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Benzothiazinones Are Suicide Inhibitors of Mycobacterial Decaprenylphosphoryl-β-D-ribofuranose 2′-Oxidase DprE1

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Supporting Information

ABSTRACT: Benzothiazinones (BTZs) are antituberculosis drug candidates with nanomolar bactericidal activity against tubercle bacilli. Here we demonstrate that BTZs are suicide substrates of the FAD-dependent decaprenylphosphoryl-β-D-ribofuranose 2′-oxidase DprE1, an enzyme involved in cell-wall biogenesis. BTZs are reduced by DprE1 to an electrophile, which then reacts in a near-quantitative manner with an active-site cysteine of DprE1, thus providing a rationale for the extraordinary potency of BTZs. Mutant DprE1 enzymes from BTZ-resistant strains reduce BTZs to inert metabolites while avoiding covalent inactivation. Our results explain the basis for drug sensitivity and resistance to an exceptionally potent class of antituberculosis agents.

The increasing number of drug-resistant *Mycobacterium tuberculosis* strains that fail to respond to first- and second-line drug treatment demands the development of new antituberculosis drugs.[1−3] Benzothiazinones (BTZs) such as BTZ043 (Figure 1A) are a promising class of new compounds that kill *M. tuberculosis* in vitro, ex vivo, and in mouse models of tuberculosis.[4] The minimal inhibitory concentration (MIC) of BTZ043 against *M. tuberculosis* is 1 ng/mL, which is significantly lower than the MICs of all currently used tuberculosis drugs and drug candidates. Decaprenylphosphoryl-β-D-ribofuranose 2′-epimerase was identified as a BTZ target (Figure 1B).[4] The enzyme is constituted of DprE1 and DprE2 that together catalyze the epimerization of decaprenylphosphoryl-β-D-ribofuranose (DPR) to decaprenylphosphoryl-β-D-arabinofuranose (DPA), the arabinosyl donor for the biosynthesis of mycobacterial cell wall arabinan polymers.[4−6] The reaction is believed to proceed via the keto intermediate decaprenylphosphoryl-D-2′-keto-*erythro*-pentofuranose (DPX) (Figure 1B).[5] The Cys387Gly and Cys387Ser point mutations in DprE1 result in 250- and 10 000-fold increases in the MIC, respectively.[4] We previously isolated a covalent adduct of

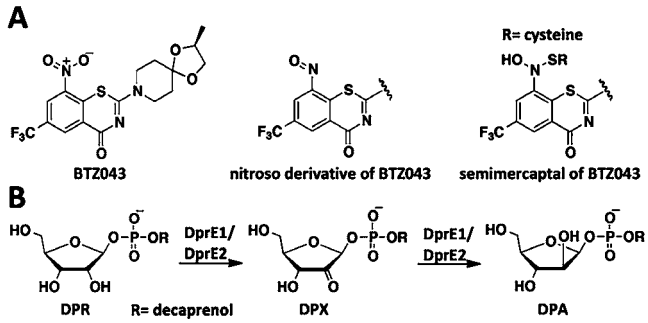

Figure 1. BTZs and decaprenylphosphoryl-β-D-ribofuranose 2′-epimerase. (A) Structures of BTZ043 and its nitroso and semimercaptal derivatives. (B) Epimerization of decaprenylphosphoryl-β-D-ribofuranose (DPR) to decaprenylphosphoryl-β-D-arabinofuranose (DPA) via decaprenylphosphoryl-D-2′-keto-*erythro*-pentofuranose (DPX).

DprE1 and BTZ043 from mycobacteria incubated with BTZ043 and proposed a mechanism of action involving reduction of the essential nitro group of BTZ043 to a nitroso group that then reacts with Cys387 of DprE1 to form a stable semimercaptal (Figure 1A).[7] However, the mechanism of action of BTZs still poses numerous questions, as it is unclear how BTZs are activated and what the basis of the specificity of the proposed nitroso derivative for DprE1 is. Furthermore, it has not been elucidated whether the observed modification of DprE1 indeed affects the activity of DprE1 or functions by inhibiting the activity of DprE2 (or both); neither have the exact roles of DprE1 and DprE2 in the epimerization reaction been validated.

A more detailed characterization of the mechanism of action of BTZs requires the availability of pure DprE1 and DprE2. As our previous attempts to purify recombinant DprE1 and DprE2 of *M. tuberculosis* H37Rv in their active form were unsuccessful,

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we cloned the orthologous genes *MSMEG_6382* and *MSMEG_6385* of *Mycobacterium smegmatis* strain mc^{2}155 for heterologous expression in *Escherichia coli (E. coli)*. The corresponding *M. smegmatis* proteins DprE1$_{SM}$ and DprE2$_{SM}$ share 81% and 85% sequence identity with their *M. tuberculosis* H37Rv counterparts DprE1 and DprE2, respectively [Figure S1A,B in the Supporting Information (SI)].

Furthermore, mutation of Cys394 in DprE1$_{SM}$ (corresponding to Cys387 in DprE1 of *M. tuberculosis* H37Rv) results in resistance of *M. smegmatis* toward BTZs. DprE1$_{SM}$ and DprE2$_{SM}$ were expressed in *E. coli* and purified to >95% homogeneity (Figure S1C,D). DprE1$_{SM}$ was isolated to ~45% with bound flavin adenine dinucleotide (FAD), confirming its annotation as a FAD-dependent oxidoreductase (Figure S2). The activity of the purified DprE1$_{SM}$ and DprE2$_{SM}$ was confirmed by epimerization of decaprenylphosphoryl-[^{14}C]-β-D-ribofuranose (^{14}C-DPR; see the SI) to ^{14}C-DPA (Figure 2A, lane 1).

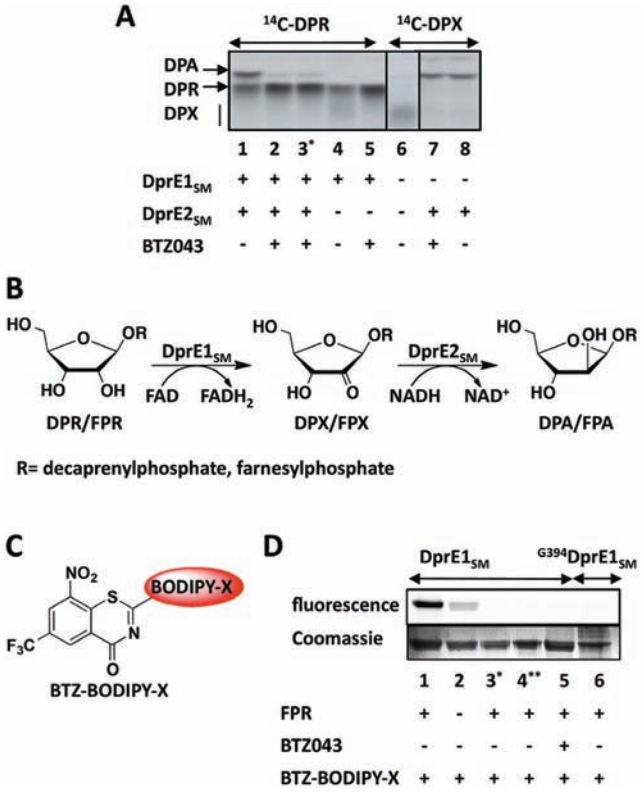

Figure 2. Characterization of DprE1$_{SM}$ and DprE2$_{SM}$ and their interactions with BTZ043. (A) TLC analysis of the conversion of ^{14}C-DPR or ^{14}C-DPX to ^{14}C-DPA by DprE1$_{SM}$ and/or DprE2$_{SM}$ in the presence or absence of BTZ043 (58 μM). All reactions were done at pH 7.9 in the presence of NAD$^+$ and NADH (both at 50 μM). Visualization of ^{14}C-labeled substrates was effected by means of autoradiography. Lane 2: Enzymes were incubated with BZT043 for 1 h before substrate was added. * denotes lane 3 as in lane 2 but without BTZ preincubation. (B) Functions of DprE1$_{SM}$ and DprE2$_{SM}$ in the epimerization of DPR (FPR) to DPA (FPA). (C) Structure of the fluorescent derivative BTZ-BODIPY-X. (D) Fluorescence labeling of DprE1$_{SM}$ and ^{G394}DprE1$_{SM}$ by BTZ-BODIPY-X (10 μM). Labeling was analyzed by SDS-PAGE and in-gel fluorescence scanning. Coomassie staining of the gel is shown for comparison. * denotes the use of heat-denatured DprE1$_{SM}$; ** denotes post-treatment with 20 mM DTT/7 M urea.

Epimerization required the presence of both proteins and nicotinamide adenine dinucleotide (NADH) as a cofactor (Figure S3A). In the absence of DprE2$_{SM}$, partial conversion of ^{14}C-DPR to ^{14}C-DPX was observed (Figure 2A, lane 4). Addition of NAD$^+$ or NADH was not required for the conversion of ^{14}C-DPR to ^{14}C-DPX by DprE1$_{SM}$ (Figure S3B). In the absence of DprE1$_{SM}$ and the presence of NADH, DprE2$_{SM}$ efficiently reduced ^{14}C-DPX to ^{14}C-DPA (Figure 2A, lane 8; Figure S3B, lane 4). Together, these data demonstrate (i) that DprE1$_{SM}$ oxidizes ^{14}C-DPR to ^{14}C-DPX using FAD as a cofactor, (ii) that DprE2$_{SM}$ reduces ^{14}C-DPX to ^{14}C-DPR using NADH, and (iii) that the two enzymes can function independently (Figure 2B). We then examined the effect of BTZ on the epimerization of ^{14}C-DPR by DprE1$_{SM}$ and DprE2$_{SM}$. BTZ043 inhibited the epimerization of ^{14}C-DPR into ^{14}C-DPA (Figure 2A, lanes 2 and 3). In contrast, BTZ did not inhibit the reduction of ^{14}C-DPX to ^{14}C-DPA by DprE2$_{SM}$ (Figure 2A, lane 7). Furthermore, inhibition of the partial conversion of ^{14}C-DPR into ^{14}C-DPX by BTZ was observed (Figure 2A, lane 5). These data suggest that BTZs target DprE1 without affecting the activity of DprE2$_{SM}$ and that DprE1$_{SM}$ is responsible for the activation of the drug.

The difficulties in producing sufficient amounts of ^{14}C-DPR as well as its analysis precluded its use in more detailed mechanistic studies. We found that farnesylphosphoryl-β-D-ribofuranose (FPR), which can be readily synthesized in greater quantities (see the SI), is an alternative substrate that is also epimerized by DprE1$_{SM}$ and DprE2$_{SM}$ (Figure S3C). Incubation of DprE1$_{SM}$ with FPR and analysis of the reaction mixture by HPLC and mass spectrometry (MS) permitted the isolation of a main reaction product with an exact mass of 431.1838 Da [M − H]. This corresponds to the mass of the 2-keto derivative farnesylphosphoryl-D-2′-keto-*erythro*-pentose (FPX) with a mass of 431.1835 Da [M − H] (Figure S3D). The data lend further support to an epimerization mechanism in which DprE1$_{SM}$, using FAD as a cofactor, independently oxidizes FPR or DPR to the corresponding D-2′-keto-*erythro*-pentose, which is then reduced by DprE2$_{SM}$ to FPA or DPA using NADH (Figure 2B).

For the detection of the proposed covalent BTZ−DprE1 adduct, we synthesized a fluorescently labeled BTZ derivative, BTZ-BODIPY-X (Figure 2C) (see the SI). Only weak fluorescent labeling of DprE1$_{SM}$ was observed upon incubation of DprE1$_{SM}$ with BTZ-BODIPY-X at 10 μM (Figure 2C, lane 2). However, the efficiency of the fluorescent labeling was increased about 8-fold upon addition of the DprE1$_{SM}$ substrate FPR to the reaction mixture (Figure 2D, lane 1). Furthermore, fluorescent labeling could be suppressed or reversed by (i) heat inactivation of DprE1$_{SM}$, (ii) prior incubation with BTZ043, or (iii) subsequent incubation with an excess of thiols under denaturing conditions (Figure 2D, lanes 3−5). Together, thesedata suggest that DprE1$_{SM}$ is capable of reducing BTZ-BODIPY-X to the corresponding electrophilic nitroso derivative, which then specifically reacts with DprE1$_{SM}$ to form a covalent complex. We assume that reduction of the nitro group of BTZ-BODIPY to the nitroso derivative requires the presence of FADH$_2$. This then explains the dependence of the labeling on the presence of FPR, which is needed to generate FADH$_2$. There is literature precedent for the reduction of nitro groups by FAD-dependent enzymes: bacterial oxygen-insensitive nitroreductases such as NfsA and NfsB from *E. coli* and NfnB from *M. smegmatis* use flavin as redox cofactor for the reduction of nitro-aromatic compounds.[8−10]

The reversal of the labeling by incubation of denatured, labeled DprE1$_{SM}$ with excess thiols is in agreement with the formation of a semimercaptal from the nitroso derivative of

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BTZ-BODIPY-X and Cys394 of $DprE1_{SM}$, as semimercaptals are labile to thiols.[11−13] To lend further support to the hypothesis that fluorescent labeling of $DprE1_{SM}$ occurs on Cys394, we prepared the mutant Cys394Gly ($^{G394}DprE1_{SM}$) and incubated it with BTZ-BODIPY-X and FPR. No significant labeling of $^{G394}DprE1_{SM}$ under these conditions could be detected. Finally, no fluorescent labeling of $DprE2_{SM}$ by BTZ-BODIPY-X could be detected (Figure S4).

To confirm the proposed formation of a semimercaptal between BTZ043 and $DprE1_{SM}$, we analyzed the covalent complex by MS. Analysis of the covalent drug−protein complex required its isolation under nonreducing conditions, and we showed previously that its MS analysis is facilitated by mutation of a second, nonessential cysteine residue, Cys136, to glycine in $DprE1_{SM}$ ($^{G136}DprE1_{SM}$). $^{G136}DprE1_{SM}$ was incubated with BTZ043 as well as FPR and after buffer exchange subjected to MS analysis. We detected a protein with a mass of 51 656 Da, which corresponds to the mass of 51 657 Da expected for the semimercaptal adduct, as well as unmodified $^{G136}DprE1_{SM}$ (Figure 3A). On the basis of peak intensities, the ratio of labeled $^{G136}DprE1_{SM}$ to unlabeled $^{G136}DprE1_{SM}$ was 3.5:1.

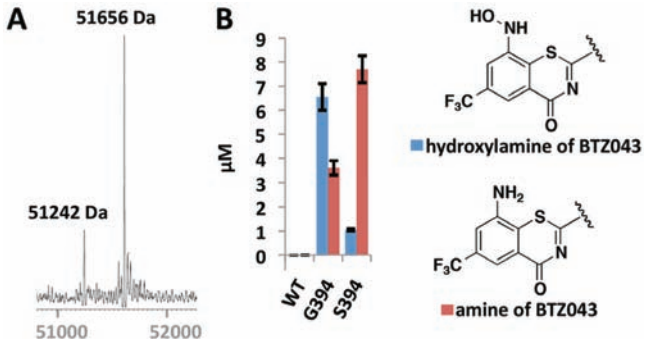

Figure 3. Interactions of $DprE1_{SM}$, $^{G136}DprE1_{SM}$, $^{G394}DprE1_{SM}$, and $^{S394}DprE1_{SM}$ with BTZ043. (A) MS of $^{G136}DprE1_{SM}$ after incubation with FPR (200 μM) and BTZ043 (20 μM) for 1 h at 37 °C. (B) Concentration of BTZ043 metabolites identified after incubation of $DprE1_{SM}$, $^{G394}DprE1_{SM}$, or $^{S394}DprE1_{SM}$ (5 μM) with BTZ043 (25 μM) in the presence of FPR (200 μM). Blue bars: hydroxylamine derivative of BTZ043; red bars: amine derivative of BTZ043. Error bars represent standard deviations of two independent experiments.

The incubation of semimercaptals with an excess of thiols is known to yield the corresponding hydroxylamines and amines.[11] We therefore analyzed the products resulting from incubation of the covalent BTZ043−$DprE1_{SM}$ complex with an excess of thiols. In agreement with the proposed formation of a semimercaptal between BTZ043 and $DprE1_{SM}$, we detected the hydroxylamine and amine metabolites of BTZ043 in a ratio of 1.2:1 (Figure S5B). Furthermore, ~0.72 equiv of BTZ metabolites was detected per molecule of $DprE1_{SM}$ (Figure S5B).

The $DprE1_{SM}$ mutations Cys394Gly and Cys394Ser result in resistance of *M. smegmatis* toward BTZ043,[4] and $^{G394}DprE1_{SM}$ is not modified by BTZ043 (Figure 2D). We therefore speculated that $^{G394}DprE1_{SM}$ and $^{S394}DprE1_{SM}$ could function as multiturnover nitroreductases. Overnight incubation of $^{G394}DprE1_{SM}$ or $^{S394}DprE1_{SM}$ with 25 μM BTZ043 and 200 μM FPR, subsequent protein precipitation, and analysis of the supernatant by HPLC resulted in the detection of the corresponding hydroxylamine and amine in yields of 4−30% (Figure 3B). For both mutants, no significant amounts of reduction products were detected in the absence of FPR (Figure S6). Furthermore, wild-type $DprE1_{SM}$ yielded no significant amount of either the amine or hydroxylamine under these conditions (Figure 3B). This indicates that the suicide inhibition of $DprE1_{SM}$ is highly efficient. A possible explanation for the observed difference between the product ratios for $^{G394}DprE1_{SM}$ and $^{S394}DprE1_{SM}$ might be attributable to mutations of the active-site residue Cys394 that affect the substrate specificity of $DprE1_{SM}$.

In summary, we have reported on the characterization of $DprE1_{SM}$ and $DprE2_{SM}$ from *M. smegmatis*, key enzymes for cell-wall biogenesis, and the inactivation of $DprE1_{SM}$ by BTZ043. The high degree of sequence identity of $DprE1_{SM}$ and $DprE2_{SM}$ with the orthologous proteins from *M. tuberculosis* and the fact that the same DprE1 mutations render both bacteria resistant to BTZs indicate that the mechanism of action of BTZs is identical in these two cases. To the best of our knowledge, BTZs represent the first example of suicide substrates that are activated through reduction of a nitro group (Figure 4). It is noteworthy that the trapping of the nitroso

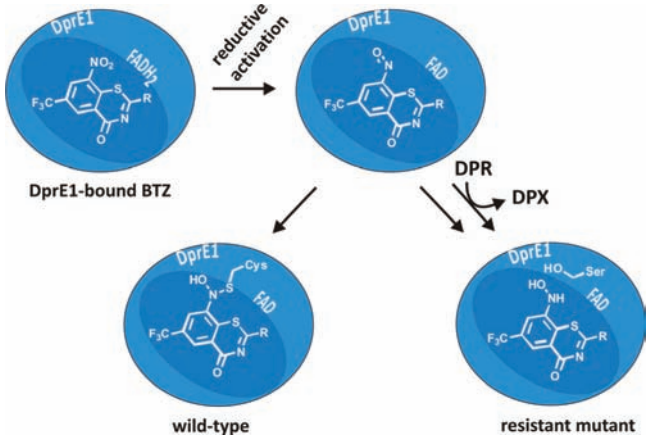

Figure 4. Mechanism of BTZ sensitivity and resistance. Reduction of the nitroso intermediate by BTZ-resistant DprE1 mutants requires regeneration of $FADH_2$, i.e., through oxidation of DPR to DPX.

derivative by the active-site residue of $DprE1_{SM}$ occurs in a near-quantitative manner, providing a rationale for the extremely low MIC of BTZ043. Our data thus provide detailed insights into the mechanisms of BTZ sensitivity and resistance and represent an important step in the development of BTZs as tuberculosis drugs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures and complete refs 4 and 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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