Original Article

Wien Klin Wochenschr (2007) 119/9–10: 297–302
DOI 10.1007/s00508-007-0784-5

WIENER KLINISCHE WOCHENSCHRIFT
The Middle European Journal of Medicine
Printed in Austria

Comparative activities of antibiotics against intracellular non-typeable *Haemophilus influenzae*

Christina Kratzer[1], **Wolfgang Graninger**[1], **Karin Macfelda**[2], **Astrid Buxbaum**[1], and **Apostolos Georgopoulos**[1]*

[1] Department of Internal Medicine I, Division of Infectious Diseases and Tropical Diseases, Medical University of Vienna, Vienna, Austria
[2] Department of Biomedical Research, Medical University of Vienna, Vienna, Austria

Received July 20, 2006, accepted after revision November 7, 2006

Vergleichende Wirksamkeit antimikrobieller Substanzen gegenüber intrazellulärem nicht-typisierbaren *Haemophilus influenzae*

Zusammenfassung. *Einleitung:* Nicht-typisierbarer Haemophilus influenzae (NTHi) ist ein wichtiger bakterieller Erreger ambulant erworbener Infektionen der Atemwege und ist in der Regel extrazellulär zu finden. Studien haben jedoch gezeigt, dass NTHi die Fähigkeit besitzt in menschliche Epithelzellen einzudringen, wo er sowohl vor den Angriffen des lokalen Immunsystems als auch zum Teil vor der Abtötung durch Antibiotika geschützt ist. Ziel der vorliegenden Studie war es, die Fähigkeit der 5 klinisch häufig eingesetzten Antibiotika Ampicillin, Azithromycin, Telithromycin, Ciprofloxacin und Moxifloxacin zu bewerten, NTHi innerhalb von bronchialen Epithelzellen abzutöten.

Methoden: Konfluierende humane bronchiale Epithelzellen wurden mit NTHi 77, einem besonders invasiven klinischen Stamm infiziert. Die extrazellulären Bakterien wurden mittels Gentamicin abgetötet. Die intrazellulären Bakterien wurden mit Antibiotika in einer Konzentration von 1 mg/l oder 10 mg/l für 4 h oder 8 h behandelt. Lebensfähige intrazelluläre Bakterien wurden nach Lyse der Epithelzellen gezählt.

Ergebnisse: Mit Ausnahme von Ampicillin erreichten alle Antibiotika eine signifikante Reduktion der intrazellulären Bakterien in einer Konzentration von 10 mg/l und einer Expositionszeit für 4 h sowie bei 1 mg/l für 8 h. Bei einer Konzentration von 1 mg/l eliminierte Moxifloxacin 94% der intrazellulären NTHi nach 4 h und 98% nach 8 h. Ciprofloxacin, Azithromycin und Telithromycin erreichten nur Killing-Indices < 75 nach 4 h, jedoch eine 86–90%ige Abtötung nach 8 h. Bei 10 mg/l erreichten Moxifloxacin, Ciprofloxacin, Telithromycin and Azithromycin nach einer Expositionszeit von 4 h eine 99,7%, 96,3%, 86,7% und 74,7%ige Eradikation der intrazellulären Bakterien.

Schlussfolgerung: Die Ergebnisse demonstrieren die rasche antibakterielle Effektivität von Moxifloxacin gegenüber intrazellulärem NTHi in vitro. Moxifloxacin, welches hohe extrazelluläre und intrazelluläre Wirksamkeit kombiniert, könnte eine vorteilhafte Substanz in der Behandlung von rezidivierenden Atemwegsinfektionen darstellen.

Schlüsselwörter: Nicht-typisierbarer Haemophilus influenzae, Bronchiale Epithelzellen, Invasion, Intrazelluläre Wirksamkeit von Antibiotika.

Summary. *Introduction:* Non-typeable *Haemophilus influenzae* (NTHi) is a major bacterial pathogen of community-acquired respiratory tract infection and is usually found extracellularly, although studies have revealed that NTHi may possess the ability to invade human epithelial cells where it is then protected against attack by the local immune system and partly against the effect of antibiotics. The aim of the present study was to assess the ability of ampicillin, azithromycin, telithromycin, ciprofloxacin and moxifloxacin, five antibiotics in common clinical use, to kill NTHi within bronchial epithelial cells.

Methods: Confluent human bronchial epithelial cells were infected with NTHi 77, a particularly invasive clinical strain. Extracellular bacterial cells were killed with gentamicin and the intracellular bacteria were incubated with antibiotics at concentrations of 1 mg/l or 10 mg/l for 4 h or 8 h. Viable intracellular bacteria were counted after lysis of the epithelial cells.

Results: With the exception of ampicillin, all the antibiotics caused significant reduction of intracellular bacteria at concentrations of 10 mg/l and exposure for 4 h or at 1 mg/l for 8 h. At 1 mg/l, moxifloxacin eliminated 94% of intracellular NTHi after 4 h and 98% after 8 h; ciprofloxacin, azithromycin and telithromycin only achieved killing indices below 75 after 4 h but 86–90% killing after 8 h. At 10 mg/l, moxifloxacin, ciprofloxacin, telithromycin and azithromycin were able to achieve

99.7%, 96.3%, 86.7% and 74.7% eradication of intracellular bacteria, respectively, after exposure for 4 h.

Conclusion: These results demonstrate the rapid antibacterial efficacy of moxifloxacin against intracellular NTHi in vitro. Moxifloxacin, which combines high extracellular and intracellular activities, could be an important tool in the treatment of recurrent respiratory tract infections.

Key words: Non-typeable *Haemophilus influenzae*, bronchial epithelial cells, invasion, intracellular activity of antibiotics.

Introduction

Community-acquired respiratory tract (RT) infections are among the most prevalent infectious diseases worldwide. Non-encapsulated, non-typeable *Haemophilus influenzae* (NTHi) is a major RT pathogen implicated in otitis media, sinusitis, conjunctivitis, exacerbated chronic bronchitis and pneumonia, particularly in patients with underlying diseases [1]. Invasion studies have revealed that NTHi, usually regarded as an extracellular mucosal pathogen, may possess the ability to invade and survive inside human epithelial cells [2–4], but the clinical significance of these observations has remained unclear. However, intracellular localization of NTHi in respiratory tissue of the upper and lower RT seems to play a role in the pathogenesis of chronic bronchitis. In a study of patients with chronic bronchitis, Bandi et al. [5] detected the presence of intracellular NTHi in the lower RT of 33% of clinically stable patients and 87% of those who were acutely ill, whereas healthy volunteers yielded no NTHi. Recently, molecular typing of culture-negative sputum has revealed persistent colonization by NTHi in patients with chronic obstructive pulmonary disease [6].

Once inside the epithelial cells, *H. influenzae* is protected against attack by the local immune system and partly against the effect of aminoglycosides and beta-lactams [7], whereas other antibiotics such as macrolides and fluoroquinolones are able to concentrate in eukaryotic cells and remain active against intracellular NTHi [8]. Nevertheless, as shown by the rising number of case reports of failure of macrolide antibiotics to eradicate *H. influenzae* [9], high intracellular concentration alone does not allow accurate prediction of the intracellular killing index of an antibiotic. Thus, investigation of the intracellular activity of an antibiotic is an important component in the evaluation of currently used antibiotics and in the development of new ones.

The aim of the present study was to compare the ability of the commonly used antibiotics ampicillin, azithromycin, telithromycin, ciprofloxacin and moxifloxacin to kill intracellular NTHi in human bronchial epithelial cells, using the invasion assay.

Materials and methods

Antibacterial agents and susceptibility testing

To investigate the intracellular activity of different antibiotics against NTHi, ampicillin (Biochemie, Kundl, Austria), azithromycin (Pfizer, Vienna, Austria), telithromycin (Aventis, Vienna, Austria), ciprofloxacin and moxifloxacin (Bayer Austria, Vienna, Austria) were studied. Gentamicin (Biochemie, Kundl, Austria) was used for killing extracellular NTHi. Extracellular minimal inhibitory concentrations (MICs) were determined using the broth microdilution reference method according to the guidelines of the Clinical Laboratory Standard Institute (formerly NCCLS) [10]. Haemophilus test medium (HTM) was prepared from Mueller-Hinton broth (Oxoid Ltd, Basingstoke, UK) supplemented with HTM supplement (Oxoid) and yeast extract (Oxoid). Antibiotics were serially diluted in HTM broth. The final inoculum of NTHi was ~5×10^5 cfu/ml and *H. influenzae* ATCC 49247 was used as quality control. MIC endpoints were read as the lowest concentration of antibiotic that totally inhibited macroscopically visible growth of the inoculum. Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration of each drug resulting in > 99% reduction in growth and were determined by culturing each dilution on chocolate agar (bioMérieux, Marcy l'Etoile, France) at 37°C in an atmosphere of 5% CO_2.

For the invasion experiments, all antibiotics were diluted in bronchial epithelial invasion medium consisting of bronchial epithelial basal medium (Clonetics Corporation, San Diego, CA, USA) supplemented with recombinant human epidermal growth factor (0.5 µg/l), insulin (5 g/l), transferrin (10 mg/l), epinephrine (0.5 mg/l), triiodothyronine (6.5 mg/l), bovine pituitary extract (52 mg/l) and retinoic acid (0.1 µg/l). The antibiotics were tested at final concentrations of 1 mg/l and 10 mg/l.

Epithelial cells

Normal human bronchial epithelial (NHBE) donor cells were obtained from Cambrex Inc. Lung tissues from two donors were disease free and neither donor was a smoker: Donor 1: NHBE 8699, lot number 1F1811, a 13-year-old boy; donor 2: NHBE 9533, lot number 2F1142, a 24-year-old woman. The epithelial cells were cultured in bronchial epithelial invasion medium. For the invasion assay, NHBE cells of the 3rd to 6th passage were seeded into 24-well tissue culture plates (1×10^5 cells/well).

Bacteria

To identify a particularly invasive clinical strain of NTHi, 22 strains isolated in 2003 from the sputa of adult patients with acute exacerbation of chronic bronchitis were randomly selected from our own collection. Identification of all strains was confirmed by standard microbiological procedures including biochemical identification (API NH) and serotyping (slide agglutination assay with antisera specific for types a–f) [11]. All *H. influenzae* isolates were grown overnight on chocolate agar at 37°C in an atmosphere of 5% CO_2. Prior to each invasion experiment, a few colonies of NTHi were inoculated into 10 ml of brain-heart infusion broth (Oxoid) supplemented with nicotinamide adenine dinucleotide and hemin and incubated overnight at 37°C, followed by dilution and further incubation in fresh pre-warmed bronchial epithelial invasion medium as described previously [4].

Invasion assay

To examine NTHi invasion, confluent monolayers of NHBE cells were washed three times with PBS, stationary-phase bacteria (10 µl $1–5 \times 10^9$ cfu/ml) were added to each well and infection was allowed to proceed for 4 h. Gentamicin was then added at a final concentration of 100 mg/l for 2 h to eliminate extracellular bacteria, as described by Ahrén et al. [4]. The monolayer was rinsed with PBS to remove gentamicin

from the culture. NHBE cells were detached using 0.025% trypsin/0.01% EDTA solution (Clonetics Corporation), resuspended in 1 ml of PBS, transferred to a glass tube containing eight glass pearls and mechanically lysed by vigorous vortexing for 1 min. To quantify the number of viable intracellular bacteria, tenfold dilutions of the lysates were made in PBS and 100 µl aliquots spread on chocolate agar; incubation was at 37°C in 5% CO_2. Colony forming units (cfu) were counted after 24 h and the invasion efficiency (percent invasion) was calculated as (bacteria recovered/bacteria inoculated) × 100.

Extracellular and intracellular antibiotic concentrations

The extracellular and intracellular concentrations of the five antibiotics were determined using the microbioassay agar-diffusion technique as described by Harold et al. [12]. Uninfected cells were incubated with ampicillin, azithromycin, telithromycin, ciprofloxacin or moxifloxacin at a final concentration of 10 mg/l for 2 h, 4 h and 8 h. The extracellular medium was then decanted and the intracellular fluid was obtained by detachment and mechanical lysis of the NHBE cells. Suspensions of different test organisms were used in the diffusion plates: *Bacillus subtilis* ATCC 6633 (for ampicillin), *Sarcina lutea* ATCC 9341 (for azithromycin and telithromycin) and *Escherichia coli* 10536 (for ciprofloxacin and moxifloxacin). Test organisms were inoculated into sterile melted agar cooled to and maintained at 42°C. Aliquots (6 ml) of inoculated agar were poured into Integrid petri dishes (Becton Dickinson), allowed to solidify, and stored at 4°C for 1 h. Wells 6.5 mm in diameter were punched into the agar and each was filled with 20 µl of an antibiotic standard or an extracellular or intracellular sample. The dishes were incubated overnight at 37°C. The inhibition zone was measured in mm and a standard curve was prepared to determine the antibiotic concentration in the extracellular and intracellular samples.

Invasion assay in combination with antibiotics

NHBE cells were infected with stationary-phase NTHi 77 (10 µl 1–5 × 10^9 cfu/ml) for 2 h. Extracellular bacteria were then killed with gentamicin, after which the monolayer was rinsed with PBS to remove gentamicin from the culture. To determine the number of viable intracellular bacteria at time 0 (before antibiotic treatment), the epithelial cells in three single wells were treated with trypsin, lysed and plated on chocolate agar. The remaining wells were incubated with fresh bronchial epithelial invasion medium alone (negative control) or supplemented with ampicillin, azithromycin, telithromycin, ciprofloxacin or moxifloxacin at final concentrations of 1 mg/l and 10 mg/l for a further 4 h and 8 h. Monolayers were then washed three times with PBS, treated with trypsin-EDTA solution and mechanically lysed. Lysates were diluted tenfold and 100 µl aliquots were plated on chocolate agar. Cfu were counted after incubation for 24 h at 37°C.

Statistical analysis

Reduction of the number of viable intracellular NTHi was described by arithmetic means and standard deviation of three individual experiments in triplicate for each tested antibiotic at concentrations of 1 mg/l and 10 mg/l, in comparison with the antibiotic-free control, after 4 h and 8 h of incubation. The intracellular survival index (SI) and the antibiotic killing index (KI) at a defined time point t were determined according to the formula described by Nielsen et al. [13]:

$$SI_t = [(cfu/ml)_t/(cfu/ml)_0] \times 100;$$
$$KI_{t;antibiotic} = [(SI_{t;control} - SI_{t;antibiotic})/SI_{t;control}] \times 100$$

Differences between the antibiotics and the antibiotic-free control were assessed with Student's *t*-test for independent samples. If significance was achieved, the Bonferroni-Holm correction was used for multicomparison of means. The multicomparison significance level was ≤ 0.05. SPSS statistical software system 12.0 for Windows (SPSS Inc., Chicago, IL) was used for calculations.

Results

Invasiveness of NTHi strains in NHBE cells

All clinical isolates of NTHi exhibited an invasion efficiency of > 0.01% in bronchial epithelial cells: 41% of the tested strains reached an invasiveness of > 0.1% and 14% of strains > 1% invasiveness after infection for 4 h. Isolate 77 was the most invasive strain of NTHi: ~300,000 cfu/well (3.3% of the original inoculum) were detected within NHBE cells.

Extracellular susceptibility

The MICs and MBCs of the tested antibiotics against NTHi 77 are shown in Table 1. Low MIC values were detected for ciprofloxacin (0.03 mg/l) and moxifloxacin (0.06 mg/l); higher MICs of 1–2 mg/l were detected for ampicillin, azithromycin and telithromycin. NTHi 77 was susceptible to all tested antibiotics with MBCs four times higher than the MICs. Published mean peak serum concentrations (C_{max}) of ampicillin, ciprofloxacin and moxi-

Table 1. Extracellular minimal inhibitory concentrations (MICs), minimal bactericidal concentrations (MBCs), peak serum concentrations (C_{max}) and intracellular-to-extracellular (C/E) ratios in bronchial epithelial cells (NHBE) for antibiotics against NTHi 77

Antibiotics	MIC (mg/l)[a]	MBC (mg/l)[a]	C_{max} (mg/l)[b]	C/E ratio[a] after exposure for		
				2 hours	4 hours	8 hours
Ampicillin	1	4	7.8 [23]	<0.1	<0.1	<0.1
Azithromycin	2	8	0.4 [24]	24.1	66.7	71.5
Telithromycin	2	8	1.7 [25]	27.1	20.1	18.9
Ciprofloxacin	0.03	0.12	1.9 [26]	5.6	6.1	6.3
Moxifloxacin	0.06	0.24	4.3 [27]	6.8	7.9	8.0

[a] Results obtained in the present study. [b] References are given in square brackets.

floxacin were above the determined MBCs; peak serum concentrations for azithromycin and telithromycin were below the MBC.

Extracellular and intracellular antibiotic concentrations

Extracellular antibiotic concentrations decreased minimally during incubation for 8 h in bronchial epithelial invasion medium. Intracellular/extracellular (C/E) ratios after 2 h, 4 h and 8 h are shown in Table 1. Intracellular concentrations of ampicillin in NHBE cells were not detectable by the microbioassay agar-diffusion method, whereas higher intracellular than extracellular concentrations were detected for the other antibiotics: after 8 h, azithromycin showed the highest intracellular level (C/E ratio 71.5), followed by telithromycin (C/E ratio 18.9), moxifloxacin (C/E ratio 8.0) and ciprofloxacin (C/E ratio 6.3).

Intracellular killing of NTHi 77

At time 0 (after gentamicin treatment), NHBE cells harbored ~23,000 viable NTHi per well. After 4 h control NHBE cells loaded with bronchial epithelial invasion medium without antibiotics contained ~86,000 viable NTHi per well and after 8 h ~13,000 NTHi per well. After 8 h, ampicillin at 10 mg/l did not influence the intracellular persistence of NTHi when compared with the antibiotic-free control ($P = 0.9$). The fluoroquinolones, azithromycin and telithromycin at 10 mg/l (Fig. 1B) and 1 mg/l (Fig. 1A) caused significant reduction of intracellular NTHi after 4 h and 8 h. Moxifloxacin at 10 mg/l achieved > 99% killing of intracellular NTHi after 4 h ($P = 0.014$); at 1 mg/l, 94% ($P = 0.022$) were killed after 4 h and 98% ($P = 0.001$) after 8 h (Table 2). Ciprofloxacin, azithromycin and telithromycin showed delayed activity in killing intracellular NTHi: at 1 mg/l they achieved a killing index < 75 after 4 h and 86–90% killing ($P = 0.002$–0.003) after 8 h. At 10 mg/l ciprofloxacin killed 96% ($P = 0.014$) of the intracellular NTHi after 4 h, whereas azithromycin and telithromycin eliminated < 88% ($P = 0.02$–0.04). After 8 h, the killing index of these latter two antibiotics climbed to 94 ($P = 0.001$).

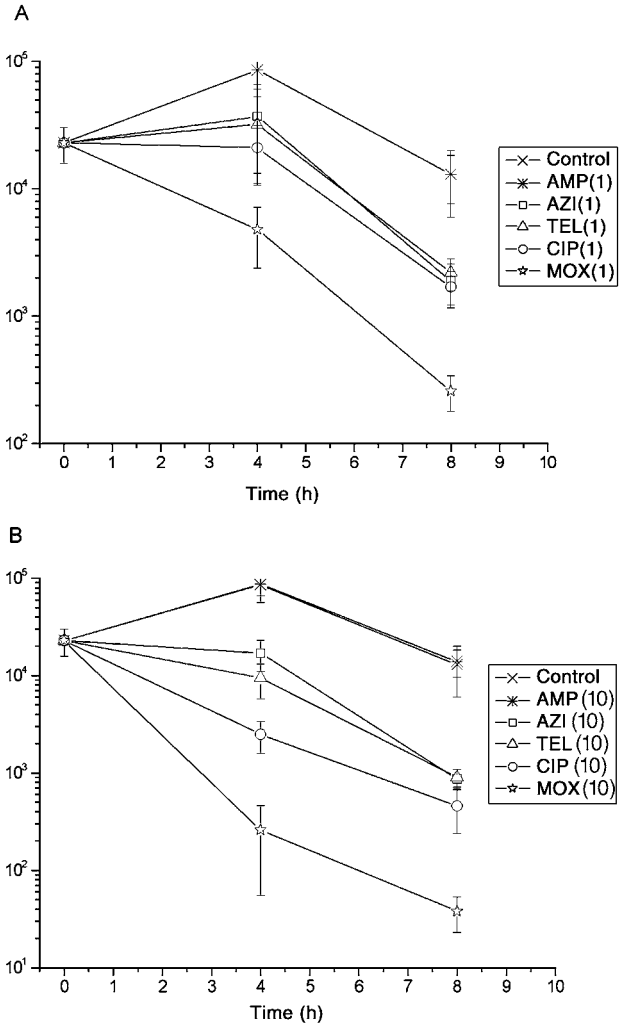

Fig. 1. Time-dependant bacterial reduction of intracellular non-typeable *Haemophilus influenzae* 77 in bronchial epithelial cells after exposure to 1 mg/l (**A**) or 10 mg/l (**B**) ampicillin (star), azithromycin (open square), telithromycin (open triangle), ciprofloxacin (open circle) and moxifloxacin (open asterisk) for 4 h and 8 h in comparison with a control without active substance (cross), as determined with the invasion assay. Error bars represent the average of three samples ± 1 standard deviation

Discussion

Infectious diseases, particularly those of the RT, remain a major cause of morbidity and a significant socioeconomic burden worldwide. The ability to predict the likelihood of a satisfactory outcome during antibacterial chemotherapy is an important aspect of treatment, the primary aim of which is to achieve sufficient drug concentration at the site of infection for an adequate length of time to ensure bacterial eradication and optimize clinical success. This is particularly an issue for obligate or facultative intracellular agents in RT infections.

Intracellular localization of NTHi might serve as a reservoir, promoting recurrent infections especially in patients with chronic obstructive pulmonary disease [5, 6]. It is therefore of great interest to evaluate the intracellular activity of antibiotics frequently used in RT infections.

The invasion assay is a simple method for assessing the intracellular killing properties of antibiotics in vitro [8, 13, 14]. Serum-free medium without albumin or other plasma proteins that influence protein binding of antibiotics was used in the present study, and the pH in all cell cultures remained stable between 7.2 and 7.4 and was not influenced by the various antibiotic agents or by incubation with the test strain.

The five antibiotics tested are clinically important members of four different antibiotic classes commonly prescribed against RT infections. As expected, the survival of NTHi in bronchial epithelial cells was not influenced by ampicillin during eight hours of incubation but was significantly impaired by the other antibiotics. Similarly, Ahrén et al. [8], when investigating the intracellular activity of antimicrobial agents against NTHi, also demonstrated excellent activity of low-dose fluoroquinolones but only a limited effect of high-dose ampicillin despite

Table 2. Intracellular survival and killing of NTHi 77 in absence and presence of ampicillin (AMP), azithromycin (AZM), telithromycin (TEL), moxifloxacin (MOX) and ciprofloxacin (CIP) in comparison with an antibiotic-free control

Antibiotic (mg/l)	Survival index[a]		Killing index[b]	
	Time 4 h	Time 8 h	Time 4 h	Time 8 h
Control	344 ± 150	62.3 ± 6.0	–	–
AMP (10)	344 ± 150	61.0 ± 4.4	0	2.0 ± 2.0
AMP (1)	344 ± 150	62.3 ± 6.0	0	0
AZM (10)	75.7 ± 15	3.7 ± 0.7	74.7 ± 11	94.3 ± 0.6
AZM (1)	152 ± 58	8.4 ± 0.2	54.7 ± 4.0	86.3 ± 1.2
TEL (10)	41.3 ± 11	3.7 ± 0.7	86.7 ± 5.5	94.0 ± 1.0
TEL (1)	193 ± 46	8.1 ± 1.9	60.0 ± 4.0	86.7 ± 2.1
CIP (10)	11.7 ± 3.1	2.0 ± 0.1	96.3 ± 1.5	96.7 ± 0.6
CIP (1)	83.7 ± 20	5.9 ± 0.2	73.7 ± 7.6	90.3 ± 1.1
MOX (10)	1.1 ± 0.8	0.2 ± 0.2	99.7 ± 0.1	99.8 ± 0.2
MOX (1)	20.7 ± 5.5	1.0 ± 0.9	93.7 ± 2.3	98.3 ± 1.1

Values represent means ± standard deviation from triplicate wells of three independent experiments. [a] Survival index SI = $[(\text{cfu/ml})_t / (\text{cfu/ml})_0] \times 100$. [b] Killing index KI = $[(SI_{t;control} - SI_{t;antibiotic})/SI_{t;control}] \times 100$.

longer antibiotic exposure. Beta-lactams, including ampicillin, penetrate eukaryotic cells poorly (C/E ratios < 1) [15], whereas azithromycin, telithromycin, ciprofloxacin and moxifloxacin accumulate in eukaryotic cells and reach markedly higher levels in the intracellular compartment than in the extracellular space [16–19].

Although penetration into eukaryotic cells is an important precondition for intracellular activity, high intracellular levels do not accurately predict the level of intracellular killing, as in the case of the macrolides. Antibiotics such as moxifloxacin, that combine excellent extracellular activity, high intracellular penetration and subsequent killing, are most effective against intracellular pathogens. This was demonstrated in the present study, where moxifloxacin showed the highest bactericidal efficacy against intracellular NTHi 77: at 1 mg/l, a therapeutic concentration far above the MBC, moxifloxacin eradicated 94% of the intracellular NTHi within four hours and 98% in eight hours. Ciprofloxacin at the same concentration showed delayed antimicrobial activity, although significant reduction of intracellular NTHi was found after exposure for four hours. However, for azithromycin and telithromycin, which reached higher intracellular levels than the fluoroquinolones, lower intracellular activity corresponded with higher extracellular MICs and MBCs: at 1 mg/l ($0.5 \times$ MIC) and an exposure time of four hours, neither antibiotic achieved significant bacterial reduction, and longer incubation of eight hours was sufficient to kill only 86% of the intracellular NTHi.

A limitation of the present study was that the invasion experiments were performed with a single highly invasive strain of *H. influenzae* and therefore results could be specific for this particular strain. Additional work will be required to determine whether other strains of *H. influenzae* behave similarly.

Apart from NTHi, other RT pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes, Streptococcus pneumoniae* and *Mycoplasma pneumoniae* are able to invade epithelial cells and leukocytes [13, 20–22]. Seral et al. recently demonstrated the high in vitro activity of moxifloxacin against intracellular *S. aureus*, in contrast to ciprofloxacin, azithromycin and telithromycin which were largely ineffective [14].

Moxifloxacin, which combines high extracellular and intracellular activities, could be seen as an important tool for the treatment of recurrent RT infections. These properties will become even more important in the future, when increasing changes in etiology and resistance will limit the choice of effective antibacterials further.

Transparency declarations

None to declare.

Acknowledgements

The authors thank Helga Sigmund of the Department of Internal Medicine I for excellent technical advice.

References

1. Murphy TF (2003) Respiratory infections caused by non-typeable *Haemophilus influenzae*. Curr Opin Infect Dis 16: 129–134
2. Ketterer MR, Shao JQ, Hornick DB, Buscher B, Bandi VK, Apicella MA (1999) Infection of primary human bronchial epithelial cells by *Haemophilus influenzae*: Macropinocytosis as a mechanism of airway epithelial cell entry. Infect Immun 67: 4161–4170
3. Swords WE, Buscher BA, Ver Steeg Ii K, Preston A, Nichols WA, Weiser JN, et al (2000) Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Mol Microbiol 37: 13–27
4. Ahrén IL, Williams DL, Rice PJ, Forsgren A, Riesbeck K (2001) The importance of a beta-glucan receptor in the nonopsonic entry of non-typeable *Haemophilus influenzae* into human monocytic and epithelial cells. J Infect Dis 184: 150–158
5. Bandi V, Apicella MA, Mason E, Murphy TF, Siddiqi A, Atmar RL, et al (2001) Nontypeable *Haemophilus influ-*

enzae in the lower respiratory tract of patients with chronic bronchitis. Am J Respir Crit Care Med 164: 2114–2119
6. Murphy TF, Brauer AL, Schiffmacher AT, Sethi S (2004) Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 170: 266–272
7. Van Schilfgaarde M, Eijk P, Regelink A, van Ulsen P, Everts V, Dankert J, et al (1999) *Haemophilus influenzae* localized in epithelial cell layers is shielded from antibiotics and antibody-mediated bactericidal activity. Microbiol Pathogenesis 26: 249–262
8. Ahrén IL, Karlsson E, Forsgren A, Riesbeck K (2002) Comparison of the antibacterial activities of ampicillin, ciprofloxacin, clarithromycin, telithromycin, and quinupristin/dalfopristin against intracellular non-typeable *Haemophilus influenzae*. J Antimicrob Chemother 50: 903–908
9. Jacobs MR, Johnson CE (2003) Macrolide resistance: an increasing concern for treatment failure in children. Pediatr Infect Dis J 22 [8 Suppl]: S131–138
10. National Committee for Clinical Laboratory Standards (2003) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically – Sixth Edition: Approved Standard M7-A6. NCCLS, Wayne, PA, USA
11. Barbé G, Babolat M, Boeufgras, Monget D, Freney (1994) Evaluation of API NH, a new 2-hour system for identification of *Neisseria* and *Haemophilus* species and *Moraxella catarrhalis* in a routine clinical laboratory. J Clin Microbiol 32: 187–189
12. Simon HJ, Yin EL (1970) Microbioassay of antimicrobial agents. Appl Microbiol 19: 573–579
13. Nielsen SL, Obel N, Storgaard M, Andersen PL (1997) The effect of quinolones on the intracellular killing of *Staphylococcus aureus* in neutrophil granulocytes. J Antimicrob Chemother 39: 617-622
14. Seral C, Van Bambeke F, Tulkens PM (2003) Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Antimicrob Agents Chemother 47: 2283-2292
15. Bergan T (1982) Penetration of antibiotics into peripheral infection foci. Wien Klin Wochenschr 94: 331–340
16. Foulds G, Shepard RM, Johnson RB (1990) The pharmacokinetics of azithromycin in human serum and tissue. J Antimicrob Chemother 25 [Suppl A]: 73–82
17. Pascual A, Ballesta S, Garcia I, Perea EJ (2001) Uptake and intracellular activity of ketolides HMR 3647 in human phagocytic and non-phagocytic cells. Clin Microbiol Infect 7: 65–69
18. Dan M, Torossian K, Weissberg D, Kitzes R (1993) The penetration of ciprofloxacin into bronchial mucosa, lung parenchyma, and pleural tissue after intravenous administration. Eur J Clin Pharmacol 44: 101–102
19. Pascual A, Gracia I, Ballesta S, Perea EJ (1999) Uptake and intracellular activity of moxifloxacin in human neutrophils and tissue-cultured epithelial cells. Antimicrob Agents Chemother 43: 12–15
20. Mandell GL, Coleman EJ (2000) Activities of antimicrobial agents against intracellular pneumococci. Antimicrob Agents Chemother 44: 2561–2563
21. La Penta D, Rubens C, Chi E, Cleary PP (1994) Group A streptococci efficiently invade human respiratory epithelial cells. Proc Natl Acad Sci USA 91: 12115–12119
22. Daxboeck F, Bauer CC, Assadian O, Stanek G (2006) An unrecognized epidemic of *Mycoplasma pneumoniae* infection in Vienna. Wien Klin Wochenschr 118: 208–211
23. Davies B, Maeson F (1979) Serum and sputum antibiotic levels after ampicillin, amoxicillin and bacampicillin in chronic bronchitis patients. Infect 7 [Suppl 5]: 465–468
24. Lode H (1991) The pharmacokinetics of azithromycin and their clinical significance. Eur J Clin Microbiol Infect Dis 10: 807–812
25. Perret C, Lenfant B, Weinling E, Wessels DH, Scholtz HE, Montay G, et al (2002) Pharmacokinetics and absolute oral bioavailability of an 800-mg oral dose of telithromycin in healthy young and elderly volunteers. Chemother 48: 217–223
26. Brunner M, Stabeta H, Moller JG, Schrolnberger C, Erovic B, Holleinstein U, et al (2002) Target site concentrations of ciprofloxacin after single intravenous and oral doses. Antimicrob Agents Chemother 46: 3724–3730
27. Wise R, Andrews JM, Marshall G, et al (1999) Pharmacokinetics and inflammatory-fluid penetration of moxifloxacin following oral or intravenous administration. Antimicrob Agents Chemother 43: 1508–1510

Correspondence: Prof. DDr. A. Georgopoulos, Department of Internal Medicine I, Division of Infectious Diseases and Tropical Diseases, Medical University of Vienna, Währinger Gürtel 18–20, 1090 Vienna, Austria,
E-mail: apostolos.georgopoulos@meduniwien.ac.at