Arsinothricin: a novel arsenic-containing antibiotic

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ABSTRACT: The emergence and spread of bacterial resistance highlights the urgent need for new antibiotics. Organoarsenicals have been used as antimicrobials since Paul Ehrlich's salvarsan. Recently a soil bacterium was shown to produce the organoarsenical arsinothricin (AST), a mimetic of the herbicidal antibiotic phosphinothricin (PT). We demonstrate that AST is an effective broad-spectrum antibiotic, showing that bacteria have acquired the ability to utilize environmental arsenic to produce a potent antimicrobial. With every new antibiotic, resistance inevitably arises. The *arsN* gene, widely distributed in *ars* operons, confers PT resistance. The functional linkage of *arsN* to arsenic detoxification was unclear. Here we show that *arsN* selectively confers resistance to AST. Crystal structures of the ArsN N-acetyltransferase shed light on the mechanism of selectivity. These results may lead to development of a new class of antimicrobials and ArsN inhibitors.

1 INTRODUCTION

New antibiotics are urgently needed because the emergence of resistance has rendered nearly every clinically used antibiotic ineffectual. Recently a new arsenic-containing natural product, arsinothricin (AST), was shown to be produced by the rhizosphere microbe *Burkholderia* sp. GSRB05 (Kuramata *et al.*, 2016). AST is an arsenic mimetic of the *Streptomyces* phosphonate antibiotic phosphinothricin (PT). The use of arsenicals as antimicrobial and anticancer agents is well-established (Jolliffe, 1993). Here we show that AST is a potent broad-spectrum antibiotic and propose that AST has the potential to be the progenitor of a new class of organoarsenical antibiotics and provide a countermeasure to the return of the pre-antibiotic era.

2 METHODS

2.1 *In vivo* resistance assays

Cells were grown in LB medium to log phase, following which the cells were 100-time diluted in M9 medium containing arsenite (As(III)), methylarsenite (MAs(III)), AST or PT. Resistance was determined from the A₆₀₀ after 24 h incubation.

2.2 Gene cloning, protein purification and crystallization and enzyme assays

Pseudomonas putida KT2440 *arsN* (*PparsN*) and the *pat* gene encoding PT N-acetyltransferase from

Streptomyces viridochromogenes (*Svpat*) were cloned into pBAD expression vector and transformed into *E. coli* TOP10. His-tag PpArsN and SvPAT were purified from the induced cells using a Ni-NTA column chromatography. Crystals of apo- and AST-bound PpArsN were grown at room temperature. X-ray data were collected on beamline 22ID at the Advanced Photon Source, Argonne National laboratory. The structures were solved by molecular replacement method using PDB ID: 1YVO as a template. The enzymatic activities and kinetic parameters of the purified PpArsN and SvPAT for PT and AST were determined (Thompson *et al.*, 1987).

3 RESULTS AND DISCUSSION

3.1 AST is a potent broad-spectrum antibiotic

AST is effective on a variety of Gram-negative and Gram-positive bacterial species at a thirtieth of the concentration of PT, suggesting that AST is a much more effective broad-spectrum antibiotic than PT (Fig. 1). Two bacterial species were resistant to AST. *Burkholderia* sp. GSRB05 is the producer of AST, so it is not unreasonable that the species has a mechanism of resistance to AST and PT. As discussed below, the *arsN* gene confers resistance in *P. putida* KT2440. In *E. coli* AW3110 Δ *arsRBC*, AST was found to be considerably more inhibitory than As(III), and its inhibitory activity was similar to that of highly toxic MAs(III). These results suggest that AST-synthesizing bacteria use environmental inorganic arsenic to produce and secrete AST to kill off competitors.

Figure 1. AST is a broad-spectrum antibiotic. Cells were cultured in the absence (left) or presence of 25 μ M AST (middle) or 800 μ M PT (right). Data are the mean $\pm$ SE (n = 3).

Figure 2. PpArsN confers resistance to AST. Strains: *E. coli* AW3110 bearing control plasmid (□) or plasmid carrying the *PpArsN* gene (◇). Data are the mean $\pm$ SE (n = 3).

3.2 *ArsN* confers AST resistance

With every antibiotic, resistance inevitably arises. PT resistance is conferred by PT *N*-acetyltransferase (PAT). A gene termed *arsN* is found in many arsenic resistance (*ars*) operons that has been annotated as encoding a PAT. *ArsN* from *P. putida* KT2440 (PpArsN) has been demonstrated to inactivate PT by *N*-acetylation (4). Why an enzyme for PT resistance should be in *ars* operons was a mystery. The identification of AST as a natural product suggested that the biological function of *ArsN* could be as an AST resistance. We examined the ability of *ArsN* to confer AST resistance (Fig. 2).

AW3110 is sensitive to AST, whereas AW3110 expressing *PpArsN* became resistant to AST, supporting our hypothesis that *ArsN* confers AST resistance. Heterologous expression of *PpArsN* also conferred PT resistance. These results are consistent with the results of *P. putida* (Páez-Espino *et al.*, 2015) (Fig. 1). In addition, we demonstrated that purified PpArsN is more selective for AST over PT. We compared this with the substrate selectivity of purified PAT from the PT-synthesizing *S. viridochromogenes* (SvPAT), whose natural substrate is PT. SvPAT showed comparable activity with PT and AST. Thus *ArsN*, which is selective for AST, is different from PAT, which shows no selectivity.

Figure 3. Structure of PpArsN. A: Overall fold of apo-PpArsN. α helices are shown in dark green. B: Interaction of AST with PpArsN. The AST binding site is formed by residues from both the chains A (cyan) and B (green) of the dimer.

3.3 Crystal structure of *ArsN*

To elucidate the mechanism of PpArsN resistance and its selectivity for AST, we solved structures of apo- and AST-bound PpArsN. PpArsN belongs to the GCN5 *N*-acetyltransferase (GNAT) superfamily and like other GNAT superfamily members, the overall conformation of PpArsN is an α/β three-layer sandwich fold (Fig. 3A). PpArsN forms an asymmetric homodimer, as does PAT. Although PpArsN shares low protein sequence identity ($\sim$ 35%) with PAT, the backbone structures of these proteins are well conserved. PpArsN has two AST-binding sites, which are asymmetrically formed by amino acid residues from both Chains A and B. Both active sites are composed of nine residues: six residues from one chain and three residues from the other (Fig. 3B). Comparison of the structures of AST-bound PpArsN and PT- and CoA-bound PAT from *S. hygroscopicus* (ShPAT) (PDB ID: 5T7E) (Christ *et al.*, 2017) demonstrates similarities of the active sites of these two related *N*-acetyltransferases.

The residues involved in AST binding in PpArsN are conserved well in ShPAT, with several conservative replacements. Surprisingly, sets of amino acid residues used by ShPAT to interact with each chemical moiety in PT are different from those used by PpArsN to interact with the corresponding chemical moiety in AST. As a result, while the arsenic atom of AST overlaps with the phosphorus atom of PT, the orientation of the amino group of PT is inclined to 95° towards the acetyl-CoA binding site with respect to the AST binding site. The minor difference in composition of amino acid residues forming the substrate binding site between *ArsN* and PAT may lead in part to the variation of their substrate selectivity.

4 CONCLUSIONS

Our results demonstrate that the novel organoarsenical natural product AST is a broad-spectrum antibiotic much more potent than its phosphorus mimetic PT. We also show that *ArsN* selectively confers resistance to AST over PT, elucidating the functional linkage of *ArsN* to arsenic detoxification. Our *ArsN* crystal

structures will be useful to design new drugs that can evade resistance or enhance AST effectiveness as antibiotics.

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