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Intracellular activity of antibiotics against *Coxiella burnetii* in a model of activated human THP-1 cells

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Abstract

We evaluated antibiotic activity against the intracellular bacterium *Coxiella burnetii* using an activated THP-1 cell model of infection. At clinically-relevant concentrations, the intracellular bacterial load was reduced 300-fold by levofloxacin and finafloxacin, 40-fold by doxycycline, 4-fold by ciprofloxacin, and was unaffected by azithromycin. Acidification of the culture media reduced antibiotic activity with the exceptions of doxycycline (no change) and finafloxacin (slight improvement). This model may be used to select antibiotics to be evaluated *in-vivo*.

Coxiella burnetii is the causative agent of Q fever, which can cause infections in humans following inhalation of contaminated aerosols (1). First line therapy is doxycycline (1), but azithromycin or the fluoroquinolones (known to accumulate in host cells), may constitute useful alternatives due to the obligate intracellular nature of this bacterium. In this work, we compared the intracellular activity of doxycycline with that of azithromycin and of three fluoroquinolones (ciprofloxacin, levofloxacin, and finafloxacin) in a model of activated THP-1 cells infected by the attenuated variant Nine Mile, Phase II RSA439, Clone 4 of *C. burnetii* (2). This strain is a relevant surrogate for fully virulent Phase I *C. burnetii,* with similar growth kinetics in THP-1 cells (3). *C. burnetii* thrives in acidic vacuoles sharing similarities with phagolysosomes (4,5), making relevant the comparison between finafloxacin, which shows lower MICs at acidic pH *vs* neutral pH against other bacterial species (related to a higher accumulation in bacteria at acidic pH), and conventional fluoroquinolones, which have better activity at neutral pH (6,7). Moreover, because *C. burnetii* causes infection of the lungs where the pH of the extravascular space is acidic (8), or result in endocarditis which may be accompanied by metabolic acidosis (9), we compared infected cells incubated in media at neutral or acidic pH.

MICs were determined by microdilution in ACCM-2 medium according to a published method (10) and are reported in footnote[e] to Table 1. PMA-differentiated THP-1 human monocytes were infected following a previously described protocol (11; see Figure S1 for the development of the model), except that *C. burnetii* and the antibiotic stocks were diluted in RPMI supplemented by 10% fetal calf serum and 2 mM glutamine. Infected cells were exposed for 72 h to each antibiotic over a wide range of concentrations (0.003-100xMIC), then harvested, and the lysates used to determine cfu counts following plating on ACCM-2 agar (10). The protein content was also determined. Data (expressed as change in cfu from the post-phagocytosis inoculum, normalized to the protein content of the samples) were used to fit a Hill equation (Figure 1 for individual data for each antibiotic and Figure S2 for antibiotic comparisons) and calculate the

pharmacodynamic parameters. The antibiotic efficacy ($E_{max\ and}$ E at C_{max}) and apparent relative potency (C_s and C_{-1log}, see footnotes to Table 1 for a definition of these parameters) were determined (Table 1). The study has been approved by the Biosafety Office of the Université catholique de Louvain on May 22, 2019.

Following 72 h of incubation, all antibiotics demonstrated concentration-dependent killing, with azithromycin and doxycycline resulting in a lower (less negative value) E_{max} (reduction of approx. 1.5 $\log_{10}$ CFU) than the fluoroquinolones (reduction of approx. 2.5 $\log_{10}$ CFU). E_{max} was not influenced by the pH of the medium. At an extracellular concentration corresponding to the human C_{max} of each antibiotic, levofloxacin and finafloxacin were the most effective in media at neutral pH, causing a reduction in CFUs close to their respective E_{max}, followed by doxycycline, ciprofloxacin, and azithromycin which was ineffective. In acidic media, the efficacy of finafloxacin and doxycycline remained unaffected while that of ciprofloxacin and levofloxacin was reduced. C_s was lower than the MIC for all drugs in media at neutral pH, and shifted to significantly higher values at acidic pH for azithromycin, ciprofloxacin, and levofloxacin. The concentration required to result in a 90% reduction in bacterial load (C_{-1log}) was achieved at clinically-relevant concentrations for doxycycline, levofloxacin, and finafloxacin in media at neutral pH. In acidic media, the C_{-1log} were shifted to higher values for azithromycin, ciprofloxacin and levofloxacin, but to a lower value for finafloxacin.

Although *C. burnetii* is an intracellular pathogen, few studies have examined antibiotic activity intracellularly and only semi-quantitatively or in a few conditions of exposure (11-14). Thanks to the establishment of appropriate axenic cultures in liquid or solid media (10), we were able to quantify *C. burnetii* intracellularly in control conditions and following exposure to selected antibiotics.

We found that fluoroquinolones are the most effective drugs among those evaluated, with levofloxacin and finafloxacin (but not ciprofloxacin) able to reduce the intracellular bacterial inoculum 400-fold at clinically-relevant concentrations. This is probably related to their known bactericidal activity, as previously suggested (14). The reason why ciprofloxacin was less active in this model remains to be established, but this data is consistent with its recently demonstrated inactivity *in-vivo*, contrasting with the remarkable efficacy of levofloxacin (11). Levofloxacin and finafloxacin also displayed similar relative potencies intracellularly, failing to determine a possible advantage of finafloxacin, despite its lower MIC. One possible explanation resides in the lower accumulation of finafloxacin *vs.* levofloxacin in THP-1 cells (2.5-fold *vs*. 10-fold respectively [7,15]). This hypothesis is further substantiated by the opposite effect of the pH of the culture media on the intracellular potency of finafloxacin *vs.* the conventional fluoroquinolones. Finafloxacin is the only fluoroquinolone for which potency was increased in acidic media (concentration-response curve shifted to the left), most probably because its uptake is increased in THP-1 cells at acidic pH (related to a higher proportion of zwitterionic form; Table S1), as opposed to that of other fluoroquinolones (7).

Doxycycline, considered as a first line of treatment, was less effective in our model, but remained highly potent, as it was still capable of reducing the intracellular bacterial inoculum 40-fold at clinically relevant concentrations. Previous work showed that the potency of doxycycline could be further increased by neutralizing infected vacuoles (12,13). However, an acidification of the extracellular media did not affect its intracellular activity, suggesting that its uptake mechanism in THP-1 cells is pH-independent in the range of pH investigated, below the isoelectric point of this molecule (Table S1). Finally, azithromycin was inactive both in axenic culture and intracellularly, despite its high cellular accumulation. The potency of azithromycin is markedly reduced in acidic environments (16) where it is fully protonated (Table S1); this includes the vacuoles where *C. burnetii* is thriving. Its uptake by THP-1 cells is reduced in acidic media (16), explaining the even lower potency observed in these conditions.

In conclusion, we have demonstrated the utility of a *C. burnetii in-vitro* intracellular infection model which has allowed pharmacological comparisons of antibiotic activity. The data collected for doxycycline, ciprofloxacin and levofloxacin are consistent with those obtained *in vivo* with the Phase I virulent variant RSA493 (11,17), suggesting that the attenuated variant, Phase II, is an appropriate, easier to handle, surrogate for larger scale screening of novel Q-fever therapeutics.

Data availability

Data will be made available upon request (via the corresponding author).

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Table 1: Pharmacological parameters and statistical analysis of the concentration-response curves of antibiotics against *Coxiella burnetii* in activated THP-1 cells incubated in media at neutral or acidic pH

Drug	E_{max} [a] (Δlog cfu from time 0)	E^b at C_{max} [c] (Δlog cfu from time 0)	Cs [d]		C_{-1log} [f]	
			x MIC [e]	mg/L	x MIC	mg/L
Media at pH 7.4						
Doxycycline (DOX)	-1.64 (-0.70 to -2.58) (A)	-1.61 (-0.71 to -2.5) (A)	0.24 (-005-0.53)	0.01 (-0.01-0.02) (A)	2.47 (0.56-4.39)	0.07 (0.02-0.13) (A)
Azithromycin (AZI)	-1.57 (3.39 to -6.5) (A)	+0.50 (0.25 to 0.75) (B)	0.15 (-0.95-1.25)	19.3 (-121.1-160.0) (B)	1.31 -0.74-3.35)	169 (-94.1-428.8) (B)
Ciprofloxacin (CIP)	-2.40 (-2.30 to -2.50) (B)	-0.59 (-0.48 to -0.70) (C)	0.56 (0.31-0.81)	1.12 (0.62-1.62) (C)	2.42 (2.23-2.60)	4.84 (4.47-5.21) (C)
Levofloxacin (LVX)	-2.84 (-1.81 to -3.86) (B)	-2.54 (-2.32 to -3.39) (D)	0.05 (-0.02-0.11)	0.18 (-0.07-0.43) (D)	0.18 (0.15-0.21)	0.72 (0.61-0.84) (D)
Finafloxacin (FIN)	-2.67 (-1.89 to -3.45) (B)	-2.50 (-1.88 to -3.13) (D)	0.59 (-0.02-1.20)	0.07 (-0.01-0.14) (D)	3.35 (2.31-4.38)	0.42 (0.29-0.55) (D)
Media at pH 5.5						
Doxycycline (DOX)	-1.6 (-1.25 to –1.97) NS (A)	-1.56 (-1.23 to -1.89) NS (A)	0.47 (-0.10-1.05)	0.01 NS (-0.01-0.03) (A)	3.99 NS (3.72-4.26)	0.12 NS (0.11-0.13) (A)
Azithromycin (AZI)	-1.55 (-1.01 to -2.09) NS (A)	+0.61 (0.23 to 0.98) NS (B)	2.32* (-0.4-5.08)	296.4* (-57.8-650.6) (B)	>8***	>1000*** (B)
Ciprofloxacin (CIP)	-2.70 (-2.37 to -3.03)* (B)	0.14 (-0.17 to 0.44)*** (C)	1.78** (0.95-2.62)	3.57** (1.90-5.23) (C)	6.70*** (6.43-6.96)	13.40*** (12.87-13.92) (C)
Levofloxacin (LVX)	-2.96 (-2.32 to -3.60) NS (B)	-2.12 (-1.75 to -2.49)* (D)	0.15** (0.09-0.21)	0.59** (0.37-0.81) (D)	0.56*** (0.44-0.67)	2.24*** (1.78-2.70) (D)
Finafloxacin (FIN)	-2.60 (-2.56 to -2.65) NS (B)	-2.54 (-2.45 to -2.64) NS (E)	0.24 (-0.13-1.20)	0.03 NS (-0.02-0.08) (A)	1.39* (-0.40-3.18)	0.17* (-0.05-0.39) (A)

[a] maximal efficacy: CFU reduction (in log_{10} units, with confidence interval) at 72 h from the corresponding initial post-phagocytosis bacterial inoculum as extrapolated from the Hill equation of the concentration-effect response for an infinitely large antibiotic concentration.

[b] CFU reduction (in log_{10} units, with confidence interval) at 72 h from the corresponding initial post-phagocytosis bacterial inoculum as calculated from the Hill equation of the concentration-effect response for an antibiotic concentration corresponding to the human C_{max}.

[c] Human C_{max} for selected doses (mg/L): DOX: 2 (100 mg bd); AZI 0.4 (500 mg od); CIP: 2.8 (500 mg bd); LVX: 8.7 (500 mg od); FIN 9.0 (800 mg od) (selected doses based on the British National Formulary for inhalation anthrax [CIP, LVX], the recommended dose for Q Fever (1) [DOX], the conventional dose [AZI] or the dose used in clinical trials [FIN]; human C_{max} based on EUCAST rationale documents (CIP, LVX, DOX, AZI) or (18) [FIN].

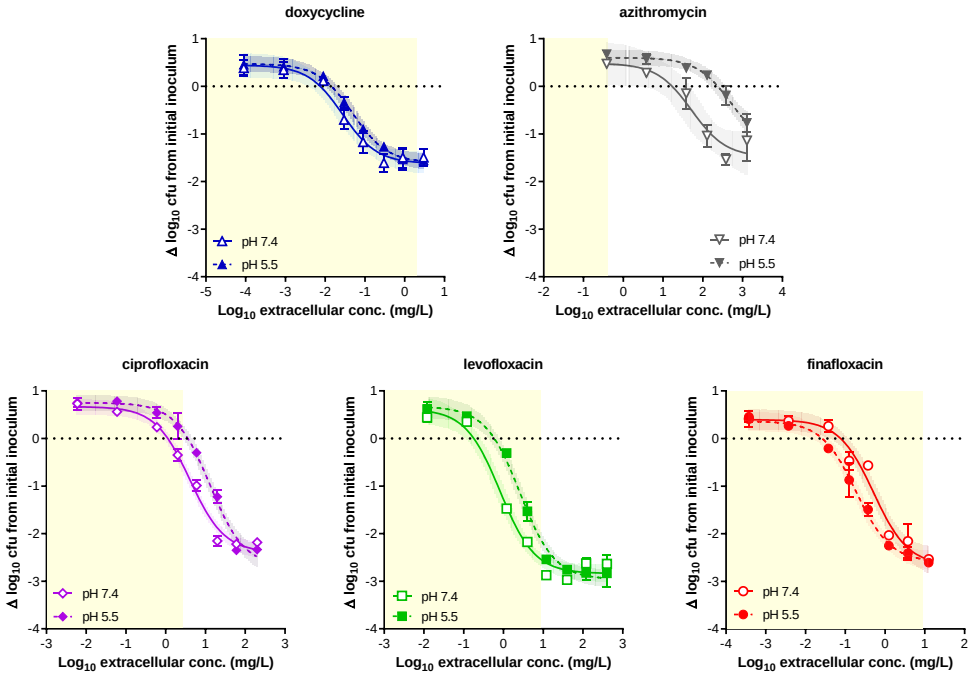
[d] Static concentration: extracellular antibiotic concentration (with confidence intervals) resulting in no apparent bacterial growth from the post-phagocytosis bacterial inoculum following 72 h of incubation, as calculated from the Hill equation of the concentration-response curve.

[e] MIC (mg/L): DOX: 0.03; AZI >128 (128 taken as MIC in calculations); CIP: 2; LVX: 4; FIN: 0.125.

[f] extracellular antibiotic concentration (with confidence intervals) resulting in a reduction of 90% of CFU (1 $\log_{10}$) from the post-phagocytosis bacterial inoculum following 72 h of incubation, as calculated from the Hill equation of the concentration-response curve.

Statistical analysis: comparison between antibiotics at a given pH: 1-way ANOVA with Tukey post hoc test: values with different letters are different from one another ($p < 0.05$); comparison for each antibiotic between data in media at pH 7.4 and 5.5 : multiple t-test: ^{NS}: $p > 0.05$; *: $p<0.05$; **: $p< 0.01$; ***: $p<0.001$. Statistics on C_s and C_{-1log} were performed on their $\log_{10}$ values (symmetrical distribution). Data are shown as the mean (95% confidence interval) of three independent experiments.

Figure 1: Concentration-response curves of antibiotics against intracellular *C. burnetii* in a model of PMA-activated THP-1 human monocytes. The abscissa shows the extracellular concentrations. The ordinate shows the change in the number of cfu from the initial post-phagocytosis inoculum following 72 h of incubation. The horizontal dotted line corresponds to the initial inoculum and allows the apparent static effect (Cs) of each antibiotic to be calculated. The data were used to fit Hill curves, represented with their 95% confidence area. All data are means ± SEM of 3 independent experiments. The yellow shaded area corresponds to the zone of effects obtained at clinically-relevant concentrations (see footnote[c] to Table 1 for C_{max} values). Open symbols and plain lines: data in media at pH 7.4; closed symbols and dotted lines: data in media at pH 5.5.



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