IC_{50} values were not calculated.

Among compounds **15**–**17**, only **16** showed a good urease inhibitory activity with an IC_{50} value of 23.6 ± 1.3 μM,

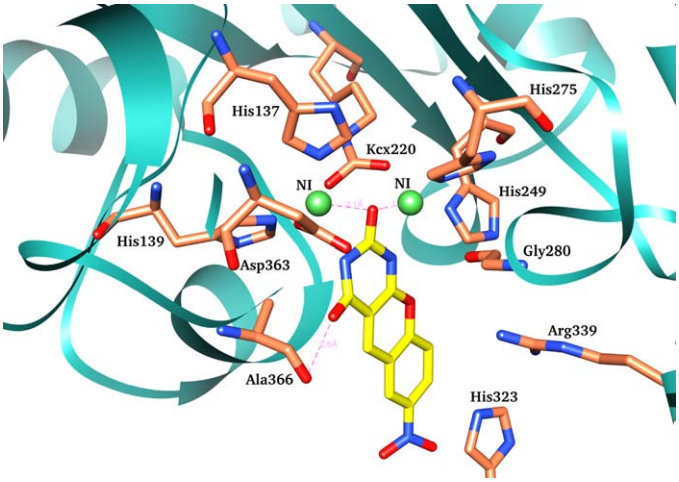
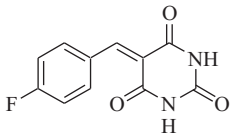
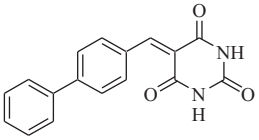
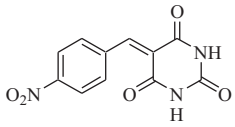
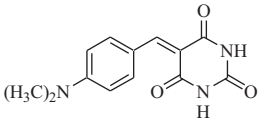
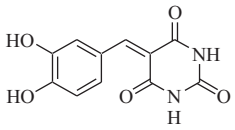
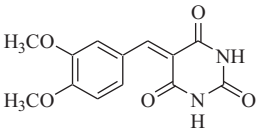
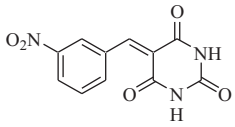
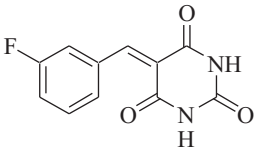
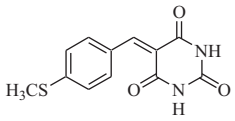
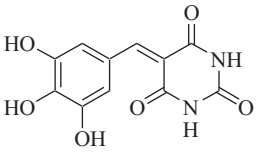
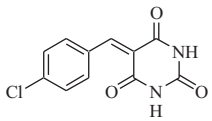
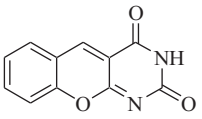
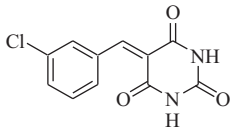
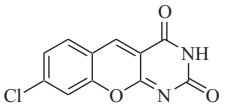
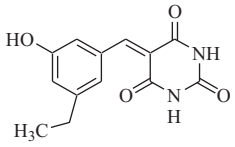
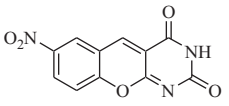
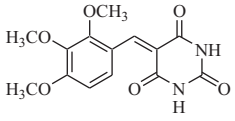
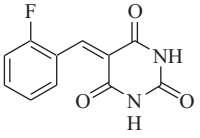


Fig. 2. Predicted binding mode of compound 17 (carbon atoms depicted in yellow) to urease. The carbon atoms in several key residues in urease are colored gold, while nitrogen and oxygen atoms are colored in blue and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
In vitro Bacillus pasteurii urease inhibition activity.

No.	Compound	IC_{50} + SEM[a] (μM)	No.	Compound	IC_{50} + SEM[a] (μM)
1		13.0 ± 1.2	**10**		NA
2		NA	**11**		17.6 ± 1.3
3		NA	**12**		NA
4		NA	**13**		19.1 ± 1.6
5		NA	**14**		NA
6		25.9 ± 0.9	**15**		41.6 ± 1.2
7		49.3 ± 0.6	**16**		23.6 ± 1.3
8		NA	**17**		66.7 ± 0.3
9		NA	**18**		23.5 ± 1.0
St.	Thiourea	21.1 ± 0.3			

[a] SEM is the standard error of the mean; NA, not active; St, standard.

while compound **15** had a moderate inhibitory potential (IC_{50} = 41.6 ± 1.2 μM).

p-Fluoro substituted arylidene barbiturate **1** is an excellent inhibitor with an IC_{50} value of 13.0 ± 1.2 μM. Its *o*-fluoro **18** and *m*-fluoro **13** analogs also have an excellent inhibitory potential with IC_{50} values of 19.1 ± 1.6 and 23.5 ± 1.0 μM, respectively. Among the fluoro-substituted arylidene barbiturate analogs, *p*-fluoro analog was the most potent one.

Compound **11**, the *p*-*N*,*N*-dimethylamino analog, was found to be the second most active urease inhibitor with an IC_{50} value of 17.6 ± 1.3 μM. Its corresponding *p*-chloro analog **6** also showed a comparable activity with an IC_{50} value of 25.9 μM.

These results clearly demonstrate that the *p*-substituted arylidene barbiturates **1**, **6**, and **11** are potent inhibitors of urease, as compared to their *ortho*- or *meta*-analogs **7**, **13**, and **18**.

Our study has resulted in the development of novel pharmacophores with dual inhibitory potential, i.e., urease inhibition and antioxidant. Antioxidant compounds **13** and **15** were identified as good urease inhibitors. Compound **13** showed an IC_{50} value of 92.9 μM for DPPH radical scavenging activity, while its urease inhibitory activity (IC_{50} = 19.1 μM) is also significant. Similarly compound **15** showed both, DPPH radical scavenging and urease inhibitory activities with IC_{50} values 151.7 μM and 41.6 μM, respectively. Conclusively, compounds **13** and **15**, with their dual inhibitory potential, can serve as lead molecules for further studies.

Moreover, bioactive compounds are often toxic to *Artemia salina* (leach, brine shrimp) larvae. The brine-shrimp lethality assay is a rapid and inexpensive assay for the screening and monitoring of physiologically active compounds. In this assay etoposide is used as standard drug (LD_{50} = 0.178 μg/mL), while derivatives **1**–**18** showed no toxicity to brine shrimps (*Artemia salina*).

3.1. Molecular docking

In order to examine the interaction of compounds **1**–**18** with urease, these compounds were docked into the binding pocket of *Bacillus pasteurii* (PDB code: 4UBP). Analysis of the docking results showed that all the synthetic compounds fit well within the binding site of the urease (Fig. 1). In most of the molecules, one of the carbonyl groups coordinates with both nickel atoms, while the other one is involved in the formation of hydrogen bonds with important active site residues, e.g., Ala170, Gly280 and Arg339. Nickel coordination might be one of the important determinants for the activities of these compounds, as mostly known urease inhibitors also interact with nickel ions [26–29]. Predicted potential ligands **1**, **6**, **7**, **11** and **13** are anchored in the active site by nickel coordination while establishing additional interactions by forming two hydrogen bonds with Gly280 and Arg339. Chromenopyrimidine diones **15**–**17**, though have interaction with bimetallic nickel center but formed only one hydrogen bond with Ala170 (Fig. 2). The extra hydrogen bonding of ligands **1**, **6**, **7**, **11** and **13** might be one of the reasons of higher activity, as compared to chromenopyrimidine diones **15**–**17** (Table 1). The synthetic compounds showed different activities with different substitution on phenyl ring, however, the docking studies were not able to explain this behavior.

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