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

A R T I C L E   I N F O

Article info

Article history:
Received 9 October 2008
Received in revised form 21 November 2008
Accepted 26 November 2008
Available online 10 December 2008

Keywords:
Quinol oxidase
Inhibitor
Natural antibiotic
Escherichia coli
Trypanosoma brucei
Alternative oxidase

A B S T R A C T

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 δ (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-Z1272 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.

© 2008 Elsevier B.V. All rights reserved.

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 oxidase 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 IC50 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 inhibitory 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,
δ, ε and ζ have been originally isolated from an imperfect fungus Fusarium sp. as inhibitors for the growth of the protist Tetratymena pyriformis [17]. Ilicicolin A, B, D, C, and F isolated from the fungus Cylindrocladium ilicicola [18] are also identical to LL-Z1272. Ilicicolin A, B, D, C, and F isolated from the fungus Fusarium δisp. as inhibitors for the growth of the protist Fusarium δisp. are also identical to LL-Z1272. LL-Z1272 was overproduced membranes from E. coli Δcyd·Δcyo·ΔcydΔtetR, which can overproduce bα-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·Δcyd/cyo·Ampβ), as described previously [22]. Trypanosome AOX-overproduced membranes were isolated from E. coli FN102 (BL21 (DE3) ΔhemA)/ 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.

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

Cytochrome bd-overproduced membranes were isolated from E. coli ST4683/pNG2 (Δcyo·Δcyd/cyd·Tetα), 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·Δcyd/cyo·Ampβ), as described previously [22]. Trypanosome AOX-overproduced membranes were isolated from E. coli FN102 (BL21 (DE3) ΔhemA)/ 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 (QH₂) 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]. IC₅₀ values in the presence of 100 μM QH₂ were estimated by using the equation for the relative residual activity: v = 1/(1+([Inhibitor]/IC₅₀ⁿ)), 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 QH₂ 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-Z1272δ, ε, δ, ε and χ, prenylphenols isolated from Verticillium sp. FO-2787 [16], and found that LL-Z1272δ 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 cyclohexane ring of LL-Z1272δ slightly increased the binding affinity to cytochrome bd (Table 1). The 50% inhibitory concentrations (IC₅₀) for LL-Z1272δ 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 ε (chlorinated derivatives) (Table 1). The IC₅₀ 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.

Fig. 1. Structures of antibiotics LL-Z1272 and related natural compounds.
data was analyzed by using the equation respectively, for 2.3. Dose

**Table 1**
Summary on IC₅₀ values of quinol oxidase inhibitors for the *E. coli* cytochrome bd and bo and *T. vivax* AOX

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cytochrome bd[^a^]</th>
<th>Cytochrome bo[^a^]</th>
<th>trypanosome AOX[^a^]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-Z172β[^b^]</td>
<td>2.1±0.1[^c^]</td>
<td>1.2±0.1</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>LL-Z172γ[^b^]</td>
<td>81±1.7</td>
<td>0.082±0.016</td>
<td>0.015±0.001</td>
</tr>
<tr>
<td>LL-Z172b[^c^]</td>
<td>3.2±4</td>
<td>0.28±0.02</td>
<td>0.046±0.004</td>
</tr>
<tr>
<td>LL-Z172c[^c^]</td>
<td>11.0±0.1</td>
<td>7.2±0.7</td>
<td>0.65±0.09</td>
</tr>
<tr>
<td>LL-Z172[^c^]</td>
<td>85±7</td>
<td>0.37±0.02</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>Ascofuranone[^c^]</td>
<td>47±10</td>
<td>0.002±0.003</td>
<td>0.0049±0.0002</td>
</tr>
<tr>
<td>Aurachin C 1–10</td>
<td>0.0003±0.0003</td>
<td>0.002±0.0001</td>
<td>0.0049±0.0002</td>
</tr>
</tbody>
</table>

[^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-Z172β and ε

Effects of LL-Z172β and ε on the Q₁H₂ 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 Kᵢ and Vₘₐₓ values for the control were determined to 50 μM and 2364 Q₁H₂/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-Z172β acts as a noncompetitive inhibitor with Kᵢ=7.6±2.5 μM while LL-Z172ε serves as a competitive inhibitor with Kᵢ=1.00±0.03 μM (Fig. 2).

3.3. Dose–response analysis of inhibition of cytochrome bo by antibiotics LL-Z172

In contrast to bd-type oxidase, the Q₁H₂ oxidase activity of the *E. coli* cytochrome bo was more sensitive to chlorinated derivatives, LL-Z172γ, ε and ζ. IC₅₀ values for LL-Z172β, γ, δ, ε and ζ (averages from two preparations) were determined to 1.2, 0.082, 0.72, and 0.37 μM, respectively (Table 1). The IC₅₀ 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 mM 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-Z172γ is a very potent inhibitor of cytochrome bo.

3.4. Kinetic analysis of inhibition of cytochrome bo by antibiotics LL-Z172

Effects of LL-Z172β, γ, δ, and ζ on the Q₁H₂ 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 Kᵢ and Vₘₐₓ values were shifted to 23 μM and 1035 Q₁H₂/enzyme/s, respectively (Fig. 3), from 50 μM and 515 Q₁H₂/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-Z172

Because of the structural similarity of antibiotics LL-Z172 with trypanocidal AF (Fig. 1), we examined the effects of antibiotics LL-Z172 on Q₁H₂ oxidase activity of *T. vivax* AOX. From dose–response analysis with the AOX-overproduced *E. coli* membranes, we determined IC₅₀ values for LL-Z172β, γ, δ, ε, AF and aurachin C 1–10 to be 180, 15, 460, 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 (IC₅₀=8.3 and 2.3 mM 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-Z172

Effects of LL-Z172β, γ, δ, ε and ζ and AF on enzyme kinetics by *T. vivax* AOX were examined in the presence of detergents. Q₁H₂ oxidation by *T. vivax* AOX followed the Michaelis–Menten kinetics

![Fig. 2](image-url)  
**Fig. 2.** Effects of antibiotics LL-Z172 on kinetic parameters for Q₁H₂ 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-Z172β or 2 (▲) or 5 (▼) μM LL-Z172c. Control data was analyzed by using the equation v=Vₘₐₓ(1+Kᵢ/S), where Kᵢ indicates the constant for substrate inhibition. Data obtained in the presence of inhibitors were analyzed based on the Michaelis–Menten kinetics. The apparent Kᵢ (μM) and Vₘₐₓ (Q₁H₂/enzyme/s) values obtained were 50±4 and 2364±194, respectively, for the control (Kᵢ=381 μM), 79±5 and 1232±33, respectively, for 2 μM LL-Z172β, 100±5 and 826±21, respectively, for 5 μM LL-Z172β, 140±3 and 1065±12, respectively, for 2 μM LL-Z172c, 287±41 and 1113±107 Q₁H₂/enzyme/s, respectively, for 5 μM LL-Z172c, respectively.

![Fig. 3](image-url)  
**Fig. 3.** Effects of antibiotics LL-Z172 on kinetic parameters for Q₁H₂ 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-Z172β (▲), 0.2 μM LL-Z172γ (●), 0.75 μM LL-Z172b (▲), and ζ (▼). Data were analyzed based on the Michaelis–Menten kinetics. The apparent Kᵢ and Vₘₐₓ values obtained were 23±2 and 1035±28 (control), 43±4 and 841±30 (0.75 μM LL-Z172β), 64±2 and 402±4 (0.2 μM LL-Z172γ), 66±3 and 361±6 (0.75 μM LL-Z172β), 46±4 μM and 486±14 Q₁H₂/enzyme/s (0.75 μM LL-Z172c), respectively. R values were >0.997.
and apparent $K_m$ and $V_{max}$ values were determined to be 232 $\mu$M and 20 $\mu$M/protein (Fig. 4). The $K_m$ value in 0.1% sucrose monolaurate was comparable to 350 $\mu$M for T. b. brucei AOX in 0.25% n-octyl-β-D-glucopyranoside plus 0.025% EDT-20 [34], but smaller than approximately 700 $\mu$M determined for T. b. brucei AOX in the absence of detergents [13,29]. Since the $K_m$ value of T. vivax AOX for ubiquinol-2 was 116 $\mu$M (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-Z1272 (50 nM) was a non-competitive inhibitor (Fig. 4). LL-Z1272 acted as (apparently) non-competitive inhibitors (Fig. 4), as $K_m$ values for non-competitive inhibition by LL-Z1272 were 0.032 and 25.5 $\mu$M and 0.483 and 9.61 $\mu$M, respectively.

4. Discussion

From the screening of natural antibiotics of the Kitasato Institute for Life Sciences Chemical Library, we identified prenylphenols LL-Z1272, which inhibit and discriminate bacterial and trypanosomal ubiquinol oxidases (Table 1). LL-Z1272 inhibited bacterial and e (dechlorinated derivatives) inhibited cytochrome bd-type oxidase while LL-Z1272 inhibited cytochrome bo-type oxidase and trypanosome AOX. Aurachin C is a potent inhibitor of 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-Z1272 on Clostridium perfringens (minimum inhibitory concentration of 25 $\mu$g/ml), antibiotics LL-Z1272 were ineffective against S. aureus, Pseudomonas aeruginosa, Mycobacterium smegmatis, and Bacteroides fragilis. Neither LL-Z1272 nor LL-Z1272 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 phenyl ring of prenylphenol molecules within the binding pocket will determine interactions of prenyl tail and/or the cyclohexane 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 of 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 suramin, 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-Z1272 [39]. LL-Z1272 and LL-Z1272 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.

Acknowledgements

We thank Dr. M. Yamamoto (aRigen Pharmaceuticals, Inc., Tokyo) for AF, Dr. S. Yoshida (Institute of Physical and Chemical Research, Saitama) for piperidine A, Dr. Rokuro Masuma (Kitasato Institute for Life Sciences) for measurements of antibacterial activities of antibiotics LL-Z1272, Dr. K. Matsushita (Yamaguchi University) for his advice on enzyme assay and Dr. R. B. Gennis (University of Illinois) for the plasmid pNG2 and the E. coli strain G0103. This study was supported by a Grant-in-Aid for Scientific Research (20570124 to TM), Scientific Research on Priority Areas (18073004 to KK) and Creative Scientific Research (18GS0314 to KK) from the Japanese Ministry of Education, Science, Culture, Sports, and Technology.

References

T. Mogi et al. / Biochimica et Biophysica Acta 1787 (2009) 129–133

Glossary

AOX: alternative quinol oxidase
HQNO: 2-heptyl-4-hydroxyquinoline N-oxide
IC50: the 50% inhibitory concentration
Q1H2: a reduced form of Q1, ubiquinol-1