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Antibiotics LL-Z1272 identified as novel inhibitors discriminating bacterial and mitochondrial quinol oxidases

Tatsushi Mogi [a,*], Hideaki Ui [b], Kazuro Shiomi [b], Satoshi Ōmura [b], Hideto Miyoshi [c], Kiyoshi Kita [a]

[a] Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108-8641, Japan
[c] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

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ABSTRACT

To counter antibiotic-resistant bacteria, we screened the Kitasato Institute for Life Sciences Chemical Library with bacterial quinol oxidase, which does not exist in the mitochondrial respiratory chain. We identified five prenylphenols, LL-Z1272β, γ, δ, ε and ζ, as new inhibitors for the *Escherichia coli* cytochrome *bd*. We found that these compounds also inhibited the *E. coli bo*-type ubiquinol oxidase and trypanosome alternative oxidase, although these three oxidases are structurally unrelated. LL-Z1272β and ε (dechlorinated derivatives) were more active against cytochrome *bd* while LL-Z1272γ, δ, and ζ (chlorinated derivatives) were potent inhibitors of cytochrome *bo* and trypanosome alternative oxidase. Thus prenylphenols are useful for the selective inhibition of quinol oxidases and for understanding the molecular mechanisms of respiratory quinol oxidases as a probe for the quinol oxidation site. Since quinol oxidases are absent from mammalian mitochondria, LL-Z1272β and δ, which are less toxic to human cells, could be used as lead compounds for development of novel chemotherapeutic agents against pathogenic bacteria and African trypanosomiasis.

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1. Introduction

The emergence of antibiotic-resistant strains of major pathogenic bacteria such as *Staphylococcus aureus* is an increasingly serious public health concern [1]. To evade bacterial drug-resistance mechanisms, new effective chemotherapeutic agents, which have novel mechanisms of action as well as different cellular targets compared with conventional antibiotics, need to be developed [2].

Cytochromes *bo* (CyoABCD) and *bd* (CydAB) are two terminal quinol oxidases of the aerobic respiratory chain in *Escherichia coli* and many other bacteria [3,4 for reviews]. Although they are structurally unrelated, both generate proton-motive force through the oxidation of quinols coupled to dioxygen reduction. Cytochrome *bo* is a proton-pumping heme–copper terminal oxidases and is predominantly expressed under highly aerated growth conditions. In contrast, cytochrome *bd* is a predominant terminal oxidase under microaerophilic growth conditions and performs a variety of physiological functions such as microaerophilic respiration and protection against oxygen stress. Further, cytochrome *bd* and its variant cyanide-insensitive oxidase (CioAB) play a key role in survival and adaptation of pathogenic bacteria that encounter host environments where dioxygen is progressively limited [5–9].

In long slender bloodstream forms of the parasitic protist *Trypanosoma brucei*, which causes sleeping sickness in human and nagana in livestock, mitochondrial respiratory Complexes III and IV are down-regulated and alternative quinol oxidase (AOX) serves as a terminal oxidase [10,11]. AOX is a di-iron family protein bound to the matrix side of the inner membrane and cannot generate the proton-motive force. All three quinol oxidases have no counterparts in mammalian mitochondria, thus they are potential targets for novel antimicrobial chemotherapeutics. In fact, we previously identified ascofuranone (AF), a prenylphenol isolated from a phytopathogenic fungus *Ascochyta viciae* [12], as a potent inhibitor for the growth of *T. brucei* and trypanosome AOX (noncompetitive inhibition with IC$_{50}$ of 2 nM) [13,14].

By screening of hundreds of natural antibiotics in the Kitasato Institute for Life Sciences Chemical Library [15] with the *E. coli* cytochrome *bd*, we found that LL-Z1272γ has potent inhibititory activity. We extended our screening to related compounds and found that antibiotics LL-Z1272β, γ, δ, ε and ζ (Fig. 1), prenylphenols isolated from the fungus *Verticillum* sp. FO-2787 [16], are a unique set of natural compounds that can discriminate and inhibit alternative respiratory quinol oxidases. Thus, antibiotics LL-Z1272 are useful probes for understanding of molecular mechanisms of quinol oxidases and we hope that our findings contribute to the development of new antibiotics.

2. Materials and methods

2.1. Isolation or source of antibiotics and inhibitors

LL-Z1272β, γ, δ, ε and ζ were isolated from the cultured mycelium *Verticillum* sp. FO-2787 [16]. Antibiotics LL-Z1272α, β, γ,

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* Corresponding author. Tel.: +81 3 5841 8202; fax: +81 3 5841 3444.
E-mail address: tmogi@m.u-tokyo.ac.jp (T. Mogi).

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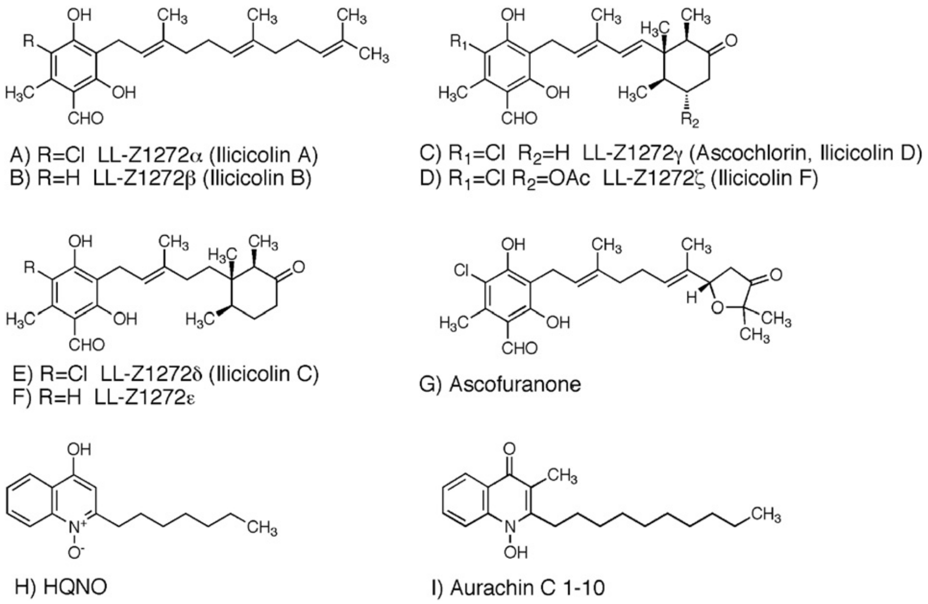

Fig. 1. Structures of antibiotics LL-Z1272 and related natural compounds.

δ, ε and ζ have been originally isolated from an imperfect fungus *Fusarium* sp. as inhibitors for the growth of the protist *Tetrahymena pyriformis* [17]. Ilicicolin A, B, D, C, and F isolated from the fungus *Cylindrocladium ilicicola* [18] are also identical to LL-Z1272α, β, γ, δ, and ζ, respectively [19]. AF and piericidin A were kind gifts from Drs. Masaichi Yamamoto (aRigen Pharmaceuticals. Inc.) and Shigeo Yoshida (Institute of Physical and Chemical Research), respectively. Synthesis of aurachin C 1-10 was described previously [20]. Antimycin A_1 and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were purchased from Sigma.

2.2. Preparation of cytoplasmic membrane vesicles and purification of cytochrome bo

Cytochrome *bd*-overproduced membranes were isolated from *E. coli* ST4683/pNG2 (Δ*cyo* Δ*cyd*/*cyd*$^+$ Tet^R), which can overproduce *bd*-type quinol oxidase as the sole terminal oxidase [21]. Heme *d* content was 2.1 ± 0.1 nmol/mg protein (*i.e.* approximately 20% of membrane proteins). Cytochrome *bo*-type quinol oxidase was purified from cytoplasmic membranes of *E. coli* GO103/pHN3795-1 (*cyo*$^+$ Δ*cyd*/*cyo*$^+$ Amp^R), as described previously [22]. Trypanosome AOX-overproduced membranes were isolated from *E. coli* FN102 (BL21 (DE3) Δ*hemA*)/pTvAOX, which can express *Trypanosoma vivax* AOX as the sole functional quinol oxidase [23]. The expression level of AOX was estimated to be ~5% of membrane proteins by SDS-polyacrylamide gel electrophoresis.

2.3. Quinol oxidase assay

The activity of the *E. coli* quinol oxidases was determined at 25 °C with a V-660 double monochromatic spectrophotometer (JASCO, Tokyo, Japan) with data acquisition at 0.05 s. The reaction mixture (1 ml) contained 50 mM potassium phosphate (pH 6.5), and 0.02% Tween 20 (protein grade, Calbiochem) [24]. Enzyme concentrations were 2.4 nM for cytochrome *bd* and 2 nM for cytochrome *bo*. Reactions were started by addition of ubiquinol-1 (Q_1H_2) at a final concentration of 100 μM, and the activity was calculated by using a molar extinction coefficient of 12,300 at 278 nm. The activity of *T. vivax* AOX was measured in 50 mM Tris–HCl (pH 7.4)–0.1% sucrose monolaurate (Mitsubishi-Kagaku Foods Co., Tokyo, Japan). Enzyme kinetics were analyzed based on the modified *ping-pong bi-bi* mechanism for cytochrome *bd* [21] or the Michaelis–Menten mechanism for cytochrome *bo* and *T. vivax* AOX, by using KaleidaGraph ver. 4.0 (Synergy Software, Reading, PA).

2.4. Dose–response analysis

Duplicate assays were performed at each concentration with two independent preparations of membranes. Dose–response data were analyzed by the nonlinear regression curve-fitting with KaleidaGraph ver. 4.0 as described previously [24]. IC_{50} values in the presence of 100 μM Q_1H_2 were estimated by using the equation for the relative residual activity; $v = 1/(1 + ([\text{Inhibitor}]/IC_{50})^n)$ where n is the Hill coefficient [24].

3. Results

3.1. Analysis of inhibition of cytochrome bd by antibiotics LL-Z1272

In the course of our screening for inhibitors against the *E. coli* cytochrome *bd*, we identified LL-Z1272γ as an antibiotic that suppressed the Q_1H_2 oxidation by the cytochrome *bd*-overproduced membranes (84% inhibition at 5 μg/ml) greater than antimycin A (50%), a non-competitive inhibitor of cytochrome *bd* [25]. We extended our screening with antibiotics LL-Z1272β, γ, δ, ε and ζ, prenylphenols isolated from *Verticillum* sp. FO-2787 [16], and found that LL-Z1272β and ε were more potent inhibitors for cytochrome *bd*. These compounds do not have a chlorine atom at position 5 of the phenol ring (Fig. 1), and the cyclohexanone ring of LL-Z1272ε slightly increased the binding affinity to cytochrome *bd* (Table 1). The 50% inhibitory concentrations (IC_{50}) for LL-Z1272β and ε (dechlorinated derivatives) were determined to be 2.1 and 1.1 μM (average values of two independent preparations), respectively, and are one-order of magnitude smaller than those of LL-Z1272γ, δ and ζ (chlorinated derivatives) (Table 1). The IC_{50} values for known inhibitors for cytochrome *bd* [20,25–27] are 10 μM for piericidin A, 5 μM for antimycin A, 1 μM for HQNO, and 8.3 nM for aurachin C 1–10.

Table 1
Summary on IC_{50} values of quinol oxidase inhibitors for the *E. coli* cytochrome *bd* and *bo* and *T. vivax* AOX

Compounds	Cytochrome *bd*[a]	Cytochrome *bo*[b]	trypanosome AOX[c]
LL-Z1272β	2.1 ± 0.1[d]	1.2 ± 0.1	0.18 ± 0.02
LL-Z1272γ	81 ± 17	0.082 ± 0.016	0.015 ± 0.001
LL-Z1272δ	32 ± 4	0.28 ± 0.02	0.046 ± 0.004
LL-Z1272ε	1.1 ± 0.1	7.2 ± 0.7	0.65 ± 0.09
LL-Z1272ζ	85 ± 7	0.37 ± 0.02	0.43 ± 0.02
Ascofuranone	47 ± 10	0.062 ± 0.003	0.0049 ± 0.0002
Aurachin C 1–10	0.0083 ± 0.0003	0.0023 ± 0.0001	28 ± 2

[a] The *E. coli* cytochrome *bd*-overproduced membranes.
[b] The purified *E. coli* cytochrome *bo*.
[c] The *T. vivax* AOX-overproduced membranes.
[d] μM.

3.2. Kinetic analysis of inhibition of cytochrome bd by LL-Z1272β and ε

Effects of LL-Z1272β and ε on the Q_1H_2 oxidation by cytochrome *bd* were further analyzed kinetically. Control data were analyzed based on the modified *ping-pong bi-bi* mechanism by assuming the stabilization of dioxygen reduction intermediates [28] and apparent K_m and V_{max} values for the control were determined to 50 μM and 2364 Q_1H_2/enzyme/s, respectively, in 50 mM potassium phosphate (pH 6.5)–0.02% Tween 20 [24] (Fig. 2). In the presence of inhibitors, reactions followed the Michaelis–Menten kinetics (Fig. 2). LL-Z1272β acts as a noncompetitive inhibitor with $K_i = 7.6 \pm 2.5$ μM while LL-Z1272ε serves as a competitive inhibitor with $K_i = 1.00 \pm 0.03$ μM (Fig. 2).

3.3. Dose–response analysis of inhibition of cytochrome bo by antibiotics LL-Z1272

In contrast to *bd*-type oxidase, the Q_1H_2 oxidase activity of the *E. coli* cytochrome *bo* was more sensitive to chlorinated derivatives, LL-Z1272γ, ε and ζ. IC_{50} values for LL-Z1272β, γ, δ, ε and ζ (averages from two preparations) were determined to be 1.2, 0.082, 0.28, 7.2 and 0.37 μM, respectively (Table 1). The IC_{50} values for known inhibitors for cytochrome *bo* [20,27,29–31] are 0.3 μM for HQNO, 0.14 μM for piericidin A, and 2.3 nM for aurachin C 1–10, showing that cytochrome *bo* is more sensitive to these quinone analogs than cytochrome *bd*. It should be noted that LL-Z1272γ is a very potent inhibitor of cytochrome *bo*.

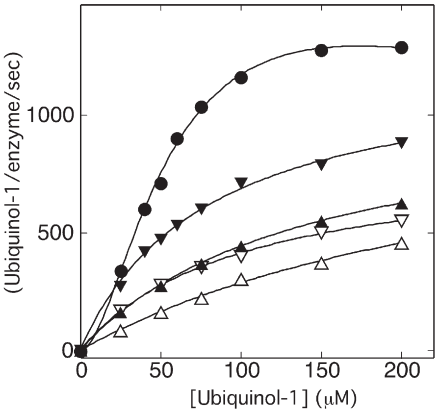

Fig. 2. Effects of antibiotics LL-Z1272 on kinetic parameters for Q_1H_2 oxidation by the *E. coli* cytochrome *bd*. Kinetic analysis was carried out in the absence of inhibitors (●) and the presence of 2 (▼) or 5 (▽) μM LL-Z1272β or 2 (▲) or 5 (△) μM LL-Z1272ε. Control data was analyzed by using the equation $v = SV_{max}/(SS(1 + S/K_S) + SK_m + K_mK_m)$ where K_S indicates the constant for substrate inhibition. Data obtained in the presence of inhibitors were analyzed based on the Michaelis–Menten kinetics. The apparent K_m (μM) and V_{max} (Q_1H_2/enzyme) values obtained were 50 ± 4 and 2364 ± 194, respectively, for the control ($K_S = 381$ μM), 79 ± 5 and 1232 ± 33, respectively, for 2 μM LL-Z1272β, 100 ± 5 and 826 ± 21, respectively, for 5 μM LL-Z1272β, 140 ± 3 and 1065 ± 12, respectively, for 2 μM LL-Z1272ε, 287 ± 41 and 1113 ± 107 Q_1H_2/enzyme/s, respectively, for 5 μM LL-Z1272ε, respectively.

3.4. Kinetic analysis of inhibition of cytochrome bo by antibiotics LL-Z1272

Effects of LL-Z1272β, γ, δ, and ζ on the Q_1H_2 oxidation by cytochrome *bo* were further analyzed kinetically at different concentrations of inhibitors. Enzyme kinetics were analyzed based on the Michaelis–Menten mechanism [29,31], and we found that the inhibition mechanism was all mixed-type (Fig. 3). It should be noted that due to changes in assay conditions apparent K_m and V_{max} values were shifted to 23 μM and 1035 Q_1H_2/enzyme/s, respectively (Fig. 3), from 50 μM and 515 Q_1H_2/enzyme/s, respectively, in 50 mM Tris–HCl (pH 7.4)–0.1% sucrose monolaurate in our previous study [32].

3.5. Dose–response analysis of inhibition of trypanosome AOX by antibiotics LL-Z1272

Because of the structural similarity of antibiotics LL-Z1272 with trypanocidal AF (Fig. 1), we examined the effects of antibiotics LL-Z1272 on Q_1H_2 oxidase activity of *T. vivax* AOX. From dose–response analysis with the AOX-overproduced *E. coli* membranes, we determined IC_{50} values for LL-Z1272β, γ, δ, ε, ζ, AF and aurachin C1–10 to be 180, 15, 46, 650, 430, 4.9 nM and 28 μM, respectively (Table 1). Our data indicate that 1) the furanone ring of AF is not essential for binding to trypanosome AOX, 2) the 5-chloride group on the phenol ring increases the binding affinity, and 3) aurachin C, the most potent inhibitor for bacterial quinol oxidases ($IC_{50} = 8.3$ and 2.3 nM for the *E. coli* cytochrome *bd* and *bo*, respectively (Table 1)) [20,27], is 2 to 4 order of magnitude less active than the prenylphenols.

3.6. Kinetic analysis of inhibition of trypanosome AOX by antibiotics LL-Z1272

Effects of LL-Z1272β, γ, δ, ε and ζ and AF on enzyme kinetics by *T. vivax* AOX were examined in the presence of detergents. Q_1H_2 oxidation by *T. vivax* AOX followed the Michaelis–Menten kinetics

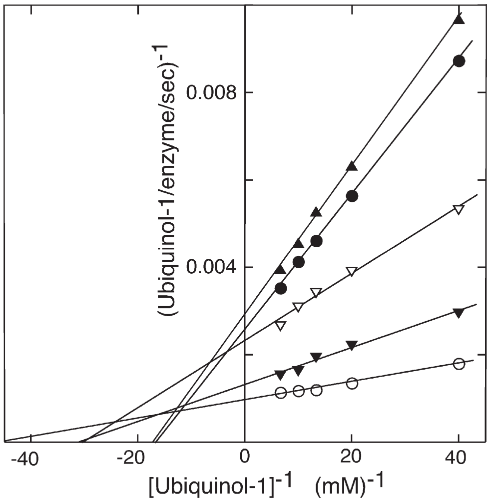

Fig. 3. Effects of antibiotics LL-Z1272 on kinetic parameters for Q_1H_2 oxidation by the *E. coli* cytochrome *bo*. Kinetic analysis was carried out in the absence of inhibitors (○) and the presence of 0.75 μM LL-Z1272β (▼), 0.2 μM LL-Z1272γ (●), 0.75 μM LL-Z1272δ (▲), and ζ (▽). Data were analyzed based on the Michaelis–Menten kinetics. The apparent K_m and V_{max} values obtained are 23 ± 2 and 1035 ± 28 (control), 43 ± 4 and 841 ± 30 (0.75 μM LL-Z1272β), 64 ± 2 and 402 ± 4 (0.2 μM LL-Z1272γ), 66 ± 3 and 361 ± 6 (0.75 μM LL-Z1272δ), 46 ± 4 μM and 486 ± 14 Q_1H_2/enzyme/s (0.75 μM LL-Z1272ζ), respectively. *R* values were >0.997.

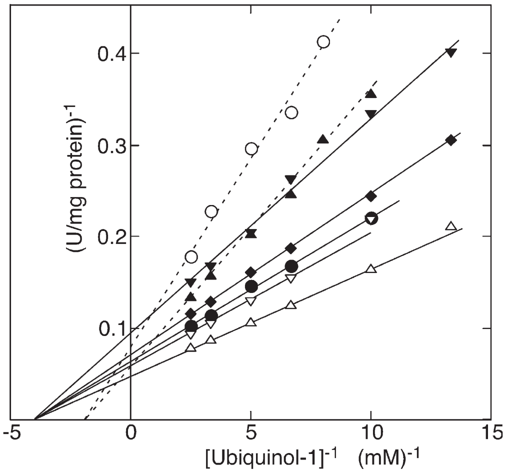

Fig. 4. Effects of antibiotics LL-Z1272 on kinetic parameters for Q_1H_2 oxidation by trypanosome AOX. Kinetic analysis was carried out in the absence of inhibitors (△) and the presence of 200 nM LL-Z1272β (▼), 20 nM LL-Z1272γ (●), 50 nM LL-Z1272δ (▲), 1 μM LL-Z1272ε (○) and 50 nM LL-Z1272ζ (▽), and 5 nM AF (◆). For clarity we showed data for one concentration of each inhibitor. The apparent K_m and V_{max} values determined for the control were 232 μM and 19.8 U/mg protein, respectively. The apparent K_i values for non-competitive inhibition by LL-Z1272β, γ, ζ, and AF were 142, 59, 203, and 2.65 nM. K_i and K_i' values for mixed-type inhibition by LL-Z1272δ and LL-Z1272ε were 0.032 and 25.5 μM and 0.483 and 9.61 μM, respectively.

and apparent K_m and V_{max} values were determined to be 232 μM and 20 U/mg protein (Fig. 4). The K_m value in 0.1% sucrose monolaurate was comparable to 350 μM for *T. b. brucei* AOX in 0.25% *n*-octyl-β-D-glucopyranoside *plus* 0.025% EDT-20 [34], but smaller than approximately 700 μM determined for *T. b. brucei* AOX in the absence of detergents [13,23]. Since the K_m value of *T. vivax* AOX for ubiquinol-2 was 116 μM (data not shown), the length of the isoprene unit may increase the binding affinity for ubiquinones [35]. The K_m of trypanosome AOX for ubiquinol-9 in *T. b. brucei* mitochondria would be comparable to the K_m value of cytochrome *bd* for ubiquinol-8 in *E. coli*.

Kinetic analysis of inhibition of *T. vivax* AOX by antibiotics LL-Z1272 revealed that LL-Z1272β (K_i = 142 nM), γ (59 nM), and ζ (203 nM) act as (apparently) non-competitive inhibitors (Fig. 4), as reported for AF [13] and salicylhydroxamic acid (SHAM, K_i = 25 μM) [34]. Since the amount of active AOX molecules in the *E. coli* membranes was difficult to estimate, we did not try kinetic analysis for tight-binding inhibitors [36]. In contrast, LL-Z1272δ and ε serve as mixed-type inhibitors with K_i and K_i' values of 0.032 and 25.5 μM and 0.483 and 9.61 μM, respectively.

4. Discussion

From the screening of natural antibiotics of the Kitasato Institute for Life Sciences Chemical Library, we identified prenylphenols LL-Z1272β, γ, δ, ε and ζ as a unique set of inhibitors, which can inhibit and discriminate bacterial and trypanosomal ubiquinol oxidases (Table 1). LL-Z1272β and ε (dechlorinated derivatives) inhibited cytochrome *bd*-type oxidase while LL-Z1272γ, δ, and ζ (chlorinated derivatives) were potent inhibitors of cytochrome *bo*-type oxidase and trypanosome AOX. Aurachin C is a potent inhibitor for both cytochrome *bo* and *bd* [20,27], while AF is more active against trypanosome AOX [13]. Since all three quinol oxidases are absent from mammalian mitochondria, prenylphenols could be used as lead compounds for development of novel chemotherapeutic agents [13,14,37]. However, except for the effect of LL-Z1272β on *Clostridium perfringens* (minimum inhibitory concentration of 25 μg/ml), antibiotics LL-Z1272 were ineffective against *S. aureus*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, and *Bacteroides fragilis*. Neither LL-Z1272γ nor LL-Z1272ε affected the aerobic growth of *E. coli* cells expressing cytochrome *bo* or *bd* as the sole terminal oxidase, likely due to the excretion by drug efflux pumps or due to the inefficient penetration through the lipopolysaccharide layer of the outer membrane.

Kinetic analysis of the inhibition of quinol oxidases by prenylphenols yielded rather complicated inhibition mechanisms (Figs. 2–4). Structural similarities of prenylphenols to ubiquinones (Fig. 1) indicate that all these compounds would act as competitive inhibitors for the quinol oxidation site. However, in many cases we found non-competitive or mixed type inhibition. In the case of tight binding inhibitors [36], Michaelis–Menten plots resemble to those of non-competitive inhibition. Alternatively, orientation of the phenol ring of prenylphenol molecules within the binding pocket will determine interactions of prenyl tails and/or the cyclohexanone ring with the protein moiety. The latter interactions would affect the former interactions. In addition, modifications of the prenyl tail (*i.e.*, the presence of the cyclohexanone or franone ring) could alter interactions with lipid bilayers and detergent micelles, which would then affect the orientation of inhibitor molecules relative to the binding pocket in quinol oxidases. Inhibition mechanisms of natural antibiotics may be inherently associated with their structural complexity, as found for inhibitors for alternative NADH dehydrogenase NDH-II [38].

Currently approved drugs for the treatment of human sleeping sickness caused by *T. b. rhodesiense* and *T. b. gambiense* are suramine, pentamidine, melarsoprol, and eflornithine [37]. They are not available for oral administration and *T. brucei* strains resistant to one or more drugs are now emerging. Thus there is an urgent need for less-toxic and more convenient new drugs against African trypanosomiasis. In parallel studies, we recently found trypanocidal activity of LL-Z1272β [39]. LL-Z1272β and LL-Z1272δ have been shown to be less toxic to human cells [18,33] and we have demonstrated that the efficacy of AF in the treatment of trypanosome-infected mice [14]. In conclusion, antibiotics LL-Z1272 are useful as probes for understanding the quinol oxidation sites of respiratory quinol oxidases and such prenylphenols are promising leading compounds for the development of new chemotherapeutic agents for African trypanosomiasis.

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Glossary

AOX: alternatice quinol oxidase
HQNO: 2-heptyl-4-hydroxyquinoline *N*-oxide
IC_{50}: IC_{50}, the 50% inhibitory concentration
Q_1H_2: a reduced form of Q_1, ubiquinol-1