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Gary Gustafson · George Davis · Clive Waldron
Amy Smith · Matthew Henry

Identification of a new antifungal target site through a dual biochemical and molecular-genetics approach

Abstract The target site of the antifungal compound LY214352 [8-chloro-4-(2-chloro-4-fluorophenoxy) quinoline] has been identified through a dual biochemical and molecular-genetics approach. In the molecular-genetics approach, a cosmid library was prepared from an *Aspergillus nidulans* mutant that was resistant to LY214352 because of a dominant mutation in a single gene. A single cosmid (6A6-6) that could transform an LY214352-sensitive strain of *A. nidulans* to LY214352-resistance was isolated from the library by sib-selection. Restriction fragments from cosmid 6A6-6 containing the functional resistance gene were identified by transformation, and sequenced. The LY214352-resistance gene coded for a protein of 520 amino acids that had a 34% identity and a 57% similarity in a 333 amino-acid overlap to *E. coli* dihydroorotate dehydrogenase (DHO-DH). The results of a series of biochemical mechanism-of-action studies initiated simultaneously with molecular-genetic experiments also suggested that DHO-DH was the target of LY214352. Assays measuring the inhibition of DHO-DH activity by LY214352 in a wild-type strain ($I_{50} = 40$ ng/ml) and a highly resistant mutant ($I_{50} > 100$ μg/ml) conclusively demonstrated that DHO-DH is the target site of LY214352 in *A. nidulans*. Several mutations in the DHO-DH (*pyrE*) gene that resulted in resistance to LY214352 were identified.

Key words: Dihydroorotate dehydrogenase · Fungicide · *Aspergillus nidulans* · Resistance gene

G. Gustafson (✉) · G. Davis · A. Smith · M. Henry
Crop Disease Management Discovery Research, DowElanco, 9330 Zionsville Road, Indianapolis, IN 46268-1054, USA

C. Waldron
Biotechnology Discovery Research, DowElanco, 9330 Zionville Road, Indianapolis, IN 46268-1054, USA

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Introduction

Agricultural antifungal agents have undergone an evolution from high-rate, multiple mechanism-of-action (MOA), surface-protectant compounds to site-specific, systemic compounds that are used at much lower rates. While the specificity and selectivity of modern antifungal agents have had positive effects on both human and environmental safety, they have also led to increasing problems with fungicide resistance. Thus, the use of phenylamide, dicarboximide, aromatic hydrocarbon and benzimidazole fungicides has been limited by the emergence of resistant fungal strains, and the most prevalent agricultural antifungal agents, sterol demethylation inhibitors, are already exhibiting decreased efficacy in some crops (Dovas et al. 1976; Cohen and Reuveni 1983; Koller and Scheinpflug 1987; Lyr 1987; Pommer and Lorenz 1987; Brent 1988).

Strategies for prolonging fungicide usefulness often involve alternating or combining two site-specific antifungal agents with different mechanisms of action. The discovery of fungicidal compounds with novel biochemical target sites has, therefore, become a priority in many industrial fungal disease-management research programs. Nevertheless, the identification of an antifungal compound with a new MOA is a relatively rare occurrence.

Traditionally, efforts to determine the MOA of antifungal compounds have been limited to morphological, histological and biochemical studies. Molecular-genetic studies now provide another potential approach to determining MOA through the isolation and identification of pesticide-resistance genes. This type of approach to MOA identification will succeed if resistant mutants can be obtained, if the resistance trait is due to a dominant mutation in the gene that codes for the biochemical target of the antifungal compound, if the mutant gene responsible for resistance can be isolated, and if the protein encoded by the resistance gene can be identified from sequence databases or from functional

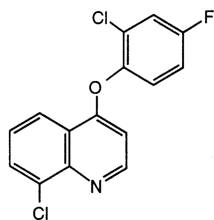

Fig. 1 Chemical structure of LY214352 [8-chloro-4-(2-chloro-4-fluorophenoxy)-quinoline]

analysis in recombinant bacteria. The results of molecular-genetic MOA studies can be used to corroborate conclusions reached from biochemical experiments, or to suggest a potential target site when biochemical studies are inconclusive.

Recently, we have been studying the antifungal activity of a series of 4-phenoxyquinoline compounds. One member of this series, LY214352 (Fig. 1), had significant disease control activity in greenhouse tests against *Botrytis cinerea* (gray mold of grape), *Venturia inaequalis* (apple scab), and several other agronomically important fungi (Coghlan et al. 1991). LY214352 also inhibited in vitro growth of a number of Ascomycete fungi including *Magneporthe grisea*, *Alternaria solani*, *Penicillium italicum*, *Cercospora beticola*, *Leptosphaeria nodorum* and *Aspergillus nidulans*, but exhibited little or no growth inhibition activity when tested in vitro against *E. coli*, *S. cerevisiae*, or fungi in the Oomycete and Basidiomycete groups.

Biochemical experiments were initiated with LY214352 to determine if this unique and active antifungal compound had a novel MOA. However, the excellent in vitro activity of LY214352 against *A. nidulans* ($I_{50} = 0.13$ μg/ml, MIC < 2.0 μg/ml), a fungus that is transformable and amenable to genetic manipulations, suggested that the MOA of LY214352 might also be approached through the isolation and identification of an LY214352-resistance gene. In an initial study to test the feasibility of this approach, six chemically induced *A. nidulans* mutants with moderate (I_{50} about 2.5 μg/ml) or high ($I_{50} > 30$ μg/ml) levels of resistance to LY214352 were isolated and characterized (Gustafson et al. 1991). Genetic analysis of the moderately resistant (52-2, 52-3, 52-5 and 52-6) and highly resistant (52-1 and 52-4) mutants indicated that resistance was always due to a dominant mutation in a single gene and that all of the resistance genes were alleles. This type of qualitative, single-gene resistance has been reported for the benzimidazole, carboxamide and phenylamide fungicides (Georgopoulos et al. 1975; Davidse 1981; Stannis and Jones 1984), and is known to be associated with target-site mutations (Davidse 1988 a, b; Keon et al. 1991). Thus, the results of the genetic analysis implied that resistance to LY214352 was due to dominant mutations in the gene that encoded its biochemical target.

Here we report on studies designed to identify the MOA of LY214352 through a typical biochemical approach and through the isolation and identification of an LY214352-resistance gene. The results confirm the strength of a dual approach to MOA studies and verify that the MOA of antifungal compounds can be determined through the identification of resistance genes. Furthermore, our findings distinguish LY214352 as the first fungicide whose site of action has been shown to be the enzyme dihydroorotate dehydrogenase (DHO-DH).

Materials and methods

Fungal strains. *Aspergillus nidulans* strains A610 (*pabaA1*, *yA2*), A612 (*riboB2*, *chaA1*; *AcrA1*) and A237 (*pabaA1*, *yA2*; *trpC 801*) were obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, Kan.). All three strains are sensitive to LY214352 ($I_{50} = 0.13$ μg/ml). *A. nidulans* strains 52-1 through 52-6 (*pabaA1*, *yA2*) are LY214352-resistant mutants which were originally derived from either strain A610 or A612 (Gustafson et al. 1991). Mutants 52-2, 52-3, 52-5 and 52-6 are moderately resistant to LY214352 (I_{50} of about 2.5 μg/ml). Mutants 52-1 and 52-4 are highly resistant to LY214352 ($I_{50} > 30$ μg/ml).

Culture conditions. Glucose minimal medium and complete medium were prepared as described (Waldron and Roberts 1974). Strain A237 was propagated on minimal media containing 4 mM tryptophan, 1 μg/ml p-aminobenzoic acid (paba) and 0.5 M NaCl. A610 and LY214352-resistant strains require only paba for growth. The growth rate of mutant 52-1 was significantly enhanced in the presence of 0.5 μg/ml of LY214352.

Vectors. Cosmid clones were prepared in the vector pKBY2 (Yelton et al. 1985) which contains the *A. nidulans trpC* gene as a selectable marker. The plasmid pUC19 (GIBCO BRL, Life Technologies, Inc., Gaithersburg, M.D.)was used to subclone cosmid DNA fragments.

Preparation of genomic DNA. A baffled flask containing 400 ml of 2× glucose minimal medium supplemented with paba was inoculated with 4×10^8 spores of strain 52-6. The culture was grown for 20 h at 37 °C and 250 rpm. The mycelium was harvested and then protoplasted by standard methods (Yelton et al. 1984). Protoplasts were lysed in 24 vol of LETS buffer (100 mM LiCl; 10 mM EDTA; 10 mM Tris-HCl, pH 8.0; 0.5% SDS; 100 μg/ml proteinase K, 20 μg/ml RNase A) and the lysate was incubated for 1 h at 50 °C. The viscous solution was gently mixed with an equal volume of phenol:chlorform:isoamyl alcohol (25:24:1) and centrifuged at 20,000 × g for 10 min at 12 °C . The aqueous phase was recovered and mixed with 0.1 volume of 2M sodium acetate (pH 5.5). Two volumes of 100% ethanol was layered over the solution, and DNA was spooled from the interface. The DNA was washed with 80% ethanol, dried under vacuum, and slowly re-suspended in 5 ml of TE buffer (1 mM EDTA; 10 mM Tris-HCl, pH 7.0).

Cosmid library preparation and cosmid pools. Genomic DNA was partially digested with the restriction enzyme *Sau*IIIA. DNA fragments of 30–40 kb were isolated as previously described (Sambrook et al. 1989) and cloned into the *Bam*HI site of pKBY2. The clones were packaged using the Packagene System (Promega Biotech, Madison, WIS.) and used to infect *E. coli* HB101. About 3000 ampicillin-resistant colonies were picked and stored in 96-well microtiter plates at −80 °C. Bacteria from each microtiter-plate well were grown individually in 5 ml cultures. The 5-ml cultures were pooled in groups of 24, and DNA was isolated from each pool.

Transformations. Protoplasts of strains A237 and A610 were isolated and transformed using standard procedures (Yelton et al. 1984). Each transformation used approximately 10^7 protoplasts and either 10 μg of pooled cosmid DNA or 2 μg of purified plasmid DNA. Transformed protoplasts were plated onto minimal media containing 1.2 M sorbitol, 1 μg/ml paba, and LY214352 to select for protoplasts transformed with the LY214352-resistance gene. Protoplast viability after transformation and transformation efficiency were estimated for each experiment involving strain A237. In addition, protoplasts transformed with pKBY2 and plated on minimal media containing LY214352 were used to estimate the potential for escapes or spontaneous resistant mutations in each transformation experiment.

Chemical reversal studies. Approximately 250 spores of the LY214352-sensitive *A. nidulans* strain A610 were applied in a volume of 1 μl to the center of a 50-mm Petri plate containing 10 ml of minimal media supplemented with p-aminobenzoic acid (paba), an I_{50} concentration of LY214352, and the chemical being tested for its ability to reverse the inhibitory effects of LY214352. Colony diameter (mm) was measured after plates had been incubated at 37 °C for 72–96 h. All tests were done in triplicate.

Isolation and assay of DHO-DH. Partially purified DHO-DH activity was isolated from an LY214352-sensitive *A. nidulans* strain (A610) and from the highly resistant mutant 52-4. Liquid minimal media (400 ml) was inoculated with 2×10^6 conidia/ml and incubated for 18 h at 37 °C and 250 rpm. Mycelium was harvested, washed and re-suspended in ice-cold buffer [50 mM Tris (pH 7.5), 10 mM EDTA] to a final volume of 175 ml. The suspended mycelium was transferred to an ice-chilled 350 ml Bead-Beater™ (Biospec Products, Bartlesville, Okla.) containing 0.5 vol of 0.5-mm diameter buffer-washed glass beads, and disrupted using four 30-s beating cycles with 1 min cooling time between cycles. The homogenate was centrifuged at low speed (1000 × g, 10 min). The supernatant was recovered and centrifuged again at high speed (100000 × g, 45 min). The resulting pellet was re-suspended with a teflon mortar and pestle in the above buffer containing 0.1% Triton X-100, quickly frozen in small aliquots in liquid nitrogen, and stored at − 80°C. DHO-DH activity was assayed essentially as previously described (Karibian 1978) Each reaction mixture contained 50 mM Tris (pH 7.5), 0.067% Triton X-100, 110 mM ubiquinone (coenzyme Q_6), 190 mM dihydroorotic acid, 43 mM 2,6-dichloroindolphenol (DCIP), 17 mM potassium cyanide, and 63 mM rotenone. LY214352 dissolved in methanol was added at various concentrations. All assays were adjusted to contain 1% methanol. Assays were initiated by the addition of an aliquot of enzyme extract to give a specific activity of 3–8 nmol/min per mg. This represented approximately 1.85 mg protein/ml for *A. nidulans.* The decrease in absorbance at 610 nm was monitored at 30 °C.

Results

Determination of the optimal LY214352 selection concentration

Concentrations of LY214352 ranging from 0.5 to 5.0 μg/ml were tested to determine their efficacy in suppressing the regeneration of 10^7 A237 protoplasts on a 100-mm Petri plate. The minimal concentration of LY214352 that completely suppressed protoplast regeneration (1.5 μg/ml) was used to select for protoplasts transformed to LY214352-resistance.

Protoplast viability and transformation efficiency

Protoplast viability after mock transformations was 2–3%. The rate at which strain A237 was transformed to *trpC* prototrophy by individual cosmid pools ranged from 15–30/μg of DNA. The transformation rate for pKBY2 with A237 protoplasts was about 75/μg. In all controls involving transformation of A237 by pKBY2, no spontaneous LY214352-resistant mutants, or colonies that escaped selection on LY214352, were observed.

Isolation of a cosmid conferring resistance to LY214352

A typical sib-selection procedure was used to isolate a cosmid containing the LY214352-resistance gene. Twenty five cosmid pools (600 cosmids) were screened before a single pool was found that transformed strain A237 to LY214352-resistance. Cosmids from that pool were initially used in groups of six, and then individually, to transform A237. Eventually, a single cosmid (6A6-6) responsible for transforming A237 to LY214352-resistance was isolated. In several experiments, an average of 36 LY214352-resistant tranformants per μg of 6A6-6 DNA was obtained. Conidiospores isolated from LY214352-resistant transformants selected on 1.5 μg/ml of LY214352 were viable when transferred to media containing as much as 40 μg/ml of LY214352.

Subcloning the LY214352-resistance gene from cosmid 6A6-6

Cosmid 6A6-6 was digested with *Sal*I, *Bam*HI or *Hind*III. Individual restriction fragments were cloned into pUC19 and used to transform strain A610. Single *Bam*HI, *Sal*I and *Hind*III fragments capable of transforming A610 to LY214352-resistance were isolated. Both the *Hind*III fragment (5300 bp) and the *Bam*HI fragment (5700 bp) mapped within the 7200-bp *Sal*I fragment (Fig. 2). The *Sal*I fragment was subcloned into 3000-bp and 4200-bp fragments by restriction with *Xho*I. Only the 3000-bp *Sal*I/*Xho*I fragment was capable of transforming A610 to LY214352-resistance. Finally, a 1000-bp *Hind*III/*Xba*I fragment that carried the resistance trait was isolated. Transformation rates for all of the subclones were much lower (2–6 resistant transformants/μg DNA) than that of the parental cosmid.

Sequencing the LY214352-resistance gene

The 1000-bp *Hind*III/*Xba*I fragment from cosmid 6A6-6 was mapped and sequenced. The sequence revealed two

S H B X Xh H B S
7200 bp
H Sp B E X
B S
H Xh
ORF 2 ORF 1
LY214352-resistance gene

Fig. 2 Partial restriction maps of the 7200-bp *Sal*I fragment that transforms *A. nidulans* to LY214352-resistance and the three overlapping DNA fragments that were used to obtain the sequence of the *A. nidulans* LY214352-resistance gene. The location of two open reading frames (ORFs) that can be joined by removal of an intron are shown. B = *Bam*HI, E = *Eco*RI, H = *Hind*III, S = *Sal*I, Sp = *Sph*I, X = *Xba*I and Xh = *Xho*I.

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   1  TCTAGATTATGATTTGATTCGAGTGTTTGGAGAGAGGACACTTGCTAAAATATGTGGTCTACATA
  66  GGTCTACAAACTCGAAACCAATCCTGCAATTCAACACTACTATATAAGCATAGACGAAGCATCGG
 131  CGCAATGGCTACGAATTCTTTCCGAAAACTCACTTTTTCAGGAGCCTCCCGTCTGGGTGGTTGTC
 196  GCCGTCTCCCACTAACCTGCAGACAACTTCGATTCGCCTCCGACAGCGGAGCCGCAGCGGCAACT
 261  ACAAAAGCAACGGCCGAATCAGCAGCCGAGTCAGCTAGTATAAACGTCAAAGAGGCACCCAAAAA
 326  GGCCGGACGGGGCCTGCGGCGCACGGTCCTGGGAACGTCGTTGGCGCTGACGCTGCTGGTTGGAT
 391  ATGTCTACGGGACGGACACCCGGGCGAGTGTGCATCGGTACGGTGTTGTGCCGCTGATTAGAGCA
 456  TTGTATCCTGATGCGGAAGATGTGCATCATATTGGTGTCGATACTTTAAAGATGCTGTATAAGTA
 521  TGGTCTGCATCCAAGGGAACGGGGGGATCCGGATGGAGATGGGGCGCTGGCGACAGAGGTGGGTG
 586  GTCTTTTATTGTGATGGAAGTTAAGGCCCTAATACTTATGGCGGATAGGTCTTTGGGTATACACT
 651  GTCAAACCCAATTGGCATATCGGGCGGCCTGGACAAGCATGCTGAGATCCCTGATCCGCTGTTCG
 716  CGATCGGTCCTGCCATTGTCGAAGTCGGGGGTACGACACCCTTACCACAGGATGGTAACCCGCGT
 781  CCTCGCGTATTCCGACTTCCATCACAGAGAGCGATGATAAACCGGTACGGCCTCAACTCCAAAGG
 846  CGCAGATCACATGGCAGCTATCTTGGAGCAACGAGTACGCGATTTTGCCTACGCAAACGGATTTG
 911  GGGCATACGATGCGGCTAAGCAGCGTGTATTGGACGGCGAAGCTGGTGTGCCACCAGGTAGTCTT
 976  CAGCCTGGTAAGCTTTTAGCTGTCCAAGTGGCAAAGAACAAGGCCACTCCTGACGGCGACATTGA
1041  AGCCATCAAGCGCGACTATGTGTATTGCGTGGACCGTGTGGCCAAATACGCTGATATTCTTGTTG
1106  TGAATGTATCGAGCCCCAACACACCCGGTCTCCGTGACCTTCAAGCCACTGCCCCGCTCACAGCT
1171  ATCTTGAGTGCTGTCGTTGGCGCGGCAAAGAGCGTGAACCGCAAGACCAAACCATATGTTATGGT
1236  CAAGGTCAGTCCGGATGAAGACTCAGATGAACAAGTCTCTGGTATCTGCGACGCCGTCCGAGCAT
1301  CCGGTGTCGACGGAGTGATTGTCGGAAACACAACAAACCGTCGCCCCGACCCTATACCCCAAGGT
1366  TACACTCTTCCGGCCAAGGAGCAGGCAACGTTGAAAGAAACCGGCGGGTATTCAGGTCCACAGCT
1431  GTTCGATCGCACAGTGGCCCTTGTGGCTCGGTACCGCTCCATGCTGGATGCGGAGTCGGAAACGG
1496  CCGGATCCGCCAAGGATTCAGCAGCGACCATAGCGCAAACAGAGCCAGGCTCGGAAAACGTTCCT
1561  CCTGTGGAAGCGCCAAGCGGACTGCCGCGCAAAGTTATCTTCGCTTCGGGTGGTATCACCAACGG
1626  GAAGCAGGCTCACGCTGTTTTAGACACAGGGGCATCTGTTGCCATGATGTACACCGGTGTGGTCT
1691  ATGGTGGCGTCGGCACTGTCACTCGAGTGAAGCAAGAACTTCGAACGGCGAAAAAGGAGTGAAAT
1756  GCCGAAAAGAGAAAACGGTGATAAGAGTACAATGAGGAGTTGATTCTGGTCCGTAGATGATAGCG
1821  AACTGTTGCAGGCACACTGGCCCTTTTCTTGTCATGCGAAAGTACCCTGCATGGTCAAATCAAAG
1886  CAGATATCACATAAATCGACAGGCTGTGCGCACAACGCCGTACCC
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Fig. 3 Complete nucleotide sequence of the dihydroorotate dehydrogenase gene from the LY214352-resistant *A. nidulans* mutant 52-6. A putative intron is *underlined*. Initiation and stop codons are in *bold type and underlined*. The sequence of the wild-type DHO-DH gene has been entered into GenBank under the accession number U47318

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A. nidu  MATNSFRKLTFSGASRLGGCRRLPLTCRQLRFASDSGAAAATTKATAESAAESASINVKE   60

A. nidu  APKKAGRGLRRTVLGTSLALTLLVGYVYGTDTRASVHRYGVVPLIR.ALYP.DAEDVHHI  118
                                                   |::| ||:  |:| :| :
E. coli                                          MYYPFVRKALFQLDPERAHEF   21

A. nidu  GVDTLKMLYKYGLHPRERGDPDGDGALATEVFGYTLSNPIGISGGLDKHAEIPDPLFAIG  178
           : |: :    : :  |      | : : :| |: ||:|: :|||| :|  |:| |:|
E. coli  TFQQLRRITGTPFEALVRQKV...PAKPVNCMGLTFKNPLGLAAGLDKDGECIDALGAMG   78

A. nidu  PAIVEVGGTTPLPQDGNPRPRVFRLPSQRAMINRYGLNSKGADHMAAILEQRVRDFAYAN  238
          : :|:|  || || || :||:|||    ::||| |:|  | |::
E. coli  FGSIEIGTVTPRPQPGNDKPRLFRLVDAEGLINRMGFNNLGVDNLV..............  124

A. nidu  GFGAYDAAKQRVLDGEAGVPPGSLQPGKLLAVQVAKNKATPDGDIEAIKRDYVYCVDRVA  298
             :  |    ||             :|:: ::||| ||   :|  | ||: |::::
E. coli  .....ENVKKAHYDG.............VLGINIGKNKDTP...VEQGKDDYLICMEKIY  163

A. nidu  KYADILVVNVSSPNTPGLRDLQATAPLTAILSAVVGAAKSVNRKTKPYVMVKVSPDED.S  357
          ||: : :|:||||||||| ||    :|  :| |:       :        || : |    | |
E. coli  AYAGYIAINISSPNTPGLRTLQYGEALDDLLTAIKNKQNDLQAMHHKYVPIAVKIAPDLS  223

A. nidu  DEQVSGICDAVRASGVDGVIVGNTTNRRPDPIPQGYTLPAKEQATLKETGGYSGPQLFDR  417
         :|::  : | :     :|||| ||| |   : ||                :||| || |  :
E. coli  EEELIQVADSLVRHNIDGVIATNTTLDR..SLVQGMKNC.......DQTGGLSGRPLQLK  274

A. nidu  TVALVARYRSMLDAESETAGSAKDSAATIAQTEPGSENVPPVEAPSGLPRKVIFASGGIT  477
           :: |   |::                                    |  |:: |||
E. coli  STEIIRRLSLELNG............................RLPIIGVGGID  299

A. nidu  NGKQAHAVLDTGASVAMMYTGVVYGGVGTVTRVKQELRTAKKE                  520
             |:  :  |||:  :| | :: |    :  :   :
E. coli  SVIAAREKIAAGASLVQIYSGFIFKGPPLIKEIVTHI                        336
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Fig. 4 Homology between the amino-acid sequences of dihydroorotate dehydrogenase from *E. coli* and the LY214352-resistant *A. nidulans* mutant 52-6. Sequences were aligned by the computer program BESTFIT. Identical (|) and conserved (:) amino-acid residues are identified

open reading frames (ORFs) separated by only a few nucleotides (Fig. 2). An examination of the region extending from the 3′-terminus of ORF 1 to the 5′-terminus of ORF 2 identified sequences commonly associated with fungal introns (Parker and Patterson 1987). When the putative 55-nucleotide intron was removed, ORFs 1 and 2 merged to create a single ORF which extended beyond the *Hind*III end of the fragment. Subclones of 6A6-6 containing the adjacent *Hind*III fragment and an overlapping *Bam*HI fragment (Fig. 2) were isolated and used to complete the sequence of the LY214352-resistance gene (Fig. 3). The complete gene coded for a polypeptide of 520 amino acids.

Identification of the LY214352-resistance gene

The computer program FastA (Pearson and Lipman 1988) was used to search the National Biomedical Research Foundation library for proteins with amino-acid sequence homology to the polypeptide encoded by the LY214352-resistance gene. The only protein with greater than random homology was *E. coli* dihydroorotate dehydrogenase (DHO-DH), an enzyme in the pyrimidine biosynthesis pathway that converts dihydroorotate to orotic acid. When aligned using the program BESTFIT (Devereux et al. 1984), the sequences of *E. coli* DHO-DH and the resistance polypeptide have a 34% identity and a 57% similarity in a 333 amino-acid overlap (Fig. 4)

Biochemical studies on the MOA of LY214352

Biochemical, physiological, morphological, chemical reversal and cross-resistance studies to determine the MOA of LY214352 were conducted simultaneously with the molecular approach to LY214352 MOA. Most of these experiments did not suggest a particular MOA for LY214352. In comparison to wild-type *A. nidulans*, LY214352-resistant mutants showed no decreased sensitivity to 20 antifungal compounds. Treatment with LY214352 perturbed cell-wall regeneration in protoplasts and caused mycelial swellings, but only in a nonspecific manner. There were no significant changes in respiration, lipid composition, amino-acid content,

protein accumulation or sterol biosynthesis in the treated mycelium. Incorporation of radio-labeled glucose, acetate and glucosamine was also unaffected.

The results of several experiments eventually led to the pyrimidine biosynthesis pathway as the most likely site of action for LY214352. First, incorporation of radio-labeled uracil into nucleic acids was found to be stimulated 2- to 4-fold by treatment with LY214352 (data not shown). Then, growth inhibition by LY214352 was shown to be slightly reversed by yeast extract. Finally, it was determined that the inhibitory effects of an I_{50} concentration of LY214352 on the growth of A610 could be almost completely reversed by 10 mM of uridine or uracil. Orotate (4 mM) also had a reversal effect, but growth inhibition caused by LY214352 could not be reversed by 10 mM dihydroorotate (DHO). In addition, the slow-growing, LY214352-resistant mutant 52-1 could be restored to a normal growth rate when supplemented with 10 mM of uridine or uracil. Thus, the results of the biochemical and chemical supplementation studies also suggested that LY214352 inhibits DHO-DH.

DHO-DH is known to have separate binding sites for DHO and ubiquinone (Hines and Johnston 1989). In supplementation studies, 10 mM of DHO had no effect on the growth of *A. nidulans* in the presence of LY214352, but 50 μg/ml of coenzyme Q_6 strongly reversed LY214352 growth-inhibition. Coenzyme Q_6 was not able to reverse the inhibitory effects of vinclozolin, benomyl or fenarimol on *A. nidulans*. Although the mechanism by which LY214352 inhibits DHO-DH activity has not yet been determined through kinetic studies, the above results suggest that LY214352 competes with ubiquinone for binding to DHO-DH.

DHO-DH assays

Enzyme assays were used to conclusively demonstrate that DHO-DH is the target site of LY214352 in *A. nidulans*. Studies on partially purified DHO-DH activity isolated from strain A610 showed that the enzyme was inhibited in a dose-dependent manner ($I_{50} = 0.04$ μg/ml) and was completely blocked at 10 μg/ml (Fig. 5). DHO-DH obtained from the resistant mutant 52-4 was about 2500-times less sensitive to LY214352 ($I_{50} > 100$ μg/ml) than the A610 enzyme. The inhibition of the isolated DHO-DH activity is clearly associated with the antifungal activity of LY214352 because the growth responses of resistant and sensitive *A. nidulans* strains to LY214352 parallels the inhibition profiles of the enzyme activity isolated from resistant and sensitive strains (data not shown).

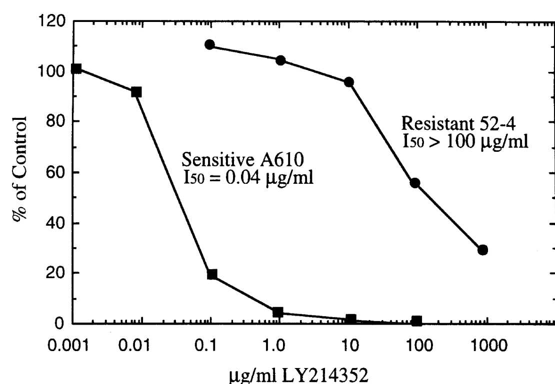

Fig. 5 Effect of LY214352 on dihydroorotate dehydrogenase activity from sensitive and resistant strains of *A. nidulans*

Sequence analysis of mutant and wild-type DHO-DH genes

DHO-DH (pyrE) genes from the parental A610 strain and the mutant 52-4 were isolated by hybridization using a DNA fragment from the 52-6 DHO-DH gene as a probe. The sequences of the A610 and 52-4 DHO-DH genes were identical except for a single-nucleotide mutation that resulted in a valine to glutamate substitution at amino acid 200 of the mutant enzyme. The DHO-DH genes from the remaining mutants were cloned by PCR and sequenced. All of the moderately resistant mutants (52-2, 52-3, 52-5, and 52-6) had the same cytosine-to-thymidine mutation which caused an alanine-to-valine substitution at position 115. Three nucleotide changes were found in the highly resistant LY214352-requiring mutant 52-1. Two of the mutations were in the same valine codon that was mutated in strain 52-4 , and caused a valine-to-aspartate substitution in the DHO-DH protein. The third nucleotide mutation was in an alanine codon and resulted in a change to threonine at position 176. The sequence data demonstrate that all of the LY214352-resistant mutants have nucleotide substitutions within the DHO-DH gene that change at least one amino acid in the LY214352-sensitive enzyme. This provides additional confirmation that DHO-DH is the target of LY214352.

Discussion

Determining the MOA of antifungal compounds is a challenging process that has traditionally relied on biochemical studies. The established genetics of some fungi, in combination with transformation and gene-cloning technology, have suggested a second approach for determining fungicide MOA that is based on the isolation and identification of fungicide-resistance genes. The results of two recent studies, both involving fungicides with MOAs previously determined by biochemical methods, have substantiated the potential of

the molecular-genetics approach in fungicide MOA research. In the first study, the isolation of a carboxin-resistance gene from *Ustilago maydis* that encoded the iron-sulphur subunit of succinate dehydrogenase (Sdh) provided verification that Sdh is the target of the carboxamide fungicides (Keon et al. 1991). In the second study, genetic analysis of *S. cerevisiae* mutants resistant to Soraphen A confirmed that Sorphen A inhibits acetyl CoA carboxylase (Vahlensieck et al. 1994).

A third study, involving the dicarboximide fungicide vinclozolin, has demonstrated the potential contribution of molecular genetics to fungicide MOA identification in cases where biochemical studies have been inconclusive (Orth et al. 1995). Extensive biochemical studies with dicarboximide fungicides have failed to clearly demonstrate a MOA for this group of antifungal compounds, although cytochrome-c-reductase has been mentioned as a possible target site (Edlich and Lyr 1987). Recently, a vinclozolin-resistance gene cloned from *U. maydis* has been shown to encode a Ser/Thr protein kinase (Orth et al. 1995). While the protein kinase has not yet been confirmed to be the target of vinclozolin, this approach has suggested a target that was not identified by biochemical MOA studies.

In the present report, the MOA of the new antifungal compound LY214352 has been successfully determined through a molecular-genetics approach. An LY214352-resistance gene was isolated and identified as *pyr*E, which codes for the enzyme dihydroorotate dehydrogenase (DHO-DH). Enzyme assays confirmed that DHO-DH is the target of LY214352 in *A. nidulans*. These findings distinguish LY214352 as the first fungicide whose site of action has been shown to be DHO-DH, and conclusively validate the use of molecular-genetics techniques in efforts to determine fungicide MOA.

DHO-DH in eukaryotes resides in the outer surface of the inner mitochondrial membrane (Chen and Jones 1976). Peptides that direct proteins to the mitochondrial intermembrane space often consist of an amino-terminal matrix-targeting portion linked to a carboxy terminal extension that has characteristics common to secretory signal peptides (von Heijne et al. 1989). The presence of seven positively charged residues in the first 31 amino acids of the DHO-DH protein suggests that this is the matrix-targeting portion of the polypeptide (Douglas et al. 1986). The amino-acid sequence following the matrix-targeting region has the characteristics of a mitochondrial intermembrane space-targeting signal peptide: a short amino-terminal positively charged region (positions 63–71), a central apolar region (positions 72–90), and a 5–6-residue carboxy terminal region (positions 91–96) with small uncharged amino acids at positions -3 and -1, counting from the cleavage site (von Heijne et al. 1989). This suggests that the histidine residue at position 97 is the N-terminal amino acid of the mature, inner-mitochondrial-membrane-associated *A. nidulans* DHO-DH protein. The homology with plasma-membrane-associated *E. coli* DHO-DH begins six amino acids downstream from this histidine residue.

DHO-DH has been previously studied as a potential target site for inhibition of infectious organisms and as a mammalian antitumor agent (DeFrees et al. 1988; Krungkrai et al. 1991; Chen et al. 1992). Mammalian DHO-DH is thought to follow a non-classical, two-site ping-pong mechanism with non-overlapping, kinetically isolated binding sites for DHO/orotate and ubiquinone (Hines and Johnston 1989). Inhibitors of mammalian DHO-DH that interact with either the DHO/orotate-or the ubiquinone-binding site have been identified (Chen et al. 1986). The mechanism by which LY214352 inhibits fungal DHO-DH has not yet been determined, but the results of chemical supplementation studies with DHO and ubiquinone suggest that LY214352 competes with ubiquinone for binding to DHO-DH. Nevertheless, the existence of a mutant (52-1) that requires LY214352 for maximum growth indicates that LY214352, like the mammalian DHO-DH inhibitor brequinar sodium (Chen et al. 1992), binds to a site on the DHO-DH enzyme that is distinct from the DHO/orotate- and ubiquinone-binding sites.

The present study clearly demonstrates the strength of a dual biochemical and molecular-genetics approach for determining fungicide MOA. Although the biochemical approach is broadly applicable, and has a proven record of success, extensive biochemical studies have failed to clearly define the MOA of some antifungal compounds. Thus, the molecular-genetics approach may prove to be a valuable alternative when fungicide resistance can be attained through target-site mutations. In this case, both approaches identified DHO-DH as the target of LY214352 in similar time frames. This novel antifungal target site can now be exploited in the development of new fungicides.

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