Identifying antibiotics based on structural differences in the conserved allostery from mitochondrial heme-copper oxidases

Antimicrobial resistance (AMR) is a global health problem [1]. Many efforts have been made to reduce the burden of AMR perils globally since 2013, yet threats from some species continue to rise regardless: drug-resistant *Neisseria gonorrhoeae* is one of five urgent threats [2]. Resistance to ceftriaxone, the last option for an empirical first-line antibiotic against *Neisseria gonorrhoeae* in most countries, has been reported and continues to emerge globally [3]. The gonococcal infection could become untreatable due to a high degree of AMR, which would increase serious complications: infertility, ectopic pregnancy, and increased transmission of HIV. The emergence of resistant pathogens to currently available antibiotics is very alarming; thus, the development of treatment options is imperative to tackle AMR. Therefore, the development of a novel mechanism and narrow-spectrum antibiotics is seriously required.

The respiratory chain has recently gained scientific attention as a target of antibiotics [4]. Heme-copper oxidases (HCOs) are terminal oxidases in the electron transfer chain and the essential enzymes in life, thereby being a prospective target of antibiotics. However, the structural similarity and substrate commonality with host proteins are risks for cross-reactivity. On this point, an allosteric inhibitor is a feasible choice to avoid cross-reactivity because allosteric sites are evolutionarily less conserved than orthosteric sites.

We identified a novel allosteric inhibitor of mammal HCO, mitochondrial cytochrome c oxidase (mtCcO), using a high-throughput screening. We determined the crystal structure of mtCcO complexed with the inhibitor solved at a resolution of 2.2 Å (X-ray diffraction data were collected at SPring-8 **BL26B1** using D-cha and SPACE) (Fig. 1) [5]. The inhibitor binding pocket was different from the binding site for molecular oxygen or cytochrome c, or the route for electron transfer pathway, proton pathway, or oxygen accessing channel (Fig. 2). But we could not conclude the inhibitory mechanism from the structure. To elucidate the allosteric inhibition mechanism, we performed molecular dynamics simulation and resonance Raman spectroscopy followed by stopped-flow spectroscopy. Taken together, we conclude that the inhibitor binding in the novel allosteric site of mtCcO obstructs the oxygen channel.

The allosteric site in mtCcO is on the surface of the core structure conserved in pathogenic bacteria, and it is covered by additional helices only in mammals (Fig. 3). We hypothesized that this additional helix in mtCcO makes the inhibition pockets distinct from bacterial HCOs. This structural difference will lead us to identify specific allosteric antibiotics with a narrow spectrum.

We used *E. coli* bo_3 ubiquinol oxidase (bo_3 UqO) to prove the conservation of the allostery as a model bacterial HCO. Firstly, we established a custom compound library by *in silico* screening from mtCcO inhibitors. We identified a specific and allosteric inhibitor for bo_3 UqO. We confirmed that the allosteric effect is even conserved in bo_3 UqO by cryo-EM analysis (Micrograph data were collected at SPring-8 **EM04CT**) (Fig. 3) and mutational analysis followed by stopped-flow analysis.

Then, we determined to tackle quinol-dependent NO reductase, qNOR, from a pathogenic bacteria. qNOR is a distant family member of HCO. The

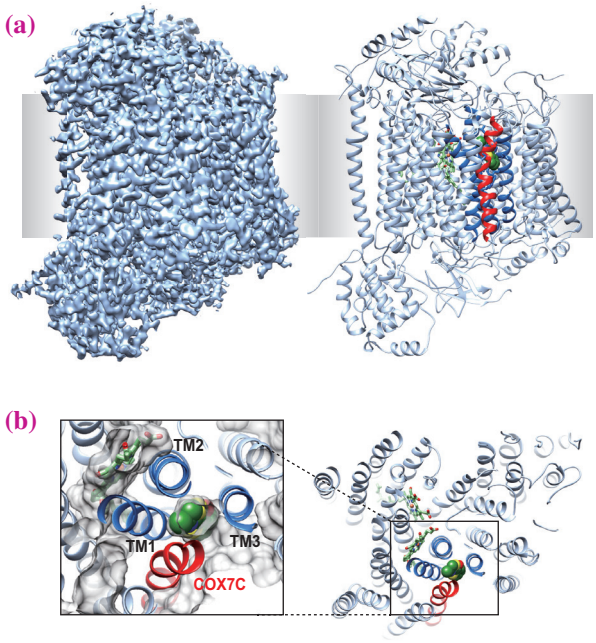

Fig. 1. The allosteric site for T113 is buried inside eukaryotic mtCcO. **(a)** X-ray structure of mtCcO with T113. The electron density map ($2Fo-Fc$), contoured at 1σ, is shown in the left. The ribbon model of mtCcO with T113 in sphere is shown in the right. T113 was covered by COX7C (red), hidden from the surface. **(b)** T113 was surrounded by 4 transmembrane helices (TM1-3, COX7C) and buried from the surface, viewed from the inter-membrane space. Protein molecular surface is shown as gray in the close-up view. Three helices of subunit I surrounding the allosteric site are shown as dark blue, the other helices of subunit I as pale blue, subunit COX7C in mtCcO is shown as red.

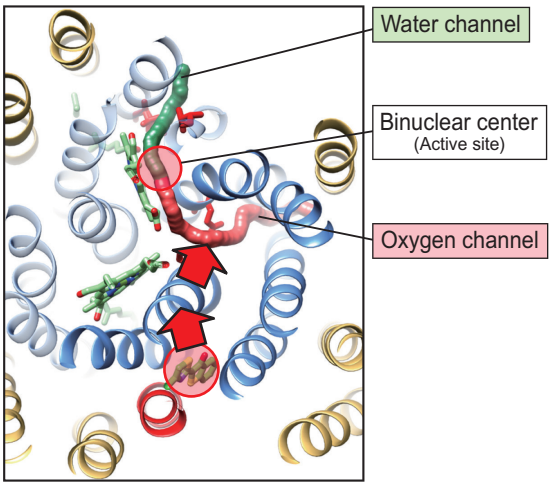

Fig. 2. Scheme of the allosteric inhibition mechanism of mtCcO. The inhibitor binding in the novel allosteric site obstructs the oxygen channel as domino effect. Helices of subunit I, are shown as blue, subunit COX7C is shown as red, and the other helices as yellow. Inhibitor is shown as a green stick.

spreading of multi-drug-resistant *Neisseria gonorrhoeae* is one of the most significant global health concerns. We performed a similar approach as bo_3 UqO and identified qNOR-specific allosteric inhibitors. Finally, we demonstrated the antimicrobial effect against a clinically isolated super-resistant *N. gonorrhoeae*.

In summary, we focused on the allosteric site on the surface of the conserved core structure of HCOs. Based on the difference in structure of the inhibitor binding site, which is covered by an additional subunit in mammals, we succeeded in identifying pathogenic bacterial HCO-specific inhibitors, which have therapeutic potential against AMR. Generally, the core structures of fundamental proteins, not only respiratory enzymes, have acquired additional subunits that modulate their function along molecular evolution. They could likely contain allostery at the boundary of the structures between eukaryotes and bacteria, making us expect that our approach can be applied to other therapeutic targets. Thus, in conclusion, this study will open fresh avenues in protein science and therapeutic development, especially for antibiotics with different mechanisms of action.

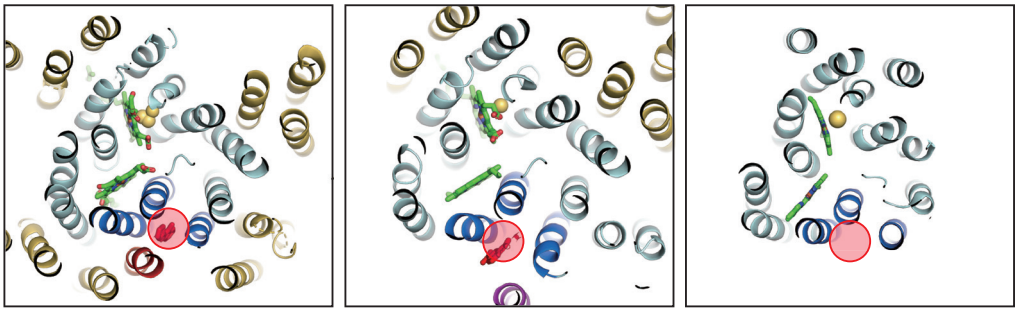

Fig. 3. The allosteric sites of mtCcO (left, pdb 7xmb, complex structure with an inhibitor) is buried by a eukaryotic-specific subunit, though those of *E. coli* bo_3 UqO (middle, pdb 7xmd, complex structure with an inhibitor) and *N. meningitidis* qNOR (right, pdb 6fwf) are opened up. Helices of subunit I, conserved subunit in HCOs, are shown as blue, subunit COX7C in mtCcO is shown as red, transmembrane helix 0 (TM0) of subunit I as purple, and the other helices as yellow. Inhibitors are shown as red stick, and the allosteric sites are shown as red circles.

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