

Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant *Mycobacterium tuberculosis*

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SUMMARY

SETTING: Pyrazinamide (PZA) is an effective sterilising drug in tuberculosis, but its mode of action is controversial.

OBJECTIVE: To test the bactericidal activity of 1.56–100 g/ml PZA in Hu/Coates models of dormant and rifampicin (RMP) tolerant *Mycobacterium tuberculosis*.

METHODS: In model 1, bactericidal activity was tested in pH 5.5 medium against 4-day, 30-day or 100-day static, hypoxic cultures. In models 2 and 3, 100 g/ml RMP was added to a 100-day culture and PZA was added either during incubation with RMP in model 3, or after resuspension in RMP-free medium in model 2.

RESULTS: Model 1: cfu counts on the 100-day and 30-day cultures fell by a maximum of about 1.6 log cfu/ml with increasing culture age, PZA concentration and in-

cubation period, while counts on the 4-day culture showed little change. Model 2: cfu counts at the end of 7 days of recovery showed little bactericidal activity. Model 3: viable bacilli were almost completely eliminated. Bactericidal activity in these models increased with decreasing metabolic bactericidal activity, as measured by the uptake of [³H] uridine into bacterial RNA.

CONCLUSION: PZA differs from other anti-tuberculosis drugs in showing greater bactericidal activity the slower the bacillary metabolic activity, hence its great value as a sterilising drug, likely to remain as an effective companion drug with newer sterilising drugs.

KEY WORDS: *Mycobacterium tuberculosis*; pyrazinamide; dormancy; rifampicin-tolerant

IN A SERIES of randomised controlled trials (RCTs) of the treatment of pulmonary tuberculosis, each of the two major anti-tuberculosis drugs, rifampicin (RMP) and pyrazinamide (PZA), has been shown to increase the sterilising activity of the regimen, i.e., the rate at which persisting tubercle bacilli are killed in the lesions.¹ When given together in RCTs, sterilising activity is increased further, demonstrating that they have bactericidal synergism.^{2–4} While there are explanations for the sterilising activity of RMP,⁵ less is known about PZA. We explored this issue in the three Hu/Coates patented models of drug sterilising action against old, anaerobically adapted cultures of *Mycobacterium tuberculosis*.^{6,7} These models have been used extensively in drug discovery. In two of the three models, bactericidal activity is measured against a special population of bacilli that survive (are tolerant to) the action of a high concentration of RMP,⁸ and may therefore be relevant to the synergism between PZA and RMP in the lesions of patients.

The best model for describing the action of PZA is due to Y Zhang.⁹ In this model, PZA, a pro-drug, is converted to pyrazinoic acid (POA) by the amidase

(pncA) in the bacterial cytoplasm.¹⁰ It is then excreted slowly from the bacilli and diffuses in again passively through the cell wall as the protonated molecule (HPOA) when the pH of the bacterial environment is acid, according to the Henderson-Hasselbach equation. HPOA is toxic to the cell membranes, but there does not appear to be any specific genetic site yet located at which it acts. HPOA can only be removed by an inefficient bacterial pump.¹¹ Slowing of bacterial metabolism does not alter passive diffusion of HPOA into the cell, but it reduces the already inefficient activity of the pump, allowing accumulation of toxic HPOA. Bactericidal activity thus increases with a fall in bacterial metabolism, increasing acidity of the bacterial environment and increasing concentration of PZA. Our findings in the Hu/Coates models can only be logically explained in the light of this model.

METHODS

Antibacterials and cultures

PZA and RMP were obtained from Sigma (Southampton, UK). The media from Difco (Becton & Dickinson,

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Sparks, MD) were 7H9 liquid medium containing 0.05% Tween 80, supplemented with 10% w/v albumin oleic acid, dextrose and catalase complex and 7H11 plates, supplemented with oleic-acid, albumin, dextrose and catalase complex. Cultures of *M. tuberculosis* strain H37Rv in 10 ml volumes of 7H9 medium in screw-capped 28-ml plastic 'Universal' containers, were initially started with an inoculum of 1 ml of a log phase culture, and sets of these tubes were then incubated at 37 C with caps screwed tight and without disturbance for 4 days, exactly 30 days ('30-day cultures') or 100–115 days ('100-day cultures'). To obtain evenly dispersed bacilli prior to experimental treatment, the sedimented clumps of bacilli in the bottom of each 10 ml of 30-day or 100-day culture were broken up by vortexing with 2 mm diameter glass beads for 5 min, followed by ultra-sonication in a water bath sonicator (Branson Ultrasonics, Slough, UK) for 5 min. After sonication, the 10 ml volumes from each of the set were pooled (without the glass beads) and then distributed again in 10 ml volumes in Universal containers so as to obtain the same conditions in each of the containers.

Model 1

The bacilli in 4-day cultures and in sonicated 30-day and 100-day cultures were washed by centrifugation and resuspended to similar densities of 0.8 by spectroscopy with a WPA CO8000 cell density meter (Fisher Scientific, Loughborough, UK) at 600 nm in fresh medium at pH 5.5 containing PZA in final concentrations increasing by two-fold steps from 1.56 to 100 g/ml. Each PZA concentration was in a separate 10 ml culture. Colony forming unit (cfu) counts were set up on day 0 after re-suspension and after incubation of the acid medium for 7 and 14 days. Colonies were counted after 2.5 weeks' incubation and their position marked on the back of the plate. They were recounted after a further 10 days' incubation to check that additional colonies had not appeared (Figure 1).

Model 2

RMP 100 g/ml was added to each of a set of sonicated and resuspended 100-day cultures, which were then incubated for 5 days. After the first day of incubation in this model, no colonies could be obtained on plates inoculated from the culture. After washing three times with phosphate buffered saline (PBS) by centrifugation, fresh RMP-free liquid 7H9 medium at pH 5.5 was added to make up the volume to 10 ml, and PZA was added in the same concentrations as in model 1. After further incubation for 7 days, cfu counts were set up. Control studies have shown that plate counts begin to yield colonies on subculture after about 4 days of incubation of RMP-free cultures at pH 7 (Figure 1).

Model 3

A set of 100-day cultures was sonicated and re-suspended in 7H9 medium at pH 5.5 containing RMP

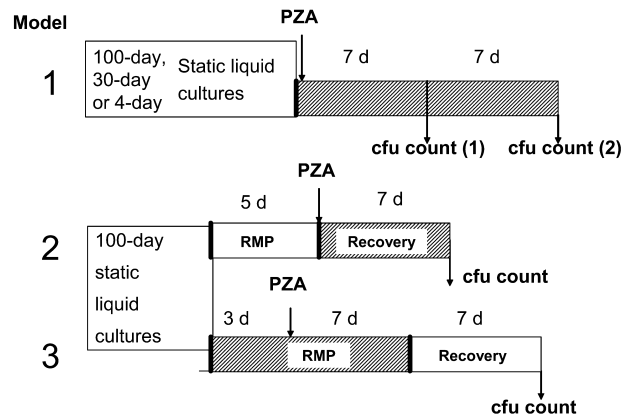


Figure 1 Diagrams of the three models of RMP persisters. Hatched areas represent 7H9 culture medium at pH 5.5. Black verticals indicate bacteria washed. RMP presence of 100 g/ml RMP in culture medium. Model 1 studied the action of PZA on 30-day and 100-day cultures with a 4-day logarithmic phase culture as a control. In models 2 and 3, a 100-day culture was exposed to 100 g/ml RMP. This left a small residual population that was tolerant to the RMP and was also able to grow only in liquid medium. During 7 days of liquid medium culture it recovered the ability to grow on plates, but did not multiply. In model 2, the PZA was added just after completion of the recovery phase. In model 3, it was added during the exposure to RMP. RMP rifampicin; PZA pyrazinamide; cfu colony forming units; RMP rifampicin.

100 g/ml. After incubation for 3 days to allow complete adaptation to the presence of RMP, additions of PZA in a range of concentrations were made and incubation was continued for a further 7 days (10 days in all). Note that broth counts of viable bacilli and [³H] uridine uptake into bacillary RNA remained constant throughout the period of exposure to RMP in earlier experiments.⁸ The cultures were then washed three times, resuspended in medium at pH7 and incubated for 7 days to allow recovery before plating for cfu counts. A control culture at pH 7 was exposed to RMP for 10 days, and was then washed and allowed to recover in the same way, then plated for cfu counts. Each of the experiments was performed twice, with closely similar results in each pair (Figure 1).

Statistics

The mean of the \log_{10} cfu/ml in the two identical experiments was calculated for each model. The mean count for each concentration of PZA was subtracted from the count for the control culture containing no PZA to give the fall (Δ) in \log_{10} cfu/ml at various PZA concentrations. The standard deviation (SD) of a cfu count and of Δ was calculated from the differences between the duplicate \log_{10} cfu counts.

RESULTS

PZA in Hu/Coates models

In model 1, the \log_{10} cfu counts/ml in control drug-free medium were 7.126 with the 4-day culture, 8.110

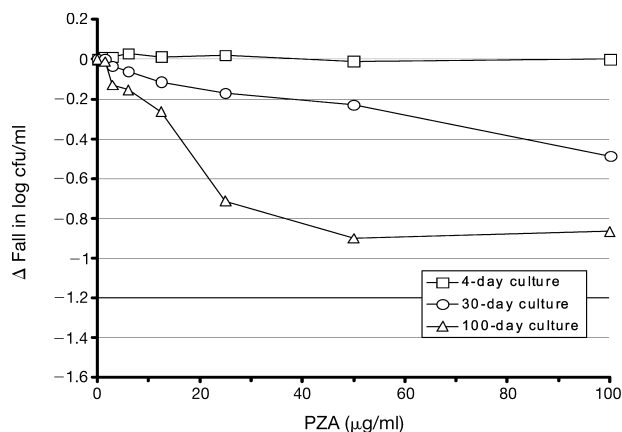


Figure 2 Model 1: Fall in cfu counts at 7 days, after 4-day, 30-day and 100-day cultures had been exposed at pH 5.5 to PZA. cfu colony forming units; PZA pyrazinamide.

with the 30-day culture and 6.279 with the 100-day culture after adjustment for equal opacity. After exposure to PZA for 7 days, there was no fall in the counts of the 4-day culture, while there was a small bactericidal effect on the 30-day culture and a larger effect on the 100-day culture, as a kill of nearly 1 log unit had been achieved with 100 g/ml PZA (Figure 2). The bactericidal effects increased as the concentration of PZA was increased. After a further 7 days of incubation, totalling 14 days (Figure 3), there was now a small bactericidal effect on the 4-day culture, amounting to a kill of about 0.4 log units with 100 g/ml PZA, and a much larger kill of about 1.5 log units in both the 30-day and the 100-day cultures. These results clearly indicate the important roles of the age of the culture and the concentration of PZA in determining the bactericidal action of PZA. They also show the slow onset of bactericidal effects over the 14 days of exposure.

In model 2, the control cfu count after exposure to acid medium in the recovery phase but not to PZA

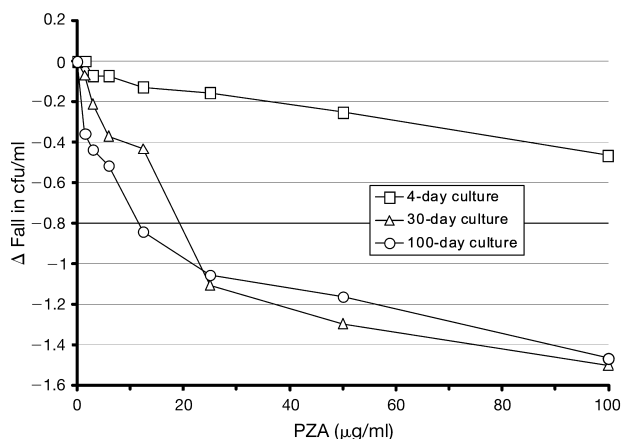


Figure 3 Model 1: Fall in cfu counts at 14 days, after 4-day, 30-day and 100-day cultures had been exposed at pH 5.5 to PZA. cfu colony forming units; PZA pyrazinamide.

was $\log_{10} 3.114/\text{ml}$, a usual value in this system, indicating that there had been no lethal damage to the plate-negative bacilli by the additional exposure to acid conditions. However, the exposure to PZA during the recovery phase, after exposure to RMP in model 2, produced virtually no kill even with 100 g/ml PZA (Figure 4). In model 3, there were two controls. The first yielded a cfu count of $3.008 \log_{10}/\text{ml}$, again a usual value in this system, after exposure at pH 7 to RMP only for 10 days. The second was a similar count of $\log_{10} 2.427/\text{ml}$ when the medium was at pH 5.5, with RMP but no PZA, for the 10 days. The decrease in counts indicates a small bactericidal effect of the acid medium on the RMP persisters. When PZA was added to the culture containing RMP in model 3, there was a substantial concentration-dependent kill, amounting to about 1.5 log units with 100 g/ml PZA (Figure 4). The cultures of the tubes containing 50 and 100 g/ml PZA yielded only 8–17 colonies in the duplicate experiments, so that almost the entire residual population had been killed. The estimated SDs of the \log_{10} cfu counts were $\log_{10} 0.0326/\text{ml}$ in model 1, $\log_{10} 0.00776/\text{ml}$ in model 2 and $\log_{10} 0.0645/\text{ml}$ in model 3. The estimated SDs of the falls in log cfu counts were 0.0461 in model 1, 0.0110 in model 2 and 0.0913 in model 3.

Bacterial metabolism in the models

Estimates of the uptake of [^3H] uridine into bacterial RNA by the method of Mangan et al.,¹² a measure of metabolic activity, in the various models is shown in the Table, together with viable counts on plates, and in liquid medium by the serial dilution method. The data have been obtained from the text and Table 2 of reference 8 and from other experiments with the models. Although representative of the findings with these models, they were obtained from experiments that are separate from those described here. All the media for these estimates were at pH 7.0. Although similar counts were obtained in plate and liquid me-

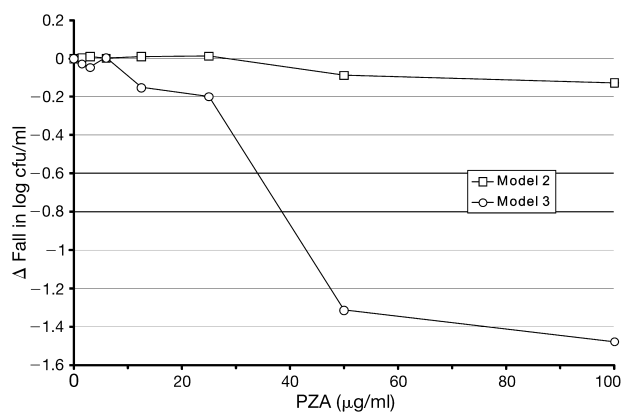


Figure 4 Fall in cfu counts in models 2 and 3. cfu colony forming units; PZA pyrazinamide.

Table Counts of viable bacilli and [³H] uridine uptake in persister models

Phase in models	Model	Plate count cfu/ml	Liquid medium count mpn*/ml	Uptake of [³ H] uridine per viable cell % of log phase
Log phase	—	1.30 × 10 ⁵	1.40 × 10 ⁵	100
100-day stationary	1	6.6 × 10 ⁵	3.8 × 10 ⁷	15
Start of recovery	2	0	1.73 × 10 ³	100
Exposed to RMP	3	0	1.73 × 10 ³	23

* Most probable number of viable cells.
cfu colony forming units; RMP rifampicin.

dium counts on log phase cultures, the liquid medium counts on 100-day cultures were higher (3.8 × 10⁷/ml) than the plate counts (6.6 × 10⁵/ml), presumably due to the presence of a majority subpopulation capable of growth in liquid medium but not on solid medium plates.^{7,8} When the culture was exposed to 100 g/ml RMP, all plate cultures were negative after exposure for 1 day, but a small residual subpopulation of 1.73 × 10³/ml bacilli, a number that remained constant during at least 5 days exposure to RMP, was detectable by liquid medium culture only. The uptake of [³H] uridine dropped to about 15% of that obtained with log phase cultures in the 100-day starter culture, and to a similar value of about 23% in the culture exposed to RMP. This uptake probably represents the metabolic activity of the persister population in both, although, under the slightly bactericidal acid conditions of model 3, it might have been even lower. As soon as the bacilli were washed and transferred to fresh RMP-free liquid medium, there was an abrupt rise in uridine incorporation to a level approximating that found in log phase cultures. There is, however, no evidence of bacterial cell division, as the mean of log₁₀ 3.094/ml (SD 0.074) in two liquid medium counts initiated immediately after transfer are similar to the plate counts of log₁₀ 3.120/ml (SD 0.017) in four plate counts obtained at the end of the 7-day recovery period (*P* = 0.7 for the difference between means).

DISCUSSION

There is already much evidence that the pH of the culture medium and the concentration of PZA are key factors in PZA activity.^{9,13–15} The results of our study demonstrate the decisive role of the metabolic activity of the bacilli. There was bactericidal activity in model 1 with 30-day starting cultures and even greater activity with 100-day cultures, when RNA incorporation was estimated as only 15% of the log phase rate. However, there was little activity against log phase cultures. When PZA was added to the RMP-tolerant bacterial population of model 3, which had an estimated rate of RNA incorporation of 23% of log phase bacilli, PZA had substantial bactericidal activ-

ity (Figure 3). However, in the recovery phase after RMP had been removed from the cultures in model 2, there was immediate recovery of the full rate of RNA incorporation, and PZA now had no bactericidal activity. The increase in activity of PZA as metabolic bacillary rates are reduced has previously been shown by its high activity against *M. tuberculosis* in the stationary growth phase¹⁶ and in anaerobic culture.¹⁷ This behaviour is in contrast to other anti-tuberculosis drugs, whose antibacterial activity decreases or disappears as metabolic activity is reduced, for instance by starvation, reduction in incubation temperature or acidity of the culture medium.^{5,18} Other drugs often act by selective inhibition of vital processes such as transcription, protein formation or production of adenosine triphosphate. As the bacterial cell goes into dormancy, inhibition of these processes becomes less lethal. Hence, synergism between them becomes less effective, making it difficult to kill those organisms that are most dormant. However, PZA appears to be the only drug whose bactericidal activity increases as the cell becomes more dormant, so that it should remain as an essential companion drug to give with current or newer drugs. Unfortunately, PZA is only active during the first 8 weeks of treatment,¹⁹ probably because an acid reaction in the environment of the bacilli, due to the presence of active inflammation, is only present during this period.²⁰ Because it has low activity on rapidly dividing bacilli and because of the slow onset of its bactericidal activity, it is only bactericidal at much the same rate as other drugs during the first 2 weeks of treatment.²¹ Its high sterilising activity must therefore take place between 2 and 8 weeks. This limitation on the period during which PZA is effective is the single mechanism that limits its efficacy. In the search for new drugs, we need to explore other molecules that might have the same important property as PZA, namely a pro-drug whose product accumulates as a non-specific toxic molecule within the bacterial cell, but without the necessity of an acid environment.²²

Acknowledgements

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RÉSUMÉ

CONTEXTE : Le pyrazinamide (PZA) est un médicament stérilisant efficace dans la tuberculose, mais son mode d'action reste toujours controversé.

OBJECTIF : Tester l'activité bactéricide de 1,56–100 g/ml de PZA dans des modèles Hu/Coates de *Mycobacterium tuberculosis* dormant et tolérant la rifampicine (RMP).

MÉTHODES : Dans le modèle 1, l'activité bactéricide a été testée dans un milieu à pH 5,5 contre des cultures hypoxiques de 4 jours, de 30 jours ou de 100 jours. Dans les modèles 2 et 3, on a ajouté 100 g/ml de RMP à une culture de 100 jours et on a rajouté le PZA, soit au cours de l'incubation avec la RMP dans le modèle 3, soit après resuspension dans un milieu sans RMP dans le modèle 2.

RÉSULTATS : Modèle 1 : les décomptes de ufc/ml dans les cultures de 100 jours et de 30 jours ont chuté d'un maximum d'environ 1,6 log ufc/ml avec l'accroissement

de l'âge de la culture, la concentration du PZA et la période d'incubation, alors que les décomptes dans les cultures à 4 jours ne se sont que légèrement modifiés. Modèle 2 : les décomptes de ufc/ml à la fin de 7 jours de récupération ont montré peu d'activité bactéricide. Modèle 3 : les bacilles viables étaient quasi complètement éliminés. L'activité bactéricide dans ces modèles a augmenté avec la décroissance de l'activité métabolique bactéricide mesurée par l'absorption de l'uridine [³H] dans l'ARN bactérien.

CONCLUSION : Le PZA diffère des autres médicaments antituberculeux car il montre une activité bactéricide d'autant plus importante que l'activité métabolique bacillaire est plus lente. D'où sa grande valeur comme médicament stérilisant, susceptible de rester un médicament d'accompagnement efficace pour de nouveaux médicaments stérilisants.

RESUMEN

MARCO DE REFERENCIA : La piracinamida (PZA) constituye un medicamento eficaz para la esterilización de las lesiones tuberculosas, pero su mecanismo de acción es fuente de controversia.

OBJETIVO : Verificar la actividad bactericida de la PZA en concentraciones entre 1,56 y 100 g/ml contra *Mycobacterium tuberculosis* latente y tolerante a la rifampicina (RMP), mediante los modelos de Hu y Coates.

MÉTODOS : En el modelo 1 se evaluó la actividad bacte-

ricida de la PZA en un medio con pH 5,5, contra cultivos anaerobios, estáticos de 4, 30 y 100 días de crecimiento. En los modelos 2 y 3 se añadieron 100 g/ml de RMP a un cultivo de 100 días y la PZA se agregó durante la incubación con RMP en el modelo 3 y después de resuspender el cultivo en medio sin RMP, en el modelo 2.

RESULTADOS : En el modelo 1, se observó una disminución máxima del recuento de ufc/ml de 1,6 en escala logarítmica en los cultivos de 100 y 30 días ; la dismi-

nución fue superior, a mayor edad del cultivo, más alta concentración de PZA y mayor tiempo de incubación. El recuento de colonias en el cultivo de 4 días mostró poca variación. En el modelo 2, los recuentos de ufc al final de 7 días de recuperación demostraron poca actividad bactericida. En el modelo 3, se eliminaron casi totalmente los bacilos viables. En estos modelos, la actividad bactericida fue mayor en la medida en que disminuía la actividad metabólica de las bacterias, evaluada medi-

ante la incorporación de uridina marcada (^3H) al ARN bacteriano.

CONCLUSIÓN: La PZA difiere de otros medicamentos antituberculosos, en que ejerce una mayor actividad bactericida entre más lenta sea la actividad metabólica de los bacilos. Por esta razón, es de gran utilidad para esterilizar las lesiones tuberculosas y probablemente seguirá siendo eficaz administrada en pautas que contengan los nuevos medicamentos con acción esterilizadora.
