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Benzothiazinones: Prodrugs That Covalently Modify the Decaprenylphosphoryl-β-D-ribose 2′-epimerase DprE1 of *Mycobacterium tuberculosis*

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Abstract: Benzothiazinones (BTZs) form a new class of potent antimycobacterial agents. Although the target of BTZs has been identified as decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE1), their detailed mechanism of action remains obscure. Here we demonstrate that BTZs are activated in the bacterium by reduction of an essential nitro group to a nitroso derivative, which then specifically reacts with a cysteine residue in the active site of DprE1.

1,3-Benzothiazin-4-ones (benzothiazinones, BTZs; Figure 1a) are tuberculosis (TB) drug candidates with high activity against *Mycobacterium tuberculosis in vitro* and *in vivo*.[1] A minimal inhibitory concentration (MIC) of 1 ng/mL against *M. tuberculosis* H37Rv was measured for the most promising BTZ, BTZ043; this MIC is a factor of 20 below that of the frontline TB drug, isoniazid. Genetic and biochemical analysis revealed that the target of BTZs is the enzyme decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE1, Rv3790).[1] DprE1, together with DprE2 (Rv3791), catalyzes the epimerization of decaprenylphosphoryl-β-D-ribose (DPR) to decaprenylphosphoryl-β-D-arabinose (DPA; Figure 1b), which is a central precursor for the synthesis of cell-wall arabinans.[2,3] Due to difficulties in isolating active DprE1, details of the mechanism of action of BTZs are scarce. It is known that a single point mutation in DprE1 (Cys387Ser or Cys387Gly) results in a more than 250-fold increased MIC (Table 1).[1]

Furthermore, the nitro group of BTZs is essential for activity; its reduction to either the amine or the hydroxylamine increased the MIC at least 500-fold.[1] Following the discovery of BTZs, another class of compounds that target DprE1 was identified:[4] dinitrobenzamide DNB1 and its derivatives (Figure 1a). As observed for BTZs, point mutations of Cys387 of DprE1 resulted in resistance to DNB1, and reduction of one of the nitro groups to the amine or the hydroxylamine abolished activity.[4] These data suggest that BTZs and dinitrobenzamides operate by the same mechanism of action.

Both BTZs and the dinitrobenzamides are nitroaromatic compounds that contain an electron-withdrawing substituent in the position meta to the nitro group. Given the prevalence of such structures in organic chemistry, we searched the literature for related compounds with reported antimycobacterial activity and identified the xanthone derivative 1-methyl-2,4,7-trinitroxanthone (MTX; Figure 1a)[5] and another class of dinitrobenzene derivatives[6] (here abbreviated as DNDs; Figure 1a).

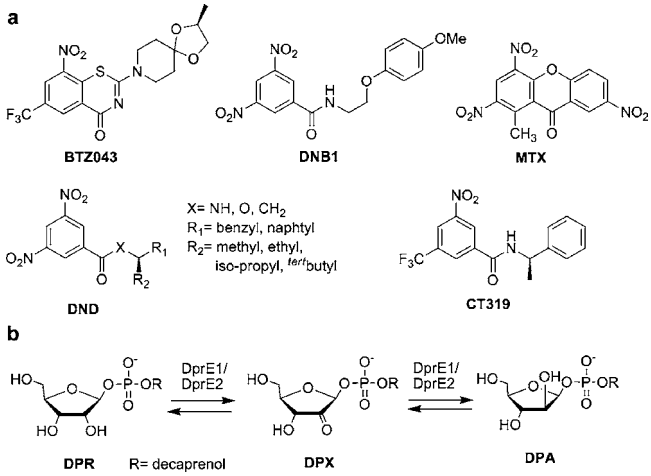

Figure 1. BTZs and DprE1. (a) BTZ043 and other compounds used in this study. (b) Epimerization of decaprenylphosphoryl-β-D-ribose (DPR) via decaprenylphosphoryl-2-keto-D-erythro-pentofuranose (DPX) to decaprenylphosphoryl-β-D-arabinose (DPA).[2] Both DprE1 and DprE2 are required for the epimerization of DPR.

Table 1. MIC Values of Compounds Used in This Study against Different Mycobacterial Strains and *M. smegmatis* mc²155 Overexpressing Different DprE1 Mutants

		MIC (μg/mL)		
strain	residue 387 in DprE1	BTZ043	MTX	CT319
M. tuberculosis H37Rv	Cys	0.001	0.125	0.31
M. bovis BCG	Cys	0.002	n.d.	0.15
M. bovis BCG BN2[a]	Ser	16	n.d.	>100
M. smegmatis mc²155	Cys	0.004	0.125	0.062
M. smegmatis MN47[a]	Gly	4	>100	>100
M. smegmatis MN84[a]	Ser	16	>100	>100
His-DprE1[b]	Cys	0.05	n.d.	7.81
His-^{G129}DprE1[b]	Cys	0.05	n.d.	7.81
His-^{G129,G387}DprE1[b]	Gly	12.5	n.d.	n.d.

[a] Strains described in ref 1. [b] Protein coexpressed with DprE2 in *M. smegmatis* mc²155. n.d., not determined.

We first tested the activity of MTX against BTZ-sensitive and BTZ-resistant mycobacteria and found that mutations in DprE1 also confer resistance to MTX (Table 1). This confirms our hypothesis that MTX and BTZ share the same mechanism of action. Comparing the structures of the reported DNDs with those of BTZs and dinitrobenzamides, we predicted that, in the DNDs, only one of the nitro groups is essential for antimycobacterial activity. We

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therefore synthesized CT319, a DND derivative in which one of the nitro groups is replaced by a trifluoromethyl group (Figure 1a; see also Supporting Information), and measured its activity against different mycobacteria (Table 1). As predicted, CT319 showed high activity against *M. smegmatis* and *M. tuberculosis* H37Rv but no significant activity against BTZ-resistant strains with the mutations Cys387Ser or Cys387Gly in DprE1. Furthermore, reduction of the nitro group to the amine abolished all antimycobacterial activity (compound CT318; Supporting Information). These data reveal that CT319, MTX, BTZs, and DNBs, despite their structural diversity, share a common mechanism of action and thereby constitute a new family of antimycobacterial agents.

Electron-deficient nitroaromatic compounds are known to be readily reduced in biological systems, yielding first the corresponding nitrosoarene, which is subsequently further reduced to the corresponding hydroxylamine and then to the amine.[7] Accordingly, it has been observed that BTZs are reduced to the corresponding amines in bacteria and mice.[1] Nitrosoarenes are electrophiles that readily react with thiols to form semimercaptals (Figure 2a)[8−10] and also can covalently modify cysteine residues in proteins.[11,12] In the absence of an excess of thiols, the semimercaptals generated from electron-deficient nitrosoarenes are relatively stable and only slowly rearrange to the corresponding sulfinamide.[9] As the mutation of Cys387 in DprE1 leads to BTZ resistance, we speculated that the nitroso derivatives of BTZs or related compounds form a semimercaptal with Cys387 (Figure 2b). To test this hypothesis, we first attempted to study the reaction of a nitroso derivative of BTZs with thiols. While the preparation of the nitroso derivative of BTZ043 proved to be difficult, we succeeded in preparing the nitroso derivative of CT319, CT325, and analyzed its reaction with glutathione (Figure S1 and discussion in the Supporting Information). Mixing equimolar solutions of glutathione and CT325 resulted in the immediate and almost quantitative formation of the corresponding semimercaptal (Figure S1 and discussion in the Supporting Information). Neither CT319 nor the amine CT318 reacted with glutathione under these conditions.

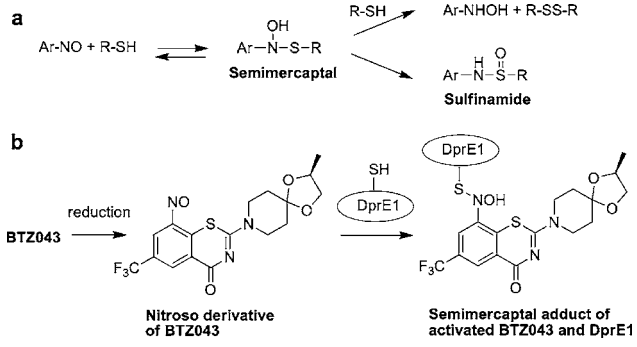

Figure 2. Nitrosoarenes and thiols. (a) Reaction of nitrosoarenes (Ar−NO) with thiols (R−SH). The semimercaptal can react with another thiol to form the hydroxylamine or rearrange to the sulfinamide. (b) Proposed mechanism of action of BTZs.

To investigate if the nitroso derivative of BTZ043 specifically reacts with Cys387 of DprE1 in cells, we envisioned incubating *M. smegmatis* coexpressing DprE1 and DprE2 from *M. tuberculosis* with BTZ043 and isolating the protein for further analysis. Overexpression of DprE1 and DprE2 from *M. tuberculosis* in *M. smegmatis* mc^{2}155 results in a decreased sensitivity relative to wild-type *M. smegmatis* mc^{2}155.[1] The decreased sensitivity is due to an increased concentration of the drug target, represented by overexpressed DprE1 and DprE2 from *M. tuberculosis* and the natively present orthologous DprE1 and DprE2 from *M. smegmatis*.[1] As our attempts to purify native DprE1 failed, we expressed DprE1 with an N-terminal His-tag (His-DprE1) and purified the protein under denaturing conditions. In addition to Cys387, His-DprE1 possesses another cysteine, Cys129. The relatively low stability of the proposed semimercaptal formed between BTZs and DprE1 required its isolation in the absence of thiols (*vide infra*), and the mutation of Cys129 to glycine facilitated experiments under such conditions (Supporting Information). Coexpression of either His-^{G129}DprE1 or His-DprE1 with DprE2 in *M. smegmatis* decreased the sensitivity toward BTZ043 relative to wild-type *M. smegmatis* to the same extent (Table 1), indicating that His-^{G129}DprE1 from *M. tuberculosis* is functional in *M. smegmatis*. *M. smegmatis* strains coexpressing His-^{G129}DprE1 and DprE2 were incubated for 4 h with low concentrations of BTZ043 (0.5 μg/mL) and subsequently lysed in the presence of 8 M urea and purified by affinity chromatography. His-DprE1 was isolated, together with GroEL1 as a major contaminant (Figure 3a). Subsequent analysis by mass spectrometry permitted the detection of a protein with a mass of 51 786 Da (Figure 3b), which corresponds to a semimercaptal formed from the nitroso derivative of BTZ043 and His-^{G129}DprE1 (calculated mass of 51 786.6 Da). Reducing the sample prior to mass analysis using DTT permitted only the detection of unmodified His-^{G129}DprE1 (Figure 3b). The sensitivity of the enzyme−drug adduct toward thiols is in agreement with the known reactivity of semimercaptals.[9] When the experiment was repeated without incubation of the mycobacteria with BTZ043 and without reduction of the sample prior to analysis by mass spectrometry, His-^{G129}DprE1 could not be detected (Figure 3b). We assume that oxidation of unmodified Cys387 of His-^{G129}DprE1 during lysis and purification prevents the detection of His-^{G129}DprE1 by mass spectrometry. The significance of this experiment is that the failure to detect unmodified His-^{G129}DprE1 in the presence of BTZ043-modified His-^{G129}DprE1 therefore cannot be interpreted as evidence for a complete labeling of His-^{G129}DprE1. We furthermore incubated *M. smegmatis* coexpressing His-^{G129}DprE1 and DprE2 with four BTZ derivatives with masses different than BTZ043, and in each case a protein with the mass of the adduct between His-^{G129}DprE1 and the corresponding nitroso derivative was detected (Table S4 and Figures S3−S6). In comparison, the copurified GroEL1, which does not possess a cysteine residue, was not modified by any of the BTZ derivatives and was detected in the presence and in the absence of thiols (Figure 3b). To demonstrate that the observed labeling depends on Cys387, we mutated Cys387 in His-^{G129}DprE1 to glycine, yielding His-^{G129,G387}DprE1. Coexpression of His-^{G129,G387}DprE1 with DprE2 in *M. smegmatis* results in high-level resistance to BTZ043 (Table 1), indicating that also His-^{G129,G387}DprE1 from *M. tuberculosis* is functional. Analysis of His-^{G129,G387}DprE1 by mass spectrometry after incubation of *M. smegmatis* with BTZ043 as described above allowed only the detection of unmodified His-^{G129,G387}DprE1 (Figure 3b). Together, these experiments support our hypothesis of the formation of a semimercaptal between Cys387 and a BTZ nitroso derivative. Furthermore, they reveal a remarkable efficiency of this reaction inside the bacterium.

To independently confirm the observed covalent modification of DprE1 by BTZs, we synthesized a ^{14}C-labeled BTZ derivative (BTZ003; Figure 3c and Supporting Information). *M. smegmatis* strains coexpressing His-^{G129}DprE1 and DprE2 were incubated with radioactive BTZ003, and His-^{G129}DprE1 was purified as described above. The degree of labeling of His-^{G129}DprE1 with BTZ003 after purification was determined to be 25% (Figure 3c and Supporting Information). When these experiments were repeated with His-^{G129,G387}DprE1, the degree of labeling of His-^{G129,G387}DprE1 was 20-fold lower than that of His-^{G129}DprE1 (Figure 3c).

DprE1 belongs to the family of vanillyl alcohol oxidases (VAO), a family of flavin adenine dinucleotide (FAD)-dependent oxidoreductases consisting of an FAD-binding domain and a substrate-binding domain.[13]

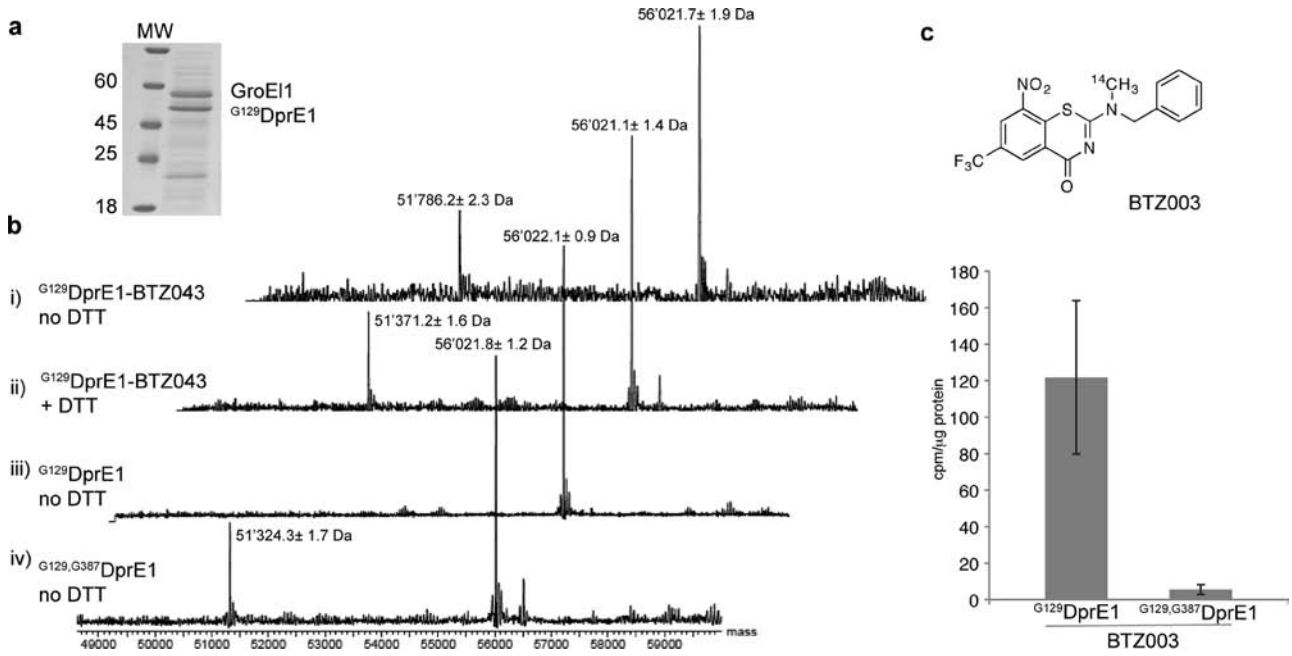

Figure 3. Covalent binding of BTZs to DprE1. (a) SDS−PAGE of isolated His-^{G129}DprE1. *M. smegmatis* strains coexpressing His-^{G129}DprE1 and DprE2 were incubated for 4 h with BTZ043 (0.5 μg/mL), lysed in buffer containing 8 M urea, and His-^{G129}DprE1 was purified by Ni-NTA affinity chromatography. The copurification of GroEL1 is due to a string of histidine residues naturally occurring at its C terminus. Proteins were stained with Coomassie blue. (b) Mass spectrometric analysis of different DprE1 samples: (i) sample as described in (a); (ii) sample as described in (a) but analyzed after incubation with 10 mM dithiothreitol (DTT); (iii) sample as described in (a) but without BTZ043 incubation; (iv) sample as described in (a) but using *M. smegmatis* expressing His-^{G129,G387}DprE1. Samples were analyzed on a Q-TOF Ultima spectrometer (Waters). Calculated masses: His-^{G129}DprE1, 51 371.4 Da; His-^{G129}DprE1-BTZ043 semimercaptal, 51 786.6 Da; His-^{G129,G387}DprE1, 51 325.4; GroEL1, 56 021.3 Da. (c) Quantification of radioactive labeling of His-^{G129}DprE1 and His-^{G129,G387}DprE1 after incubation of *M. smegmatis* expressing either His-^{G129}DprE1 or His-^{G129,G387}DprE1 with ^{14}C-labeled BTZ003 and purification of the protein as described in (a).

Among the enzymes of this family with known structure, alditol oxidase possesses the highest sequence identity to DprE1 (18%).[14] To gain further insight into the interaction of BTZs with DprE1, we built a structural model of DprE1 by homology modeling (Supporting Information). In our model, Cys387 points into the substrate binding pocket of DprE1 (Figure S2). Thus, modification of Cys387 should block the substrate binding site of the enzyme. The mechanism of action of BTZs proposed also raises the question of the activation of the prodrug inside the mycobacterium. One possibility would be that the nitroso derivative is generated through reduction of BTZ by an unknown enzyme and then reacts with DprE1. Alternatively, DprE1 itself might reduce BTZ to the nitroso derivative, which then directly undergoes a reaction with Cys387. This hypothesis is supported by the observation that bacterial oxygen-insensitive nitroreductases are also flavin-dependent enzymes.[15] More experiments are needed to characterize the activation and to explain the remarkable specificity of BTZs, but it is noteworthy that another exciting TB drug candidate, the bicyclic nitroimidazole PA-824,[16,17] is also a prodrug that is activated by reduction of an essential nitro group. In the case of PA-824, the mechanism of action is based on the resulting intracellular release of nitric oxide,[17] and DprE1 is not a target (our unpublished data).

In summary, BTZs and other electron-deficient nitroaromatic compounds form a new family of antimycobacterial prodrugs defined by a unique mechanism of action. The mechanistic insights presented here represent an important step in the further development of BTZs as TB drug candidates.

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Supporting Information Available: Detailed description of experiments, additional analytical data, and complete refs 1 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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