A Randomized Controlled Trial of Atorvastatin in Patients With Bronchiectasis Infected With Pseudomonas Aeruginosa

A Proof of Concept Study

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Procedures

I. Clinical Studies

We conducted assessments at baseline, 3 months, 4.5 months and at 7.5 months.

We assessed cough with the LCQ. This questionnaire is a 19-item, self-completed, quality-of-life measure of chronic cough, with scores from 3 to 21 (a lower score indicates more severe cough). The minimum clinically important difference in LCQ score is 1·3 units. The LCQ is repeatable over 6 months in stable disease (intraclass correlation coefficient 0·96, 95% CI 0·93–0·97; p<0·0001) (16).

We measured prebronchodilator FEV₁, FVC, and FEV₁:FVC by spirometry, followed by an incremental shuttle-walk test; an externally paced, 10 m, field-walking test incorporating an assessment of dyspnoea before and after, with results recorded on the Borg scale (a rating of perceived exertion) (17). Health-related quality of life was assessed with the SGRQ. This questionnaire is a 50-item self-administered test with a total score ranging from 0 to 100 (a higher score indicates poorer health-related quality of life). The minimum clinical important difference in SGRQ score is 4 units (18).

We induced sputum with hypertonic (3%) saline for 10 min and gathered samples for bacteriological analysis and neutrophil assessments (19). We determined samples as being suitable for processing if more than 25 polymorphonuclear leucocytes and fewer than ten squamous cells were present on Gram stain with low-power magnification (×20). We used 1 mL of the sputum sample for qualitative and quantitative microbiological analyses. Briefly, we homogenised sputum and liquefied it with an equal volume of dithiothreitol. To achieve dilution factors of 10⁻¹ to 10⁻⁴, we serially diluted the liquid samples with sterile 0·85% saline. We inoculated plates of Pseudomonas isolation agar (Difco; BD Biosciences, Oxford, UK), chocolate blood agar containing bacitracin (Oxoid, Basingstoke, UK), and horse blood agar (Oxoid) with 100 µL of each dilution and incubated plates at 37°C for 48 h. We counted colonies of pathogens to ascertain the sputum bacterial density (expressed as log₁₀ colony-forming units [cfu] per mL).

We divided the remainder of the sputum sample equally into two portions. To assess total cell numbers, we treated one part with 0·1% Sputolysin (Calbiochem, Hertfordshire), washed the sample twice with phosphate-buffered saline, centrifuged it at 2000 g for 10 min at 4°C, and filtered the sample once, then did cytocentrifugation at 75 g for 3 min at room temperature. We
calculated cell-differential counts by counting 400 cells per sample after cytocentrifugation (20). We confirmed apoptosis by the colour and shape change of the neutrophil nuclei on cytospins of sputum samples, as observed under light microscopy (magnification ×1000). The second portion was ultracentrifuged at 50,000 g for 90 min at 4°C (21). The colloidal solution phase was stored at −70°C until needed for analysis of the activity of myeloperoxidase, free neutrophil elastase, and CXCL8. We measured myeloperoxidase activity (22) with a chromogenic substrate assay and free elastase activity by spectrophotometry with a synthetic substrate (methoxysuccinyl-Ala-Ala-Pro-Val paranitroanilide; Sigma, Gillingham, UK), and we assayed interleukin 8 using commercially available specific ELISAs (R&D Systems, Oxford, UK) (21,23).

We took 30 mL of venous blood to obtain a full-blood count; to measure the erythrocyte sedimentation rate; to ascertain amounts of C-reactive protein, urea, electrolytes, and creatine kinase; and to do liver-function tests. We centrifuged 5 mL of blood at 750 g for 10 min, collected the supernatant, and stored it at −70°C until it was needed for measurement of amounts of proinflammatory and anti-inflammatory cytokines and chemoattractants by cytometric bead array (BD Biosciences) and ELISA’s as per manufacturers protocols (CXCL8 and ICAM 1- R&D systems).

We assessed patients for the presence or absence of side-effects at all study visits. If activity of alanine aminotransferase was greater than five times the normal value, or concentrations of creatine kinase were greater than three times the upper limit of normal, we stopped the assigned study treatment. We recorded all side-effects on a patient diary card. We defined exacerbations according to British Thoracic Society guidelines and treated them according to baseline sputum bacteriological findings and administered 14 days of oral antibiotic treatment.

II. In vitro studies

Isolation of neutrophils: Freshly drawn blood was collected from healthy volunteers, into 3.8% sodium citrate. Granulocytes were subsequently isolated by dextran sedimentation and discontinuous Percoll gradient, as described (24). Cells were re suspended at desired concentrations in media and counted using a haemocytometer. Purity of neutrophil preparations were evaluated by cytocentrifuge preparation analysis and only >95% pure neutrophil populations were used in experiments.

Neutrophil activation: As there was a reduction in serum ICAM-1 (see results section), we investigated the role of statins in CD11b expression, which functionally regulates neutrophil adhesion. We also assessed expression of CD62L which is key for leukocyte rolling prior to migration. After isolation of neutrophils as described above, neutrophils (10^7/ml in 75 µL PBS
containing Ca\(^{2+}\)/Mg\(^{2+}\) in 2 ml eppendorf tubes, atorvastatin (at final concentrations of 1 nM, 10 nM, 100 nM, 1 µM and 10 µM) was added and kept at 37°C for 30 minutes. Next the eppendorf tubes were put on a 37°C shaking heat block (300 rpm), before 100 nM formyl methyl leucyl phenylalanine (fMLF) or PBS was added, and incubated for a further 30 minutes. FITC- labelled CD11b antibodies and PE- labelled CD62L antibodies were added for 30 minutes and kept on ice in the dark. The supernatants were discarded and the cell pellets were resuspended and samples analysed by flow cytometry (25).

**Intracellular calcium flux:** As Ca\(^{2+}\) ions serve as important second messengers in signal transduction in neutrophils, we investigated the role of statins in regulating neutrophil calcium flux. Neutrophils were loaded with fura-2/AM (2 µM; Invitrogen) for 30 min in HBSS without divalent cations, washed, and re suspended at 2 x 10\(^6\)/ml in HBSS with divalent cations. Intracellular calcium flux was quantified using a spectrofluorimeter (Perkin Elmer, Waltham, MA, USA), in response to fMLF, with or without a 30 min pretreatment with atorvastatin at varying concentrations, as described (26).