Identification of a novel structure-specific endonuclease AziN that contributes to the repair of azinomycin B-mediated DNA interstrand crosslinks

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ABSTRACT

DNA interstrand crosslinks (ICLs) induced by the highly genotoxic agent azinomycin B (AZB) can cause severe perturbation of DNA structure and even cell death. However, Streptomyces sahachiroi, the strain that produces AZB, seems almost impervious to this danger because of its diverse and distinctive self-protection machineries. Here, we report the identification of a novel endonuclease-like gene aziN that contributes to drug self-protection in S. sahachiroi. AziN expression conferred AZB resistance on native and heterologous host strains. The specific binding reaction between AziN and AZB was also verified in accordance with its homology to drug binding proteins, but no drug sequestering and deactivating effects could be detected. Intriguingly, due to the high affinity with the drug, AziN was discovered to exhibit specific recognition and binding capacity with AZB-mediated ICL structures, further inducing DNA strand breakage. Subsequent in vitro assays demonstrated the structure-specific endonuclease activity of AziN, which cuts both damaged strands at specific sites around AZB-ICLs. Unravelling the nuclease activity of AziN provides a good entrance point to illuminate the complex mechanisms of AZB-ICL repair.

INTRODUCTION

DNA interstrand crosslinks (ICLs) are highly cytotoxic lesions induced by endogenous and environmental genotoxic agents that covalently tether two bases on opposite strands of the DNA double helix (1,2). This kind of lesion seriously impairs DNA replication and transcription by blocking DNA strand separation, resulting in chromosomal mutations, rearrangements, instability, and even cell death (3). Rapidly dividing cells, such as tumour cells, are hypersensitive to ICL damage, which has led to the extensive clinical application of many well-known ICL inducers in cancer treatment (4,5). Unlike the repair of simple DNA damage, a large number of proteins responsible for multiple specialized DNA repair mechanisms, including nucleotide excision repair (NER), homologous recombination, translesion synthesis, mismatch repair and base excision repair (BER), can all be recruited to repair ICLs (3,6). Different ICL repair proteins are involved depending on the structure of the ICL, the cell cycle phase and the biological species. New factors implicated in ICL repair continued to be discovered with sustained advances in the research of these complex mechanisms in recent years.

ICLs are serious challenges to the ability of all living cells to maintain the chemical integrity of their genome. However, ICL agent-producing microorganisms can easily mitigate the danger by employing diverse self-protection machineries. For instance, the clinical anticancer drug mitomycin C (MMC) producer, Streptomyces lavendulae, is known to be protected by the encoded products of three genes, merA, mrd and mct, via reoxidation of the active reduced MMC, retention/sequestration of the native pro-drug, and drug efflux, respectively (7–9). The drug binding protein BlmA and the N-acetyltransferase BlmB confer resistance to bleomycin in Streptomyces verticillus, the bleomycin-producing strain, through drug sequestration and inactivation (10). In addition to the proteins acting on drugs, DNA repair enzymes, such as the UvrA-like proteins (SgcB2 for C-1027 resistance, DrrC for daunorubicin resistance, and CmrX for chromomycin resistance) and the DNA glycosylase homologue (YtKR2 for yatakemycin resistance) derived from the biosynthetic gene clusters of DNA-targeting drugs, have been recently reported to be implicated in the drug self-resistance of the producing strains (11–15).

Azinomycin B (AZB), a highly genotoxic agent with two reactive groups, which includes an active epoxide moiety and an unusual aziridine ring (5,16–20), has prominent in vivo antitumor activity and in vitro cytotoxicity comparable to that of MMC (21). Its excellent biological activity appears to be derived from the formation of covalent ICLs in the major groove between guanine and purine residues in
the duplex DNA sequence 5’-d(GNPy)-3’ via tandem electrophilic attacks of C10 in the aziridine ring and C21 in the epoxide moiety (16) (Supplementary Figure S1). Our previous research identified a new type of DNA glycosylase, Orf1, also known as Azi36 and AlkZ, which unhooks AZB-ICLs by enzymatic cleavage of the N-glycosidic bond at the crosslinking site and thus confers self-resistance on the AZB producer, Streptomyces sahachiroi (22,23). Herein, we report another new structure-specific nucleoside cleavage enzyme identified from the AZB self-resistance system, which is a product encoded by orf3/azi38 (renamed aziN, GenBank accession number MK453398) located near the orf1/alkZ gene. Initially, AziN was considered to be involved in self-resistance by drug sequestration. However, upon further analysis, it was demonstrated to specifically recognize the AZB-ICLs by drug binding and unhook them via cleaving the phosphodiester bonds on at least one side of the lesion. The cleavage mode of this newly discovered endonuclease was later determined by a series of in vitro experiments.

MATERIALS AND METHODS

Strains and media

Streptomyces sahachiroi ATCC 33158 and its series of mutant strains were grown on PS5 medium (5 g/l cotton seed meal, 5 g/l soluble starch, 20 g/l agar, pH 7.0) for spores formation, and on GYM medium (4 g/l glucose, 4 g/l yeast extract, 10 g/l malt extract, 2 g/l CaCO3, 20 g/l agar, pH 6.8) for fermentation. Streptomyces lividans ZX1 and its derivative strains for heterologous expression strains were cultured on MS medium (20 g/l soybean meal, 20 g/l mannitol, 20 g/l agar, pH 7.2). Escherichia coli DH5α and BL21 (DE3) were grown in LB or on LB solid medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 20 g/l agar, pH 7.0) plate at 37°C. The other strains, plasmids and primers used in this study were presented in Supplementary Table S1 and Table S2.

Production and isolation of AZB

For AZB production, S. sahachiroi ATCC 33158 was inoculated on GYM medium and incubated at 30°C for 3 days. After fermentation, AZB was isolated and purified according to the previous literatures (24,25).

Fluorescence titration assay

Since the intrinsic fluorescence of AziN is mainly derived from tryptophan residues, the excitation wavelength was 295 nm and emission spectra were collected between 305 and 500 nm with a bandwidth of 5 nm. The dissociation constant (Kd) was measured in three independent replicate experiments by fluorimetric titration of 1 μM AziN with the addition of 3 mM different ligands (AZB, MMC, or metal ions) prepared in Tris–HCl buffer. The fluorescence intensity at 320 nm was chosen to measure the binding constant. The data were nonlinear fit to the quadratic equation using Origin 8.6 Software:

\[
F_{\text{obs}} = F_0 + \Delta F \cdot \frac{P_0 + L_0 + K_d - \sqrt{(P_0 + L_0 + K_d)^2 - 4 \cdot P_0 \cdot L_0}}{2 \cdot P_0}
\]

where \(F_{\text{obs}}\) is the obtained fluorescence, \(F_0\) is the initial fluorescence of protein before added the ligands, \(\Delta F\) stands for the whole fluorescence change in the titration, \(P_0\) represents the protein concentration, \(L_0\) is the ligand concentration.

Microscale thermophoresis binding assay

The ligands (AZB or MMC) was diluted to desired concentrations (range from 30 nM to 0.5 mM), and then incubated with 0.1 μM purified protein for 5 min at room temperature in PBS buffer. The samples were loaded into the Nano Temper glass capillaries and detected by the microthermophoresis with 20% LED power and 20% MST. The binding constants were determined using Nano Temper software.

Electrophoretic mobility shift assay

DNA fragment pre-incubated with or without AZB was treated with various amounts of AziN at 30°C for 20 min in a mixture solution containing 10 mM Tris–HCl, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 60 mM KCl. The reaction mixture was detected by electrophoresis in a 4% polyacrylamide gel with 0.5× Tris–borate–EDTA buffer.

DNA crosslinking assay

For AZB crosslinking reaction, DNA was treated with AZB as described by Wang et al. (22) in a reaction buffer (25 mM triethanolamine, 1 mM EDTA, pH 7.2) at 37°C for 2 h and then analyzed by gel electrophoresis. The ICL derived from an abasic (Ap) site with an adenine residue on the antisense strand were prepared as previously described (26). A 2’-deoxyuridine (dU)-containing oligodeoxynucleotide duplex (AU, Supplementary Table S3) was designed according to Price et al. (26). Through hydrolysis of uracil DNA glycosylase (UDG), dU sites were converted into Ap sites to give the Ap-containing duplexes which were 3’-end-FAM labelled on the forward strand (Ap/A-1) and the reverse strand (Ap/A-2), respectively. The Ap-A ICL products were generated by incubation of the Ap-containing duplexes in a reaction buffer (50 mM HEPES, pH 7, 100 NaCl) at 37°C for 120 h.

DNA cleavage assay

The crosslinked DNA purified by chloroform was reacted with AziN at 30°C for 20 min and subsequently electrophoresed after treatment with 1 mg/ml proteinase K at 37°C for 15 min.

RESULTS

AziN is closely related to AZB production

For a comprehensive analysis of the toxin resistance mechanism in the ICL agent producer S. sahachiroi, all open reading frames within and adjacent to the AZB biosynthetic gene cluster were investigated. In addition to the previously reported orf1/alkZ, orf3/aziN also attracted our attention. Although deletion of the gene aziN from the S. sahachiroi chromosome (∆aziN, Supplementary Figure S2A) did not
completely abolish the production of AZB, the yield of this compound was significantly reduced, and no intermediate with a new structure has been detected thus far. When aziN was cloned into the expression vector pWS052 and introduced into the gene deletion mutant strain ΔaziN, the AZB yield was partially restored in the gene complementation strain aziN/Cp (Figure 1A and Supplementary Figure S2B). No obvious difference was observed between the wild-type strain and the two mutant strains, ΔaziN and aziN/Cp, in morphological phenotypic characteristics of growth rate, colony and spore formation, except for the AZB yield. It is apparent that AziN is implicated in AZB biosynthesis and has a remarkable effect on the efficiency of the production of this drug.

**AziN specifically binds to AZB**

A BLASTP search indicated that AziN shows homology to some unidentified proteins of the glyoxalase/bleomycin resistance/dioxygenase family. The well-known proteins of this superfamily include type I extradiol dioxygenase, methylmalonyl-CoA epimerase, glyoxalase I and a group of antibiotic resistance proteins (27). The bleomycin-binding protein (BlmT) encoded by the transposon Tn5 and the mitomycin-binding protein (MRD) derived from the MMC biosynthetic gene cluster are typical representatives of these antibiotic resistance determinants, conferring resistance by drug sequestration (10,28,29). Another kind of antibiotic resistance protein in this superfamily, the fosfomycin resistance protein encoded by the transposon Tn 2921, renders resistance protein in this superfamily, the fosfomycin resistance proteins, its capacity to bind and inactivate drugs (23−32%).

Drug binding proteins are generally considered to confer resistance to AZB between ΔaziN and the wild type strain was undetectable. Considering the essential role of AlkZ in AZB self-resistance, another aziN gene deletion mutant strain ΔaziU3ΔalkZΔaziN was constructed based on the alkZ gene triple deletion strain ΔaziU3ΔalkZ (22) (Supplementary Figure S2C) and used for the disc diffusion susceptibility test. The triple gene deletion mutant showed greater sensitivity to AZB than the original double gene deletion strain ΔaziU3ΔalkZ (Figure 2A). After eliminating the interference effect of alkZ on drug resistance, the resistance function of AziN against AZB began to unfold.

**In vivo expression of AziN confers resistance to AZB**

Given that drug binding proteins can usually provide resistance to host cells from drug toxicity, the *in vivo* activity of AziN was analysed by heterologous expression of the protein in *Streptomyces lividans* ZX1, an AZB-sensitive strain. The expression plasmid pWS052-aziN and the empty vector pWS052 were introduced into *S. lividans* ZX1 to yield the expression strains *S. lividans::aziN* and *S. lividans::pWS052*, respectively. In both the disc diffusion test and the cross streak assay (Figure 2A), the strain expressing AziN exhibited remarkably enhanced viability in the presence of AZB, indicating the specific drug resistance function of AziN.

To further confirm the physiological function of AziN in the wild-type strain *S. sahachiroi*, the single gene deletion mutant strain ΔaziN was employed in cross streak assay as well. Unexpectedly, the difference in sensitivity to AZB between ΔaziN and the wild type strain was undetectable. Considering the essential role of AlkZ in AZB self-resistance, another aziN gene deletion mutant strain ΔaziU3ΔalkZΔaziN was constructed based on the alkZ gene triple deletion strain ΔaziU3ΔalkZ (22) (Supplementary Figure S2C) and used for the disc diffusion susceptibility test. The triple gene deletion mutant showed greater sensitivity to AZB than the original double gene deletion strain ΔaziU3ΔalkZ (Figure 2A). After eliminating the interference effect of alkZ on drug resistance, the resistance function of AziN against AZB began to unfold.

**In vitro binding of AziN to AZB does not affect drug activity**

Drug binding proteins are generally considered to confer resistance by drug sequestration and inactivation (33,34), therefore, the antibacterial and crosslinking activities of AZB bound with AziN were checked. The inhibitory effect of AZB on the growth of *Bacillus subtilis* 168 gradually decreased as incubation time increased due to drug degradation at room temperature. However, no difference in antibacterial activity between samples with and without AziN was observed (Figure 2B). Moreover, crosslinking activity of the AziN–AZB complexes was detected after the free drug molecules were removed by ultrafiltration and washing. As shown in Figure 2C, DNA was crosslinked by the protein–drug complexes (the remnant of reaction 3), while in the control sample without protein (reaction 2), the same amount of drugs was transferred to the filtrate, and no crosslinking activity of the remnant in the ultrafilter was found. The results of both experiments demonstrated that the binding of AziN to AZB neither blocked nor inactivated the active groups of the antibiotic molecule, in contrast to most known drug binding proteins.
Figure 1. The drug binding protein AziN has remarkable effect on efficiency of the AZB production. (A) Bioassay (up) and LC–MS analysis (down) of the AZB production in the wild-type (WT) and mutant strains, ΔaziN and aziNCP. (B) Equilibrium fluorescence titration assay to detect the interaction of AziN with two different ICL agents, AZB and MMC. (C) Microscale thermophoresis binding assay to monitor the binding isotherm of two ICL agents, AZB and MMC, with AziN.

AziN specifically recognizes and incises the AZB–ICL DNA

To determine the real resistance mechanism of AziN, the possibility of interaction between the protein and DNA, the target of the drug, was then investigated by an electrophoretic mobility shift assay (EMSA) after its influence on drug activities was excluded. Because no conserved domain for DNA binding was found in AziN, a 154 bp DNA fragment from the chromosome of another Streptomyces strain was randomly used for the experiment. After incubation with or without AZB at 37°C for 2 h, AziN was subsequently added to the DNA solution at 30°C for 20 min of incubation. With increasing concentrations of AziN, a retardation band of the protein-DNA complex started to appear gradually in ICL DNA samples, while no change was observed in native DNA (Figure 3A). The drug was supposed to mediate the binding of AziN to AZB–ICL DNA. Surprisingly, however, a significant DNA degradation phenomenon was observed in ICL DNA samples with a further increase in AziN concentration, while native DNA remained unchanged under the same conditions. This result suggested that the interaction between AziN and ICL DNA might lead to DNA degradation.

The DNA repair capacity of AziN was detected by crosslinking assay of the double-stranded circular DNA of the plasmid pOJ260. The DNA distortion induced by AZB–ICL led to conformation changes in supercoiled DNA, which were manifested by decreased migration speed in electrophoresis (Supplementary Figure S6). Once the crosslinking was removed, the DNA configuration resumed, as did the speed of DNA migration (20). After treatment with AziN, a new migration band of decrosslinked DNA appeared between ICL DNA and ocDNA (open circular DNA), and its intensity increased with the protein concentration (Figure 3B, lanes 2 and 3), indicating the ability of AziN to repair ICL DNA. Moreover, the new shift band exhibited different mobility from the product repaired by the DNA glycosylase Orf1/AlkZ (22) (lane 6), implying distinct incision modes between the two proteins. DNA degradation was also found in the sample with a higher concentration of AziN (lane 4), providing further evidence for DNA strand breakage induced by the repair of the protein.

To confirm the pattern of cleavage, the linearized plasmid pBluescript SK(–) DNA was then used in the ICL repair assay (Figure 3C). Since the conformation change in linear DNA is not obvious, alkali denaturation was employed to distinguish between crosslinked and uncrosslinked DNA prior to agarose gel analysis. The basic double helix structure of repaired ICL DNA was preserved due to the base-pairing hydrogen bonds before alkali denaturation (lane 4). When the base pairing of repaired ICL DNA was disrupted via alkali denaturation, a large amount of low-molecular weight DNA appeared (lane 10), which verified the incision of AziN at the phosphodiester backbone of ICL DNA (Figure 3D).

The substrate specificity of AziN has been investigated by using DNA containing different types of distortions (Supplementary Figure S7). A 29-bp oligodeoxynucleotide duplex with a single AZB–ICL site of 5′-GCC-3′ was used to generate AZB–ICL DNA as the positive control. The other type of ICL structure derived from the reaction between an abasic (Ap) site and an adenine (A) residue on the opposite strands of DNA helix, was prepared (26) and validated by
its resistance to digestion by the Ap endonuclease Endo IV (lane 4 in Supplementary Figure S7B and D). While AZB-ICL DNA was distinctly cleaved to produce a small fragment, no incision was observed in the Ap-A ICL DNA samples after treatment with AzIN at the same dose. Similarly, the protein did not act on mismatched DNA (MIS) (Supplementary Figure S7C). These results established the specific cleavage activity of AzIN on AZB-ICL DNA and excluded distractions of possible contaminant nucleases in the protein preparation.

The lack of crystallographic data of highly homologous proteins as modelling templates makes the structure prediction of AzIN and the identification of potential active sites an arduous task. Therefore, we used both SWISS-MODEL and PredictProtein servers to analyse the protein sequences, and 17 relatively conserved amino acid residues of AzIN were selected for site-directed mutagenesis (Supplementary Table S4) to investigate their roles in incision and binding to AZB-ICL DNA. In vitro cleavage assay showed that most of mutants harboured near wild-type cleavage activities, except that mutations of L17A, W21A and R82D showed ~20% of wild-type activity. The results of EMSA showed that L17A and R82D were devoid of the ability to bind ICL DNA while W21A retained weak binding to ICL-DNA (Supplementary Figure S8), suggesting that they mediate catalysis through ICL DNA binding.

Cleavage sites for AzIN

For precise identification of the cleavage sites for AzIN in ICL DNA, a 21-bp oligodeoxynucleotide duplex containing one 5’-GCC-3’ site located with AT tracts (named GCC2, Supplementary Table S3) was designed according to the previous literature (22,35–37). The forward strand of GCC2 was 3’-end-FAM labeled (in Figure 4D) and then served as the substrate for the DNA cleavage assay. The first alkylation reaction induced by AZB occurred mostly at 3’-CGG-5’ of the reverse strand, but the FAM dye was labeled on the forward strand of GCC2. Hence, mono-alkylation products were invisible in this case, and the product with lower mobility than native DNA in 20% denatured polyacrylamide gel was supposed to be the crosslinked oligodeoxynucleotides...
Figure 3. Identification of the nuclease-like activity of AziN. (A) EMSA analysis of the binding of AziN to ICL DNA. A 154 bp DNA fragment was pre-incubated with or without AZB, and subsequently treated with various amounts of AziN (0, 1.5, 3, 4.5 mg/mL). The reaction mixtures were analyzed by 4% native PAGE. (B) Characterization of the cleavage activity of AziN. The crosslinked circular DNA was incubated with different concentration of AziN (0.67, 1.3, 2.7 mg/mL) at 30°C for 20 min and subsequently treated with 1 mg/mL proteinase K at 37°C for 15 min prior to gel electrophoresis. (C) Detection of the incision pattern of AziN. The crosslinked linear DNA was incubated without or with AziN (1.3 mg/mL) and then denatured by alkaline treatment before gel analysis. The inactivated AziN was used as a protein control (lanes 3, 6, 9 and 12) and the native linear DNA was used as a DNA control (lanes 1, 5–7, 11 and 12). (D) The proposed status of ICL DNA before (b) and after (a) the cleavage of AziN under alkaline treatment.

(lane 2 in Figure 4A). As increasing concentrations of AziN were added, the intensity of the ICL DNA band decreased, and a new DNA fragment with higher mobility than the denatured native DNA appeared (lanes 4–5 in Figure 4A), which was supposed to be the cleavage product of AziN. To determine the size of this fragment, two pairs of oligodeoxynucleotides (GCC3 and GGC5, Supplementary Table S3) containing a single SaqAI site were designed and 3′-end-FAM labeled on reverse strand. After digestion with SaqAI, the DNA duplexes 4-/6-nt-FAM from GCC3 and 8-/10-nt-FAM from GCC5 (Supplementary Figure S9) were produced and used as the DNA molecular weight standards M1 and M2, respectively. As shown in Figure 4B, the small cleavage fragment of GCC2 was identical in mobility to M2 in denatured PAGE, which indicated that AziN catalysed hydrolytic cleavage of the phosphodiester bond at the 3′ end of the crosslinking site on the forward strand to produce a 10-mer DNA fragment containing the FAM label at its 3′ end (Figure 4D).

When FAM dye was labelled at the 3′ end of the reverse strand of GCC3, both mono-adducts and ICL DNA were visibly displayed in lane 2 of Figure 4C. After AziN was added to the mixture, the migration band of ICL DNA disappeared, and a unique shift band with mobility between the mono-adducts and ICL DNA was generated (lanes 4 and 5). No DNA fragment smaller than native DNA, suggesting that there was no incision in the 3′ flanking region of the crosslinking site on the reverse strand. Thus, the new band presented in lanes 4 and 5 of Figure 4C was regarded as the residual cleavage product after removal of the 3′ incised sequence in the forward strand (Figure 4E). In addition, the intensity of the mono-adduct band was almost untouched by AziN, implying that the protein incubated IC DNA rather than the mono-alkylation product.

To verify the above results and obtain a more comprehensive analysis of the AziN cleavage mode, a longer 29-mer oligodeoxynucleotide duplex (GCC6, Supplementary Table S3) was 3′-end-FAM labeled on the forward strand (GCC6-1), reverse strand (GCC6-2), and both strands (GCC6-3). When FAM dye was labeled at the 3′ end of the forward strand (GCC6-1), a smaller band (fragment I) than native DNA was observed in both denatured and native PAGE (Figure 5C and D), while a larger band (fragment II) than mono-adduct DNA was traced in the cleavage products of the ICL DNA with a 3′-end-FAM label on the reverse strand (GCC6-2). Two cleavage products (fragments I and II) were detected simultaneously when both strands of GCC6-3 were 3′-end-FAM labelled (Figure 5F and G). The mobility of fragment I was identical to that of the DNA marker M4 (18-nt-FAM/16-nt) in denatured PAGE (Figure 5C and F), while it was slightly higher than that of M4 in native PAGE (Figure 5D and G). It is evident that AziN could cut both strands of ICL DNA, and the cleav-
age site on the reverse strand should be in the 5′ flanking region of the crosslinking site. For precise localization of the cleavage sites, both ends of one DNA strand (GCC6-4 labelled on the forward strand and GCC6-5 labeled on the reverse strand) were labelled with 5′-end-TAMRA and 3′-end-FAM simultaneously. In addition to the large band of fragment II, a small band of 18-nt-FAM, regarded as the forward sequence of fragment I (lane 4 in Figure 5H), and a small band of 11-nt-TAMRA, regarded as the reverse sequence of fragment I (lane 4 in Figure 5I), were detected in the cleavage assay of crosslinked GCC6-4 and GCC6-5, respectively. The cleavage site on the reverse strand is supposed to be between the fifth and sixth nucleotides near the 5′ end of the cross-linking site. Thus, fragment II is most likely the other half of the cleavage products, which still contain crosslinking structures after a double stranded break (Figure 5B). Due to its high affinity with the drug, AziN can specifically recognize and bind the AZB-ICL DNA. Moreover, it can further lead to hydrolytic cleavage of the phosphodiester bonds adjacent to the crosslinked structure on both strands.

**DISCUSSION**

In this study, another new determinant of AZB resistance, aziN, was discovered in the vicinity of the essential AZB resistance gene orf1/alkZ through gene deletion and heterologous expression experiments. BLASTP analysis showed that AziN has homology and similarity with many unidentified members of the vicinal oxygen chelate (VOC) metalloenzyme superfamily, but no putative conserved domains have been detected. In the sequence analysis of the Pfam database, AziN was classified as a member of the Glyoxalase_6 (PF18029) family of proteins due to the presence of a glyoxalase-like domain. This family of proteins contains a diverse set of domains related to the glyoxalase domain, and the specifics of their structure and function are uncertain. There are a few antibiotic resistance proteins in this superfamily, such as MRD, the bleomycin resistance proteins (Blms) and the fosfomycin resistance proteins (FosA/FosB/FosX) (27). Through either sequestration or inactivation, drug activities are prevented by these known resistance proteins (38–40). Unlike them, the specific binding of AziN with Cu²⁺ had no influence on the antibacterial and crosslinking activities of the drug, suggesting the particular function of AziN. Although the interaction of AziN with Cu²⁺ was detected, the drug binding ability and the cleavage activity of AziN are both independent of metal ions (Supplementary Figure S4, S5 and S7). We suggest that AziN descended from an ancient metalloenzyme, and its catalytic activity changed after a natural evolutionary process while preserving metal-ion-binding capability, as previously reported as a possible explanation for the functional switching of MRD (41).
It is suspected that drug-bind proteins can cooperate with
drug-transporting proteins for intracellular drug efflux by
capturing and delivering drugs. The drug efflux system,
which serves as an elementary resistance mechanism against
exogenous and endogenous toxins, is widespread in the
cells of most living organisms and particularly plentiful in
antibiotic-producing strains and drug-resistant pathogens
(14,42,43). In the AZB biosynthetic gene cluster, the trans-
membrane export protein AziE was verified to play a key
role as an efflux pump in resistance to AZB. The single het-
erologous expression of AziE or AziN can improve AZB
resistance in the sensitive strain S. lividans ZX1. AZB pro-
duction decreased more markedly in the aziN gene deletion
mutant than in the aziE gene deletion mutant (Xiaorong
Chen and Jing He, unpublished work). Obviously, the func-
tions of the two resistance proteins are not closely linked to
each other. AziN was proven exert its drug resistance func-
tion by its involvement in ICL repair rather than through
drug sequestration and efflux.

The first identified AZB binding protein AziR, homolo-
gous to aminoglycoside phosphotransferases, can also pro-
vide resistance against AZB toxicity to the heterologous

hosts *S. lividans* TK24 and *E. coli* (31). No chemical modification of AZB was found in the interaction of AzIR and the drug, but regrettably, the potential drug sequestration effect of canonical binding proteins has not been tested. Except for azIR, the other three resistance determinants, aziE, alkZ and aziN, are all located within the AZB biosynthetic gene cluster and associated with drug production. Both alkZ and aziN can specifically recognize and excise AZB-ICL DNA. The DNA glycosylase AlkZ catalyses the hydrolysis of the N-glycosidic bond of the alkylated nucleobase to repair ICLs by triggering a BER-like pathway (22), while AzIN induces hydrolytic cleavage of the phosphodiester bonds around ICL sites. Without conserved DNA binding domains, the interaction of AzIN with ICL DNA is mediated by the drug. AzIN has selectivity and specificity for AZB–ICL DNA, a kind of specific DNA structure, rather than for a DNA sequence, behaving as a structure-specific endonuclease. This kind of endonuclease usually resolves specific DNA secondary structures such as single-stranded flaps, stem-loops, Holliday junctions and DNA adducts that arise during DNA damage repair, DNA recombination, DNA replication and transcription stress processes (44). ICL repair also relies on structure-specific endonucleases to cut DNA strands at lesion sites (45–47). Despite the lack of similarity to known endonucleases, the structure-specific endonuclease activity of AzIN to cut the damaged strands was verified by an *in vitro* assay in this study. The repair of ICLs is a complex, multi-step process requiring the cooperation of many DNA repair proteins (1,3). Our research has shown that the protein AzIN is involved in the damage recognition and incision steps of the ICL repair process, most likely in cooperation and coordination with other nucleases and regulation factors. The resulting DNA gap is generally filled by downstream translesion synthesis and homologous recombination before the remnant ICL structure is completely removed by NER (Figure 5J). Future intensive studies on the enzymatic and structural characteristics of AzIN and screening of its collaborators in ICL repair will set a basis to elucidate the action mode and regulatory mechanism of this new type of structure-specific endonuclease.

**DATA AVAILABILITY**

The protein sequence of Orf3/Azi38 that renamed as AzIN has been submitted to the GenBank database with accession number MK453998.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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