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Novel fungicide quinofumelin shows selectivity for fungal dihydroorotate dehydrogenase over the corresponding human enzyme

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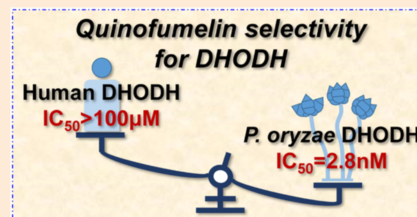
Supplementary material

The species selectivity of class 2 dihydroorotate dehydrogenase (DHODH), a target enzyme for quinofumelin, was examined. The *Homo sapiens* DHODH (HsDHODH) assay system was developed to compare the selectivity of quinofumelin for fungi with that for mammals. The IC_{50} values of quinofumelin for *Pyricularia oryzae* DHODH (PoDHODH) and HsDHODH were 2.8 nM and >100 μM, respectively. Quinofumelin was highly selective for fungal over human DHODH. Additionally, we constructed recombinant *P. oryzae* mutants where *PoDHODH* (*PoPYR4*) or *HsDHODH* was inserted into the *PoPYR4* disruption mutant. At quinofumelin concentration of 0.01–1 ppm, the *PoPYR4* insertion mutants could not grow, but the *HsDHODH* gene-insertion mutants thrived. This indicates that HsDHODH is a substitute for PoDHODH, and quinofumelin could not inhibit HsDHODH as in the HsDHODH enzyme assay. Comparing the amino acid sequences of human and fungal DHODHs indicates that the significant difference at the ubiquinone-binding site contributes to the species selectivity of quinofumelin.



Keywords: species selectivity, dihydroorotate dehydrogenase, quinofumelin, fungicide.

Introduction

Fungicides play a vital role in plant disease management. They help improve crop yield and quality. However, the emergence of resistant fungal strains has threatened the effectiveness of fungicides.[1)] We await new compounds with novel modes of action or with novel target sites to manage the resistance to the existing fungicides and provide more effective options for managing plant diseases.

Quinofumelin, 3-(4,4-difluoro-3,3-dimethyl-3,4-dihydroisoquinolin-1-yl) quinoline, (Fig. 1A) developed by Mitsui Chemicals Agro, Inc. showed potent fungicidal activity against a broad range of ascomycete fungi like *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) and *Botrytis cinerea*. In our previous study, the target site of quinofumelin was shown to be class 2 dihydroorotate dehydrogenase (DHODH).[2)] Thus, quinofumelin with a novel mode of action is expected to contribute more effective options for managing plant diseases.

DHODH, which catalyzes the reaction from L-dihydroorotate to orotate, is an essential step in the *de novo* pathway of the pyrimidine biosynthesis of various eukaryotes.[3,4)] DHODH has been actively studied as a drug discovery target.[4–6)] The inhibitors of *Plasmodium falciparum* DHODH[7)] have also been investigated as antimalarial drugs, and selective inhibitors of *P. falciparum* DHODH have been successfully discovered.[8)] It is also crucial for agrochemical usage to have high selectivity for target species over mammals. Therefore, in this study, we investigated the inhibition selectivity of quinofumelin between *P. oryzae* DHODH and *H. sapience* DHODH using *in vitro* DHODH assays and recovery tests of the recombinant *DHODH* gene-inserted *P. oryzae*.

Materials and methods

1. *Chemicals, culture media, and strain of* P. oryzae

Quinofumelin (Fig. 1A) used in this study was synthesized at

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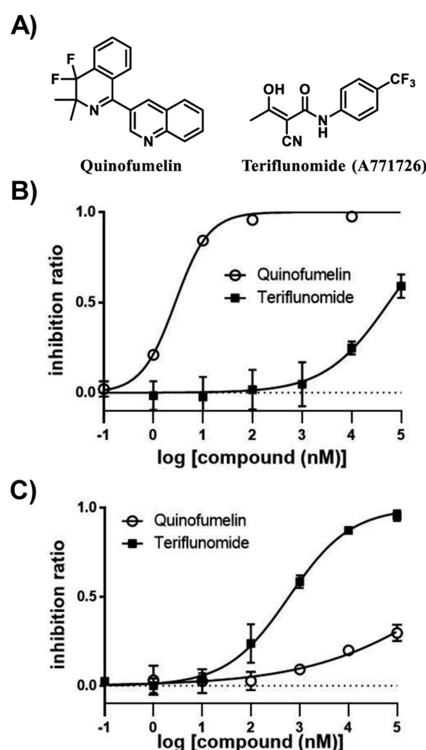

Fig. 1. Structure of quinofumelin and teriflunomide (A). Effects of quinofumelin and teriflunomide on recombinant PoDHODH (B) and ΔN-HsDHODH (C). The inhibition ratios of PoDHODH and ΔN-HsDHODH are presented by open circles (quinofumelin) and solid squares (teriflunomide), respectively. Average and standard deviations are calculated from three independent experiments conducted in quadruplicate.

Agrochemicals Research Center, Mitsui Chemicals Agro, Inc. (Chiba, Japan). Teriflunomide (A77-1726) (Fig. 1A) was purchased from Cosmo Bio Corporation (Tokyo, Japan). Other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA: 4-g potato starch from infusion, 20-g dextrose, and 15-g agar per liter formula as natural medium agar), Czapek solution agar (CZA: 30-g saccharose, 2-g sodium nitrate, 1-g dipotassium phosphate, 0.5-g magnesium sulfate, 0.5-g potassium chloride, 0.01-g ferrous sulfate, and 15-g agar as per liter formula as minimal medium agar), yeast extract, and Luria-Bertani (Miller) Broth (LB) were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Restriction enzymes and plasmid vectors were purchased from TaKaRa (Shiga, Japan) and TOYOBO (Osaka, Japan), respectively. *P. oryzae* Ina-86-137 (MAFF 151011) and fungal disruption plasmids pETHG and pCAMBIA-Bar-RfA were obtained from Dr. Yoko Nishizawa of the Genetically Modified Organism Research Center, National Institute of Agrobiological Sciences.

2. DHODH assay

Preparation of histidine-tagged recombinant *P. oryzae* DHODH (PoDHODH) and N-terminal truncated *H. sapience* DHODH (ΔN-HsDHODH) protein, that the N-terminal 1-28 amino acids truncated from full length HsDHODH takes a role of import and proper location and fixation of the enzyme in the inner mitochondrial membrane,[9] is described in the supplementary material. The DHODH activity was measured using PoDHODH and ΔN-HsDHODH proteins following the previously described protocol.[2,8,10] The oxidation of the substrate dihydroorotate with the quinone co-substrate was coupled to reduce the chromogen 2,6-dichloroindophenol (DCIP). One hundred microliters of reaction mixture containing 50-mM Tris-HCl (pH 8.0), 150-mM NaCl, 0.1% (w/v) TritonX-100, 200-μM DCIP, 2-mM dihydroorotate, 100-μM decylubiquinone (QD), approximately 10-μg/mL recombinant PoDHODH or ΔN-HsDHODH protein suspension, and various concentrations of test compounds dissolved in 1% DMSO (or no compound control) were incubated at 30°C for 20–30 min. After incubation, 10-μL 10% sodium dodecyl sulfate was added to each sample and mixed well to stop the reaction. Then, absorbance at 595-nm was measured. The inhibitory rate was calculated as (1-T/C), where C and T represent the decreased absorbance quantity at 595-nm with the control and test samples, respectively. The IC_{50} (half-inhibition concentration) values for the test compounds on PoDHODH and ΔN-HsDHODH were determined using a four-parameter logistic curve-fitting program (GraphPad Prism 6.00), in which two parameters were constrained (*i.e.*, the top and bottom were fixed as 1 and 0).

3. Recovery test

The construction of the *PoPYR4* or *ΔN-HsDHODH* gene-insertion of *P. oryzae* into *the PYR4* gene-disruption mutant is described in the supplementary materials (Fig. S1). *P. oryzae* cv. Ina-86-137, its *PYR4* disruption-mutant *ΔPopyr4* (PoDKO), *PoPYR4* gene-insertion mutant (PoD#1 to PoD#3), and *ΔN-HsDHODH* gene-insertion mutant (HsD#1 to HsD#3) were preincubated on a natural medium agar plate (PDA plate) to form a mycelial colony. The resultant mycelial colony disk (4 mm in diameter) was removed and inoculated onto a fresh minimal medium agar plate (CZA plate) containing quinofumelin, ranging from 0.01–1 ppm. The plates were subsequently incubated at 25°C for 9 days, after which we visually observed the growth of the mycelial colony.

4. Sequence alignment and identification of amino acid residues at binding sites

The HsDHODH amino acid sequence was aligned with those of *B. cinerea* and *P. oryzae* DHODHs using the alignment sequence module in the Discovery Studio 2020 (BIOVIA, Dassault Systèmes) software package. Two X-ray crystal structures of the N-terminally truncated human DHODH, 3U2O,[11] and 6FMD[12] were used to assign α-helical and β-sheet regions as well as the binding sites of ubiquinone, flavin mononucleotide (FMN) and orotate. After aligning the sequences with default settings, gaps in the α-helical and β-sheet regions were manually removed. Then, the sequences were aligned again with the condition

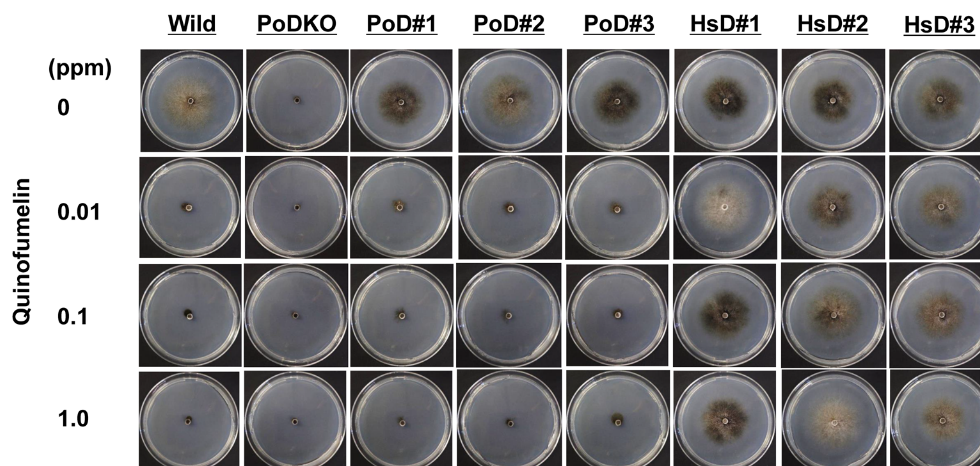

Fig. 2. Mycelial growth test of recombinant *P. oryzae* on a minimal medium agar plate. Mycelial growth was tested by adding quinofumelin in the concentration range of 0–1 ppm. Clone abbreviations: Wild, Ina-86-137; PoDKO, *P.oryzae PYR4* gene disruption mutant (*ΔPopyr4*); PoD#1 to#3, *P.oryzae PYR4* gene insertion mutants into *ΔPopyr4*; HsD#1 to#3, *H.sapience* ΔN-HsDHODH gene insertion mutants into *ΔPopyr4*.

that the alignment of α-helical and β-sheet regions was fixed. The results of amino acid sequence alignment of DHODHs are shown in the supplementary materials (Fig. S2). Amino acids, within 5-Å from FMN or orotate and directly in contact with them in the crystal structures of 3U2O or 6FMD, were defined as FMN and orotate binding site residues. X-ray crystal structures of DHODH in complex with inhibitors, including 3U2O or 6FMD, indicate that the inhibitors target the ubiquinone binding site.[13,14] Thus, amino acids, within 5-Å from the inhibitors in 3U2O or 6FMD and are in direct contact with them, were defined as ubiquinone binding site residues.

Results and discussion

DHODH is present in many species. Thus, fungal/mammalian selectivity at target sites is important for fungicides to reduce toxicity risk. The inhibitory activity of quinofumelin and teriflunomide, the active metabolites of the immunosuppressive drug leflunomide were measured using PoDHODH and ΔN-HsDHODH enzymes to examine species-selectivity. Quinofumelin strongly inhibited the enzymatic activity of PoDHODH, with an IC_{50} value of 2.80-nM. Teriflunomide inhibits the enzymatic activity of PoDHODH with an IC_{50} value of 56.4-μM (Fig. 1B), and teriflunomide also inhibits the enzymatic activity of ΔN-HsDHODH with an IC_{50} value of 600-nM, whereas quinofumelin poorly inhibits the enzymatic activity of ΔN-HsDHODH (Fig. 1C). The IC_{50} value of quinofumelin for ΔN-HsDHODH was over 100-μM (IC_{50}>100-μM). Quinofumelin shows about 10^5-fold greater selectivity for fungal DHODH than for human DHODH, whereas teriflunomide shows 94-fold selectivity for human DHODH over that of fungal DHODH.

Next, to examine the *in vivo* selectivity of quinofumelin, we constructed recombinant *P. oryzae* mutants, in which either *ΔN-HsDHODH* gene or *PoPYR4* gene was randomly inserted into the *PoPYR4* gene-disruption mutant. The mycelial growth of these mutants on a minimal medium agar plate (CZA plate) was examined. The mycelia of *P. oryzae* Ina-86-137 (wild type) and the *PoPYR4* gene-disruption mutant PoDKO could not grow on the CZA plate when quinofumelin was added at a concentration of 0.01–1 ppm (Fig. 2). The mycelia of *PoPYR4* gene random insertion mutants (PoD#1–PoD#3) grew on the CZA plate without quinofumelin, but could not grow with 0.01-ppm quinofumelin (Fig. 2). The recovery of mycelial growth of PoD#1–PoD#3 indicated that the randomly inserted *PoDHODH* gene functioned properly. The mycelia of *ΔN-HsDHODH* gene-insertion mutants (HsD#1–HsD#3) grew on the CZA plate, and quinofumelin could not inhibit their mycelial growth even at 1-ppm (Fig. 2). It was found that the enzyme function of the *PoDHODH* gene was complemented by the *ΔN-HsDHODH* gene, which is consistent with the result of the mycelial growth examination using *ΔN-HsDHODH* gene-inserted *U. maydis*.[15] Quinofumelin up to 1-ppm could not inhibit the mycelial growth of the ΔN-HsDHODH gene-insertion mutant. This result indicated that quinofumelin, which failed to inhibit the enzymatic activity of ΔN-HsDHODH up to 100-μM, could not inhibit ΔN-HsDHODH in *P. oryzae* enough to inhibit the mycelial growth. Quinofumelin shows the *in vivo* selectivity for fungal DHODH than for human DHODH.

To investigate the fungal/mammalian selectivity at target sites of DHODH, the similarity of DHODH amino acid sequences was compared among HsDHODH, PoDHODH, and *B. ceneria* DHODH (BcDHODH). The amino acid residues of FMN and orotate binding site and the ubiquinone binding site are shown in Fig. S2. The FMN and orotate binding site residues of HsDHODH exhibited 75.9% and 72.4% identity with those of PoDHODH and BcDHODH, respectively, whereas the ubiquinone binding site residues of HsDHODH exhibited only 29.6% and 33.3% identity with those of PoDHODH and BcDHODH, respectively (Fig. 3). PoDHODH exhibited high amino acid sequence identity with BcDHODH at both the FMN and orotate site and the ubiquinone site. The amino acid sequence compari-

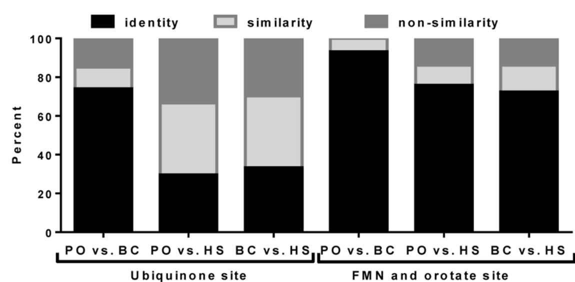

Fig. 3. Multiple alignment of the amino acid sequences among human and fungal DHODHs. The amino acid sequences were obtained from the NCBI protein database or PDB: BC (*B. cinerea*; XP_024553432.1), PO (*P. oryzae*; XP_003719157.1), and HS (*Homo sapiens*; 3U2O_A). Sequence similarity is defined by the following amino acid groupings in one-letter code: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW.

son revealed that the FMN and orotate binding sites were conserved between human and fungal DHODHs, whereas the ubiquinone binding sites were poorly conserved between human and fungal DHODHs. The analysis of X-ray crystal structures revealed that the DHODH inhibitors with various species targeted the ubiquinone binding site.[13,14] Based on these X-ray crystal structure analyses, the binding site of quinofumelin also seems to be the ubiquinone site. The difference in ubiquinone binding site residues between HsDHODH and PoDHODH would give quinofumelin a fungal DHODH selectivity. Therefore, further X-ray crystal structure analyses of the DHODHs with quinofumelin are needed to elucidate the selectivity between HsDHODH and PoDHODH. Additionally, quinofumelin seems to be a promising fungicide because of its low toxicity risk at the target site. The similarity of whole amino acid sequences of fungal DHODH was compared with other species (mammals, birds, plants, insects, protozoa, and bacteria). According to phylogenetic analysis using fast-tree (Fig. S3), the fungal DHOHDs (*B. cinerea* and *P. oryzae*) differed most from the mammalian ones. The comparison of whole amino acid sequences also indicated that DHODH is expected to show selectivity between fungi and mammals. The high species-selectivity indicates the risk of development of resistant strains carrying mutations that maintain enzymatic activity and reduce the inhibitory activity of quinofumelin. Thus, effective resistance management is critical for preserving the long-term utility of quinofumelin.

In this study, quinofumelin showed a high selectivity for fungal DHODH over human DHODH. Quinofumelin failed to inhibit the mycelial growth of the *ΔN-HsDHODH* gene insertion mutants, which further confirmed the strong fungal selectivity of quinofumelin. The difference in ubiquinone binding sites between fungal and human DHODHs seems to cause the strong species-selectivity. Highly fungal selective quinofumelin with a novel mode of action is expected to provide more effective options for managing plant diseases.

Acknowledgements

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Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Table S1, and Supplemental Figs. S1–S3), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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