

Iron enhances the antituberculous activity of pyrazinamide

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Received 12 September 2003; returned 16 October 2003; revised 23 October 2003; accepted 29 October 2003

Background: Pyrazinamide is a paradoxical frontline tuberculosis drug characterized by high *in vivo* sterilizing activity but poor *in vitro* activity. This separation in pyrazinamide activity reflects differences between the *in vivo* tissue environment and *in vitro* culture conditions. The well-known acid pH requirement for pyrazinamide activity was discovered previously based on such reasoning but does not completely explain the discrepancy between *in vivo* and *in vitro* activity of pyrazinamide. This study examined the effect of iron, which could potentially be elevated in local inflammatory lesions, on pyrazinamide activity *in vitro*.

Materials and methods: The effect of iron on the activity of pyrazinamide or its active derivative pyrazinoic acid against *Mycobacterium tuberculosis* was assessed in liquid medium in a drug exposure assay or in solid medium with pyrazinamide plus iron or pyrazinamide alone. The effect of iron on pyrazinamide or pyrazinoic acid was expressed as percentage of growth inhibition.

Results: We have shown that iron enhances the activity of pyrazinamide and pyrazinoic acid against *M. tuberculosis* in both liquid and solid media at acid pH 5.6. Iron enhanced the activity of pyrazinoic acid but not pyrazinamide against the naturally pyrazinamide-resistant *Mycobacterium bovis* BCG. Other metal ions such as magnesium, calcium and zinc did not enhance the activity of pyrazinamide or pyrazinoic acid.

Conclusions: Iron increased the activity of pyrazinamide or pyrazinoic acid against *M. tuberculosis* *in vitro*. These findings may have implications for the study of mechanism of action of pyrazinamide and possible iron supplement for improving the activity of pyrazinamide.

Keywords: pyrazinamide, iron, drug susceptibility, *Mycobacterium tuberculosis*

Introduction

Pyrazinamide is an important front-line tuberculosis (TB) drug that forms the most effective TB chemotherapy along with isoniazid, rifampicin and ethambutol.¹ Pyrazinamide plays a key role in shortening the TB therapy from previously 9–12 months to 6 months.² The ability of pyrazinamide to shorten the therapy seems to correlate with its ability to kill a special bacterial population with low metabolic activity residing in acidic pH environments² that are not killed by other TB drugs and also its ability to kill old non-growing bacilli more effectively than young bacilli.³ Pyrazinamide is a paradoxical TB drug. Despite its high sterilizing activity *in vivo*,^{4,5} surprisingly, pyrazinamide has no activity under normal culture conditions at close to neutral pH.⁶ This discrepancy between the *in vitro* and *in vivo* activity of pyrazinamide reflects possible differences between *in vivo* and *in vitro* conditions that affect the drug activity. McDermott and colleagues reasoned that one of the differences was the acid pH, which is present *in vivo* at local lesions owing to production of lactic acid by infiltrating inflammatory cells. This reasoning led to the discovery

that acid pH increases the activity of pyrazinamide.⁷ Even at acid pH, pyrazinamide kills tubercle bacilli ineffectively, killing no more than 76% of bacilli with a high pyrazinamide concentration of 1000 mg/L at pH 4.8 over a period of 2 weeks.⁸ The MIC of pyrazinamide is 50–100 mg/L at acid pH of 5.5–6.0.⁹ This MIC is somewhat higher than the serum pyrazinamide concentration of 30–60 mg/L.⁹ Thus, it appears that acid pH cannot completely explain the discrepancy between high *in vivo* activity and poor *in vitro* activity of pyrazinamide. Various factors besides acid pH,¹⁰ such as inoculum size, serum albumin, age of cultures and efflux inhibitors, could also influence the activity of pyrazinamide.³ During our ongoing study of factors that potentially influence the activity of pyrazinamide, we tested the effect of iron on the activity of pyrazinamide and its active derivative pyrazinoic acid. We reasoned that another possible difference besides acid pH between *in vitro* and *in vivo* conditions is the local iron concentration in the lesions, which could be increased during inflammation.¹¹ We show in this study that iron can indeed enhance the activity of pyrazinamide and pyrazinoic acid in various *in vitro* tests.

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Iron enhancement of pyrazinamide activity

Materials and methods

Drugs and chemicals

Pyrazinamide, pyrazinoic acid, ferrous sulphate, ferrous ammonium sulphate, ferric chloride, ferric ammonium citrate, magnesium sulphate and tetrazolium redox dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) were obtained from Sigma–Aldrich Co. Pyrazinamide was dissolved in sterile deionized water at 10 mg/mL and filter-sterilized. Pyrazinoic acid was dissolved in DMSO at 50 mg/mL. Various iron salt and MTT solutions were freshly prepared before use by dissolving in sterile deionized water at 20 mM and 2 mg/mL, respectively, and filter-sterilized before use.

Effect of iron on the activity of pyrazinamide and pyrazinoic acid as assessed in liquid medium

To assess the effect of iron on pyrazinamide or pyrazinoic acid activity, a 3-week-old *Mycobacterium tuberculosis* H37Ra culture grown in 7H9 medium with albumin-dextrose-catalase (ADC) supplement (Difco) was diluted at 1:200 in Sauton's medium (pH 5.6) containing ferrous sulphate or ferric chloride at 1.2, 0.6, 0.3, 0.15 or 0 mM and pyrazinoic acid at 60, 30, 15 or 7.5 mg/L in a 96-well microtitre plate. The microtitre plate was incubated at 37°C for 7 days before the viability of the bacteria was assessed by MTT redox dye using the method described previously.¹² MTT OD readings were recorded at OD₅₉₀. The degree of growth inhibition was calculated by the following equation: [(MTT OD value of control–MTT OD value of the sample)/MTT OD value of control] × 100.

The effect of iron or other divalent cations on pyrazinamide activity was also assessed in a drug exposure experiment followed by cfu assay. A 10-day-old H37Ra culture was centrifuged at 7000 r.p.m. for 10 min at 4°C and the cells were resuspended in 7H9 medium at pH 5.5 to a cell density of about 10^{6–7} bacilli/mL. The cell suspensions were treated with nothing (control) or pyrazinamide (100 mg/L), ferrous sulphate (1 mM), or pyrazinamide plus ferrous iron, followed by incubation at 37°C for 5 days. A control that received no drug was also included. The cells were washed with 7H9 medium and plated on 7H11 agar plates, which were then incubated at 37°C for 3–4 weeks when the cfu was determined. The effect of other divalent cations such as zinc, magnesium and calcium was also assessed in a similar manner as above.

Effect of iron on the activity of pyrazinamide and pyrazinoic acid as assessed on 7H11 agar

M. tuberculosis H37Ra, H37Rv or BCG cultures were grown in 7H12B (Becton Dickinson, Sparks, MD, USA) in the BACTEC 460 system. When a growth index of 999 was achieved (which indicates saturation of growth or stationary phase), 200 µL aliquots from the BACTEC 12B (Becton Dickinson) cultures were used to prepare 10-fold serial dilutions (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) in sterile distilled water for inoculation. Middlebrook 7H11 plates at pH 5.6 with ADC supplement were prepared with the addition of pyrazinamide (with concentrations of 50, 25, 12.5 and 6.25 mg/L) plus ferrous ammonium sulphate or ferric ammonium citrate (with concentrations of 1.0 and 0.5 mM), or pyrazinoic acid (with concentrations of 50, 25, 12.5 and 6.25 mg/L) plus ferrous ammonium sulphate or ferric ammonium citrate (with concentrations of 1.0 and 0.5 mM). Control plates (pH 5.6) with the indicated concentrations of pyrazinamide, pyrazinoic acid, ferrous ammonium sulphate, and ferric ammonium citrate alone and plates lacking these compounds were also included. Finally, 30 µL aliquots of the four dilutions (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) of the H37Ra or H37Rv were used to inoculate each quadrant of the 7H11 plates. All plates were incubated at 37°C in an incubator with an atmosphere of 5% CO₂ and 95% air for 3 weeks before cfu determination. The enhancement effect of ferrous or ferric iron on pyrazinamide or

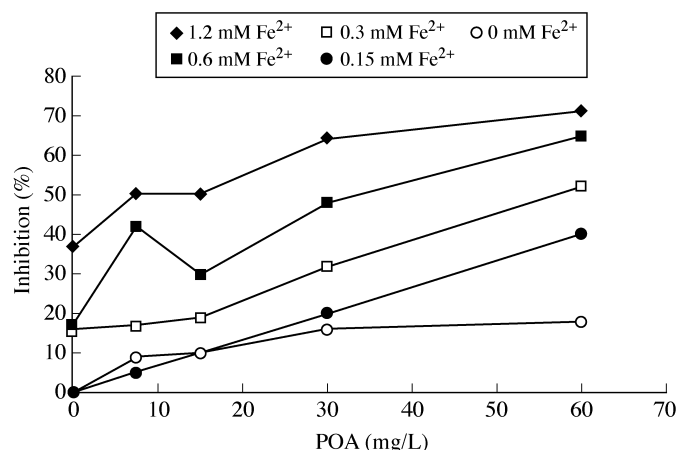


Figure 1. Effect of ferrous iron on pyrazinoic acid activity against *M. tuberculosis* H37Ra expressed as inhibition rate (%). H37Ra cells (about 10⁶ cells) from a 3-week-old culture were incubated in liquid Sauton's medium (pH 5.6) containing ferrous sulphate at 1.2, 0.6, 0.3, 0.15 or 0 mM and pyrazinoic acid at 60, 30, 15 or 7.5 mg/L at 37°C for 7 days when the bacterial viability was assessed by the MTT method. The degree of growth inhibition was calculated using the equation as described in Materials and methods.

pyrazinoic acid was expressed as the percentage of growth inhibition by the equation: [(cfu of control–cfu of sample)/cfu of control] × 100.

Results and discussion

We initially tested the effect of iron on pyrazinamide or pyrazinoic acid activity against *M. tuberculosis* H37Ra in liquid Sauton's medium at pH 5.6. The results for pyrazinoic acid are shown in Figure 1. The antituberculous activity of pyrazinoic acid was enhanced at ferrous sulphate concentrations of 0.15–0.6 mM. This was shown by a greater degree of inhibition by pyrazinoic acid plus ferrous iron than by pyrazinoic acid or iron alone. Although the concentration of ferrous iron at 1.2 mM was high, showing considerable growth inhibition by itself, the iron enhancement effect could still be seen at this concentration (Figure 1). Using similar experimental conditions, we assessed the effect of ferrous iron on the activity of pyrazinamide with lower iron concentrations. Indeed, ferrous iron at 0.15–0.6 mM also enhanced the activity of pyrazinamide (Figure 2). However, one difference was that ferrous iron enhancement of pyrazinamide activity was seen at somewhat higher pyrazinamide concentrations of 25 and 50 mg/L, but not at 12.5 and 6.25 mg/L (Figure 2). This presumably results from the fact that only a portion of the prodrug pyrazinamide was converted into the active component pyrazinoic acid that reacted with ferrous iron to show activity. We also tested the effect of ferrous iron on pyrazinamide and pyrazinoic acid activity using naturally pyrazinamide-resistant BCG in a similar experiment as described above. *Mycobacterium bovis* BCG is known to have a characteristic single point mutation in its *pncA* gene which is responsible for its natural pyrazinamide resistance,¹³ but remains susceptible to pyrazinoic acid.⁹ Ferrous iron at 0.15–0.6 mM increased the activity of pyrazinoic acid, but not pyrazinamide against BCG as expected (Table 1). In a similar type of experiment, ferric iron (ferric chloride) was also found to enhance the activity of pyrazinoic acid and pyrazinamide (Table 2). It is worth noting that the enhancement effect of ferric iron on pyrazinoic acid activity was more obvious when the pyrazinoic acid concentrations were below its MIC (50 mg/L)

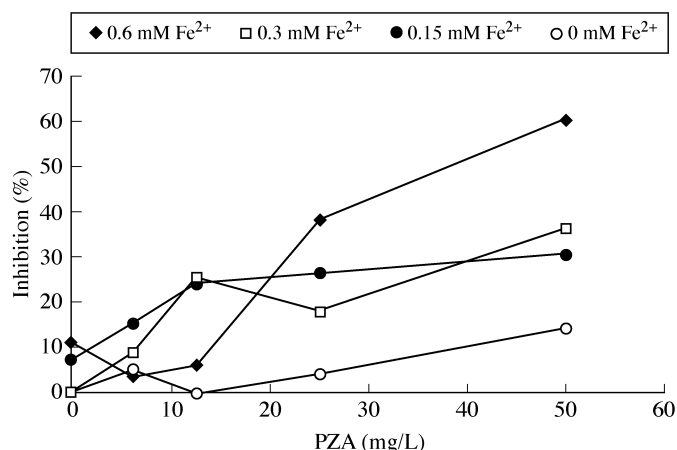


Figure 2. Effect of ferrous iron on pyrazinamide activity against H37Ra expressed as inhibition rate (%). The effect of ferrous iron on pyrazinamide activity was assessed in a similar manner as described in Figure 1.

Table 1. Effect of ferrous (Fe²⁺) iron on pyrazinoic acid and pyrazinamide activity against BCG expressed as inhibition rate (%)

Fe ²⁺ (mM)	POA (mg/L)				PZA (mg/L)				
	50	25	12.5	6.2	50	25	12.5	6.2	0
0.6	84	72	50	28	0	13	-19	-2	-9
0.3	57	38	6	-3	11	19	11	21	-4
0.15	61	33	22	4	20	16	18	0	-13
0	50	4	-4	-3	0	16	-5	-17	0

(Table 2). This is because at the MIC of 50 mg/L, pyrazinoic acid showed a significant growth inhibitory effect by itself and the effect of iron was not as evident as when pyrazinoic acid concentrations were at 25, 12.5 and 6.25 mg/L, where there was a much higher iron enhancement effect (Table 2).

We also tested the effect of iron on pyrazinamide activity in a drug exposure assay after incubation for 5 days followed by cfu count on 7H11 plates. As can be seen in Figure 3, ferrous iron had a significant enhancement effect on pyrazinamide activity with a more than 10-fold reduction in cfu compared with pyrazinamide alone. The difference was statistically significant ($P < 0.05$) when comparing pyrazinamide plus iron with either pyrazinamide alone or with iron alone. Unlike iron, zinc chloride as a control divalent cation did not have any enhancement effect on pyrazinamide activity (Figure 3). Other divalent cations such as magnesium or calcium also did not enhance the activity of pyrazinamide (data not shown).

To confirm the above results obtained in liquid medium, the effect of iron on pyrazinoic acid and pyrazinamide activity against *M. tuberculosis* H37Ra or BCG was also assessed on 7H11 plates (pH 5.6) containing iron and pyrazinamide or pyrazinoic acid. Although 1 mM ferrous ammonium sulphate alone had some growth inhibitory effect, it caused a more significant enhancement of pyrazinoic acid activity against H37Ra, with the enhancement effect being more obvious at 25 mg/L (no visible growth) than at 12.5 mg/L (faint growth) pyrazinoic acid (Figure 4a). Similarly, ferrous

Table 2. Effect of ferric (Fe³⁺) iron on pyrazinoic acid and pyrazinamide activity against H37Ra expressed as inhibition rate (%)

Fe ³⁺ (mM)	POA (mg/L)				PZA (mg/L)				
	50	25	12.5	6.2	50	25	12.5	6.2	0
0.6	81	70	52	38	58	39	22	19	16
0.3	61	19	16	15	43	17	25	-2	15
0.15	67	32	16	12	41	33	31	26	19
0	60	20	7	-7	17	5	-4	0	0

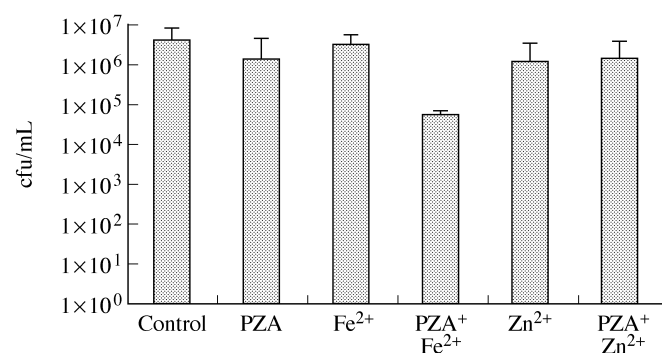


Figure 3. Effect of ferrous iron and zinc on pyrazinamide activity against *M. tuberculosis* H37Ra. The H37Ra cells were treated with pyrazinamide (100 mg/L) in the presence or absence of ferrous sulphate (1 mM) or zinc chloride (1 mM) for 5 days when the cfu was determined on 7H11 agar plates. For details of the experiment, see Materials and methods.

ammonium sulphate at 0.5 mM increased the activity of pyrazinoic acid against BCG by causing a 10-fold reduction in cfu compared with pyrazinoic acid alone (Figure 4b). In separate experiments with *M. tuberculosis* H37Ra, when the cfu on plates containing pyrazinamide (25 mg/L) plus ferrous ammonium sulphate (0.5 mM) was compared with that on plates containing 25 mg/L pyrazinamide alone, the presence of ferrous iron caused a 70% reduction in cfu (Table 3). In contrast, comparison of cfu of the plates containing only ferrous ammonium sulphate (0.5 mM) showed a lower level (12%) of growth inhibition over the control with no ferrous ammonium sulphate or pyrazinamide (Table 3). No visible growth could be observed on the plate containing pyrazinamide (25 mg/L) plus 1 mM ferrous ammonium sulphate, whereas ferrous ammonium sulphate alone (1 mM) resulted in a weaker inhibitory effect (64% versus the control without ferrous iron or pyrazinamide) (Table 3). A similar enhancement effect of ferrous iron on pyrazinamide activity was also observed for pyrazinoic acid. Pyrazinoic acid (25 mg/L) and ferrous ammonium sulphate (0.5 mM) together produced an 87% growth reduction versus pyrazinoic acid alone (25 mg/L), whereas the growth reduction with ferrous iron alone (0.5 mM) versus the control (with no pyrazinoic acid or ferrous ammonium sulphate) was 42% (Table 3). As with pyrazinamide, the mutual growth inhibitory effect of pyrazinoic acid (25 mg/L) and ferrous ammonium sulphate (1 mM) caused complete growth inhibition, whereas ferrous ammonium sulphate alone at 1 mM showed a lower (69%) growth inhib-

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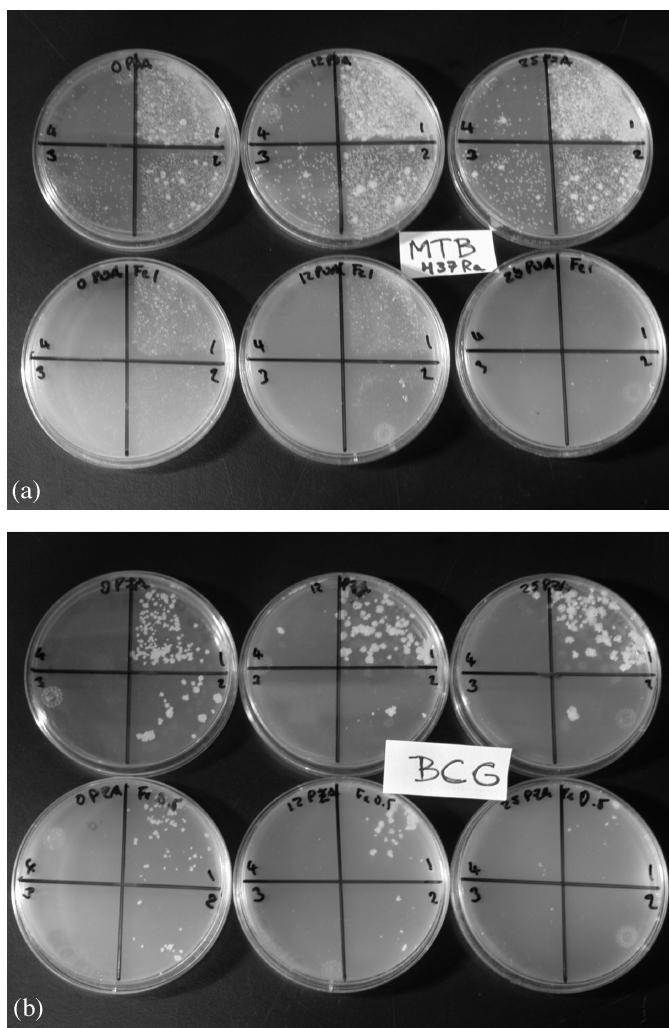


Figure 4. Effect of ferrous iron on enhancing the activity of pyrazinoic acid against *M. tuberculosis* H37Ra (a) and BCG (b) in 7H11 agar. Ferrous ammonium sulphate and pyrazinoic acid were incorporated into 7H11 agar (pH 5.6) at different concentrations. Thirty microlitres of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions of 3-week-old H37Ra or 6-week-old BCG cultures were spread onto the 7H11 agar plates, which were incubated for 3–4 weeks before the cfu was counted. The top row are plates containing 0, 12.5 and 25 mg/L pyrazinoic acid, and the bottom row are plates with ferrous iron (1 mM for H37Ra and 0.5 mM for BCG) and 0, 12.5 and 25 mg/L pyrazinoic acid from left to right.

ition (Table 3). Ferric iron had a similar effect as ferrous iron on enhancing pyrazinamide and pyrazinoic acid activity against *M. tuberculosis* H37Ra in the 7H11 agar method (data not shown). Similar effects of ferrous and ferric iron on enhancement of pyrazinamide and pyrazinoic acid activity were also found for *M. tuberculosis* H37Rv strain (data not shown).

The high *in vivo* sterilizing activity, but poor *in vitro* activity of pyrazinamide reflects potential differences in *in vitro* and *in vivo* environments that impact pyrazinamide activity. In 1954, the discovery by McDermott and colleagues of acid pH that increases pyrazinamide activity was based on this reasoning.⁷ During active inflammation in the lesions *in vivo*, there is an acidic pH environment, as a result of lactic acid production by inflammatory cells. However, acid pH does not appear to completely explain the separa-

Table 3. Effect of ferrous (Fe^{2+}) iron on pyrazinamide and pyrazinoic acid activity against H37Ra in 7H11 agar plates expressed as inhibition rate (%)

Fe^{2+} (mM)	PZA (0)	PZA (25 mg/L)	POA (0)	POA (25 mg/L)
1	64	NG	69	NG
0.5	12	70	42	87
0	0	8	0	57

NG, no growth.

tion of pyrazinamide activity *in vitro* versus *in vivo* as the MIC of pyrazinamide (50–100 mg/L at pH 5.5–6.0) is higher than the serum pyrazinamide concentration (30–60 mg/L). It is worth noting that iron can be released from iron binding proteins (e.g. transferrin, lactoferrin, ferritin, haem-containing enzymes) at acid pH during active inflammation,¹¹ which could potentially cause increased local iron concentration in tuberculous lesions. It is significant that we have shown in this study that iron could enhance the activity of pyrazinamide and pyrazinoic acid against *M. tuberculosis*. The elevated iron concentration in the lesions could be another factor besides acid pH that can potentiate pyrazinamide activity. However, there is no information available on iron concentrations in local tuberculous lesions, and future studies are needed to assess this in the context of iron enhancement of pyrazinamide activity.

We have shown in this study that various iron salts with different anion groups complexed with iron such as ferrous sulphate, ferrous ammonium sulphate, ferric chloride and ferric ammonium citrate could all enhance the activity of pyrazinamide or pyrazinoic acid. This indicates that it is the iron that is important for the activity, not the anion group in complex with the iron.

The mechanism of iron enhancement of pyrazinoic acid and pyrazinamide activity is not known. It is possible that iron causes lipid peroxidation of the *M. tuberculosis* membrane and membrane damage, leading to disruption of membrane potential through increased permeability to protons or inhibition of membrane-embedded respiratory chain enzymes such as H^+ -ATPase, NADH dehydrogenase and cytochrome *c* oxidase involved in membrane potential and energy production. The iron-mediated disruption of membrane potential could then serve as a ‘weak point’ for attack by pyrazinoic acid, which we have shown to decrease membrane potential at acid pH.¹⁴ Further studies are needed to address these possibilities and to determine whether iron can be used as a supplement for enhancement of pyrazinamide activity in animal models of TB infection. However, one must be cautious regarding the iron supplement used for enhancement of pyrazinamide activity, as iron could also serve as a bacterial growth factor.¹⁵

Acknowledgements

The work was supported by research funding from NIH (AI44063 and AI49485).

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