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Recent Advances in Bioinorganic Chemistry of Bismuth

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Abstract

Bismuth has been used in medicine for over two centuries for the treatment of various diseases, in particular for gastrointestinal disorders, owing to its antimicrobial activity. Recent structural characterization of bismuth drugs provides an insight into assembly and pharmacokinetic pathway of the drugs. Mining potential protein targets inside the pathogen via metallomic/metalloproteomic approach and further characterization on the interactions of bismuth drugs with these targets laid foundation in understanding the mechanism of action of bismuth drugs. Such studies would be beneficial in rational design of new potential drugs.

Introduction

Bismuth compounds have been used in medicine for more than 200 years for the treatment of various diseases including syphilis, hypertension, infections, skin conditions, and gastrointestinal disorders [1, 2•]. The discovery of *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterium from gastric mucosa which is responsible for gastric and duodenal ulcers [3], has further promoted both research and medical applications of bismuth. Currently, three bismuth drugs i.e. bismuth subsalicylate (Pepto-Bismol®), colloidal bismuth subcitrate (De-Nol®), and ranitidine bismuth citrate (Tritec® and Pylorid®) are being used worldwide in combination with antibiotics to eradicate *H. pylori* infection. In addition, many new bismuth compounds with different structures and activities as well as bismuth nanotubes have been synthesized [2•, 4, 5].

For the past few years, enormous efforts have been made towards understanding the structures [6, 7••,8] as well as the mechanism of actions of the bismuth drugs [2•]. Recent advances in biophysics and molecular biology have provided invaluable tools necessary to study the

bioinorganic chemistry of bismuth. For example, time-resolved inductively coupled plasma mass spectrometry enables bismuth antiulcer drugs to be tracked in single *Helicobacter pylori* cells (ca. $1.0x10^6$ Bi atoms/cell). The uptake of bismuth by *Helicobacter pylori* is retarded by ferric ions (Fe^{3+}) suggesting that bismuth drugs may utilize certain iron-transport pathways in the pathogen [9]. A number of protein targets of bismuth drugs, in particular those from the *H. pylori* have been identified by metallomic and metalloproteomic approach [10•, 11]. Several comprehensive reviews summarized the chemistry and biological chemistry of bismuth as well as its medical applications are available recently [1, 2•, 6, 11, 12]. Here we will only focus on some recent advances in the structures and protein targets of bismuth drugs as well as design of potentially active new bismuth complexes.

Structural models of bismuth drugs

In spite of extensive clinical usage of bismuth drugs, the structures of these drugs have not been unveiled until the last two decades. Various bismuth citrate complexes with different bismuth to citrate ratios have been crystallized at different pH values. Among these, the structure obtained under acidic condition (pH 3) most likely represents real situation in the stomach [13]. Colloidal bismuth subcitrate (CBS) is likely to assemble into sheets and then three-dimensional polymers using bismuth citrate dinuclear units $[Bi(cit)_2Bi]^{2-}$ as building blocks to form a protective coating on the ulcer craters [13].

Crystal structures of four bismuth citrate complexes obtained at the acidic pH values in the presence of either ethylenediamine or pyridine [14] reveal that bismuth citrate dimeric units $[Bi(cit)_2Bi]^{2-}$ (Figure 1A) serve as the basic building blocks leading to polymeric structures with regular meshes and internal cavities. The protonated ethylenediamine, despite unobservable in the structure (Figure 1B), and pyridine moieties (Figure 1C) are embedded probably by diffusion or electrostatic interaction in the polymeric framework to achieve charge balance since all citrate anions found in the structures are deprotonated leading to citrate tetrannions ($[C_6H_4O_7]^{4-}$). The composition of the bismuth citrate complex frameworks depends on the size and level of protonation of the inserted cations, the pH values as well as the time of crystallization. The structural model of ranitidine bismuth citrate (RBC) was constructed based on the framework of crystal structure of complex **1** [14]. Ranitidine molecules can be readily embedded into the

cavities perpendicular to the *bc* plane with H-bonds formed between ranitidine and the bound citrate ligands, Figure 1D. In addition, the sulfur atoms of ranitidine form H-bonds with a water molecule that coordinates to bismuth. Based on X-ray crystal structures as well as ESI-MS of bismuth citrate complexes, it is likely that bismuth citrate-based drugs degrade under gastric acidic condition (pH ~3), from 3-D polymer framework to 2-D polymeric sheets and finally to a basic dimeric unit such as $[Bi(cit)_2Bi]^{2-}$ which can be absorbed or transported by membrane receptors.

Bismuth subsalicylate (Pepto-Bismol®, BSS) is one of the most commonly used bismuth drugs [11]. Structural characterization of bismuth carboxylates is a challenging due to difficulties in controlling hydrolysis of these complexes or the formation of coordination oligomers/polymers. Recently, structures $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O_2)]\cdot(Me_2CO_4)$ and $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]\cdot(Me_2CO)_{1.5}$ (Figure 1E) may provide an insights into the nature of BSS and a basis for studies the mode of action of the drug [8]. The crystals of $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]\cdot(Me_2CO)_{1.5}$ initially predominate with only relative small amounts of the large cluster with 38 bismuth atoms which appeared to be the least soluble and most thermodynamically stable form when extending crystal growth times. The structure contains a basic building block $[Bi_6O_8]^{2+}$ polyhedron as found in other bismuth oxo clusters [15], which have six octahedral Bi atoms with the eight O atoms located over all of the triangular faces. The cores of the two structures are the Bi_9 clusters comprised of a central Bi_6 octahedron but with only seven of the eight trigonal faces capped by an O atom as shown in Figure 1F. In both structures, bismuth also coordinated to the solvent molecules of acetone, which can be replaced by other solvent molecules such as DMF (dimethyflormamide) when crystallized in this solvent [7••]. Interestingly, the core of $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]\cdot(Me_2CO)_{1.5}$ lies at the heart of $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O_2)]\cdot(Me_2CO_4)$ combined with the presence of Bi-coordinated solvent molecules in these structures reveals a possible process for hydrolysis and core formation, and indicates that the complex $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]\cdot(Me_2CO)_{1.5}$ gave rise to the complex $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O_2)]\cdot(Me_2CO_4)$.

The first structure of BSS without organic auxiliaries obtained from X-ray diffraction data of bismuth disalicylate powder [7••] gave rise to two-dimensional polymers held by Bi-O linkages

and O-H···O hydrogen bonds with one bismuth atom, one salicylate mono-anion and one salicylate dianion in each unit. The monoanionic salicylate coordinates to a single Bi^{3+} ion through its carboxylate group only and the dianionic salicylate employs the phenoxide oxygen atoms as bridging ligands to form four-membered Bi_2O_2 rings. An additional oxygen atom from a water molecule is bonded to bismuth. Such a structure of BSS resembles bismuth complexes with substituted benzoic acids [16].

New bismuth complexes and their activities

Over the last decade, significant work has been devoted into the development of new bismuth drugs [2, 17, 18]. Various new bismuth-containing complexes have been synthesized and showed promising *in vitro* activities against *H. pylori* [17, 18]. Importantly, some bismuth-containing complexes have been demonstrated to exhibit new *in vitro* activities including antifungal, antiviral or even anticancer activities [19-24].

Bismuth drugs such as BSS, CBS and RBC are effective in treating and eradicating *Helicobacter pylori* together with antibiotics. However, these non-steroidal anti-inflammatory drugs (NSAIDs) may also cause gastrointestinal damage. Bismuth derivatives of NSAIDs exhibited good *in vitro* activity against the three strains of *H. pylori* with the minimum inhibitory concentrations (MIC) $\geq$ 6.25 μg/mL, which are better than commercially used BSS (8 μg/mL), laboratory prepared bismuth salicylate (≥12.5 μg/mL) and CBS (≥12.5 μg/mL) [18]. Therefore, these compounds may have great potential in the treatment of *H. pylori* infection while allowing the concomitant therapeutic benefits of NSAID treatment. Similarly, heteroleptic bismuth sulfosalicylate complexes $[PhBi(HSsal)H_2O]_\infty$ and $[PhBi(HSsal)H_2O]_\infty$ as well as *bis*-phenylbismuth sulfonates $[Ph_2Bi(O_3SR)]_\infty$ (R=*p*-toly, mesityl or *S*-(+)-10-camphoryl) [17, 25] showed significant activities against *H. pylori* with MIC< 6.25 μg/mL. Some cyclic organobismuth compounds bearing a nitrogen or sulfur atoms as additional ring member also exhibit various antibacterial activities including gram-positive and gram-negative bacteria [26]. Although these bismuth-containing complexes showed *in vitro* activities against *H. pylori* and other pathogens, there appears to be lack of *in vivo* data and more works are warranted to promote medical application of these complexes.

Beside antibacterial activity, bismuth complexes of substituted benzoic acids also exhibit significant anti-*Leishmanial* activity against the promastigotes of *L. major V121* [27]. Heterocyclic organobismuth compounds [ClBi(5-R-C_6H_3-2-$SO_2C_6H_4$-1´-) (R=Me, Ph, MeO, Cl, H, *t*-Bu, CF_3, F, Me_2N) exert antifungal activities against *Saccharomyces cerevisiae* and the activity depends on the lipophilicity of the compounds: the higher the lipophilicity, the lower the antifungal activity [19]. Some organobismuth compounds have been also found to exhibit anti-tumor potentials [2, 24]. Bismuth xanthate complexes [Bi($S_2COR)_3$] (R=Et, *i*-Pr, cyclohexyl) were shown to exert cytotoxic activities against Calu-6 (lung adenocarcinoma) with a similar IC_{50} values of cisplatin [28], indicating that these compounds have potency comparable to cisplatin. Moreover, these bismuth complexes also exerted cytotoxic activities against cisplatin-insensitive MCF-7 (mammary carcinoma) [28]. Bismuth dithiocarbamate complexes with general formula of [Bi(S_2CNR_2)] were also demonstrated to exhibit potent *in vitro* cytotoxicity against a panel of seven human cancer cell lines [23]. Some heterocyclic organobismuth compounds have potent cytotoxic activities against various cancer cell lines with IC_{50} in the range of 0.059-5.1 μM, in particular, are sensitive towards leukemic cell lines [20, 29]. These bismuth compounds such as bi-chlorodibenzo[*c*,*f*][*1*,*5*]thiabismocine may induce apoptosis in HL-60 cells through the activation of caspase, production of ROS, and perturbation of mitochondria [20], they may also target tubulin to induce G2/M arrest in HeLa cells probably by interacting with the colchicines-binding site through its thiolate (-SH) groups [29]. This type of organobismuth compounds may be utilized as antimitotic agents and in the treatment of refractory acute promyelocytic leukemia similar to arsenic trioxide.

The antiviral activity of bismuth complexes was explored over the severe acute respiratory syndrome coronavirus (SARS-CoV), an enveloped, single-stranded RNA positive-strand virus that killed hundreds of people worldwide when is broken out in 2003 [30]. A series of bismuth complexes including bismuth nitrilotriacetate Bi(NTA), bismuth tricysteine Bi($Cys)_3$, ranitidine bismuth citrate (RBC), bismuth ethylenediaminetetraacetate (Bi(EDTA)) as well as bismuth complexes with various N,O-containing chelate ligands including bismuth porphyrin complexes were examined against the SARS-CoV helicase [21, 22], which was postulated to be the potential target for anti-SARS therapy [30]. The majority of bismuth complexes tested exhibited activities against SARS-CoV helicase ATPase and duplex-unwinding activities with the highest

activities found for RBC and bismuth porphyrin complexes at IC_{50} values of less than 1 μM. Bi(EDTA) and other two bismuth complexes with N, O-containing chelate ligands showed almost no activity, indicating that different inhibition activities of these complexes are correlated to their different coordination environments, i.e. the higher affinity of bismuth towards the chelate ligands, the lower activities. The treatment of SARS-CoV infected cells by bismuth-containing complexes such as RBC and Bi_2-hTF (hTF=human transferrin) confirmed an inhibitory role of bismuth during later stages of the replicative cycle [22]. The studies represent the first attempt in using bismuth as anti-virus agent although further work is required to elucidate such activities.

Potential protein targets of bismuth drugs

The mechanism of action of bismuth drugs is complicated and not fully understood. It is generally believed that bismuth drugs are taken up into gastric mucus to form a protective coating probably as BiOCl and bismuth citrate complexes on the ulcer crater. They may inhibit *Helicobacter pylori* adherence and also bind strongly to connective tissue proteins, mucus glycoproteins and enzymes [1, 2]. Accumulative studies demonstrated that proteins (peptides) are likely to be the potential targets of the drugs. Bismuth drugs have been shown to interact with a range of proteins such as human serum transferrin [31, 32], lactoferrin [33], serum albumin [32, 34], and metallothionein [35]. The binding of bismuth to lactoferrin may deprive of iron acquisition of *H. pylori* since the bacteria utilize host-specific lactoferrin for iron acquisition [36]. The level of bismuth uptake by *H. pylori* single cell is also found to be reversibly correlated to the level of iron [9]. Bismuth drugs have also been demonstrated to inhibit several enzymes from *H. pylori*. It inhibits the activity of yeast alcohol dehydrogenase by interfering with the zinc site and altering enzyme native structures [37]. Once bismuth is up-taken by the pathogen *H. pylori*, it may target several proteins inside the pathogen to inhibit synthesis of essential enzymes such as urease and hydrogenease or other nickel-binding proteins, which are critical for bacterial survival. Very recently, the metallomics/metalloproteomics approach has been used to investigate the role of metals in biological systems [38•, 39•] and to search for putative binding proteins (targets) of bismuth drugs inside the bacterium [10, 40, 41]. The recent advances in this area are updated since other information has been summarized elsewhere [1, 2, 6, 11].

Urease, accountable for up to 10% of the total cellular proteins, is an essential nickel-containing enzyme for *H. pylori* colonization and virulence. It catalyzes the hydrolysis of urea to yield carbamate and ammonia and thus neutralizes its immediate environment of the bacterium to aid its survival under acidic conditions of the gastric lumen and mucosa. Bismuth complexes such as, RBC as well as some triarylbismuthanes can inhibit urease activity [42, 43]. Inhibition of jack bean urease by the triarylbismuthanes compounds is in good agreement with observed antibacterial activity of the compounds against *H. pylori* [43]. Both Bi(EDTA) and $Bi(Cys)_3$ are competitive inhibitors of jack bean urease, while RBC is a non-competitive inhibitor. Kinetic analysis demonstrated that Bi(EDTA) is both a competitive inhibitor and a time-dependent inactivator of the recombinant *Klebsiella areogenes* urease. Such an inhibition is likely due to the binding of bismuth to the cysteine residues of the enzyme (Cys319 in *Klebsiella areogenes* and probably Cys592 in jack bean urease) at the entrance of the active site [42].

The survival of *H. pylori* requires a constant supply of Ni^{2+} ions for the synthesis and activities of the Ni^{2+}-containing enzymes. Like other bacteria, *H. pylori* has to be able to strike a delicate balance between the import of Ni^{2+} ions, their efficient intracellular storage and delivery to Ni^{2+}-dependent metalloenzymes when required. *H. pylori* is able to synthesize a small histidine-rich cytoplasmic protein Hpn (28 histidine out of 60 amino acids) and histidine-rich glutamine-rich proteins Hpnl (18 histidine and 30 glutamine out of 71 amino acids), which may play roles of storage of nickel ions as a 'reservoir', donation of nickel to other proteins and detoxification via sequestration of excess nickel ions depending on the exogenous nickel levels [44-46]. Both *in vitro* and *in vivo* experiments suggested that Hpn and Hpnl sequester nickel ions at neutral pH but donate them probably for urease activation under acidic condition [44, 45, 47]. *H. pylori* lacking *hpn/hpnl* gene, cultured *in vitro*, are more susceptible to Ni^{2+} and Bi^{3+} than the wild-type strain. Both essential metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+} and therapeutic metal ions e.g. Bi^{3+} bind to Hpn *in vitro*, indicating that Hpn may serve as a potential target for bismuth therapy. Hpn binds ca. 4.8±0.2 Ni^{2+} and 3.8±0.2 Bi^{3+} per monomer probably via multiple imidazole groups and four Cys residues with dissociate constants (K_d) of 7.1 and 11.1 μM respectively [47, 48], while Hpn-like (Hpnl) binds ca. 2.0±0.1 Ni^{2+} per monomer (K_d of 3.8±0.2 μM) and two histidine residues (His29 and His31) were identified to play a critical role in binding of Ni^{2+} [45, 49]. Whether Hpnl interacts with bismuth drugs is not known and may warrant for studies. Recently,

fluorescence resonance energy transfer (FRET) approach has allowed studies of metal binding properties of Hpn (where the protein sequence is inserted between the FRET partners CFP and YFP) both *in vitro* and *in vivo*. It was found that when Hpn-FRET was expressed in a model system e.g. *E. coli*, FRET change was only observed with addition of bismuth subsalicylate (Pepto Bismol) but not Ni^{2+} and Zn^{2+}, clearly indicating that Hpn is a potential target for bismuth *in vivo* [50••]. The binding of bismuth to the protein may therefore interfere with its normal functions.

Systematic identification of potential targets of metallodrugs is achievable nowadays by metallomic/metalloproteomic approach as shown in Figure 2A. Immobilized-metal affinity chromatography (IMAC) in combination with two dimensional electrophoresis (2-DE) and MALDI-TOF mass spectrometry is a particularly useful tool for elucidating the metabolism of intracellular metal ions and identifying molecular targets or binding proteins associated with the disease etiology and pathology for metallodrugs [10, 40]. This approach has been used for the first time to search the potential protein targets of bismuth drugs in *H. pylori* on a genome-wide scale. A comparative proteomic analysis of *H. pylori* cells before and after treatment of colloidal bismuth subcitrate (CBS) was performed [41]. Eight proteins were found to be significantly up- or down-regulated, Figure 2B. These proteins are mainly involved in either cellular processes (HspA, HspB, putative alkyl hydroperoxide reducase TsaA and neutrophil-activating protein NapA), or oxidative stress resistance (thioredoxin), and hemoglobin. The up-regulated expression of thioredoxin, a low redox potential reductant, may reflect the response of *H. pylori* to the high level of oxidative stress. Using immobilized-bismuth affinity chromatography (Bi-IMAC), seven bismuth-binding proteins were subsequently identified from *H. pylori* cell extracts Figure 2C. Interestingly, the intracellular levels of four proteins e.g. HspA, HspB, NapA, TsaA were decreased upon addition of CBS, Figure 2C, suggesting that these proteins directly bind to bismuth. The other bismuth-binding proteins identified include fumarase and urease subunit UreB and a translational factor Ef-Tu. Furthermore, it was found that bismuth led to around 8-fold decrease in cellular protease activity and elevated the levels of lipid hydroperoxide and hemin in the whole cell extract of bismuth-treated *H. pylori* cells than those untreated cells [41].

To validate the potential targets of bismuth drugs identified by metalloproteomic approach subsequent studies have been carried out [51,52••,53]. For example, fumarase, an enzyme which catalyses the reversible hydration of fumarate to malic acid, binds one Bi^{3+} per monomer, leading to an apparent non-competitive inhibition of the enzyme [53]. HspA, a member of the GroES chaperonin family, is a small protein with a unique histidine/cysteine-rich domain at the C terminus with the sequence shown in Figure 3A. Apart from the normal function of GroES e.g. aiding protein folding in conjunction with GroEL, it also plays a role in nickel regulation: to facilitate nickel acquisition by donation of nickel to appropriate proteins (and enzymes), and to detoxificate excess nickel [51]. Recombinant HspA from *H. pylori* was found to bind about two Bi^{3+} ions (or two Ni^{2+}) per monomer of the protein, Figure 3B, and the C-terminal Cys-Cys motif and histidine residues are likely involved in the binding, similar to Hpn [44, 48, 51, 54]. The potential roles of the His- and Cys-rich terminus *in vivo* was examined by comparison of the growth of *E. coli* cells expressing the wild type or C-terminal deletion mutant in M9 minimal medium supplemented with metal ions in a concentration-dependent manor [51]. *E. coli* cells expressing the C-terminal deletion mutant were more susceptible to increasing concentrations of nickel ions. In contrast, Bi^{3+} retarded the growth of the wild type-containing cells [51]. Surprisingly, apart from the C-terminal metal binding domain, three residues (His45, Cys51, and Cys53) are found to be critical for Zn^{2+} binding *in vivo* but not for other transition metal ions such as Ni^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+} [52••]. These residues are located at the N-terminal apical domain and were probably originated from negative selection (Figure 3D) and make up an oxidation-sensitive zinc binding site since the Zn^{2+}-bound *Hp*GroESΔMBD restored the activity of enzyme alkaline phosphatase (AP). Equilibrium dialysis study showed that bismuth binds to the C-terminal deleted *Hp*GroES (*Hp*GroESΔMBD) in a similar pattern to Zn^{2+} with an affinity 40-fold higher than that bismuth binding to the C-terminal metal binding domain [52••]. Competition experiments between Bi^{3+} and Zn^{2+} reacting with *Hp*GroESΔMBD indicated that bismuth is able to replace the bound zinc from the protein, Figure 3E. Significantly, the binding of bismuth to GroES altered its quaternary structure from its native heptamer to a dimer, which is attributable to bismuth binding to the N-terminal zinc-site, not the C-terminal metal binding domain, Figure 3F. Taken together, once bismuth drugs entered the *H. pylori*, they may interfere with nickel homeostasis by binding to nickel storage proteins (Hpn/Hpnl), and accessory proteins (HspA)

responsible for hydrogenease or urease synthesis. The bismuth drugs are also likely to inhibit protease and urease activity; and modulate cellular oxidative stress.

Conclusions and perspectives

Bismuth drugs have been used clinically for its antimicrobial activity. Structural characterization of bismuth drugs shed light on assembly and decomposition of the drugs. Extensive studies have been carried out to explore other potentials of bismuth drugs and amazingly, bismuth also exhibits *in vitro* activities such as anti-Leishmanial activity, anti-tumor activity that both antimony and arsenic possess. Currently, no bismuth-containing compounds are in clinical trial for treatment of either cancer or viral infection. Nevertheless, some recent *in vitro* data are quite encouraging [20]. Further extensive explore may be needed in future. Moreover, pretreatment of bismuth drugs can reduce the side-effects of anticancer drugs such as cisplatin [55], which may also offer a potential for the application of bismuth in the treatment of cancer.

In spite of extensive studies, our knowledge on the mechanism of action of bismuth drugs is still very limited. Accumulated data indicated that proteins (and enzymes) are likely to be the targets of bismuth drugs. The interactions of bismuth drugs with a number of proteins have been characterized individually. Recently, an integrated view on potential bismuth drugs inside *H. pylori* was given with the aid of metallomics/metalloproteomics approach. The strategy can readily be extended to study the mechanism of other metallodrugs as well as to evaluate uptake, storage and excursion of transition metal ions [10, 38].

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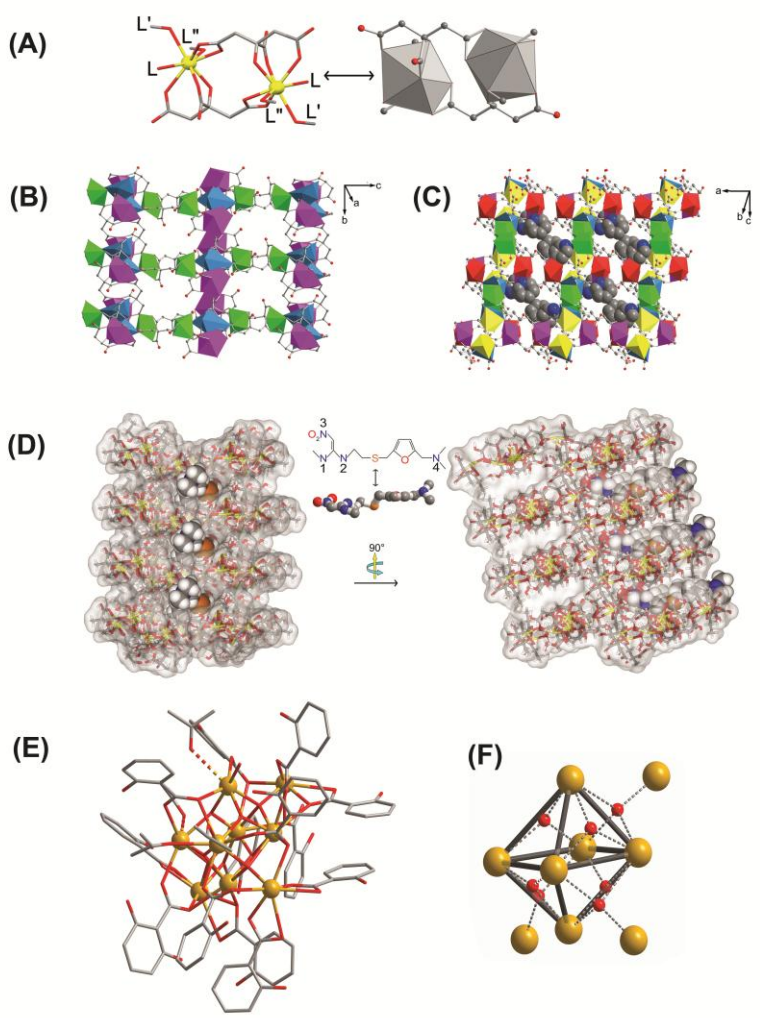

Figure 1 Structures and structural models of bismuth citract and bismuth sub-subsalicylate (BSS). (A) Dimeric bismuth citrate building block with ball-stick model and polyhedral; (B) the polymeric framework of bismuth citrate linked by three types of bismuth citrate building blocks represented by adjacent green, blue and purple polyhedral respectively; (C) the two-dimensional polymeric structure of the polymeric framework with pyridine rings in the channels (represented as space-filling mode); (D) structural model of rantidine bismuth citrate with ranitidine inserted in the channel; (E) Core structure of bismuth sub-subsalicylate $[Bi_9O_7(HSal)_{13}((CH_3)_2CO)_5]$ showing the shrouding of the Bi_9O_7 core by 13 salicylate ligands (stick structures) and (F) structure of the Bi_9O_7 core showing the octahedral arrangement of the bismuth atoms (ark brown balls) with seven octahedral faces capped by an oxygen atom (red balls).

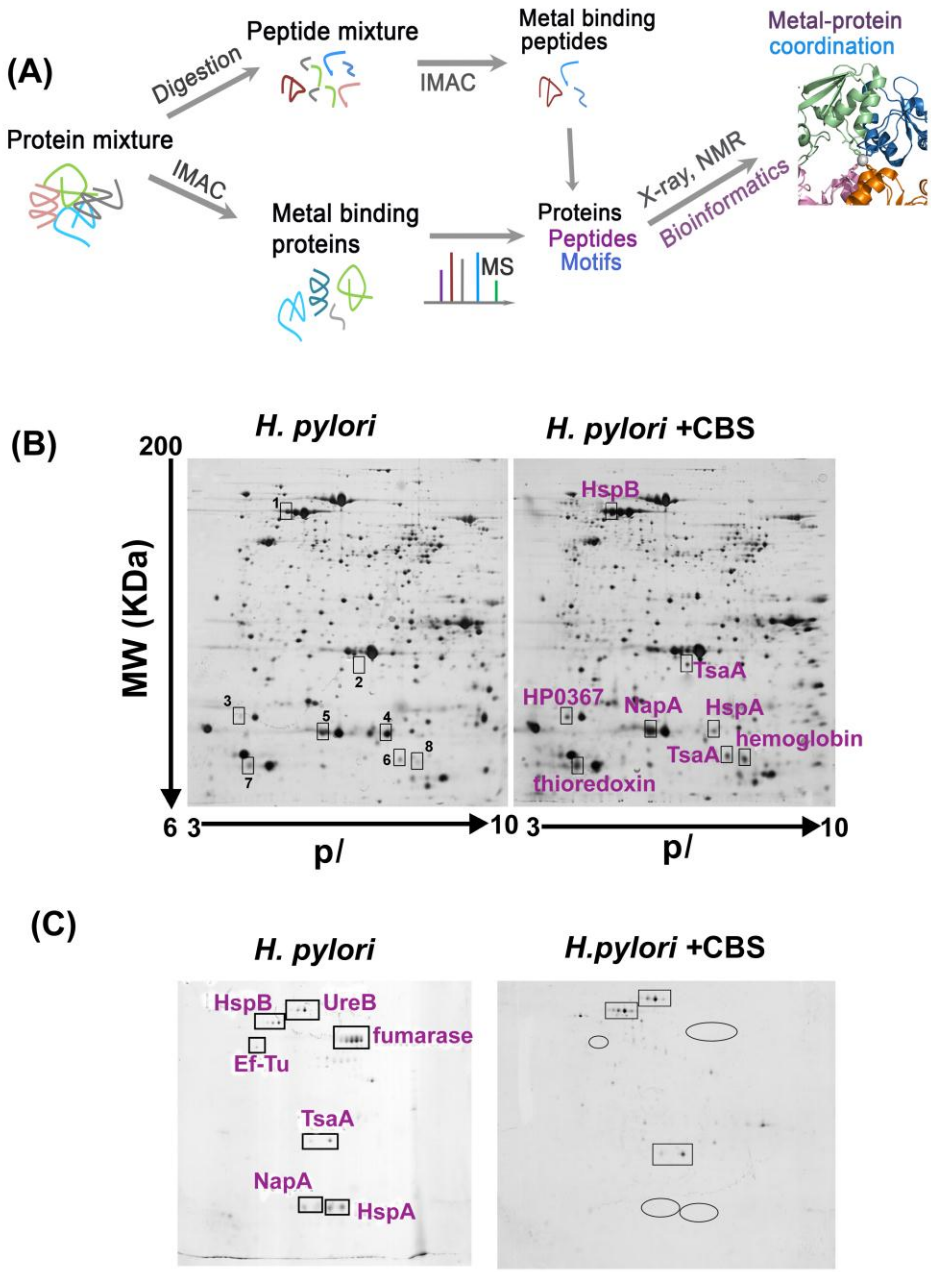

Figure 2 (A) Schematic chart of metalloproteomics in identification of bismuth-binding (including bismuth) proteins and motifs. (B) Two-dimensional gel electrophoresis image of cell extracts of *H. pylori* (strain 11637) in the absence and presence of bismuth drugs. (C) Two-dimensional gel electrophoresis image of fractions of cell extracts eluted from bismuth column from untreated and bismuth drug treated *H. pylori* cells, showing bismuth binding proteins, i.e. UreB, HspA, HspB, TasA, NapA and fumerase.

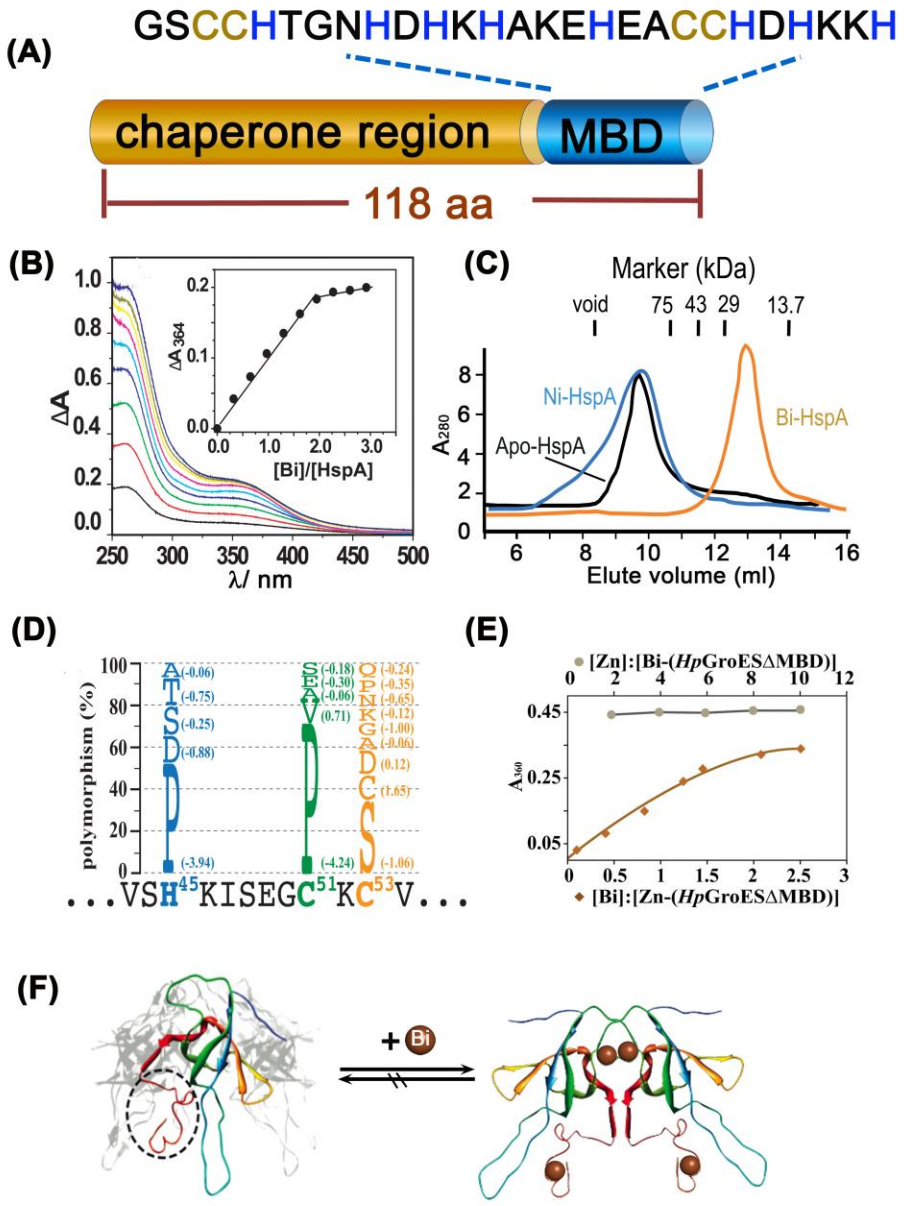

Figure 3 (A) Scheme of HspA with the putative metal binding domain highlighted. (B) Binding of bismuth to the protein was demonstrated by difference UV-vis spectra. (C) Bismuth but not nickel induces HspA from a heptamer to a dimer. (D) Negative selection of the three metal-binding residues, His49, Cys51 and Cys53 as demonstrated in the position-specific polymorphy. The substitution modes are identified from the alignment of multiple Hspa (GroES) homologs, and the corresponding proportions of each substitute are shown by the height of the letters. (E) Proposed scheme showing the oligomeric state of HspA in the presence of bismuth. For clarity, only the front subunit is highlighted, and the rest are shaded in gray.