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Activity of *n*-Propyl Pyrazinoate against Pyrazinamide-Resistant *Mycobacterium tuberculosis*: Investigations into Mechanism of Action of and Mechanism of Resistance to Pyrazinamide

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The mechanism of action of pyrazinamide (PZA) is not known. One hypothesis is that PZA functions as a prodrug of pyrazinoic acid. Susceptibility to PZA correlates with amidase activity of the *Mycobacterium tuberculosis* isolate in question. PZA-resistant isolates retain susceptibility in vitro to pyrazinoic acid and *n*-propyl pyrazinoate. Esters of pyrazinoic acid appear to circumvent the requirement for activation by mycobacterial amidase. The MICs of *n*-propyl pyrazinoate for *M. tuberculosis* isolates are lower than those of pyrazinoic acid. Further studies to assess the effects of modifications of the alcohol and pyrazine moieties of pyrazinoate esters on in vitro and in vivo antituberculosis activity are under way. This may lead to a candidate compound with enhanced activity against both PZA-susceptible and PZA-resistant *M. tuberculosis* isolates suitable for clinical development.

Pyrazinamide (pyrazinoic acid amide) (PZA) is a first-line agent for the treatment of tuberculosis (1, 5) and an essential element of experimental preventive therapy regimens (7, 12). Despite a lack of demonstrable in vitro activity with media at pH 6.6, this nicotinamide analog was shown to have activity in animal models of tuberculosis (6, 14). Subsequent demonstration of activity in vitro required the use of an acid medium, generally at pH 5.0 to 5.5 (16). PZA was first used in the treatment of pulmonary tuberculosis in humans in 1949 (20).

The mechanism of action of PZA is not known. It appears that PZA functions as a prodrug of pyrazinoic acid (PA) and is converted to PA intracellularly. However, the biochemical basis for the antituberculosis activity of PA has not been established (8, 13, 16). While PZA-susceptible and PZA-resistant isolates are generally susceptible to PA in vitro, the acid does not appear to have activity in vivo (10).

Susceptibility to PZA correlates with amidase activity of the *Mycobacterium tuberculosis* isolate in question (2, 10, 15). The majority of PZA-resistant isolates of *M. tuberculosis* have decreased or no amidase activity and are presumably unable to convert PZA to the active agent. One purpose of the present study was to assess the qualitative and quantitative amidase activities of a series of *M. tuberculosis* isolates. Isolates were chosen to encompass a range of in vitro susceptibilities to PZA. Selected nontuberculous mycobacterial isolates were also evaluated for amidase activity.

A second purpose of the present study was to further evaluate the in vitro activity of *n*-propyl pyrazinoate (*n*PPA) against a series of PZA-susceptible and PZA-resistant isolates of *M. tuberculosis*. A series of esters of PA previously synthesized demonstrated enhanced in vitro activity against both PZA-susceptible and PZA-resistant *M. tuberculosis* isolates (3). We hypothesized that PA esters would not require activation by amidase but would be converted to PA by mycobacterial esterase. Susceptibility to *n*PPA was evaluated in parallel with susceptibilities to PZA and PA. Evaluation of amidase activity of mycobacterial isolates coupled with determination of in vitro susceptibilities to PZA, PA, and *n*PPA may help delineate a possible mechanism(s) of action and potential mechanisms of resistance to PZA.

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MATERIALS AND METHODS

Mycobacterial isolates. Isolates of *M. tuberculosis*, *M. bovis*, and *M. microti* were kindly provided by Margaret Weginbothom (National Tuberculosis Reference Laboratory, Cardiff, Wales), Leonid B. Heifets (Mycobacteriology Clinical Reference Laboratory, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.), Adalbert Laszlo (National Reference Center for Tuberculosis, Ottawa, Canada), and Salman H. Siddiqi (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). *M. tuberculosis* DOL, *M. kansasii* S, and *M. kansasii* P were obtained as clinical isolates from patients at the Veterans Affairs Medical Center and the State University of New York Health Science Center, Syracuse, N.Y. Additional strains of *M. bovis* (ATCC 27289 and ATCC 35720) and *M. tuberculosis* (ATCC 25618, ATCC 35828, ATCC 27294, and ATCC 35801) were obtained from the American Type Culture Collection, Rockville, Md.

Drugs. PZA was obtained from Sigma Chemical Company, St. Louis, Mo. PA was obtained from Aldrich Chemical Company, Milwaukee, Wis. *n*PPA was synthesized as previously described (3).

Stock solutions were prepared by dissolving each compound in modified 7H10 broth (7H10 agar formulation with agar and malachite green omitted), pH 5.8, with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, Detroit, Mich.) at a concentration of 2,048 μg/ml. Stock solutions were sterilized by passage through a 0.22-μm-pore-size membrane filter. Stock solution of PA was adjusted to pH 5.8 with 1 N KOH prior to sterilization. Serial twofold dilutions of each compound were made in modified 7H10 broth (concentrations ranged from 2,048 to 0.5 μg/ml).

Susceptibility testing. Mycobacteria were grown in modified 7H10 broth, pH 6.6, with 10% OADC enrichment and 0.05% Tween 80 (18). Cell suspensions were diluted in modified 7H10 broth, pH 5.8, to yield 2 Klett units/ml (Klett-Summerson colorimeter; Klett Manufacturing, Brooklyn, N.Y.) or approximately 10^6 CFU/ml. A 0.1-ml volume of culture suspension was added to each tube containing drug in 1.9 ml of modified 7H10 broth, pH 5.8, yielding a final inoculum of approximately 5×10^4 CFU/ml. Susceptibility testing was performed with modified 7H10 broth, pH 5.8, because some isolates of *M. tuberculosis* grow poorly at pH 5.6, the standard pH used for susceptibility testing in agar. Inoculum size was determined by titration and counting from duplicate 7H10 agar plates (BBL Microbiology Systems, Cockeysville, Md.). A tube without drug was included for each isolate as a positive control. Tubes were incubated on a rotary shaker (190 rpm) at 37°C for 2 to 4 weeks. The MIC was defined as the lowest concentration of drug that yielded no visible turbidity.

Qualitative niacin test. Tubes containing 10 ml of 7H10 agar (BBL Microbiology Systems) with 0.05% glycerol, 0.25% L-asparagine (Sigma Chemical Company), and 10% OADC enrichment were prepared (9, 21). The agar was allowed

to solidify with the tubes in an upright position. Each tube was inoculated with 0.1 ml of heavy culture suspension. Tubes were incubated at 37°C for 4 weeks.

A 1.5-ml volume of distilled water was added to each tube to permit extraction of niacin. After 30 min, a 0.6-ml aliquot of the water was removed and placed in a test tube (16 by 100 mm). A niacin test strip (Difco Laboratories) was placed in each test tube and allowed to react for 15 min. Development of a yellow color indicated production of niacin (9, 21). Known niacin-negative and niacin-positive culture suspensions were included with each experiment as controls.

Qualitative pyrazinamidase test. Tubes containing 10 ml of 7H10 agar with 0.05% glycerol and 0.1% PZA were prepared for pyrazinamidase detection by the Wayne method (19). Each tube was inoculated with 1 ml of heavy culture suspension, and tubes were incubated at 37°C for 4 days.

A 1-ml volume of freshly prepared 1% ferrous ammonium sulfate solution was added to each tube. Samples were allowed to stand at 4°C for 4 h. The appearance of a pink band in the agar indicated pyrazinamidase activity. Positive and negative controls were included with each experiment.

HPLC assays for esterase and pyrazinamidase. The high-performance liquid chromatography (HPLC) system consisted of a model AS-100 automatic sampler, a model CR501 Chromatopac integrator (both from Bio-Rad Laboratories, Hercules, Calif.), an LDC miniPump (LDC Analytical, Riviera Beach, Fla.), and a Dynamax UV-C absorbance detector (Rainin Instrument Co., Inc., Woburn, Mass.). A μBondapak C_{18} column (Waters Associates, Inc., Milford, Mass.) was used to achieve separation of the desired compounds. A 50% methanol isocratic mobile phase was used at a flow rate of 1 ml/min. PA and other compounds containing the PA moiety were detected by A_{270}. Standard curves from 1 to 100 μg/ml were made in modified 7H10 broth, pH 6.6, for each analyte (detection limit, 1 μg/ml). Injection volumes were 50 μl in all experiments.

Mycobacterial suspensions were grown in modified 7H10 broth to between 100 and 400 Klett units/ml. Titers of aliquots of cell suspensions were determined in duplicate on 7H10 agar to ascertain the initial viable cell count. A 1-ml sample of a stock solution (1 mg/ml) of either PZA or *n*PPA was added to 9 ml of culture suspension. Culture tubes were incubated on a rotary shaker at 37°C. Aliquots were taken at 0.5 h for esterase assay and at 6 h for pyrazinamidase assay. Samples were passed through a 0.22-μm-pore-size membrane filter and frozen at −20°C until analyzed. Results are expressed as nanomoles per hour per 10^8 CFU.

RESULTS

In vitro susceptibility. The broth dilution MICs of PZA, PA, and *n*PPA for the *M. tuberculosis* isolates ($n = 30$) are shown in Table 1. The MIC range for PZA was from ≤4 to >2,048 μg/ml. The MIC ranges for PA and *n*PPA were from ≤1 to 32 μg/ml and from ≤0.5 to 32 μg/ml, respectively.

Qualitative niacin test. Results of the niacin test for the *M. tuberculosis* isolates are shown in Table 1.

Qualitative pyrazinamidase tests. Results of the qualitative pyrazinamidase tests, done by the Wayne agar method and HPLC assay, are shown in Table 1. There was good agreement between the two methods. Only isolate CDC89 tested amidase negative by the agar method and had amidase activity detected by HPLC.

HPLC assays for esterase and pyrazinamidase. All *M. tuberculosis* and nontuberculous isolates exhibited esterase activity as detected by HPLC (data not shown). A comparison of the breakdown rates of PZA and *n*PPA by PZA-susceptible *M. tuberculosis* ATCC 27284 demonstrated that ester hydrolysis was significantly more rapid than PZA deamidation (data not shown).

Results for quantitative pyrazinamidase activity determined by HPLC assay are noted in Table 2. The average pyrazinamidase activity is inversely related to the MIC of PZA for *M. tuberculosis* isolates. Neither PZA nor *n*PPA was hydrolyzed when assayed in 7H10 broth in the absence of mycobacterial cells.

Evaluation of nontuberculous mycobacterial isolates. The broth dilution MICs of PZA, PA, and *n*PPA for the six nontuberculous isolates are shown in Table 3. Results for niacin tests and pyrazinamidase tests are noted in the same table.

TABLE 1. MICs of drugs used in this study and results of pyrazinamidase and niacin tests

M. tuberculosis strain	MIC (μg/ml)[a] of:			Result of test for:		
				Pyrazinamidase		Niacin
	PZA	PA	*n*PPA	Agar	HPLC	
CDC89	≤4	1	≤0.5	−	+	+
CDC72	8	≤1	≤0.5	+	+	+
M142	8	8	4	+	+	−
T2603	16	16	8	+	+	−
T2614	32	8	4	+	+	+
DOL	32	32	4	+	+	+
H6777	32	4	1	+	+	−
ATCC 27294	32	32	4	+	+	+
ATCC 25618	64	16	8	+	+	+
M4597	64	32	2	+	+	+
ATCC 35801	128	32	8	+	+	+
F7091	256	32	16	+	+	+
H6128	256	32	16	+	+	+
W6126	256	32	16	+	+	−
DHMH 444	256	32	4	−	−	+
CA516	512	8	1	−	−	−
W11053	512	8	1	−	−	+
H3652	1,024	≤8	≤8	−	−	−
M4812	2,048	16	8	−	−	+
DHMH 5207	>2,048	16	1	−	−	−
DHMH 4319	>2,048	16	2	−	−	+
W4693	>2,048	32	32	−	−	−
T5721	>2,048	32	32	−	−	+
ATCC 35828	>2,048	32	32	−	−	+
M4809	>2,048	32	8	−	−	+
VA 205	>2,048	32	16	−	−	+
DHMH 6577	>2,048	32	4	−	−	+
BDDIS 20	>2,048	32	16	−	−	+
CDC-BP-98	>2,048	32	16	−	−	+
T494	>2,048	128	4	−	−	+

[a] Determined in modified 7H10 broth.

DISCUSSION

The MICs of PA and *n*PPA for *M. tuberculosis* are more favorable than are those of PZA. PZA-resistant isolates (except T494) retain susceptibility in vitro to PA and *n*PPA. This supports the hypothesis that esters of PA can circumvent the requirement for activation by mycobacterial amidase. The MICs of *n*PPA for *M. tuberculosis* are the same or one- to fivefold lower than those of PA. This suggests that the biochemical basis for antituberculosis activity of PA (and hence PZA) is not simply the reduction in pH caused by the PA moiety (7). An alternate explanation could be that PA and *n*PPA are transported more efficiently than PZA into the mycobacterial cell.

TABLE 2. Relationship between MIC of PZA and pyrazinamidase activity

MIC of PZA (μg/ml)	*n*	Avg pyrazinamidase activity[a]
≤4	1	50
8	2	45
16	1	34
32	4	30
128	1	20
256	5	5
≥512	16	0

[a] Expressed as nanomoles of PA per hour per 10^8 CFU.

TABLE 3. Results for nontuberculous mycobacteria

Isolate	MIC (μg/ml) of:			Result of test for:		
				Pyrazinamidase		Niacin
	PZA	PA	*n*PPA	Agar	HPLC	
M. bovis 35720	2,048	8	32	−	+	−
M. bovis 27289	>2,048	32	32	−	−	−
M. bovis T7941	>2,048	16	8	−	−	−
M. kansasii S	>2,048	256	128	−	+	−
M. kansasii P	2,048	512	128	−	+	−
M. microti NT206	128	32	32	+	+	+

Mycobacterial amidase (pyrazinamidase or nicotinamidase) catalyzes the conversion of PZA to PA and nicotinamide to nicotinic acid (niacin) (17). The crucial mycobacterial amidase has not been isolated or characterized. Results of the qualitative niacin test do not agree with the results of the pyrazinamidase assays. Four PZA-susceptible *M. tuberculosis* isolates (isolates M142, T2603, H6777, and W6126, all with detectable pyrazinamidase activity) were niacin negative. Conversely, 12 of 16 PZA-resistant isolates (MIC, ≥512 mg/ml), none with detectable pyrazinamidase activity, produced niacin. The niacin test detects niacin and nicotinamide (11). Furthermore, the niacin test measures traffic over the NAD pathway rather than a specific enzymatic step in the NAD pathway (nicotinamide deamination) as measured by the nicotinamidase or pyrazinamidase assay.

Results of the pyrazinamidase tests, done by the Wayne agar method and HPLC assay, are in good agreement. Only *M. tuberculosis* isolate CDC89 tested amidase negative by the agar method and had amidase activity detectable by HPLC. Quantitation of pyrazinamidase activity shows an inverse relationship between the MIC of PZA and average pyrazinamidase activity as measured by production of PA.

The results for the nontuberculous mycobacterial species are of interest. Isolates of *M. bovis* and *M. kansasii* are resistant to PZA in vitro, the MIC being ≥2,048 μg/ml. Despite the in vitro resistance, pyrazinamidase activity was detectable by the HPLC method for one of three *M. bovis* isolates and for both *M. kansasii* isolates. This suggests an alternative mechanism of resistance to PZA for the nontuberculous species, such as decreased transport (4) or enhanced efflux. Each of the *M. bovis* and *M. kansasii* isolates tested was niacin negative. Both PA and *n*PPA are active in vitro against PZA-resistant *M. bovis* and *M. kansasii*. Results noted for *M. microti*, a PZA-susceptible species closely related to *M. tuberculosis*, are similar to those for PZA-susceptible *M. tuberculosis.*

Further studies to assess the effects of modifications of the alcohol and pyrazine moieties of the pyrazinoate esters on in vitro antituberculosis activity are under way. This work may lead to a candidate compound with enhanced activity against both PZA-susceptible and PZA-resistant *M. tuberculosis* isolates, in addition to further delineating the mechanism of action of PZA and potential mechanisms of resistance to PZA.

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REFERENCES

1. **Bass, J. B., Jr., L. S. Farer, P. C. Hopewell, R. O'Brien, R. F. Jacobs, F. Ruben, D. E. Snider, Jr., and G. Thornton.** 1994. Treatment of tuberculosis and tuberculosis infection in adults and children. Am. J. Respir. Crit. Care Med. **149:**1359–1374.
2. **Butler, R. W., and J. O. Kilburn.** 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob. Agents Chemother. **24:**600–601.
3. **Cynamon, M. H., S. P. Klemens, T.-S. Chou, R. H. Gimi, and J. T. Welch.** 1992. Antimycobacterial activity of a series of pyrazinoic acid esters. J. Med. Chem. **35:**1212–1215.
4. **David, H. L.** 1981. Basis for lack of drug susceptibility of atypical mycobacteria. Rev. Infect. Dis. **3:**878–884.
5. **Davidson, P. T., and H. Q. Le.** 1992. Drug treatment of tuberculosis—1992. Drugs **43:**651–673.
6. **Dessau, F. L., R. L. Yeager, and M. Kulish.** 1952. Pyrazinamide (aldinamide) in experimental tuberculosis of the guinea pig. Am. Rev. Tuberc. **65:**519–522.
7. **Grosset, J. H.** 1990. Present and new drug regimens in chemotherapy and chemoprophylaxis of tuberculosis. Bull. Int. Union Tuberc. Lung Dis. **65:**86–91.
8. **Heifets, L. B., M. A. Flory, and P. J. Lindholm-Levy.** 1989. Does pyrazinoic acid as an active moiety of pyrazinamide have specific activity against *Mycobacterium tuberculosis*? Antimicrob. Agents Chemother. **33:**1252–1254.
9. **Kilburn, J. O., and G. Kubica.** 1968. Reagent impregnated paper strips for detection of niacin. Am. J. Clin. Pathol. **50:**530–532.
10. **Konno, K., F. M. Feldmann, and W. McDermott.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. **95:**461–469.
11. **Konno, K., R. Kurzmann, and K. T. Bird.** 1957. The metabolism of nicotinic acid in mycobacteria. Am. Rev. Tuberc. **75:**529–537.
12. **Lecoeur, H. F., C. Truffot-Pernot, and J. H. Grosset.** 1989. Experimental shortcourse preventive therapy of tuberculosis with rifampin and pyrazinamide. Am. Rev. Respir. Dis. **140:**1189–1193.
13. **Mackaness, G. B.** 1956. The intracellular activation of pyrazinamide and nicotinamide. Am. Rev. Tuberc. **74:**718–728.
14. **Malone, L., A. Schurr, H. Lindh, D. McKenzie, J. S. Kiser, and J. H. Williams.** 1952. The effect of pyrazinamide (aldinamide) on experimental tuberculosis in mice. Am. Rev. Respir. Dis. **65:**511–518.
15. **McClatchy, J. K., A. Y. Tsang, and M. S. Cernich.** 1981. Use of pyrazinamidase activity in *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. Antimicrob. Agents Chemother. **20:**556–557.
16. **McDermott, W., and R. Tompsett.** 1954. Activation of pyrazinamide and nicotinamide in acidic environments *in vitro*. Am. Rev. Tuberc. **70:**748–754.
17. **Tarnok, I., H. Pechmann, U. Krallmann Wenzel, E. Rohrscheidt, and Z. Tarnok.** 1979. Nicotinamidase and the so-called pyrazinamidase in mycobacteria; the simultaneous occurrence of both activities. Zentralbl. Bakteriol. **244:**302–308. (In German.)
18. **Vestal, A. L.** 1969. Procedures for the isolation and identification of mycobacteria, p. 113–115. Public Health Service publication no. 1995. Laboratory Division, National Communicable Disease Center, Atlanta.
19. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am. Rev. Respir. Dis. **109:**147–151.
20. **Yeager, R. L., W. G. C. Munroe, and F. L. Dessau.** 1952. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. Am. Rev. Tuberc. **65:**523–546.
21. **Young, W. D., Jr., A. Maslansky, M. S. Lefar, and D. P. Kronish.** 1970. Development of a paper strip test for detection of niacin produced by mycobacteria. Appl. Microbiol. **20:**939–945.