CrossMark

NOTE

Biotransformation and molecular docking of cyazofamid by human liver microsomes and cDNA-expressed human recombinant P450s

H. Lee[1,2] · J.-H. Kim[3] · E. Kim[1] · Y. Shin[1] · J.-H. Lee[1] ·
H. Jung[4] · Y. Lim[4] · H. S. Lee[3] · J.-H. Kim[1]

Abstract The purpose of this study was to understand the formation of metabolites from the metabolic reaction of cyazofamid with human liver microsomes. Human liver microsomal incubation of cyazofamid in the presence of NADPH produced one metabolite, 4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)*N,N*-dimethyl-1H-imidazole-1-sulfonamide (CCHS). An incubation study using cDNA-expressed human recombinant P450s (rCYPs) demonstrated that cyazofamid-derived CCHS is mediated by CYP2B6, 2C9, and 2C19 at different reaction rates. The crystal structure of cyazofamid was obtained using single-crystal X-ray diffraction. According to a molecular modeling study of the crystal structure of cyazofamid with the rCYPs 2B6, 2C9, 2C19, and 3A4, the metabolic reactivities (2B6 > 2C19 > 2C9) were well-correlated to the distances between heme irons of CYPs and 4-methylphenyl group of cyazofamid.

Keywords Crystal · Cyazofamid · Human liver microsomes · Metabolism · Molecular docking

✉ J.-H. Kim
kjh2404@snu.ac.kr

1 Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea

2 Geum River Environmental Research Center, National Institute of Environmental Research, Okcheon gun 29027, Republic of Korea

3 College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea

4 Division of Bioscience and Biotechnology, BMIC, Konkuk University, Seoul 05029, Republic of Korea
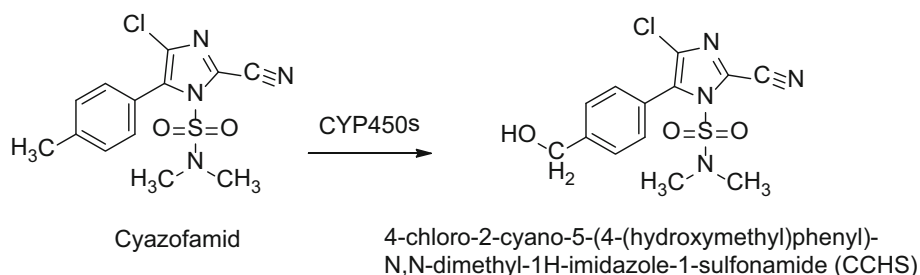
Introduction

Cyazofamid (4-chloro-2-cyano-*N,N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide, Fig. 1), a sulfonamide fungicide (Tomlin 2009), has been used to protect several vegetables and fruits from various diseases, such as tomato late blight (*Phytophthora infestans*) and downy mildew (*Pseudoperonospora cubensis* of cucumber) (Mitani et al. 2001; Tomlin 2009). It is known to inhibit the Qi site (ubiquinone reducing site) of cytochrome bc1 in complex III (ubiquinol-cytochrome c reductase) of the mitochondrial respiratory chain (Mitani et al. 2001; Tomlin 2009).

In general, pesticides are biotransformed to metabolites in biosystems by a variety of metabolic reactions, such as major phase I and phase II reactions. Phase I reactions primarily produce oxidized compounds, while phase II reactions produce conjugates with glucuronic acid, glucose, glutathione and other metabolites (Hodgson and Rose 2008; Abass et al. 2014). Such metabolic reactions usually detoxify harmful xenobiotic compounds. However, in a number of cases, parent compounds are bioactivated to create even more toxic metabolites or reactive intermediates (de Graaf et al. 2005). In phase I reactions, the cytochrome P450 (CYPs) group of enzymes plays a major role, and those CYPs are found in high concentrations in the liver, while small amounts are found in the lung, kidney, gastrointestinal tract, nasal mucosa, skin, brain, heart, and placenta (Hodgson and Rose 2008; Zanger and Schwab 2013; Abass et al. 2014; Lee et al. 2014). For metabolism studies of xenobiotics, including pesticides, pooled human liver microsomes (HLMs), which contain CYPs and a variety of cDNA-expressed human recombinant cytochrome P450s (rCYPs), have become commercially available in recent years. For example, in vitro metabolism

Cyazofamid

4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)-
N,N-dimethyl-1H-imidazole-1-sulfonamide (CCHS)

studies of various pesticides by HLMs, such as benfuracarb (Abass et al. 2014), flucetosulfuron (Lee et al. 2014), bifenthrin, S-bioallethrin, bioresmethrin, β-cyfluthrin, cypermethrin, and permethrin (Scollon et al. 2009), were conducted to identify metabolites that could be produced in liver, their reaction kinetics, and the CYP isoforms responsible for metabolite formation.

As the formation of metabolites is a result of interaction between molecules and CYPs, in silico molecular docking methods are a useful method of investigating the binding properties of molecules to CYPs (Marechal et al. 2006). The molecular mechanisms of biological activities, such as metabolism (Sousa et al. 2013), inhibition (Marechal et al. 2006) and drug–drug interactions (Qiu et al. 2015), can be elucidated using in silico docking studies.

In the present study, metabolism of cyazofamid with HLMs was carried out to identify the metabolite, and the metabolic reactivity and rCYPs isoforms responsible for formation of the metabolite were investigated with 10 types of rCYPs. Additionally, the crystal structure of cyazofamid was obtained by a molecular docking study with reactive rCYPs in an attempt to understand differences in metabolic reactivity between these molecules.

Materials and methods

Chemicals and reagents

Cyazofamid was purchased from Fluka™ (St. Louis, MO, USA). Pooled HLMs and 10 different cDNA-expressed human recombinant P450s (rCYPs), including CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Supersomes™) were purchased from BD Gentest (Woburn, MA, USA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP$^+$), nicotinamide adenine dinucleotide phosphate reduced (NADPH), potassium phosphate monobasic/dibasic, and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents (HPLC grade) were obtained from Burdick and Jackson® (Ulsan, Korea).

In vitro metabolism of cyazofamid by HLMs and rCYPs

To determine metabolite formation from cyazofamid, incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), 10 mM magnesium chloride, pooled HLMs (0.5 mg/mL), NADPH-generating system (1 mM NADP$^+$, 5 mM glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, and 1 mM NADPH), and 100 μM cyazofamid were prepared in a total incubation volume of 500 μL. The reaction mixtures were incubated at 37 °C for 0, 30, 60, and 120 min in a shaking water bath before the reaction was terminated by the addition of 500 μL of acetonitrile on ice. The reaction mixture was centrifuged at 13,000 rpm for 7 min at 4 °C, and 2 μL of supernatant was subsequently analyzed using HPLC and LC–MS/MS. The analytical settings of the instruments used for the reaction mixture and metabolite identification were described earlier (Lee et al. 2016). Control incubations were conducted in the absence of an NADPH-generating system or by denaturing the HLMs at 80 °C. To confirm whether FMOs were involved in metabolite formation, the HLMs were heated for 30 min at 45 °C prior to the incubation.

To identify the rCYPs isoforms that were responsible for metabolite formation, metabolic reactions were performed for 10 min with 10 different rCYPs isoforms (10 pmol) and 10 μM of cyazofamid.

X-ray crystallography and molecular docking

Single crystals of cyazofamid were obtained by slow evaporation of the solvents (acetone and hexane). Examination of the cyazofamid structure was performed by single crystal X-ray diffraction with Mo Kα1 radiation (λ = 0.71073 Å) on a RIGAKU R-ASXIS RAPID diffractometer.

Cyazofamid and CYP crystal structure docking experiments were performed on an Intel Core 2 Quad Q6600 (2.4 GHz) Linux PC using Sybyl 7.3 software (Tripos, USA). Three dimensional (3D) structures of CYP 2B6, 2C9, 2C19, and 3A4 were adapted from the Protein Data

Bank (3IBD, 1OG5, 4GQS, and 2J0D, respectively). The binding pocket was determined from 3IBD.pdb, 1OG5.pdb, 4GQS.pdb, and 2J0D.pdb using LigPlot (Wallace et al. 1995), and all 3D images were constructed using PyMOL (PyMOL Molecular Graphics System, Version 1.0r1, Schrödinger, LLC.).

Results and discussion

Metabolite formation from the reaction of cyazofamid with HLMs and rCYP isoforms

Incubation of cyazofamid with HLMs in the presence of NADPH resulted in the formation of a single metabolite, while no metabolites were observed with denatured HLMs without the NADPH generating system, with denatured HLMs (at 80 °C for 10 min) or with the heated HLMs (at 45 °C for 30 min). The metabolite was identified by LC–MS/MS ($[M+H]^+ = m/z$ 341) as 4-chloro-2-cyano-5-(4-(hydroxymethyl)phenyl)N,N-dimethyl-1H-imidazole-1-sulfonamide (CCHS, Fig. 1), which was also observed as CM3 in a previous study (Lee et al. 2016).

In metabolic reactions of cyazofamid with 10 different rCYP isoforms, only three isoforms (CYP2B6, 2C19, and 2C9) produced a single metabolite CCHS and did so at different rates (Fig. 2). CYP3A4, the most abundant isoform (Abass et al. 2012), was not involved in metabolite production. In work by Abass et al. (2012), a metabolism study of 63 pesticides using rCYP isoforms showed that CYP 2C19 was involved in metabolism at a level of 15 %,
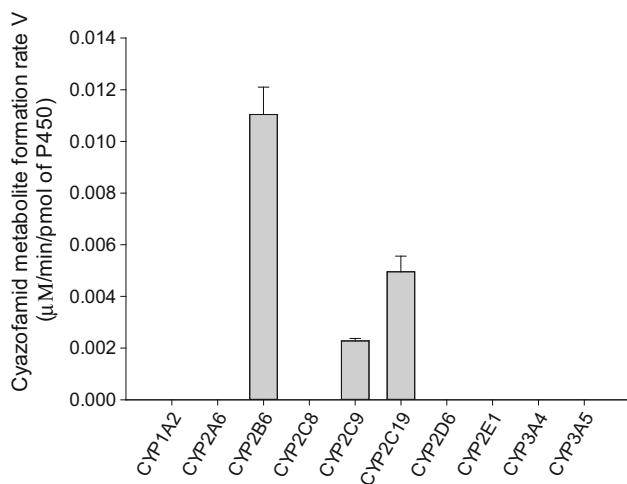
Fig. 2 Formation of metabolite from cyazofamid by cDNA-expressed P450 isoforms when incubated with 20 μM of cyazofamid at 37 °C for 10 min. Data represent the averages of triplicate experiments

CYP2B6 was involved at a level of 12 %, CYP2C9 at a level of 10 %, and CYP3A4 at a level of 24 %. The relative importance of CYP2B6, which showed the highest reactivity in our study, has only recently become apparent in drug and pesticide metabolism. Recent studies have demonstrated that CYP2B6 is an important isoform in human metabolism of pesticides, including alachlor, metolachlor, acetochlor, butachlor (Coleman et al. 2000) and endosulfan (Lee et al. 2006).

Single crystal structure of cyazofamid and molecular docking with rCYPs

In crystal structure, the monoclinic cell parameters and calculated volumes of cyazofamid crystal were $a = 6.8160(10)$ Å, $b = 13.396(2)$ Å, $c = 16.462(2)$ Å, and $\beta = 92.993(3)^o$, while the torsion angle between the benzene and imidazole rings was 79° (Fig. 3A). These crystallographic characteristics were similar to the reported data (Ning et al. 2010).

The crystal structure of cyazofamid was docked with rCYPs 2B6, 2C9, 2C19, and 3A4 using Sybyl 7.3 software to elucidate the reactivity differences between rCPY isoforms.

In CYP2B6, the binding pocket of 3IBD.pdb included Arg98, Ile114, Phe297, Ala298, Glu301, Thr302, Leu363, Arg434, and Cys436. All of the residues except Thr302 and Ile114 participated in hydrophobic interactions with cyazofamid, and two residues formed hydrogen bonds (Fig. 3B). The residues of 1OG5.pdb that neighbored the binding site in CYP2C9 were Ile99, Phe100, Ala103, Leu208, Gln214, Asn217, Ser365, Leu366, Pro367, Asn474, Phe476, and Ala477. All of the residues except Gln214 and Asn217 showed hydrophobic interactions with cyazofamid, and two residues formed hydrogen bonds. In the case of CYP2C19, the residues of 4GQS.pdb that neighbored the binding site were Leu102, Val208, Ile205, Leu233, Gly296, Ala297, Glu300, Thr301, and Phe476, and all of the residues showed hydrophobic interactions with cyazofamid. The distances between the carbon of the methyl group at C13 of cyazofamid (Fig. 3A), which was oxidized by rCYPs to form the metabolite CCHS, and the iron center of a heme in the rCYPs were calculated because the distance between two molecules is the most important parameter for their metabolic reaction. Those distances were 4.949, 14.209, 14.266, and 15.397 Å for CYP2B6:-cyazofamid, CYP2C19:cyazofamid, CYP2C9:cyazofamid, and CYP3A4:cyazofamid, respectively. Cyazofamid was closest to the heme of CYP2B6 (Fig. 3C) and was farthest from the heme of CYP3A4. This result correlated well with the different metabolism reaction rates of the three rCYPs (Fig. 2) and CYP3A4.

Fig. 3 Packing diagram (A) for cyazofamid and the interaction of cyazofamid with rCYP2B6. (B) LigPlot analysis. (C) Protein structure with the heme group (a *white* ball-and-stick) and cyazofamid (a *pink* carbon ball-and-stick)
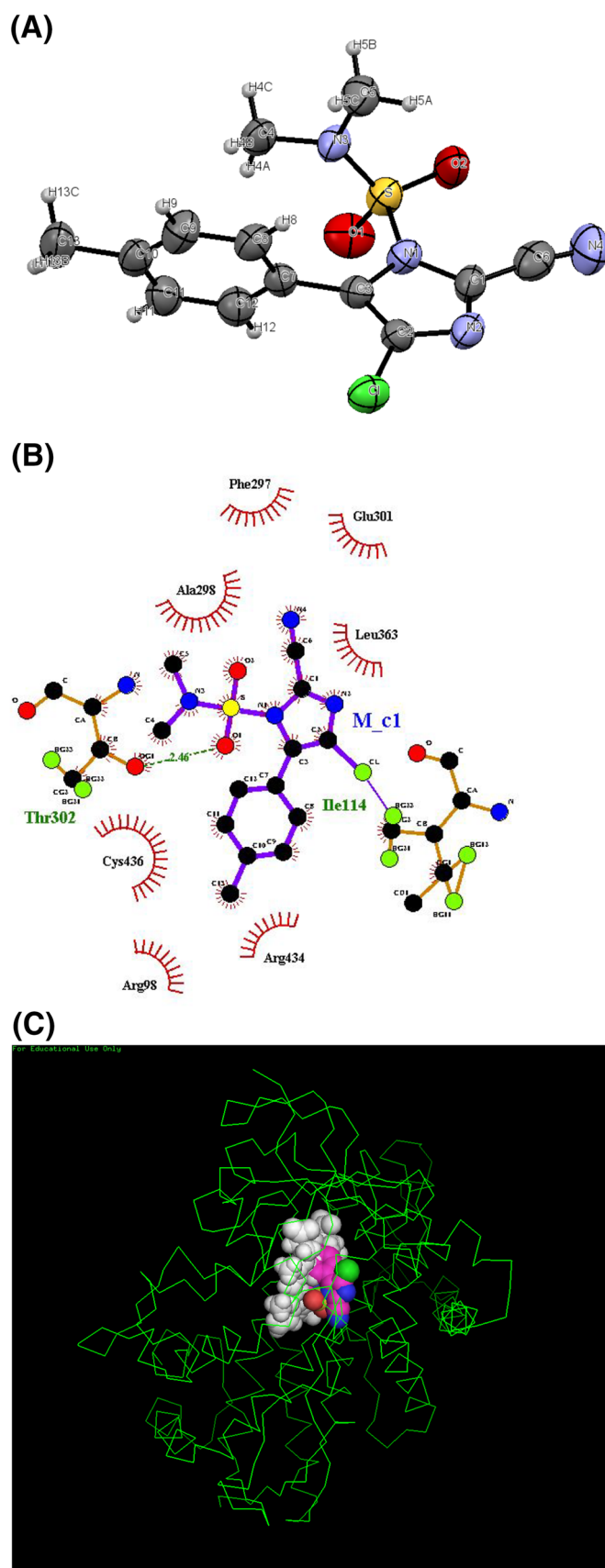
(A)

(B)

(C)

References

Abass K, Turpeinen M, Rautio A, Hakkola J, Pelkonen O (2012) In Insecticides-advances in integrated pest management. InTech, New York

Abass K, Reponen P, Mattila S, Rautio A, Pelkonen O (2014) Human variation and CYP enzyme contribution in benfuracarb metabolism in human in vitro hepatic models. Toxicol Lett 224:300–309

Coleman S, Linderman R, Hodgson E, Rose RL (2000) Comparative metabolism of chloroacetamide herbicides and selected metabolites in human and rat liver microsomes. Environ Health Perspect 108:1151–1157

de Graaf C, Vermeulen NP, Feenstra KA (2005) Cytochrome p450 in silico: an integrative modeling approach. J Med Chem 48:2725–2755

Hodgson E, Rose RL (2008) Metabolic interactions of agrochemicals in humans. Pest Manag Sci 64:617–621

Lee HK, Moon JK, Chang CH, Choi H, Park HW, Park BS, Lee HS, Hwang EC, Lee YD, Liu KH, Kim JH (2006) Stereoselective metabolism of endosulfan by human liver microsomes and human cytochrome P450 isoforms. Drug Metab Dispos 34:1090–1095

Lee YS, Liu KH, Moon JK, Ko BJ, Choi H, Hwang KS, Kim E, Kim JH (2014) In vitro metabolism of flucetosulfuron by human liver microsomes. J Agric Food Chem 62(14):3057–3063

Lee H, Kim E, Shin Y, Lee J-H, Hur H-G, Kim J-H (2016) Identification and formation pattern of metabolites of cyazofamid by soil fungus *Cunninghamella elegans*. Appl Biol Chem 59:9–14

Marechal JD, Yu J, Brown S, Kapelioukh I, Rankin EM, Wolf CR, Roberts GC, Paine MJ, Sutcliffe MJ (2006) In silico and in vitro screening for inhibition of cytochrome P450 CYP3A4 by comedications commonly used by patients with cancer. Drug Metab Dispos 34:534–538

Mitani S, Araki S, Takii Y, Ohshima T, Matsuo N, Miyoshi H (2001) The biochemical mode of action of the novel selective fungicide cyazofamid: specific inhibition of mitochondrial complex III in *Phythium spinosum*. Pest Biochem Physiol 71:107–115

Ning B-K, Xu C, Li Z-Y, Wang Y-M, Sun Q-N (2010) Synthesis, characterization and crystal structure of cyazofamid. Appl Chem Ind 39:1351–1354

Qiu JX, Zhou ZW, He ZX, Zhang X, Zhou SF, Zhu S (2015) Estimation of the binding modes with important human cytochrome P450 enzymes, drug interaction potential, pharmacokinetics, and hepatotoxicity of ginger components using molecular docking, computational, and pharmacokinetic modeling studies. Drug Des Devel Ther 9:841–866

Scollon EJ, Starr JM, Godin SJ, DeVito MJ, Hughes MF (2009) In vitro metabolism of pyrethroid pesticides by rat and human hepatic microsomes and cytochrome p450 isoforms. Drug Metab Dispos 37:221–228

Sousa MC, Braga RC, Cintra BAS, de Oliveira V, Andrade CH (2013) In silico metabolism studies of dietary flavonoids by CYP1A2 and CYP2C9. Food Res Int 50:102–110

Tomlin C (2009) In the pesticide manual: a world compendium. British Crop Protection Council, Alton

Wallace AC, Laskowski RA, Thornton JM (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng 8:127–134

Zanger UM, Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 138:103–141