



The Repurposed Drug Disulfiram Inhibits Urease and Aldehyde Dehydrogenase and Prevents *In Vitro* Growth of the Oomycete *Pythium insidiosum*

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ABSTRACT *Pythium insidiosum* is an oomycete microorganism that causes a life-threatening infectious disease, called pythiosis, in humans and animals. The disease has been increasingly reported worldwide. Conventional antifungal drugs are ineffective against *P. insidiosum*. Treatment of pythiosis requires the extensive removal of infected tissue (i.e., eye and leg), but inadequate surgery and recurrent infection often occur. A more effective treatment is needed for pythiosis patients. Drug repurposing is a promising strategy for the identification of a U.S. Food and Drug Administration-approved drug for the control of *P. insidiosum*. Disulfiram has been approved to treat alcoholism, but it exhibits antimicrobial activity against various pathogens. In this study, we explored whether disulfiram possesses an anti-*P. insidiosum* activity. A total of 27 *P. insidiosum* strains, isolated from various hosts and geographic areas, were susceptible to disulfiram in a dose-dependent manner. The MIC range of disulfiram against *P. insidiosum* (8 to 32 mg/liter) was in line with that of other pathogens. Proteogenomic analysis indicated that several potential targets of disulfiram (i.e., aldehyde dehydrogenase and urease) were present in *P. insidiosum*. By homology modeling and molecular docking, disulfiram can bind the putative aldehyde dehydrogenase and urease of *P. insidiosum* at low energies (i.e., -6.1 and -4.0 Kcal/mol, respectively). Disulfiram diminished the biochemical activities of these enzymes. In conclusion, disulfiram can inhibit the growth of many pathogenic microorganisms, including *P. insidiosum*. The drug can bind and inactivate multiple proteins of *P. insidiosum*, which may contribute to its broad antimicrobial property. Drug repurposing of disulfiram could be a new treatment option for pythiosis.

KEYWORDS *Pythium insidiosum*, disulfiram, drug repurposing, pythiosis, susceptibility

Pythium insidiosum is an oomycete microorganism that infects humans and animals and causes a life-threatening disease called pythiosis (1–3). Affected humans and animals have been increasingly reported worldwide, especially in the tropical and subtropical countries (3). Several serological and molecular assays have been developed to assist a rapid and accurate diagnosis of pythiosis (4–11). The use of conventional antifungal drugs is usually ineffective against *P. insidiosum* because the organism lacks the drug target enzymes in the sterol biosynthetic pathway (2, 12). Immunother-

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apy (i.e., administration of the *P. insidiosum* protein extract) shows limited efficacy in the treatment of pythiosis (2, 13). It requires the extensive removal of an infected organ (i.e., eye and leg) to eradicate the infection. However, adequate surgical intervention is not possible in many pythiosis patients, leading to recurrent infection and increased morbidity and mortality (2, 14). There is an urgent need for a more effective treatment for the control of *P. insidiosum*.

The development of a novel drug requires significant effort and time. In addition, diseases that are prevalent in lower-income countries receive less attention for drug development. Drug repurposing (or repositioning) is a strategy to identify an existing drug designed for one disease and use it in the treatment of a different disease (15–17). Success stories of the repurposing strategy include drug discoveries for infectious diseases. For example, auranofin (approved indication: rheumatoid arthritis), loperamide (diarrhea), tamoxifen (breast cancer), and chlorcyclizine (allergy) are repurposed as a new treatment option for infections caused by bacteria, parasites, fungi, or viruses (15). Drug repurposing is therefore a promising strategy for rapidly identifying a potential, ready-to-use, and safe compound (i.e., U.S. Food and Drug Administration [FDA]-approved drugs) for the control of other infectious diseases, including pythiosis.

Disulfiram is an FDA-approved drug used in the treatment of chronic alcoholism for decades (18). Several reports have shown that disulfiram markedly inhibits various pathogens, including *Staphylococcus aureus* (19), *Pseudomonas aeruginosa* (20), *Giardia lamblia* (21), *Mycobacterium tuberculosis* (22), and nontuberculous mycobacteria (23). The antimicrobial property of disulfiram could result from the inactivation of target enzymes, such as aldehyde dehydrogenase (ALDH), urease (URE), carbamate kinase (CK), and dopamine beta-hydroxylase (DBH) (21, 24–26). Because disulfiram possesses the broad-spectrum antimicrobial property, it is an open question whether the drug can be repurposed for the control of other pathogens, including *P. insidiosum*. In the present study, we performed an *in vitro* susceptibility assay and showed that all 27 *P. insidiosum* strains tested were susceptible to disulfiram. In addition, we identified some putative target enzymes in the genome of *P. insidiosum* (27, 28) and initially assessed a potential inhibitory mechanism of disulfiram against the pathogen by homology modeling, molecular docking, and biochemical analyses.

RESULTS

Susceptibility of *P. insidiosum* to disulfiram. In this study, the *P. insidiosum* strains were isolated from various sources (i.e., humans, $n = 15$; animals, $n = 9$; and the environment, $n = 3$) and geographic origins (i.e., Asia, $n = 20$; Americas, $n = 6$; and Australia, $n = 1$) (Table 1). The ribosomal DNA (rDNA) sequence analysis assigned these strains into three genotypes: clade I ($n = 5$), clade II ($n = 18$), and clade III ($n = 4$) (29) (Table 1). The *in vitro* susceptibility assays (i.e., agar- and broth-based methods) showed that all 27 *P. insidiosum* strains were sensitive to disulfiram in a dose-dependent manner (Table 1; Fig. 1). The MICs of disulfiram against *P. insidiosum* varied, depending on the strains and susceptibility methods. The agar susceptibility method exhibited an MIC range of 16 to 128 mg/liter, an MIC₅₀ of 32 mg/liter, and an MIC₉₀ of 64 mg/liter. The broth susceptibility method showed an MIC range of 8 to 32 mg/liter, an MIC₅₀ of 16 mg/liter, and an MIC₉₀ of 32 mg/liter.

Identification of putative disulfiram targets in *P. insidiosum*. The known target proteins of disulfiram (i.e., ALDH, DBH, CK, and URE) served as the query sequences and were subjected to a BLAST search against the genome-derived proteome of *P. insidiosum* (14,962 proteins) (27, 28). The DBH and CK sequences failed to match any *P. insidiosum* proteins. The URE sequence matched one protein, PINS00920015C (E value, 0.0; identity, 62%; similarity, 75%) (Table 2). The ALDH sequence matched 11 different proteins (Table 2), and among them PINS02440013A showed the best BLAST scores (E value, 3E–120; identity, 43%; similarity, 60%). All 12 matched proteins showed significant BLAST hits against the *P. insidiosum* transcriptome. Five proteins (i.e., PINS00920015C, PINS00560032A, PINS00450059A, PINS00850003A, and PINS00030096) can be mapped with 13 to 40 peptides of the *P. insidiosum* mascot proteome (Table 2).

TABLE 1 Twenty-seven strains of *Pythium insidiosum* used in the *in vitro* susceptibility assays^a

Strain ID	Reference ID	Host (tissue)	Country of origin	Clade	Agar MIC (mg/liter)	Broth MIC (mg/liter)
Pi01	CBS 578.85	Equine	Costa Rica	I	32	16
Pi02	CBS 579.85	Equine	Costa Rica	I	32	16
Pi08	CBS 580.85	Equine	Costa Rica	I	32	32
Pi09	CBS 101555	Equine	Brazil	I	32	32
Pi10	ATCC 200269	Human (skin)	USA	I	32	16
Pi12	SIMI 149-41	Human (artery)	Thailand	II	32	16
Pi23	MCC 10	Human (gut)	Thailand	II	32	32
Pi25	P19	Human (eye)	Thailand	II	32	16
Pi29	P27	Human (artery)	Thailand	II	32	16
Pi36	ATCC 64221	Equine	Australia	II	32	16
Pi42	CR02	Environment	Thailand	II	32	16
Pi43	LP04	Environment	Thailand	II	64	32
Pi20	MCC 18	Human (eye)	Thailand	II	32	8
Pi35	Pi-S	Human (artery)	Thailand	II	128	8
Pi33	P36	Human (artery)	Thailand	II	32	32
Pi34	P37	Human	Thailand	II	32	32
Pi52	P38	Human (artery)	Thailand	II	64	32
Pi53	P39	Equine (nose)	Thailand	II	64	16
Pi54	P40	Human (artery)	Thailand	II	32	32
Pi40	CBS 777.84	Mosquito larva	India	II	64	16
Pi38	CBS 101039	Human (eye)	India	II	32	16
Pi39	CBS 702.83	Equine	Japan	II	32	8
Pi37	ATCC 28251	Equine	Papua New Guinea	II	64	16
Pi51	NAN06	Environment	Thailand	III	32	16
Pi47	SIMI 2921-45	Human (eye)	Thailand	III	16	16
Pi49	SIMI 7695-48	Human (artery)	Thailand	III	32	16
Pi50	ATCC 90586	Human (skin)	USA	III	32	16

^aThe strain/reference identification numbers (ID), the hosts (tissues) and countries where the organisms were isolated, the rDNA-based genotypes (i.e., clades I, II, and III), and the disulfiram MICs determined by agar and broth susceptibility methods are indicated.

Homology modeling of the target proteins and molecular docking of disulfiram. The best matched *P. insidiosum* proteins for URE (PINS00920015C) and ALDH (PINS02440013A) were subjected to homology modeling, using the Jack bean URE (PDB ID 3LA4) and the *Pseudomonas* γ -hydroxymuconic semialdehyde dehydrogenase (PDB ID 4GO4) as the prototypic templates, respectively. Disulfiram can be docked into the binding pockets of the *P. insidiosum* URE (amino acid residues: ALA432, HIS511, HIS537, CYS584, HIS585, ARG601, ALA628, and MET629), with a binding affinity of -4.0 Kcal/mol, and ALDH (amino acid residues: ARG101, VAL109, TYR159, TRP166, TYR283, VAL288, and TRP458) with a binding affinity of -6.1 Kcal/mol (Fig. 2). The most poses

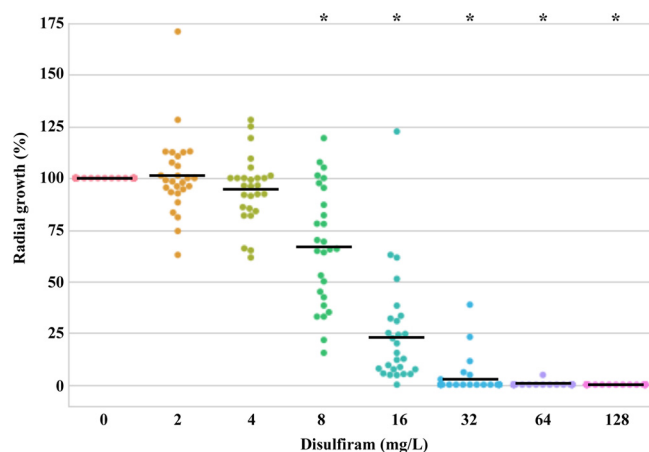


FIG 1 Agar-based susceptibility of *P. insidiosum* against disulfiram. The radial growths (%) of the organism (27 strains) after exposure to various concentrations of disulfiram (range, 0 to 128 mg/liter) are plotted. A straight line indicates mean growth. An asterisk represents significant growth reduction in relation to the no-drug control.

TABLE 2 Putative target proteins of disulfiram identified in the genome-derived proteome of *Pythium insidiosum*^a

Target proteins of disulfiram	Accession no. of query protein sequence	BLASTP against <i>P. insidiosum</i> proteome			TBLASTN against transcriptome (E value)	No. of mapped peptide(s) in mascot library
		Matched protein ID	E value	Accession		
Dopamine beta-hydroxylase (DBH)	P09172	No match				
Carbamate kinase (CK)	4OLC	No match				
Urease (URE)	4H9M	PINS00920015C	0.00	GAX98700.1	0.00	40
Aldehyde dehydrogenase (ALDH2)	P05091	PINS02440013A	3E-120	GAY03418.1	0.00	No match
		PINS00560032A	4E-66	GAX96905.1	0.00	13
		PINS00240041A	7E-66	GAX94676.1	5E-179	No match
		PINS00460028A	7E-61	GAX96345.1	0.00	No match
		PINS00450059A	2E-51	GAX96237.1	0.00	21
		PINS00090050A	1E-43	GAX93307.1	0.00	No match
		PINS00450060A	4E-43	GAX96236.1	0.00	No match
		PINS00530007C	6E-30	GAX96738.1	0.00	No match
		PINS01590003A	1E-22	GAY01314.1	0.00	No match
		PINS00850003A	5E-22	GAX98398.1	0.00	14
		PINS00030096A	5E-09	GAX92574.1	0.00	18

^aThe query protein sequences are deposited in the PDB (i.e., CK and URE) or UniProt (i.e., DBH and ALDH2) database. The transcriptome and in-house mascot proteome of *P. insidiosum* were used to validate the matched proteins.

of docking could dock into the same binding pockets. The disulfiram-derived metabolites diethyldithiocarbamic acid (DDC), diethylamine (DEA), and diethyldithiomethylcarbamate (Me-DDC) can also be docked into the binding pocket of the *P. insidiosum* URE with the binding affinities of -3.4 , -2.8 , and -3.7 Kcal/mol, respectively (see Fig. S1 to S3 in the supplemental material). Likewise, these metabolites can be docked into the binding pocket of the *P. insidiosum* ALDH with binding affinities of -3.4 , -2.8 , and -3.6

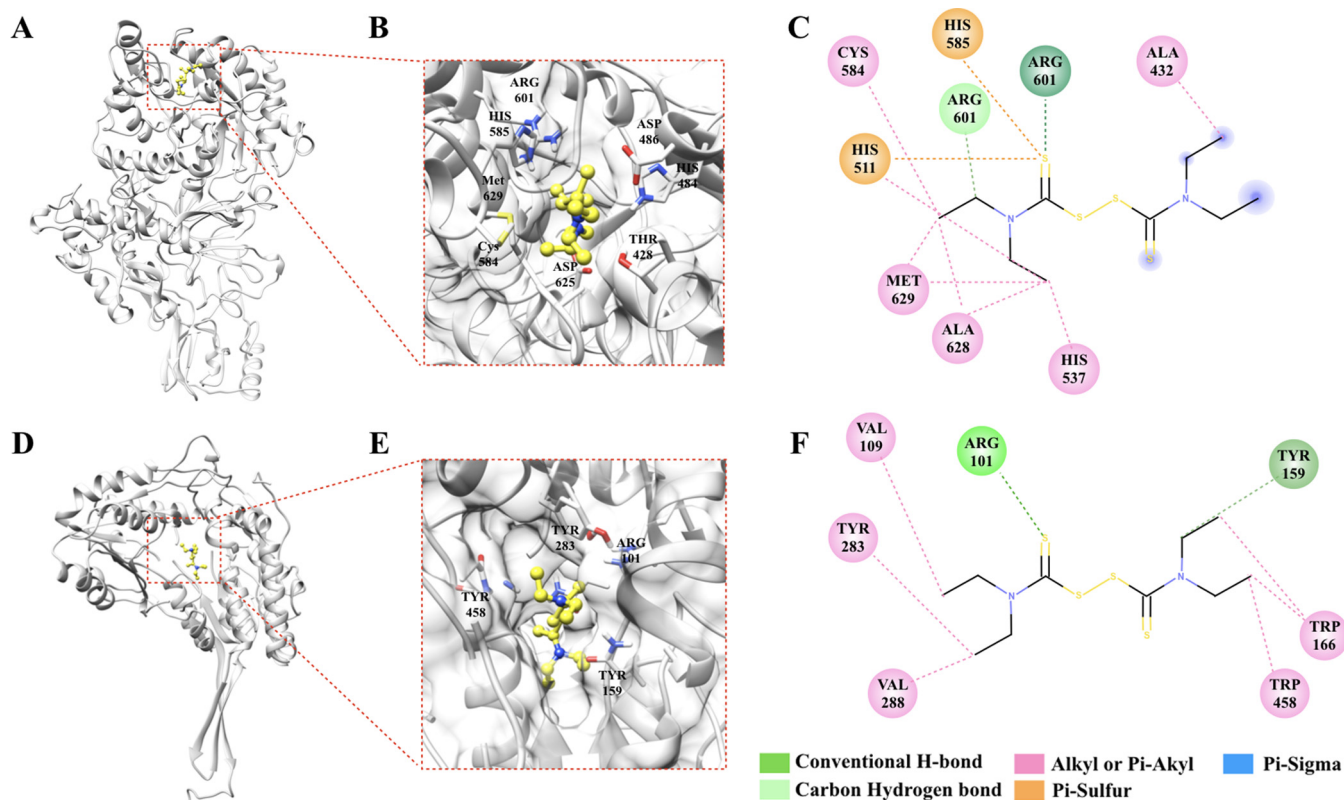


FIG 2 Complexes of the ligand “disulfiram” and the predicted 3D structures of the urease (A to C) and aldehyde dehydrogenase (D to F) of *P. insidiosum*. Panels A and D show ribbon structure models, while panels B and E depict enlarged structure showing molecular docking of disulfiram. Panels C and F show the 2D interactions of disulfiram and the predicted binding sites of urease and aldehyde dehydrogenase, respectively. The interacting amino acids and types are shown in figures B, C, E, and F. The structures and complex interaction images were generated using the UCSF Chimera and Discovery Studio Visualizer (see Materials and Methods).

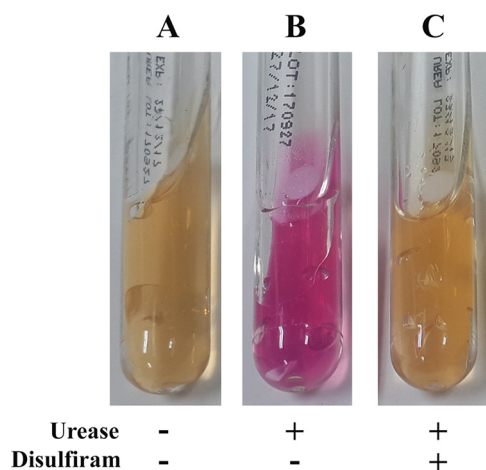


FIG 3 Biochemical effect of disulfiram on urease activity. The urease test tube assays were tested against DMSO (a diluent of disulfiram; this served as a control) (A), Jack bean urease alone (0.1 mg/ml) (B), and a preincubated mixture of Jack bean urease (0.1 mg/ml) and disulfiram (128 mg/liter) (C).

Kcal/mol, respectively (see Fig. S1 to S3 in the supplemental material). Drug-target interactions (i.e., conventional H-bond, carbon hydrogen bond, alkyl or Pi-alkyl bond, Pi-sulfur bond, Pi-Sigma bond, and Vander Waals bond) of the tested chemicals and the specific amino acid residues of the *P. insidiosum* URE and ALDH are shown in Fig. 2 (disulfiram), as well as Fig. S1 (DDC), S2 (DEA), and S3 (Me-DDC) in the supplemental material.

Disulfiram inhibits the URE and ALDH activities. To demonstrate whether disulfiram inhibits the activity of URE, the mixtures of the Jack bean URE (concentration, 0.1 mg/ml) and the disulfiram (concentrations, 0 or 128 mg/liter) were assessed for biochemical activity, using the URE test tube assay. The assay was read positive in the absence of disulfiram (the agar color turned pink; Fig. 3B) but read negative in the presence of the drug (the agar color remained yellow; Fig. 3C). Likewise, to determine whether disulfiram can inactivate ALDH, the mixtures of the recombinant human ALDH2 (concentration, 0.1 mg/ml) and the disulfiram (concentrations, 0 or 128 mg/liter) were tested for the ALDH activity using a colorimetric assay. In the absence of disulfiram, the ALDH activity was 443.2 nmol of NADH/min/ml, whereas, in the presence of the drug, the ALDH activity was 6.8 nmol of NADH/min/ml.

DISCUSSION

No standardized drug susceptibility method has been established for *P. insidiosum*. However, some investigators perform the microdilution-based susceptibility testing of *P. insidiosum* by adapting the CLSI M38-A2 method and using the inocula prepared from zoospores (asexual stage of the organism) (30, 31). In our hands, production of the zoospores is time-consuming and usually provides inadequate yield for the subsequent drug susceptibility assays. Alternatively, Brown et al. (32) and Lerksuthirat et al. (12) use the inocula prepared from agar plugs with actively growing *P. insidiosum* hyphae and performed the drug susceptibility assay by measuring the radial growth of the organism on a drug-containing agar plate. Their agar-based susceptibility method is easy, robust, and reproducible. For these reasons, we adopted the agar-based method for *in vitro* susceptibility testing of *P. insidiosum* against disulfiram. We also performed a broth-based susceptibility assay, which is a modified version of the agar-based susceptibility method that uses drug-containing broth rather than agar.

Pythiosis has high rates of morbidity and mortality. Most conventional antifungal drugs show unfavorable response against *P. insidiosum*. Drug repurposing (15, 16) could be a strategy to identify a potential FDA-approved drug for the control of *P. insidiosum*. Disulfiram (originally approved to treat alcoholism) exhibits antimicrobial activity against various pathogens (18–23). We explored whether disulfiram possesses

any anti-*P. insidiosum* effect. As shown here, disulfiram inhibited the growths of all 27 *P. insidiosum* strains isolated from various hosts and geographic areas, in a dose-dependent manner (Table 1 and Fig. 1). The MIC range (8 to 32 mg/liter), the MIC₅₀ (16 mg/liter), and the MIC₉₀ (32 mg/liter) of the broth-based susceptibility method were lower than the MIC range (16 to 128 mg/liter), the MIC₅₀ (32 mg/liter), and the MIC₉₀ (64 mg/liter) of the agar-based susceptibility method (Table 1). In the broth-based method, *P. insidiosum* was completely immersed in disulfiram-containing liquid, whereas, in the agar-based method, the organism exposed the drug only on one side of the colony. The extent of drug exposure could explain the relatively lower MIC values of the broth-based method. Compared to other microorganisms, the MIC range of disulfiram against *P. insidiosum* (8 to 32 mg/liter [broth-based method]) was in line with that of the antibacterial drug-resistant *S. aureus* (8 to 32 mg/liter) (19) and the nontuberculous mycobacteria (32 mg/liter) (23) but markedly higher than that of *M. tuberculosis* (1.56 mg/liter) (22). Thus, disulfiram has an extended antimicrobial spectrum that covers the oomycete microorganisms, including *P. insidiosum*.

In an attempt of measuring disulfiram in blood, Brown et al. reported that 12 h after a single 200-mg dose, the blood level of the drug was approximately 4.5 mg/liter (33). Moreover, after oral administration of the daily dose of 200 mg for 14 days, the blood level of disulfiram increased to 8.3 mg/liter (34, 35). Since the recommended daily dose of disulfiram could be optimized to 500 mg (36), it is possible that the blood concentration of the drug can reach a level that potentially inhibits the susceptible organisms (i.e., *P. insidiosum*) that have an MIC₅₀ of >8 mg/liter. Regarding toxicity, disulfiram has a 50% lethal dose (LD₅₀) of 8.6 g/kg (orally in rats [<https://www.drugbank.ca/>]), and thus it is a practically nontoxic drug, based on the Hodge and Sterner criteria (<https://www.ccohs.ca/oshanswers/chemicals/ld50.html>). However, a future susceptibility experiment in an animal model of pythiosis should provide insightful information on *in vivo* anti-*P. insidiosum* effect and toxicity of disulfiram.

In other organisms, inactivation of the enzyme ALDH, URE, CK, or DBH contributes to the chemotherapeutic or antimicrobial activities of disulfiram (21, 24–26). We initially assessed these targets of disulfiram in *P. insidiosum*. The BLAST search only identified ALDH (11 copies) and URE (one copy), but not CK and DBH, in the genome-derived proteome of *P. insidiosum* (27, 28) (Table 2). Interestingly, ALDH and URE are considered a microbial virulence determinant or essential enzyme that can promote an infection (37–40), suggesting that these proteins are potential drug targets. By homology modeling and molecular docking, disulfiram can bind the putative ALDH and URE of *P. insidiosum* at the relatively low energies of –6.1 and –4.0 Kcal/mol, respectively (Fig. 2). In addition, sequence homology analysis revealed that the *P. insidiosum* URE contains “VCHHL” (amino acid residues 583 to 587), which is a conserved region found in all bacterial, plant, and fungal UREs and plays a crucial role in URE function (24). Disulfiram can form protein-ligand interactions at this conserved region of the *P. insidiosum* URE, covering the amino acid residues CYS584 and HIS585 (Fig. 2), which suggests a potential anti-URE mechanism of the drug in this organism.

The human gastrointestinal tract absorbs at least 80% of the ingested disulfiram into the blood circulation, where the drug can transform to DDC by the endogenous thiols and the glutathione reductase of red blood cells (41). The DDC then forms CS₂, DEA, Me-DDC, and other metabolites (41). Disulfiram and its metabolites circulate and distribute to various tissues, such as the liver, kidney, heart, lung, brain, testes, spleen, and intestine (42). Drug metabolism is a clinical concern for the repurposed use of disulfiram in cancer, as it may decrease the effective form and lead to an unfavorable response of the drug against cancer cells (43). Metabolism of disulfiram is also a concern for infectious diseases, since the formation of various metabolites might compromise the antimicrobial activities of the drug. To address this issue, Chen et al. proposed the use of disulfiram-loaded lipid emulsion, which minimizes degradation and improves the chemical stability of the drug in the blood (43). However, since the inhibitory property of the disulfiram-derived metabolites against *P. insidiosum* is concerned, we performed the molecular docking and demonstrated that DDC, DEA, and

Me-DDC are capable of binding *P. insidiosum* URE and ALDH at low energies of less than -2.8 Kcal/mol (Fig. S1 to S3). In agreement with the docking analysis, disulfiram inhibited the biochemical activities of both ALDH (represented by the recombinant human ALDH2) and URE (represented by Jack bean URE). Taken together, disulfiram and its metabolites can bind and inactivate multiple proteins of *P. insidiosum* (i.e., ALDH and URE), that may contribute to the broad-spectrum antimicrobial property of this FDA-approved chemical.

Not only does *P. insidiosum* cause systemic infection, but it also causes a serious local infection of the eye (2). Topical administration of disulfiram (which has limited water solubility) is possible in the treatment of ocular pythiosis because an ophthalmic formulation of the drug can be prepared at a high concentration (i.e., 0.5% [vol/wt]) by using solid nanoparticles (44). Such ophthalmic formulation shows good corneal penetration and undetectable toxicity in the treatment of glaucoma in a rabbit model (44). In contrast to the systemic infection, metabolism or decomposition of disulfiram should be less concerned in the ocular infection due to a lower chance of the drug to expose the glutathione reductase system of red blood cells.

Deferasirox (originally approved to treat chronic iron overload) has been also repurposed for the control of *P. insidiosum* (30). The MIC range of deferasirox against *P. insidiosum* (12.5 to 50 mg/liter) is slightly higher than that of disulfiram (8 to 32 mg/liter). Micafungin, when combined with deferasirox, as opposed to being tested alone, shows a synergistic antimicrobial effect in most *P. insidiosum* strains tested (45). Likewise, vancomycin, when combined with disulfiram, shows an enhanced antimicrobial activity against *S. aureus* (19). A combination of disulfiram and other drugs might provide a synergistic anti-*P. insidiosum* effect, which requires a further study.

In conclusion, disulfiram (approved for the alcoholism treatment) inhibits the growth of many pathogenic microorganisms, including *P. insidiosum*, by inactivating several proteins, i.e., ALDH, URE, CK, or DBH (21, 24–26). Various strains of *P. insidiosum* from different hosts and geographic origins were susceptible to disulfiram in a dose-dependent manner and at MICs similar to those for other pathogens. ALDH and URE are present in *P. insidiosum* and serve as potential binding targets of disulfiram. Evaluation of disulfiram in an animal model of pythiosis could provide more information on the *in vivo* drug efficiency against *P. insidiosum*. Disulfiram is an FDA-approved drug that could be repurposed as a new treatment option for pythiosis.

MATERIALS AND METHODS

Microorganisms. Twenty-seven strains of *P. insidiosum*, isolated from different hosts and geographic areas (Table 1), were recruited for the *in vitro* susceptibility test against disulfiram. Identities of the organisms were verified by rDNA sequence analysis. All strains were maintained on Sabouraud dextrose (SD) agar (pH 7.2) and subcultured once a month until use.

In vitro susceptibility assays. The growths of 27 *P. insidiosum* strains (Table 1), upon exposure to disulfiram, were measured using the agar-based (12, 32) and broth-based (see below) susceptibility methods. The experiments were performed in duplicate. SD agar plugs (5 mm in diameter) with actively growing mycelium were excised from a *P. insidiosum* colony (~7 days of age) and served as the inocula for the susceptibility assays. An agar plug with the organism was placed on the SD agar or immersed in the SD broth containing various concentrations (i.e., 0, 2, 4, 8, 16, 32, 64, and 128 mg/liter) of disulfiram (purity, $\geq 98\%$; solvent, dimethyl sulfoxide [DMSO]; manufacturer, Unidrug Innovative Pharma Technologies, Ltd., India) and incubated at 37°C for 2 days. For the agar-based susceptibility method (12, 32), two perpendicular diameters of a *P. insidiosum* colony were averaged, subtracted by the agar plug diameter (5 mm), and divided by half. The actual radial growth of a disulfiram-exposed strain was calculated to the “percent growth” in relation to that of the same strain without drug exposure (control). The percent growths of all *P. insidiosum* strains tested with different disulfiram concentrations were compared, using the Stata version 10 (StataCorp LLC) and a paired *t* test. Dot plots representing the percent growths of *P. insidiosum* was generated, using the Python program version 3.7.1 (<https://www.python.org>) and a modified script from Seaborn (<https://seaborn.pydata.org>). For the broth susceptibility method (which is a modified version of the agar-based susceptibility method that uses drug-containing broth rather than agar), an agar plug with a growing organism was immersed in a test tube containing 3 ml of SD broth with or without disulfiram. After incubation at 37°C for 2 days, each strain was checked for growth by naked eyes and reported as susceptible (if both replicates showed no growth) or resistant (if at least one replicate grew).

Proteogenomic analysis of putative targets of disulfiram. The protein sequences of ALDH (UniProt ID [P05091](#)) and DBH (UniProt ID [P09172](#)) were retrieved from the UniProt database

(<https://www.uniprot.org>), whereas those of CK (PDB ID 4OLC) and URE (PDB ID 4H9M) were retrieved from the Protein Data Bank (<https://www.rcsb.org>). To identify homology sequences, the retrieved proteins were BLASTP searched against the *P. insidiosum* genome-derived proteome (27, 28), using the locally installed blast 2.2.28+ program (<https://www.ncbi.nlm.nih.gov>; E value cutoff, $<10^{-6}$). The in-house mascot proteomic library and transcriptome of *P. insidiosum* were used to validate the matched proteins and their encoding genes, as described previously (28, 46).

Homology modeling of *P. insidiosum* proteins. The X-ray crystallography-derived three-dimensional (3D) structures of two proteins, the Jack bean URE of *Canavalia ensiformis* (PDB ID 3LA4) and the γ -hydroxymuconic semialdehyde dehydrogenase of *Pseudomonas* species strain WBC-3 (PDB ID 4GO4), were retrieved from the Protein Data Bank. These 3D structures were used as protein templates for generating 3D structures of the URE (accession no. BBE00775.1) and the betaine ALDH (accession no. GAY03418.1) of *P. insidiosum*, respectively. Binding pockets of the obtained 3D structures were predicted using the I-TASSER Suite (47). The UCSF Chimera program, v1.10.2 (<http://www.cgl.ucsf.edu/chimera/>) (48), was used to visualize the predicted 3D structures of the *P. insidiosum* proteins.

Molecular docking. The 3D structures of disulfiram (PubChem ID 3117), DDC (PubChem ID 8987), and DEA (PubChem ID 8021) were downloaded from the PubChem server (<http://pubchem.ncbi.nlm.nih.gov/>). The 3D structure of Me-DDC was constructed based on the DDC structure using the PreADMET server (<https://preadmet.bmdrc.kr/introduction/>). Molecular docking analyses of these chemical 3D structures (i.e., disulfiram, DDC, DEA, and Me-DDC) and the *P. insidiosum* proteins (i.e., URE and betaine ALDH) were performed using the AutoDock Vina program (49) and the PyRx suite open-source software version 0.9.7, which is a knowledge-based empirical scoring function of a ligand-protein complex (50). The input files were converted to the pdbqt file format by the AutoDock Vina program. The grid center (X:Y:Z) and dimension (X:Y:Z) of URE were 90:52:84 and 40:35:35, respectively, while those of ALDH were 75:80:76 and 22:23:25. The docking analyses were relied on the Lamarckian generic algorithm (GA) using the following default parameters: 20 GA runs, 150 individuals in the population, 270,000 maximum numbers of energy evaluation, and a 0.02 gene mutation rate and a 0.8 crossover rate. The outputs of 10 best binding poses for each docking run were stored for further evaluation of protein-ligand interaction, using the Discovery Studio Visualizer version 17.2.0 (Dassault Systemes Biovia Corp.).

Inhibitory activities of disulfiram. Inhibitory activities of disulfiram against the known URE and ALDH were evaluated. The Jack bean URE (Sigma-Aldrich) (final concentration, 0.1 mg/ml) and disulfiram (final concentrations, 0 or 128 mg/liter) were mixed at the final volume of 30 μ l and incubated at room temperature for 10 min. The mixture was then added onto a 6-mm-diameter grade-AA disc paper (Whatman), already placed in a URE test tube (CLINAG, Thailand). After incubation at 37°C for 7 days, the URE test was read as positive if the agar color was changed to pink or as negative if the agar color remained unchanged (yellow). The recombinant human ALDH2 (Sigma-Aldrich) (final concentration, 10 μ g/ml) and disulfiram (final concentrations, 0 or 128 mg/liter) were mixed at the final volume of 50 μ l and incubated at room temperature for 10 min. The ALDH activity (reported as the nmol of NADH/min/ml) of the mixture was measured by using an ALDH activity colorimetric assay kit (Sigma-Aldrich).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00609-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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