Inhibition Mechanism of Urease by Au(III) Compounds Unveiled by X-ray Diffraction Analysis

On the inhibition mechanism of urease by Au(III) compounds unveiled by X-ray diffraction analysis

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KEYWORDS: urease, nickel, gold compounds, X-ray structure, ureolytic pathogens, metal-based drugs

ABSTRACT: The nickel-dependent enzyme urease is a virulence factor for a large number of critical human pathogens, making this enzyme a potential target of therapeutics for the treatment of resistant bacterial infections. In the search for novel urease inhibitors, five selected coordination and organometallic Au(III) compounds containing N^N or C^N and C^N^N ligands were tested for their inhibitory effects against *Canavalia ensiformis* (jack bean) urease. The results showed potent inhibition effects with IC_{50} values in the nanomolar range. The 2.14-Å resolution crystal structure of *Sporosarcina pasteurii* urease inhibited by the most effective Au(III) compound $[Au(PbImMe)Cl_2]PF_6$ (PbImMe = 1-methyl-2-(pyridin-2-yl)-benzimidazole) reveals the presence of two Au ions bound to the conserved triad αCys322/αHis323/αMet367. The binding of the Au ions to these residues blocks the movement of a flap, located at the edge of the active site channel and essential for enzyme catalysis, completely obliterating the catalytic activity of urease. Overall, the obtained results constitute the basis for the design of new gold complexes as selective urease inhibitors with future antibacterial applications.

Several metals and metal-based compounds have been used for centuries as anti-infective agents on a simple empirical basis with some appreciable results. For instance, gold cyanide was proposed by Koch as an antitubercular agent in the pioneering times of modern pharmacology.[1] Since then, remarkable progress has been made on the development of gold complexes as therapeutic agents, starting from the anti-arthritis agent auranofin (AF) [2,3,4,6-tetra-o-acetyl-L-thio-*β*-D-glucopyranosato-S-(triethyl-phosphine)Au(I)]. Recently, this drug is receiving increasing attention for its potential to be repurposed as anticancer, antiparasitic and antibacterial agent,[2-4] leading to the development of novel gold-based antibacterial agents acting as specific inhibitors of several important enzymes.[4-12]

For example, a family of Au(I)-based phosphine complexes were tested and compared with AF toward a representative panel of pathogens that included Gram-positive, Gram-negative and *Candida* strains,[13] and showed activity on Gram-positive strains. More recently, organometallic Au(I) N-heterocyclic carbene (NHC) complexes were reported as effective antibacterial agents towards Gram-positive bacteria.[9, 14-16] Despite an increasing number of studies, the precise mechanism of the antimicrobial action of Au(I) complexes and their biomolecular targets are unknown. Due to the reported inhibition on the mammalian seleno-enzyme thioredoxin reductase (TrxR) by AF and Au(I) NHCs complexes, with formation of a stable Au-selenol adduct at the active site of the protein,[17] it was hypothesized that this enzyme could also be responsible for the observed antibacterial effects. However, the bacterial TrxRs lack the 'aurophilic' selenol active site,[18] and this may account for the reduced affinity of Au(I) binding with respect to mammalian TrxRs.

Within this framework, only rare studies on the possible use of Au(III) complexes as targeted inhibitors of bacterial enzymes have appeared so far. For example, phosphorous dendrimers bearing iminopyridino-end groups coordinating to Au(III) ions were reported to inhibit the growth of both Gram-positive and Gram-negative bacterial strains.[19] Moreover, moderate antibacterial activity of Au(III) complexes with different L-histidine-containing dipeptides was described,[20] but no mechanistic investigation was conducted to rationalize the observed biological effects. In general, Au(III) complexes have less affinity and selectivity for TrxRs binding,[21] while they appear to target different types of mammalian proteins, including zinc finger proteins,[22, 23] water/glycerol channels,[24, 25] the proteasome[26] and phosphatases,[27] among others.

An emerging target for bacterial infections is urease (urea amidohydrolase, E.C. 3.5.1.5), a nickel-dependent enzyme found in a large variety of organisms[28-32] and featuring a bimetallic Ni(II)-containing reaction site.[29, 30, 32] Urease is involved in the global nitrogen cycle, catalyzing the rapid hydrolytic decomposition of urea to eventually yield ammonia and carbonate,[33, 34] consequently causing a pH increase that has negative effects both on agriculture[35] and human health.[36] For instance, ten of the twelve antibiotic-resistant priority pathogens listed in 2017

by the World Health Organization (WHO) are ureolytic bacteria for which urease is a virulence factor.[37] Moreover, mixed species infections are more difficult to treat because of an increased tolerance to antimicrobials.[36] The general high significance given by the WHO to the antimicrobial-resistance priority, supported by the Global Antimicrobial Resistance Surveillance System (GLASS),[38] imposes urease to the attention of the researchers as a target to develop new drugs for the treatment of important bacterial infections acting as a threat to public health worldwide. Moreover, the very high structure conservation of ureases from plants and bacteria warrants the possibility to extend the results obtained in the pharmaceutical and medical applications to the agro-environmental field, for which an excessive urease activity also represents a negative aspect.[28-32]

A large number of urease inhibitors such as β-mercapto-ethanol,[39] phosphate,[40] sulfite,[41] fluoride,[42] as well as hydroxamic,[43] citric[44] and boric[45] acids, 1,4-benzoquinone[46] and catechol,[47] diamido-phosphate and monoamido-thiophosphate originated, respectively, by urease-catalyzed hydrolysis of phenylphosphorodiamidate (PPD)[48] or *N*-(*n*-butyl)thiophosphoric triamide (NBPT)[49] have been studied and the structural details of their modes of action have been elucidated. Additionally, metal ions such as Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Pb(II), Hg(II) and Ag(I) are also good urease inhibitors.[50-52] Within this context, the efficacy of Ag(I) as a urease inactivator has been recently rationalized by the structural determination of its adduct with *Sporosarcina pasteurii* (*S. pasteurii*) urease (SPU).[53] Ag(I) deactivates urease by forming a dinuclear cluster of two Ag(I) ions bound to the largely conserved triad αCys322/αHis323/αMet367, thus blocking the movement of a structural motif (mobile flap), essential for urease activity, consequently inhibiting the catalytic activity of the enzyme. Remarkably, no Au compound has ever been tested as urease inhibitor so far.

We report here on a urease inhibition activity screening of selected Au(III) complexes with bidentate N-donor ligands or cyclometalated C^N and C^N^N scaffolds (compounds **1**-**5**, Figure 1). In detail, two coordination complexes with (pyridyl)benzimidazole type ligands – [Au(PbIm)Cl_2] (**1**) (PbIm = 2-(pyridin-2-yl)-benzimidazole)[54] and [Au(PbImMe)Cl_2]PF_6 (**2**) (PbImMe = 1-methyl-2-(pyridin-2-yl)-benzimidazole),[24] respectively, were selected. Moreover, three organometallic Au(III) compounds featuring the C^N cyclometalated 2-benzylpyridine (py^b-H) ligand (**3**)[55] and *N*-phenylpyridin-2-amine ($phepy^a$) ligand (**4**),[56] as well as the tridentate C^N^N ligand 6-(1,1-dimethylbenzyl)-2,2'-bipyridine ($bipy^{dmb}$) (**5**)[57] were also examined. The compounds were synthesized according to previously reported procedures.[24, 54-57] The Au(III) compounds were tested for their inhibitory effects against *Canavalia ensiformis* (jack bean) urease (JBU) *in vitro*, and the structure of the adduct obtained upon incubation of compound **2** with SPU was determined by X-ray diffraction crystallography. Model coordinates and structure factors were deposited in the Protein Data Bank (PDB) under the accession code 6I9Y.

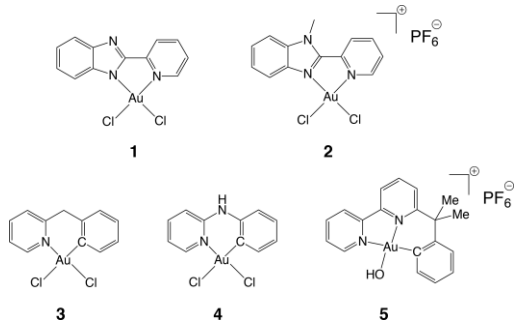

Figure 1. Schematic structures of Au(III) complexes tested for urease inhibition.

Kinetics of JBU inhibition with Au(III) compounds. The efficiency of urease inhibition by compounds **1**-**5** was tested by incubating JBU for 1 h in the presence of increasing concentrations of the different compounds, in order for the enzyme-inhibitor complex to reach a steady-state condition. Subsequently, the activity of urease was determined (see Supplementary Information for experimental details). Plots of the reaction rate after incubation, normalized *vs*. the reaction rate of the non-treated enzyme, as a function of inhibitor concentration, show a sigmoidal distribution (Figure 1-SI). A fit of the data to the canonical equation to obtain the inhibitor concentrations bringing about 50% inactivation of the enzyme (IC_{50}) yielded similar values for compounds **1**-**5**, in the low-nanomolar concentration range (Table 1). The most potent inhibitor is the cationic coordination complex **2**, while the bidentate neutral N^N compound **1** and the cyclometalated C^N derivatives **3** and **4** are ca. 2-fold less active. This observation suggests that non-covalent gold complex-urease adduct formation, involving electrostatic interactions between the two partners, may be instrumental to facilitate Au(III) coordinative binding.[58] In line with previous studies on the reactivity of cyclometalated complexes with proteins,[59] the Au(III) C^N^N complex **5** is the least efficient of the series. Notably, the urease inhibition strength of the Au(III) compounds is comparable to that observed for other metal ions (namely, Hg(II), Ag(I) and Cu(II)) and two-three orders of magnitude greater than in the case of Zn(II), Cd(II), Ni(II), Pb(II) and Co(II).[50-52] However, the advantage of using coordination or organometallic compounds allows the fine-tuning of the properties of the respective Au(III) complexes, reduces their speciation in aqueous solution, and allows for further optimization of the selectivity properties for a certain target.

Table 1. Values of IC_{50} for the inhibition of jack bean urease (JBU) by compounds 1-5 in Figure 1

Compound	Type of ligand	IC_{50} (nM)
1	N^N	18 ± 1
2	N^N	9.0 ± 0.2
3	C^N	14.9 ± 0.5
4	C^N	16.4 ± 0.5
5	C^N^N	31 ± 1

X-ray structure of SPU inactivated by a selected coordination Au(III) complex. The X-ray crystal structure of SPU co-crystallized in the presence of the best urease inhibitor, specifically the cationic Au(III) compound **2** (PDB code 6I9Y, see Supplementary Information for experimental details; data collection, processing and refinement statistics for Au-inhibited SPU are provided in Table 1-SI) shows the well-described heteropolymeric nature of *S. pasteurii* urease, consisting of an $(\alpha\beta\gamma)_3$ quaternary structure. The similarity of the protein scaffold with respect to native urease (PDB code 4CEU)[42] is confirmed by the RMSD between their Cα atoms (0.29, 0.25 and 0.20 Å for the α, β and γ subunits, respectively). A more detailed analysis of the Cα RMSD (Figure 2-SI) reveals that the β and γ subunits show a largely invariant backbone with respect to that of the native enzyme, whereas three portions of the α subunit, containing the Ni-bound active-site, are affected by significantly larger displacements: *i)* a region including residues 390-400, located on a surface patch showing a large conformational variability among the SPU structures determined so far, with RMSD values up to ca. 0.9 Å, *ii)* a region including residues 310-340, which corresponds to the mobile helix-turn-helix motif (mobile flap) responsible for the substrate access into the active site of urease, with RMSD up to ca. 1.4 Å, and *iii)* the region including residues 548-555, which forms a solvent exposed loop at the C-terminal portion of the α subunit, with RMSD up to ca. 1.2 Å.

The overall framework of the Ni-containing active site region of the refined model is highly conserved with respect to the native enzyme,[42] as revealed by the well-defined electron density represented in Figure 2.

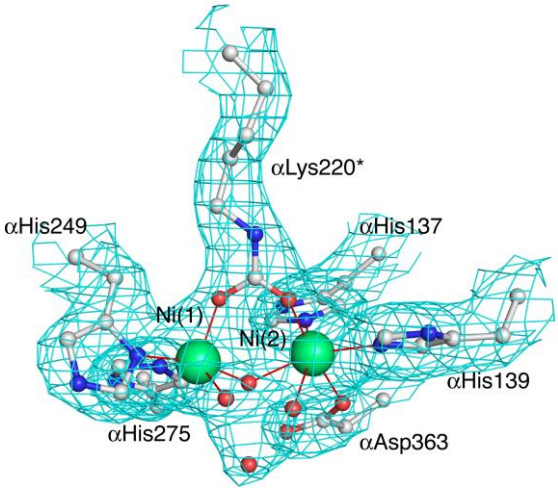

Figure 2. Atomic model of the active site of SPU inhibited in the presence of compound **2**. The nickel-coordination environment is shown super-imposed on the final $2F_o - F_c$ electron density map contoured at 1σ (cyan). Carbon, nitrogen, oxygen and nickel atoms are light grey, blue, red and green, respectively.

The two Ni(II) ions, distant 3.6 Å apart, are bridged by the carboxylate group of the carbamylated αLys220* residue, which is bound to Ni(1) and Ni(2) by Oθ1 and Oθ2, respectively. Ni(1) is additionally coordinated by αHis249 Nδ and by αHis275 Nε, whereas Ni(2) is further bound to αHis137 Nε, αHis139 Nε and αAsp363 Oδ1. The tetrahedral cluster of four water molecules in the vicinity of the Ni(II) ions that, in the native urease, are substituted by substrate urea during the enzyme catalytic cycle, are also not perturbed in the presence of the inhibitor. The distances and angles around the Ni(II) ions in the SPU inhibited by compound **2** are largely indistinguishable from those observed in native and Ag(I)-inactivated SPU structures[53] (Table 2-SI).

The unbiased omit electron density map reveals two additional spherical electron densities located in the vicinity of the mobile flap, around the αCys322 thiol, the αHis323 imidazole, and the αMet367 thioether functionalities (Figure 3A). The strong anomalous difference Fourier electron density maps associated to those $2F_o$-F_c electron densities suggested the presence of two Au atoms in this region (Figure 3A). The coordination environment around the two Au atoms bound to the His-Cys-Met triad (Figure 3A and 3B) involves αHis323 Nδ and to αCys322 Sγ for Au(1), and αCys322 Sγ and αMet367 Sδ for Au(2) (Figure 3B; all distances and angles around the Au atoms are reported in Table 3-SI). The two Au ions are bridged by Cys322 Sγ and are separated by 3.27 Å.

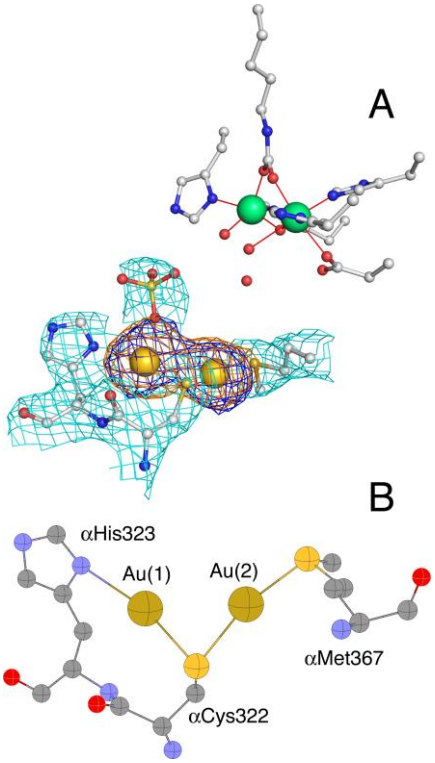

Figure 3. (**A**) Atomic model of the Ni(II)-containing active site of SPU inhibited in the presence of compound **2**, together with the region affected by Au(I) binding (PDB code 6I9Y). The model for the latter region is shown superimposed onto the final $2F_o - F_c$ electron density map contoured at 1σ (cyan). The unbiased $F_o - F_c$ omit map is shown contoured at 3.0 σ (orange). The anomalous difference electron density map contoured at 3.0 σ is also shown (dark blue). (**B**) Coordination environment of the Au(I) dinuclear cluster in the structure of SPU inhibited in the presence of compound **2**. The occupancy of the gold atoms is 0.85. Carbon, nitrogen, oxygen, nickel and gold atoms are light grey, blue, red, green and dark grey, respectively.

The quasi-linear geometric arrangement of the ligands is compatible with the presence of two Au(I) ions. This conclusion is also supported by previously reported electrochemical studies of compound **2**, which features a reduction potential for the $Au^{III/I}$ reduction process E_{red} = -0.33 V,[24] demonstrating the possibility for the complex to undergo reduction in biological environment. The short Au ••• Au distance (3.27 Å), the small

Au(1) - αCys322 Sγ – Au(2) angle (73.2°) and the slight distortion of the αHis323 Nδ – Au(1) – αCys322 Sγ and αCys322 Sγ – Au(2) – αMet367 Sδ angles (166.4° and 159.5°, respectively), caused by a bowing effect of the two Au atoms towards each other, are strong indications of the presence of an aurophilic interaction between the two Au(I) ions.[60]

A third electron density was observed close to αCys555 Sγ in the C-terminal portion of the α chain, also featuring a strong anomalous signal suggesting the presence of a third Au atom in that region. This Au(3) atom resides in a position 25-27 Å distant from the Ni(II) ions in the active site, with no evident influence on the catalytic mechanism. The oxidation state of this atom cannot be established with certainty based on the coordination geometry, because no electron density clearly attributable to ligands other than the side chain thiol sulfur atom can be observed, even though the presence of additional ligands, such as disordered water molecules, cannot be excluded.

The protein backbone of urease in the vicinity of the active site flap is strongly affected by the presence of the two Au ions, compared to the structure of the native enzyme (Figure 4), in a similar manner to that of Ag(I)-inhibited urease.[53] Specifically, the region around the Au-binding αCys322 and αHis323, residues belonging to the mobile flap, features RMSD values for the backbone Cα atoms with respect to the corresponding atoms of native SPU that are significantly larger than the average for the unbound enzyme (Figure 2-SI). The thiolate Sγ atom of αCys322 and the imidazole ring of αHis323 are shifted by ca. 2.3 Å and 1.2 Å, respectively, in order to coordinate the two Au ions (Figure 4). The Au(2) ion also provides the establishment of a covalent bridge between the mobile flap, with αCys322 and αHis323, and the more rigid portion of the protein that hosts αMet367, with its Sδ shifted by 0.3 Å (Figure 3).

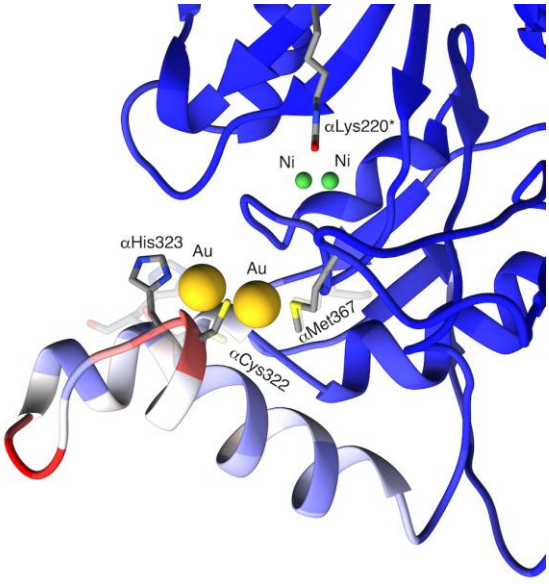

Figure 4. Ribbon diagram of a close-up of the structure of SPU inhibited in the presence of compound **2** (PDB code 6I9Y) in the vicinity of the active site region, showing the position of the mobile flap; the ribbon is colored according to the RMSD calculated with respect to the native enzyme (PDB code 4CEU) (red: RMSD > 1; pink: 0.5 < RMSD < 1; white: RMSD = 0.5; blue: RMSD < 0.5). The Ni and Au atoms are shown as green and gold spheres, respectively. The transparent atoms show the conformation of the side chain of the residues αCys322, αHis323 and αMet367 in the native enzyme.

Overall, we have shown here that the Au(III) complexes **1**-**5** are able to potently inhibit urease. Moreover, we have also structurally characterized by X-ray diffraction, for the first time, the adduct of Au ions with essential residues in the vicinity of the urease active site, elucidating the mechanism of inhibition at a molecular level. In the case of coordination compounds with bidentate N^N ligands, the binding directly involves residues likely to impair the enzymatic mechanism *via* alterations of the overall protein structure. The lack of the organic ligand in the crystal structure of SPU inhibited by compound **2**, leaving only two naked Au ions bound to the conserved Cys-His-Met triad, suggests the presence of several successive steps of ligand substitutions and metal reduction leading to the final structure observed in the solid state. As observed for previously reported Au(III)/protein adducts, this type of compounds favors exchange of the original ligands and further possible reduction to Au(I) species.[61-63]

It is worth mentioning that so far only a few X-rays structures of gold complexes (Au(I) and Au(III)) with proteins have been reported,[64] where these metal ions bind to Cys,[65 66] Lys,[67] or His residues.[68] Further studies are necessary to validate the binding mode of the cyclometalated Au(III) complexes to urease. In this case, retention of the cyclometalated ligands may be envisaged upon protein adduct formation,[23] which would be essential to design urease-targeted inhibitors. Finally, our results hold promise for the screening of the antibacterial properties of Au(III) complexes in ureolytic microorganisms, including *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Yersinia pestis* and *Cryptococcus neoformans*.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details of the kinetics measurements of JBU inhibition by compounds **1**-**5**. Experimental details of the crystallization of the adduct between SPU and compound **5**, X-ray diffraction data collection, processing and refinement, and final refinement statistics. Geometric parameters for Ni and Au coordination environments.

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Author Contributions

The manuscript was written through contributions of all authors.

Funding Sources

LM is supported by a fellowship from the University of Bologna and by CIRMMP (Consorzio Inter-universitario di Risonanze Magnetiche di Metallo-Proteine). MP is a Ph.D. student supported

by the University of Bologna. The University of Bologna and Cardiff University are acknowledged for funding and infrastructure support.

ACKNOWLEDGMENTS

X-ray diffraction data were collected under the beam time award number MX-1949 from the European Synchrotron Radiation Source (ESRF, Grenoble, France).

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