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Clinical Isolates of *Mycobacterium tuberculosis* in Four European Hospitals Are Uniformly Susceptible to Benzothiazinones▽†

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The new antitubercular drug candidate 2-[2-*S*-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one (BTZ043) targets the DprE1 (Rv3790) subunit of the enzyme decaprenylphosphoryl-β-D-ribose 2′-epimerase. To monitor the potential development of benzothiazinone (BTZ) resistance, a total of 240 sensitive and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from four European hospitals were surveyed for the presence of mutations in the *dprE1* gene and for BTZ susceptibility. All 240 strains were susceptible, thus establishing the baseline prior to the introduction of BTZ043 in clinical trials.

The rapid spread of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) and extensively drug-resistant *M. tuberculosis* (XDR-TB) strains demands new antitubercular drugs that overcome the problem of drug-resistant strains (4). Recently, two different classes of promising new antitubercular agents, the benzothiazinones (BTZ) and dinitrobenzamides (DNB), were found to be highly active against *M. tuberculosis*-resistant and -sensitive strains, including XDR and MDR strains (2, 3). The lead compound belonging to the BTZ class, 2-[2-*S*-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one (or BTZ043), has been used in this study. Interestingly, both BTZ and DNB drugs have the same target, the heterodimeric decaprenylphosphoryl-β-D-ribose 2′-epimerase, encoded by the *dprE1* (*Rv3790*) and *dprE2* (*Rv3791*) genes (3). DprE1 and DprE2 are involved in the transformation of decaprenylphosphoryl-D-ribose to decaprenylphosphoryl-D-arabinose, which is the sole precursor for arabinogalactan and lipoarabinomannan synthesis in the mycobacterial cell wall (3, 7). Moreover, *dprE1* is essential for *M. tuberculosis* growth (5).

In all the spontaneous BTZ-resistant laboratory mutants, the Cys387 codon within *dprE1* was replaced by a Ser or Gly codon. Furthermore, the Cys387 codon was shown to be highly conserved in several actinobacterial species, with the exception of *Mycobacterium avium*, which was found to be naturally resistant to BTZ043, due to replacement of the Cys387 by an Ala codon (3).

The pharmaceutical industry usually estimates the likelihood of the development of resistance against a new drug by focusing on the mutation resistance rate, on the assumption that this rate is a major determinant of resistance development in clinical settings (1). Therefore, drug targets for which the resistance mechanisms have the most negative effect on fitness are expected to show a low resistance development rate (1). It is noteworthy that *in vitro M. tuberculosis* BTZ-resistant mutants were rare, arising at a frequency of $<10^{-8}$ (3).

The aim of this work is to investigate whether any of the present *M. tuberculosis* clinical isolates are BTZ resistant.

A total of 240 *M. tuberculosis* clinical isolates (including MDR- and XDR-TB strains) from four different European hospitals were screened for mutations in the Cys387 codon of *dprE1* and for BTZ sensitivity. Seventy-eight *M. tuberculosis* clinical isolates came from the National Institute for Infectious Diseases (INMI) L. Spallanzani hospital in Rome, Italy; 118 strains were isolated at the Sondalo Division of the Valtellina and Valchiavenna, Italy, hospital authority, 32 clinical isolates were from the Ambroise Paré Hospital in Boulogne-Billancourt, France, and 12 strains were isolated at the Central Institute of Tuberculosis in Moscow, Russia. All strains were isolated between 2003 and 2009 (see Table S1 in the supplemental material).

Of the 240 *M. tuberculosis* clinical strains isolated from the

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TABLE 1. MICs of BTZ043 for *M. tuberculosis* clinical isolates

Isolate source and group	No. of isolates (%)	No. of HIV-positive isolates (%)	BTZ043 MIC (ng/ml) or resistance status[a]
Sondalo Hospital			
Sensitive	78 (66.1)	1 (1.2)	0.75–3
MDR	16 (13.6)	0	0.75–3
Resistant	24 (20.3)	0	0.75–3
Total	118	1 (8.4)	0.75–3
Spallanzani Hospital			
Sensitive	36 (46.2)	1 (2.8)	3–6
XDR	1 (1.2)	0	3–6
MDR	13 (16.8)	2 (7.7)	3–6
Resistant	28 (35.8)	2 (7.4)	3–6
Total	78	5 (5.1)	3–6
Ambroise Paré Hospital			
Sensitive	28 (87.5)	1 (3.5)	<15*
MDR	2 (6.25)	0	<15
Resistant	2 (6.25)	0	<15
Total	32	1 (3.1)	<15*
Central Institute of Tuberculosis			
MDR	7 (58.6)	0	3–6
Resistant	5 (41.4)	0	3–6
Total	12	0	3–6
Total			
Sensitive	142 (59.2)	3 (2.1)	S
XDR	1 (0.4)	0	S
MDR	38 (15.8)	2 (5.2)	S
Resistant	59 (24.6)	2 (3.4)	S
Total	240	7 (2.9)	S

[a] * indicates three strains with MICs of 30 ng/ml. S, sensitive.

different European centers, 1 was XDR and 38 (15.8%) had an MDR phenotype. Moreover, some MDR strains were resistant to other first- and second-line drugs, namely, 12 MDR isolates from Rome (strains P25, P36, P43, P75, P105, P670, P713, P717, P877, P878, P889, and P939), 16 from Sondalo (strains S1, S4, S46, S64, S67, S105, S128, S138, S173, S180, S183, S199, S232, S245, S246, and S257), 2 from Boulogne-Billancourt (strains F21 and F35), and 5 from Moscow (strains R18, R19, R20, R51, and R87). These 35 MDR strains were sensitive to only a few drugs. Some *M. tuberculosis* clinical isolates were resistant to one or more drugs but did not meet the MDR definition (24.6%) (see Table S1 in the supplemental material). Seven *M. tuberculosis* clinical strains were isolated from HIV-positive patients; among these strains, there were two monoresistant (isoniazid [INH] or rifampin [RIF]), two MDR, and three sensitive strains (see Table S1 in the supplemental material). MDR- and XDR-TB in HIV patients result in increased morbidity and mortality (6).

An internal fragment of *dprE1*, spanning the Cys387 codon, was amplified by PCR with the MDR1 (5′-TATCG CGGCAAGGTCCAGAA-3′) and MDR2 (5′-AGCGTCGG GCCATGTCGGA-3′) primers and directly sequenced from 228 *M. tuberculosis* clinical isolates (the strains from Italian and French hospitals) to search for possible mutations. None of the *M. tuberculosis* isolates had mutations in the *dprE1* gene, and hence, these isolates were presumably sensitive to BTZ. This result confirms that BTZ resistance mutations are not present in the strains of *M. tuberculosis* currently circulating.

From these results, it is reasonable to hypothesize that the Cys residue has a fundamental role in BTZ sensitivity, possibly in BTZ binding.

To correlate the PCR results with BTZ043 sensitivity, we compared the BTZ043 MIC ranges for a large number of drug-susceptible and MDR-TB clinical isolates and for the XDR-TB strain (see Table S1 in the supplemental material). Table 1 shows that all the *M. tuberculosis* clinical isolates tested were sensitive to BTZ043, with values in the range of 0.75 to 30 ng/ml, without differences between strains sensitive and resistant to other drugs. The differences in the BTZ MIC ranges are likely due on the one hand to strain differences and on the other to operational differences in the different settings. The positive controls for resistance, *M. tuberculosis* strains NTB1 and NTB9 (3), routinely displayed MICs of >1,000 ng/ml. This result confirms that BTZ043 is very active against both sensitive and resistant strains of *M. tuberculosis*, including MDR and XDR strains.

Two clinical isolates of *Mycobacterium bovis* from France were shown to be sensitive to BTZ043 and to lack mutations in *dprE1* (data not shown). In contrast, on sequencing of *dprE1*, all five *Mycobacterium avium* clinical isolates from Rome, Italy, displayed the same Cys-Ala polymorphism previously described (3) and were fully resistant to BTZ043 (data not shown).

In all the *dprE1* sequences of BTZ-sensitive mycobacteria, the codon for this cysteine is conserved, thus confirming the correlation between the presence of cysteine and benzothiazinone sensitivity and *vice versa*. These results have very important implications for future clinical trials. Specifically, it will be possible to perform an easy and rapid diagnostic test for BTZ resistance in clinical isolates simply by sequencing the *dprE1* gene around the Cys387 codon or by using a real time-PCR assay, thus bypassing the need for systematic MIC determination.

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