Chlamydia pneumoniae growth inhibition in human monocytic THP-1 cells and human epithelial HEP-2 cells by a novel phenoxazine derivative

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In this study the effects of 2-amino-phenoxazine-3-one (phenoxazine derivative, Phx-3) on Chlamydia (Chlamydophila) pneumoniae growth in human monocytic THP-1 cells as well as human epithelial HEP-2 cells were examined. Cells were infected with bacteria at an m.o.i. of 10 by centrifugation. After washing to remove any remaining bacteria, the cells were incubated with or without Phx-3 in the presence or absence of tryptophan for 72 h. The bacteria in cells were assessed by staining of chlamydial inclusions with FITC-labelled anti-chlamydial antibody, electron microscopic analysis, real-time RT-PCR specific for C. pneumoniae 16S rRNA and propagation on HEP-2 cells. Treatment with Phx-3 significantly inhibited growth of C. pneumoniae in THP-1 and HEP-2 cells. A decrease in the number of bacterial 16S rRNA transcripts was also confirmed in both cell lines by real-time RT-PCR. Electron microscopic studies revealed that treatment with Phx-3 induces bacterial destruction in most of the inclusion bodies in these cells. Addition of tryptophan to the culture slightly blocked the growth inhibition of C. pneumoniae by Phx-3. The reagents did not show any cytotoxicity to the cells at the concentrations used. The results suggest that Phx-3 inhibits C. pneumoniae replication in human monocytic cells as well as epithelial cells, partially depending on the tryptophan-metabolic pathway of host cells. Thus, Phx-3 might be a useful compound for controlling C. pneumoniae growth in cells and may be an alternative conventional therapy.

INTRODUCTION

Chlamydia (Chlamydophila) pneumoniae is an obligate intracellular bacterium that causes a common human respiratory infection (Saikku et al., 1985; Grayston et al., 1986). Interestingly, previous studies have shown that the chlamydial infections tend to become chronic (Aldus et al., 1992; Saikku, 1992; Von Hertzen et al., 1995), which is clinically important with regard to not only persistent respiratory diseases but also blood vessel inflammatory diseases such as atherosclerosis (Hahn et al., 1991).

C. pneumoniae preferentially infects monocytes as well as respiratory tract epithelial cells (Gaydos et al., 1996; Airenne et al., 1999; Lin et al., 2000). Although the pathogenic potential of this pathogen in the respiratory system is well established, several studies indicate that the organism may disseminate from this site to the blood stream through monocytes (Moazed et al., 1998). Thus, susceptibility of C. pneumoniae to antibiotics after invasion of monocytes is critical in controlling the spread of the organism from the lungs to possible sites of chronic infection. However, recent studies have suggested that the elimination of C. pneumoniae from persistently infected monocytes is extremely difficult, even though it is relatively easy to exclude the bacteria from epithelial cells or endothelial cells (Gieffers et al., 2001; Yamaguchi et al., 2003). Therefore, it is very important to investigate new approaches that do not rely on antibiotics in order to improve persistent chlamydial infection, which can cause serious chronic diseases, including atherosclerosis. The

Abbreviations: IDO, indoleamine-2,3-dioxygenase; Phx-3, 2-amino-phenoxazine-3-one.
mechanisms leading to complete exclusion of persistent *C. pneumoniae* infection are not clear. However, it is known that bacteria under surveillance by the host immune system can escape through down-regulation of apoptosis in the host cell and securing an intracellular pool of tryptophan; and thus control of these cellular metabolic activities in host cells is a critical event (Shemer & Sarov, 1985; Byrne et al., 1986; Beatty et al., 1993; Summersgill et al., 1995; Rottenberg et al., 2002).

Phenoxazine is a unique compound found in various insects and among mould metabolites (Tomoda et al., 1986, 1992), and is useful as a biosensor for detecting nicotinamide adenine dinucleotide (Vasilescu et al., 2003). The phenoxazine derivative, 2-amino-phenoxazine-3-one (Phx-3), is known to possess an anti-tumour activity and acts via the inhibition of cellular proliferation (Tomoda et al., 1986, 1992; Ishida et al., 1996; Abe et al., 2001; Azuine et al., 2004). Interestingly, recent studies have shown that Phx-3 inhibits the proliferation of viruses such as poliovirus and human immunodeficiency virus *in vitro* (Holmes & Gait, 2003; Iwata et al., 2003). However, the mechanism of antibacterial activity by Phx-3 has not been defined, and a possible involvement of Phx-3 on controlling obligate intracellular bacterial replication and survival in host cells is still not understood. It is also known that *Chlamydia* infection causes inhibition of apoptosis through the blockade of mitochondrial cytochrome c release and caspase activation (Fan et al., 1998). Thus the prevention of apoptosis in *C. pneumoniae*-infected cells is critical for bacterial survival. Therefore, in the present study, the effects of Phx-3 on *C. pneumoniae* growth in human monocytic THP-1 cells and epithelial HEP-2 cells were examined.

**METHODS**

**Bacteria.** *C. pneumoniae* TW183 strain was kindly provided by G. Byrne of the University of Wisconsin, Madison, WI, USA. Bacteria were propagated in a HEP-2 cell culture system as described previously (Aldus et al., 1992; Yamaguchi et al., 2002). Briefly, infected cells were harvested on day 3 and were disrupted by freezing, thawing and ultrasonication. After centrifugation at 500 g for 30 min in order to remove cellular debris, bacteria were concentrated by high-speed centrifugation at 30 000 g for 30 min. Pellet bacterial pellets were resuspended in sucrose-phosphate-glutamic acid buffer [0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, 5 mM l-glutamic acid (pH 7.4)] and were then stored at −80 °C until use. Organisms resuspended in either Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium (DMEM complete medium for HEP-2 cells, RPMI complete medium for THP-1 cells) containing 10% heat-inactivated foetal calf serum (FCS) and antibiotics (gentamicin sulfate, 10 μg ml⁻¹; vancomycin, 10 μg ml⁻¹; amphotericin B, 1 μg ml⁻¹) (Sigma) at 37 °C were used for experiments. The number of infectious *C. pneumoniae* was determined as inclusion-forming units (i.f.u.) by counting chlamydial inclusions formed in HEP-2 cells using a FITC-conjugated monoclonal anti-chlamydia antibody specific to chlamydia lipopolysaccharide (Denka Seiken) (Saikku et al., 1985). Bacterial suspensions were confirmed to be mycoplasma-free by PCR, as reported elsewhere (Ossewaarde et al., 1996).

**Cell lines.** The human epithelial cell line HEP-2 and human monocytic cell line THP-1 were kindly provided by R. Widen of the Tampa General Hospital, Tampa, FL, USA. Cells were cultured in either DMEM or RPMI complete medium at 37 °C 5% CO₂.

**Chemicals.** Phx-3 was synthesized and purified as described previously (Tomoda et al., 1986, 1992). The drug is made up of similar elemental components to actinomycin D (Tomoda et al., 1986, 1992; Abe et al., 2001), is very small (molecular mass, 212) and has a hydrophobic nature. One litre of bovine haemolysates was mixed with 100 ml aliquots of 50 mM α-aminophenol hydrochloride, which was previously neutralized with 0.1 M NaOH solution, and then incubated at pH 7.0 and 37 °C for 6 days. The reaction mixture was treated with 100% methanol in order to denature haemoglobin and proteins. After 1 h, the mixture was centrifuged at 1000 g for 5 min. The supernatant was dried to powder using an evaporator (Tokyo Rika). The powder was dissolved in 100% methanol and was applied to a column of Sephadex LH 20 (Amersham-Biosciences), which was previously equilibrated with 50% ethanol. Elution was performed by addition of 50% ethanol, and the third fraction was collected and evaporated. The remaining powder was dissolved in a small amount of 100% methanol, applied to a column of Sephadex LH 20, previously equilibrated with 50% ethanol, and eluted with 50% ethanol. The major eluates exhibiting absorption spectra comparable to that of questimycin A were collected and identified as Phx-3 based on UV and visible spectra, 1H NMR and 13C NMR spectra, and IR spectra (Tomoda et al., 1986). Phx-3 was dissolved in ethanol at a stock concentration of 25 mM and was stored at 4 °C until use.

**C. pneumoniae infection.** Cultured cells were infected with *C. pneumoniae* at m.o.i. 10 for 1 h at room temperature by centrifugation at 800 g. Then the cells were resuspended in medium. After washing twice with medium, cultures were further resuspended in medium and spread with 5 × 10⁴ cells per well (96-well plate), 5 × 10⁵ cells per well (24-well plate) and 1·5 × 10⁶ cells per well (six-well plate) for cell viability assay, and for i.f.u. assay, and for real-time RT-PCR and electron microscopic study, respectively. Cultures were then incubated for up to 72 h in the presence of Phx-3 (1, 10, 25 or 50 μM) and/or tetracycloph (1-type, Sigma) (100 or 400 μg ml⁻¹). Infected cells without any treatment were used as controls, and ethanol, finally diluted to 1:500 in the complete medium, was also used as an ethanol control. The reagent was diluted for working concentration with either complete medium.

**Cell viability assay.** The effects of Phx-3 (1, 10, 25 and 50 μM) on *C. pneumoniae*-infected HEP-2 cell viability were determined by viable cell count. Briefly, 2, 24, 48 and 72 h after Phx-3 treatment, *C. pneumoniae*-infected HEP-2 and THP-1 cells were washed with PBS and detached with trypsin-EDTA (Sigma). Cells were resuspended with either complete medium. The number of viable cells was determined using 2% trypan blue in a haemocytometer. To confirm in more detail the cell viability in the presence of Phx-3, we used the calcine-AM colorimetric assay cell-counting kit –F (Dojindo Laboratories). The cells were incubated with calcine-AM (excitation wavelength, 485 nm; emission wavelength, 535 nm) provided in the assay kit according to the manufacturer’s manual. The fluorescence intensity of the cell lysates was determined by a fluorescence microplate reader.

**Assessment of infective progeny.** In order to assess the infective progeny in the culture during the cultivation periods, cultures were frozen and thawed, as described elsewhere (Roblin et al., 1992; Summersgill et al., 1995; Gaydos et al., 1996). The lysates, in 10-fold serial dilutions, were inoculated onto HEP-2 cell monolayers in 96-well plates, centrifuged at 800 g for 1 h and then incubated in medium with cycloheximide (1 μg ml⁻¹) for 72 h at 37 °C. The number of i.f.u. in the cells was then assessed by staining with FITC-conjugated anti-
chlamydia antibody (Roblin et al., 1992; Summersgill et al., 1995; Gaydos et al., 1996).

**Total RNA extraction and real-time RT-PCR.** The presence of *C. pneumoniae* in cultures was assessed by real-time RT-PCR with primers specific for *C. pneumoniae* 16S rRNA (Berger et al., 2000; Yamaguchi et al., 2004). Total bacterial RNA was extracted from cultures using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions for bacterial cells. Total RNA was treated with DNAse (DNA-free, Ambion) in order to eliminate contaminating DNA. The resulting DNA-free RNA was confirmed by PCR without RT. RT was performed with 2 μg RNA using avian myeloblastosis virus reverse transcriptase with random primers in a commercial reaction mixture (20 μl; Reverse Transcription System; Promega). The efficiency of cDNA synthesis and the reproducibility based on the quantity of input RNA were also confirmed by performing real-time RT-PCR for G3PDH mRNA using an aliquot of each target sample. The resulting cDNAs (2 μl) were then subjected to real-time PCR with the master mixture (QuantiTect SYBR green PCR kit; Qiagen) containing primers specific for *C. pneumoniae* 16S rRNA (sense, 5’-GGACCTTAGCTGACCTGACATG-3’; antisense, 5’-CCATGCGACCTGTATCTG-3’) in a GeneAmp 5700 sequence detection system. The results of a BLAST search showed that the primers used for real-time RT-PCR were specific for *C. pneumoniae* detection. The thermal cycling conditions were 95°C for 10 min and 50 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 20 s. The melting temperature profiles were assessed for each PCR run in order to confirm the specificity of the PCR products. As a standard for *C. pneumoniae* 16S rRNA, a series of diluted *C. pneumoniae* DNA samples extracted from *C. pneumoniae*-infected HeP-2 cells were used. The relative concentration of *C. pneumoniae* 16S rRNA copies (number of copies per PCR) was calculated from the standard curve. Each cDNA sample was tested by PCR at least three times. To prevent carryover contamination, an aerosol-resistant tip was used in all steps.

**Electron microscopic study.** The infected cells were collected and washed with PBS (pH 7.4). Then the cells were pre-fixed by 2.5% glutaraldehyde in PBS for 2 h at 4°C, followed by post-fixation with 1% osmium tetroxide in PBS (pH 7.4) for 2 h at 4°C. The specimens were embedded in Epon812 and sectioned with a Poter-Blum ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate, and were examined with a JEM 1010 electron microscope (JOEL).

**Statistical analysis.** Statistical analysis was performed using the unpaired Student *t* test.

**RESULTS**

**Effects of Phx-3 on *C. pneumoniae*-infected HeP-2 and THP-1 cell viability**

In order to determine the cytotoxicity of the reagent in HeP-2 and THP-1 cells, cells infected with *C. pneumoniae* were incubated with various concentrations of Phx-3 (1, 10, 25 and 50 μM), and the viability of *C. pneumoniae*-infected cells was then assessed by trypan blue dye exclusion. Concentrations of less than 10 μM did not show any cytotoxicity to either cell line. In addition, ethanol exhibited no cytotoxic effects in either cell line. To confirm more detailed cell viability in the presence of Phx-3, we performed the calcein-AM colorimetric cell-counting assay. As shown in Fig. 1, concentrations of 10 μM or less did not show any cytotoxicity to either cell line infected with *C. pneumoniae* as was seen for the results of trypan blue dye exclusion. No cytotoxic effect of concentrations of less than 10 μM of Phx-3 on the uninfected cells was also confirmed (data not shown).

**Effects of Phx-3 on *C. pneumoniae* growth in THP-1 and HeP-2 cells by i.f.u. assay**

In order to determine the effects of Phx-3 on *C. pneumoniae* growth, bacterial growth in both cell lines was assessed by i.f.u. assay. As shown in Fig. 2, treatment with Phx-3 resulted in a significant decrease in *C. pneumoniae* growth in both cell lines at 72 h after cultivation. The concentration required for a significant inhibition of bacterial growth was more than 1 μM. Phx-3 at a concentration of 50 μM was more effective on *C. pneumoniae* growth inhibition by phenoxazine than 1 μM Phx-3. Therefore, a concentration of up to 10 μM of Phx-3 was selected for the following experiments.

**Number of *C. pneumoniae* 16S rRNA transcripts in culture**

Since it is known that the *C. pneumoniae* aberrant body, which is thought to be a persistent form, lacks the ability to infect host cells and is not detected by standard i.f.u. assay (Shemer & Sarov, 1985; Byrne et al., 1986; Summersgill et al., 1995; Beatty et al., 1993; Rottenberg et al., 2002), it may be possible that aberrant bodies are induced by Phx-3 treat-
ment. Quantification of bacterial transcripts in infected cells by real-time RT-PCR is appropriate for analysing not only actively growing but also persistent C. pneumoniae organisms in cells. Analysis of bacterial transcripts, such as C. pneumoniae 16S rRNA, in infected cells treated with Phx-3 showed a marked reduction of bacterial transcript levels in Phx-3-treated THP-1 cells and HEP-2 cells at 72 h after infection (Fig. 3).

Transmission electron microscopy (TEM) analysis of C. pneumoniae-infected HEP-2 and THP-1 cells with treatment of Phx-3

In order to analyse the morphological effects of Phx-3 on C. pneumoniae-infected cells, infected cells treated with Phx-3 were examined by TEM. Fig. 4 shows representative electron micrographs of chlamydia inclusion bodies. At 48 h after treatment with Phx-3, no divided bacteria were observed in inclusion bodies in either HEP-2 or THP-1 cells (data not shown). Moreover, at 72 h after treatment with Phx-3, chlamydial cell walls were detached from the bacterial body in almost all inclusions, thus suggesting bacterial destruction (Fig. 4B, D). In contrast, C. pneumoniae in the untreated cells grew normally at 72 h after infection (Fig. 4A, C).

Effect of tryptophan on C. pneumoniae inhibition by Phx-3 treatment

In order to confirm whether Phx-3-mediated inhibition of C. pneumoniae growth in THP-1 and HEP-2 cells was caused by a host response via a cellular metabolic pathway, the effects of tryptophan on the observed C. pneumoniae growth inhibition were examined. As shown in Fig. 5, addition of tryptophan caused a slight alteration of this inhibition in both cell types. The results indicate that C. pneumoniae growth inhibition by Phx-3 might be partially mediated by host cellular activation.

DISCUSSION

The present study clearly showed that Phx-3 has the potential to control C. pneumoniae growth in these cells following bacterial destruction, and that 10 μM was the most effective concentration for inhibiting C. pneumoniae growth without leading to significant cell toxicity. Similar results were also confirmed in cells infected with three clinical isolates as well as the standard TW183 strain utilized here (data not shown). MICs based on a bioassay with cycloheximide, which is a protein synthesis inhibitor for mammalian cells, were also assessed. However, MIC values of Phx-3 against C. pneumoniae in both cell lines were more than 50 μM (data not shown). Because addition of cycloheximide blocked C. pneumoniae growth inhibition by Phx-3, it is possible that Phx-3 may inhibit bacterial growth through the host metabolic pathway.

C. pneumoniae has no tryptophan-synthesis pathway, and the decrease in intracellular pools as a consequence of enzymic degradation by indoleamine-2,3-dioxygenase...
(IDO) activation substantially reduces chlamydial survival in host cells (Shemer & Sarov, 1985; Byrne et al., 1986; Beatty et al., 1993; Summersgill et al., 1995; Rottenberg et al., 2002). We therefore predicted that up-regulation of IDO activity resulting in tryptophan depletion would be one of the mechanisms of \textit{C. pneumoniae} growth inhibition by Phx-3. Addition of excessive amounts (up to 400 \mu g ml\(^{-1}\)) of tryptophan to the cultures altered the observed \textit{C. pneumoniae} growth inhibition by Phx-3. Thus, these results indicate that the host response, which is a major mechanism for anti-\textit{C. pneumoniae} activity in cells (Shemer & Sarov, 1985; Byrne et al., 1986; Beatty et al., 1993; Summersgill et al., 1995; Rottenberg et al., 2002), may be partially involved in \textit{C. pneumoniae} growth inhibition by Phx-3. However, even though addition of tryptophan did not result in complete recovery of this inhibition, it remains unclear whether other pathways are associated with chlamydial disruption by Phx-3.

Aberrant chlamydia bodies that lack growth potential but exhibit metabolic activity are often observed in cells treated with antimicrobial agents and cytokines (Rottenberg et al., 2002). These bodies are involved in persistent infection, and the identification of sitted bacteria is impossible by general methods based on the i.f.u. assay. Detection of \textit{C. pneumoniae} transcripts as markers for viable and metabolically active bacteria by real-time RT-PCR is a useful method to confirm the effects of reagents on bacterial elimination (Esposito et al., 1999; Gerard et al., 2000; Gieffers et al., 2001). It is known that the transcription of the \textit{C. pneumoniae} 16S rRNA gene can be detected from early to late stage of bacterial growth and the relative level of this gene expression in culture reveals semiquantitative fluctuation of bacteria during infection (Shaw et al., 2000; Haranaga et al., 2002). Therefore, in the present study, the species-specific region of the 16S rRNA gene was used as a target gene for PCR detection of \textit{C. pneumoniae}. Using this method, we found that treatment of \textit{C. pneumoniae} with Phx-3 caused a significant decrease in bacterial transcripts as was seen in the results of the i.f.u.-based assay.

\textit{C. pneumoniae} preferentially infects monocytes in addition to respiratory tract epithelial cells, endothelial cells and aortic smooth muscle cells (Gaydos et al., 1996; Airenne et al., 1999; Lin et al., 2000). Bacteria in circulating human monocytes are refractory to antibiotic treatment (Gieffers et al., 2001). Our previous study also indicates that \textit{C. pneumoniae} growth in monocytes does not show uniform susceptibility to antibiotics (Yamaguchi et al., 2003). These findings suggest that successful antibiotic-mediated eradication of \textit{C. pneumoniae} from monocytes, which contribute to the pathogenesis of chronic inflammatory diseases, may be very difficult. However, Phx-3 has the ability to inhibit \textit{C. pneumoniae} proliferation in the human monocyte cells and epithelial cells used in the present study. The mechanism of uniform diffusion of Phx-3 to different cells, such as monocyte cells and epithelial cells, is not clear, but one of the reasons might be a hydrophobic characteristic of Phx-3 permitting the direct crossing of the cell membrane without interception by a receptor (Tomoda et al., 1986, 1992; Abe et al., 2001).

It is important to confirm the specificity of Phx-3 as a useful tool for bacterial elimination; treatment of other bacteria such as \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa}, \textit{Staphylococcus aureus} and \textit{Listeria monocytogenes} with Phx-3 at concentrations up to 200 \mu M resulted in no clear alterations in the bacterial growth (data not shown). Because the experiments were performed against a limited range of

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**Fig. 4.** TEM analysis of \textit{C. pneumoniae}-infected HEp-2 cells (A, B) and THP-1 cells (C, D). The cells were incubated for 72 h with (B, D) or without (A, C) Phx-3 (10 \mu M). Arrows show detached bacterial membrane. Bars, 1 \mu m.
bacteria, conclusions from the present study regarding specificity cannot be drawn. Nevertheless, the observations suggest that the inhibitory effects of Phx-3 on *C. pneumoniae* growth are a specific finding.

In conclusion, we demonstrated that Phx-3 is able to control *C. pneumoniae* growth in human monocyte THP-1 cells as well as human epithelial HEp-2 cells, and that this inhibition partially depends on the host tryptophan metabolic pathway. The mechanism of bacterial eradication remains unclear, but Phx-3 may be a useful reagent for understanding complicated aspects of *C. pneumoniae* infection through studies of tryptophan metabolism as a critical pathway for *C. pneumoniae* survival in cells.

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Fig. 5. Effect of tryptophan on Phx-3-induced *C. pneumoniae* growth inhibition in HEp-2 (a) and THP-1 (b) cells. The cells were infected with bacteria and then treated with or without Phx-3 (1 or 10 μM) in the presence or absence of tryptophan (100 or 400 μg ml⁻¹). The cell lysates were prepared at 72 h after infection and then inoculated onto HEp-2 cell monolayers for assessment of infective progeny. The lysates were prepared at 72 h after infection and then inoculated onto HEp-2 cell monolayers for assessment of infective progeny. The numbers of i.f.u. at 72 h after infection per culture of THP-1 cells treated with 0, 100 and 400 μg tryptophan ml⁻¹ in the absence of Phx-3 were 7.5 × 10⁵ ± 4.5 × 10⁵, 9.8 × 10⁵ ± 5.0 × 10⁵ and 1.1 × 10⁶ ± 5.2 × 10⁴, respectively. The numbers of i.f.u. at 72 h after infection per culture of THP-1 cells treated with 0, 100 and 400 μg tryptophan ml⁻¹ in the absence of Phx-3 were 1.2 × 10⁵ ± 4.5 × 10⁵, 0.7 × 10⁴ ± 3.4 × 10⁴ and 1.1 × 10⁵ ± 1.0 × 10⁴, respectively. *P < 0.05 vs untreated control at each concentration of Phx-3. Bars: white, control; grey, 1 μM Phx-3; black, 10 μM Phx-3.


