Research and development of new inhibitors of urease, a nickel-enzyme involved both in pathologies caused by antibiotic-resistant ureolytic bacteria and in the management of global nitrogen resources in the agro-environmental context

RESEARCH PROJECT

Urease (urea aminohydrolase, EC 3.5.1.5) is a nickel-dependent non-redox enzyme [1] found in bacteria, fungi, plants, algae and invertebrates [2-5]. Its catalytic function is the hydrolysis of urea to eventually yield ammonia and bicarbonate. The overall hydrolysis of these products causes an increase of pH of the surrounding milieu, causing negative consequences both in medical and in agricultural settings [2-7]. Urease requires the presence of Ni(II) in its fully conserved active site [1].

Several and often deadly human pathogenic bacteria rely on the activity of urease enzyme (urea amidohydrolase; EC 3.5.1.5) to survive and colonize the infected organism [8]. In particular, this enzyme represents the main virulence factor for Helicobacter pylori [9], Mycobacterium tuberculosis [10], Yersinia enterocolitica [11], Cryptococcus neoformans [12], and Proteus mirabilis [13]. Helicobacter pylori (Hp) is the only bacterium classified as a class-I carcinogen by the WHO, and relies on the activity of urease to survive in the extreme pH conditions of human guts. Finally, 10 out of the 12 bacterial species recently released by the WHO for which new antibiotics are urgently needed are ureolytic [14], depending on the activity of urease to colonize and survive inside the infected host.

On the other hand, urease has an important environmental impact related to agricultural soil management: the use of organic nitrogen fertilizers such as urea, the most largely used fertilizer in the world, and the consequent high pH reached during its hydrolysis induced by the ureolytic activity of soil bacteria such as Sporosarcina pasteurii, is the main cause for the loss of nitrogen as ammonia, which in turn causes root and leaf damage in the sprouts, in addition to contributing negatively to atmospheric pollution, with very significant economic losses and environmental damage [15, 16].

In all these instances, a tight control of urease activity is therefore required to counteract its deleterious effects. Several classes of molecules have been proposed and tested, both in medicine and agriculture, as urease inhibitors [4, 17-20]. However, all the inhibitors tested so far exhibit either toxicity or low stability and are therefore not optimal for clinical or agronomic purposes [8], and the development of new urease inhibitors is urgently needed. This endeavor requires the knowledge of the molecular details of the enzyme structure, catalytic mechanism, and inactivation modes of the inhibitors so far developed.

Knowledge about the structure of the nickel-containing active site cavity has been derived by structural studies on native ureases from several sources [21-24] (Figure 1A,B), which revealed a conservation in the coordination environment around the two Ni(II) ions and suggested a common catalytic mechanism. On the basis of the structural information obtained from the crystal structures of native S. pasteurii urease (SPU) [20, 22] and of its complexes with several inhibitors, such as β-mercaptoethanol [25], acetohydroxamic acid [26], phosphate [27], analogues of the substrate (e.g. boric acid) [28] and analogues of the transition state or an intermediate state (e.g. diamidophosphate) [22], as well as citrate [29], fluoride [20], sulfate [30], benzoquinone [31] and catechol [32], a general scheme of the catalytic mechanism of ureases has been proposed [1, 5, 22, 33-36]. In this mechanism, the nickel-bridging hydroxide acts as the nucleophilic group that attacks the urea molecule bound to the bimetallic Ni(II) cluster by an oxygen atom and a nitrogen atom. An extended network of second shell hydrogen bonds stabilizes the substrate binding during the catalytic process. In addition, a flexible flap carrying a conserved and druggable cysteine residue, essential for catalysis, changes the active site channel from an open to a closed conformation (Figure 1C).

Figure 1. (A) Ribbon scheme of the biological assembly of native SPU (PDB code 4CEU). The structure is a homo-trimer of hetero-trimers (αβγ). One αβγ subunit has been colored in light yellow, while the α, β, and γ chains of another subunit are in dark yellow, orange, and, red respectively. The last αβγ subunit has been colored accordingly to the B-factor with the less ordered regions colored in red and the most ordered in blue. The Ni(II) are reported as green spheres. (B) Detail of the active site of native SPU. Atoms are colored accordingly to the atom type. (C) Superimposition of the open (blue ribbons, 4CEU) and closed (yellow ribbons, PDB code: 3UBP) conformation of the flexible flap. Ni(II) ions are shown as green spheres. The αCys322 side chain is reported as "sticks". (D) Multiple sequence alignment of the flap region of the ureases for which a crystal structure is available: SPU, Klebsiella
The focus of the present research is the development of new urease inhibitors, and relies on the structure of urease and its complexes with inhibitors to develop new and more efficient inhibitors of urease.

This research will be carried out in three parts. The first step of the project will involve an in silico virtual screening of drug-like molecular databases that will suggest new potential urease ligands. The second part of the project will consist in the biochemical and structural characterization of the interaction between the enzyme and the most promising molecules identified by the virtual screening. In the same step, known urease inhibitors and their derivatives not studied so far will be also tested. This will pave the way towards the structure-based drug design of new urease inhibitors performed in the third step of the project, by improving the inhibitory strength of the best molecules identified in the second part.

The sources of urease will be Sporosarcina pasteurii (a widespread ureolytic soil microorganism), H. pylori (HPU), and Canavalia ensiformis (jack bean, a urease plant source). The experimental methodologies will include X-ray crystallography as a technique of choice for the structural characterization of urease-ligand complexes. Crystals of SPU and HPU, grown in the presence of new inhibitors of the enzyme, will be obtained by following standard laboratory protocols. Calorimetric as well as spectrophotometric and titration methods will provide the biochemical information on the inhibition mode of the testing molecules. The structures of urease-inhibitor complexes are expected to clarify the role of the new molecules on the urease catalytic mechanism at the molecular level, helping the further development of new classes of urease inhibitors for medical and agro-environmental applications.

Having established the kinetic and structural features of known enzyme inhibitors on urease, the next endeavor will be to discover new and possibly more efficient urease inhibitors relative to NBPT ($K_i = 0.1$ µM). This investigation could lead to a new set of molecules usable in agriculture as well as in human health for urease-related pathologies.

The project scheme generally entails:

i) in silico virtual ligand screening;

ii) biochemical evaluation in vitro to assess the potency of the newly identified molecules;

iii) chemical synthesis campaigns aimed at improving the inhibitory activity against urease.

Using the information coming from the available X-ray structures of several inhibitors solved in complex with the urease enzyme, we will carry out a structure-based virtual ligand screening campaign using state-of-the-art computational methodologies for drug discovery. In detail, the ZINC15 database [37], which contains over 100 million purchasable compounds, will be considered as the starting database; these molecules will be initially filtered according to their physicochemical parameters to discard chemically unstable and reactive molecules. The remaining compounds will be filtered retaining only those molecules that could be lodged acceptably well into the urease active site. Subsequently, after a careful visual inspection of all the molecules, we will retain a total number of about 100-150 molecules for in vitro biological evaluation. These molecules will be purchased and tested for urease inactivation power, leading to the identification of hit compounds with inhibition potencies at least in the micromolar range.

The next steps will be carried out through a structure-based optimization of the lead fragments thus identified, taking advantage of the X-ray structures of urease-inhibitor complexes. In particular, the milestones of the project are schematically reported hereafter:

1. Identify, among commercially available drug-like fragments, those derivatives that best fit into the urease-binding pocket - milestone at month 3
2. Generate preliminary structure-activity relationships (SARs) - milestone at month 5
3. Carry out extensive and in depth SAR studies using chemical synthesis - milestone at month 12
4. Patentability and freedom to operate in terms of intellectual properties will be carefully and collaboratively considered through chemical and patent databases - milestone at month 12
5. Solubility and chemical stability assessment of the new compounds will be carried out - milestone at month 15
6. Biochemical characterization of the identified best compounds in terms of urease inhibition mechanism, kinetics of inhibition, time-course of the reaction; the evaluation will proceed in parallel with the SAR studies - milestone at month 15
7. Structural characterization of urease bound to the most promising compounds, thus providing feedbacks for the chemical features needed for urease inhibition - milestone at month 24
8. Preliminary metabolic and toxicological profile evaluation - milestone at month 24

In this way, we aim at designing, synthesizing and biochemically characterizing about 50-70 novel compounds with the objective to obtain novel and potent urease inhibitors with improved stability, good solubility, and reduced human and environmental toxicity. This could lead to modern agricultural applications by enhancing the efficiency of nitrogen soil fertilization, in addition to novel therapeutic strategies for the treatment of infections by ureolytic bacterial pathogens.

Notes:

1) This plan realistically covers a time span of 24 months
2) This plan, depending on the outcomes, might need a time extension;
3) The plan requires the hiring of two personnel units: one devoted to deal with the chemical evaluation and synthesis of the new molecules, and the other to characterize the biochemical effects of the new urease inhibitors as well as the structural biology of the urease-inhibitor complexes;
4) The costs of the first personnel unit will be covered by available funds, while the cost of the second personnel unit will be covered by one postdoctoral research fellow ("Assegno di Ricerca") position which is the object of the current application.
TRAINING PLAN

The winner of the present "Assegno di Ricerca" will learn and handle several methodologies, either biochemical and computational, in order to deal with the aspects of structural biology applied to metallo-enzymes and proteins. He/She will use spectrophotometric, calorimetric and titration techniques in order to determine the kinetic parameters of the interaction between urease and new potential inhibitors. He/She will apply X-ray crystallography techniques for the determination of the structural bases of the inhibition of urease by the testing molecules. Finally, the postdoctoral fellow will collaborate with experts in other methodologies, such as biomolecular NMR spectroscopy, Small Angle X-ray Spectroscopy, as well as molecular biologists and computational and pharmaceutical chemists. In particular, the laboratory of the proponent has a has large expertise in structural biology and in biophysical characterization of metallo-proteins, applying high resolution NMR spectroscopy, biocrystallography, and other techniques such as X-ray absorption spectroscopy, circular dichroism, fluorescence spectroscopy, bioelectrochemistry, light-scattering, and calorimetry, as well as in molecular simulations and structural bioinformatics.

Moreover, the laboratory of the proponent currently collaborates with the following national and international structures and facilities, in order to guarantee technological and scientific support for the postdoctoral fellow:
1) Department of Pharmacy and Biotechnology, University of Bologna, for the in silico computational screenings as well as biochemical and biophysical characterization of protein – ligand interactions;
2) Consorzio Interuniversitario di Risonanze Magnetichie di Metallo-Proteine (CIRMMP), Department of Chemistry, University of Florence, for biomolecular NMR Spectroscopy.
3) European Molecular Biology Laboratory c/o DESY, Hamburg (Germany) for X-ray crystallography.
4) The European Synchrotron (ESRF), Grenoble (France) for X-ray crystallography.
5) ELETTRA Synchrotron, Trieste (Italy) for X-ray crystallography.

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