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Effects of low incubation temperatures on the bactericidal activity of anti-tuberculosis drugs

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Objectives: To explore the effect of low incubation temperatures and the consequent slowing of bacterial metabolism on the bactericidal action of anti-tuberculosis drugs against *Mycobacterium tuberculosis*.

Methods: Counting of surviving bacteria during exposure of static cultures to 1 mg/L isoniazid, 2 mg/L rifampicin, 0.5 or 2 mg/L TMC207 and 40 or 160 mg/L pyrazinamide, usually for periods of 21 days at temperatures of 37, 25, 22, 19, 16 or 8°C.

Results: The bactericidal activities of isoniazid and rifampicin were progressively reduced at 25 and 22°C, and were minimal at lower temperatures. TMC207 was immediately bactericidal at 37°C, in contrast to the early static phase reported with log phase cultures, and showed less change in activity as incubation temperatures were reduced than did rifampicin or isoniazid. Pyrazinamide was more bactericidal when incubation temperatures were reduced below 37°C and when the static seed cultures were most dormant.

Conclusions: These results can be explained by the surmise that at low temperatures bacterial energy is at a low level with only just sufficient ATP to maintain homeostasis, making the bacteria more susceptible to the blocking of ATP synthesis by TMC207. Insufficient ATP at low temperature would also hinder the export of pyrazinoic acid, the toxic product of the pro-drug pyrazinamide, from the mycobacterial cell by an inefficient efflux pump that requires energy.

Keywords: pyrazinamide, TMC207, low temperature, ATP availability

Introduction

Perhaps the most important issue in understanding the chemotherapy of tuberculosis is why it takes so long to kill *Mycobacterium tuberculosis* in the lesions of patients when axenic cultures can be sterilized in much shorter periods. The tolerance of these persistent bacilli to antibacterial drugs is characterized by the cessation of multiplication and a slowing of metabolism, so that inhibition of the cellular biochemical machinery after drug exposure is less effective in killing the cell.^{1,2} A variety of *in vitro* model systems have been proposed to mimic *in vivo* *M. tuberculosis* populations and interrogate drug action,^{3–5} but none has transcriptional signatures identical to those found by global gene expression profiling of the 'fat and lazy' bacilli encountered in the sputum of patients with pulmonary tuberculosis⁶ or from bacilli isolated from human lung sections.⁷ Furthermore, most model systems use culture media at the conventional pH of 6.6–6.8 whereas lesions must be more acid

in the range of pH 5.5–6.0 to account for the known bactericidal activity of pyrazinamide whilst allowing multiplication of *M. tuberculosis*.^{8,9} In the search for inexpensive model systems that would be more representative of persistent bacilli and yet easy to use in assays extending for several weeks, we decided to explore the effects of lowering the incubation temperature from 37 to 25°C, where multiplication ceases but metabolism continues, to lower temperatures that likely reduce metabolism further. We used a static stationary phase culture, with characteristic adaptations of persistence including anaerobic respiration and energy production from β -oxidation of fatty acids,^{10,11} at pH 6.0 to allow the action of pyrazinamide to be explored. The other drugs tested were the first-line drugs isoniazid and rifampicin together with the diarylquinoline TMC207 (previously R207910), which inhibits mycobacterial ATP synthase, because it is the first of the new drugs being developed and has been shown to be promising both in its characteristics¹² and in the preliminary results of clinical trials.¹³

Methods

Bacterial strain

M. tuberculosis, strain H37Rv.

Chemicals

TMC207 was a gift from Tibotec Pharmaceuticals (Beerse, Belgium). Other antibacterial compounds were purchased from Sigma. All other chemicals were obtained from VWR International (Magna Park, Leicestershire, UK) except where specified otherwise.

Media

Dubos broth buffered to pH 5.95 was prepared as follows. To 850 mL of distilled water, 8.2 g of KH_2PO_4 and 3.2 g of K_2HPO_4 were added and mixed until dissolved. Then 50 mL of glycerol and 6.5 g of Dubos broth base (Difco 238510; Becton-Dickinson, Sparks, MD, USA) were added and mixed on a magnetic stirrer until dissolved. The pH was adjusted to 5.90 with 1 M citric acid (approximately 5 mL), to give a final pH of 5.95 ± 0.05 . The medium was sterilized through a $0.2 \mu\text{m}$ filter. An aliquot of 100 mL of Dubos Medium Albumin (Difco 230910; Becton-Dickinson) was added to complete the medium.

Serial 10-fold dilutions of Dubos broth cultures in 100 μL volumes were plated onto Mycobacteria 7H11 agar (Difco 283810; Becton-Dickinson) supplemented with 100 mg/L oleic acid/albumin/dextrose enrichment (BBL212260; Becton-Dickinson) and 50 mg/L carbendazim (Aldrich), a broad-spectrum antifungal.

Plates were incubated in the dark at 37°C for 28 days, after which colonies were counted.

Antibacterial compounds

TMC207 5 mg/mL stock solution in sterile DMSO was added at final concentrations of 0.5 and 2.0 mg/L. Pyrazinamide 10 mg/mL stock solution in sterile distilled water was added at final concentrations of 30, 40 and 160 mg/L. Rifampicin 10 mg/mL stock solution in sterile absolute methanol was added at a final concentration of 2 mg/L. Isoniazid 10 mg/mL stock solution in sterile distilled water was added at a final concentration of 1 mg/L. The drug concentrations used correspond to concentrations near peak and near trough obtained in patients under treatment¹⁴ except that 160 mg/L pyrazinamide is well above peak concentration, to see what effect higher concentrations would have.

Culture systems

The seed inoculum was prepared by growing *M. tuberculosis* in Dubos broth to an opacity of 0.4–0.6 at 580 nm and storing aliquots frozen in liquid nitrogen. Aliquots were pre-tested for the presence of contaminants. In each experiment, a series of 10 mL volumes of buffered Dubos broth at pH 5.95 were dispensed into 28 mL glass screw-capped tubes, which were inoculated with 100 μL of seed culture and either, in procedure 1, incubated undisturbed for 30 days or, in procedure 2, incubated until they reached standard opacity (equivalent in turbidity to a 2 McFarland standard) and then inoculated at a dilution of 1:4 by volume into fresh buffered Dubos broth at pH 5.95 and incubated undisturbed for 30 days. The cultures from either procedure were then vortex-mixed to create an even suspension, antibacterial compounds were added and serial samples were taken for colony counting at 3, 7, 14 and 21 days post-exposure after incubation at various temperatures. At least two replicates of each antibacterial compound concentration were tested.

Incubation temperatures

The 28 mL screw-capped tubes were incubated either in a small conventional incubator with an adjustable temperature or in a Boekel PCB2 cooling incubator (Grant Instruments, Cambridge) at temperatures of 8, 16, 19, 22, 25 and 37°C.

Statistics

Counts and linear regression coefficients were calculated in Excel and were further examined by analysis of variance in Stata 8 (Stata Corp., College Station, TX, USA).

Results

Drug-free cultures

Control cultures (no drug) grown at 37°C increased their cfu count by about 1 log unit over 21 days, whereas cultures

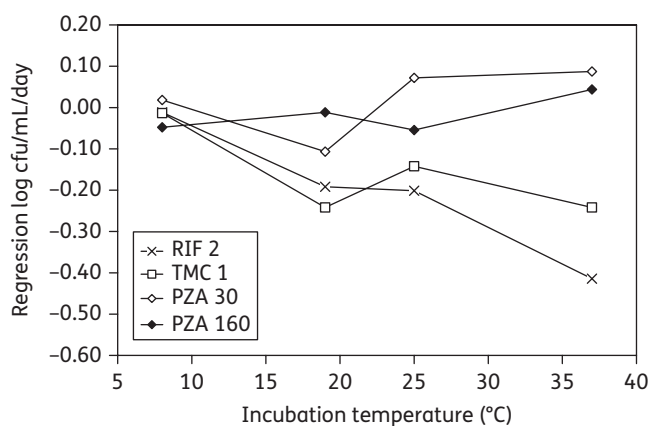


Figure 1. Speed of bactericidal activity of 2 mg/L rifampicin (RIF 2), 1 mg/L TMC207 (TMC 1) or 30 or 160 mg/L pyrazinamide (PZA 30 and PZA 160) against 10 day static cultures of *M. tuberculosis* at incubation temperatures of 37, 25, 19 and 8°C. Speed is represented by the linear regression coefficients for log cfu/mL/day.

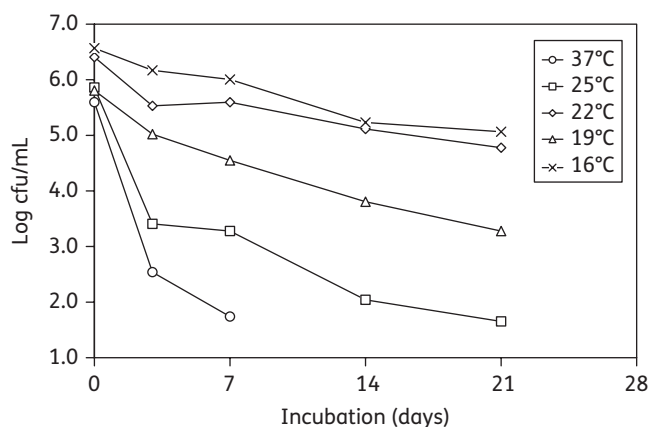


Figure 2. Survival curves for 30 day static cultures of *M. tuberculosis* exposed to 2 mg/L rifampicin for 21 days at temperatures ranging from 37 to 16°C.

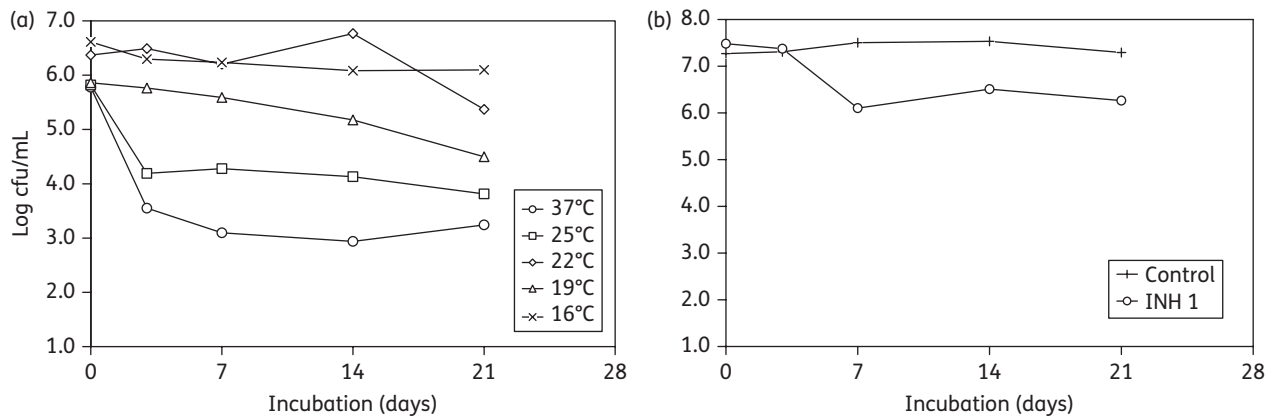


Figure 3. (a) Survival curves for 30 day static procedure 1 cultures of *M. tuberculosis* exposed to 1 mg/L isoniazid for 21 days at temperatures ranging from 37 to 16°C. (b) Survival curves for 30 day static procedure 2 cultures exposed to 1 mg/L isoniazid (INH 1) at 37°C.

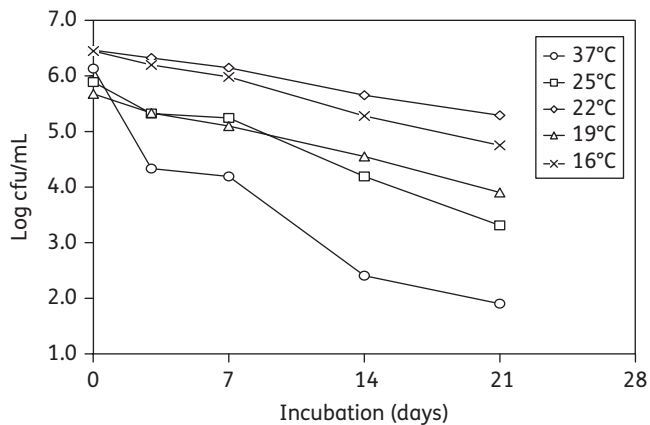


Figure 4. Survival curves for 30 day static cultures of *M. tuberculosis* exposed to 2 mg/L TMC207 for 21 days at temperatures ranging from 37 to 16°C.

grown at lower temperatures, including 25°C, showed no change in their counts over this incubation period.

Inoculum of 10 day static cultures

We first exposed static 10 day cultures to 2 mg/L rifampicin, 1 mg/L TMC207 or 30 or 160 mg/L pyrazinamide at 37, 25, 19 and 8°C. Linear regression coefficients were calculated from cfu counts over 7 days of incubation (Figure 1). None of the drugs had bactericidal activity at 8°C. The bactericidal activity of rifampicin was greatest at 37°C, but declined sharply at 25 and 19°C. TMC207 was less bactericidal than rifampicin at 37°C, but its activity dropped more slowly at 25 and 19°C. The difference in behaviour between rifampicin and TMC207 was highly significant ($P < 0.001$). Both concentrations of pyrazinamide allowed slight growth at 37°C, but were marginally bactericidal at 25 and 19°C.

Inoculum of 30 day static cultures using procedure 1

To extend these findings of a drug-specific effect of incubation temperature, we exposed static 30 day cultures for 21 days to 2 mg/L rifampicin, 1 mg/L isoniazid, 0.5 or 2 mg/L TMC207 or

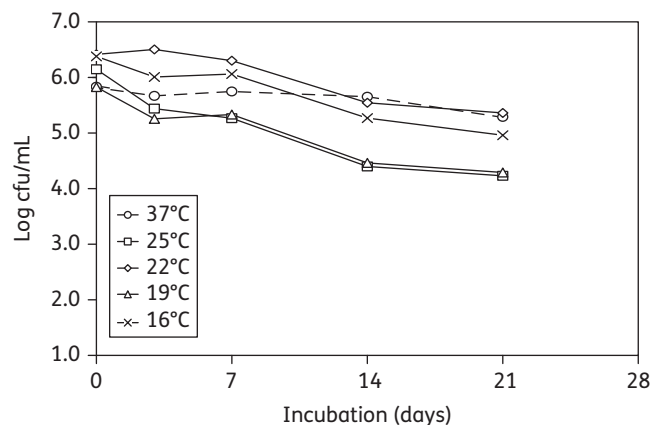


Figure 5. Survival curves for 30 day static cultures (procedure 1) of *M. tuberculosis* exposed to 160 mg/L pyrazinamide for 21 days at temperatures ranging from 37 to 16°C.

40 or 160 mg/L pyrazinamide at 37, 25 and 19°C. Cultures were also exposed to the same drugs at 22°C and at 16°C in additional follow-up experiments. The counts for rifampicin (Figure 2) show an almost exponential decline, most rapid at 37°C and less rapid at 25°C, and similar, slower rates of killing at 22, 19 and 16°C. The counts for isoniazid (Figure 3a) at 37°C showed a rapid decline of 3 log units during the first 7 days of exposure followed by no loss of viability to 21 days. At 25°C, the initial fall was about 2 log units, while at lower temperature only a very slight decline occurred throughout. The counts for 2 mg/L TMC207 showed an exponential fall at 37°C (Figure 4), starting with an initial substantial fall during the first 3 days, and with a slight reduction, much less than with either rifampicin or isoniazid, in the rate of fall at the lower temperatures. The counts for 160 mg/L pyrazinamide showed little change over the 21 days at 37°C, but increased bactericidal activity with similar steady falls at 19 and 25°C (Figure 5). Linear regressions of the viable counts, which estimate overall bactericidal activity, were calculated for isoniazid over 0–7 days, during which period all bactericidal activity occurred, and for the remaining drugs over 0–21 days since their bactericidal activity was more prolonged (Figure 6). The loss of bactericidal activity as temperature

was lowered was most evident with rifampicin and isoniazid. The corresponding fall in activity for TMC207 at both concentrations was smaller than with rifampicin or isoniazid. In contrast to the other drugs, pyrazinamide at both concentrations showed increased bactericidal activity with temperatures below 37°C.

Inoculum of 30 day static cultures using procedure 2

To confirm the enhanced efficacy of pyrazinamide at low temperature in a model in which isoniazid-mediated killing was not so prominent, we adopted a second culture system (procedure 2) and re-tested pyrazinamide and isoniazid killing. In procedure 1 for preparation of the test inoculum, isoniazid at 37°C caused a drop of approximately 3 log units during the first 7 days (Figure 3a), whereas in procedure 2 cultures only a 1 log unit reduction in cfu was detected after isoniazid exposure (Figure 3b). Drug-free control cultures grew successfully at 37°C, and there was no change in viable count over time at 25 or 22°C, as expected. The viable counts during exposure to 40 and

160 mg/L pyrazinamide demonstrate unequivocally that a reduction in incubation temperature from 37 to 25°C resulted in a striking increase in bactericidal activity, which was even greater at 22°C (Figure 7). At 37°C, pyrazinamide at either concentration had negligible bactericidal activity, but at 22°C the counts for cultures exposed to 40 mg/L pyrazinamide dropped from log 7.7 to log 5.4 cfu/mL (2 log kill) over 21 days, while those exposed to 160 mg/L pyrazinamide dropped even further to log 4.3 cfu/mL (3 log kill).

Discussion

The most striking finding of this study was the increase in bactericidal activity of pyrazinamide at temperatures below 37°C, particularly evident when the test cultures had been prepared by procedure 2 (Figure 7). This preparation procedure was hypothesized to increase the resemblance of the test cultures to persistent *M. tuberculosis* in sputum and presumably also in lesions because isoniazid exposure caused an initial drop of about 1 log unit in counts (Figure 3b). This is similar to the drop in viability demonstrated during the first week of early bactericidal studies on patients given isoniazid monotherapy,¹⁵ and is consistent with evidence suggesting that isoniazid tolerance may be a feature of persistent bacilli.^{5,16} This drop of 1 log unit using procedure 2 is appreciably smaller than the reduction of 3 log units using bacilli prepared by procedure 1 (Figure 3a), and is, as predicted, inversely correlated with pyrazinamide efficacy in these models. An explanation for the increase in bactericidal activity of pyrazinamide at low temperatures is provided by the Zhang hypothesis for the mode of action of pyrazinamide.⁸ According to this hypothesis, pyrazinamide is deaminated in the cytoplasm of bacilli and the resulting pyrazinoic acid is pumped out to the micro-environment. It is then passively reabsorbed in a pH-dependent manner into the bacilli. Once inside, bacilli are required to actively pump pyrazinoic acid out again. If the mycobacterial energy supply is diminished, pyrazinoic acid accumulates within the cell, eventually killing it.¹⁷ Thus, the elimination of pyrazinoic acid is energy-dependent. If ATP levels are diminished by sustained incubation at low temperature, pyrazinoic acid will accumulate more rapidly and killing will occur more readily. The finding that bactericidal activity

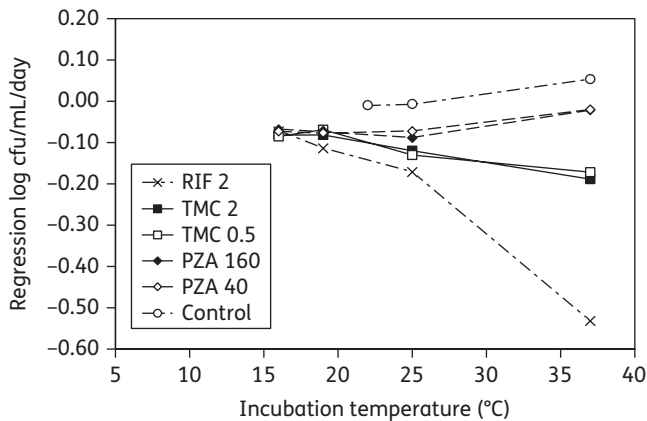


Figure 6. Speed of bactericidal activity of 2 mg/L rifampicin (RIF 2), 0.5 or 2 mg/L TMC207 (TMC 0.5 and TMC 2) or 40 or 160 mg/L pyrazinamide (PZA 40 and PZA 160) against 10 day static cultures of *M. tuberculosis* at incubation temperatures of 37, 25, 19 or 16°C. Speed is represented by the linear regression coefficients for log cfu/mL/day.

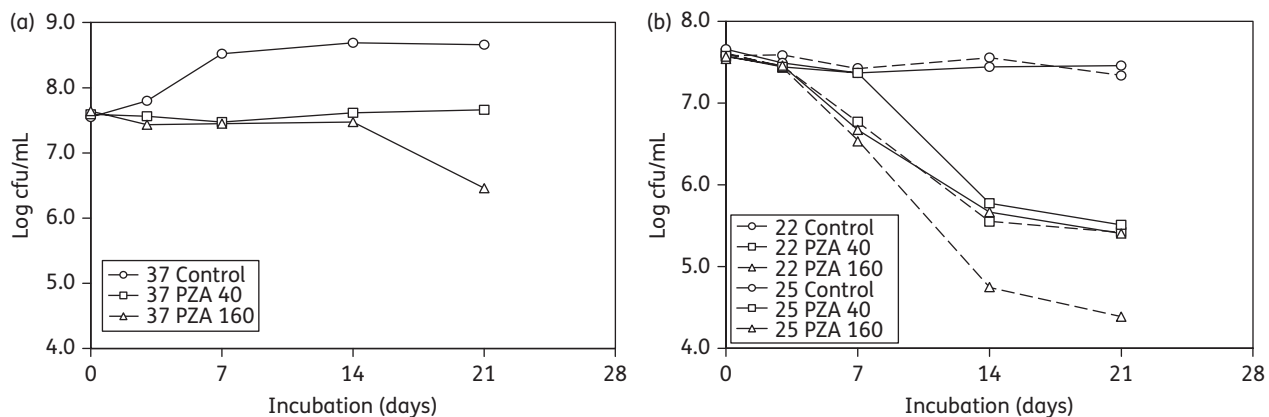


Figure 7. Survival curves for 30 day static cultures (procedure 2) of *M. tuberculosis* exposed to 40 or 160 mg/L pyrazinamide (PZA 40 and PZA 160) for 21 days at temperatures of (a) 37°C or (b) 25°C (symbols joined by continuous lines) or 22°C (symbols joined by broken lines).

was greater in procedure 2 than in procedure 1 test cultures indicates that less energy, perhaps in the form of ATP, was available in the non-replicating procedure 2 cultures. This hypothesis is consistent with ATP measurements and transcriptional signatures of energy production and ribosomal biosynthesis, showing a reduction in ATP levels in hypoxic non-replicating *in vitro* models of persistence.^{6,10,18–20} The bactericidal activity of pyrazinamide is therefore at a maximum when the energy sources available to the bacilli are at their lowest, either because metabolism is limited by adaptation to the *in vivo* environments encountered or by low incubation temperatures.

The second finding of interest was the rapid onset of the bactericidal activity of TMC207 at 37°C, evident during the first 3 days of exposure. This rapid onset is in contrast to the existence of an initial phase lasting 7–14 days when no change in the cfu count occurs after exposure of log phase bacilli to TMC207.^{12,21} The results from log phase organisms gave rise to the view that TMC207 has time-dependent but not dose-dependent activity.¹² We suggest that the gradual use of the energy store available in log phase bacilli is responsible for the static phase, which is eventually followed by rapid dose-dependent killing when ATP levels are low and the effects of the ATP synthase inhibitor become cidal. A further feature of interest was the small decrease in the bactericidal activity of TMC207 as the incubation temperature was reduced compared with the larger effects on the bactericidal activities of rifampicin and isoniazid (Figure 6). Since bacterial metabolism is likely to progressively decrease as the temperature goes down, TMC seems to retain its activity even when metabolism is low. This indicates its potential value as a drug capable of sterilizing the more persistent bacterial populations, and identifies energy generation as important for the maintenance of cell viability in bacilli isolated from *in vitro* models of persistence.^{18–20} This effect may be noticeable because TMC207 was considerably less bactericidal at 37°C than rifampicin or isoniazid, but this greater kill caused by isoniazid and rifampicin may not be reflected by a similar greater activity in treating patients because the half-lives of isoniazid and rifampicin and therefore the period of exposure to the drugs in lesions is considerably shorter than that of TMC207.^{12,14}

In brief, the effects of reducing the incubation temperature are particularly illuminating in exploring the action of drugs, such as pyrazinamide and TMC207 whose activity is greatly dependent on the energy resource status of bacilli.

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Transparency declarations

None to declare.

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