Recent advances in design of new urease inhibitors: A review

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Introduction

Being the first organic compound synthesized by Friedrich Wöhler from inorganic components [1] urea has a unique role in history. Urea is an endogenous product of protein and amino acid catabolism. For example, approximately 20–35 g of urea is excreted in human urine per day. Urea is also used in huge quantities as fertilizer (being an exogenous source of ammonia for plants). This compound is hydrolytically stable and the half-life of non-enzymatic hydrolysis of urea is equal 3.6 years and the mechanism of this simple process is still disputable [2,3]. In Nature it is hydrolyzed by an enzyme urease (urea aminohydrolase E.C.3.5.1.5), a multi-subunit nickel dependent metalloenzyme that catalyzes the hydrolysis of urea at a rate approximately 10^{14} times the rate of the un-catalyzed reaction [4,5]. It is worth to express that the latter process is proceeding via different mechanism than this catalyzed by urease. This key enzyme of global nitrogen cycle converts urea to ammonia and carbamate, which in turn spontaneously generate carbon dioxide and next molecule of ammonia. Urease is the first enzyme, which was ever crystallized in 1926 by James B. Summer, who reported that a pure protein might function as an enzyme [6].

Bacteria, fungi, yeast, and plants produce urease where it catalyzes the urea degradation to supply these organisms with a source of nitrogen for growth. Urease is also a virulence factor found in various pathogenic bacteria. Therefore, it is not surprising that it is essential in colonization of a host organism and in maintenance of bacterial cells in tissues. Its activity leads to several implications such as appearance of urinary stones, catheters blocking, pyelonephritis, ammonia encephalopathy, hepatic coma as well as gastritis [7]. One of the most frequently studied bacterial urease is that from H. pylori, a causative agent of gastritis and peptic ulceration and stomach cancer [8,9].
Ruminal microbial urease plays an important role in the nitrogen metabolism in ruminants such as cattle and sheep. The urea from diet or recycled from blood to rumen is hydrolyzed to ammonia by bacteria residing in this stomach. This causes poor nitrogen accumulation when diets contain a high urea content [10,11].

Urea accounts significantly in total nitrogen fertilizers consumption worldwide. Its application is accompanied with large losses in ammonia, which is released upon action of bacterial ureases by its volatilization [12,13].

Variable and important role of urease stimulate that this enzyme continued to be the focus of researchers around the world, in the fields of genetics, biochemistry and physiology [14–16]. Strategies based on urease inhibition are considered as a promising mean to treat the diseases caused by bacteria producing urease, as well as a mean to diminish nitrogen loss from urea used as fertilizer. Therefore, it is not surprising that inhibitors of urease have been recently reviewed [17–21]. In this paper the most recent discoveries leading to inhibitors of this enzyme will be reviewed in some detail.

Crystal and molecular structure of urease

Enzymes, especially those vital for pathogenesis, are considered to be the most effective and promising targets for small molecule interventions in human and animal therapy, as well for design of pesticides [22]. The process of development of new inhibitor of an enzyme is challenging, time consuming, expensive, and requires consideration of many aspects. To fulfill these challenges, several multidisciplinary approaches are required, which collectively would form the basis of rational design. Structure-guided methods are an integral part of such development with three-dimensional structure of a target enzyme, bound to its natural ligand or an effector of its activity (determined either by X-ray crystallography or by NMR), serving as a template to produce new inhibitors.

Plant and fungal ureases are homo-oligomeric proteins of 90-kDa identical subunits, while bacterial ureases are multimers of two (αβ) or three (αβγ) subunits of different molecular mass forming various complexes. Number of urease subunits is varied according to their sources. For example, Klebsiella aerogenes and Sporosarcina pasteurii enzymes are composed of an (αβγ)3 trimer with each α-subunit having an (αβ)8-barrel domain containing a bi-nickel active center [23]. Staphylococcus saprophyticus urease consists of these three subunits of (αβγ)4 stoichiometry [24], whereas urease from Helicobacter pylori consists of only two subunits (α and β) forming a spherical assembly of (αβ)12 stoichiometry [25]. There are an impressive number of papers dealing with determination of structures of ureases from various sources [26–28]. They revealed that, despite the difference in number of sub-units, the structure of the active site in the vicinity of the nickel (II) ions is conserved and induces the same mechanism of catalytic activity [27,29].

Also molecular modeling was used to understand better the mechanism of action of this enzyme [30]. The studies on two bacterial enzymes (Klebsiella aerogenes and Helicobacter pylori) have revealed experimentally unobserved wide-open flap state that, unlike the well-characterized closed and open states of the enzyme, allows ready access of inhibitors to the metal cluster in the active site [31,32]. Molecular modeling was also used to predict the three-dimensional structure of Arabidopsis thaliana enzyme complexed with urea [33].

Crystal structures of ureases complexed with various ligands

Rational design of urease inhibitors is strongly enforced by the knowledge of crystal structures of this enzyme in its complexes with various inhibitors. Such structures have been determined and deposited in Protein Data Bank. The most of them consider Sporosarcina pasteurii urease complexes with the following ligands: β-mercaptoethanol (PDB 1UPB) [34], acetylhydroxamate (PDB 4UPB) [35], phenylphosphorodiamide (PDB 3UPB) [36], phosphate (PDB 1IE7) [37] (N-(n-butyl)thiophosphoric triamide (PDB 4CU) [38], fluoride (PDB 4CEX) [39], sulfite (PDB SA6T) [28], citrate (PDB 2UPB, Fig. 1) [27], boric acid (PDB 1S3T) [40], catechol (PDB 5G4H) [41] and 1,4-benzoquinone (PDB 5FSE) [42]. Other crystal structures are scarce and consider acetylhydroxamate inhibited ureases from Helicobacter pylori urease complexed with acetohydrazodic acid (PDB 1E9Y) [25] and Klebsiella aerogenes (PDB 1FWE) [43] and jack bean urease complexed with phosphate (PDB 3LA4) [26].

The crystal structures published recently indicate requirement for three indispensable elements for effective inhibitor: presence of nickel-complexing moiety alongside with properly placed

Fig. 1. Structural scheme (left panel) and model (right panel) of urease from S. pasteurii (pdb 4AC7) showing the requirements for the good inhibitor of the enzyme.
network of hydrogen-bond donors and acceptors attached to flexible scaffold. Additionally, special attention should be paid to the proper protonation states of the designed ligands [27].

The process of design of urease inhibitors is also strongly dependent on their possible role – if considering potential drugs molecular scaffold of could be structurally complex since the drug might be expensive, whereas in the case of inhibition of decomposition of urea in soil inhibitor has to be of simple structure and thus substantially cheap.

**Inhibitors bearing fragment of urea in their structures**

Urea is a small molecule and natural substrate of urease. On the other hand, as indicated by crystallographic studies, the enzyme is quite flexible and is able to bind big scaffolds [27]. Therefore, compounds containing fragment of urea or thiourea are of natural choice for the construction of inhibitors of this enzyme. Such an example is 1-(4-chlorophenyl)-3-palmitoylthiourea (compound 1), the most potent amongst a series of effective inhibitors of jack bean urease obtained recently [44]. It appears to be uncompetitive inhibitor and its binding determined by molecular modeling is different than this expected since it is bound in a quite long distance from nickel ions (Fig. 2).

Barbiturates and thiobarbiturates could be also treated as compounds bearing urea fragment in their structures (see Fig. 3 for representative structures: compounds 2, 3, 4 and 5). They appeared to be moderate inhibitors, with inhibition constants in micromolar range. They are bound by ureases from jack bean and *S. pasteurii* in a manner analogous to the substrate with urea or thiourea fragment being complexed by two nickel (II) ions [45–48].

Representative structures of iminothiazolines (compound 6) [49], cyanoacetamides (compound 7) [50] and hydrazones (compound 8) [51], possessing structural fragments mimicking urea, are shown in Fig. 3. They appeared, however, to be weak to moderate uncompetitive or mixed inhibitors of jack bean and *Helicobacter pylori* enzymes, and have no practical value.

**Quinolones**

Quinolone antibiotics constitute an important class of large group of synthetic broad-spectrum antibacterial agents, which are nowadays the most successful clinically synthetic antibacterial drugs [52]. They inhibit DNA synthesis. Nearly all quinolone antibiotics in modern use are fluoroquinolones. Their two popular representatives – Levofloxacin and Ciprofloxacin (compounds 9 and 10, Fig. 4) [53,54], as well as their analogs [55], appeared to be quite promising inhibitors of *Helicobacter pylori* and *Proteus mirabilis* enzymes. Molecular modeling suggests their binding with carboxylic group interacting with active site nickel ions. However, mechanism of additional covalent interaction with the enzymatic cysteine similar to this observed for simple quinones, cannot be ruled out [56]. Acetohydroxamic acid is a prescription medicine (Litostat) that is used in patients with chronic urea-splitting urinary infection to prevent the excessive build-up of ammonia in the urine. It inhibits urease by complexing nickel ions and thus is also one of the compounds most intensively studied as the potential therapeutics for the treatment of ulcer caused by *H. pylori* [57]. Therefore, it is not surprising that modification of carboxylic group of fluoroquinolones by their conversion into hydroxyamic acid (compound 11, Fig. 4), hydrazide and amide yielded interesting classes of inhibitors of this enzyme [58].

Recently Moxifloxacin (compound 12) have been used for capping of silver and gold nanoparticles and appeared to be exceptional inhibitor of urease, more potent than antibiotic itself [59].

**Flavonoids**

It is well known that structural diversity and complexity within natural products stimulates research on their use as lead compounds for various diseases. Extracts of various plants, including green tea and cranberries often have been used to treat gastritis or urinary tract infections. This effect is believed to result from the action of (+)-catechin and (−)-epigallocatechin gallate as urease inhibitors [60]. Also flavonoids isolated from other plants: Daphne retusa (daphnetusic acid), *Pistacia atlantica* (transililin and dihydro luteolin) and cotton (gossypol, gossypolone and apogossypol) appeared to be micromolar inhibitors of urease from jack bean [61–63]. These studies stimulated the efforts to analyze inhibitory potential of flavonoids in some detail. Thus, 11 natural and 19 synthetic compounds were screened against *H. pylori* urease [64]. They appear to be moderate competitive (micromolar range) to weak inhibitors of the enzyme with synthetic compounds

![Fig. 2. Structure of 1-(4-chlorophenyl)-3-palmitoylthiourea (1) and the mode of its binding by jack bean urease as remodeled by authors of this paper.](Image)
and 14, and quercetin (compound 15) (Fig. 5) [65] being the most active. Docking of the most active compound (13) into the crystal structure of *H. pylori* urease performed by the AutoDock program revealed the mode of binding of this inhibitor. In detail, the compound is oriented with its benzopyrone moiety in proximity to urea binding cavity, letting phenyl ring to locate at the mouth of the cavity. The channel to the active site for urea is therefore blocked off. Since catechol moiety of flavonoids does not bind nickel ion(s) there is a possibility of covalent interaction of this fragment of the molecule with one of cysteine residues present in the binding site. Such a mechanism has been determined and detail studied in the case of simple catechol [41].

Radix Scutellariae, known as “Huang-Qin” in Chinese, is originated from the dried root of *Scutellaria baicalensis*. Its major bioactive compounds are flavone glycosides baicalin and scutellarin (Fig. 5, compounds 16 and 17). Baicalin was found to be a competitive, slow-binding and concentration-dependent inhibitor of jack bean and *H. pylori* ureases [66–68]. Kaempferol-3-O-β-D-glucopyranoside (compound 18) and kaempferol-3-O-α-L-rhamnopyranoside (Fig. 5, compound 19), isolated from the fruits of *Syzygium alternifolium*, appeared more potent inhibitors of *H. pylori* enzyme [69].

Molecular modeling revealed that these compounds are bound differently than flavonoids, with catechol being involved in complexation of nickel ion. However, the most important for inhibition seems to be interaction with cysteine located at the mobile flap covering the active site through its S–H...π interactions with aromatic fragment of these molecules (Fig. 6). The active site of ureases is of relatively small volume (related to the size of urea) and is covered by a movable flap. This flap contains a cysteine residue that could be targeted by inhibitors. This cysteine, besides being directly involved in the architecture of the active site, plays a vital role in positioning other key residues in the active site appropriately for the catalysis.

Other natural products

Natural products (mostly secondary metabolites) have been the most successful source of potential drug leads so far. Even if these efforts somewhat decline in interest they continue to provide unique structural diversity of potential enzyme inhibitors. This is also the case if considering research on urease. In last several years there are several reviews on action of plant extracts [70–72] and isolated natural compounds [20,73] towards this enzyme.

Representative examples of natural products of recently determined inhibitory action against urease are: boswellic acid (Fig. 7, compound 20) a component of African medicinal plant *Boswellia carterii* [74], palmatine (compound 21) and epiberberine (compound 22) from *Coptis chinensis* [75–77], a plant traditionally used in China for the treatment of gastrointestinal diseases, andrographolide (compound 23), the major diterpenoid lactone and the primary effective constituent of Chinese medicinal plant *Andrographis paniculata* [78] and a popular antibiotic from garlic – allicin (compound 24) [79,80].
Docking of palmitine to the ureases from jack bean and *H. pylori* revealed that this alkaloid well fills the active pockets of these ureases, tightly anchoring the helix-turn-helix motif over the active-site cavity (Fig. 8). This prevents the flap of the urease active-site cavity from backing to the close position, which results in the inhibition of its activity.

It is worth to mention that there are quite intensive studies on influence of various honeys [81–83], honey fractions [84] and their combination with plant extracts [85] on the activity of urease from *H. pylori*. These papers seem to indicate that regular daily consumption of these honeys can prevent gastric ulcers.

**Heterocyclic compounds**

The practice of random testing of a large number of newly synthesized molecules in hope to find a new drug candidate is still the most popular approach. This process of screening, though inefficient, has led to the identification of many new lead compounds. Aromatic heterocycles yielded the most interesting activity against ureases. All the compounds reported recently appear to be micromolar inhibitors of *H. pylori* or jack bean ureases. As suggested by molecular modeling, they are bound within the active site of the enzymes and their activity results from interaction of side chain of cysteine or methionine with $\pi$ electrons of aromatic fragment of the molecule. In Fig. 9 the most representative examples of inhibitory benzimidazole (compound 25) [86], oxadiazole (compound 26) [87], ethyl tiazolidine-4-carboxylate (compound 27) [88] and dihydropyridone (compound 28) [89,90]. Also thiadiazoles were considered as inhibitors of *H. pylori* urease, however enzymatic studies have not been carried out and this assumption was derived from their antibacterial activity supported by molecular modeling against this enzyme [91]. The combination of two inhibitory scaffolds, namely of benzimidazole with triazole (compound 29) or oxadiazole (compound 30) [92], as well as aminopyridine with carbazole (compound 31) [93] did not result in elevation of inhibitory activity.

**Inhibitors, which bind covalently to urease**

These inhibitors are compounds designed to bind covalently to a specific molecular target and thereby suppress its biological function. They exhibit crucial advantage resulting from strong binding to the target and thus higher potency, extended duration of action and lower dose. However, they are also often considered as less attractive drug candidates because of drawbacks as general toxicity, immunogenicity and problems associated with degradation.
of inhibited proteins, issues that are of great concern. Therefore, it is not surprising that such inhibitors of urease have been scarcely studied.

Good candidates for such inhibitors are Michael acceptors. Thus, forty relatively simple molecules containing functional groups of various geometries (E and Z isomers) of substituted double bonds or containing linear triple bonds or allenes were screened for their inhibitory activities against *S. pasteurii* urease. This led to several compounds exhibiting potency in the nanomolar range [94]. All groups that are controlling the chemical reactivity of double/triple bonds contained carbonyl groups (carboxylic acids, their esters or ketones), with compounds 32 and 33 (Fig. 10) being the most potent. As shown by molecular modeling, compound 33 is the first example of an interesting mode of binding, which combines the formation of a covalent bond with the cysteine residue and interactions with two nickel ions (Fig. 10). Such a mode of binding seems to promote selectivity of the inhibitors toward this enzyme.
Another example of covalent inhibitor of urease is Disulfiram (compound 34, Fig. 11), a drug used to support the treatment of chronic alcoholism by inhibiting acetaldehyde dehydrogenase. Kinetic experiments suggest that it carbamylates Citrullus vulgaris urease active site flap Cys695 in a manner similar to its action on dehydrogenase (Fig. 11) [95].

Also novel selenoorganic bacterial urease inhibitors based on a 1,2-benzisoselenazol-3(2H)-one scaffold are acting by binding this sensitive cysteine in H. pylori and S. pasteurii enzymes [96]. The most active appeared to be ebselen (Fig. 12, compound 35), an agent of anti-inflammatory, anti-oxidant and cytoprotective activity studied as a potential drug against reperfusion injury, stroke, hearing loss, tinnitus and bipolar disorder. Molecular modeling had shown its preferable binding resulting from both complexation of nickel ion by carbonyl atom of the molecule and formation of sulfur-selenium bond with cysteine 322 (Fig. 12).

Organophosphorus compounds as transition state analogs

Competitive inhibition of urease by phosphate was first described as far as in 1934 [97] and intensively studied up to 2001 when its binding mode to urease from S. pasteurii was determined by crystallography [37]. It is a relatively weak inhibitor, whereas its amides (phosphoramidates) rank amongst the most active ones with their high efficiency being well justified by the crystal structures of complex of diamidophosphoric acid with S. pasteurii urease (compound 36, Fig. 13) [35]. This analysis had shown that high activity of this compound is apparently related to its close similarity to the transition state of the enzymatic reaction and tight binding to the active metallocenter.

Urea is a primary solid nitrogen fertilizer in the market because of the restriction against the use of ammonium nitrate, which may be employed as explosives, and the high price of ammonium sulfate. Its hydrolysis by bacterial ureases results in the loss of ammonia, which, besides the economic significance for the farmers, may have negative ecological impact on atmospheric quality. Since phosphoramidates are relatively cheap compounds they are considered as agents reducing the losses of ammonia from urease fertilizers. This is well exemplified by introduction of new formulation of an old inhibitor – N-(n-butyl)thiophosphoric triamide (NBPT, compound 37, ARM U™) to agriculture in 2017 [98,99]. Recently evaluated binding of this inhibitor to S. pasteurii urease showed that NBPT, after binding to the enzyme, is hydrolyzed yielding monoamidothiophosphoric acid (MATP, compound 38), which is effectively bound to the two Ni(II) ions in the active site (Fig. 13) [38]. Thus, NBPT may be classified as suicide substrate of this enzyme.

Quite recently a big library of structurally variable phosphoramidates was prepared and studied against jack ban urease. Structure–activity relationship analyses suggest that the presence of cyclohexylamine group (see the structure of representative compound 39, Fig. 13) is an important feature associated with enhanced activities [100].

Unfortunately, the phosphoramidate P—N bond is not stable in aqueous solutions, which limits their further applications. Recently, compounds containing a carbon-to-phosphorus bond linkage (phosphonates and phosphinates) emerged as an alternative to overcome this hydrolytic liability. If considering that simple phosphoramidate (36) mimics the tetrahedral transition state of urea hydrolysis aminomethyl(P-methyl)phosphinic acid (Fig. 14, compound 40) might be treated as its extendent analog. Similarly to phosphoramidate 36 it appeared to be weak inhibitor of ureases from Proteus vulgaris and S. pasteurii. Further, enhanced by molecular modeling, modifications of its structure were done by derivatization of its amino moiety [101]. Indeed, Simple N-methylation of the parent structure to compound 41 gave a 20-fold increase in the

Fig. 10. Two most potent Michael acceptor inhibitors of S. pasteurii urease and the mode of binding of compound 32.

Fig. 11. Structure of Disulfiram and its reaction with active site cysteine of urease.
Fig. 12. Structure of ebselen and the mode of its binding by *S. pasteurii* urease.

Fig. 13. Structures of phosphoramidates 36, 37, 38 and 39 and the mode of the binding of compound 36 by *S. pasteurii* urease.
inhibitory activity. Further modifications of the parent structure 40 resulted in several big libraries of phosphinate inhibitors with compounds 42, 43, 44 and 45 (Fig. 14) being the most potent, submicromolar inhibitors of the enzyme [102–105].

The biological relevance of these inhibitors was verified in vitro against an ureolytically active Escherichia coli Rosetta host that expressed H. pylori urease and against a reference strain, H. pylori J99 [104]. The majority of the studied compounds exhibited urease-inhibiting activity in these whole-cell systems with bis(N-methylaminomethyl)phosphinic acid (Fig. 14, compound 46) being the most effective.

Basing on the results presented in a study describing the crystal structure of S. pasteurii urease complexed with citrate [27] a new scaffold of phosphonate (phosphinate)/carboxylate was proposed. It imitates the 1,2-dicarboxylate portion of citrate (Fig. 1). As a result, one of the most potent organophosphorus inhibitors of urease, α-phosphonomethyl-p-methylcinnamic acid (Fig. 15, compound 47), was identified [106].

Molecular modeling has shown that it is so highly complementary to the enzyme active site that any modification of its structure resulted in diminished activity (Fig. 15).

### Coordination complexes

Complexes of simple organic molecules with metal ions are applied as inhibitors of enzymes on the premise that they may either act through substitution of one of the ligands by specific amino acid side chains of the enzyme or by such preorganization of relatively simple molecules into complex scaffold that is complementary to the structure of binding sites of the enzyme. Most likely, in the case of urease, only this second mean has been used.

Complexation of copper (II) and zinc (II) ions by Schiff bases formed between simple analogs of salicylic aldehydes and phenylethylamines resulted in formation of either polymeric structures (these are not useful as inhibitors) or dimeric ones, in which two molecules of ligand are bound to central copper ion (see the representative structure 48 in Fig. 16) [107]. The latter ones appeared far more effective inhibitors of jack bean urease than parent Schiff bases. Simple ternary cobalt (II) complexes with 1,2-bis(2-methoxy-6-formylphenoxy)ethane (obtained by reacting of vanillin with 1,2-dibromoethane) and phenylalanine, tryptophan (compound 49, Fig. 16) or methionine also appeared to be moderate inhibitors of jack bean urease [108]. Molecular modeling proved that they are well fitting to the binding cavity of this urease.

Quite complex structure is a ternary chelate composed of two copper (II) ions with four molecules of (E)-3-[(3,4-dihydrobenzo[b][1,4]dioxin-6-yl)acrylic acid (simple derivative of cinnamic acid) and two molecules of DMSO. It is potent, submicromolar inhibitor of jack bean urease [109].

For the construction of various supramolecular structures, silver as a d10 metal is quite frequently used because of its flexible coordination sphere and the fluid nature of interaction between silver and multifunctional ligands. Recently silver (I) carboxylate complexes based on the substituted trans-cinnamic acids, 1,4-benzodioxane-6-carboxylic acid and propyl-substituted imidazole-4,5-dicarboxylic acid (compound 50), which are the promising candidates for urease inhibitors [110–112]. In solution they form a polymeric structure and the mode of their binding do the enzyme was not evaluated.
Conclusions

Because of medicinal and agricultural importance of ureases the search for their inhibitors is quite extensive. In order to achieve this goal all he standard techniques of inhibitor design were applied. In many cases they were enforced by the application of computer-assisted inhibitor design. Despite of the detailed knowledge of the architecture of active and binding sites of ureases, the design, synthesis and evaluation of new inhibitors is still challenging and difficult. It is well illustrated by the fact that the most active ones exhibit submicromolar inhibitory constants. This results from that the binding sites are quite spacious and flexible and thus variable and difficult to predict mechanisms of inhibition might be utilized. The future perspective seems to relay on better understanding of binding preferences of the enzymes from different sources and on the application of computer-aided prediction of potentially active compounds.

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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