J Infect Chemother 25 (2019) 322–324

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Note

Gene expression analysis in the potent bactericidal activity of sitafloxacin against *Streptococcus pneumoniae*☆

Intetsu Kobayashi [a, *], Izumo Kanesaka [a], Akiko Kanayama Katsuse [a], Hiroshi Takahashi [a], Ryo Okumura [b], Yasuhiro Nakanishi [c], Akihiro Kaneko [c]

[a] Department of Infection Control and Prevention, Toho University Faculty of Nursing, 4-16-20, Omori-Nishi Ota-ku, Tokyo 143-0015, Japan
[b] Rare Disease Laboratories, Daiichi Sankyo Co. Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
[c] Department of Oral and Maxillofacial Surgery, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa, 253-1193, Japan

ARTICLE INFO

Article history:
Received 11 June 2018
Received in revised form
3 September 2018
Accepted 25 September 2018
Available online 23 October 2018

Keywords:
Gene expression
Bactericidal activity
Streptococcus pneumoniae
Sitafloxacin
Cmax concentration

ABSTRACT

We investigated the degree of expression of *Streptococcus pneumoniae* genes associated with bacteriolysis and cell death in relation to the rapid bactericidal activity of sitafloxacin. *S. pneumoniae* ATCC 49619 was added to brain heart infusion containing sitafloxacin and garenoxacin concentrations equivalent to the Cmax achieved with the usual single dose and 4 h post-Cmax concentration. RNA was extracted and cDNA was prepared using reverse transcriptase. Following RNA extraction and cDNA synthesis, quantitative PCR was performed to determine the amount of gene expression for 13 genes associated with cell death.

Of the 13 genes analyzed, *S. pneumoniae* exposed for 10 min to a sitafloxacin concentration of 4 h post-Cmax showed 3.9 times increased expression of *lytA* compared to the control strain. Furthermore, we observed a slightly increased expression for *cibA* encoding a competence induced bacteriocin.

Our study suggests that the induction of a lytic enzyme and bacteriocin may reflect gene expression in response to sitafloxacin accounting for part of its rapid bactericidal activity against *S. pneumoniae*.

© 2018 Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Sitafloxacin (STFX) is a fluoroquinolone with high affinity to DNA gyrase and topoisomerase enzymes that has shown clinical efficacy in respiratory infections [1]. STFX exhibits strong bactericidal activity against organisms resistant to other fluoroquinolones [2]. We previously reported on the superior and more rapid bactericidal activity of STFX compared to other respiratory fluoroquinolones against fluoroquinolone-resistant *Streptococcus pneumoniae*, oral streptococci and anaerobes [3,4]. Furthermore, we observed a non-protein synthesis dependent mechanism contributing to the rapid and potent bactericidal activity of STFX against *S. pneumoniae* [3]. Our findings are consistent with the additive effect described by Drlica et al. [5] in which cell death was not dependent on protein synthesis.

While there have been other studies evaluating gene expression of selected genes in response to bactericidal and bacteriolytic antimicrobials, almost no previous studies have comprehensively examined gene expression levels. In this study, we evaluated simultaneous expression of 13 genes associated with bacteriolysis and cell death in relation to STFX's rapid bactericidal activity.

S. pneumoniae ATCC 49619 STFX MIC; 0.03 μg/mL, garenoxacin (GRNX) MIC; 0.06 μg/mL [3] was added to brain heart infusion (BHI) containing a STFX concentration equivalent to the blood-level Cmax (1 μg/mL) achieved with the usual single oral administration dose and 4 h post-Cmax (0.47 μg/mL) [3]. The initial concentration of *S. pneumoniae* was 10^6 cfu/mL. Inoculated tubes were incubated at 35 °C. At 0, 10, 30 and 60 min after addition of *S. pneumoniae*, RNA was extracted from a 50 mL aliquot of BHI and cDNA was synthesized using SuperScript VILO Master Mix (Life technologies, CA) from total RNA (500 ng) extracted using TRIzol Max Bacterial RNA Isolation Kit (Life technologies). The cDNA samples were then pre-amplified with TaqMan PreAmp Master Mix (Life technologies). Real-Time Quantitative Reverse Transcription PCR was performed using Taqman primers/probes (Table 1), BioMark 48.48 Dynamic Array (Fluidigm, CA) and Taqman Fast Advanced Master Mix (Life technologies) according to each manufacturer's instructions. In our experiments, 16S rRNA was used as a reference gene for normalization of expression of respective target genes. Data was analyzed using BioMark Gene Expression Data Analysis software (Fluidigm)

☆ All authors meet the ICMJE authorship criteria.

* Corresponding author.
E-mail address: kobatora@med.toho-u.ac.jp (I. Kobayashi).

https://doi.org/10.1016/j.jiac.2018.09.012
1341-321X/© 2018 Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Table 1
Primers and probes used in this study for gene expression.

	Forward Primer	Reverse Primer	Dye	Probe	Quencher
spxB	AGCCAACGAAGTGGTCTTTGAA	TGCTTCGTAAACTTCAGCAAATGC	FAM	CCTTGGTTCAAACTTC	NFQ
clpP	AGGCAGATGTCCAAACCATTGTTAT	TGATGCGATGACTGTTCCCATAG	FAM	ATGCAGCCATTCCC	NFQ
lytA	CGTTGACCCTTATCCATATCTTGCT	GCCGTTCTCAATATCATGCTTAAACTG	FAM	CTCACGGCTAATGCC	NFQ
cibA	TTGACATTCTTGACAATCAATTTTTATCCTT	CCTTCCAAGATACTGCTACTCCAAA	FAM	ACCGCCATCAATATCT	NFQ
cibB	TCTAATAAGGAATTGCAAGAAATCAAGGGT	ACCTGCCATAAATAAAGCGATACCAA	FAM	CACCTACACCAAAGCC	NFQ
cbpD	CTGCCTATAATGGAAGCTATCGTTATGT	GTTGAAGAAAGAACAGAATTACCTAGAGGAT	FAM	CACAGCCTCCAATTGA	NFQ
recA	GTTTGCAGGCTCGTATGATGAG	GGTTGATAAAAATGGCAATTGTTTTGGTT	FAM	CCATGCGTAAACTTG	NFQ
sulA	GCGACCTCAGCACCCTAG	CTCTGTAAAAGCTTGACCAAAACCA	FAM	CTCGCTCATCTTCCC	NFQ
relA	AAGACCAGGCTGATGATGCTAAG	AAACGTAAATCTCCTCAGCCAAATAGTT	FAM	CAGAGTCCACAAATTC	NFQ
gyrA	GCTAGACCATATCGACGAAGTGATT	GACGTTCAGAAAGCTTAAACTTGCT	FAM	TCCGCATCCGTTTCAC	NFQ
parC	GCCAAGGAAAACCTCAAAGTTAGC	ACGGTACAGTTGCAAAGTTACGATA	FAM	CTCAGCCTGTTCTTCC	NFQ
rpoS	TGGTATGCAGTTCCTTGACTTGATT	TCAAACTTGTCAACCGCCTTCA	FAM	TCAAGCCCATATTTCC	NFQ
ply	AGAGTGGAAGCAGATTTTGGACAAT	CTTGGGTCGCCCCCTAAAATAA	FAM	CCGCCTTCACTTCTG	NFQ
16S rRNA	CCCCTTATGACCTGGGCTACA	CGGCTTGCGACTCGTTGT	VIC	CGTGCTACAATGGCT	NFQ

to obtain Ct values. Relative gene expression values were determined using the $2^{-\Delta\Delta}$CT method and shown as a fold change relative to respective genes at 0 h [6].

cDNA was prepared using reverse transcriptase. Quantitative PCR was performed to determine the level of gene expression for 13 genes (*spxB, clpP, lytA, cibA, cibB, cbpD, recA, sulA, relA, gyrA, parC, rpoS, ply*) associated with bacteriolysis and cell death [7].

Gene expression results for STFX were compared with GRNX at concentrations equivalent to the Cmax (7.19 μg/mL) achieved with the usual single dose and 4 h post-Cmax (5 μg/mL), as reported previously [3].

Of the 13 genes associated with cell death, *S. pneumoniae* exposed for 10 min to a STFX concentration equivalent to 4 h post-Cmax showed 3.9 times increased expression of pneumococcal autolysin *lytA* compared to the control strain (Table 2). Furthermore, after 60 min STFX exposure, we observed 1.7 to 1.8 times increased expression of *lytA*, *cibA* encoding a competence induced bacteriocin A (Fig. 1). Compared to STFX, no significant gene expression was observed when *S. pneumoniae* was exposed to GRNX.

In this study, we investigated gene expression levels in 13 genes associated with *S. pneumoniae* cell death in studying the bactericidal properties of STFX and GRNX.

Within 60 min of *S. pneumoniae* exposure to STFX, we observed increased gene expression of *lytA* and *cibA* which are associated with allolysis. When *lytA* gene expression to STFX C4 at 30 min was compared to expression levels at 10 and 60 min, expression level was lower although slightly higher than the control. Furthermore, in comparison to GRNX, the level of gene expression was higher after exposure to STFX.

Accelerated *lytA* and *cibA* gene expression was detected at relatively short time-intervals of 10, 30 and 60 min after exposure to STFX concentrations at Cmax (1 μg/mL) and 4 h post-Cmax (0.47 μg/mL).

The strong bactericidal activities of antimicrobials against *S. pneumoniae*, such as penicillin and vancomycin, are felt to be associated with LytA-induced lysis [8,9]. In time-kill studies of induction of autolysis in *S. pneumoniae* by quinolones, Okumura et al. showed that *lytA* mutants exhibited slower killing compared to *lytA*-positive strains [10].

This finding suggests that, as with penicillin and vancomycin, those quinolones with enhanced bactericidal activity against *S. pneumoniae* exhibiting short-term bactericidal activity also induce autolysis as the result of accelerated LytA-induced autolysis. On the other hand, CibAB bacteriocin-mediated triggering of allolysis is felt to be a general property of *S. pneumoniae*. Therefore, rapid *S. pneumoniae* cell death may be the result of *lytA*-induced cell lysis as well as *lytA*-induced lysis being a secondary effect of CibAB allolysis.

The gene expression results clearly suggest that STFX influences on 2 genes (*lytA*, *cibA*) associated with cell death. We have previously reported on the potent bactericidal properties of STFX [3]. The findings from this study point to a potential mechanism of action explaining the very interesting finding of rapid bacteria count decline within a short one-hour time frame. The observation of accelerated expression of those genes associated with cell death, within the observed 10–60 min period, would be consistent with increased gene expression from the beginning of exposure to STFX leading to rapid decline in bacterial count.

Table 2
Relative gene expression value.

	STFX Cm[a]			STFX C4[b]			GRNX Cm			GRNX C4			Control			
min.	10	30	60	10	30	60	10	30	60	10	30	60	0	10	30	60
spxB	0.29	0.45	0.65	0.64	0.96	0.98	0.19	0.21	0.34	0.20	0.34	0.48	1	1.49	1.55	1.46
clpP	0.48	1.37	1.48	0.98	1.08	2.02	0.47	0.45	0.85	0.30	0.67	1.03	1	1.62	1.59	1.48
lytA	0.14	1.65	1.11	3.87	0.47	1.80	1.28	0.14	0.13	0.67	0.50	0.37	1	0.47	0.31	0.11
cibA	0.11	0.77	0.58	1.05	0.49	1.68	0.34	0.07	0.12	0.10	0.22	0.28	1	0.75	0.66	0.69
cibB	0.50	0.43	0.60	0.37	0.61	0.71	0.26	0.25	0.38	0.15	0.29	0.74	1	1.51	1.03	1.59
cbpD	0.69	0.88	1.15	0.86	1.10	1.25	0.51	0.61	1.01	0.54	0.75	1.02	1	1.72	1.35	1.44
recA	0.82	1.75	1.70	1.34	1.02	1.45	1.20	0.89	0.92	0.99	1.30	1.22	1	1.16	0.79	0.34
sulA	0.30	0.44	0.60	0.58	0.78	0.83	0.24	0.25	0.30	0.26	0.35	0.52	1	1.43	1.17	1.16
relA	0.22	0.66	0.63	0.86	0.61	1.35	0.30	0.14	0.14	0.15	0.20	0.28	1	1.28	1.17	1.06
gyrA	0.56	0.95	1.51	1.15	1.37	1.05	0.82	0.79	1.20	0.80	1.17	1.57	1	1.30	1.21	0.25
parC	0.28	0.63	0.77	0.73	0.81	1.19	0.21	0.26	0.50	0.24	0.42	0.57	1	1.50	1.23	1.41
rpoS	0.63	1.07	1.41	0.85	1.19	1.53	0.68	0.95	1.61	0.59	1.02	1.32	1	1.71	1.40	2.22
ply	0.66	0.69	0.77	0.65	0.73	0.53	0.65	0.50	0.44	0.63	0.49	0.86	1	1.05	0.43	1.18

[a] Cm: Cmax.
[b] C4: 4 h post-Cmax.

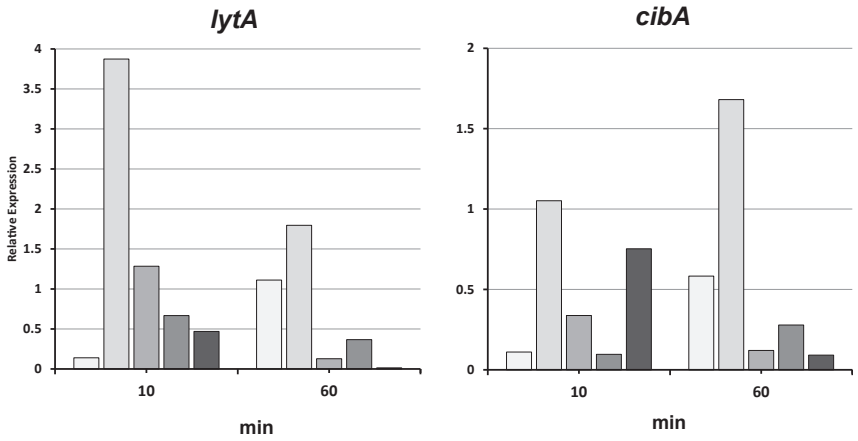

Fig. 1. Comparison of *lytA* and *cibA* gene expression of *Streptococcus pneumoniae* after treatment with sitafloxacin and garenoxacin. □STFX Cm(Cmax), □STFX C4(4 hours post-Cmax), ▒GRNX Cm, ▒GRNX C4, ■Control.

The concentrations used in the study was the Cmax achieved with normal dosage and 4 h post-Cmax which corresponds to a STFX concentrations of 1 μg/mL and 0.47 μg/mL, respectively, and GRNX concentrations of 7.19 μg/mL and 5 μg/mL, respectively. The difference in gene expression cannot be accounted for the relative MIC differences between the two fluoroquinolones.

The MICs of the *S. pneumoniae* strain used in the study were 0.03 μg/mL, and 0.06 μg/mL to STFX and GRNX, respectively, reflecting only a one 2-fold dilution difference. The antibiotic concentration equivalent to 4 h post-Cmax and Cmax/MIC ratios for STFX were 15 and 30 times compared to 80 and 120 times for GRNX, respectively.

Our results suggest that the MIC is not directly related to gene expression in those antimicrobials with rapid bactericidal properties. Rapid bactericidal activity may be more associated with the unique mechanisms of actions of an antimicrobial. Furthermore, although the basis for this is not understood, even for the same antimicrobial, gene expression may not be directly related to antibiotic concentration.

The observation of different gene expression profiles associated with the respective bactericidal activity of STFX and GRNX against *S. pneumoniae* is of great significance and requires further study.

Furthermore, a strong association between rapid bactericidal activity of STFX and gene expression levels was observed. In studying the determinants of bactericidal activity, the data suggests that the rapid bactericidal activity of STFX against *S. pneumoniae* is related to its effect on the genes associated with cell death. Since our observation is restricted to an ATCC strain, there is a need to reproduce our findings using clinical isolates as well as strains missing these genes. Our study suggests that STFX might induce expressions of lytic enzyme and bacteriocin to account for part its rapid bactericidal activity against *S. pneumoniae.*

Conflicts of interest

The authors declare no conflict of interest. This work was supported in part by Daiichi Sankyo Co.,Ltd.

References

[1] Tanaka M, Onodera Y, Uchida Y, Sato K, Hayakawa I. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV purified from *Staphylococcus aureus*. Antimicrob Agents Chemother 1997;41:2362–6.
[2] Okumura R, Hirata T, Onodera Y, Hoshino K, Otani T, Yamamoto T. Dual-targeting properties of the 3-aminopyrrolidyl quinolones, DC-159a and sitafloxacin, against DNA gyrase and topoisomerase IV: contribution to reducing in vitro emergence of quinolone-resistant *Streptococcus pneumoniae*. J Antimicrob Chemother 2008;62:98–104.
[3] Kobayashi I, Kanayama A, Hasegawa M, Kaneko A. Bactericidal activity of sitafloxacin and other new quinolones against antimicrobial resistant *Streptococcus pneumoniae*. Jpn J Antibiot 2013;66:25–35.
[4] Kobayashi I, Watanabe D, Mizusawa N, Kubota H, Suzuki T, Denda Y, et al. Bactericidal activity of sitafloxacin and other new quinolones against anaerobes and oral *Streptococcus*. Oral Ther Pharmacol 2013;32:136–43.
[5] Drlica K, Malik M, Kerns RJ, Zhao X. Quinolone-mediated bacterial death. Antimicrob Agents Chemother 2008;52:385–92.
[6] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. Methods 2001;25:402–8.
[7] Rice KC, Bayles KW. Molecular control of bacterial death and lysis. Microbiol Mol Biol Rev 2008;72:85–109.
[8] Normark BH, Normark S. Antibiotic tolerance in pneumococci. Clin Microbiol Infect 2002;8:613–22.
[9] Tomasz A, Waks S. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc Natl Acad Sci USA 1975;72:4162–6.
[10] Okumura R, Hoshino K, Otani T, Yamamoto T. Quinolones with enhanced bactericidal activity induce autolysis in *Streptococcus pneumoniae*. Chemotherapy 2009;55:262–9.