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In Vitro Activities of the New Antitubercular Agents PA-824 and BTZ043 against *Nocardia brasiliensis*

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The *in vitro* activity of PA-824 and BTZ043 against 30 *Nocardia brasiliensis* isolates was tested. The MIC_{50} and MIC_{90} values for PA-824 were both >64 μg/ml. The same values for BTZ043 were 0.125 and 0.250 μg/ml. Given the MIC values for benzothiazinone (BTZ) compounds, we consider them good candidates to be tested *in vivo* against *N. brasiliensis*.

Nocardia brasiliensis is a natural inhabitant of the soil that in some cases gains entry to human skin by trauma with splinters or wood material contaminated with this bacterium (17). Once in the subcutaneous tissues, the bacteria proliferate, producing local inflammation, abscesses, and fistulae, and may affect subjacent organs, depending on the topographic localization of the lesions. The production of chronic inflammation and scarring of tissue makes it difficult for antimicrobials to penetrate and act against the bacteria. Several antimicrobials, including sulfonamides, aminoglycosides, beta-lactams, etc., have been used in the treatment of actinomycetoma (2, 5, 17). However, in some cases cure is not obtained, making it important to evaluate *in vitro* and *in vivo* the activity of new antimicrobials.

Given the close phylogenetic relationship among actinobacteria, it is possible that some antitubercular agents are active against nocardiae. Among the most recently developed antitubercular compounds, PA-824 {(S)-2-nitro-6-[4-(trifluoromethoxy)benzyloxy]-6,7-dihydro-5H-imidazo[2,1-b][1,3] oxazine} has shown the best and most promising results (4, 12). *In vitro*, those compounds present MIC values for *Mycobacterium tuberculosis* isolates similar to those of isoniazid (MIC of PA-824, 0.015 to 0.25 μg/ml; MIC of isoniazid, 0.03 to 0.06 μg/ml) (12). PA-824 acts as a prodrug activated through a bioreduction process within the *M. tuberculosis* cell, and it is efficient against both latent and replicating *M. tuberculosis*. Transcriptional analysis has revealed a mixed potential mechanism of action that operates both by affecting cell wall synthesis and by chemical poisoning. The latter is achieved by increasing the intracellular amount of toxic nitric oxide (NO) (7, 10, 11). The development of *M. tuberculosis* mutants and its whole-genome resequencing showed the importance of a gene named *ddn* (*Rv3547*) in PA-824 resistance. This gene encodes a 151-amino-acid protein, a deazaflavin-dependent nitroreductase (Ddn); orthologous genes have been found in other actinobacteria (7).

1,3-Benzothiazin-4-one (benzothiazinone [BTZ]) compounds have been recently described that have excellent activity against actinobacteria, including *Corynebacterium*, *Mycobacterium*, *Rhodococcus*, and *Nocardia* (6). They are particularly active against *Mycobacterium tuberculosis in vitro* and *in vivo*, with BTZ043 showing a MIC of 1 ng/ml for the control strain *M. tuberculosis H37Rv*. This value is far below that of other active drugs, including rifampin and isoniazid. The biochemical target, the decaprenyl-phosphoribose-2′-epimerase (encoded by gene *dprE1*), is commonly distributed among actinobacteria (6, 13).

In the present work, we analyze the susceptibility of 30 *N. brasiliensis* isolates from human mycetoma to these compounds by a broth microdilution method.

We studied 30 isolates from the collection of the Laboratorio Interdisciplinario de Investigación Dermatológica (LIID) of the Servicio de Dermatología, Hospital Universitario, Universidad Autónoma de Nuevo León (UANL), including *N. brasiliensis* HUJEG-1 utilized previously in other *in vitro* and *in vivo* assays (1, 9, 15). All the isolates came from human cases of actinomycetoma and were identified as *N. brasiliensis* by biochemical methods and by nucleotide sequence analysis of a fragment of the 16S rRNA gene as described before (14).

PA-824 was kindly donated by the Global Alliance for TB Drug Development; BTZ043 was provided by one of the authors of the present study.

The broth microdilution method based on the CLSI M24-A2 document that we used has been described before (3). As external controls, we used *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. PA-824 was tested at concentrations of 64 to 0.25 μg/ml. In the case of BTZ043, the lowest concentration used was 0.0015 μg/ml. *M. tuberculosis* H37Rv was used also as a control.

The *dprE1* gene from *N. brasiliensis* was obtained by comparing the *dprE1* (locus *Rv3790*) gene sequence of *M. tuberculosis* H37Rv to the entire genome sequence of *N. brasiliensis* HUJEG-1 obtained by our group (16) by using the BLAST program available at the NCBI Internet site. To establish the presence of a putative *ddn* (Rv3547) ortholog in *N. brasiliensis*, we utilized the *M. tuberculosis* H37Rv nucleotide sequence published in GenBank and compared it to the complete chromosomal sequence of *N. brasiliensis* HUJEG-1 by the use of the BLAST program.

The MIC_{50} and MIC_{90} values of PA-824 for the *N. brasiliensis* isolates were >64 μg/ml in both cases. *N. carnea* ATCC 6847 and *N. transvalensis* ATCC 6865 showed the same MIC value. As a resistant control, we tested *M. smegmatis* LR222, for which the

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N. brasiliensis HUJEG-1       SALNVFKLFGAGNPAPLSFPMPGWNICVDFPIKPGLNELVSEL
N. farcinica nfa1970          SFLNVFKYFGQGNQAPLSFPMPGWNVCLDFPIKPGLNEFVTEL
M. tuberculosis (Rv3790)      SFLNVFKLFGPRNQAPLSFPIPGWNICVDFPIKDGLGKFVSEL
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FIG 1 Alignment of the BTZ resistance-determining region of *N. brasiliensis* HUJEG-1 with the corresponding DPR protein sequences of *N. farcinica* and *M. tuberculosis*, both BTZ-sensitive organisms. In red we show the Cys387 amino acid related to resistance to this antimicrobial.

drug MIC was >64 μg/ml. The MIC value for the susceptible control, *M. tuberculosis* H37Rv, was 0.125 μg/ml.

The BTZ043 MIC_{50} and MIC_{90} values were 0.125 and 0.25 μg/ml, respectively. The MIC for *N. carnea* ATCC 6847 was 0.003 μg/ml, for *N. transvalensis* ATCC 6865 was 0.003 μg/ml, for *N. brasiliensis* NCTC10300 was 0.03 μg/ml, and for *N. brasiliensis* HUJEG-1 was 0.125 μg/ml. The MIC value for *M. tuberculosis* H37Rv was 0.000976 μg/ml. The MIC values of both PA-824 and BTZ-043 were >64 μg/ml for *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 29213.

Comparing the *M. tuberculosis dprE1* gene sequence to the complete sequence of *N. brasiliensis*, we found a sequence with 74% homology encoding a 493-amino-acid protein (accession number ZP_09840034.1) similar to the FAD binding 4 superfamily proteins of other actinobacteria, including *Nocardia*, *Gordonia*, *Mycobacterium*, *Amycolatopsis*, *Corynebacterium*, etc. In Fig. 1 we show the multiple alignment of the part of the DPR protein associated with BTZ043 resistance (6). The *N. brasiliensis* sequence presents a Cys (cysteine) in position 387 (of the *M. tuberculosis* H37Rv gene), which corresponds to a susceptible genotype.

Analyzing the *M. tuberculosis ddn* gene sequence (Rv3547) by the use of the BLAST program against all the sequences reported in GenBank, only a few pertinent sequences were found, mostly from slowly growing mycobacteria. However, we could not find a *ddn* gene in the *N. brasiliensis* HUJEG-1 genome. PA-824 is one candidate for use in human cases of tuberculosis; a recent study in humans has demonstrated good early bactericidal activity and safety (4). Unfortunately, our results indicate a low susceptibility of *N. brasiliensis* isolates.

The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* has prompted the development of new antituberculosis compounds. Recently, drugs belonging to the class of benzothiazinones (BTZs) have been demonstrated to be highly active against *M. tuberculosis in vitro*, *ex vivo*, and *in vivo* (6). BTZs act by binding covalently to the enzyme DprE1(Rv3790) (13). This enzyme and DprE2 catalyze the conversion of decaprenylphosphoryl-D-ribose (DPR) to decaprenylphosphoryl-D-arabinose (DPA), which is essential for the building of arabinogalactan and lipoarabinomannan, important components of the mycobacterial cell wall. BTZ compounds bind to a cysteine residue at position 387 in the DprE1 active site, leading to the formation of a covalent complex. In *M. tuberculosis* BTZ-resistant mutants and in other mycobacteria that are naturally resistant, Cys387 of DprE1 is replaced by serine or glycine. In our case, we observed a susceptible genotype, demonstrating that the target is largely conserved among actinobacteria, and this raises the possibility that this drug is also active against *N. brasiliensis ex vivo* and *in vivo* in the experimental model of infection.

PA-824 was selected because it is being tested in clinical trials and may thus become available for tuberculosis patients. Unfortunately, the *in vitro* MIC values were too high against *Nocardia* spp., thereby decreasing the possibility of any *in vivo* activity. Resistance to PA-824 has been associated in *M. leprae* with the lack of an orthologous gene encoding Ddn, a nitro reductase (8); Ddn is essential for the anaerobic killing of *M. tuberculosis* by PA-824, which acts as a NO donor. Our group has recently sequenced the complete genome of *N. brasiliensis* HUJEG-1, which allowed us to search for a similar gene, but we did not find such a gene. The antimicrobial susceptibility of actinobacteria can be associated with common biochemical pathways such as cell-wall biogenesis (like BTZs) or with very specific genes such as *ddn*, found only in some mycobacteria. However, as we describe here, availability of the genome sequence can help in predicting antimicrobial susceptibility.

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