Inhibition of virulence-promoting disulfide bond formation enzyme DsbB is blocked by mutating residues in two distinct regions

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Disulfide bonds contribute to protein stability, activity, and folding in a variety of proteins, including many involved in bacterial virulence such as toxins, adhesins, flagella, and pili, among others. Therefore, inhibitors of disulfide bond formation enzymes could have profound effects on pathogen virulence. In the Escherichia coli disulfide bond formation pathway, the periplasmic protein DsbA introduces disulfide bonds into substrates, and then the cytoplasmic membrane protein DsbB reoxidizes DsbA’s cysteines regenerating its activity. Thus, DsbB generates a protein disulfide bond de novo by transferring electrons to the quinone pool. We previously identified an effective pyridazinone-related inhibitor of DsbB enzymes from several Gram-negative bacteria. To map the protein residues that are important for the interaction with this inhibitor, we randomly mutagenized by error-prone PCR the E. coli dsbB gene and selected dsbB mutants that confer resistance to this drug using two approaches. We characterized in vivo and in vitro some of these mutants that map to two areas in the structure of DsbB, one located between the two first transmembrane segments where the quinone ring binds and the other located in the second periplasmic loop of DsbB, which interacts with DsbA. In addition, we show that a mutant version of a protein involved in lipopolysaccharide assembly, LptDAsv, is synthetically lethal with the deletion of dsbB as well as with DsbB inhibitors. This finding suggests that drugs decreasing LptD assembly may be synthetically lethal with inhibitors of the Dsb pathway, potentiating the antibiotic effects.

Protein disulfide bonds are sulfur-sulfur chemical bonds that result from an oxidative process in which two electrons are removed from a protein, linking non-adjacent cysteines of the protein. Disulfide bonds contribute to protein stability, activity, and folding (1, 2). In bacteria, proteins containing structural disulfide bonds are rarely, if at all, found in cytoplasmic compartments; they are usually present in the cell envelope or the extracellular milieu (1). Many proteins involved in bacterial virulence (such as toxins, adhesins, flagella, fimbriae, pili, and types II and III secretion systems) require disulfide bonds (3). Pathways involved in catalyzing disulfide bond formation are therefore attractive targets for identifying small molecule inhibitors, because loss of such systems can undermine the activity of numerous bacterial virulence factors as do null mutations of the genes for these enzymes (4–11).

The enzymes that promote formation of protein disulfide bonds in Gram-negative bacteria are in the cell envelope. The periplasmic enzyme DsbA, a member of the thioredoxin family, oxidizes pairs of cysteines in substrate proteins through its Cys-Xaa-Xaa-Cys active site (12). The resulting reduced DsbA is reoxidized by the cytoplasmic membrane protein DsbB, regenerating DsbA’s activity (13). DsbB itself is reoxidized by membrane-embedded quinones, from which electrons are transferred to the electron transport chain (14). However, in many of the Actinobacteria and Cyanobacteria the membrane protein VKOR (vitamin K epoxide reductase) instead of DsbB is required for the reoxidization of DsbA (15). Although VKOR has no overall amino acid sequence homology with DsbB, both proteins encode two extracytoplasmic soluble domains containing essential pairs of cysteines and are capable of reoxidizing DsbA fundamentally by the same mechanism (15, 16).

We have previously generated a methodology for identifying specific inhibitors of both bacterial DsbBs and a VKOR that is based on the functional homology between the two proteins (17). The assay for inhibition of disulfide bond formation utilizes a disulfide-sensitive β-galactosidase (β-GalAsvä)4 assay. This approach allowed us to identify inhibitors of either enzyme by a single high-throughput screening procedure. By this approach, we have found a family of pyridazinone-related molecules that are effective inhibitors of DsbB proteins of sev-

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eral Gram-negative bacteria but do not inhibit a bacterial VKOR.

Because we have sought to develop DsbB inhibitors as anti-

virulents/antibiotics, we wanted to understand how resistance
to these compounds might arise in vivo as well as how pyridazi-

nones inhibit DsbB. To this end, we report here mutants of

DsbB that confer resistance to that inhibition. We used two

methods for direct selection of spontaneous mutants of DsbB

resistant to one of the strongest pyridazinone inhibitors. These

selections have failed to yield any mutants that have altered

DsbB. However, when we randomly mutagenize by error-prone

PCR a dsbB gene, which is carried on a high copy number plas-

mid, DsbB mutants resistant to our inhibitors can be isolated.

Characterization of these DsbB mutant proteins shows that

they all exhibit lower affinity toward ubiquinone and menadi-

one, and two of them show higher turnover numbers. Our stud-

ies suggest that resistance of DsbB to pyridazinone inhibitors is
difficult to obtain by spontaneous selections perhaps due to the

effects of inhibitor-resistant mutations on the normal function-

ing of DsbB. The location within the DsbB protein of the amino

acid changes that do confer resistance provides suggestions as
to the mechanism of inhibition and regions of the protein that

influence quinone binding.

Results

Selection of mutations that confer resistance to compound 12,
a pyridazinone inhibitor of DsbB

We have developed a genetic selection for mutants resistant
to inhibitors of DsbB that uses an Escherichia coli strain further

sensitized to the inhibitor by the presence of an additional

mutation (lptD4213). In Gram-negative bacteria, a set of lpt

genes encodes proteins required for the transport and assembly

of lipopolysaccharides (LPS) into the outer leaflet of the outer

membrane. LptD is an outer membrane β-barrel protein, which requires
two disulfide bonds for its proper assembly and function, and it

is involved in the last steps of LPS assembly (Fig. 1A) (18).
Despite the essentiality of LptD, strains lacking a functional disulfide bond formation pathway remain viable under aerobic conditions presumably because background oxidation can lead to sufficient spontaneous disulfide bond formation in LptD and other essential proteins (19). We considered the possibility that a strain carrying the \textit{lptD} \textit{dsbB} mutant allele (\textit{lptD} \textit{dsbB}) \textit{CL380} strain encoding the \textit{lptD} \textit{dsbB} allele (CL337 strain). We then attempted to transduce a deletion of the genomic copy of \textit{dsbB} into the \textit{lptD} \textit{dsbB} strain. Although \textit{ΔdsbB} transductants were readily obtained when a second copy of \textit{dsbB} was present, we were not able to isolate transductants in the strain encoding only one copy of \textit{dsbB} unless cystine was added to the medium, which yielded a lower frequency than having two \textit{dsbB} copies. Cystine is an oxidant that can mediate disulfide bond formation in the absence of the disulfide bond formation pathway (13). Furthermore, the strain carrying the plasmid with \textit{dsbB} under an arabinose promoter (\textit{lptD} \textit{dsbB} \textit{PBAD} \textit{CL380} strain) was able to grow on LB, although it did not grow on minimal media unless 0.2% arabinose was added (Fig. 1B). Thus, \textit{dsbB} and \textit{lptD} \textit{dsbB} are synthetically lethal combinations.

We then asked whether the \textit{lptD} \textit{dsbB} mutant strain was sensitive to a particularly potent \textit{E. coli} \textit{dsbB} inhibitor, compound 12 (4,5-dichloro-2-[2-chlorophenyl]methylpiridazin-3-one, Fig. 1C). We have shown that compound 12 forms a covalent bond with the second cysteine of \textit{dsbB} (Cys-44) (17). Ordinarily, quinones, the direct source of oxidation of \textit{dsbB}, form a charge-transfer complex with Cys-44 of \textit{dsbB} during the process of electron transfer between \textit{DsbA} and \textit{DsbB} (25). We have proposed that the inhibition of \textit{DsbB} activity by pyridazinone compounds, including compound 12, results from the competition with quinone for the quinone-binding site leading to the covalent reaction with Cys-44, thus inactivating the protein (17). We observed that, unlike the strain with wild type \textit{lptD}, the \textit{lptD} \textit{dsbB} strain was highly sensitive to compound 12 as demonstrated by the inhibition of growth in a concentration-dependent manner (black circles, Fig. 1D). Because we have demonstrated that compound 12 targets \textit{DsbB} (by interfering with \textit{dsbB} reduction, exhibits a decrease in motility and an increase in the disulfide bond-sensitive β-galactosidase) (17), these data also indicated that the combination of the \textit{lptD} \textit{dsbB} and \textit{dsbB} inhibition results in a synthetic lethal interaction. In addition, the conditionally lethal strain \textit{lptD} \textit{dsbB} \textit{dsbB} \textit{PBAD} was even more sensitive to compound 12 when no arabinose was present in liquid minimal media where disulfide bond formation is partly dependent on air oxidation (red circles, Fig. 1D).

The findings above suggested that selecting for growth of a strain that contains the \textit{lptD} \textit{dsbB} allele and is exposed to the \textit{DsbB} inhibitor, compound 12, could yield inhibitor-resistant mutants. We therefore plated the \textit{lptD} \textit{dsbB} strain on M63 minimal media with 10 μM compound 12, a concentration of drug ~10-fold higher than the minimal inhibitory concentration (MIC). Although mutants were obtained at a very low frequency (~10^{-9}) when exposed to the compound, none of them mapped to the \textit{dsbB} gene but rather to the gene \textit{bamB}. Twenty two of 51 colonies analyzed by PCR yielded a larger than expected product for the \textit{bamB} region, and the sequence of all these indicated an insertion of an IS1 element in the gene. Whole-genome sequencing was performed in three of the colonies in which the \textit{bamB} product was similar in size to wild type. Two of these encoded mutations within \textit{bamB} (BamB_{1240} and BamB_{252-255}). Both mutations, \textit{bamB}:SI1 and BamB_{252-255} are known to be loss of function mutants of BamB that confer similar phenotypes (22). Therefore, these mutations most likely inactivated the outer membrane lipoprotein BamB, a scenario known to bypass the assembly defect of \textit{lptD} \textit{dsbB} (22, 26–28).

**Selection of mutations that confer resistance to compound 12 by PCR mutagenesis of the \textit{dsbB} gene**

Because our initial selection for \textit{dsbB} mutants resistant to compound 12 did not yield any mutations in that gene, we decided to use the same \textit{lptD} \textit{dsbB} strain to select for mutants resistant to compound 12 using a randomly mutagenized \textit{dsbB} library. To do this, we mutagenized \textit{dsbB} via error-prone PCR and cloned the resultant PCR products into a plasmid in which \textit{dsbB} expression is under the control of an IPTG-inducible promoter. This pool of plasmids was then transformed into the conditionally lethal strain \textit{lptD} \textit{dsbB} \textit{dsbB} \textit{PBAD} selecting for the presence of the plasmid using the antibiotic marker. The transformation yielded ~4,500 independent colonies carrying both a plasmid with an arabinose-inducible wild type \textit{dsbB} and a plasmid with an IPTG-inducible mutated \textit{dsbB}. The colonies were scraped up, pooled together, and plated on selection plates of M63 glucose with 10 μM compound 12, which is ~10-fold higher than the MIC observed for the strain carrying the two plasmids expressing wild type \textit{dsbB}. Because glucose represses transcription of wild type \textit{dsbB} from the \textit{PBAD} promoter, these conditions select for resistant \textit{DsbB}s expressed from the mutant library. We obtained 20 colonies and sequenced only the mutagenized \textit{dsbB} gene from the IPTG-inducible plasmid (see “Materials and methods”). We found that 9 of 20 colonies (45%) had mutations in \textit{dsbB} (Fig. 2A) and 6 of these 9 colonies encoded a change of Leu-25 to Pro in combination with a second change of Cys-44 to Pro (see “Materials and methods”). We found that 9 of 20 colonies (45%) had mutations in \textit{dsbB} (Fig. 2A) and 6 of these 9 colonies encoded a change of Leu-25 to Pro in combination with a second change of Cys-44 to Pro (see “Materials and methods”). We found that 9 of 20 colonies (45%) had mutations in \textit{dsbB} (Fig. 2A) and 6 of these 9 colonies encoded a change of Leu-25 to Pro in combination with a second change of Cys-44 to Pro (see “Materials and methods”).
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- Compound 12 inhibits anaerobic growth of an E. coli wild type strain at 1 μM (17). We therefore sought to isolate mutants resistant to this inhibitor using a selection for anaerobic growth in the presence of 2 μM compound 12. We again observed that spontaneous resistant mutations arose at a very low frequency (<10⁻⁷), and none of them mapped to the dsbB gene. However, whole-genome sequencing of four of these resistant mutants indicated that all of them encoded mutations in the gene encoding thioredoxin reductase (TrxBP16L, TrxBD287Y, TrxBS143F, and TrxB231–236, V237I). TrxB is a critical component in the disulfide bond isomerization pathway, and mutations in this pathway have been shown to partially restore disulfide bond formation (30).

We again made use of the same library of plasmids containing the PCR-mutagenized dsbB and transformed them into an E. coli dsbB strain, selecting aerobically for the presence of the plasmid using the antibiotic marker. The transformation yielded ~3,000 independent colonies. This mutant pool was plated anaerobically on solid media containing M63 glucose with 40 mM fumarate, 2 μM compound 12 and solidified with 1% agarose. This concentration of compound 12 is twice the MIC normally seen under these conditions. From this selection, we isolated 82 resistant colonies and sequenced the dsbB gene of each (Fig. 2A). Most (92%) encoded mutant dsbB alleles. The most frequently isolated mutation was DsbBL25P similar to our lptD selection, which could indicate a mutational hot spot that caused enrichment for this mutant in our library or a more effective resistance.

Characterization of five DsbB mutants

We observed that the mutations encoding resistance to compound 12 localized to two regions in the structure of DsbB, the quinone-binding site in the region of the first two transmembrane helices of DsbB and a segment of the periplasmic loop of the protein that interacts with DsbA during DsbA-DsbB complex formation (Fig. 2B).

We selected five of the mutants to study further (Fig. 3A) as follows: L25P (which was found in two different selections), A29V, K39E, P100S, and F106L, which included alterations of the two distinct regions, i.e. near the cysteines that bind to quinone and near the cysteines that bind to DsbA located in the periplasmic loop. We assessed the DsbB levels in the mutants to verify that the resistance to the drug was not due to an increased amount of DsbB. Four of the five mutants showed no difference in the amount of DsbB expression when 1 mM IPTG is added (Fig. 2C). The K39E mutant exhibited a 2-fold increase in DsbB levels for reasons that are not clear.

To gain insights into the resistance displayed by DsbB mutants, we purified the proteins and analyzed their enzyme kinetics using an ubiquinone reduction assay (31). We observed that although the affinity toward ubiquinone (Km) of the wild do not grow. Compound 12 inhibits anaerobic growth of an E. coli wild type strain at 1 μM (17). We therefore sought to isolate mutants resistant to this inhibitor using a selection for anaerobic growth in the presence of 2 μM compound 12. We again observed that spontaneous resistant mutations arose at a very low frequency (~10⁻⁷), and none of them mapped to the dsbB gene. However, whole-genome sequencing of four of these resistant mutants indicated that all of them encoded mutations in the gene encoding thioredoxin reductase (TrxBP16L, TrxBD287Y, TrxBS143F, and TrxB231–236, V237I). TrxB is a critical component in the disulfide bond isomerization pathway, and mutations in this pathway have been shown to partially restore disulfide bond formation (30).

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We also measured the inhibition of DsbB by compound 12 using an in vitro assay with purified components in the ubiquinone reduction assay (Table 1, 8th column). DsbB_{A29V} displayed a 50-fold increase in the IC_{50}, whereas DsbB_{L25P} and DsbB_{K39E} showed a 5- and 2-fold increase, respectively, under saturating concentrations of ubiquinone and DsbA. Under these conditions, neither DsbB_{P100S} nor DsbB_{F106L} showed an increase in the IC_{50} (see under “Discussion”).

Mutations isolated anaerobically conferred resistance aerobically to LptD_{4213} strain

We then asked whether the DsbB mutants obtained in the anaerobic selection also conferred resistance when tested in our aerobic model using the LptD_{4213} strain. We transformed the IPTG-inducible dbsB mutant plasmids obtained anaerobically into the lptD_{4213}ΔdbsB strain carrying a plasmid with an arabinose-inducible wild type dbsB. We then determined...


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Table 1

| Mutant          | Ubiquinone | Menadione | Ubiquinone | Menadione | Ubiquinone | Menadione | Ubiquinone | Menadione |
|-----------------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|
|                 | $K_{\text{cat}}$ | $K_{m}$ | $K_{\text{cat}}$ | $K_{m}$ | $K_{\text{cat}}$ | $K_{m}$ | $K_{\text{cat}}$ | $K_{m}$ |
| DsbB<sub>WT</sub> | 2.8 ± 0.07 | 1.9 ± 0.05 | 0.94 ± 0.1 | 35.8 ± 2.8 | 2.99 | 5.3 × 10<sup>-2</sup> | 0.033 (0.029–0.039) | 1.14 (1.09–1.18) |
| DsbB<sub>ΔH11003</sub> | 2 ± 0.03 | 0.54 ± 0.05 | 2.3 ± 0.1 | 174 ± 25 | 0.88 | 0.31 ± 10<sup>-2</sup> | 0.173 (0.157–0.192) | 4.06 (3.84–5.52) |
| DsbB<sub>ΔH11006</sub> | 3 ± 0.2 | 1.4 ± 0.05 | 10.8 ± 1.5 | 90.5 ± 6.5 | 0.28 | 1.5 × 10<sup>-2</sup> | 1.697 (1.54–1.79) | 1.47 (1.39–1.56) |
| DsbB<sub>ΔH11007</sub> | 5.5 ± 0.2 | 0.8 ± 0.1 | 3 ± 0.4 | 201 ± 38 | 1.81 | 0.39 × 10<sup>-2</sup> | 0.071 (0.065–0.079) | 3.01 (2.75–3.30) |
| DsbB<sub>ΔH11009</sub> | 2.1 ± 0.06 | 0.66 ± 0.03 | 3.6 ± 0.4 | 47 ± 5.1 | 0.58 | 1.4 × 10<sup>-2</sup> | 0.033 (0.030–0.037) | 2.01 (1.93–2.1) |
| DsbB<sub>ΔH11016L</sub> | 6.2 ± 0.09 | 0.69 ± 0.08 | 3.6 ± 0.2 | 61.8 ± 15 | 1.73 | 1.1 × 10<sup>-2</sup> | 0.035 (0.049–0.063) | 1.2 (1.05–1.37) |

<sup>a</sup> $K_{\text{cat}}$ expressed as nanomoles of ubiquinone-1 or menadione per nmol of DsbB per s.

<sup>b</sup> $K_{m}$ values represent ubiquinone-1 or menadione concentrations.

<sup>c</sup> In vitro inhibition was measured using 10 nM DsbB, 10 μM ubiquinone-1, and 20 μM reduced DsbB.

<sup>d</sup> In vivo inhibition was measured by growth inhibition of strain ltpD<sub>ΔdsbB</sub>dsbBP<sub>ΔH11004</sub> (CL409 – 410, CL416 – 417, and L118 –19 strains) in the presence of drugs.

whether these mutants were able to support growth of ltpD<sub>ΔdsbB</sub> strain by curing the plasmid encoding the arabinose-inducible wild type dsbB (see “Materials and methods”). All DsbB mutants were able to support growth of ltpD<sub>ΔdsbB</sub> strain indicating that the mutants selected anaerobically are also functional aerobically. These strains were then tested for growth in the presence or absence of compound 12 in minimal medium. The results are shown in Table 1 (9th column). DsbB<sub>L25P</sub> and DsbB<sub>K39E</sub> exhibited a 3–4-fold increase in the IC<sub>50</sub> for growth in the presence or absence of compound 12 in minimal medium. The results are shown in Table 1 (9th column).

In the oxidation pathway that introduces disulfide bonds into proteins in the bacterial periplasm, DsbA cysteines need to be reoxidized to start a new catalytic cycle. The cytoplasmic membrane protein DsbB performs this task. DsbB is a cellular machine that generates a protein disulfide bond de novo at the expense of electrons to be transferred to ubiquinone (14, 32). During the transfer and interaction of DsbA with DsbB, the latter undergoes conformational changes (29). In this work, we have selected DsbB mutants that confer resistance to a pyridazinone inhibitor and are located in two prominent areas in the structure of DsbB, one located between the two first transmembrane segments where the quinone ring fits. These mutants, DsbB<sub>L25P</sub>, DsbB<sub>A29V</sub> and DsbB<sub>F106L</sub> showed an almost 2-fold increase in the IC<sub>50</sub> for at least two of the inhibitors tested (Table 4). Thus, we observed at least some level of cross-resistance to pyridazinones for all DsbB mutants.

Discussion

In the oxidation pathway that introduces disulfide bonds into proteins in the bacterial periplasm, DsbA cysteines need to be reoxidized to start a new catalytic cycle. The cytoplasmic membrane protein DsbB performs this task. DsbB is a cellular machine that generates a protein disulfide bond de novo at the expense of electrons to be transferred to ubiquinone (14, 32). During the transfer and interaction of DsbA with DsbB, the latter undergoes conformational changes (29). In this work, we have selected DsbB mutants that confer resistance to a pyridazinone inhibitor and are located in two prominent areas in the structure of DsbB, one located between the two first transmembrane segments where the quinone ring fits. These mutants, DsbB<sub>L25P</sub>, DsbB<sub>A29V</sub> and DsbB<sub>F106L</sub> show a higher $K_{m}$ value for quinones as one might expect given that they are in the region of the quinone binding (Fig. 2B). Surprisingly, the other area is located in the second periplasmic loop of DsbB known to interact with DsbA. It has been shown that this segment from Pro-100 to Phe-106 is accommodated deep in the hydrophobic groove of DsaA’s structure (29, 33). The fact that we find mutants in this region and that DsbB<sub>P100S</sub> and DsbB<sub>F106L</sub> mutants exhibit an increase in the $K_{m}$ value for quinone despite not being located within the quinone-binding site suggests that this region also shapes the DsbB-quinone interaction. This model is in agreement with the fact that this segment of DsbB has to be mobile because it contains the Cys-104 residue that must to be mobile because it contains the Cys-104 residue that participates in the exchange of disulfides with Cys-41–Cys-44 of DsbA and oxidize DsbA (34).

Levels of DsbB were assessed in the mutants to demonstrate that the resistance to compound 12 is not due to changes in DsbB amount (Fig. 2C). Although levels remained unchanged
in four of the five mutants, due to unknown reasons DsbB<sub>K39E</sub> showed a 2-fold increase in DsbB. Although we cannot rule out the possibility that the increase in DsbB levels may contribute to the resistance of DsbB<sub>K39E</sub>, the purified mutant displayed significantly different kinetics than the wild type enzyme, suggesting that the resistance conferred by the mutation is at least partially due to its effects on enzyme activity. This study highlights the decrease in quinone affinity rendering the mutants less susceptible to inhibition. The mutations may selectively inhibit access of the compound to Cys-44 while allowing limited passage of quinone. However, two mutants DsbB<sub>K39E</sub> and DsbB<sub>F106L</sub> also show an increase in \( k_{cat} \) implying that the reac-

### TABLE 2

| ID Number | Structure | Inhibition Ratio (\( IC_{50 \text{ Compound} 12/IC_{50 \text{ Compound} 4} \)) | \( IC_{50} \) Compound 12 (M) |
|-----------|-----------|-------------------------------------------------|-------------------------------|
| 36 (G1-4) | ![](structure.png) | 6.4 | 121 |
| 12        | ![](structure.png) | 1 | 17 |
| 37 (G1-7) | ![](structure.png) | 0.55 | 10 |
| 38 (G1-3) | ![](structure.png) | 0.5 | 10 |
| 39 (G1-8) | ![](structure.png) | 0.11 | 10 |
| 40 (G1-9) | ![](structure.png) | 0.05 | 10 |
| 41 (G1-10)| ![](structure.png) | 0.022 | 10 |
| 42 (G1-11)| ![](structure.png) | 0.019 | 10 |
| 43 (G1-12)| ![](structure.png) | 0.015 | 10 |
| 44 (G1-13)| ![](structure.png) | 0.011 | 10 |
| 45 (G1-14)| ![](structure.png) | 0.008 | 10 |
| 46 (G1-15)| ![](structure.png) | 0.005 | 10 |
| 47 (G1-16)| ![](structure.png) | 0.00002 | 10 |

* The \( IC_{50} \) values were obtained using \( \beta \)-galactosidase activity, which is a measure of the inhibition of DsbB in E. coli expressing \( \beta \)-Gal<sup>act</sup>. The more DsbB inhibition of a drug the more \( \beta \)-galactosidase activity will be observed in cells, so one can calculate the concentration that gives 50% of inhibition (\( IC_{50} \)) of the total activity observed in a \( \Delta \text{dbsB} \) strain and use that concentration to get the fold-increase by dividing the \( IC_{50} \) of compound 12 (0.16 \( \mu \text{M} \), 95% confidence interval 0.13–0.20 \( \mu \text{M} \)) between the \( IC_{50} \) of the tested drug. Thus, a drug more potent than compound 12 will have a higher ratio and vice versa. The results were obtained using data of at least three independent experiments.

### TABLE 2—continued

| ID Number | Structure | Inhibition Ratio (\( IC_{50 \text{ Compound} 12/IC_{50 \text{ Compound} 4} \)) | \( IC_{50} \) Compound 12 (M) |
|-----------|-----------|-------------------------------------------------|-------------------------------|
| 48 (G1-6) | ![](structure.png) | <0.00001 | 1 |
| 49 (G1-8) | ![](structure.png) | <0.00001 | 1 |
| 50 (G1-10)| ![](structure.png) | <0.00001 | 1 |
| 51 (G1-12)| ![](structure.png) | <0.00001 | 1 |
| 52 (G1-14)| ![](structure.png) | <0.00001 | 1 |
| 53 (G1-16)| ![](structure.png) | <0.00001 | 1 |
| 54 (G1-18)| ![](structure.png) | <0.00001 | 1 |
| 55 (G1-20)| ![](structure.png) | <0.00001 | 1 |

### TABLE 3

| Compound | Structure | Compound's MW (Da) | Measured MW of DsbB<sub>K39E</sub> complex | Mass increase upon incubation with compound (Da) | Mass loss (Da) |
|----------|-----------|--------------------|---------------------------------------------|-----------------------------------------------|--------------|
| No compound | - | - | 43184.5 ± 3.2 | - | - |
| 12 | ![](structure.png) | 289.5 | 43437.1 ± 1.7 | 252.8 | 36.7 |
| 36 | ![](structure.png) | 378.44 | 43481.8 ± 2.2 | 297.5 | 80.9 |
| 37 | ![](structure.png) | 333.99 | 43437.0 ± 2.0 | 252.6 | 81.4 |
| 38 | ![](structure.png) | 333.99 | 43481.5 ± 2.3 | 297.2 | 36.8 |
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| ID | Structure | In vivo IC₅₀ (mM) | In vitro IC₅₀ (mM) |
|----|-----------|------------------|------------------|
| 36 | DsbB     | 0.31             | 0.91             |
|    | DsbB_DsbA| 0.98             | 0.45             |
|    | DsbB     | 0.35             | 0.35             |
|    | DsbB_DsbA| 0.35             | 0.35             |
|    | DsbB     | 0.39             | 0.39             |
|    | DsbB_DsbA| 0.39             | 0.39             |
| 37 | DsbB     | 0.61             | 1.35             |
|    | DsbB_DsbA| 0.58             | 1.58             |
|    | DsbB     | 0.64             | 0.64             |
|    | DsbB_DsbA| 0.64             | 0.64             |
| 38 | DsbB     | 0.42             | 2.2              |
|    | DsbB_DsbA| 0.45             | 2.45             |
|    | DsbB     | 0.45             | 0.45             |
|    | DsbB_DsbA| 0.45             | 0.45             |
| 45 | DsbB     | 23.02            | >64              |
|    | DsbB_DsbA| 23.28            | 23.79            |
|    | DsbB     | 23.39            | 23.39            |
|    | DsbB_DsbA| 23.39            | 23.39            |
| 42 | DsbB     | 41.97            | >100             |
|    | DsbB_DsbA| 39.43            | 46.67            |
|    | DsbB     | 46.67            | 46.67            |
|    | DsbB_DsbA| 46.67            | 46.67            |
| 40 | DsbB     | 3.48             | 8.69             |
|    | DsbB_DsbA| 2.13             | 7.0             |
|    | DsbB     | 3.87             | 10.73            |
|    | DsbB_DsbA| 3.87             | 10.73            |
| 41 | DsbB     | 10.09            | 22.76            |
|    | DsbB_DsbA| 8.96             | 20.34            |
|    | DsbB     | 11.36            | 25.47            |
|    | DsbB_DsbA| 11.36            | 25.47            |
| 43 | DsbB     | 25.43            | >100             |
|    | DsbB_DsbA| 23.63            | 54.67            |
|    | DsbB     | 27.36            | 97.70            |
|    | DsbB_DsbA| 27.36            | 97.70            |

* In vivo inhibition was measured by growth inhibition of strain lptD4213 ΔdsbB
dsbBrov205 (CL409–10, CL416–7 and LI18–19 strains) in the presence of drugs.

In vivo resistance was measured as a 2- to 4-fold increase in the IC₅₀ for the DsbB protein compared with wild type. Underlined numbers have 1.8–1.9-fold increase of IC₅₀, and boldface numbers have more than a 2-fold increase. From the 52 DsbB protein sequences analyzed (that share 90% or greater identity), the five residues presented in this work were conserved and similar to wild type DsbB (data not shown). One additional observation is that these two mutants conferred in vivo resistance to the lptD4213 strain to compounds that are less potent inhibitors than compound 12, with the exception of compound 38 (Table 4).

We asked whether there exist variants of DsbB enzymes that might be resistant to pyridazinones by doing a bioinformatic search among the different E. coli-sequenced genomes available. From the 52 DsbB protein sequences analyzed (that share 90% or greater identity), the five residues presented in this work were conserved and similar to wild type DsbB (data not shown). We also looked at the conservation of these five residues among other DsbB proteins from Gram-negative bacteria, specifically the ones that we know from our previous work are inhibited by pyridazinone-related molecules (17). The identity between DsbB proteins from Salmonella enterica sv. typhimurium, Klebsiella pneumoniae, Vibrio cholerae, and Haemophilus influenzae ranges from 85 to 41% when compared with E. coli DsbB. Among these organisms, four of the five residues were conserved overall when aligned to wild type E. coli DsbB, except for Lys-39. However, DsbB proteins from Pseudomonas aeruginosa, Acinetobacter baumannii, and Francisella tularensis, which share ~20% of identity with E. coli DsbB, demonstrated little or no conservation in the five residues studied. Moreover, P. aeruginosa DsbB1 encodes a Val-29 variant; similarly, P. aeruginosa DsbB2 and A. baumannii DsbB encode a Glu-39-resistant variant studied in this work. Nevertheless, we have shown that these proteins are still sensitive to compound 12 and related pyridazinones (17). Thus, it is possible that each enzyme may have slight differences in the structure and therefore differences in
binding to pyridazinone drugs, which is in agreement with our previous observation that the extent of inhibition changes among different pyridazinone inhibitors (17).

All mutant DsbBs were able to functionally complement the lptD<sub>4213</sub>ΔdsbB<sup>−</sup> strain for growth, indicating that the mutants are functional enzymes not only anaerobically but also aerobically. Similarly, the DsbB mutants were also able to complement two other dsbB<sup>−</sup> phenotypes. They restored motility of the dsbB mutant, and they also lacked β-galactosidase activity when the β-Gal<sup>dsbB</sup> is expressed in the strain (data not shown). However, all of the mutants obtained displayed lower catalytic efficiencies than the wild type enzyme.

Our finding that the combination of an lptD<sub>4213</sub> mutation and a dsbB null mutation are synthetically lethal leads us to suggest that any mutation or drug that decreases LptD assembly may also be synthetic lethal with the Dsb pathway (dsbA or dsbB mutants). Consequently, this finding suggests that combinations of drugs that target these two pathways can potentiate their antibiotic effect. This also suggests that inhibitors of the Dsb pathway may help to study LptD assembly by searching for mutations that confer resistance to these small molecules in order to identify additional genetic factors involved in LptD assembly (22).

The mutants studied here have a modest level of resistance (2–5-fold increase in IC<sub>50</sub>) to pyridazinone molecules in E. coli growth. It may be that greater changes in resistance are costly to the enzyme and thus to bacterial growth. Two different spontaneous genetic selections for resistance to the pyridazinone inhibitor, anaerobic selection for growth and growth of the lptD<sub>4213</sub> mutant, indicate that the frequency with which resistance arises is quite low. Obtaining such mutations was only made possible by PCR mutagenesis of a plasmid-encoded dsbB, which artificially increased the mutation rate. Although the environment in infections may generate different conditions for selection, these initial results raise the possibility that resistance problems during infections may possibly be avoided. Our findings may provide insights to the development of more effective pyridazinone drugs that do not bind covalently and are also important for understanding the nature of resistance, which may also hold some clinical relevance. This suggests that further development of pyridazinones as potential antivirulents/antibiotics may be warranted.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Tables 5 and 6, respectively. Standard molecular biology techniques and P1 transduction were used for the construction of strains and expression vectors (37, 38). All strains were grown in LB Miller agar or in M63 0.2% glucose liquid and agar media at 37 °C. Minimal M63 with 0.2% glucose and 40 mM fumarate solidified with 1% agarose plates were prepared for anaerobic growth experiments by placing in a Coy anaerobic chamber (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>) to equilibrate for several days before use. The antibiotic concentrations used were as follows: ampicillin 100 μg/ml (for plasmid copy), 25 μg/ml (for chromosomal copy), or 10 μg/ml (for LptD<sub>4213</sub> strain), kanamycin 40 μg/ml, tetracycline 10 μg/ml, and chloramphenicol 10 μg/ml.

dsbB mutagenesis and construction of mutant library

A mutagenic PCR of the dsbB gene using primers CI13 and CI14 was generated using the first seven mutagenic conditions of Diversify mutagenesis kit (Clontech) that on average generates 2–5.8 mutations/kb. The amplification conditions used were 94 °C (30 s) as denaturing temperature, 55 °C (30 s) as annealing, and 68 °C (30 s) as extension repeated for 25 cycles. The products were reamplified using Taq platinum (Thermo Fisher Scientific) to produce more of the PCR product. PCR products of all reactions were then mixed, column-purified, Ncol-Sacl-digested, and ligated to a digested pDSW204 plasmid (39). 1 μl of the ligation reaction was transformed into highly competent XL1-Blue cells (Agilent Technologies). A sample of the colonies obtained after selection on ampicillin plates was collected for plasmid preparation used to confirm efficiency of ligation by PCR and digestion. Given that 9 of 10 colonies did have the expected insert, the rest of the ligation reaction (49 μl) was transformed into DH10β highly competent cells (New England Biolabs). The transformation yielded ~3,000 colonies, which were scraped up and grown overnight in M63 glucose for plasmid preparation. Plasmid preparations were frozen at −20 °C until use.

Construction of a conditionally lethal strain lptD<sub>4213</sub>ΔdsbB

lptD<sub>4213</sub> (amino acid deletion from 330–352) mutant was constructed in E. coli MC1000 strain by transducing the mutation from the MC4100 strain, NR698 (22). First, the lptD gene was linked to a tetracycline resistance cassette (at carB gene, 20–25% linkage) by making a P1 lysate from the GC208 strain (40). This lysate was then used to infect the NR698 strain (lptD<sub>4213</sub> mutation) selecting for transductants in tetracycline plates. The lptD<sub>4213</sub> transductants linked to tetracycline cassette were verified by the size of the PCR product of part of lptD gene (1.5 kb), and the mutants have a 68-bp smaller PCR product due to the deletion of 23 amino acids using primers CI84 and CI85. It was also noticed that all small colonies had lptD<sub>4213</sub> mutation, and the regular size colonies had wild type lptD. Thus, this was used in later selections to distinguish between them. A P1 lysate from one verified transductant in the previous step was prepared to infect HK295 (MC1000) strain. After verifying the presence of lptD<sub>4213</sub> mutation in HK295 strain, the tetracycline cassette linker was removed from the strain by P1 transduction of wild type strain and selecting on minimal M63 media, because the carB mutation makes the cells arginine and uracil auxotrophs on minimal media (41). The colonies that grew in minimal glucose media were again verified by PCR and sequenced to have the lptD<sub>4213</sub> mutation; one colony was selected for further experiments (CL337 strain).

To construct the conditionally lethal strain lptD<sub>4213</sub>ΔdsbB ΔdsbB<sub>PRAD</sub> first a plasmid expressing dsbB under the regulation of arabinose promoter (pCL67) was transformed into the lptD<sub>4213</sub> strain (CL337). The deletion of dsbB gene from HK310 strain was then P1-transduced to the lptD<sub>4213</sub> strain selecting on LB kanamycin plates supplemented without or with 0.2%
arabinose. Kanamycin-resistant colonies were obtained in both cases, and the transduction of the dsbB deletion was verified by PCR using primers Cl55–56; all checked colonies did have the correct product size (1-kb dsbB<sub>WT</sub> versus 1.6-kb dsbB<sub>:Km</sub>). This result indicated that the basal levels of expression from arabinose promoter were enough to complement growth in rich medium. One colony with confirmed deletion was isolated and used for further work (CL380 strain). When the dsbB deletion was transduced to lptD<sub>4213</sub> with no other copy of the dsbB gene, no colonies with the correct deletion of dsbB were obtained unless the transductants were plated on LB with 1 mM dsbB.

The growth of CL380 strain was tested in minimal media plates. M63 glucose with 0.2% arabinose allowed growth of the CL380 strain, whereas the strain was not able to grow on M63 minimal media plates lacking arabinose. However, this strain is able to grow in liquid M63 minimal media with no arabinose under shaking conditions where oxygen may contribute to background oxidation.

**Selection of DsbB mutants using lptD<sub>4213</sub> strain**

For spontaneous resistant mutants, CL337 cells from over-night culture were washed twice with M63 minimal media, and ~10<sup>9</sup> cells were plated in M63 glucose media plates with 10 µM compound 12 (10-fold higher the MIC). Plates were incubated for 2 days at 37 °C. 51 colonies were purified in M63 minimal media plates to characterize them. We amplified and sequenced of these gave a wild type sequence. We noticed that some of the selected mutants did confer resistance to bile salts, and because these mutations had been previously studied (21), we amplified and sequenced also 22 of 51 colonies analyzed by PCR did have a higher size product. Two of these did have mutations in the bamB gene, and the sequence of all these indicated an insertion of IS1 element in the gene. Whole-genome sequencing was performed in three of the colonies that did not indicate an insertion of IS1 element in the gene. The transformation gave around 4,500 independent colonies and sequencing of these gave a wild type sequence.

### Table 5

| Strain | Genotype | Reference |
|--------|----------|-----------|
| N6698  | MC4100 LptD<sub>4213</sub> | 22        |
| GC208  | MC4100 carB<sub>1</sub>T<sub>1</sub>n<sub>10</sub>(T<sub>C</sub>) | 40        |
| J6 (FSH94) | BL21 C43 (DE3) ΔdsbB (K<sub>M</sub>) | 42        |
| J7 (FSH95) | BL21 C43 (DE3) ΔdsbB (K<sub>M</sub>) | 42        |
| FSH69 | Lemo21(DE3, C<sub>M</sub>) pFL39 (6HisDsbA, K<sub>M</sub>) | 17        |
| CL337  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pCL67 (dsbB<sub>pBAD</sub>, C<sub>M</sub>) | This study |
| CL380  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pCL23 (dsbB<sub>pBAD</sub>, Ampr) | This study |
| CL410  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pBOM230 (DsbB<sub>2,5G</sub> under P<sub>trc</sub>, Ampr) | This study |
| CL417  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pBOM252 (DsbB<sub>2,5V</sub> under P<sub>trc</sub>, Ampr) | This study |
| CL19   | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pBOM253 (DsbB<sub>2,5V</sub> under P<sub>trc</sub>, Ampr) | This study |
| CL416  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pBOM228 (DsbB<sub>2,5G</sub> under P<sub>trc</sub>, Ampr) | This study |
| CL409  | HK295 LptD<sub>4213</sub> ΔdsbB (K<sub>M</sub>) pBOM231 (DsbB<sub>2,5G</sub> under P<sub>trc</sub>, Ampr) | This study |
| CL591  | HK295ΔdsbB αatt::DsbB<sub>WT</sub> (P<sub>trc</sub>, Ampr) | This study |
| CL592  | HK295ΔdsbB αatt::DsbB<sub>2,5G</sub> (P<sub>trc</sub>, Ampr) | This study |
| CL594  | HK295ΔdsbB αatt::DsbB<sub>2,5V</sub> (P<sub>trc</sub>, Ampr) | This study |
| CL595  | HK295ΔdsbB αatt::DsbB<sub>2,5V</sub> (P<sub>trc</sub>, Ampr) | This study |
| CL593  | HK295ΔdsbB αatt::DsbB<sub>2,5G</sub> (P<sub>trc</sub>, Ampr) | This study |
| CL596  | HK295ΔdsbB αatt::DsbB<sub>2,5G</sub> (P<sub>trc</sub>, Ampr) | This study |

### Plasmids

| Plasmid | Description |
|---------|-------------|
| pTrc99A | Expression vector, pBR322 origin, Amp<sup>+</sup> |
| pDSW204 | Promoter down mutation in −35 of pTrc99A (P<sub>trc</sub>), (Amp<sup>+</sup>) |
| pBAD45  | Arabinose-inducible vector (P<sub>BAD</sub>, pSC101 origin, Cm<sup>+</sup>) |
| pET28a  | Expression vector, T7lac promoter, N-terminal and C-terminal His tag, EMD |
| pWM76   | Expression vector, pBR322 origin, Km<sup>+</sup>, 6-His (Ampr) |
| pFL39   | pET28a 6-His-DsbA cloned at NdeI-Xhol |
| pCl23   | pDSW204-dsbB cloned at Ncol-Sacl, DsbB<sub>WT</sub> (MV-DsbB<sub>2,5G</sub>−<sub>176</sub>) |
| pCL67   | pBAD45-dsbB cloned at EcoRI-HindIII, DsbB<sub>pBAD</sub> |
| pBOM228 | pDSW204-dsbB<sub>2,5G</sub> cloned at Ncol-Sacl, DsbB<sub>2,5G</sub> |
| pBOM225 | pDSW204-dsbB<sub>2,5V</sub> cloned at Ncol-Sacl, DsbB<sub>2,5V</sub> |
| pBOM253 | pDSW204-dsbB<sub>2,5G</sub> cloned at Ncol-Sacl, DsbB<sub>2,5G</sub> |
| pBOM230 | pDSW204-dsbB<sub>2,5G</sub> cloned at Ncol-Sacl, DsbB<sub>2,5G</sub> |
| pBOM231 | pDSW204-dsbB<sub>2,5G</sub> cloned at Ncol-Sacl, DsbB<sub>2,5G</sub> |
| pLI1   | pWM76-dsbB<sub>TRC</sub> (P<sub>trc</sub>) |
| pLI2   | pWM76-dsbB<sub>2,5G</sub> (P<sub>trc</sub>) |
| pLI3   | pWM76-dsbB<sub>2,5G</sub> (P<sub>trc</sub>) |
| pLI4   | pWM76-dsbB<sub>2,5G</sub> (P<sub>trc</sub>) |
| pLI6   | pWM76-dsbB<sub>2,5G</sub> (P<sub>trc</sub>) |
dent colonies, which were scraped and saved in glycerol stocks. After growing the library on LB broth, cells were washed twice with M63 minimal media, and 10^8 cells were plated on M63 glucose minimal media containing 10 mM compound 12 to select for resistant mutants. After 2 days of growth at 37 °C, colonies appeared and were purified on LB plates with no antibiotic. A PCR product of the mutagenized dsbB gene was amplified using primers Cl24–25 (prime only to pDSW204) to sequence.

**Anaerobic selection of DsbB mutants**

Purified plasmids from the mutagenized library were transformed into dsbB cells (HK320) and plated aerobically on LB with ampicillin. The transformation yielded around 3,000 colonies, which were scraped and saved as glycerol stocks to use for further selection. The mutant library obtained in dsbB mutant was grown aerobically in M63 0.2% glucose to an A600 of 0.6. Cells were washed, and 10^7 cells were plated on M63, 0.2% glucose, 40 mM fumarate, 1% agarose plates with 2 mM compound 12. Plates were then incubated at 37 °C in a Coy anaerobic chamber (85% N2, 10% H2, 5% CO2) for 3 days. The resistant colonies were purified under the same conditions and then cultured aerobically to isolate plasmids. Plasmids were transformed back into dsbB cells, and growth of the resultant transformants was tested anaerobically under selective conditions to confirm that the plasmid carried the resistance mutation. The dsbB gene was then sequenced with primers Cl24–Cl25 to identify the mutations.

**Using lptD4213 strain to confirm resistance of the studied DsbB mutants**

To confirm resistance of the five selected mutations, the plasmids pBOM228, -30, -31, -52, and -53 were used to transform the CL380 strain. The resultant strains were then plated on LB with 0.4% arabinose plates to select for cells cured of the plasmid with the wild type copy of dsbB (pCL67). Because the overexpression of dsbB causes cell toxicity, those cells able to grow under arabinose are most likely the cells that have lost the arabinose-inducible plasmid. Purified colonies were checked for loss of chloramphenicol resistance and were verified by PCR with primers Cl24 and Cl25 that prime only to pDSW204 but not to pBAD plasmid and with primers Cl6 and Cl8 that prime only to pBAD but not to pDSW204 plasmid. The dsbB mutations were confirmed by sequencing.

**Growth assays of lptD4213 dsbB mutants in the presence of various pyridazinone drugs**

Strains were grown overnight in minimal M63 0.2% glucose media with 5 μM IPTG (Enzo Life Sciences Inc.) to induce the expression of dsbB. Overnight cultures of bacteria were diluted to an A600 of 0.02 in M63 0.2% glucose minimal media, and 200 μl of diluted cultures were aliquoted in 96-well plates (Thermo Fisher Scientific). Serial dilutions of the drug or DMSO were added in a volume of 2 μl (1% DMSO final concentration). The plates were covered with breathable films (VWR Scientific) and

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**Table 6**

| ID | Sequence | Restriction site |
|----|----------|-----------------|
| C6 | ATGCCCATAGCATTTTATCC | NcoI |
| C8 | GATTAACCTGTTGACTAGG | |
| C13 | CTCAGCGGATTCGAGTGTGTTTTGCAACATAGTACGTATT | |
| C14 | CGAGCTGAGGACCGACGAGATACC | |
| C24 | GCCGACCTCCGGTCTGGAATATGG | |
| C25 | GTCGACGGTAGTGCTGGAAAGCAGATGAGGTA | |
| C55 | CATCCTCGCGCTGCCCTTATATG | |
| C56 | GGGAATCCAGCAACAATGGCAGATGAA | |
| C84 | TGAGTTCTACCTGCCATATTACTGG | |
| C85 | TTTCGCCACCGTGACGATGAGGTA | |
| C105 | CTGGTGAATTCGAGTGTGTTTTGCAACATAGTACGTATT | EcoRI |
| C110 | CTGAACGGTTTTAAGCCAGAGATCAGTATT | HindIII |
| C118 | GGGTGAAAGGOGGCGGCGATATTGGGGA | |
| C119 | GTTGAGGTTTTTAGCGACGATGAGGTA | |
| C120 | AGGAGCAGGATTATGGAATGCGCGG | |
| C129 | TACTGCGGACACGAGGCTGGGA | |
| C130 | GGAGAGCGGGAACGAGGGA | |
| C131 | CTCCTCGCGCTGCCCTTATATG | |
| C132 | GATGCGGTTCTACGGTCAAGATGATT | |
| C155 | GCAACCGGGAACGAGGGA | |
| C165 | CAGTCAATGCGCGCC | |
| K39E-f | AGTTTGATCAGTGGGGA | |
| K39E-r | GTCTGCGGACACGAGGCTGGGA | |
| P100S-f | CTAATCTCGGACGACGACG | |
| P100S-r | GCAGTTGACGACGACGACG | |
| F106L-f | GACAAGCGGGAACGAGGGA | |
| F106-r | GAACTGCGGGAACGAGGGA | |
| CI225 | CGAGGTGACGACGACGACG | |
| CI226 | CAAACGAGGTGACGACGACG | |
| CI230 | CCAACGAGGTGACGACGACG | |
| CI230 | CCAACGAGGTGACGACGACG | |
| CI230 | CCAACGAGGTGACGACGACG | |
| CI230 | CCAACGAGGTGACGACGACG | |
| CI231 | GTCGACATTTTCTGACGATGAGGTA | |
| CI240 | CCCGACAAAGGAGGTTGCGCCCTTG | |

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DsbB mutations resistant to pyridazinone-related molecules

then incubated for 19 h at 37 °C and 900 rpm in an orbital shaker (Multitron ATR). The A₆₀₀ from at least three independent experiments was read to determine the growth, and this was used to calculate the IC₅₀ values (concentration that gives 50% inhibition of growth without drug) with 95% confidence intervals using GraphPad Prism (La Jolla, CA) in the function of non-linear regression (log inhibitor versus response with variable slope, normalized response).

Purification of DsbB proteins and enzyme kinetics

The five mutations in DsbB were generated by site-directed mutagenesis of plasmid pWM76 using the primers listed in Table 2. Then DsbB proteins were purified as described before (42). Purified proteins were at least 90% as judged from SDS-PAGE (supplemental Fig. 1). Determination of kinetic properties and IC₅₀ values was done as described before with slight changes (17). Briefly, various amounts of inhibitors were mixed with 10 nM DsbB in phosphate buffer (pH 6.5) containing 0.1% n-dodecyl-β-D-maltopyranoside (Affymetrix Inc.), 100 mM NaCl and ubiquinone-5 (Sigma, 1–50 μM for kinetic constants and 10 μM for inhibition assays) or menadione (Sigma, 0.5–128 μM). Reactions were started at room temperature by the addition of small amounts of highly concentrated DsbA solution to give a final concentration of 20 μM. Initial velocities of DsbB-catalyzed quinone reduction were measured at 275 nm for ubiquinone and 260 nm for menadione.

Structure-activity relationship approach of related pyridazinones

Given that a substructure analysis with pyridazinones helped us previously to identify more effective inhibitors such as compound 12 (17), we decided to explore more variations in the core of the drug to validate our understanding of the drug inhibition and to find more effective inhibitors. The molecules were designed first by substituting the chlorine atoms at positions 4 and 5 of the pyridazinone ring by other halogen atoms such as bromine and by other groups that unlike halogens could act as nucleophile (electron donor) rather than electrophile (electron acceptor), i.e. methyl groups. Second, we substituted the benzyl group at position 2 by different rings such as thiophene and naphthalene (2017) 292(16) 6529–6541 and to find more effective inhibitors. The molecules were synthesized by Sundia MediTech Co., Ltd. (China, purity over 95% analyzed by LC-MS). The chemical synthesis protocols are presented at the end of supplemental Information. Compound 12 was purchased from Enamine (Ukraine, purity over 95% analyzed by LC-MS).

To test inhibition, all compounds were tested in vitro with DsbB in minimal media with 1 mM IPTG until log phase. The lack of IPTG makes DsbB levels undetectable when dsbB is under trc204 promoter (data not shown). Proteins were TCA-precipitated, run on reducing SDS-PAGE, and immunoblotted against anti-DsbB (44). DTT was used for reducing disulfide bonds.

DsbB immunoblot

Each plasmid containing dsbB mutants was integrated into the chromosome of the strain HK320 by AlnCh method generating strains CL591–596 (43). To determine DsbB expression levels, strains CL591 to CL596 were grown aerobically in M63 minimal media with 1 mM IPTG until log phase. The lack of IPTG makes DsbB levels undetectable when dsbB is under trc204 promoter (data not shown). Proteins were TCA-precipitated, run on reducing SDS-PAGE, and immunoblotted against anti-DsbB (44). DTT was used for reducing disulfide bonds.

Author contributions—C. L. performed lptD and substructure experiments. B. M. M. performed anaerobic selection. L. M. and C. L. performed β-gal and growth assays. L. I. and F. H. purified proteins and performed in vitro and mass spectrometry assays. N. Q. T. purified a protein. D. B. performed bioinformatics analysis. C. L., B. M. M., D. B., and J. B. discussed the data. C. L. and J. B. wrote the paper.

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