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MODEL SYSTEMS

Bactericidal activity of the diarylquinoline TMC207 against *Mycobacterium tuberculosis outside and within cells*

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SUMMARY

The bactericidal activities of the diarylquinoline TMC207 in a liquid culture medium started with a bacteriostatic phase lasting about 7 days and then continued with a dose-related bactericidal phase. In comparison, its intra-cellular activity in primary mouse peritoneal macrophages (PM) and in the J774 macrophage-like cell line had little or no static phase so that the bactericidal kill was evident by 5–7 days presumably due to low bacterial ATP levels. Bactericidal activities in the three systems were compared by estimating [1] the rate of bacterial killing (K) during exposure to 0.12–1.0 μg/ml TMC207 which were similar at, −0.35 in the J774 cells and −0.27 in mouse PM ($p = 0.6$) with each lower than −0.11 in extra-cellular cultures ($p < 0.001$) and [2] the TMC207 concentration at the intersection between the curve relating cfu count to TMC207 concentration and the cfu count at day-0, defined as the static concentration. Static concentrations were 0.22 μg/ml for extra-cellular cultures, 0.17 μg/ml for mouse PM and 0.06 μg/ml for J774 cells, significantly lower than the extra-cellular value ($p < 0.001$). Thus, the intra-cellular activity of TMC207 is clearly greater than its extra-cellular activity mainly because the preliminary static phase was a shorter or absent.

1. Introduction

The diarylquinoline, TMC207 (formerly R207190), is a new anti-tuberculosis drug[1] under assessment in a phase II clinical trial in which it was added to a background regimen for the treatment of multi-drug resistant (MDR) pulmonary tuberculosis.[2] In this trial, sputum conversion to negativity has occurred after 8 weeks of treatment in 48% of 23 patients on TMC207 + background drugs compared to 9% of 24 on placebo + background drugs. Peak plasma concentrations of 3.2 μg/ml and 1.7 μg/ml and trough concentrations of 1.0 and 0.7 μg/ml were found during dosage with 400 mg daily or 200 mg thrice weekly, respectively. These encouraging results were obtained essentially as a result of its action on the extra-cellular bacilli that comprise the majority of the bacterial population in cavitary pulmonary tuberculosis.[3,4] Evidence of its intra-cellular activity is also necessary to assess its effect on the intra-cellular component of the bacterial population particularly because the bacilli that best survive chemotherapy and cause relapse may well be those resident in macrophages.[5] Since TMC207 acts by selective inhibition of mycobacterial ATP synthase,[1] the factors that will affect its antibacterial activity in comparing intra-cellular and extra-cellular activity are (1) the concentration of ATP and the energy pool present in each of the bacterial populations at the start of its activity, (2) the MIC and MBC against each population and (3) the available concentration of TMC207. We therefore compared the extra-cellular activity of TMC207 with its activity on bacilli within mouse peritoneal macrophages and the J774 macrophage-like cell line, using dual criteria [1] the rate ($K_{(c)}$) at which the concentration c of TMC207 kills, which will be affected by all of these factors and [2] the approach of earlier studies,[6,7] in which the concentration that just inhibits growth is taken as the static concentration (SC) which is likely to reflect the MIC but would not be affected by the available bacterial ATP.

2. Materials and methods

2.1. Culture media and drugs

All tissue culture media and supplements were obtained from Invitrogen Ltd (Paisley, UK). The broth used in extra-cellular studies was Middlebrook 7H9 plus ADC supplement (Becton Dickinson UK

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Ltd, Oxford UK). Colony counts were done on Middlebrook 7H11 oleic acid-agar medium (Becton Dickinson). *Mycobacterium tuberculosis,* strain H37Rv, was kept as stock strain in liquid nitrogen and serially transferred at weekly intervals in 7H9 broth + ADC. Pure powder TMC207, a gift from Johnson & Johnson, was dissolved and diluted in dimethyl sulphoxide (VWR International Ltd, Lutterworth, UK) at a concentration of 10 mg/ml and stored at −20 °C. Final dilutions in the range 0.006–1.0 μg/ml or 0.25–32 μg/ml, as well as a control without drug were made in the culture media.

2.2. Extra-cellular activity

A 6 ml volume of a 7-day culture of *M. tuberculosis,* H37Rv in 7H9 broth was added to 200 ml 7H9 broth. After incubation for 4 days, the inoculated medium was dispensed into 30-ml screw capped universal containers and TMC207 added Immediately (day-0). After 7, 15 and 22 days of incubation at 37° C, samples were ultrasonicated briefly and viable counts set up.

2.3. Primary mouse peritoneal macrophages (mouse PM)

For the preparation of macrophage monolayers, the peritoneal cavities of recently killed specific pathogen free BALB/c mice (Harland UK Ltd, Bichecster, UK) weighing 18–20 g were washed out with RPMI 1640 medium containing heparin (5 U/ml; Sigma–Aldrich). Pooled washings were centrifuged at 150× g for 10 min and the cells were resuspended in RPMI 1640 + 10% fetal calf serum (FCS). From this suspension, 5×10^5 cells were put into 24 well plates (16 mm diameter VWR International) and these were incubated at 37 °C with 5% CO_2 for 2 h to allow adherence. Non-adherent cells were removed by washing three times with RPMI 1640 + 10% FCS and the resulting monolayer was cultured in RPMI 1640 + 10% FCS. For infection and addition of drug, a 7-day culture of *M. tuberculosis* in 7H9 broth was washed twice by centrifugation at 1000 g for 15 min and resuspended in RPMI 1640 + 10% FCS and then ultrasonicated for 21 s (Rinco Ultrasonics UK Ltd, London, UK). The infective inoculum was prepared to give a bacteria to cells ratio of 1:1. After a phagocytosis period of 2.5 h, monolayers were washed four times to remove unattached mycobacteria and overlaid with RPMI 1640 + 10% FCS + TMC207 (day-0). The medium and drug over the monolayers were replaced daily for 2 days left unchanged for 2 days and changed again on day-5. Cells were removed by adding 0.25% SDS (Sigma–Aldrich) into the 24 well plates and aspirating the cell suspensions into tissue culture tubes. A final volume of 1 ml per cell was made up with RPMI 1640 + 10% FCS to abrogate the toxic effect of SDS. These were briefly ultrasonicated to disrupt cell clumps and used for counting colonies at day-5.

2.4. J774 cell line

The J774A.1 cell line derived from mouse macrophages was obtained from Dr Barry Walker (National Institute and Biological Safety Standards, Potters Bars UK). This was kept at stock concentrations in liquid nitrogen, thawed at 37° C and aliquoted into filtered 25 cm flasks (Fisher Scientific UK, Loughborough, UK) with warmed RPMI 1640 + 10% FCS medium. Cells were allowed to adhere until confluent and the flasks split twice a week to maintain the cell line. Splitting was carried out once the cells were confluent by discarding the medium and adding 4 ml of RPMI 1640 + 10% FCS. The cells were gently taken off the flask by pipetting up and down till the medium becomes cloudy and the back of the flask became clear. From this suspension, 0.5 ml was added into a new flask with medium for maintenance. A cell count was carried out on this suspension and 5×10^5 cells were added into 24 well plates and allowed to adhere for 48 h with daily medium changes before infecting with *M. tuberculosis*. Drugs were added (day-0) after phagocytosis for 2.5 h. The culture medium and drugs were changed as for the mouse PM. Colony counts were done after incubation for 5 and 7 days.

2.5. Experimental design

There were three experiments in each of which a comparison was made between the activities of TMC207 in the range of 0.006–1.0 μg/ml on *M. tuberculosis* in each of the three conditions: extra-cellular, in the J774 cell line and in primary mouse PM. In further single experiments in each condition, the range of TMC207 was increased to 0.25–32 μg/ml. In each experiment, test cultures were set up in duplicate for extra-cellular cultures and in triplicate with the J774 cells and peritoneal macrophages. Serial 10-fold dilutions were made from each culture, and an inoculum of 0.1 ml from each was added to a one-third segment of a 7H11 plate in duplicate. Plates were packed into polyethylene bags and incubated for 3–4 weeks at 37 °C before colony forming units (cfu) were counted. Colony counts were recorded on an Excel sheet and converted to $\log_{10}$ cfu/ml using a standard formula.

2.6. Statistical methods

Rates of kill (K) were calculated by fitting a mixed effects linear regression model to the mean log ctu/ml counts over time. Counts on extra-cellular preparations at day-7 were omitted since with some concentrations bacteriostasis occurred during the 0–7 day period. Static concentrations (SC) were calculated for each replicate (two replicates extra-cellular and three replicates in the J774 cells and in peritoneal macrophages) and for each of three experiments using trigonometric methods with both colony count and drug concentration log transformed. Given the highest concentration, x on the log scale, with a cfu count, cfu_x, greater than the cfu count at day-0, cfu_0, and the lowest concentration, y, with a cfu count, cfu_y, smaller than the cfu_0, the formula for the SC used was:

Log (SC) = $-R((cfu_y - cfu_0) - y/R)$, where $R = (y - x)/(cfu_y - cfu_x)$. Using these estimates of SC, mixed effect models were used to estimate the mean SC for each type of culture condition.

3. Results

3.1. Bactericidal activities of TMC207 against bacteria in extra-cellular and intra-cellular locations

The bactericidal activity of extra-cellularTMC207 was slow in onset with stasis or slight growth at low concentrations during the first 7–14 days (Figure 1). Only by 21 days was there a clear distinction between concentrations of 0.006–0.12 μg/ml that allowed growth and concentrations of 0.25–32 μg/ml that were bactericidal in a dose-related manner. Reduction of the cfu count below the limit of detection (LOD) occurred with concentrations of 16–32 μg/ml The curves showed acceleration of killing during the 21-day exposure period. The corresponding results with J774 cells containing *M. tuberculosis* (Figure 2) showed earlier starts to bactericidal action with no initial static phase. The distinction in allowing growth became clear by 7 days with growth occurring at 0.006–0.025 μg/ml and inhibition in a concentration dependent manner at 0.12–32 μg/ml. The LOD was reached with 8–32 μg/ml by 5 days, more rapidly than in extra-cellular cultures. Again, the rate of kill accelerated between 5 and 7 days. The results with infected primary mouse peritoneal macrophages were similar to those obtained with the J774 macrophage cell line, except that counts were obtained only after incubation for 5 days since the