Mechanism of Resistance to an Antitubercular 2-Thiopyridine Derivative That Is Also Active against *Burkholderia cenocepacia*

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The discovery of new compounds that are able to inhibit the growth of *Burkholderia cenocepacia* is of primary importance for cystic fibrosis patients. Here, the mechanism of resistance to a new pyridine derivative already shown to be effective against *Mycobacterium tuberculosis* and to have good activity toward *B. cenocepacia* was investigated. Increased expression of a resistance-nodulation-cell division (RND) efflux system was detected in the resistant mutants, thus confirming their important roles in *B. cenocepacia* antibiotic resistance.

Respiratory tract infections are the main cause of mortality in cystic fibrosis (CF) patients ([1]). *Burkholderia cenocepacia* is frequently associated with reduced survival and a high risk of developing cepacia syndrome, a fulminating pneumonia ([2]).

Up to now, a few antibiotics have been effective for treating *B. cenocepacia* infections, such as meropenem plus ceftazidime, chloramphenicol, trimethoprim-sulfamethoxazole, and aztreonam ([3], [4]). In particular, combination therapy is recommended, since the bacteria causing these infections show a high level of intrinsic resistance ([5]). These treatments have had a substantial impact on the quality of life and survival of CF patients; however, the increasing diffusion of resistant bacteria requires new and alternative drugs for treatment.

Our laboratory is focused on the mechanisms of resistance mediated by resistance-nodulation-cell division (RND) efflux systems. These proteins have been shown to play a prominent role in drug resistance in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, while their role in *B. cenocepacia* drug resistance has been only partially described ([6], [7]). In particular, we demonstrated that the deletion of the RND-4 system (BCAL2820 to BCAL2822) led to increased sensitivities to different antimicrobial compounds, suggesting its involvement in intrinsic drug resistance ([8], [9]).

This work describes a promising new molecule, a 2-thiopyridine derivative named 1-oxido-5-(trifluoromethyl)pyridin-2-yl thiocyanate (11026103) (Fig. 1), which is particularly active against *B. cenocepacia* growth and was recently shown to be effective against *Mycobacterium tuberculosis* ([10]). This compound was selected after a screening of >60 compounds, which were tested against *B. cenocepacia* strain J2315. For each compound, the MIC was evaluated, but only 11026103 was found to have promising antibacterial activity, with an MIC of 16 µg/ml (Table 1).

B. cenocepacia J2315 cells were spread on plates containing the 11026103 molecule at concentrations ranging from 4 to 10× the MIC. Two spontaneous resistant mutants were isolated through direct selection on solid medium containing 64 µg/ml of compound (4× the MIC) (Table 1). These mutant strains were named CU4 and CU14. The mutation frequency toward 11026103 was 10^{-10}.

In order to understand which mutations were responsible for the resistance of these mutants, the genomic DNA of the mutant strains was extracted and sequenced by an Illumina Solexa method. The obtained reads were then mapped against the *B. cenocepacia* J2315 reference genome. The mutations with ≥60% frequency of variant alleles and leading to a change to a different amino acid or to the synthesis of a truncated protein were analyzed. In both mutants, one mutation was identified in the *BCAL2823* gene. The *BCAL2823* (*bpeR*) gene encodes a 213-amino acid protein that belongs to the TetR family of transcriptional regulators, localized next to the RND-4 efflux system (Fig. 1A). The mutation identified in the CU4 strain is the deletion of A250, which caused a frameshift in the nucleotide sequence, thus resulting in an early stop codon and a truncated protein of only 92 amino acids. In the CU14 mutant, the mutation was the substitution T458C, which led to the amino acid change L153P, with the two amino acids having different chemical properties.

In order to determine whether the resistance to 11026103 was associated with an upregulation of the RND-4 system due to mutations in its negative regulator, quantitative real-time PCR (RT-PCR) experiments were performed on both resistant strains. The results showed that compared to the wild type, the *RND-4* (*BCAL2821*) gene was upregulated 4-fold in CU4 and 2-fold in CU14, respectively. As the overexpression detected in the CU14 mutant is very low, only CU4 was considered for further analyses.

Since the overexpression of efflux pumps can cause resistances to different compounds ([11]), the MICs of the most commonly used antibiotics were assessed for the CU4 strain. In particular, the MICs of chloramphenicol and nalidixic acid increased 8-fold in the mutant compared to the wild type, the MIC of levofloxacin increased 4-fold compared to the wild type, and the MICs of ciprofloxacin, norfloxacin, and sparfloxacin were 2-fold higher in the mutant. These results confirm that RND-4 overexpression is able to confer resistances to different compounds, as was already
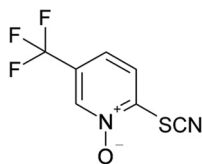
FIG 1 Chemical structure of compound 11026103.

FIG 2 Genetic organization and EMSAs with BCAL2823 and BCAL2823$_{CU4}$. (A) Genetic organization of *RND-4* operon and BCAL2823. RND-4 transcriptional start site position is indicated by the smaller arrow. The mutations in BCAL2823 are marked with asterisks. (B) A 3.39 nM 6-FAM-labeled probe was used, with increasing amounts of BCAL2823 (indicated above each lane). The bound BCAL2823 is indicated by arrows. −, control. (C) A 3.39 nM 6-FAM-labeled probe was used, with increasing amounts of BCAL2823$_{CU4}$ (indicated above each lane) or 35 pmol BCAL2823. − and + indicate absence or presence, respectively, of BCAL2823 protein.
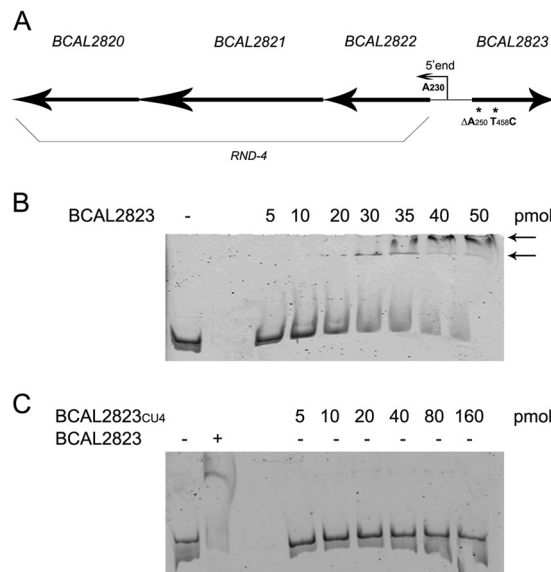
shown ([8]), and they suggest that this transporter is also involved in the 11026103 efflux.

To further confirm this hypothesis, the MIC of 11026103 was also assessed for the RND-4 deleted strain, which was 2-fold less resistant than the wild-type strain (Table 1).

To characterize the mechanism of resistance to the 11026103 drug, electrophoretic mobility shift assay (EMSA) experiments were performed using the TetR repressor (BCAL2823) purified from both *B. cenocepacia* J2315 and the CU4 mutant. The *BCAL2823* coding region was cloned into the expression vector pET-28a (Novagen) and transformed in *Escherichia coli* BL21(DE3). Protein expression was obtained with the ZYP-5052 autoinducing medium ([12]). The recombinant proteins were purified by affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen).

The 400-bp intergenic region located between the *BCAL2822* and *BCAL2823* genes (Fig. 2A) was used for DNA-binding assays and 5′-labeled with 6-carboxyfluorescein (6-FAM). The 20-μl binding reaction buffer was composed of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1.25 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 3 μg bovine serum albumin, 1 μg salmon sperm DNA, and 10% glycerol. The labeled DNA region was used at 3.39 nM, with increasing concentrations of both BCAL2823 and BCAL2823$_{CU4}$ (Fig. 2B and C). Specificity was demonstrated by performing the experiment with increasing concentrations of unlabeled probe (data not shown). The results showed that the BCAL2823 protein purified from the wild-type strain was able to bind to the 400-bp intergenic region at a concentration of 30 pmol (Fig. 2B, arrows), while the truncated protein purified from the CU4 mutant was unable to bind the same intergenic region even at higher concentrations (160 pmol) (Fig. 2C). Hence, as hypothesized, if BCAL2823 does not work correctly as a transcriptional repressor, an ineffective negative regulation might allow overexpression of the *RND-4* operon, leading to drug resistance.

The transcriptional start site of the *RND-4* operon was then mapped by 5′-Rapid amplification of cDNA Ends (RACE) technique. The reaction was made with ImProm-II reverse transcriptase (Promega) using an adaptor primer (5′-GACCACGCGTAT CGATGTCGACT$_{16}$-3′) and a specific primer. The 5′ end resulted in the A being located 230 bp upstream of the translational start site of the *BCAL2822* gene (Fig. 2A).

To further confirm that RND-4 was responsible for resistance

to 11026103, a wild-type copy of *BCAL2823* was cloned into pAP20 constitutive expression vector (kindly provided by Miguel Valvano). The *B. cenocepacia* CU4 mutant was transformed with the recombinant vector by triparental mating ([13]), and the MIC of 11026103 was determined for both the CU4 strain transformed with the empty vector and for the same strain carrying the *BCAL2823*/pAP20 vector. The MIC of the mutant transformed with the wild-type copy of *BCAL2823* was the same as that of the wild-type *B. cenocepacia* J2315, indicating that complementation occurred. This was further confirmed by a quantitative RT-PCR experiment, which was conducted also on the complemented strain, and the value achieved was the same as for the wild type.

In conclusion, a new compound, which has been shown to be effective against *M. tuberculosis*, has been demonstrated to be promising also for the treatment of *B. cenocepacia* infections. This is very important for CF patients, as the current therapy is not effective. Moreover, the RND-4 transporter was confirmed to have a prominent role as an efflux transporter in this bacterium, and in the future, it will be important to find drugs that are able to block this pump and not be extruded by this protein.

TABLE 1 MICs of 11026103

B. cenocepacia strain	MIC (μg/ml)
J2315 (WT[a])	16
CU4	128
CU14	128
ΔRND-4[b]	8

[a] WT, wild type.
[b] See Buroni et al. ([8]).

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