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Efficacy of Nitazoxanide against Clinical Isolates of *Mycobacterium tuberculosis*

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Nitazoxanide (NTZ) has bactericidal activity against the *H37Rv* laboratory strain of *Mycobacterium tuberculosis* with a MIC of 16 μg/ml. However, its efficacy against clinical isolates of *M. tuberculosis* has not been determined. We found that NTZ's MIC against 50 clinical isolates ranged from 12 to 28 μg/ml with a median of 16 μg/ml and was unaffected by resistance to first- or second-line antituberculosis drugs or a diversity of spoligotypes.

Treatment of drug-sensitive tuberculosis (TB) currently involves treatment with four drugs for 2 months and two drugs for another 4 months. Failure to adhere to this regimen or ingestion of diluted or counterfeit drugs may lead to emergence of drug-resistant TB. TB most often occurs in resource-poor settings that lack the health care system necessary to ensure adherence (1, 2). HIV infection increases the risk for developing active TB and is fueling the TB epidemic, particularly in places where both infections are prevalent. TB remains one of the leading causes of death in AIDS patients, and treatment of TB in such patients requires longer duration of therapy and is associated with a high recurrence rate.

Moreover, primary drug-resistant TB is rarely recognized as such when patients first present, owing to the lack of facilities for drug sensitivity testing (DST). As a consequence, multidrug-resistant (MDR) TB, defined as *Mycobacterium tuberculosis* disease that is resistant to the first-line drugs isoniazid and rifampin, is increasing in prevalence (3–6). Also increasing is extensively drug-resistant (XDR) TB caused by strains of *M. tuberculosis* that are also resistant to the second-line quinolones and injectable aminoglycoside and peptide antibiotics (7, 8). The discovery of new TB drugs is therefore a public health priority (9, 10).

Nitazoxanide (NTZ) (Alina; Romark Laboratories) is a widely used anti-infective that is remarkable for both the breadth of its clinical indications and its record of safety (11, 12). NTZ, a synthetic nitrothiazolyl salicylamide, is deacetylated in the gastrointestinal tract to the active metabolite tizoxanide (13, 14). NTZ is approved for the treatment of giardiasis and cryptosporidiosis (15, 16) and has broad-spectrum activity against other protozoa, helminths, and the anaerobic or microaerophilic bacteria *Clostridium difficile* and *Helicobacter pylori*, as well as showing effectiveness in infections caused by rotavirus and hepatitis C (14, 17–22).

It was recently reported that NTZ and tizoxanide killed the H37Rv reference strain of *M. tuberculosis in vitro*, both when *M. tuberculosis* was replicating and when its replication was blocked by physiologic conditions of acidity and nitrosative stress (23). The ability of a given compound to kill both replicating and nonreplicating *M. tuberculosis* is uncommon (24). The mycobactericidal activity of NTZ was both dose and time dependent but minimally inoculum dependent (23). No resistant mutants were identified in multiple experiments that implied a frequency of resistance of $<10^{-13}$, suggesting that nitazoxanide may have multiple targets (23). The aim of the present study was to evaluate the MIC of NTZ against clinical isolates of *M. tuberculosis* with various drug resistance patterns.

Sputum specimens for *M. tuberculosis* culture were collected at Le Groupe Haïtien d'Etude du Sarcome de Kaposi et des Infections Opportunistes (GHESKIO) in Port-au-Prince, Haiti, stored at 4°C, and processed within 3 days. Samples were decontaminated with *N*-acetyl cysteine-sodium hydroxide, washed, resuspended, cultured in Bactec MGIT growth indicator tubes according to the manufacturer's instructions (Becton Dickenson), and incubated in a Bactec 960 MGIT device. Aliquots were frozen at −70°C in 30% glycerol.

Conventional DSTs were performed on positive cultures using the Bactec MGIT 960 SIRE kit with 1.0 μg/ml streptomycin, 0.1 μg/ml isoniazid, 1.0 μg/ml rifampin, and 5.0 μg/ml ethambutol. Pyrazinamide (PZA) susceptibility testing was performed using Bactec MGIT 960 PZA kits at pH 6.0 with 100 μg/ml pyrazinamide. Ofloxacin susceptibility testing was performed using a concentration of 2.0 μg/ml with a proportion method on 7H10 agar as recommended by the Clinical and Laboratory Standards Institute (25).

Spoligotyping was performed using standard PCR-based methods determining variations in the presence or absence of 43 direct-repeat interspacers (26). Spoligotyping was performed using a Luminex platform for high-throughput detection of multiple simultaneous DNA sequences. The Luminex system incorporates microspheres containing two fluorochromes, with oligonucleotide probes attached to each microsphere as previously described (27). Results were referenced against the SITVITWEB international database to assign spoligotype number and lineage (28).

The present study used 50 typed isolates, including 20 pan-

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sensitive specimens, 3 specimens resistant to isoniazid but sensitive to other first-line drugs, and 27 isolates resistant to both isoniazid and rifampin, with various resistances to streptomycin (21), ethambutol (22), and pyrazinamide (14). Two of the MDR specimens were also resistant to ofloxacin.

In the GHESKIO biosafety level 3 laboratory, *M. tuberculosis* isolates were grown in Bactec MGIT 960 as described above. Cultures were vortexed for 10 s and subcultured in Difco Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) and tyloxapol to an optical density at 580 nm (OD_{580}) of 0.01 and then subcultured to an OD_{580} of 0.6 to 0.8. Cultures were diluted in 7H9/OADC/tyloxapol to an OD_{580} of 0.01, and 200 μl was added to 96-well plates whose outer wells were filled with phosphate-buffered saline (PBS)/tyloxapol to minimize evaporative losses. NTZ (2 μl) was prediluted in dimethyl sulfoxide (DMSO) to appropriate concentrations and added to produce final concentrations of 0 to 36 μg/ml in 4-μg/ml increments (final DMSO concentration ≤ 1%). Assays were performed in triplicate. After 10 days of incubation at 37°C, MICs were determined by visual inspection. Each set of experiments included a MIC determination for rifampin on a pansensitive strain as a positive control.

MICs for NTZ ranged from 12 to 28 μg/ml with a median of 16 μg/ml and a mean of 17.6 μg/ml (Table 1). There was no significant difference in MICs between the drug-sensitive strains and the drug-resistant strains ($P = 0.22$; Student's *t* test). The MICs for the 20 pansensitive isolates ranged from 12 to 28 μg/ml with a median of 18 μg/ml and a mean of 17.9 μg/ml. The MICs for the 30 drug-resistant isolates ranged from 12 to 28 μg/ml with a median of 16 μg/ml and a mean of 17.5 μg/ml.

Twenty-two different spoligotypes from 5 different *M. tuberculosis* lineages were represented among the isolates (29). Spoligotype did not appear to affect the MIC, but the sample size was insufficient for statistical analysis. MICs from H and LAM lineages did not show significant variance ($P = 0.91$).

Thus, MICs of NTZ for clinical isolates of *M. tuberculosis* were not significantly different from that seen for the *H37Rv* laboratory strain and were not affected by resistance to first- and second-line TB drugs or by spoligotype. Plasma NTZ levels of 30.7 μg/ml have been observed in human volunteers following the administration of 1g NTZ twice daily (b.i.d.) with minimal side effects (30). These concentrations are nearly double the median MICs observed among sputum samples shown in this report as well as concentrations shown to be bactericidal against *H37Rv* (23).

NTZ and tizoxanide accumulate in *M. tuberculosis* and disrupt *M. tuberculosis*'s membrane potential and intrabacterial pH homeostasis (31). Neither of these mechanisms accounts for NTZ's synergistic mycobactericidal activity with reactive nitrogen intermediates (31), and NTZ's molecular targets within *M. tuberculosis* are unknown. In protozoa, NTZ inhibits pyruvate-ferredoxin oxidoreductase (PFOR). PFOR is an essential enzyme in some anaerobic and microaerophilic microbes but has not been identified in *M. tuberculosis* (32).

NTZ has host targets as well, leading to immune modulatory effects that may augment its direct antimicrobial actions. For example, NTZ activates the double-stranded RNA (dsRNA)-dependent protein kinase PKR, leading to increased phosphorylation of eukaryotic initiation factor 2α (eIF2α) (17, 22, 33). NTZ inhibits the quinone reductase NQO1, which contributes to suppression of signaling by the mammalian target of rapamycin (mTORC1)

TABLE 1 Nitazoxanide MICs on 50 clinical isolates with multiple drug resistance patterns and spoligotypes[a]

Specimen no.	Spoligotype	*M. tuberculosis* lineage	Drug resistance	Mean NTZ MIC (μg/ml)
1	42	LAM	None	16
2	4	ND	None	17.3
3	ND	ND	None	20
4	5	T	None	24
5	42	LAM	None	18.7
6	42	LAM	None	16
7	70	X	None	16
8	ND	ND	None	20
9	163	LAM	None	16
10	106	ND	None	20
11	51	T	None	20
12	50	Haarlem	None	20
13	2	Haarlem	None	16
14	51	T	None	20
15	168	Haarlem	None	28
16	17	LAM	None	14.7
17	168	Haarlem	None	20
18	42	LAM	None	12
19	34	S	None	12
20	50	Haarlem	None	12
21	53	T	H	26.7
22	53	T	H	18.7
23	183	Haarlem	H	24
24	909	ND	H, R	16
25	20	LAM	H, R	20
26	53	T	H, R, E	17.3
27	20	LAM	H, R, E	20
28	17	LAM	H, R, E	16
29	390	Haarlem	H, R, S	16
30	137	X	H, R, E, S	16
31	137	X	H, R, E, S	14.7
32	20	LAM	H, R, E, S	16
33	50	Haarlem	H, R, E, S	20
34	50	Haarlem	H, R, E, S	14.7
35	2281	LAM	H, R, E, S	12
36	2	Haarlem	H, R, S, Z	20
37	34	S	H, R, E, Z	16
38	119	X	H, R, S, Z	20
39	50	Haarlem	H, R, E, S, Z	16
40	93	LAM	H, R, E, S, Z	16
41	34	S	H, R, E, S, Z	12
42	455	T	H, R, E, S, Z	21.3
43	93	LAM	H, R, E, S, Z	16
44	47	Haarlem	H, R, E, S, Z	13.3
45	455	T	H, R, E, S, Z	24
46	93	LAM	H, R, E, S, Z	17.3
47	50	Haarlem	H, R, E, S, Z	16
48	17	LAM	H, R, E, S, Z	16
49	93	LAM	H, R, E, S, Ofx	16
50	93	LAM	H, R, E, S, Z, Ofx	16

[a] The MICs of nitazoxanide (NTZ) against 20 pansensitive and 30 drug-resistant clinical isolates of *Mycobacterium tuberculosis* are shown. Resistance to isoniazid (H), rifampin (R), streptomycin (S), ethambutol (E), pyrazinamide (Z), and ofloxacin (Ofx) is indicated. Assays were run in triplicate. The mean is reported, with the standard error of the mean ranging from 0 to 1.9. Spoligotypes are assigned a numerical code according to international standards (29). Isolates not coded in the international database are reported as not defined (ND). Lineage designation also follows international standards.

and is associated with stimulation of autophagy within macrophages, enhancing their mycobactericidal activity (34).

In summary, NTZ has significant bactericidal activity against replicating and nonreplicating *H37Rv M. tuberculosis*, exhibits an ultralow frequency of resistance, and has an excellent clinical safety record when used as an approved antimicrobial agent for other indications. NTZ is quantitatively glucuronidated in mice, and the glucuronide is inactive against *M. tuberculosis* (L. P. Sorio de Carvalho and C. Nathan, unpublished observations), so studies of NTZ in mouse models of tuberculosis have not been informative. Given the safety record of NTZ in humans and the present demonstration that NTZ was comparably effective *in vitro* against *M. tuberculosis* from 50 clinical isolates with various drug resistance patterns and spoligotypes at clinically relevant concentrations, we believe that trials of the 2-week early bactericidal activity of the drug are warranted for the experimental treatment of drug-resistant TB.

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