Coadministration of the cyanobacterial lipopolysaccharide antagonist CyP with antibiotic inhibits cytokine production by an *in vitro* meningitis model infected with *Neisseria meningitidis*

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**Objectives:** In this study, the objective was to determine the anti-inflammatory properties of CyP, a cyanobacterial lipopolysaccharide (LPS) antagonist, used in combination with antibiotic chemotherapy during infection of an *in vitro* meningitis model infected with *Neisseria meningitidis* (meningococcus).

**Methods:** Monocultures of human meningioma cells and meningioma–primary human macrophage co-cultures were infected with meningococci (10²–10⁸ cfu/monolayer) or treated with isolated outer membranes or purified LPS (0.1–100 ng/monolayer) from *N. meningitidis*. CyP (1–20 mg/monolayer) was added at intervals from \( t = 0 \) to 4 h, with and without benzylpenicillin (1–20 mg/monolayer). The antagonistic effect of CyP and its adjunctive properties to benzylpenicillin administration was determined by measuring cytokine levels in culture supernatants after 24 h.

**Results:** CyP significantly inhibited \((P<0.05)\) the secretion of interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1 and RANTES (‘regulated upon activation, normal T cell expressed and secreted’) (overall reduction levels from 50% to >95%) by meningioma cell lines and meningioma–macrophage co-cultures challenged with either live meningococci or bacterial components. Inhibition was effective when CyP was added within 2 h of challenge \((P<0.05)\) and was still pronounced by 4 h. In the co-culture model, CyP alone partially inhibited IL-1β secretion, but did not prevent tumour necrosis factor (TNF)-α secretion, whereas penicillin alone inhibited IL-1β and TNF-α but conversely did not reduce MCP-1 and RANTES secretion. However, coadministration of CyP and penicillin in both models had an additive effect and restored the overall inhibitory profile.

**Conclusions:** CyP inhibits cytokine production in an *in vitro* meningitis model and augments the anti-inflammatory response when combined with benzylpenicillin. Administration of an LPS antagonist with anti-biotic merits consideration in the emergency treatment of patients presenting with meningococcal infection.

**Keywords:** meningococcus, *Oscillatoria planktothrix*, inflammation

**Introduction**

The Gram-negative proteobacterium *Neisseria meningitidis* (meningococcus) is a major causative organism of sepsicaemia and meningitis, which are, respectively, acute compartmentalized intravascular and intracranial inflammatory responses, characterized by the presence of elevated serum and CSF cytokine levels. Meningococcal meningitis is a classical leptomeningitis, i.e. the organism shows a predilection for associating with the arachnoid, pia mater and trabeculae (collectively the leptomeninges) that traverse the CSF-filled subarachnoid space (SAS).¹ The CSF of patients presenting with acute meningococcal meningitis typically contains raised levels of the pro-inflammatory cytokines interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-6, the CXC chemokine IL-8, and the CC chemokines monocyte chemoattractant protein (MCP)-1 and RANTES (regulated upon activation, normal T cell expressed and secreted).² ³ These leptomeninges play an active role in the
innate inflammatory response to meningococcal infections, most likely aided by the small population of resident meningeal macrophages.

It is generally accepted that the *N. meningitidis* lipopolysaccharide (Nm-LPS), which is present in the outer membrane (OM) of the bacterium, is largely responsible for inducing intracranial inflammation. The mechanism is probably similar to that of other Gram-negative LPS, through interaction with and activation of the innate CD14-Toll like receptor (TLR4)-MD2 recognition receptor complex. Emergency treatment of meningitis patients involves the rapid administration of penicillin-based antibiotics, followed by intensive care management and steroid therapy. Anti-biotic treatment does reduce LPS-induced cytokine secretion, principally by reducing bacterial numbers in the blood and CSF, but regardless of prompt treatment, the mortality rates from meningococcal infection can still be as high as 20%–50%. This highlights the need for adjunctive therapies that could further inhibit the activation of myeloid and non-myeloid cells by liberated Nm-LPS.

Recently, an LPS-related molecule derived from the cyanobacterium *Oscillatoria planktonthrix* FP1 and termed CyP has been shown to act as a selective TLR4-MD2 receptor antagonist. Several reports have now shown the utility of CyP in inhibiting the LPS stimulation of inflammatory responses in animals and ex vivo cell culture models. CyP protected mice from endotoxic shock caused by *Salmonella abortus* LPS and was itself nontoxic. CyP was also shown to act as a competitive inhibitor of Escherichia coli LPS binding to the receptor complex on human dendritic cells ex vivo, and to subsequently suppress downstream gene transcription and cytokine production. Recently, we reported that CyP was a potent antagonist of Nm-LPS, inhibiting cytokine production in an *in vitro* whole blood model of meningococcal septicemia. Similarly, CyP was reported to be an efficient inhibitor of E. coli LPS-induced cytokine production in porcine whole blood and also with isolated human polymorphonuclear leucocytes (PMNLs).

Although cyanobacterial LPS (CyP) is basically similar to LPS from Gram-negative bacteria, including Nm-LPS, there are differences in chemical and biological properties between the two. For the current study, we tested the hypotheses that CyP could be used (i) to reduce or completely inhibit the cytokine response induced by the interactions of live meningococci, isolated OMs and pure Nm-LPS with the meninges, and (ii) as an effective adjunct to benzylpenicillin therapy. In order to test these hypotheses, we used an established in vitro model consisting of cells derived from benign human meningiomas, which we have shown are morphologically and cytologically similar to normal leptomeningeal cells. Importantly, these cells also express TLR4, are responsive to Nm-LPS and react with meningococci similarly.

In addition, the model was extended by mimicking the presence of resident meningeal macrophages in the SAS by the addition of human primary macrophages to the meningioma cell cultures.

### Materials and methods

**Bacteria and growth conditions**

*N. meningitidis* strain MCS8 (B:15:P1.7,16b) was isolated from an outbreak of meningococcal infections that occurred in Stroud, Gloucestershire, UK in the mid-1980s. Meningococci were grown at 37°C in an atmosphere containing 5% (v/v) CO2 on supplemented proteose peptone (Becton Dickinson, Oxford, UK) agar.

**Preparation of meningococcal OMs and Nm-LPS**

OMs were prepared by the extraction of wild-type MCS8 whole cells with lithium acetate. Meningococci were harvested from the confluent growth of bacteria on 60 14.5-cm agar plates directly into 40 mL of 0.2 M lithium acetate (pH 5.8) and extracted at 45°C with stirring in the presence of glass beads. The OM fraction was recovered by differential centrifugation at 4°C, initially at 15000 g for 30 min to sediment debris, followed by centrifugation of the supernatant at 150000 g for 2 h (Beckman Coulter Optima L-80XP, Palo Alto, CA, USA). The resulting pellet was suspended in sterile water and stored at −20°C. Nm-LPS was purified from MCS8 by extraction with hot phenol as follows. The confluent growth of meningococci from 40 14.5-cm agar plates was harvested into 6.5 mL of water at 70°C and 6.5 mL of 90% (v/v) molecular biology-grade phenol (BDH, VWR International Ltd, Lutterworth, Leicestershire, UK) added. The suspension was homogenized for 15 min in a water bath at 70°C and then centrifuged at 10000 g for 30 min to separate the aqueous and phenol phases. The phenol and interface phases were extracted again with a further 6.5 mL volume of water. The aqueous phases were pooled and dialysed against running tap water for 2 days at room temperature. Sodium EDTA (Sigma−Aldrich, Poole, UK) was then added to a final concentration of 5 mM, followed by dialysis overnight against deionized water. The dialysate was then transferred to a glass container and a few crystals of MgCl2 added, followed by 2 volumes of cold acetone and storage at 4°C overnight. The resulting precipitate was centrifuged and washed with 50% (v/v) acetone and desiccated under vacuum. The pellet was suspended in 5 mL of 0.25 M Tris-acetate buffer, pH 7.2, and dissolved with homogenization. MgCl2 was added to a final concentration of 1 mM, followed by RNase and DNase enzymes. The mixture was stirred at room temperature for 4 h and then dialysed against 0.25 M Tris-acetate buffer, pH 7.2, overnight at 37°C. Lastly, the suspension was centrifuged at 10000 g for 15 min and the supernatant then centrifuged at 90000 g for 2 h. The final pellet was suspended in water and dried. The quantification of Nm-LPS and OMs was based on the dry weight and protein content as determined by the bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Cramlington, Northumberland, UK), respectively. OMs and Nm-LPS were prepared in sterile, endotoxin-free PBS prior to dilution in culture medium for infection experiments.

**CyP**

CyP was supplied by Bluegreen Biotech s.r.l. (Milan, Italy) and was extracted from the freshwater cyanobacterium *O. planktonthrix* FP1 by a phenol−guanidinium thiocyanate-based method, as described previously. Bacteria were collected by centrifugation and were suspended in TRI Reagent (Sigma−Aldrich, St Louis, MO, USA). After centrifugation, the aqueous phase was recovered and the extracts precipitated with 10 mM sodium acetate buffer and 2 volumes of acetone. Pellets, obtained after centrifugation, were washed with 70% (v/v) ethanol and then treated with DNase (20 μg/mL) and RNase (10 μg/mL) in 50 mM Tris buffer, pH 7.5, containing 10 mM MgCl2, for 2 h at room temperature. Proteinase K (100 μg/mL) was then added and the mixture incubated overnight at 37°C. The sample was re-extracted with TRI Reagent, purified by ion-exchange chromatography and then freeze-dried. The product was visualized by deoxycholate (DOC)−PAGE and silver staining, or by DOC−PAGE and staining with Pro-Q−Emerald 300 PBS gel stain kit (Invitrogen, Monza, Italy). The specific activity of CyP was determined by the inhibition of TNF-α production in culture supernatants of human THP1 cells stimulated with E. coli LPS (Sigma−Aldrich). No cytotoxic
effects were observed using CyP at high doses (100 μg/mL) in vitro. Protein and nucleic acid contamination of CyP samples was evaluated by the Bradford method and reading of the absorbance at λ = 260/280 nm, respectively; protein and nucleic acid contamination was <3% and <2%, respectively. CyP was dissolved in sterile, endotoxin-free PBS prior to addition to cell cultures.

Cell culture

Human meningioma cells

The culture and characterization of human meningioma cell lines was carried out as described previously. Briefly, fresh meningioma tissue was obtained from surgically removed tumours and necrotic tissue, and blood clots were removed. The tissue was cut into small pieces (1 mm³) and digested with 0.125% (w/v) trypsin/0.02% (w/v) EDTA in Hanks balanced salt solution. Following digestion, the cells were centrifuged (10 min, 300 g), and suspended in Dulbecco’s modified Eagle’s medium (DMEM) containing GlutaMAX™-1 and sodium pyruvate (DMEM/GlutaMAX™; Lonza Biologics, Slough, UK) and supplemented with 10% (v/v) decomplemented fetal calf serum (dFCS; Lonza Biologics). Cells were cultured in flasks or 24-well cell culture plates in humid environment at 37 °C

Isolation and culture of monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized human blood by layering on Lymphoprep® (Axis-Shield, Oslo, Norway) and centrifuging according to the manufacturer’s instructions. Monocytes were isolated from PBMCs using CD14 + microbeads (Miltenyi Biotec, Bisleby, UK), according to the manufacturer’s instructions. Isolated monocytes (>95% CD14 + as assessed by flow cytometry) were differentiated in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mg/mL L-glutamine, 0.05 U/mL penicillin, 50 μg/mL streptomycin, 0.5 mg/mL amphotericin B (all from Invitrogen, Paisley, UK) and 10 ng/mL macrophage-colony stimulating factor (R&D Systems, Abingdon, UK) for 7 days. Cells were harvested from culture dishes using Non-Enzymatic Cell Dissociation Solution (Sigma–Aldrich, Poole, UK) prior to suspension in basal DMEM and cell counting using a haemocytometer. The collection of blood samples for this study was approved by the Southamptom and South West Hampshire Research Ethics Committee (reference: 08/H0504/138).

Meningioma cell–macrophage co-culture

Human meningioma cell lines M43 and M53 were grown to confluence (~3.3 × 10⁴ cells/monolayer) in 24-well plates and washed with warm sterile PBS to remove antibiotics. Macrophage suspensions of 10³ cells/mL were then added to the wells in fresh DMEM containing 10% (v/v) dFCS without antibiotics. The co-cultures were maintained at 37°C with 5% (v/v) CO₂ for 24 h to allow macrophages to settle prior to treatment.

Infection experiments

Meningioma cell lines M43 and M53 from passages 5–10 were grown to confluence on collagen-coated 24-well cell culture plates. The medium was removed, and the cell monolayers were washed in PBS containing 0.1% (v/v) dFCS (wash buffer) and then challenged with concentrations of bacteria, prepared in DMEM/GlutaMAX™ containing 1.0% (v/v) dFCS (1 mL per monolayer), ranging from 1 × 10⁷ to 10⁸ (moi = 0.2). 1 × 10⁸ (moi = 20) and 1 × 10⁹ (moi = 2000) cfu/mL. Meningioma cells were also treated with varying concentrations of isolated OMs (0.1–100 ng/monolayer) and Nm-LPS (0.1–100 ng/monolayer). For the meningioma cell–macrophage co-cultures, the culture medium was removed and the cells were infected with 10⁶ cfu of bacteria/culture in DMEM/GlutaMAX™ containing 1.0% (v/v) dFCS.

Meningococcal infection of meningioma cell and macrophage monolayers and meningioma cell–macrophage co-cultures was also done in the presence of varying concentrations of CyP (1–20 μg/culture) and/or benzylpenicillin (1 μg/culture; Sigma–Aldrich, Poole, UK) added at various times (0–4 h). For controls, monolayers of meningioma cells and macrophages and co-cultures were left untreated with and without the addition of CyP and/or benzylpenicillin.

Measurement of cytokine production

The levels of pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α), chemokine (MCP-1, IL-8 and RANTES) and anti-inflammatory cytokine (IL-10) proteins were quantified by sandwich immunoassay kits from E Bioscience (Hatfield, UK) and R&D Systems, according to the manufacturers’ instructions. Briefly, Nunc Maxisorp 96-well plates (Nunc A/S, Roskilde, Denmark) were coated with purified anti-human cytokine capture antibodies, overnight at 4°C. After washing with PBS containing 0.05% (v/v) Tween 20, the wells were blocked with proprietary assay buffer for 1 h at room temperature. Supernatant samples and cytokine standards were serially diluted in assay buffer for testing, and the plates stored at 4°C overnight to allow maximum binding. After washing, bound cytokines were detected by incubation for 1 h at room temperature with biotin-conjugated anti-human cytokine antibodies followed by 30 min incubation with avidin–horseradish peroxidase conjugate. After washing, a proprietary substrate solution containing tetramethylbenzidine was added to the wells for 10 min and the reaction stopped with the addition of 1 M H₂SO₄. The absorbance was read at λ = 450 nm using an iMark plate absorbance reader (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). An unpaired two-sample t-test was used to compare the mean levels of cytokine secretion from n=3 wells following particular treatments, with P<0.05 considered as significant.

Results

CyP inhibits cytokine production by human meningioma cells infected with live meningococci and treated with isolated OMs and Nm-LPS

For our first series of experiments, we investigated the effects of various concentrations of CyP (1–20 μg) on cytokine production by human meningioma cell lines M43 and M53 infected with various concentrations of live N. meningitidis (10²–10⁸ cfu/monolayer). CyP was added at the time of infection (t=0 h) and cytokine secretion was measured after 24 h. IL-6, IL-8, MCP-1 and RANTES secretion was induced by all doses of bacteria, compared with uninfected control monolayers (P>0.05), and in general cytokine levels increased with increasing doses of bacteria. All doses of CyP inhibited IL-6 secretion induced by 10⁴–10⁸ bacteria...
by ~50% to >99% (Figure 1), compared with infected monolayers without CyP (P<0.05). In the case of IL-8, the highest dose of CyP (20 µg) inhibited secretion induced by 10^2–10^6 bacteria by ~70%–80%, and lower doses were still effective to between ~30% and 50% (P<0.05). However, with the highest dose of bacteria tested, only a 25% reduction in IL-8 was observed with 10–20 µg of CyP. For MCP-1, secretion induced by 10^2–10^6 bacteria was inhibited by CyP (dose range of 1–20 µg) by 40%–60%, whereas with the higher dose of 10^8 meningococci, a dose range of 5–20 µg only inhibited between 15% and 40% of secretion. For RANTES, the dose range of CyP tested inhibited secretion induced by all doses of bacteria by ~50%–75% (P<0.05).

During infection, meningococci shed OM vesicles or ‘blebs’ into the CSF, and these blebs contain Nm-LPS that can interact with meningeval cells. The median concentration of purified or membrane-bound Nm-LPS in the CSF of patients with meningococcal meningitis has been measured as 2.5 ng/mL, which is equivalent to a CSF bacterial load of ~2.5×10^6 cfu/mL live N. meningitidis. In the current study, isolated Nm-LPS-replete OMs induced the secretion of IL-6, IL-8, MCP-1 and RANTES, which was significantly inhibited by all doses (1–20 µg) of CyP added at 0 h. At the highest doses of 10–100 ng/monolayer of OMs, >90% inhibition of IL-6 secretion was observed with doses of 1–20 µg of CyP, and ~60%–90% for IL-8 and MCP-1, compared with OM-treated cell cultures alone (P<0.05) (Figure 1). For RANTES, the dose range of CyP tested inhibited secretion induced by all doses of bacteria by ~60%–85% (P<0.05).

To confirm that CyP was acting as an antagonist of Nm-LPS stimulation, meningioma cell monolayers M43 and M53 were treated with various doses of pure Nm-LPS (0.1–100 ng/monolayer) in the presence (1–20 µg) of the antagonist (Figure 1). For RANTES, the dose range of CyP tested inhibited secretion induced by all doses of Nm-LPS, since increasing

Figure 1. CyP inhibits cytokine production by human meningioma cells infected with live meningococci and treated with isolated OMs and Nm-LPS. Human meningioma cell lines M43 and M53 were infected with live meningococci (10^2–10^8 cfu/monolayer) or treated with OMs (0.1–100 ng/monolayer) or Nm-LPS (0.1–100 ng/monolayer) in the presence (1–20 µg/monolayer) or absence of CyP, added at t=0 h. Culture medium was removed at 24 h and tested for cytokines. The data shown are representative of experiments carried out with cell line M53 (n=4 experiments) and similar data were obtained with meningioma cell line M43 (n=2 experiments). Individual points report mean cytokine levels of triplicate wells and the error bars are the standard deviations. *Statistically significant inhibition compared with untreated monolayers, 0 CyP (P<0.05).
concentrations of CyP resulted in an additive effect on chemokine secretion. For RANTES, the dose range of CyP tested inhibited the secretion induced by all doses of bacteria by \( \approx 60\%-75\% \) \( (P, 0.05) \).

Next, we investigated whether the efficacy of CyP was dependent on the time of administration. Meningioma cell line M43 was infected with meningococci \( (10^6 \text{ cfu/monolayer}) \) or treated with OMs \( (10 \text{ ng/monolayer}) \) or Nm-LPS \( (10 \text{ ng/monolayer}) \) in the presence \( (20 \mu\text{g/monolayer}) \) or absence of CyP added at various timepoints. Culture medium was removed at 24 h and tested for cytokines. The data are representative of \( n=2 \) experiments, with individual columns reporting mean cytokine levels of triplicate wells and the error bars the standard deviations. *Statistically significant inhibition compared with monolayers with no CyP \( (P<0.05) \).

Effect of CyP when used as an adjunct to penicillin for inhibiting cytokine secretion

Administration of antibiotics during meningococcal infection has been reported to reduce cytokine levels as a result of inhibiting bacterial growth.\(^\text{10}\) In our current study, we tested the hypothesis that CyP would act as an adjunct to antibiotic treatment and result in a further reduction in cytokine secretion. Thus, we carried out a series of experiments \( (n=3) \) with meningioma cell line M53 infected with meningococci \( (10^9 \text{ cfu/monolayer}) \) and treated with CyP \( (5 \text{ or } 20 \mu\text{g dose}) \) and benzylpenicillin \( (1 \mu\text{g dose}) \) alone and together, and added at 0 and 2 h after infection. A dose of 1 \( \mu\text{g/mL} \) was used in order to mimic the concentration of antibiotic measured in the CSF of patients with purulent meningitis.\(^\text{11}\) The addition of the antibiotic at 0 or 2 h resulted in a 96\%-99\% reduction in the meningococcal cfu within 2 h of administration, as judged by the counting of viable bacteria in the culture medium \( (Figure \ 3a) \). CyP alone shows no antimicrobial activity against meningococci.\(^\text{16}\)
controls, infected and uninfected cultures with and without CyP and/or benzylpenicillin treatments at both timepoints were included.

CyP or penicillin alone when added at 0 h inhibited IL-6 secretion by ~90% (P<0.05) and by 70%–90% when added after 2 h of infection (Figure 3b). When CyP and penicillin were given together, inhibition of IL-6 secretion increased to >98% (P<0.05). Similarly, for IL-8, CyP and penicillin alone inhibited secretion by 70%–80% and 50%–70% at 0 and 2 h, respectively, but this increased to 80%–95% when used together (Figure 3b). However, subtle differences were observed for the chemokines MCP-1 and RANTES. CyP administered at 0 h reduced MCP-1 and RANTES secretion by 60% and 70%, respectively. When administered at 2 h, MCP-1 and RANTES secretion was reduced by 40% and 50%, respectively. In contrast, penicillin administered alone was less effective: MCP-1 secretion was inhibited

Figure 3. (a) Benzylpenicillin kills meningococci when added to infected meningioma cell cultures. Monolayers of human meningioma cell line M53 were infected with 10⁶ cfu of meningococci and treated with CyP (20 μg dose) and benzylpenicillin (1 μg dose), added at 0 and 2 h (n=2 experiments). Viable counts were made of bacteria in the culture over time. The symbols define the mean bacterial counts from triplicate wells. (b) CyP is an effective adjunct to penicillin for inhibiting cytokine secretion by human meningioma cells infected with meningococci. Monolayers of human meningioma cell line M53 were infected with meningococci (10⁶ cfu/monolayer) and treated with CyP (20 μg dose) and benzylpenicillin (1 μg dose) alone and together at 0 and 2 h after infection. For controls, infected and uninfected cultures with and without CyP and/or benzylpenicillin treatments at both timepoints were included. The data shown are from a representative experiment (n=3), and the columns report mean cytokine levels of triplicate wells and the error bars are the standard deviations. *Statistically significant inhibition compared with monolayers with no CyP/benzylpenicillin (P<0.05). PEN, benzylpenicillin.
CyP with antibiotic inhibits cytokine production

by only 33% and 5% when administered at 0 and 2 h, respectively, whereas RANTES secretion was not inhibited (Figure 3b). However, administration of CyP with penicillin inhibited MCP-1 and RANTES secretion to the levels that were observed with the use of CyP alone (Figure 3b). Identical findings for the inhibition of cytokine secretion were obtained using both a lower concentration of CyP (5 μg) with benzylpenicillin (1 μg) or increased doses of antibiotic (20 μg) with CyP (20 μg), suggesting a broad effective range (data not shown).

**Effect of CyP and antibiotic on cytokine production from a human meningioma cell–macrophage co-culture infected with N. meningitidis**

We next extended the model for testing CyP by mimicking the presence of resident meningeal macrophages in the SAS, by the addition of human primary macrophages to the meningioma cell culture lines. To our knowledge, the exact percentage ratio of resident meningeal macrophages to meningeal cells in the sterile human leptomeninges is not known, but it has been reported to be ~1% in the meninges associated with the human embryonic optic nerve. In the current study, co-cultures of human macrophages with meningioma cells (M53) were established using ~2% (10^6) macrophages to meningioma cells. In pilot experiments (n = 2) a minimum concentration of 10^6 macrophages was necessary to produce detectable cytokine responses to infection with 10^6 meningococci and, moreover, the addition of CyP alone (20 μg) could inhibit cytokine production when added at 0 and 2 h (data not shown). In the subsequent experiments, CyP (20 μg) and benzylpenicillin (1 μg) were tested in combination at 0 and 2 h in this co-culture model for their ability to inhibit the cytokine secretion induced by infection with 10^6 meningococci (Figure 4).

In the co-culture model, the addition of CyP at 0 and 2 h resulted in a significant reduction of IL-6 secretion by 65% and 50%, respectively (P < 0.05), compared with untreated co-cultures, whereas penicillin alone was slightly more effective (~85%–90% inhibition; P < 0.05) (Figure 4). For IL-8, CyP added at either time point reduced secretion by ~50% and the reduction was in the region of 65%–75% with penicillin alone (P < 0.05). For RANTES, CyP reduced secretion by ~70% and 60% at 0 and 2 h, respectively, but penicillin alone was less effective, reducing secretion by 50% and 40%, respectively (P < 0.05). However, a combination of CyP and penicillin showed an additive effect at both 0 and 2 h administration, with inhibition of IL-8, IL-6 and RANTES secretion rising to > 75%–97% (Figure 4), compared with untreated co-cultures (P < 0.05). Differences were observed for the pro-inflammatory cytokines IL-1β and TNF-α, and the chemokine MCP-1. For IL-1β, CyP inhibited secretion by ~50% and 20% at 0 and 2 h, whereas penicillin alone reduced the cytokine to non-detectable levels (P < 0.05). In contrast, although CyP could inhibit MCP-1 secretion by ~45% compared with untreated co-cultures, penicillin alone was ineffective (P > 0.05) in the co-cultures (Figure 4). Furthermore, whereas CyP showed inhibitory effects for all the above cytokines, it was ineffective when used alone for inhibiting TNF-α secretion by the co-cultures (Figure 4). Penicillin showed an efficacy of 70% and 50% in reducing TNF-α secretion at 0 and 2 h, but the combination of both CyP and antibiotic was additive, reducing TNF-α levels by 97% and 81%, respectively (P < 0.05; Figure 4). No significant IL-10 was detected in any of the cultures.

Included as controls within the same co-culture experiments, we also assessed the effects of CyP and/or penicillin combination on cytokine secretion by macrophages alone (Figure 4). Macrophages in co-culture specifically secreted TNF-α and IL-1β in response to meningococcal infection and contributed to IL-6, IL-8 and RANTES secretion, but did not produce MCP-1 (Figure 4). CyP alone added at 0 h inhibited IL-6, IL-8, RANTES and IL-1β by ~40%, 30%, 20% and 80%, respectively, compared with untreated cells (P < 0.05), but failed to inhibit TNF-α secretion (P > 0.05). Penicillin alone added at 0 h reduced IL-6, IL-8, RANTES, IL-1β and TNF-α by ~50%, 30%, 40%, 100% and

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**Figure 4.** CyP in combination with antibiotic inhibits cytokine production in human meningioma cell–macrophage co-cultures infected with N. meningitidis. Co-cultures of human macrophages and monolayers of meningioma cell line M53 were established and infected with 10^6 meningococci and treated at 0 and 2 h with CyP (+C; 20 μg dose) and benzylpenicillin (+P; 1 μg dose) alone or in combination. Monocultures of macrophages (10^6 cells) were treated similarly. Culture supernatants were collected at 24 h and tested for cytokine secretion. The data are from a representative experiment with cell line M53 (from n = 4 experiments carried out with four independent donors for macrophage isolation), and the columns report the mean cytokine levels of triplicate wells and the error bars are the standard deviations. Cytokine levels were non-detectable for control, uninfected meningioma–macrophage co-cultures and macrophage monolayers, with or without CyP/benzylpenicillin. *Statistically significant inhibition compared with infected co-cultures or monolayers without CyP/benzylpenicillin (P < 0.05).
80%, respectively (P<0.05). A combination of CyP and penicillin resulted in inhibition levels ranging from 30% for IL-8 up to ≥90% for the other cytokines (Figure 4).

Discussion

In many CNS infections it is the pathogen-induced inflammatory response that causes much of the damage seen. In meningococcal meningitis there is a clear correlation between increasing Nm-LPS concentration and intracranial cytokine levels, leucocyte recruitment and CNS-induced injury, and the clinical presentations of headache, nuchal rigidity, reduced consciousness, cerebral oedema and brainstem herniation causing death. The key role that Nm-LPS plays in the pathophysiology of meningococcal meningitis makes it a logical target for novel adjunctive therapies. But, to our knowledge, no anti-inflammatory reagents that target the direct effects of Nm-LPS have been used for treating meningococcal meningitis solely. In contrast, several anti-LPS strategies have been tested for their ability to reduce the LPS-induced inflammation seen in bacterial sepsis. In clinical trials, the administration of recombinant human bactericidal/permeability-increasing protein (rBPI21), which inhibits activation by directly binding to LPS, resulted in reduced morbidity in meningococcal sepsis patients, but survival rates were unaffected. In contrast, in a Phase II trial of eritoran (E5564), a synthetic LPS that functions as a TLR4/MD2 antagonist in much the same way as CyP, there was an observed trend towards a lower mortality rate in patients with severe bacterial sepsis and with a predicted high rate of mortality. In the current study, we explored the effect of CyP on inflammation in an in vitro model of meningococcal meningitis. We demonstrated that CyP exerted a significant inhibitory effect upon cytokine production by meningioma cell lines challenged with up to 10⁶ cfu/mL live meningococci and 10–100 ng/mL pure Nm-LPS and isolated OMs. Thus, in vitro, CyP significantly inhibited the inflammatory cytokine secretion stimulated by the levels of bioactive Nm-LPS most commonly seen in the CSF of patients with meningococcal meningitis. Notably, this inhibition was similar to that observed in an in vitro whole blood model of meningococcal septicemia. In the current study, CyP alone showed no cytocytotoxicity towards meningioma cells or primary human macrophages. This is consistent with reports of the non-toxicity of the compound in animal studies, and with whole blood and other cell lines in vitro. In addition, when different batch preparations of CyP were tested, the cytokine inhibitory effect was very consistent. For example, in experiments in which meningioma cells were infected with live meningococci and comparing three independent batches of CyP used at a dose of 20 μg, the mean percentage inhibition of IL-6 (with 95% confidence limits in parentheses) was 93% (88%–99%); for IL-8, 81% (71%–92%); for MCP-1, 61% (52%–72%); and for RANTES, 73% (58%–93%). CyP also did not stimulate meningial cell lines or macrophages. In particular, CyP did not up-regulate the secretion of chemo-attractant cytokines such as IL-8 and MCP-1, which has been seen previously upon addition of CyP to human whole blood or isolated PMNLs.

The inhibitory effect of CyP upon cytokine production was dependent upon the time of addition after bacterial challenge. In this study, CyP significantly inhibited cytokine secretion when administered up to 2 h after challenge with Nm-LPS, OMs or live meningococci. Notably, the inhibitory effect for some cytokines was still apparent when CyP was administered at 4 h. This is an improvement in efficacy when compared with treating sepsis in vitro, wherein CyP was only effective at inhibiting cytokine production in whole blood if added within 1 h of meningococcal stimulation and no later. This difference is likely to be due to the rapid activation of the heterogeneous myeloid cell population in whole blood in vitro, compared with a cell monoculture, but suggests that CyP has a longer time window for administration to produce a significant anti-inflammatory response during meningeeal irritation without septicemia. This is consistent with the clinical observations on the use of eritoran, which was administered to sepsis patients within an 8 h time period of confirmation of sepsis, with two dose regimes (45 and 105 mg) given at 12 h intervals over 6 days, which afforded median plasma drug levels of 2.2–4.3 μg/ml.

In the current study, the combination of CyP and penicillin had an additive effect on inhibiting the secretion of cytokines (IL-6, IL-8 and IL-1β) by both meningioma cell monocultures and macrophage–meningeal cell co-cultures. For some cytokines, e.g. MCP-1 and RANTES, CyP was more effective than penicillin alone in inhibiting secretion and the poorer efficacy of the antibiotic could be overcome by the presence of CyP. In contrast, CyP was ineffective when used alone for inhibiting TNF-α secretion by either co-cultures or macrophages alone and inhibition only occurred with the administration of penicillin. Clearly, these findings show that both CyP and the antibiotic have anti-inflammatory properties, but with subtle differences in their ability to reduce the secretion of different cytokines. However, a combination of the two treatments augments the overall anti-inflammatory effect and overcomes the individual failings of each treatment. In the Phase II clinical trial of eritoran, all patients received sepsis management that included antibiotics, so no conclusions could be drawn as to whether such a cooperative effect was occurring in vivo. However, it is interesting to note that a related LPS antagonist, E5531, not only protected mice from E. coli LPS-induced lethality, but also in cooperation with the antibiotic latamoxef protected mice from a lethal infection of live E. coli.

In response to meningococcal infection, meningeal cells specifically secrete IL-6, IL-8, MCP-1 and RANTES. In the co-culture system, the presence of sentinel macrophages at levels approximating those found in the meninges contributed by specifically secreting the pro-inflammatory cytokines IL-1β and TNF-α and by generally increasing the levels of IL-6, IL-8 and RANTES. As expected, the levels of MCP-1 were not increased. These in vitro findings correlate with the cytokine profiles of patients with meningococcal meningitis and now identify the individual cellular sources of these molecules. Indeed, increasing the number of macrophages in our co-culture system to ~10⁶ (~20% cell ratio to meningeal cells) resulted in further increases in all cytokine levels (R. Oliver and M. Christodoulides, unpublished observations). This particular observation correlates with increased cytokine production in the CSF of patients with significant meningeal irritation, which leads to the influx of neutrophils followed by monocytes in the reconstitution phase. This cellular infiltrate increases the concentration of CD14–TLR4–MD2 available for Nm-LPS interaction, thereby exacerbating the intracranial inflammatory response. In the current study, the CyP concentrations
used were sufficient to inhibit cytokine production by both meningococcal lipopolysaccharides in human pathology. J Endotox Res 2001; 7: 2163–7.


