Impact of a low oxygen environment on the efficacy of antimicrobials against intracellular Chlamydia trachomatis

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Emergence of chronic inflammation in the urogenital tract induced by *Chlamydia trachomatis* (C. trachomatis) infection in females is a long-standing concern. To avoid the severe sequela of *C. trachomatis* infection such as pelvic-inflammatory diseases (PID), ectopic pregnancies and tubal infertility, antibiotic strategies aim to eradicate the pathogen even in asymptomatic and uncomplicated infections. Although first-line antimicrobials have been proven successful for the treatment of *C. trachomatis* infection, treatment failures have been observed in a notable amount of cases. Due to the obligate intracellular growth of *C. trachomatis*, reliable antimicrobial susceptibility assays have to consider environmental conditions and host cell-specific factors. Oxygen concentrations in the female urogenital tract are physiologically low and further decrease during an inflammatory process. We compared minimum inhibitory concentration (MIC) testing and time-kill curves (TKC) for doxycycline, azithromycin, rifampin and moxifloxacin under hypoxia (2% O₂) and normoxia (21% O₂). While low oxygen availability only moderately decreased antichlamydial activity of azithromycin in the conventional MIC testing (0.08 µg/mL vs. 0.04 µg/mL, \( p < 0.05 \)), TKC analyses revealed profound divergences for antibiotic efficacies between both conditions. Thus, *C. trachomatis* was significantly less rapidly killed under hypoxia by doxycycline and azithromycin, whereas efficacies of moxifloxacin and rifampin remained unaffected using concentrations of therapeutic serum levels. Chemical inhibition of the multi-drug resistance protein (MDR-1), but not multi-drug resistance associated protein (MRP-1), restored doxycycline activity against intracellular *C. trachomatis* under hypoxia. We suggest careful consideration of tissue-specific characteristics including oxygen availability when testing antimicrobial activities of antibiotics against intracellular bacteria.
INTRODUCTION

Genital tract infections with Chlamydia trachomatis (C. trachomatis) are the most common sexually transmitted disease (STD) in the United States (1), affecting mostly adolescents and adults under 25 years (1). Ninety two million people are estimated to be infected worldwide (36). Severe clinical sequelae such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility develop through chronic inflammatory processes of persistently infected tissues (11,23).

First-line drugs such as doxycycline and macrolides have been successfully proven for the treatment of asymptomatic and uncomplicated C. trachomatis infections (1). However, treatment failures resulting in persisting or recurrent C. trachomatis infections with subsequent chronic tissue damage have been frequently observed (4,21). Defining accurate treatment strategies for intracellular chlamydial infections are difficult, as standardized assays for in vitro testing of chlamydial isolates are hardly established and limited by the fact that specific environmental conditions are not considered. The minimum inhibitory concentration (MIC) test determines antimicrobial activity against freshly inoculated cells, but does not reflect the potency of the antibiotic against pathogens in different intracellular developmental stages (31). In contrast, determination of time-kill curves (TKC) for antimicrobials against C. trachomatis allows simulating clinical treatment conditions that are more closely related to the in vivo situation (31).

Hypoxia is known to modulate central host cell signaling pathways involved in metabolism and survival and it regulates the expression of multidrug resistance transporters via the hypoxia-inducible factor-1α (HIF-1α) (29). We and others showed that chlamydiae well adapted to a low oxygen environment and directly interfered with the stabilization of HIF-1α, the central mammalian oxygen sensor, to replicate (17,26). In addition, IFN-γ could not control intracellular growth of C. trachomatis in human fallopian tube cells in a low oxygen environment that can be found in the urogenital tract of women (25).

Reduced effectiveness of chemotherapeutical agents within oxygen-restricted areas is a well known phenomenon (10). While a diminished efficacy of antimicrobials against intracellular bacteria in cells overexpressing the multi-drug resistance protein (MDR-1) has been reported, no direct correlation between oxygen availability and intracellular effectiveness of antimicrobials has been observed so far (20,22,30). Therefore we investigated whether intracellular activities of recommended and alternative antimicrobials against C. trachomatis are maintained in a low oxygen environment (16,34).
MATERIALS AND METHODS

Bacterial strains and epithelial cell culture. *C. trachomatis* serovar L2 (ATCC VR-902B) and HEp-2 cells (ATCC CCL-23) were used in the present study. A total of 5 x 10⁴ cells per well in 24-well plates (Greiner bio-one, Frickenhausen, Germany) or 2.5 x 10⁵ cells per well in 6-well plates (Greiner bio-one) were grown in RPMI 1640 medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Karlsruhe, Germany), non essential amino acids (PAA Laboratories), 2 mM glutamine (PAA Laboratories), 2 µg/mL amphotericin B (PAA Laboratories) and 100 µg/mL Gentamicin (PAA Laboratories) under normoxic (21% O₂) and hypoxic (2% O₂) conditions (Toepffer lab systems, Göppingen, Germany).

Chemicals. Doxycycline, azithromycin, rifampin, Cyclosporine A (CsA) and probenecid were purchased from Sigma Aldrich (Deisenhofen, Germany) and moxifloxacin was purchased from Bayer Vital GmbH (Leverkusen, Germany).

Determination of the MIC for *C. trachomatis*. A total of 5 x 10⁴ HEp-2 cells per well was seeded in 24-well plates and cultured for 24 h under normoxic and hypoxic conditions. Culture medium was changed subsequently to RPMI 1640 medium supplemented with 5% fetal bovine serum and cells were infected with *C. trachomatis* serovar L2 with 3 x 10⁵ inclusion-forming units (IFUs)/mL with or without respective antibiotics in different concentrations. MICs were determined after 48 h incubation by immunofluorescence staining with a mouse anti-chlamydial lipopolysaccharide (LPS) antibody (kindly provided by Dr. Helmut Brade, Borstel, Germany) and a polyclonal rabbit FITC-labeled anti-mouse IgG antibody (Dako, Hamburg, Germany) for the detection.

TKC. TKC was performed as described previously with minor modifications (31). Briefly, 5 x 10⁴ HEp-2 cells per well were seeded in 24-well plates and cultured for 24 h under normoxic and hypoxic conditions. Culture medium was changed subsequently to RPMI 1640 medium supplemented with 5% fetal bovine serum and cells were infected with *C. trachomatis* serovar L2 with 3 x 10⁵ IFUs/mL with or without respective antibiotics in different concentrations. Reported serum levels (serum protein unbound antibiotics) were selected as working concentrations for doxycycline (2 µg/mL), azithromycin (0.5 µg/mL), moxifloxacin (3 µg/mL).
and rifampin (8 µg/mL) (9,31,35). After the indicated time points cells were washed twice with medium to remove the remaining antibiotic and subsequently cultured for 48 h for determination of the recoverable chlamydiae as described before (31). Recoverable chlamydiae at the indicated time points were normalized to the untreated controls (0 h p.i.) under normoxia and hypoxia, respectively and displayed as the percentage of the controls. Mean of duplicate experiments from four independent experiments were used for calculation of the statistical significance.

**Real-Time PCR.** A total of $2.5 \times 10^5$ HEp-2 cells/well was seeded in 6-well plates overnight and cultured in RPMI 1640 medium with 5% fetal bovine serum for up to 24 h under normoxic and hypoxic conditions. Extraction of total RNA, reverse transcription to cDNA and PCR amplification using the LightCycler Detection System (Roche Molecular Biochemicals, Mannheim, Germany) were performed at indicated time points as described previously (27). Relative quantification of MDR-1 or multi-drug resistance associated protein (MRP-1) mRNA expression levels (37) were performed against the endogenous control β-actin gene (6). The primer sequences were as follows: MDR1 forward, 5´-CCCATCATTGCAATAGCAGG-3´; MDR1 reverse, 5´-GTTCAAACTTCTGCTCCTGA-3´; MRP1 forward, 5´-GGACCTGGACTTCGTTCTCA-3´; MRP1 reverse, 5´-CGTCCAGACTTCCCTTCATCCG-3´; β-actin forward, 5´-CCTGGCACCCAGCACAAT-3´; β-actin reverse, 5´-GGGCCGGACTCGTCATAC-3´.

**MDR-1 and MRP-1 inhibition assay.** A total of $5 \times 10^4$ HEp-2 cells per well was seeded in 24-well plates and cultured for 24 h under hypoxic condition. Culture medium was changed subsequently to RPMI 1640 medium supplemented with 5% fetal bovine serum and cells were infected with *C. trachomatis* serovar L2 with $3 \times 10^5$ IFUs/mL with or without the inhibitor for 8 h. Doxycycline efficacy with/without inhibitory treatment was analyzed 2h and 4h p.i. and normalized to the inhibitory effect of CsA or probenecid 8 h p.i..

**Statistics.** Data are indicated as mean ± standard error of the mean (SEM). Data were evaluated using unpaired student *t*-test. *P*-values ≤ 0.05 were considered as statistically significant.
RESULTS

MIC testing of antimicrobials against *C. trachomatis* under normoxia and hypoxia. The MICs for doxycycline, azithromycin, moxifloxacin and rifampin against *C. trachomatis* were calculated under normoxic (21% O$_2$) and hypoxic (2% O$_2$) conditions (Table 1). No significant differences were observed for doxycycline, moxifloxacin and rifampin. Interestingly, azithromycin showed significantly higher MIC values (0.08 µg/mL vs. 0.04 µg/mL; n=3, p<0.05) for *C. trachomatis* when the cells were cultured under the hypoxic condition in comparison to the normoxic condition (Table 1, Fig. 1).

TKC of antimicrobials against *C. trachomatis* under normoxia and hypoxia. To uncover differences in the activities of antimicrobials against intracellular chlamydial growth under normoxic and hypoxic conditions, we performed TKC. *C. trachomatis* growth without antibiotic treatments was not significantly different in normoxia compared to hypoxia (5x10$^8$±4x10$^7$ IFUs/mL vs. 5x10$^8$±6x10$^7$ IFUs/mL, n=7, p=0.4) (Fig. 2A) confirming what has been reported by Juul *et. al.* (17). In contrast to MIC testing, TKC revealed significant differences in the antichlamydial activities of all tested antibiotics between normoxic and hypoxic conditions. Thus, doxycycline (2 µg/mL) and azithromycin (0.5 µg/mL) treatment were significantly less effective against early intracellular chlamydial growth when the cells were grown in hypoxia (Fig. 2 and 3). When the doxycycline concentration was reduced (0.5 µg/mL) significantly less chlamydiae were killed in hypoxia compared to normoxia whereas an increase to 8 µg/mL resulted in complete eradication of chlamydiae under both conditions (Fig. 2B). The same trend was observed for azithromycin (Fig. 3B). In contrast, both moxifloxacin and rifampin were equally efficient in eradicating intracellular *C. trachomatis* at concentrations of 3 µg/mL and 8 µg/mL, respectively (data not shown). However, when the concentrations of moxifloxacin and rifampin were reduced to 0.5 µg/mL *C. trachomatis* was significantly more effectively eradicated in normoxia than in hypoxia (Fig. 4 A/B).

MDR-1 and MRP-1 mRNA expression levels under normoxic and hypoxic conditions. We could demonstrate that antibiotic efficacy was reduced under hypoxia compared to normoxia by TKC testing. It is known that intracellular activities of antimicrobials strongly depend on cellular accumulation and are tightly regulated by multi-drug resistance transporters. We therefore investigated transcriptional activities of the MDR-1 and MRP-1 genes under normoxia and...
hypoxia. Within 24 h both MDR-1 (2.0±0.2-fold, \( p<0.05 \)) and MRP-1 (1.4±0.1-fold, \( p<0.05 \)) were significantly up-regulated under hypoxia compared to normoxia (Fig. 5). Stabilization of the hypoxia-inducible factor-1 \( \alpha \) (HIF-1\( \alpha \)) was observed by Western blot analysis in cells that were incubated under hypoxia but not under normoxia (data not shown).

**Functional relevance of MDR-1 and MRP-1 expression on reduced antichlamydial activity of doxycycline under hypoxia.** To test whether hypoxia-induced up-regulation of MDR-1 and MRP-1 expression is responsible for reduced activity of doxycycline under hypoxia we blocked MDR-1 with cyclosporine A (CsA) and MRP-1 with probenecid. Control studies were also conducted to verify that each inhibitor, at the concentrations employed, did not affect epithelial cell viability over the assay period (data not shown). *C. trachomatis* growth was slightly inhibited by 10 \( \mu \)M CsA and 2.5 mM probenecid. To analyze the impact of the inhibitors on doxycycline efficacy in hypoxia, we normalized respective values against the rate of recoverable chlamydiae with/without CsA or probenecid treatment in the absence of doxycycline. Inhibition of MDR-1 by CsA significantly increased antichlamydial activity of doxycycline (2 \( \mu \)g/mL) under hypoxic conditions compared to the untreated control cells (Fig. 6A). In contrast, inhibition of MRP-1 using probenecid did not restore antichlamydial activity of doxycycline, but rather attenuated the efficacy of doxycycline on chlamydial growth under hypoxic condition (Fig. 6B).

**DISCUSSION**

Infection with *C. trachomatis* is the most common bacterial sexually transmitted disease (STD) with more than 2.8 million new cases estimated to occur each year in the United States (2,3). Reinfections from non- or inadequately treated sex partners and treatment failures perpetuated the high prevalence over the last years. Doxycycline (100 mg orally twice a day for 7 days) and azithromycin (1000 mg orally in a single dose) are the preferred treatment regimens for uncomplicated urogenital tract infections with *C. trachomatis* in women (1,3). However, treatment failures are frequently reported, suggesting inadequate eradication of chlamydiae from the sites of the infection (21). *C. trachomatis* treatment failure has been observed in up to 14% of the patients whereas only 2% of patients seems to fail initial treatment in *M. tuberculosis* infections (4,7). In addition, the propensity of *C. trachomatis* to induce persistent infections when sub-inhibitory antibiotic concentrations are applied is of major concern. In the persistent
state of chlamydiae, the pathogen remains viable in an atypical intracellular inclusion and resists high doses of otherwise effective antimicrobials (13).

The use of MIC testing to predict the therapeutic relevance of the antimicrobial activity against intracellular pathogens is questionable because a MIC test does not take into account host-cell specific and environmental factors (21). TKC therefore evolved as a suitable tool to evaluate antimicrobial growth inhibition of bacteria at different intracellular developmental stages (15,31). In this study we investigated the impact of low oxygen that can be observed in the female urogenital tract under physiological conditions, on antichlamydial activities of doxycycline, azithromycin, rifampin and moxifloxacin. While the MICs for the respective substances, except for a slight increase for azithromycin, remained unchanged when the tests were performed under hypoxia, significant changes between the antimicrobial activities in the time-dependent killing of intracellular chlamydiae were observed. Reduced effectiveness against intracellular chlamydial growth under hypoxia was observed for doxycycline and azithromycin at therapeutic serum concentration levels, whereas moxifloxacin and rifampin lost antichlamydial activity exclusively at reduced concentrations. Therapeutic serum concentrations reflect concentrations of unbound antibiotics and it is important to take the binding of antibiotics by serum proteins in the experimental setting into account (12,24,32,38). As protein binding is almost proportional to the percentage of serum in the medium, 5% serum as used in this study, can be expected to have almost no effect on the concentrations of unbound antibiotics in our experiments (38). Our findings are not only of interest for the treatment of chlamydial infections but could also have an impact on treatment strategies for other obligate or facultative intracellular bacteria. With regard to recent findings in humans and animal studies changes in local oxygen levels are linked to the transition between active and latent M. tuberculosis infections (14,18,28). Furthermore, it has been shown in a guinea pig model of tuberculosis that M. tuberculosis resisting antibiotic treatment are found in hypoxic areas of the granuloma (18).

Intracellular activity of antimicrobials requires efficient drug penetration and accumulation within the cell and is constraint by excretion, metabolism and inactivation resulting in reduced bioavailability (33). Although various drug transporters are found in different cells (5), active drug efﬂux pumps such as the ATP-binding cassette (ABC) transporter proteins MDR-1 and MRP-1 have an important role in the regulation of intracellular drug concentrations of antimicrobials (20,22,30). Localized at the plasma membrane of most cell types, MDR-1 and
MRP-1 are distributed almost ubiquitously in numerous organs (19). In macrophage and epithelial cells, several groups have demonstrated that expression of MDR-1 and MRP-1 affected intracellular concentrations of macrolides and quinolones (20,30). Furthermore, higher concentrations of doxycycline, macrolides, rifampin and quinolones were required to kill *Listeria monocytogenes* in MDR-1 overexpressing cells (22). To test whether MDR-1 and MRP-1 were responsible for the reduced effectiveness of doxycycline and azithromycin in cells that were cultured in hypoxia, we analyzed hypoxia-induced expression and functional relevance of both proteins. Hypoxic cells that were characterized by increased stabilization of HIF-1α showed enhanced transcription of MDR-1 and MRP-1. It was shown previously that HIF-1α directly regulates MDR-1 expression via a hypoxia responsive element (HRE) within the gene promoter region (8). Our data showing that incubation with CsA but not with probenecid restored antichlamydial activity of doxycycline suggest a functional role of MDR-1 in the reduced effectiveness under hypoxia.

Taken together our data indicate that intracellular activities of antimicrobials against *C. trachomatis* depend on the local oxygen availability. Reduced efficacies of first-line antimicrobials in hypoxic areas might account for insufficient eradication and subsequent persistence of *C. trachomatis* within the diseased tissue. Improvement of the currently available in *vitro* models and establishment of human tissue models of *C. trachomatis* infection for antibiotic testing will help to predict the successful outcome of treatment strategies more precisely.
FIGURE LEGENDS

Figure 1. Comparison of the MIC values for azithromycin (AZM) under normoxia (21% O₂) and hypoxia (2% O₂) 48 h p.i.. Immunofluorescence staining with mouse anti-chlamydial LPS antibody visualized chlamydial inclusions (green). Evans blue counterstaining of host cells (red) was used for better characterization of intracellular inclusions. Under normoxia, no visible chlamydial growth was observed at a concentration of 0.04 µg/mL whereas complete eradication of chlamydiae was obtained at a concentration of 0.08 µg/mL under hypoxia (Nox: normoxia, Hox: hypoxia). Representative images of three independent experiments are shown.

Figure 2. TKC of doxycycline (DOX) against C. trachomatis under normoxic and hypoxic conditions. Chlamydial growth was not significantly different between normoxic and hypoxic conditions (n=7) (A). Immunofluorescence stainings showed reduced efficacy of DOX (2 µg/mL) against C. trachomatis under hypoxic conditions (B). Significant differences in the antichlamydial activity of doxycycline between normoxia and hypoxia were also observed when the concentration of the antimicrobial was reduced (0.5 µg/mL) but not when the concentration was increased (8 µg/mL) (C). Recoverable chlamydiae at indicated time points were calculated as the percentage of the untreated control under normoxia and hypoxia, respectively. (n=4; mean ± SEM, *p<0.05. Nox: normoxia, Hox: hypoxia).

Figure 3. TKC of azithromycin (AZM) against C. trachomatis under normoxic and hypoxic conditions. Immunofluorescence stainings showed reduced efficacy of AZM (0.5 µg/mL) against C. trachomatis under hypoxic conditions (A). Differences in the efficacy of AZM in early intracellular C. trachomatis eradication with respect to the oxygen availability were maintained when the concentration was decreased to 0.04 µg/mL and even more pronounced when the concentration was enhanced to 0.5 µg/mL (B). Recoverable chlamydiae at indicated time points were calculated as the percentage of the untreated control under normoxia and hypoxia, respectively. (n=4; mean ± SEM, *p<0.05. Nox: normoxia, Hox: hypoxia).

Figure 4. TKC of rifampin (RIF) and moxifloxacin (MXF) against C. trachomatis under normoxic and hypoxic conditions. 0.5 µg/mL RIF (A) and 0.5 µg/mL MXF (B) showed reduced antichlamydial activity under hypoxia. Recoverable chlamydiae at indicated time points were
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Figure 5. Quantification of the MDR-1 and MRP-1 mRNA expression under normoxic and hypoxic conditions. Data were normalized to normoxic values of 0 h and 24 h. A significant increase of the MDR-1 and MRP-1 mRNA expression under hypoxia was observed within 24 h (n=6; mean ± SEM, *p<0.05. Nox; normoxia, Hox; hypoxia).

Figure 6. MDR-1 but not MRP-1 inhibition restores antichlamydial activity of doxycycline under hypoxia. Incubation for 8 h with 10 µM CsA (A) but not with 2.5 mM probenecid (B) resulted in an increased intracellular eradication of C. trachomatis when the cells were treated with doxycycline under hypoxic condition. Doxycycline efficacy with/without inhibitory treatment was analyzed 2h and 4h p.i. and normalized to the inhibitory effect of CsA or probenecid 8 h p.i. (n=4 for CsA, n=3 for probenecid; mean ± SEM, *p<0.05).

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Table 1. MIC of antibiotics against *C. trachomatis* under normoxia and hypoxia (*p*<0.05)

<table>
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<th>Hypoxia (2% O&lt;sub&gt;2&lt;/sub&gt;)</th>
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REFERENCES


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