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A quinol peroxidase inhibitor prevents secretion of a leukotoxin from *Aggregatibacter actinomycetemcomitans*

E. Takashima[1], H. Yamada[1], A. Yajima[1], K. Shiomi[2], S. Ōmura[2], K. Kiyoshi[1]

[1]Department of Microbiology, School of Life Dentistry at Tokyo, The Nippon Dental University, Chiyoda-ku, Tokyo, Japan and [2]Kitasato Institute for Life Sciences, Graduate School of Infection Control Sciences, Kitasato University, Minato-ku, Tokyo, Japan

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Background and Objective: Quinol peroxidase (QPO) catalyzes peroxidase activity using quinol in the respiratory chain as a substrate. Quinol peroxidase is essential for the secretion of leukotoxin (LtxA), which destroys leukocytes and erythrocytes in humans and is one of the major virulence factors of *Aggregatibacter actinomycetemcomitans*, which is associated with localized aggressive periodontitis. In the present study, we aimed to find a highly potent QPO inhibitor to attenuate the virulence of *A. actinomycetemcomitans*.

Material and Methods: For screening of QPO inhibitors, QPO activity was measured kinetically by SpectraMax Plus with 96-well UV plates. Three hundred compounds in the Kitasato Institute for Life Sciences Chemical Library were screened. Secretion of LtxA in the culture supernatant was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cytotoxicity against human promyelocytic leukemia cell line (HL-60) cells from the culture supernatant was measured by Trypan Blue exclusion test.

Results: The present study characterized ascofuranone as a highly potent inhibitor of QPO (K_i = 9.557 ± 0.865 nM). Ascofuranone inhibited secretion of LtxA by *A. actinomycetemcomitans* in a dose-dependent manner, making *A. actinomycetemcomitans* less pathogenic to HL-60 cells.

Conclusion: Quinol peroxidase inhibitors are promising candidates as alternative drugs for the treatment and prevention of the onset of localized aggressive periodontitis. Using ascofuranone as a seed compound, further study of QPO inhibitors could provide novel chemotherapeutic strategies for controlling localized aggressive periodontitis.

Eizo Takashima, PhD, Department of Microbiology, School of Life Dentistry at Tokyo, The Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan
Tel: +81 3 3261 8763
Fax: +81 3 3261 8763
e-mail: eizo.takashima@gmail.com

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Aggregatibacter actinomycetemcomitans is a facultative anaerobic, CO_2-requiring, gram-negative human pathogen that has been associated with localized aggressive periodontitis, a severe disease in adolescents that is characterized by rapid bone and tissue destruction and ultimately the loss of teeth (1). Leukotoxin (LtxA) is secreted from *A. actinomycetemcomitans* as an approximately 113 kDa soluble protein that destroys leukocytes and erythrocytes in humans (2–4). An LtxA high-production strain of *A. actinomycetemcomitans* is correlated with the onset of localized aggressive periodontitis (5), and LtxA has been recognized to be one of the major virulence

factors of *A. actinomycetemcomitans* (6–8).

Recently, we characterized a quinol peroxidase (QPO), a 53.6 kDa tri-heme *c* membrane enzyme of *A. actinomycetemcomitans* that catalyzes peroxidase activity using quinol in the respiratory chain as the physiological electron donor to reduce hydrogen peroxide (9). Quinol peroxidase is the only characterized peroxidase containing three hemes and is also the only characterized bacterial peroxidase with a transmembrane region. Since the amino acid sequence of the C-terminal region of QPO shares ~43% identity with di-heme bacterial cytochrome *c* peroxidase, we grouped them together into a single enzyme family, which we named the bacterial multi-heme peroxidase family (10). Our resent study also showed that a QPO null mutant (QPS003) exhibits an endogenous oxidative stress phenotype, suggesting that QPO functions physiologically as an antioxidant enzyme to scavenge endogenous oxidative stress in *A. actinomycetemcomitans* (11).

Interestingly, LtxA is degraded upon exposure to reactive oxygen species (12). Consistent with this, QPS003 exhibited a secretion defect of LtxA, probably owing to its failure to protect LtxA against endogenous oxidative stress (11). Since LtxA is one of the major virulence factors of *A. actinomycetemcomitans*, QPO may serve as a drug target to attenuate the virulence of this bacterium for the prevention and treatment of localized aggressive periodontitis. For this reason, in the present study, we characterized a compound that inhibits QPO activity with high potency.

Material and methods

Bacterial strains and culture conditions

A. actinomycetemcomitans IDH781 (leukotoxic, serotype d strain; 4), *A. actinomycetemcomitans* ATCC29522 (leukotoxic, serotype b strain; 13), *Streptococcus gordonii* DL1 and *Escherichia coli* K-12 were used as wild-type strains. QPS003, a QPO null mutant derived from IDH781, and QPS003 (pVJTqpo), which harbors a plasmid with the full-length sequence of *qpo*, was generated during a previous study (9).

All bacterial cell cultures were grown at 37°C in an air atmosphere containing 5% CO_2. Bacterial culture medium was purchased from Difco, BD Diagnostic Systems, Sparks, MD, USA. *A. actinomycetemcomitans* strains were cultured in *A. actinomycetemcomitans* growth medium (AAGM; 3) containing appropriate antibiotics [50 μg/mL spectinomycin for QPS003 and QPS003 (pVJTqpo); 2 μg/mL chloramphenicol for QPS003 (pVJTqpo; 9,11)]. *S. gordonii* DL1 and *E. coli* K-12 were cultured in brain–heart infusion and Luria-Bertani broth (1% tryptone, 0.5% yeast extract, and 1% NaCl), respectively. The growth of the *A. actinomycetemcomitans* strains was monitored in 200 μL liquid cultures in 96-well cell culture plates by measurement of the optical density at 600 nm ($o.d._{600nm}$) using SpectraMax Plus (MDS Analytical Technologies, Sunnyvale, CA, USA). Before of the measurements, we mixed the plates using a vortex mixer to remove adherent cells.

Minimal inhibitory concentrations (MIC) of ascofuranone (AF) against *A. actinomycetemcomitans*, *S. gordonii* and *E. coli* were determined with liquid cultures in 96-well cell culture plates as in a previous report (14). Two hundred microlitres of appropriate medium containing serial diluted AF were dispensed in each well, and $\sim 10^5$ test bacteria were inoculated into the each well. The plates were cultured for 1 day in the conditions described above. The highest dilution at which no growth occurred ($o.d._{600nm} < 0.05$) was defined as the MIC. All antibiotics were obtained from Sigma (St Louis, MO, USA).

Kinetic analysis

Quinol peroxidase was purified from *A. actinomycetemcomitans* ATCC 29522 (9). Quinol peroxidase activity was measured as in a previous report (9) with slight modification. Quinol peroxidase activity was measured at 25°C in a buffer containing 100 mM Tris-HCl (pH 7.5), 0.1% (w/v) sucrose monolaurate (SM-1200; Nacalai Tesque, Inc., Kyoto, Japan), ubiquinol-1 (32, 40, 53, 80 and 160 μM) and AF (5.7, 10.9, 17.1 and 22.8 nM). Ubiquinone-1 was a kind gift from Eisai (Tokyo, Japan) and the reduced form (ubiquinol-1) was prepared as previously described (15). The reaction was initiated by the addition of 80 μM H_2O_2. Oxidation of ubiquinol-1 was monitored at 278 nm using an extinction coefficient of 10/mM/cm. The kinetic parameters were calculated using GRAPHPAD PRISM (Graphpad Software, San Diego, CA, USA) by non-linear least-squares analysis.

Quinol peroxidase inhibitor screening

Screening of QPO inhibitors was conducted in 96-well UV plates (Corning, NY, USA). Total assay volume was 100 μL per well. Quinol peroxidase activity was kinetically measured by SpectraMax Plus.

Detection of oxidatively modified proteins

A. actinomycetemcomitans strains were grown to the late-log phase. The cultures were harvested and solubilized using 6% (w/v) of sodium dodecyl sulfate. Carbonyl groups in the protein side-chains were detected by the Oxyblot kit (Chemicon, Temecula, CA, USA) with SuperSignal West Pico detection reagents (Pierce, Rockford, IL, USA), as previously reported (11).

Cytotoxicity assay

The cell toxicity of the culture supernatant from *A. actinomycetemcomitans* against human promyelocytic leukemia cell line (HL-60) cells was determined using the Trypan Blue dye exclusion test as previously reported (16). The HL-60 cells were washed once in assay buffer (0.5 mM $CaCl_2$ and 0.2 M sodium phosphate buffer, pH 5.7) and resuspended in the buffer at a cell density of $\sim 5 \times 10^5$ cells/mL. The *A. actinomycetemcomitans* culture supernatants were obtained by centrifugation at 15,000 *g* for 10 min at 4°C. To induce cytotoxic activity, the

culture supernatants from late-log phase (equivalent to 5 μL of the culture at o.d.$_{600nm}$ = 1; typically, 3.5–6.0 μL of the culture supernatants) were added to 100 μl of cell suspension per well of a 96-well plate, then incubated at 37°C for 2 h in an atmosphere containing 5% CO_2. Heat-treated culture supernatant (70°C, 60 min) was used as a negative control.

Miscellaneous methods

Secreted proteins in the late-log phase of the *A. actinomycetemcomitans* culture supernatants (equivalent to 250 μL of culture at o.d.$_{600nm}$ = 1; typically 180–300 μL of the culture supernatants) were concentrated by cold ethanol and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue staining, as previously reported (3,11).

Results

Ascofuranone inhibited QPO activity with high potency

We screened QPO inhibitors out of approximately 300 compounds, comprising metabolites of bacteria and fungi, in the Kitasato Institute for Life Sciences Chemical Library (17). Among of them, fortunately, ascofuranone (AF, Fig. 1A) strongly inhibited QPO activity. Ascofuranone is a prenylphenol compound that is well known as a strong inhibitor of trypanosome alternative oxidase (TAO), a terminal oxidase of the respiratory chain of *Trypanosoma brucei brucei* (18). Ascofuranone cures mice infected with *T. b. brucei* by intraperitoneal injection; thus it is a promising drug candidate for African trypanosomiasis (19).

To explore the mechanism of action of AF against QPO, we measured QPO activity in the presence of various concentrations of ubiquinol-1 and AF. As shown by double-reciprocal plots in Fig. 1B, AF exhibited a mixed-type inhibition for QPO with respect to ubiquinol-1. The mixed-type mechanism of inhibition is described by the equilibrium shown in Fig. 1D, and the velocity equation for this is given by the formula shown in Fig. 1E (20). The Michaelis–Menten plot of the data and the non-linear fitting curves using this formula are shown in Fig. 1C [k_{cat} = 729.7 ± 30.1/s, K_m = 108.4 ± 8.10 μM, K_i = 9.557 ± 0.865 nM and α = 3.15 ± 1.03 (± S.D.)]. Ascofuranone is the first characterized QPO inhibitor. The K_i value is comparable to the value determined for purified recombinant TAO (1.69 nM), although AF inhibited TAO in a competitive manner (21).

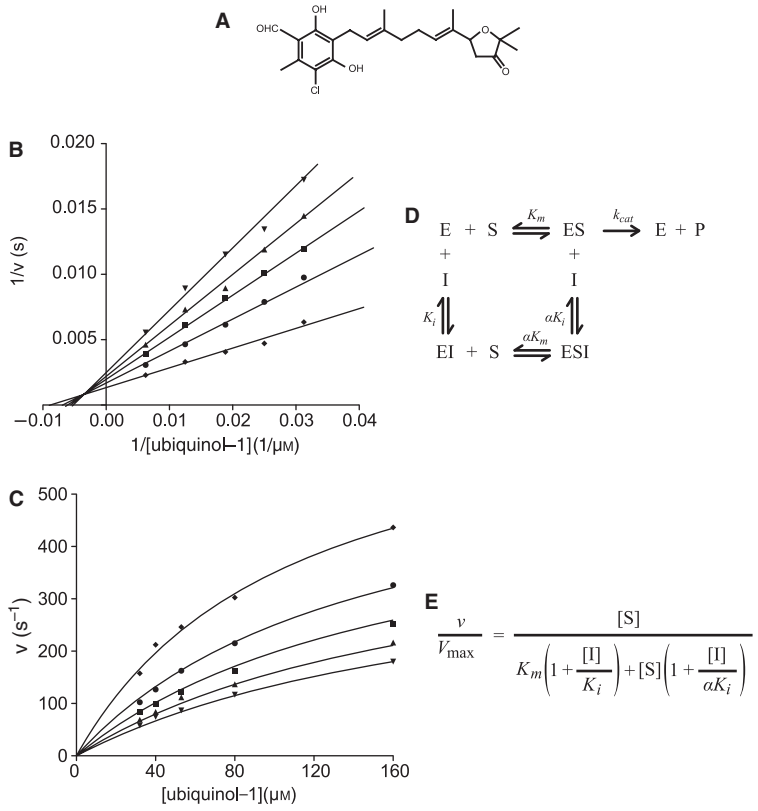

Fig. 1. (A) Chemical structure of AF. The molecular mass of AF is 420.926 Da. (B,C) Quinol peroxidase activity at various concentrations of ubiquinol-1 with AF at the concentration indicated by following symbols: ♦, 0 nM; ●, 5.7 nM; ■, 10.9 nM; ▲, 17.1 nM; and ▼, 22.8 nM. (B) Double reciprocal-plot with linear-regression lines. (C) Michaelis–Menten plot with non-linear curve fitting using the formula described in (E). (D) Mixed-type mechanism of inhibition described by the equiblium. (E) The velocity equation for the mixed-type inhibition.

Ascofuranone reduced the growth rate of *A. actinomycetemcomitans*

We previously showed that QPS003 grew slower than IDH781 (11). Consistent with this, the addition of 20 μg/mL of AF reduced the growth rate of IDH781 (Fig. 2A). The fact that the growth curves of QPS003 and IDH781 with 20 μg/mL AF were very similar suggests that the major target of AF in the bacteria is QPO. The fact that QPS003 grew slightly slower when it was cultured with 20 μg/mL AF implies that an unknown minor target of AF may exist.

Ascofuranone induced endogenous oxidative stress in *A. actinomycetemcomitans*

QPS003 exhibits endogenous oxidative stress phenotypes, suggesting that QPO plays an important role in scavenging reactive oxygen species that are endogenously produced (11). For this reason, it was assumed that AF would induce oxidative stress for *A. actinomycetemcomitans*. Regarding this, we conducted Western blotting analysis using anti-dinitrophenyl (DNP) antibody to detect

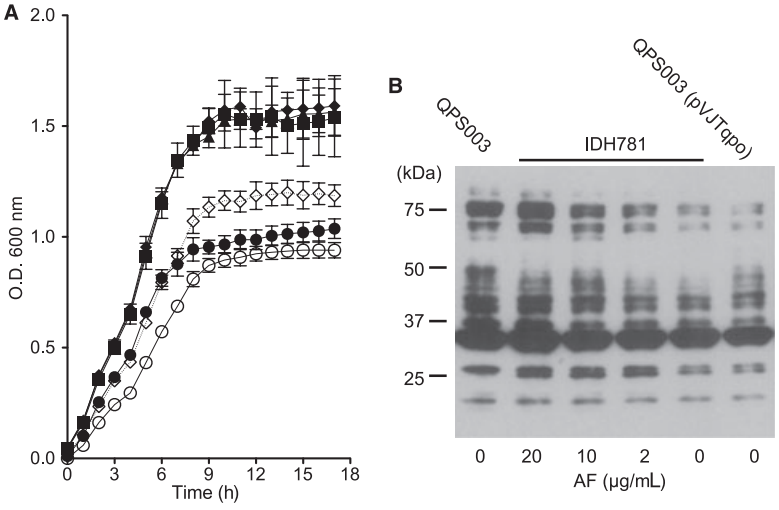

Fig. 2. (A) Growth curves of *A. actinomycetemcomitans* strains in the presence of AF. Optical densities at 600 nm of IDH781 and QPS003 cultures are shown by closed and open symbols, respectively. Error bars indicate S.D. ($n = 3$). ●, IDH781 with 20 μg/mL AF; ■, IDH781 with 10 μg/mL AF; ▲, IDH781 with 2 μg/mL AF; ♦, IDH781 with 0 μg/mL AF; ○, QPS003 with 20 μg/mL AF; and ◇, QPS003 with 0 μg/mL AF. (B) Protein oxidative damage of the strains cultured with indicated concentrations of AF. Oxidatively damaged proteins were detected by anti-DNP Western blot. Protein samples (15 μg) were loaded as indicated for each lane.

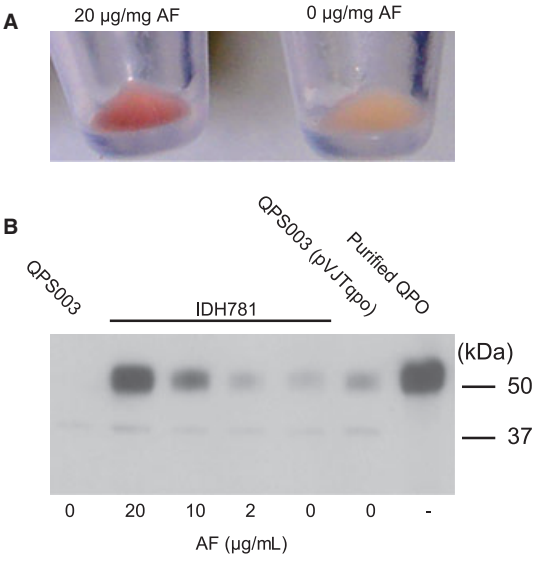

Fig. 3. Quinol peroxidase induction in the presence of AF. (A) Cell pellet of IDH781 cultured with indicated concentrations of AF. (B) Western blotting analysis using rabbit polyclonal anti-QPO antibody. The signals were visualized by SuperSignal West Pico (Pierce). Protein samples (100 ng) from *A. actinomycetemcomitans* strains cultured with the indicated concentrations of AF were loaded as indicated for each lane. As a positive control, 1 ng of purified QPO was loaded for the lane marked 'Purified QPO'.

oxidatively modified proteins from the bacteria cultured with AF. As shown in Fig. 2B, AF induced oxidative stress in *A. actinomycetemcomitans* in a dose-dependent manner. The amount of oxidatively damaged protein from IDH781 with 20 μg/mL AF was very similar to that of QPS003, suggesting that most QPO activity in the bacteria is inhibited by 20 μg/mL AF.

Interestingly, the cell pellet of IDH781 cultured with 20 μg/mL of AF exhibited a reddish color (Fig. 3A), suggesting that QPO, a reddish tri-heme *c* protein, was induced by the oxidative stress due to the presence of AF. Western blotting analysis using anti-QPO antibody clearly showed that QPO was induced by AF (Fig. 3B). Previous study shows that OxyR (22), a global regulator of antioxidant defenses, is involved in the induction of *qpo* homologue (*yhjA*) in *E. coli* (23). This might suggest that QPO would also be induced by OxyR. The gene homologue of *oxyR* in *A. actinomycetemcomitans* can be found in the Oralgen genome database (http://www.oralgen.lanl.gov/; accession number AA01513).

Ascofuranone inhibited secretion of LtxA from *A. actinomycetemcomitans*

The fact that QPS003 has a secretion defect of LtxA (11) led us to hypothesize that AF would inhibit the secretion of LtxA from *A. actinomycetemcomitans*. To measure the inhibitory effect on the secretion of LtxA by AF, we cultured *A. actinomycetemcomitans* with AF. Fortuitously, SDS-PAGE showed that the protein band of LtxA from the culture supernatant of IDH781 decreased in the presence of AF in a dose-dependent manner (Fig. 4A). As in our previous study, QPS003 did not show any LtxA band (Fig.4A).

Consistent with disappearance of the LtxA band in the SDS-PAGE, AF reduced the cytotoxic activity against HL-60 cells of the culture supernatant from IDH781 (Fig. 4B). Likewise, the cytotoxicity of the culture supernatant from *A. actinomycetemcomitans* ATCC29522, a serotype b strain, also decreased in the presence of AF in a dose-dependent manner and was abolished entirely by 20 μg/mL of AF (not shown). The culture supernatant of QPS003 did not show detectable cytotoxic activity, as expected (Fig. 4B). These results indicated that AF attenuated the virulence of the supernatant of the *A. actinomycetemcomitans* strains by decreasing the amount of LtxA from the bacteria. The fact that QPS003 does not have an altered

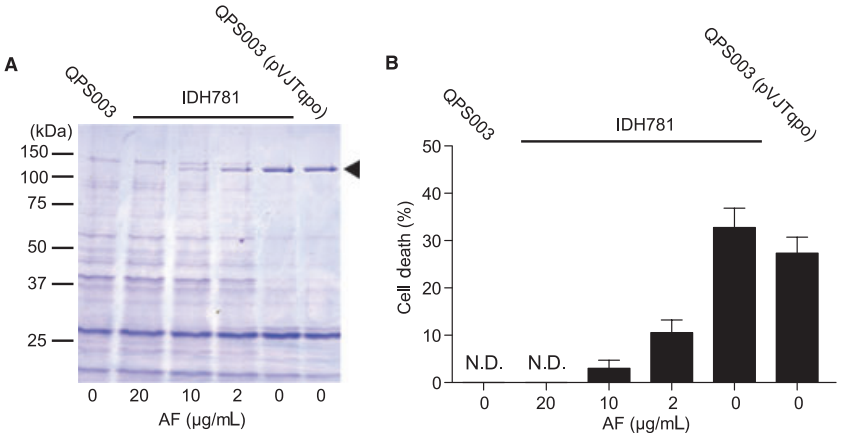

Fig. 4. (A) SDS-PAGE of the culture supernatants of *A. actinomycetemcomitans* strains cultured with the indicated concentrations of AF. Arrowhead indicates the protein band of LtxA. (B) HL-60 cell toxicity of the culture supernatants of the strains cultured with indicated concentrations of AF. Cell viability was determined by Trypan Blue exclusion test. Error bars indicate S.D. (n = 3). N.D., not detected.

transcription level of *ltxA* (11) suggests that the site of action of AF for the prevention of LtxA secretion is not the promoter activity of *ltxA*.

The MIC values of AF

Owing to the rapid progression of localized aggressive periodontitis (1), early diagnosis and prevention of the onset of localized aggressive periodontitis is important. To our knowledge, however, there are no useful drugs to prevent the onset of localized aggressive periodontitis. Since antibiotics have adverse effects (e.g. selection of antibiotic-resistant microorganisms and induction of resistance gene expression or transfer; 24), antibiotic prophylaxis is not applied for localized aggressive periodontitis.

According to this point of view, for the prevention of the onset of localized aggressive periodontitis, practically no bactericidal drug is desirable. For this reason, we measured MIC values of AF for the bacteria in the normal flora to address the impact of AF on these species. As a result, the MIC values for *S. gordonii* DL1 and *E. coli* K-12 were > 50 μg/mL, suggesting that AF is not an effective bactericidal agent at concentrations that would prevent the secretion of LtxA. Likewise, the MIC value against *A. actinomycetemcomitans* IDH781 was > 50 μg/mL. These results imply that AF would have less impact on the normal bacterial flora than antibiotics, and therefore less adverse effects.

Discussion

For the treatment of localized aggressive periodontitis, systemic administration of antibiotics confers clinical benefits (25). However, unfortunately, many systemic antibiotic therapies are unable to suppress subgingival *A. actinomycetemcomitans* consistently to undetectable levels, and single systemic therapies using metronidazole or tetracycline may markedly reduce oral *A. actinomycetemcomitans* but not eradicate the organism (25). So far, to our knowledge, there are currently no adequate drugs for the prevention of the onset of localized aggressive periodontitis. Therefore, the development of chemical agents for the prevention of localized aggressive periodontitis would greatly contribute to the periodontal health of adolescents, especially for high-risk individuals, such as those from a Moroccan immigrant family infected with highly leukotoxic *A. actinomycetemcomitans* (26).

In the present study, we showed that AF inhibited QPO activity with high potency. Ascofuranone induced *A. actinomycetemcomitans* oxidative stress. Ascofuranone is not an effective bactericidal agent but it prevented LtxA secretion by *A. actinomycetemcomitans* in a dose-dependent manner, making *A. actinomycetemcomitans* culture supernatant less pathogenic to HL-60 cells. Given these pieces of evidence, we propose that QPO inhibitors are promising candidates as drugs for the prevention of localized aggressive periodontitis. Moreover, for individuals who do not respond to conventional therapy, QPO inhibitors may provide an alternative practical approach based on attenuation of *A. actinomycetemcomitans* virulence.

Ascofuranone does not have significant cytotoxicity to primary culture of rat mesangial cells (27). The fact that oral and intraperitoneal administration of AF cures *T. b. brucei*-infected mice suggests that AF might have no significant acute toxic effects *in vivo* (28). However, its pharmacokinetics properties and details of acute/chronic toxicological effects in animals are not well known. Using AF as a seed compound, further study of QPO inhibitors could potentially provide novel chemotherapeutic strategies for controlling localized aggressive periodontitis.

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References

1. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J Clin Periodontol* 1985;**12:**1–20.
2. Lally ET, Hill RB, Kieba IR, Korostoff J. The interaction between RTX toxins and target cells. *Trends Microbiol* 1999;**7:**356–361.
3. Kachlany SC, Fine DH, Figurski DH. Secretion of RTX leukotoxin by *Actinobacillus actinomycetemcomitans*. *Infect Immun* 2000;**68:**6094–6100.
4. Balashova NV, Crosby JA, Al Ghofaily L, Kachlany SC. Leukotoxin confers beta-hemolytic activity to *Actinobacillus actinomycetemcomitans*. *Infect Immun* 2006;**74:**2015–2021.
5. Haubek D, Ennibi OK, Poulsen K, Poulsen S, Benzarti N, Kilian M. Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*. *J Dent Res* 2001;**80:**1580–1583.

6. Haubek D, Dirienzo JM, Tinoco EM *et al*. Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. *J Clin Microbiol* 1997;**35:**3037–3042.
7. van Winkelhoff AJ, Slots J. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in nonoral infections. *Periodontol 2000* 1999;**20:**122–135.
8. Haraszthy VI, Hariharan G, Tinoco EM *et al*. Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. *J Periodontol* 2000;**71:**912–922.
9. Yamada H, Takashima E, Konishi K. Molecular characterization of the membrane-bound quinol peroxidase functionally connected to the respiratory chain. *FEBS J* 2007;**274:**853–866.
10. Takashima E, Yamada H, Konishi K. Bacterial Multi-heme Peroxidase. In: Mohan RM, ed. *Research Advances in Biochemistry*. Kerala: Global research network, 2007: 21–27.
11. Takashima E, Konishi K. Characterization of a quinol peroxidase mutant in *Aggregatibacter actinomyctemcomitans*. *FEMS Microbiol Lett* 2008;**286:**66–70.
12. Balashova NV, Park DH, Patel JK, Figurski DH, Kachlany SC. Interaction between leukotoxin and Cu,Zn superoxide dismutase in *Aggregatibacter actinomycetemcomitans*. *Infect Immun* 2007;**75:**4490–4497.
13. Zambon JJ, DeLuca C, Slots J, Genco RJ. Studies of leukotoxin from *Actinobacillus actinomycetemcomitans* using the promyelocytic HL-60 cell line. *Infect Immun* 1983;**40:**205–212.
14. Takarada K, Kimizuka R, Takahashi N, Honma K, Okuda K, Kato T. A comparison of the antibacterial efficacies of essential oils against oral pathogens. *Oral Microbiol Immunol* 2004;**19:**61–64.
15. Rieske JS. Preparation and Properties of Reduced Coenzyme Q-Cytochrome *c* Reductase (Complex III of the Respiratory chain). *Methods Enzymol* 1967; **10:** 239–245.
16. Kachlany SC, Fine DH, Figurski DH. Purification of secreted leukotoxin (LtxA) from *Actinobacillus actinomycetemcomitans*. *Protein Expr Purif* 2002;**25:**465–471.
17. Ui H, Ishiyama A, Sekiguchi H *et al*. Selective and potent in vitro antimalarial activities found in four microbial metabolites. *J Antibiot (Tokyo)* 2007;**60:**220–222.
18. Minagawa N, Yabu Y, Kita K *et al*. An antibiotic, ascofuranone, specifically inhibits respiration and in vitro growth of long slender bloodstream forms of *Trypanosoma brucei brucei*. *Mol Biochem Parasitol* 1997;**84:**271–280.
19. Yabu Y, Yoshida A, Suzuki T *et al*. The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei brucei* in mice. *Parasitol Int* 2003;**52:**155–164.
20. Segel IH. Mixed-type inhibition. In: Segel IH, ed. *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Stady-State Enzyme Systems*. New York: John Wiley & Sons, Inc., 1993: 170–176.
21. Nihei C, Fukai Y, Kawai K *et al*. Purification of active recombinant trypanosome alternative oxidase. *FEBS Lett* 2003; **538:** 35–40.
22. Toledano MB, Kullik I, Trinh F, Baird PT, Schneider TD, Storz G. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. *Cell* 1994;**78:**897–909.
23. Partridge JD, Poole RK, Green J. The *Escherichia coli yhjA* gene, encoding a predicted cytochrome *c* peroxidase, is regulated by FNR and OxyR. *Microbiology* 2007;**153:**1499–1507.
24. Pallasch TJ. Antibiotic prophylaxis: problems in paradise. *Dent Clin North Am* 2003;**47:**665–679.
25. Slots J, Ting M. Systemic antibiotics in the treatment of periodontal disease. *Periodontol 2000* 2002;**28:**106–176.
26. Haubek D, Ennibi OK, Abdellaoui L, Benzarti N, Poulsen S. Attachment loss in Moroccan early onset periodontitis patients and infection with the JP2-type of *Actinobacillus actinomycetemcomitans*. *J Clin Periodontol* 2002;**29:**657–660.
27. Cho HJ, Kang JH, Kwak JY *et al*. Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK- and Ap1-dependent mechanisms. *Carcinogenesis* 2007;**28:**1104–1110.
28. Yabu Y, Minagawa N, Kita K *et al*. Oral and intraperitoneal treatment of *Trypanosoma brucei brucei* with a combination of ascofuranone and glycerol in mice. *Parasitol Int* 1998;**47:**131–137.